AUTONOMIC CONTROL OF CARDIAC FUNCTION IN HYPOXIC ZEBRAFISH (*DANIO RERIO*) LARVAE

By
Shelby L. Steele

Thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
in partial fulfillment of the requirements for the
PhD Degree in the
Ottawa – Carleton Institute of Biology

Thèse soumise à
l'Ecole d'Études Superieure et de Recherché
Université d'Ottawa
envers la réalization partielle des éxigeances du doctorat
à l'Institut de Biologie d'Ottawa-Carleton

ABSTRACT

Cardiac parasympathetic tone mediates hypoxic bradycardia in fish, however the specific cholinergic mechanisms underlying this response have not been established. In Chapter 2, bradycardia in zebrafish (*Danio rerio*) larvae experiencing translational knockdown of the M_2 muscarinic receptor was either prevented or limited at two different levels of hypoxia (PO_2 = 30 or 40 Torr). Also, M_2 receptor deficient fish exposed to exogenous procaterol (a presumed β2-adrenergic receptor agonist) had lower heart rates than similarly treated control fish, implying that the β2-adrenergic receptor may have a cardioinhibitory role in this species.

Zebrafish have a single β1-adrenergic receptor (*β1AR*), but express two distinct β2-adrenergic receptor genes (*β2aAR* and *β2bAR*). Zebrafish β1AR deficient larvae described in Chapter 3 had lower resting heart rates than control larvae, which conforms to the stereotypical stimulatory nature of this receptor in the vertebrate heart. However, in larvae where loss of β2a/β2bAR and β1/β2bAR function was combined, heart rate was significantly increased. This confirmed my previous observation that the β2-adrenergic receptor has an inhibitory effect on heart rate *in vivo*.

Fish release the catecholamines epinephrine and norepinephrine (the endogenous ligands of adrenergic receptors) into the circulation when exposed to hypoxia, if sufficiently severe. Zebrafish have two genes for tyrosine hydroxylase (*TH1* and *TH2*), the rate limiting enzyme for catecholamine synthesis, which requires molecular oxygen as a cofactor. In Chapter 4, zebrafish larvae exposed to hypoxia for 4 days exhibited increased whole body epinephrine and norepinephrine content. *TH2*, but not *TH1*, mRNA expression decreased after 2 days of hypoxic exposure.

The results of this thesis provide some of the first data on receptor-specific control of heart rate in fish under normal and hypoxic conditions. It also provides the first observations that catecholamine turnover and the mRNA expression of enzymes required for catecholamine synthesis in larvae are sensitive to hypoxia. Taken together, these data provide an interesting perspective on the balance of adrenergic and cholinergic control of heart rate in zebrafish larvae.

RÉSUMÉ

Le rythme parasympathique cardiaque régule la bradycardie induite par l'hypoxique chez les poissons. Cependant, les mécanismes cholinergiques particuliers qui régulent cette réponse ne sont pas établis. Dans le deuxième chapitre, une perte de l'expression du récepteur muscarinique M_2 induite expérimentalement a complètement ou partiellement aboli la bradycardie chez les larves du poisson-zèbre (*Danio rerio*) soumis à deux niveaux d'hypoxie (PO_2 = 30 ou 40 Torr). De plus, en absence du récepteur M_2, les poissons auxquels on a administré du procaterol (assumé d'être un agoniste du récepteur adrénergique β2) montraient une fréquence cardiaque réduite comparativement aux poissons contrôles manipulés de façon similaire, suggérant que le récepteur adrénergique β2 possède possiblement un rôle comme inhibiteur cardiaque chez cette espèce.

Les poissons-zèbres ont un seul récepteur adrénergique β1 (*β1AR*) mais ils expriment deux récepteurs adrénergiques β2 distincts (*β2aAR* et *β2bAR*). Les larves de poissons-zèbres ayant perdu la fonction du récepteur β1AR, décrites dans le chapitre trois, avaient un rythme cardiaque au repos réduit comparativement aux larves contrôles, ce qui est conforme au rôle présumé de ce récepteur comme stimulateur du cœur des vertébrés. Cependant, chez les larves ayant une perte combinée de la fonction de β2a/β2bAR et β1/β2bAR, le rythme cardiaque était significativement augmenté. Ceci confirma mes observations antérieures indiquant que les récepteurs adrénergiques β2 ont un effet inhibiteur sur le rythme cardiaque *in vivo*.

Les poissons secrètent les catécholamines épinéphrine et norépinéphrine (les ligands endogènes des récepteurs adrénergiques) dans le système circulatoire quand ils sont exposés à une hypoxie assez sévère. Les poissons-zèbres possèdent deux gènes pour la tyrosine hydroxylase (*TH1* et *TH2*), qui catalyse l'étape limitante de la synthèse des catécholamines, et qui requiert de l'oxygène moléculaire comme co-facteur. Dans le chapitre quatre, les larves de

poissons-zèbres exposées à l'hypoxie pour quatre jours démontrent une augmentation, au niveau de l'animal entier, du contenu en épinéphrine et norépinéphrine. L'expression de l'ARNm de *TH2*, mais non de *TH1*, était réduite après deux jours d'hypoxie.

Les résultats de cette thèse constituent les premières données sur le contrôle, par les récepteurs de monoamines, du rythme cardiaque chez des poissons sous des conditions normoxiques et hypoxiques. De plus, cette thèse rapporte les premières observations de la modulation du renouvellement des catécholamines et de l'expression des ARNm des enzymes requises pour leur synthèse chez des larves soumise à l'hypoxie. Ensemble, ces données présentent une nouvelle perspective de l'équilibre du contrôle du rythme cardiaque via des sources adrénergique et cholinergique chez les larves du poisson-zèbre.

ACKNOWLEDGEMENTS

First of all, I would like to thank my advisers, Drs. Steve Perry and Marc Ekker. My PhD career has had its share of ups and downs, and you have both been a great source of encouragement and inspiration through it all. Thank you for always having your doors open to me, it is important for any student to know that they are a priority to their advisers and I always felt that way with you. I couldn't have chosen better mentors – thank you for everything.

I would also like to thank my committee members, Drs. Tom Moon, Marie-Andrée Akimenko, and Ken Storey. Thank you for taking the time to act as my advisory committee and for your invaluable input. Also, thanks so much to my collaborators at the University of Innsbruck, Drs. Bernd Pelster and Thorston Schwerte, for being such great hosts during my stay in Innsbruck.

Thanks to all my wonderful lab mates, both past and present! All the laughs have definitely helped keep me going. And of course, for all your help and inspiration in the lab. I also want to thank all my close friends for supporting me during my time at Ottawa U. It has been a challenging and stressful and fun and inspiring process all rolled into one, and you guys have been there to support me through it all.

Finally I need to thank my parents, who have always respected and admired my life decisions. Particlarly my mom, it was always her dream for me to further my education and be able to do whatever I want with my life. Thank you Mom, for your unfailing support.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 2

Chapter 4

LIST OF TABLES

Chapter 2

Chapter 3

LIST OF ABBREVIATIONS

AC, adenylyl cyclase
ACh, acetylcholine
αAR, alpha-adrenergic receptor
ANOVA, analysis of variance
AR, adrenergic receptor
βAR, beta-adrenergic receptor
BCIP, 5-bromo-4-chloroindolyl phosphate
Bmax, maximal binding capacity
BSA, bovine serum albumin
cAMP, cyclic-3'5'-adenosine monophosphate
$Ca(NO_3)_2$, calcium nitrate
CCAC, Canadian Council of Animal Care
CCD, charge-coupled device
cDNA, complimentary deoxyribonucleic acid
CO, cardiac output
DβH, dopamine β hydroxylase
DHA, dihydroalprenolol
DHBA, 3,4-dihydroxybenzylalamine
DIG, digoxigenin
dNTP, deoxynucleotide triphosphate
dpf, days post fertilization
E, epinephrine
EDTA, ethylenediaminetetraacetic acid
FBS, fetal bovine serum
Gi, inhibitory G protein
GPCR, G protein coupled receptor
Gs, stimulatory G protein
h, hour(s)
HCl, hydrochloric acid
HEK293 cells, Human Embryonic Kidney 293 cells
HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpf, hours post fertilization
HPLC, high performance liquid chromatography
IgG, immunoglobulin G
KCl, potassium chloride
K_d, equilibrium dissociation constant for [^{3}H]-DHA
K_i, equilibrium dissociation constant for unlabelled ligands
KOH, potassium hydroxide
l, litre
LB, Lysogeny broth
LED, light-emitting diode
MEM, minimum essential medium
$MgCl_2$, magnesium chloride
$MgSO_4$, magnesium sulphate
min, minute(s)

MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA, messenger ribonucleic acid
MS-222, ethyl-3-aminobenzoate methanesulfonate
N, sample size
NaCl, sodium chloride
NBT, nitro blue tetrazolium
NE, norepinephrine
PBS, phosphate buffered saline
PBST, phosphate buffered saline with Tween
PCA, perchloric acid
PFA, paraformaldehyde
PI3K, phosphoinositide 3 kinase
PKA, protein kinase A
PO_2, partial pressure of oxygen
PTU, 1-phenyl-2-thiourea
PTX, pertussis toxin
PVDF, polyvinylidine fluoride
RNA, ribonucleic acid
RNAi, RNA interference
s, second(s)
SEM, standard error of the mean
SV, stroke volume
TBS, Tris buffered saline
TBST, Tris buffered saline with Tween
TH, tyrosine hydroxylase
tRNA, transfer RNA
w/v, weight per volume

CHAPTER 1.

General Introduction

Autonomic and Humoral Control of Heart Function in Vertebrates

Nervous control of heart function in vertebrates is provided, in general, by both the sympathetic and parasympathetic branches of the peripheral autonomic nervous system. Parasympathetic innervation of the heart is provided by the cardiac vagus, a branch of the vagus nerve (i.e. the tenth cranial nerve) and exerts an inhibitory effect on the rate and force of contraction. Conversely, cardiostimulatory sympathetic neurons extend from paravertebral sympathetic ganglia which are arranged in two connecting chains on either side of the spine. Innervation of peripheral tissues, including the heart, by these efferent sympathetic nerves helps regulate a wide variety of physiological processes which are mediated by adrenergic receptors in the target tissue (see Triposkiadis et al., 2009).

The anatomy of the autonomic nervous system in fishes can vary substantially from the typical vertebrate arrangement. In the ancient cyclostomes (i.e. hagfish (*Myxini*) and lampreys (*Petromyzontidae*)), there are no discrete chains of sympathetic ganglia. The vagus nerve is present, however there is no evidence of either sympathetic or parasympathetic cardiac tone in the systemic or portal hearts of these fish (e.g. Johnsson and Axelsson, 1996; Nilsson, 2010). Catecholamines are stored in chromaffin cells within the walls of the myxine systemic and portal hearts and posterior cardinal vein, and release is mediated not by autonomic innervation but possibly by humoral stimulation by other secretagogues, such as adrenocorticotropic hormone (ACTH; Bernier and Perry, 1996). Elasmobranchs, like the cyclostomes, do not appear to have sympathetic nervous innervation of the heart, but do have a well developed cardiac vagus (Taylor et al., 1977; Taylor and Butler, 1982; Nilsson, 2010). Humoral release of catecholamines from the axillary bodies (modified sympathetic ganglia) are likely an important source of cardiac adrenergic stimulation in these fish (Abrahamsson, 1979). In the dipnoans (i.e. the lungfish), chromaffin cells are dispersed within the atrium, the intercostal

arteries, and the posterior cardinal vein (for references see Perry and Capaldo, 2010). Lungfish are also presumed to lack cardiac cholinergic tone (Fritsche et al., 1993), although this observation has been recently challenged (Sandblom et al., 2010). The pattern of peripheral autonomic nerves and ganglia in teleost fish more closely matches that seen in tetrapods. They have a well developed vagus nerve with a branching cardiac vagus, as well as discrete chains of paravertebral sympathetic ganglia supplying sympathetic innervation to the heart (for review see Nilsson, 2010). Interestingly, the flatfish (*Pleuronectiformes*) represent an order of teleost fish in which cardiac adrenergic innervation seems to be absent (Donald and Campbell, 1982; Mendonça and Gamperl, 2009). Teleosts also have peripheral chromaffin tissue which is localized to the head kidney and the walls of the posterior cardinal vein and, as in mammals, the release of catecholamines from these cells is primarily mediated by cholinergic stimulation from pre-ganglionic sympathetic nerve fibres (see Reid et al., 1998).

Acetylcholine (ACh) is the primary neurotransmitter for both the pre- and post-ganglionic nerve fibres of the parasympathetic nervous system, but only for pre-ganglionic fibres of the sympathetic nervous system. Of the two ACh receptor types, nicotinic ACh receptors are primarily located in the central and peripheral nervous systems, at the synapse of sympathetic pre- and post-ganglionic nerve fibres, and at the neuro-muscular junction of the somatic musculature (for review see McGehee and Role, 1995). Muscarinic ACh receptors, however, are more widely distributed in peripheral tissues including the heart, smooth muscle, and vasculature (for review see Ichii and Kurachi, 2006). Post-ganglionic neurotransmitters released from sympathetic neurons are the catecholamines norepinephrine (primarily, in mammals) and epinephrine. Sympathetic stimulation of the adrenal medulla (in mammals) and chromaffin cells (in fish) by pre-ganglionic sympathetic nerves also causes the humoral

release of epinephrine and norepinephrine into the circulation (for references see Reid et al., 1998; Perry and Capaldo, 2010). Therefore, whereas cholinergic signaling in the heart occurs exclusively by localized release of ACh from the vagus, adrenergic receptors in the heart can be stimulated by catecholamines from either neural or humoral sources.

Adrenergic and cholinergic cardiac tone in vertebrates are facilitated primarily by a specific subset of β-adrenergic and muscarinic ACh receptors, respectively. These receptors have distinct and complementary effects on the heart, as will be described below.

Beta-adrenergic Receptors

General Overview

Biological activity of epinephrine and norepinephrine was first discovered over a hundred years ago, but the receptive elements for these compounds were poorly understood until a pivotal study by Raymond Ahlquist in 1948. Using a variety of sympathetic amines, including arterenol (i.e. norepinephrine) and epinephrine, he determined that the tissue-specific excitatory and inhibitory action of these compounds was regulated by a series of receptors which he termed “alpha” and “beta” adrenotropic receptors (Ahlquist, 1948). By reviewing a series of studies examining the cardiovascular and lipolytic action of different adrenergic ligands, Lands et al. (1967a) concluded that there were at least two β-adrenergic receptor subtypes, the β1 and the β2. In this original classification, Lands et al. (1967a) distinguished the β1 subtype as having a potent cardiostimulatory and lipolytic function compared to the β2, which functioned mainly in broncho- and vasodilation. Discovery of the cellular signaling pathway that brings about these physiological changes at the tissue level began with description of the second messenger cyclic-3’5’-AMP (cAMP) in 1958 (Sutherland and Rall, 1958). Increases in tissue levels of cAMP were reported in a variety of

tissues exposed to catecholamines, including the heart, (for review see Sutherland and Robison, 1966) and could be related to the activity of adenylyl cyclase (Sutherland et al., 1962) as well as the activation of protein kinase A (PKA; Beavo et al., 1974). Indeed, when *Xenopus* oocytes expressing either the human β1- or β2-adrenergic receptors were exposed to catecholamines (including epinephrine and norepinephrine), a dose-dependent increase in adenylyl cyclase (AC)-mediated intracellular cAMP was observed (Frielle et al., 1987). Today, we know that the adrenergic and cholinergic receptors belong to a large class of proteins called seven-transmembrane domain receptors, or G protein-coupled receptors (GPCRs). Each of these proteins has seven transmembrane domains which are highly conserved within the class, and they all associate with G proteins within the cell. Amongst the different types of G proteins, the stimulatory G proteins (Gs) and the inhibitory G proteins (Gi) interact with membrane-bound AC to modulate the downstream production of cAMP (Pierce et al., 2002). The signal transduction paradigm for β-adrenergic receptors is strongly dependent on the individual subtype and continues to be the topic of original research and debate.

The enormous scope of physiological processes mediated by β-adrenergic receptors in peripheral tissues has been the subject of many reviews and will not be taken up here. Instead, the focus will be limited to the current understanding of β1- and β2-adrenergic control of heart function, including the distribution of these receptors within the heart and the cell signaling pathways that mediate cardiac control by β-adrenergic stimulation. The scenario postulated by Lands et al. (1967b) implied that the β1-adrenergic receptor was almost exclusively responsible for increased cardiac activity following catecholamine stimulation. In some of the first experiments examining β-adrenergic receptor density with radioligands in mammalian tissues, Minneman et al. (1979) suggested that approximately 83% of β-adrenergic receptors in

the rat heart were of the β1 subtype, with the remaining 17% being β2-adrenergic receptors. Many studies have since confirmed that significant surface expression of both β1- and β2-adrenergic receptors exists in the heart with the β1 subtype as the most abundant form. The ratio β1:β2 ratio is slightly higher in the ventricle (~80:20) than in the atrium (~70:30; for review and summary see Brodde, 1991).

The mammalian β1-adrenergic receptor gene was first cloned from a human placenta cDNA library (Frielle et al., 1987), whereas the β2 subtype was cloned from hamster lung (Dixon et al., 1986) and human placenta (Kobilka et al., 1987). While obviously not limited to the heart, both β1- and β2AR mRNA are present in this tissue (e.g. Frielle et al., 1987; Sylvén et al., 1991; Lazar-Wesley et al., 1991). Together with classic pharmacological and cell culture techniques, loss of function and transgenic over-expression in animal models have expanded our understanding of β-adrenergic function and signaling in the heart. The current outlook on β1- and β2-adrenergic receptor signaling, as well as the effect of targeted gene deletion of these receptors on the heart rate of mammals, is briefly outlined below.

The β1AR

The β1-adrenergic receptor has been termed the "classic" cardiac adrenergic receptor, presumably because it is the dominant adrenergic receptor found in this tissue, but also because it is exclusively linked with stimulatory G proteins (Gs) within the cardiomyocyte. Specifically, agonist activation of the β1 receptor stimulates Gs proteins, which in turn cause an increase in AC activity thereby increasing cAMP and activating PKA. Once activated, PKA phosphorylates L-type Ca^{2+} channels, which promotes the influx of Ca^{2+} into the cardiomyocyte required for an increase in the force and rate of myocyte contraction (for reviews see Brodde and Michel, 1999; Triposkiadis et al., 2009).

Targeted gene deletion in the mouse has been one of the key tools to confirm the stimulatory nature of the β1AR in the heart. The basal heart rate of mice which lack β1AR (e.g. $\beta 1AR^{-/-}$) has been shown to either be decreased (Ecker et al., 2006) or unchanged (Rohrer et al., 1996; Rohrer et al., 1998), suggesting that other adrenergic receptors are at least partially responsible for maintaining adrenergic tone in this species. However, the increase in the rate of contraction in response to isoproterenol treatment was severely attenuated in both $\beta 1AR^{-/-}$ mice (Rohrer et al., 1996; Rohrer et al., 1998) and in cultured myocytes from $\beta 1AR^{-/-}$ mice (Devic et al., 2001). In cardiomyocytes expressing primarily β1AR (i.e., heart cells from $\beta 2AR^{-/-}$ mice), the isoproterenol-stimulated increase in contraction rate was equal to that in wildtype cardiomyocytes and was not sensitive to pertussis toxin (PTX) inhibition of Gi proteins, but was dependent on PKA activity (Devic et al., 2001). Isoproterenol also failed to initiate cAMP accumulation in the ventricle of $\beta 1AR^{-/-}$ mice (Rohrer et al., 1996) and in cultured $\beta 1AR^{-/-}$ myocytes (Devic et al., 2001). Altogether, these data suggest that the β1-adrenergic receptor is a potent mediator of increased contractile response in the heart via the classic Gs/cAMP pathway. It is clear, however, that changes in heart function in $\beta 1AR^{-/-}$ mice cannot be entirely attributed to the loss of function of this receptor. The role of other β-adrenergic receptors in cardiac control, particularly the highly expressed β2AR, must also be considered.

The β2AR

The role of the β2-adrenergic receptor in cardiac function is less clear than that of β1AR. The heart rate of $\beta 2AR^{-/-}$ mice is not significantly different from that of wildtypes (Chruscinski et al., 1999; Ecker et al., 2006). This is consistent with the absence of β2AR-mediated increases in cAMP in cultured $\beta 1AR^{-/-}$ mouse cardiomyocytes (Devic et al., 2001), or

in the ventricle of $\beta 1AR^{-/-}$ mice (Rohrer et al., 1996). However, like the β1 subtype, the β2-adrenergic receptor interacts with Gs proteins in human cardiomyocytes (e.g. Bristow et al., 1989) and in cell culture (e.g. Frielle et al., 1987; Green et al., 1992; Levy et al., 1993) to increase cAMP in response to agonist stimulation. While these results may seem contradictory, some of these discrepancies may be due to interspecific differences, or to methodological differences (e.g. loss of function versus pharmacological). However, the interaction of the β2AR with alternate signaling pathways within the cell also may explain these inconsistencies.

Unlike the β1AR, the β2-adrenergic receptor couples to Gs and/or inhibitory Gi proteins (for reviews see Xiao, 2001; Xiang et al., 2003). In $\beta 1AR^{-/-}$ cardiomyocytes, isoproterenol causes an initial increase in contraction rate followed by a significant decrease in contraction rate compared to wildtype cells. This decrease was abolished by PTX treatment but not linked to changes in cAMP and was unaffected by inactivation of PKA, suggesting this Gi mediated effect arose independently of the classic cAMP second messenger system (Devic et al., 2001; Xiang et al., 2002; Wang et al., 2008). In cells where β2ARs do interact with Gs proteins, a concurrent interaction with Gi proteins may functionally restrict this stimulatory response. β1AR stimulation seems to cause global cellular increases in cAMP in murine cardiomyocytes (for review see Xiao, 2001), while β2AR mediated increases in cAMP are locally restricted within these cells (Xiao et al., 2003; Nikolaev et al., 2006; Nikolaev et al., 2010) and in rat cardiomyocytes (Nikolaev et al., 2010). In some cases, PTX treatment caused a globalization of the β2AR mediated increase in cAMP (e.g. Xiao, 2001; Xiao et al., 2003). However, other studies have suggested that this phenomenon arises independently of Gi protein interactions (Nikolaev et al., 2006; Nikolaev et al., 2010) and may be more dependent on the β2AR localization at the cellular and tissue level. Indeed, functional β2ARs may be

restricted to transverse tubules (Nikolaev et al., 2010) or caveolae (Rybin et al., 2000; Xiang et al., 2002) within the cell membrane of the cardiomyocyte. While this is not an exhaustive overview of studies examining β2AR signaling in cardiomyocytes, it is apparent that the β1- and β2-adrenergic receptors have distinct roles in regulating cardiac function in vertebrates.

βARs in the Heart of Fish

The presence and function of cardiac β-adrenergic receptors in fish has been demonstrated primarily by pharmacological methods. Adrenergic receptor agonists generally have a positive chronotropic and inotropic effect on the fish heart, including that of the rainbow trout *Oncorhynchus mykiss*, the spiny dogfish *Squalus acanthias*, European flounder *Platichthys flesus* (Ask, 1983), the common carp *Cyprinus carpio* (Temma et al., 1985; Temma et al., 1986b), the marbled African lungfish *Protopterus aethipoicus* (Abrahamsson et al., 1979), the medaka *Oryzias latipes* (Kawasaki et al., 2008), and the zebrafish (Bagatto 2005; Schwerte et al., 2006; Denvir et al., 2008). Several studies have suggested that β-adrenergic receptors in the heart of rainbow trout (Gamperl et al. 1994), winter flounder (*Pleuronectes americanus*; Mendonça and Gamperl, 2008) and common carp (Temma et al., 1986a) are almost exclusively the β2 subtype, in sharp contrast to the mammalian condition. Interestingly, zebrafish have two distinct β2-adrenergic receptors, the β2aAR and the β2bAR. The mRNAs of both of these genes are found in the heart of zebrafish (Wang et al., 2009).

Expression of *β1AR* mRNA has been reported in the hearts of the medaka (Kawasaki et al., 2008) and of the zebrafish (Wang et al., 2009). β1AR-mediated cardiac hypertrophy due to isoproterenol exposure has been demonstrated in medaka (Kawasaki et al., 2008), and, a significant decrease in resting heart rate was observed following β1AR loss of function in zebrafish larvae (Wang et al. 2009). It is apparent that both the β1- and β2-adrenergic

receptors are functionally relevant in the fish heart. To date, however, a functional characterization of the contribution of these receptor subtypes to cardiac function in fish is lacking.

Muscarinic Acetylcholine Receptors

General Overview

The original distinction between "muscarinic" and "nicotinic" ACh receptors can be attributed to Henry Dale, who first described the different effects of muscarine and nicotine on a variety of cholinergically mediated physiological responses (Dale, 1914). Distinguishing the different muscarinic receptor subtypes began much later, with the observation that muscarinic receptors in the sympathetic ganglia (M_1) had a 30-fold higher affinity for the antagonist pirenzepine than atrial muscarinic receptors (M_2; Hammer and Giachetti, 1982). In a review bringing together the results of many studies using functional assays for cholinergic ligands, Mitchelson (1988) proposed that there were at least five different muscarinic receptor subtypes. It wasn't until the sequences of the genes coding for these receptors were determined that classification of the five distinct muscarinic ACh receptors as they are known today, M_1 through M_5, was solidified. All muscarinic receptor subtypes are expressed in the brain, with the M_1, M_4, and M_5 being almost exclusively found in different brain regions. The M_3 receptor can be found in smooth muscle and glandular tissue. The M_2 receptor is also highly expressed in the brain, and to some extent in smooth muscle. Of all the subtypes, however, the M_2 receptor is the predominating if not the exclusive muscarinic receptor expressed in the vertebrate heart (for reviews see Caulfield, 1993; Ishii and Kurachi, 2006).

The M_2 Muscarinic Receptor and the Heart

The negative chronotropic effect of vagal tone of the heart was first described in 1921 by Otto Löwi, who showed that a chemical substance released from the cardiac vagus ("Vagusstoff", later determined to be acetylcholine) caused a decrease in the contraction rate of isolated frog hearts (for references see Ishii and Kurachi, 2006). The critical involvement of the M_2 muscarinic receptor in this response was eloquently highlighted in the M_2 loss of function mouse model. The significant decrease in heart rate (i.e. bradycardia) caused by the muscarinic agonist carbachol was absent in $M_2^{-/-}$ mice (Gomeza et al., 1999; Stengel et al., 2000), but not in $M_4^{-/-}$ mice (Stengel et al., 2000). The M_2 muscarinic receptor causes a decrease in the rate of myocyte contraction due to its association with inhibitory Gi proteins, decreasing cAMP production and thereby heart cell contractility (for review see Caulfield, 1993). Clearly, therefore, cardiac cholinergic tone is somewhat antagonistic to the stimulatory nature of adrenergic tone (described above). Indeed, the balance between sympathetic and parasympathetic signaling is critical for maintaining cardiac function in vertebrates.

Muscarinic Receptors in the Heart of Fish

In fish, as in mammals, cardiac muscarinic receptors are associated with the control of vagal tone in this tissue. When the general muscarinic receptor antagonist atropine is applied to the heart of fish, parasympathetic tone is abolished (e.g. Gannon and Burnstock, 1969; Taylor et al., 1977). The M_2 receptor appears to predominate in the heart of fish, as in mammals, however other muscarinic receptors may also be present. For example, M_2 mRNA expression in the bluegill *Lepomis macrochirus* was highest in heart compared to brain and retina, whereas M_5 expression was absent in cardiac tissue (Phatarpekar et al., 2005). Tissue specific analysis of M_2, M_3, and M_5 mRNA expression in the nile tilapia (*Oreochromis*

niloticus) showed detectable levels of all three in the heart, however the M_3 mRNA was more abundant than that of the duplicated M_2 genes in this species (Seo et al., 2009). Subtype specific muscarinic contribution to vagal tone in the heart of fish is limited to one study in zebrafish (Hsieh and Liao, 2002). Similar to the mouse M_2 loss of function model (Gomeza et al., 1999; Stengel et al., 2000), zebrafish larvae lacking M_2 receptor expression, using either RNAi or morpholinos, are insensitive to the negative effect of carbachol on heart rate (Hsieh and Liao, 2002). These data strongly suggest that the M_2 receptor is involved in maintaining basal cholinergic tone in larval zebrafish hearts. However, parasympathetic tone regulates a stereotypical response of the fish heart to hypoxia – a reflex bradycardia. To date, the M_2 receptor has not been specifically implicated in promoting hypoxic bradycardia in any fish species. This distinction is particularly important considering the possible contribution of other muscarinic receptors in the fish heart (Phatarpekar et al., 2005).

Hypoxia

In the Aquatic Environment

Aquatic hypoxia is the condition in which the dissolved oxygen content of the water drops below levels of normal oxygen saturation. Within the teleost fishes, a wide range of hypoxia tolerance has been reported. Rainbow trout, for example, are sensitive and can experience high mortality in acute hypoxia (e.g. Landman et al., 2005), whereas the crucian carp (*Carassius carassius*) can survive extended periods of anoxia (e.g. Nilsson, 1989). Overall, the relative ability of fish to withstand environmental hypoxia can be related to unique physiological adaptations within this group. Some of these are exclusive to a few species (e.g. ethanol production in carp species exposed to anoxia; Shoubridge and

Hochachka, 1980) and some are more common, including (but not limited to) bradycardia and the release of catecholamines from chromaffin tissue into circulation.

Hypoxic Bradycardia in Fishes

Bradycardia is a typical response to acute hypoxia in teleost fishes and has recently been documented in the toadfish *Opsanus beta* (McDonald et al., 2010), the snapper *Pagrus auratus* (Janssen et al., 2010), and the tilapia *Oreochromis* sp. (Speers-Roesch et al., 2010). Hypoxic bradycardia has also been observed in elasmobranchs (e.g. Taylor et al., 1977), but not in hagfish (Axelsson et al., 1990; Forster et al, 1992) or lungfish (Fritsche et al., 1993; Sanchez et al., 2001; Perry et al., 2005). The physiological benefit of hypoxic bradycardia in fish has been the subject of some debate and speculation (see Perry and Deforges, 2006; Farrell, 2007). Despite the uncertainty of the physiological benefit of hypoxic bradycardia, one thing is clear; this reflex is primarily mediated by an increase of inhibitory cardiac vagal tone. Hypoxic bradycardia is prevented by cardiac vagotomy (Taylor et al., 1977; McKenzie et al., 2009) or blocking of the cardiac muscarinic receptors with atropine (Taylor et al., 1977; Leite et al., 2009; Iversen et al., 2010). Indeed, the lack of hypoxic bradycardia observed in cyclostomes and dipnoans is likely due to the absence or reduced vagal tone in these species (see above).

To date, there are no published measurements of heart rate of adult zebrafish exposed to hypoxia (most likely due to methodological constraints). However, there have been several studies examining the effect of hypoxia on heart rate of larval zebrafish. Results of these studies are somewhat conflicting, which may be related to the duration and/or severity of the hypoxic stress. For example, Jacob et al. (2002) showed that, at physiological temperatures (i.e. 28^{o}C) zebrafish larvae developed significant tachycardia at 4 and 5 dpf after being reared

in moderate hypoxia (approximately 75 Torr) from 20 hpf. A similar trend was confirmed by Barrionuevo et al. (2010), who measured slight but significant tachycardia in 20-100 dpf zebrafish reared in a similar level of moderate hypoxia (80 Torr) from less than 24 hpf. Increases in heart rate due to hypoxia have also been reported in the larvae of rainbow trout (Holeton, 1971) and Arctic char (*Salvelinus alpinus*; McDonald and McMahon, 1977).

Previous reports of hypoxic bradycardia in zebrafish larvae tended to rely on experimental conditions where aquatic oxygen was reduced to much lower levels, or where the fish were made hypoxic for longer periods of time. Barrionuevo and Burggren (1999) reported significant hypoxic bradycardia in zebrafish larvae that had been exposed to gradually decreasing levels of hypoxia down to PO_2 = 10 Torr. However, this response began only at 30 dpf. The aforementioned zebrafish larvae reared in moderate hypoxia (Barrionuevo et al., 2010) produced significant bradycardia (<50% of starting heart rate) when ambient PO_2 was progressively lowered to 10 Torr, beginning at 40 dpf. Finally, when zebrafish were reared in a very low ambient PO_2 (~20 Torr), they had consistently lower heart rates than their normoxic counterparts over a 10 day exposure period (Bagatto et al., 2005). These data suggest that, at lower ambient oxygen levels, zebrafish larvae decrease their heart rate as is seen in the adults of other fish species. The role of vagal tone and, more specifically, the dominant M_2 muscarinic receptor in promoting this response has yet to be examined.

Hypoxia and Catecholamines

Epinephrine and norepinephrine levels in the tissues and plasma of fish depend on a balance between synthesis, release, and degradation. This has been extensively reviewed (see Randall and Perry, 1992; Reid et al., 1998; Perry and Capaldo, 2010). In short, in addition to bradycardia, extreme hypoxia causes an increase in the catecholamine release into circulation.

Not only is the release of catecholamines sensitive to hypoxia, but the pathway for catecholamine synthesis can be affected. Within the pathway for catecholamine synthesis, both the rate limiting enzyme, tyrosine hydroxylase (TH), and dopamine β hydroxylase (DβH) require molecular oxygen as a cofactor, suggesting these enzymes may be sensitive to oxygen availability. In fact, TH enzyme activity, as well as mRNA and protein expression levels have been shown to change in the brain of chronically hypoxic rats (Gozal et al., 2005). Similar to the β2ARs, zebrafish have two tyrosine hydroxylase genes, *TH1* and *TH2*. The mRNA expression of both genes is differentially distributed across larval development, and between the brain, eyes, kidney, and liver of adult fish (Chen et al., 2009). *TH1* mRNA expression is higher than *TH2* throughout development, and also appears in the brain earlier than TH2 mRNA (Chen et al., 2009). These data may indicate that TH activity in neural tissue and peripheral chromaffin tissue is partitioned between the two paralogs. Therefore, these two TH genes may be differentially regulated in stressful situations where catecholaminergic pathways are affected, such as hypoxia.

Zebrafish

General Overview

The zebrafish is a freshwater tropical cyprinid, native to the Himalayan region where it can be found anywhere from fast moving streams to stagnant water bodies and rice fields. Although popular as an aquarium fish, the zebrafish began its ascent as a model organism of choice in biological research due to several key characteristics. It is a hardy species that is easily kept in large numbers in aquaria at temperatures of 22-28^{o}C. They reach sexual maturity at approximately 3 months and are constant breeders. It is the embryos and larvae of zebrafish which are the key stages used in many areas of research, because the process of

embryonic development, as well as genetic makeup, is highly conserved within vertebrates. Large scale screens to examine genetic mutations in zebrafish have been instrumental in linking genes to processes affecting development in vertebrates (for review see Grunwald and Eisen, 2002). With the emergence of reverse genetics technologies such as gene silencing by RNAi or morpholinos (for references see Eisen and Smith, 2008), or gene-specific mutagenesis by TILLING (for review see Moens et al., 2008), researchers are able to pinpoint and manipulate expression of specific genes involved in development, cognition, metabolism, and disease (to name only a few). The widespread popularity of the zebrafish as a model organism is obvious; a PubMed search at the time of this writing (September 2010) revealed almost 13,500 original published works under the keyword "zebrafish" since 1955, with approximately 1000 of those being published to date in 2010. Hence, in order to facilitate information sharing amongst zebrafish researchers, the Zebrafish Information Network (http://zfin.org) was established in 1994. This resource provides researchers with a searchable database of such things as mutant/morphant genotypes and phenotypes, gene expression profiles, atlases of embryonic development, and antibodies useful for the detection of zebrafish proteins (Sprague et al., 2008).

One characteristic of zebrafish embryos and larvae which makes them ideal for cardiovascular physiology research is their translucency. The chorion of the zebrafish egg is transparent, allowing one to visualize the developing embryo from the time of fertilization. The embryos and larvae of zebrafish remain optically clear up to 20 dpf, allowing easy visualization of the heart (for example) through the body wall of the fish (e.g. Barrionuevo et al., 2010). Not only does this persistent translucency highlight the developing organs, but qualitative mRNA and protein expression measurements (by whole mount *in situ* hybridization and immunohistochemistry) in the whole larva can be performed to establish

where and when particular genes are expressed. Indeed, when investigating the physiology of very young animals, it is important to establish at what point structures and genes become present and functional within the organism. The present thesis investigates the development of cardiac control and catecholamine production in larval zebrafish. Thus, the current state of knowledge surrounding the development of the heart, cardiac autonomic tone, and chromaffin tissue development in zebrafish larvae will be outlined briefly.

Development of the Heart

The development of the embryonic zebrafish heart and cardiovascular system, as well as the molecular mechanisms that control this process, has been extensively reviewed (e.g. Stainier and Fishman, 1994; Fishman and Chien, 1997; Roman and Weinstein, 2000; Vogel and Weinstein, 2000). Briefly, cardiac progenitor cells migrate from the developing epiblast to the midline axis beginning around 5.5 hpf, where they eventually form two tubes on either side of the midline of the developing embryo. The two tubes fuse to form the single, unlooped heart tube by approximately 19 hpf, and rhythmic contractions of this tube begin at 22-24 hpf, when blood circulation also begins. The formation of the two chambers of the heart, the atrium and the ventricle, begins at approximately 30 hpf, while the looping of the heart (to the right, as in all vertebrates), occurs at 33-36 hpf. Circulation of blood cells within the embryonic vasculature can be clearly distinguished in the head and trunk by this time. By 2 dpf, the embryonic zebrafish heart resembles that of other developing vertebrates, with venous blood flowing from the sinus venosus to the atrium, then to the ventricle which pumps blood through the bulbus arteriosus to ventral aorta. By the time the larva is 5 days old, the heart will have its adult configuration, with the atrium positioned dorsally to the ventricle.

Development of Autonomic Regulation of the Heart

The developmental timing of autonomic innervation of the zebrafish heart remains unclear. It is generally accepted that the development of the adrenergic and cholinergic receptors in the heart may precede the development of autonomic tone. The negative chronotropic action of cholinergic agonists (acetylcholine or carbachol) has been shown as early as 4 (Bagatto, 2005) or 5 dpf (Schwerte et al., 2006), whereas cardiac cholinergic tone (by treatment with the muscarinic cholinergic receptor antagonist atropine) was not detectable until 12 dpf (Schwerte et al., 2006). However, zebrafish larvae were unable to produce a cholinergically mediated decrease in heart rate in response to startle stimulus at 5 dpf when incubated in atropine (Mann et al., 2010). Furthermore, loss of function of the M_2 muscarinic receptor, the primary mediator of cardiac cholinergic tone, results in a higher heart rate in 3dpf zebrafish larvae compared to controls (Hsieh and Liao, 2002). Therefore, it seems that receptor loss of function experiments revealed the presence of cardiac vagal tone earlier in development compared to traditional pharmacological methods. Cardiac adrenergic tone has been inhibited by pharmacological methods at earlier stages than cholinergic tone, suggesting it may develop first. For example, propranolol treatment increased heart rate beginning at 5 dpf (Schwerte et al., 2006). Sympathetically mediated tachycardia was inhibited in larvae of the same age exposed to propranolol before repeated startle stress (Mann et al., 2010). Interestingly, the development of adrenergic tone in the heart has not been examined by a similar loss of function method as presented by Hsieh and Liao (2002) for the M_2 muscarinic receptor. This would be particularly interesting considering the existence of multiple β2-adrenergic receptors, β2aAR and β2bAR (Wang et al., 2009). By independently removing the function of each of these genes (by morpholino, for example), their relative contribution to cardiac control could be assessed.

Development of Chromaffin Tissue

Chromaffin cells are derived from the neural crest in both mammals (for review see Langley and Grant, 1999) and fish, including the zebrafish (An et al., 2002). By detection of DβH immunoreactivity, chromaffin cells which are in close association with the steroidogenic interrenal cells (To et al., 2007) begin to develop as early as 2 dpf in zebrafish embryos (Chai et al., 2003). At 7 dpf, clusters of TH and DβH positive chromaffin cells are present in close association with the cervical sympathetic ganglion, and by 28 dpf, they are also detected in the kidney (An et al., 2002). While it is apparent that the enzymes required for catecholamine production are present at early developmental stages, there is no documentation of the levels of epinephrine and norepinephrine in zebrafish larvae. Sallinen et al. (2009) present relative changes in norepinephrine levels in 5 dpf larvae exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) but did not provide absolute values. The overall ability of zebrafish larvae to produce catecholamines at early developmental stages and to regulate them under stressful conditions has yet to be examined.

Goals of Thesis

The present thesis aims to describe, in zebrafish larvae, the development of cardiac control by the receptors presumed to be the primary mediators of autonomic signaling; the M_2 muscarinic and the β1- and β2-adrenergic receptors. Concurrent developmental expression of the enzymes required for catecholamine synthesis, TH and DβH, will also be examined. Thus, this thesis will attempt to link the function of these receptors and of catecholamine synthesis in early development to the physiological changes brought about by an environmental stress, hypoxia.

Chapter 2 will examine the involvement of the M_2 muscarinic receptor in hypoxic bradycardia in larval zebrafish. It is hypothesized that loss of function of the M_2 muscarinic receptor using morpholinos will prevent bradycardia in larval fish reared at varying degrees of aquatic hypoxia (PO_2 = 30 or 40 Torr). In Chapter 3, the morpholino-mediated loss of function of the various cardiac type β-adrenergic receptors (β1AR, β2aAR, and β2bAR) will be carried out in zebrafish larvae to determine the relative contribution of these receptors to cardiac control. Each of these receptors will be expressed in a heterologous cell culture to determine their efficacy of AC activation. It is hypothesized that each of the β-adrenergic receptor subtypes will have a unique role in maintaining baseline cardiac function *in vivo*. Finally, in Chapter 4, zebrafish will be exposed to hypoxia (PO_2 = 30 Torr) for either 2 or 4 days to determine the concurrent changes in catecholamine content and TH expression in whole larvae. These will be linked to β-adrenergic receptor mRNA expression and the ability of zebrafish larvae to maintain a heart rate response to exogenous catecholamines after hypoxia exposure. Ultimately, these studies will form an integrated perspective on the importance of cholinergic and adrenergic control in the zebrafish heart, encompassing the effects of both larval development and hypoxic stress.

CHAPTER 2.

Loss of M_2 muscarinic receptor function inhibits development of hypoxic bradycardia and alters cardiac β-adrenergic sensitivity in larval zebrafish *Danio rerio*

Notes on Chapter

The present chapter has been published in the American Journal of Physiology, Regulatory, Integrative and Comparative Physiology as per the following citation:

Steele SL, Lo KH, Li VW, Cheng SH, Ekker M and Perry SF. Loss of M_2 muscarinic receptor function inhibits development of hypoxic bradycardia and alters cardiac β-adrenergic sensitivity in larval zebrafish (*Danio rerio*). *Am J Physiol Regul Integr Comp Physiol* 297: R412-R420, 2009.

KH Lo, VW Li, and SH Cheng were collaborators on this project, and their contribution is represented in Figure 2.7.

Abstract

Fish exposed to hypoxia develop decreased heart rate, or bradycardia, the physiological significance of which remains unknown. The general muscarinic receptor antagonist atropine abolishes the development of this hypoxic bradycardia, suggesting the involvement of muscarinic receptors. In this study, I tested the hypothesis that the hypoxic bradycardia is mediated specifically by stimulation of the M_2 muscarinic receptor, the most abundant subtype in the vertebrate heart. Zebrafish (*Danio rerio*) were reared at two levels of hypoxia (PO_2 = 30 and 40 Torr) from the point of fertilization. In hypoxic fish the heart rate was significantly lower than in normoxic controls from 2 to 10 days post fertilization (dpf). At the more severe level of hypoxia (30 Torr), there were significant increases in the relative mRNA expression of *M_2* and the cardiac type β-adrenergic receptors (*β1AR*, *β2aAR*, and *β2bAR*) at 4 dpf. The hypoxic bradycardia was abolished (at PO_2 = 40 Torr) or significantly attenuated (at PO_2 = 30 Torr) in larvae experiencing M_2 receptor knockdown (using morpholino antisense oligonucleotides). Sham injected larvae exhibited typical hypoxic bradycardia in both hypoxic regimes. The expression of *β1AR*, *β2aAR*, *β2bAR*, and *M_2* mRNA were all altered at various stages between 1 and 4 dpf in larvae experiencing M_2 knockdown. Interestingly, M_2 receptor knockdown unveiled a cardioinhibitory role for the β2-adrenergic receptor. This is the first study to demonstrate a specific role of the M_2 muscarinic receptor in the initiation of hypoxic bradycardia in fish.

Introduction

Homeostatic regulation of heart function in vertebrates relies on a variety of G-protein-coupled receptors (GPCRs), most importantly β-adrenergic and muscarinic cholinergic receptors. The M_2 subtype is the predominant muscarinic receptor found in the mammalian heart (Dhein et al., 2001), and presumably in the heart of other vertebrates including fish. Stimulation of the heart with the general muscarinic receptor agonist carbachol causes a decrease in heart rate (i.e. bradycardia) in mice (e.g. Stengel et al., 2002). In the mouse, targeted loss of function of M_2, M_3 and M_4 muscarinic receptors has been used to further tease apart the role of these subtypes in eliciting this bradycardic response. Interestingly, heart muscle preparations derived from $M_3^{-/-}$ (Stengel et al., 2002) and $M_4^{-/-}$ (Stengel et al., 2000) mice and treated with carbachol exhibit bradycardia, while preparations derived from $M_2^{-/-}$ mice do not (Stengel et al., 2000). Using larval zebrafish, Hsieh and Liao (2002) demonstrated that M_2 receptor loss of function is associated with an increase in resting heart rate and the loss of the typical decrease in heart rate upon carbachol exposure. Thus, the M_2 subtype appears to be critical for regulating pacemaker activity in both mammals and fish.

Cardiac function in fish is sensitive to a variety of environmental factors, one of which is lowered environmental oxygen, or hypoxia. Fish consistently experience bradycardia when exposed to hypoxic conditions (for references see Farrell, 2007). While the physiological function remains a mystery, hypoxic bradycardia in fish is most certainly cholinergically mediated. Injecting fish with the general cholinergic antagonist atropine partially blocks the hypoxic bradycardia in rainbow trout (Perry and Deforges, 2006) and the common carp (Stecyk and Farrell, 2006) and completely blocks it in the crucian carp (Vornanen and Tuomennoro, 1999), the short-horned sculpin (*Myoxocephalus scorpius*; MacCormack and Driedzic, 2007) and lesser-spotted dogfish (*Scyliorhinus canicula*; Taylor et al., 1977).

Vagotomized dogfish are also incapable of producing bradycardia in hypoxia (Taylor et al., 1977), suggesting parasympathetic tone is critical in this response. Until recently, studying the cholinergic regulation of hypoxic bradycardia in fish was limited to pharmacological and surgical methods such as those described above. Advances in molecular biology have lead to the discovery of a variety of gene silencing techniques, including loss of function (or "knockdown") using morpholino antisense oligonucleotides (Nasevicius and Ekker, 2000). Zebrafish reared in severe hypoxia (water PO_2 ~ 20-30 Torr) exhibit significantly lower heart rates than normoxic larvae beginning as early as 2 days post fertilization (dpf; (Bagatto, 2005). Although the M_2 receptor has been successfully silenced in zebrafish using gene knockdown techniques (Hsieh and Liao, 2002), its possible role in promoting hypoxic bradycardia has not been investigated. Indeed, there is increasing evidence for multiple cardiac muscarinic receptor subtypes, at least in mammals (Myslivecek et al., 2008; for review see Dhein et al., 2001). Therefore, the single gene silencing strategy to pinpoint the specific role of the M_2 receptor in eliciting hypoxic bradycardia provides an additional approach to pharmacological methods that use selective muscarinic receptor subtype antagonists. The primary hypothesis of this study is that the cardiac M_2 muscarinic receptor mediates the hypoxic bradycardia in larval zebrafish. Thus, it is predicted that larvae experiencing M_2 receptor knockdown will not develop hypoxic bradycardia.

Cardiac cholinergic and adrenergic receptors are functionally linked. While muscarinic receptor activity has negative chronotropic and inotropic actions, stimulation of cardiac adrenergic receptors (mainly the β1 and β2 subtypes) generally evokes the opposite effects. It is therefore not surprising that the level of activity of each branch of the autonomic nervous system on cardiac function is dependent on the other (Henning et al., 1990; for review see Levy, 1984). Indeed, when cardiac muscarinic tone is acutely blocked with atropine, β-

adrenergic mediated increases in heart rate are larger (Levine and Leenan, 1989; Schäfers et al., 1997; Bruck et al., 2003), suggesting a blunting effect of vagal tone on the adrenergic response in the heart. Currently, there are little data concerning the effects of chronic lack of vagal tone on the functioning of cardiac β-adrenergic receptors. A second hypothesis of this study, therefore, is that zebrafish larvae lacking the M_2 receptor will exhibit decreased sympathetic tone and a blunted chronotropic response to β-adrenergic agonists. Measurements of heart rate will be complemented by assessing the mRNA expression of key receptors involved in regulating heart rate, including M_2 but also the cardiac β-adrenergic receptors, β1AR and β2AR.

Materials and Methods

Animals

Adult zebrafish were purchased from Big Al's Aquarium Services (Ottawa East, ON) and maintained in 10 l acrylic tanks in multi-rack aquatic housing systems (Aquatic Habitats, Apopka, FL USA). All tanks were supplied with well aerated dechloraminated City of Ottawa tap water at 28^{o}C (for ion composition refer to Perry and Vermette, 1987). Tanks housed approximately 15-20 fish. Fish were maintained under a 14h:10h light: dark cycle and spawning occurred daily at the beginning of the light cycle. To obtain embryos, breeder tanks (1 l; Aquatic Habitats, Apopka, FL USA) were placed in each 10 l tank prior to spawning and collected after spawning was allowed to proceed for at least half an h. All experiments were performed in accordance with institutional animal care guidelines and those of the Canadian Council of Animal Care (CCAC). Experimental procedures (Protocol BL-226) were preapproved by University of Ottawa Animal Care and Veterinary Service.

Tissue Collection – Adults, Embryos and Larvae

Adult zebrafish were killed by overdose of benzocaine (Sigma E1501) and tissues were dissected and frozen on dry ice in RNase free 1.5 ml micro-centrifuge tubes. To obtain enough total RNA for subsequent analyses, each sample was a pool of tissues from 5 individuals.

Pooled samples of embryos or larvae (20-100 individuals depending on developmental stage) were placed into an RNase free 1.5 ml micro-centrifuge tube and flash frozen in liquid nitrogen. For hypoxia-reared animals, embryos or larvae were removed from the tanks and quickly screened so that each sample contained only animals of the proper developmental stage. All adult and larval tissue samples were stored at -80^{o}C for up to 1 month.

RNA Extraction, cDNA Synthesis, and Real-Time PCR

RNA extraction was performed using TRIzol reagent (Invitrogen Corp., Carlsbrad CA, USA) according to the manufacturer's directions. Total RNA was dissolved in 10-100 µl of RNase free water and quantified using a Nanodrop ND-1000 Spectrophotometer with NanoDrop ND-1000 Software v. 3.3.0 (NanoDrop Technologies LLC, Wilmington DE USA). RNA was stored at -80^{o}C for up to 1 month.

Total RNA (2 µg) was treated with DNase I Amplification Grade (Invitrogen Corp., Carlsbrad CA, USA) prior to reverse transcription with RevertAid^{TM} M-MuLV Reverse Transcriptase (Fermentas International Inc., Burlington ON, Canada) according to the manufacturer's directions. cDNA was diluted 1 in 5 for genes of interest (*M_2, β1AR, β2aAR, β2bAR*) and 1 in 1000 for 18S and stored at -20^{o}C. Sequences for all real-time PCR primers used are provided in Table 2.1. 18S was chosen as the reference gene for all real-time PCR measurements based on consistent findings in our group that the expression of this gene changes minimally with experimental treatments in multiple fish species. Also, when compared to other reference genes commonly used to validate real-time PCR data, 18S is one of the most stable across tissue types (McCurley and Callard, 2008) and developmental stages (Tang et al., 2007) in zebrafish. Real-time PCR was performed using Brilliant™ SYBR Green Mastermix (Stratagene, La Jolla CA USA) in a 12.5 µl reaction with the following components: 0.5 µl diluted template, 0.5 µl each forward and reverse primers (final concentration = 100 nM), 0.19 µl reference dye (provided with kit, diluted according to manufacturer's directions), 4.6 µl sterile water, and 6.25 µl Brilliant™ SYBR Green Master Mix. The PCR reactions were run using the following thermal profile: Initial denaturation at 95^{o}C for 10 min, then 40 cycles of 95^{o}C for 30 s, 58^{o}C for 1 min, and 72^{o}C for 1 min. Dissociation curves for all samples were run according to program defaults.

To determine the efficiencies of the PCR reactions, standard curves were constructed using serial dilutions of a pooled template sample. Amplification efficiencies for all real-time PCR reactions are listed in Table 2.1. The final real-time PCR data were analyzed using the modified delta-delta ct method described by Pfaffl (2001).

Morpholino Injection

A M_2 antisense morpholino oligonucleotide (Gene Tools, Philomath OR, USA) with the sequence 5'-CACTCAGATCGCTATTGGCAGGACG-3' (and conjugated to the fluorescent protein carboxyfluorescein) was used according to that previously described by Hsieh and Liao (2002). For injection, the M_2 morpholino was prepared to a final concentration of 4 ng nl^{-1}in 1x Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES (pH =7.6)), and 0.05% phenol red (for visualization of the injection volume). Injections were performed using a Narishige IM 300 Microinjector system (Narishige International USA Inc, Long Island NY, USA). Control fish ("sham") were injected with a mixture of injection buffer and phenol red alone. Approximately 1 nl of morpholino (4 ng $embryo^{-1}$) or sham mixture was injected into each embryo at the one cell stage. A 4 ng $embryo^{-1}$ dose was chosen as per the morpholino dose response presented by Hsieh and Liao (2002). Here, the authors showed significant changes in heart rate due to injection of 0.8, 2.4 and 8 ng, however 50% of animals at the highest dose experienced undesirable phenotypic changes. Therefore, an intermediate dose of 4 ng was used in the present study. No obvious phenotypic differences were observed between the M_2 morphants and sham injected controls produced in the present study. After injection, embryos were placed in 30 ml petri dishes containing E3 embryo medium containing 0.03‰ ethylene blue and incubated at 28^{o}C. E3 medium was replaced daily. At 24 h post fertilization (hpf), M_2

morphant embryos were screened for the presence of the carboxyfluorescein by visualizing the embryos using a Nikon SMZ1500 stereomicroscope (Nikon Instruments Inc., Melville NY, USA) supplied with fluorescent light. Only embryos that were carboxyfluorescein positive were kept and used for subsequent experiments.

Chronic Hypoxia Exposure of Embryos

Water flow was stopped and tanks (10 l) were gassed using a length of perforated aquarium tubing with mixtures of air and nitrogen to obtain a final PO_2 of 40 or 30 Torr. Nitrogen was mixed with air using a gas mixing flowmeter (Cameron Instrument Co., Port Aransas, TX USA). Normoxic control tanks were gassed with air alone. Temperature (28^oC) was maintained in the tanks by partially immersing them in a 28^oC water bath. Water O_2 levels were monitored using a fiber optic oxygen sensor probe (model no. FOXY-AL 300) supplied with a LED pulsed light source (model no. R-LS-450-2) connected to a fiber optic spectrometer (model no. S2000-UV-VIS). Data were collected from the spectrometer using OOISensors software version 1.00.09. All O_2 detecting equipment was obtained from Ocean Optics Inc., Dunedin, FL USA. Each tank was treated with 1 ml Maroxy antifungal agent (Virbac AH Inc., Fort Worth TX USA).

Embryos were placed in the normoxic and hypoxic chambers at approximately 2 hpf. Beginning at 48 hpf, 50% water changes were performed on each tank daily. Embryos were staged according to Kimmel et al. (1995). Beginning at 5 dpf, larvae were fed daily with starter ZM-000 fry food (Zebrafish Management Ltd., Manchester UK). Both of the hypoxic groups (PO_2 = 30 and 40 Torr), experienced developmental delays, with the 30 Torr group being more delayed (approximately 24 chronological h) than the 40 Torr group (approximately

16 chronological h). Therefore, all analyses (e.g. heart rates, mRNA levels) were performed at the dpf of developmental time rather than chronological time.

Heart Rate Measurements

Larvae reared in hypoxia or normoxia were placed individually in a small volume of 100 mg l^{-1} Tris buffered MS-222 (ethyl-3-aminobenzoate methanesulfonate salt, Sigma-Aldrich Inc., St. Louis MO, USA) at 28^{o}C. After 3 min, the heart rate was measured by observing the embryo under a dissecting microscope and counting heart beats for 30 s.

Baseline heart rates of M_2 morphant and sham injected larvae were measured as described above. Each larva was then placed in a fresh solution also containing 100 mg l^{-1} MS-222 and either norepinephrine (NE, general adrenergic receptor agonist), propranolol (β-adrenergic receptor antagonist), or procaterol (β2AR agonist). After 10 min of exposure to these chemicals, heart rate was measured again as described above. The dose of 100 mg l^{-1} of MS-222 was chosen because it was the minimum dose required to keep the larvae immobilized during the heart rate measurements. While it has been shown that high levels of MS-222 can decrease heart rate in zebrafish larvae (e.g. Hsieh and Liao, 2002; Denvir et al., 2008), the concentration used in the present study was similar to that used in other zebrafish studies (e.g. Schwerte et al., 2006). In the present experiments, only a dose of 160 mg l^{-1} (compared to 80 and 120 mg l^{-1}) was found to significantly decrease heart rate after 15 min of anesthesia in 4 dpf larvae (data not shown). Therefore, I am confident that the anesthesia did not cause any non-specific effects on heart rate during these experiments.

Whole Mount in situ Hybridization

A PCR product for M_2 was amplified from adult heart cDNA using the primers listed in Table 2.1. PCR conditions for amplifying this product was as follows (all reagents from Invitrogen Inc., Carlsbad CA, USA): 2 µl template cDNA, 2.5 µl 10x PCR buffer, 0.75 µl 50mM $MgCl_2$, 0.5 µl 10 mM dNTP mix, 0.5 µl each forward and reverse primers, 0.2 µl Taq DNA Polymerase, and sterile water to a final volume of 25 µl. Products were visualized on a native agarose gel (0.8%) stained with ethidium bromide (0.1 µg ml^{-1}). The band corresponding to the product of interest was excised using a razor blade and extracted using a Sigma GenElute™ gel extraction kit (Sigma-Aldrich Inc., St. Louis MO, USA). The gel extracted product was quantified using a NanoDrop spectrophotometer and software (as described above). The product was cloned into pDrive vector using Qiagen PCR Cloning Plus kit (Qiagen Inc., Valencia CA USA) according to the manufacturer's protocol. Transformed cells were plated on 30 ml of agar in LB plates containing 100 mg l^{-1} ampicillin and incubated at 38^{o}C for up to 16 h. Colonies containing plasmid were individually selected and grown in 5 ml of LB medium containing 100 mg l^{-1} ampicillin at 38^{o}C for up to 6 h. Fifty µl of this fresh culture was used to inoculate a larger volume (100 ml) of LB medium containing 100 mg l^{-1} ampicillin. After 16 h of incubation at 38^{o}C, bacteria were pelleted from the media and plasmids extracted using Qiagen HiSpeed Plasmid Midi Kit (Qiagen Inc., Valencia CA, USA) according to the manufacturers protocol. Extracted plasmids were quantified using the NanoDrop spectrophotometer as described previously.

Ten µg of plasmid DNA was linearized by restriction digest with *HindIII* restriction enzyme (Invitrogen Inc., Carlsbad CA USA). The resulting linear DNA was run on an agarose gel, excised, gel extracted, and quantified using the methods described above. One µg of the linearized plasmid was used as a riboprobe template using T7 RNA polymerase (Roche

Applied Science, Indianapolis, IN USA) according to the manufacturers protocol. Final concentration of DIG labeled RNA probes was estimated by comparison with a RiboRuler^{TM} RNA ladder (Fermentas International Inc., Burlington ON, Canada) on a native agarose gel as described above.

Embryos used for *in situ* hybridization were reared in tanks containing 0.003% 1-phenyl-2-thiourea (PTU; Sigma-Aldrich Inc., St. Louis MO, USA) to minimize pigment development. Larvae were fixed at 4 dpf in 4% paraformaldehyde (PFA; Fisher Scientific, Waltham MA, USA) in 1x PBS overnight at 4^{o}C. Fixed larvae were dehydrated in 100% methanol and kept at -20^{o}C in 100% methanol until use. Prior to hybridization, larvae were rehydrated in a graded methanol/PBS series and washed in 1x PBST (1x PBS + 0.1% Tween 20) 3 times for 5 min. Larvae were then permeabilized in 20 μg ml^{-1} Proteinase K in 1x PBST for 20 min. Larvae were washed twice for 5 min in 1x PBST and post-fixed in 4% PFA in PBS for 20 min. Larvae were then washed twice for 5 min in 1x PBST and prehybridized in a hybridization solution containing 50% formamide, 5x SSC, 0.1% Tween-20, 50 μg ml^{-1} heparin, 100 μg ml^{-1} yeast tRNA, and 9.2 mM citric acid (pH 6.0) for up to 3 h at 65^{o}C. Hybridization solution was replaced with fresh hybridization solution containing approximately 0.5 ng μl^{-1} of M_2 riboprobe or RNAse free water (no probe control) and incubated overnight at 65^{o}C.

After probe hybridization, larvae were washed in a graded hybridization mix/2x SSC series and 2 times 30 min in 0.2x SSC at 65^{o}C. Larvae were then washed in a graded 0.2x SSC/1x PBST series at room temperature. In order to block non-specific binding of the anti-DIG antibody, larvae were incubated in 1x PBST containing 10% goat serum and 2 mg ml^{-1} bovine serum albumin (BSA) for up to 4 h at room temperature. This was replaced with fresh solution containing 1:2000 anti-DIG antibody (Roche Applied Science, Indianapolis, IN USA)

and larvae were incubated overnight at 4°C. After washing (6 x 15 min) in 1x PBST at room temperature, larvae were washed in a coloration buffer containing 100 mM Tris HCl (pH 9.5), 50 mM $MgCl_2$, 100 mM NaCl, and 0.1% Tween-20 for 5 min at room temperature. Color development was performed in fresh coloration buffer containing 5-bromo-4-chloroindolyl phosphate (BCIP) and nitro blue tetrazolium (NBT; Roche Applied Science, Indianapolis, IN USA). Images of larvae were captured using a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI USA) attached to a stereomicroscope (SZX12; Olympus, Center Valley, PA USA).

Statistical Analyses

All statistical analyses were performed using SigmaStat statistical analysis software (v. 3.5; Systat Software Inc., San Jose CA, USA). Relative mRNA expression data in Figures 2.1 and 2 were compared using one-factor ANOVA and a Tukey post-hoc test. Heart rate data in Figures 2.3 and 2.4 were compared using two-factor ANOVA and a Holm-Sidak post-hoc test. Heart rate data in Figure 2.5 were compared using Student *t* test. Data in Figure 2.9 were compared using a paired *t* test.

For Figures 2.6 and 2.8, each treatment mean was compared to individual controls that were set to a value of 1. Therefore, data were compared to the numerical value of 1 using a one-sample *t* test.

Results

Developmental and Adult Tissue Gene Expression of M_2

The expression of M_2 mRNA in embryos was first detectable at the pre-hatch stage of 12 hpf (Figure 2.1), therefore all subsequent values were expressed relative to this stage. Expression at every stage up to 10 dpf was significantly higher than the 12 hpf level. Expression at 1 dpf was approximately 35-fold higher than at 12 hpf and continued to increase, peaking at about 750-fold at 6 dpf (Figure 2.1).

M_2 mRNA expression was detectable to some degree in all adult tissues that were examined, therefore all values were expressed relative to an arbitrarily chosen tissue with low mRNA expression levels (the gut; Figure 2.2). Eye, heart, and brain expression levels were significantly higher than in the gut, being 12-, 43-, and 70-fold higher, respectively (Figure 2.2).

Effectiveness of the M_2 Morpholino

There was no effect of 10^{-4} M carbachol exposure on the heart rate of either 2 or 3 dpf sham injected or M_2 morphant larvae (Figure 2.3). However, at 4 and 5 dpf, sham injected fish experienced significant bradycardia relative to anaesthetized control fish (Figure 2.3). In M_2 morphants, the carbachol-evoked bradycardia was prevented at both 4 and 5 dpf (Figure 2.3). Because the margin of difference between morphant and sham-injected larvae was greater at 4 dpf, the latter stage was chosen as the end-point for all subsequent experiments assessing the effects of M_2 receptor knockdown.

Hypoxia Exposure – Bradycardia, M_2 Morphants, and mRNA Expression Analysis

Under normoxic conditions, larval heart rates increased during development from 2 – 6 dpf and then declined until 10 dpf (Figure 2.4). Chronically hypoxic larvae (reared at a PO_2 of either 30 or 40 Torr) exhibited bradycardia relative to their normoxic control group at all stages between 2 and 10 dpf with the exception of the 3 dpf stage in the 30 Torr experiment (Figure 2.4).

M_2 morphants and sham injected controls were reared in either normoxia or hypoxia (PO_2 = 40 or 30 Torr) from 0 to 4 dpf. In the 40 Torr experiment, both groups raised in normoxia had resting heart rates of approximately 200 beats min^{-1} and were not significantly different from each other (Figure 2.5). However while morphant fish were unaffected, sham injected fish displayed bradycardia when reared in hypoxia; heart rate in 40 Torr larvae was approximately 170 beats min^{-1} or 15% lower than in sham injected fish in normoxia. In the 30 Torr experiment, the hypoxic bradycardia was attenuated but not abolished by in the M_2 morphant larvae (Figure 2.5).

Preliminary analysis of the published zebrafish genome (www.ensembl.org) revealed that two isoforms of the β2AR may exist in this species. Real-time PCR analysis revealed that both genes are expressed in the zebrafish during development and in the adult heart (Chapter 3, Figures 3.1 and 3.2). For the purpose of this study, these genes have been labeled *β2aAR* and *β2bAR*. Expression of *M_2*, *β1AR*, *β2aAR* and *β2bAR* mRNA was examined in larvae from 3 to 6 dpf reared in a PO_2 of either 30 or 40 Torr. Fish reared in PO_2 of 40 Torr showed no significant change in mRNA expression of any of the genes studied (Figure 2.6A). At PO_2 of 30 Torr, expression of *M_2*, *β1AR*, *β2aAR* and *β2bAR* mRNA was significantly higher than in normoxic controls at 4 dpf, being 2.5-, 2.0-, 2.4-, and 1.5-fold higher, respectively (Figure 2.6B). In an attempt to determine cardiac specific changes in *M_2* receptor mRNA expression during hypoxia, *in situ* hybridization was performed on 4 dpf fish reared at a PO_2 of 40 Torr.

Positive staining for *M_2* mRNA occurred mainly in the heart and brain of both normoxic and hypoxic larvae (Figure 2.7A, B). Based on visual differences in staining intensity, larvae reared in hypoxia (Figure 2.7B) appeared to have greater expression of *M_2* mRNA in brain and heart when compared to larvae reared in normoxic water (Figure 2.7A).

It should be noted that although results for two levels of hypoxia (PO_2 = 30 and 40 Torr) are presented here, all experiments carried out at a PO_2 of 40 Torr were completed before those using the lower PO_2. The second set of experiments was undertaken to determine if significantly lower heart rates could be achieved with a more extreme level of hypoxia, and if this lower PO_2 could induce changes in mRNA expression not seen at 40 Torr. Despite the fact that only the PO_2 of 30 Torr induced a significant change in mRNA levels in whole larvae, the bradycardia produced in this group was not largely different than that seen in the 40 Torr experiment (Figure 2.4).

M_2 Knockdown – Effects on β-adrenergic Receptors

Expression of *$\beta 1AR$* mRNA was significantly higher in M_2 morphants than in sham injected fish at 1 and 3 dpf (Figure 2.8). *$\beta 2aAR$* mRNA expression was higher in morphants at 1 and 3 dpf, while there was a significant 1.6-fold increase in *M_2* mRNA expression at 4 dpf (Figure 2.8). *$\beta 2bAR$* mRNA was approximately 50% lower in M_2 morphants at 2 dpf but was increased 1.6-fold at 3 dpf (Figure 2.8).

Based on the outcome of preliminary experiments using wildtype (i.e. unmanipulated) larvae, two concentrations of NE were chosen for use in this study. The highest dose (10^{-4} M) caused a maximal increase in heart rate, while 5 x 10^{-7} M NE caused a medial increase (data not shown). Exposure to 10^{-4} NE caused similar increases in heart rate in M_2 morphants and sham controls, being 6.5% and 7.6% respectively (Figure 2.9). However, when exposed to 5 x

10^{-7} M NE, only sham injected fish displayed significantly higher heart rates (3.7% higher than control values); the M_2 morphant heart rates remained unchanged (Figure 2.9). To elucidate the contribution of each βAR subtype to these responses, subtype specific agonists were used. Sham injected larvae exposed to the β2AR specific agonist procaterol (10^{-4} M) showed no change in heart rate, whereas the heart rate of M_2 morphants decreased significantly to 93% of their anaesthetized control values (Figure 2.9). Both the sham and M_2 morphants exhibited significant cardiac β-adrenergic tone. Exposure to the general βAR antagonist propranolol (10^{-4} M) caused similar decreases in heart rate in both groups (Figure 2.9).

Figure 2.1

Relative mRNA levels of the M_2 muscarinic receptor in whole zebrafish embryos and larvae. Levels are below levels of detection (BLD) at 1 and 6 h post fertilization (hpf); values are therefore relative to 12 hpf. All samples were normalized to the expression of 18S ribosomal RNA. dpf = days post fertilization. Values are mean + SEM, N = 5. All values are significantly different from 12 hpf ($p < 0.05$).

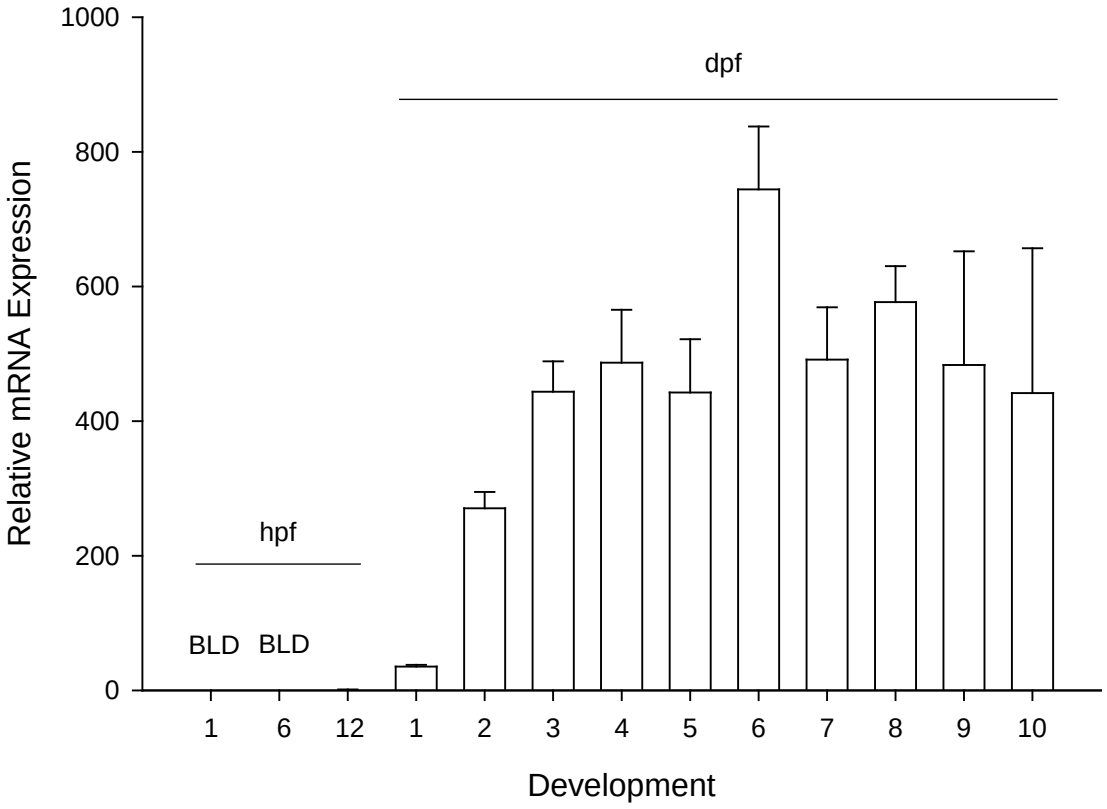

1000
800
600
400
200
0
Relative mRNA Expression
dpf
hpf
BLD
BLD
1
6
12
1
2
3
4
5
6
7
8
9
10
Development

Figure 2.1

Figure 2.2

Relative mRNA levels of the M_2 muscarinic receptor in various adult zebrafish tissues. All samples were normalized to the expression of 18S ribosomal RNA and expressed relative to the expression of M_2 in the gut. Values are mean + SEM, N = 5. * indicates significant difference from gut value ($p < 0.05$).

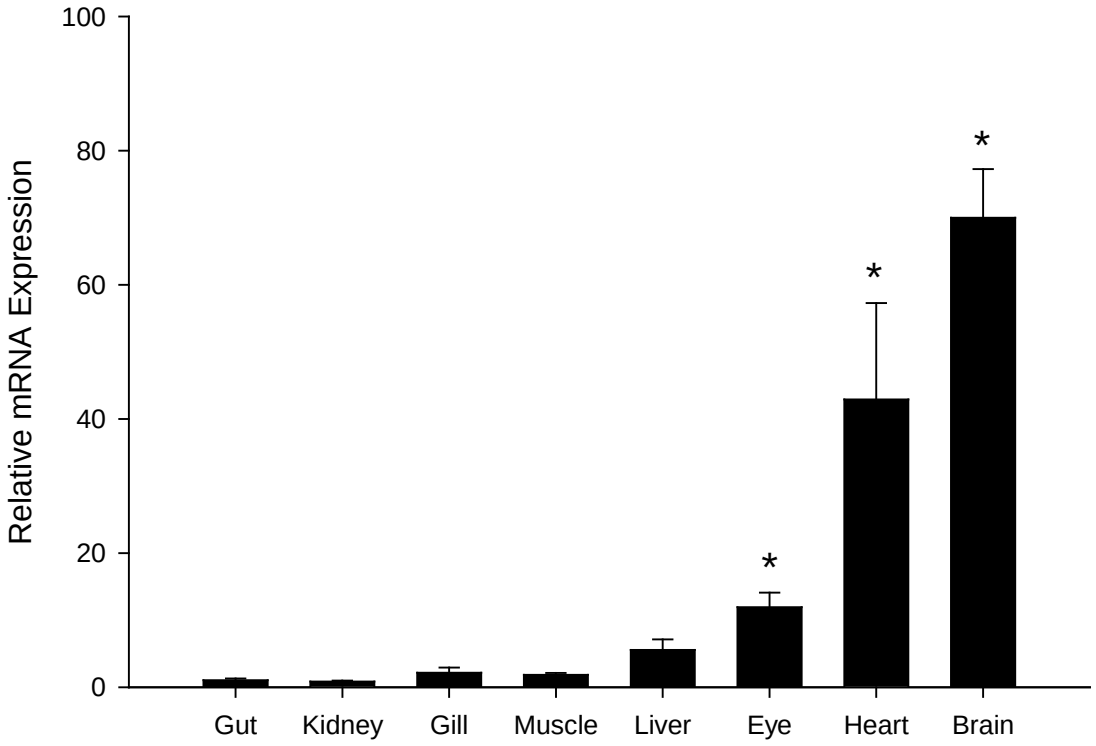

Relative mRNA Expression
100
80
60
40
20
0
*
*
*
Gut
Kidney
Gill
Muscle
Liver
Eye
Heart
Brain

Figure 2.2

Figure 2.3

Heart rate response of sham injected (black bars) and M_2 morphant zebrafish larvae (white bars) exposed to the general muscarinic receptor agonist carbachol (10^{-4} M). Values are expressed as a percent of the average heart rate of larvae which were anaesthetized only (100 mg l^{-1} MS-222). Values are mean + SEM, N = 6. * indicates significant difference from sham injected group within developmental stage. Letters (a or b) indicate significant difference between developmental stages within sham or morphant groups ($p < 0.05$).

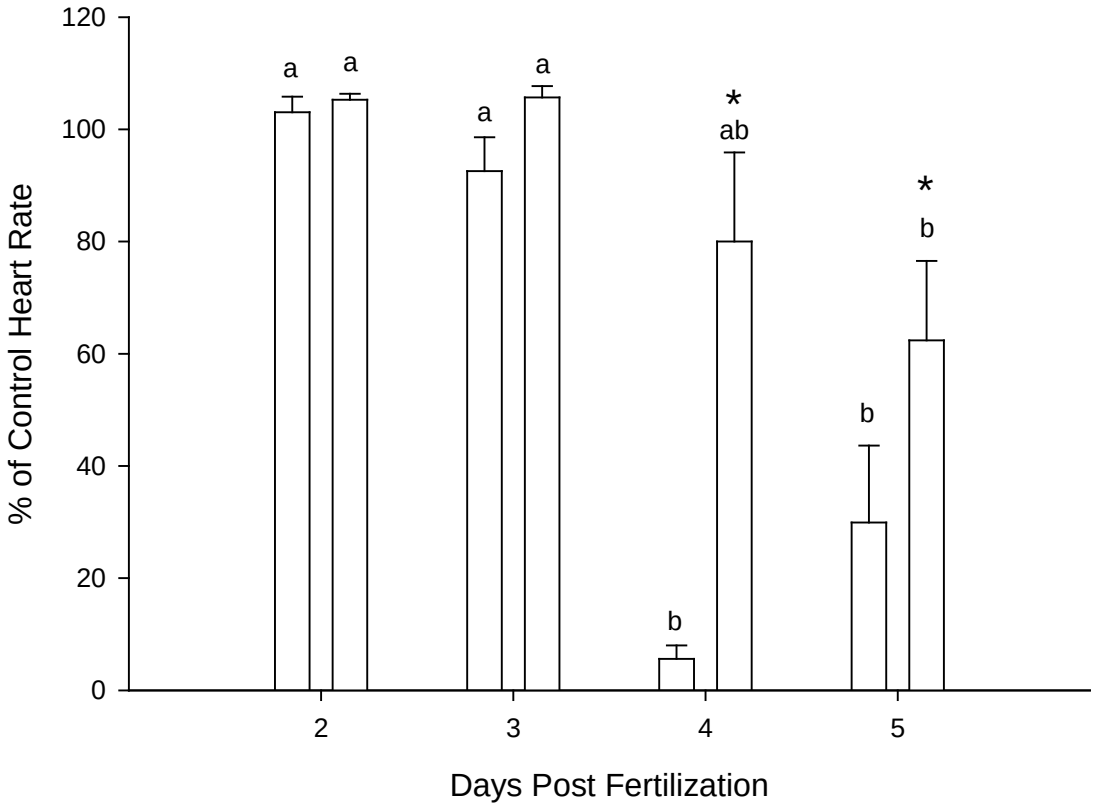

120
100
80
60
40
20
0
% of Control Heart Rate
a
a
a
a
b
*
ab
b
*
b
2
3
4
5
Days Post Fertilization

Figure 2.3

Figure 2.4

Heart rate (beats min^{-1}) of zebrafish larvae reared from conception in normoxia (PO_2 = 150 Torr; solid circles) and a PO_2 of either 40 Torr (A; open circles) or 30 Torr (B; open circles). Heart rate of fish reared at 40 and 30 Torr are significantly lower than normoxic controls at all time points except for the 3 dpf time point at 30 Torr ($p < 0.05$). Values are means ± SEM, N = 5-20.

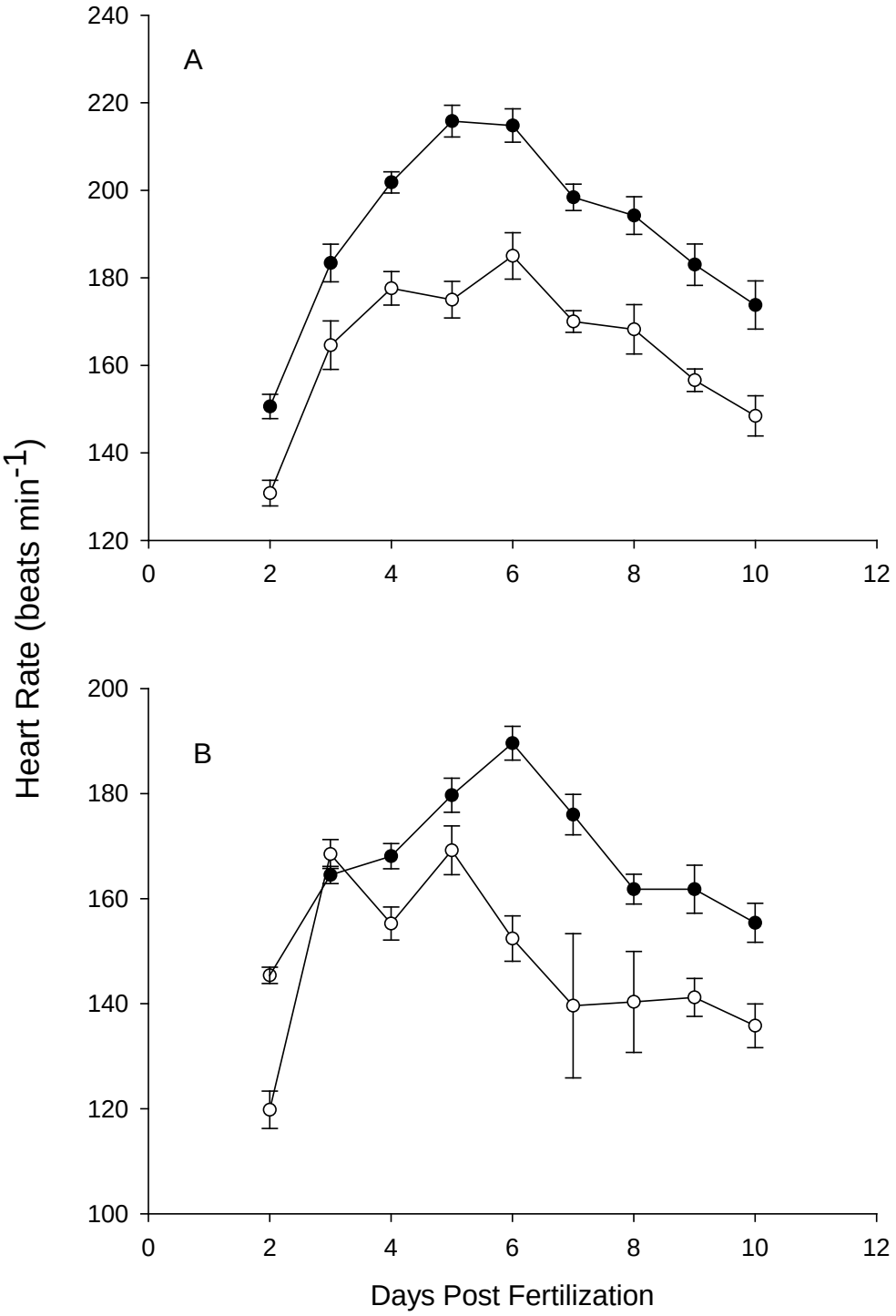

A
B
Heart Rate (beats min-1)
Days Post Fertilization
240
220
200
180
160
140
120
100
0
2
4
6
8
10
12

Figure 2.4

Figure 2.5

Heart rate (beats min^{-1}) of 4 days post fertilization (dpf) zebrafish larvae deficient in expression of the M_2 muscarinic receptor by morpholino injection versus sham injected fish reared in normoxic (PO_2 = 150 Torr) and hypoxic (PO_2 = 40 or 30 Torr) conditions. * indicates significant difference from normoxic value. + indicates significant difference between sham and M_2 morphant larvae at PO_2 = 30 Torr ($p < 0.05$). Values are mean + SEM, N = 9-10.

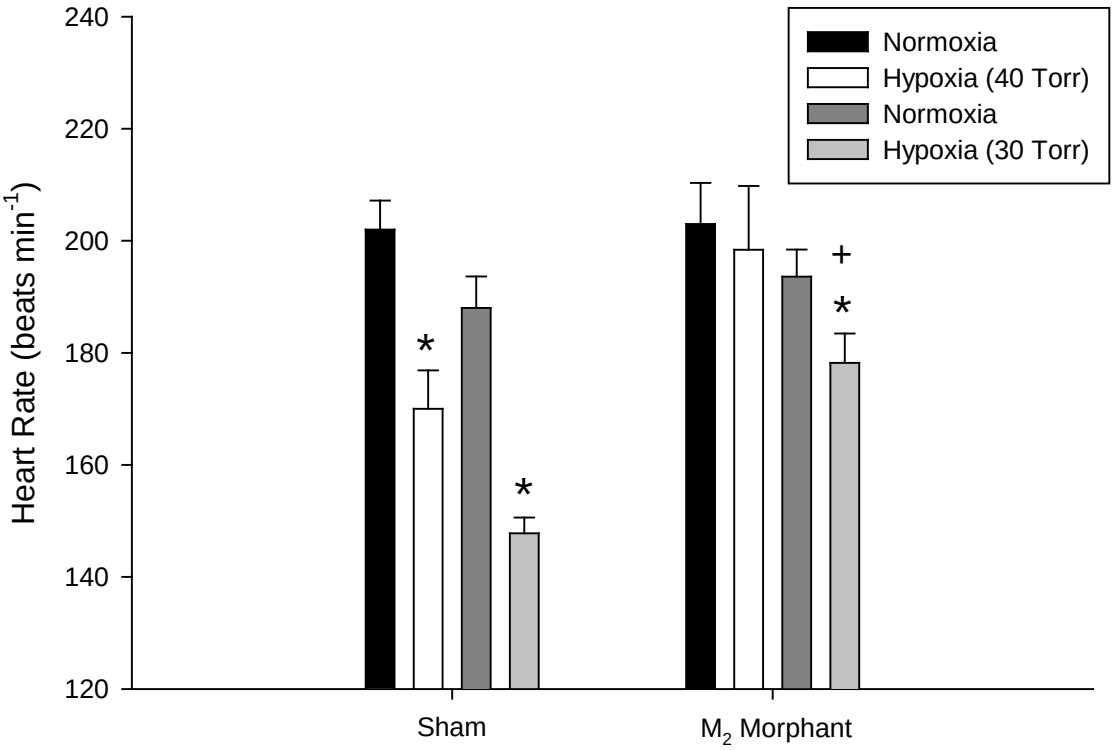

240
220
200
180
160
140
120
Heart Rate (beats min-1)
Normoxia
Hypoxia (40 Torr)
Normoxia
Hypoxia (30 Torr)
*
*
+
*
Sham
M2 Morphant

Figure 2.5

Figure 2.6

Change in mRNA expression of zebrafish β-adrenergic receptors (*β1AR*, *β2aAR*, and *β2bAR*) and the M_2 muscarinic receptor in zebrafish larvae reared in two different levels of hypoxia. (A) PO_2 = 40 Torr and (B) PO_2 = 30 Torr. All samples were normalized to the expression of 18S ribosomal RNA and values represent the fold change in expression relative to normoxic (PO_2 = 150 Torr) controls. * indicates significant difference from 1 ($p < 0.05$). Values are mean + SEM, N = 4.

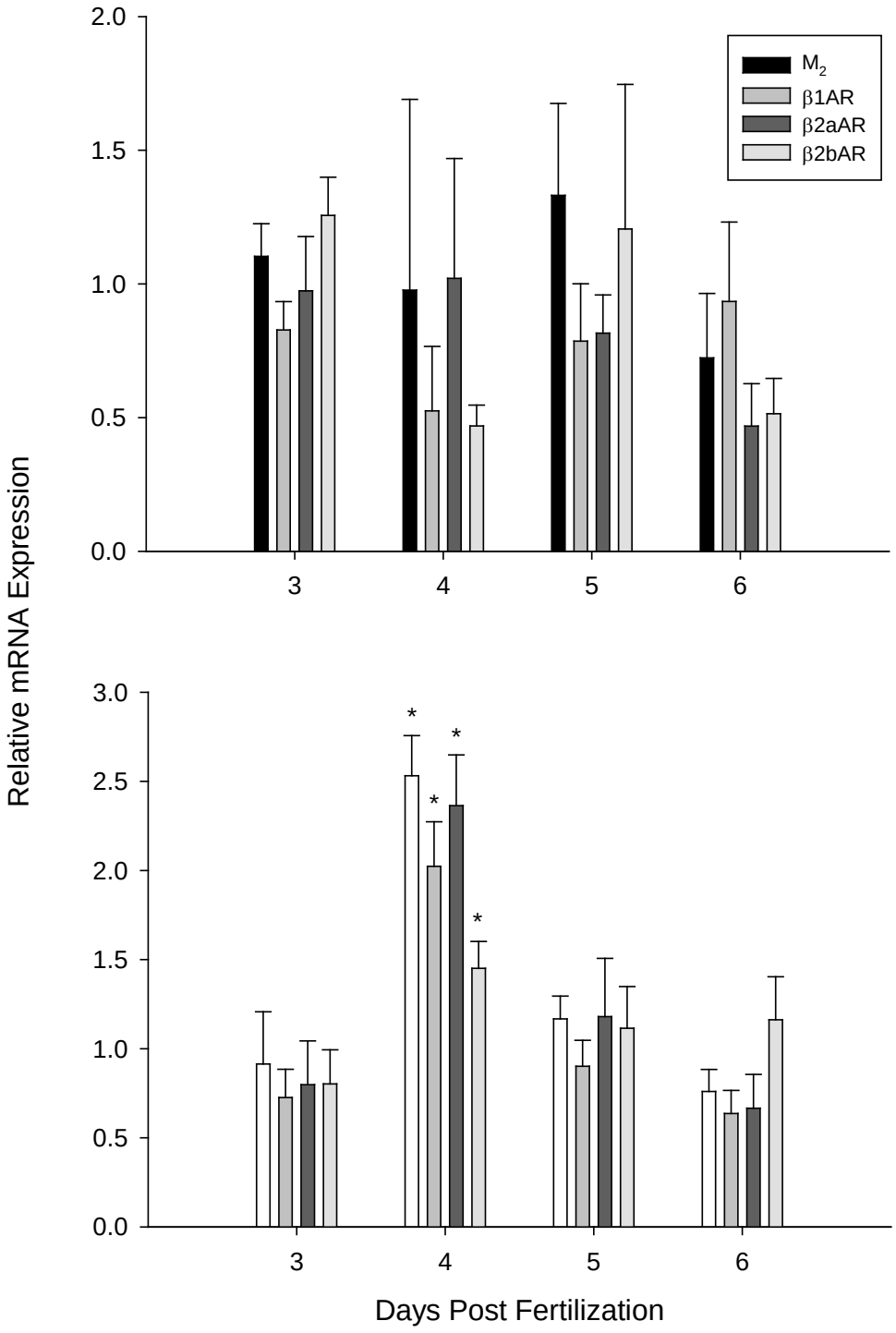

2.0
1.5
1.0
0.5
0.0
M2
β1AR
β2aAR
β2bAR
3
4
5
6
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
3
4
5
6
Relative mRNA Expression
Days Post Fertilization

Figure 2.6

Figure 2.7

Localized M_2 muscarinic receptor mRNA expression in 4 dpf zebrafish larvae reared from conception in normoxia (A, PO_2 = 150 Torr) or hypoxia (B, PO_2 = 40 Torr) by *in situ* hybridization. Heart region of each larva is indicated by the black arrows. Scale bar = 250 µm.

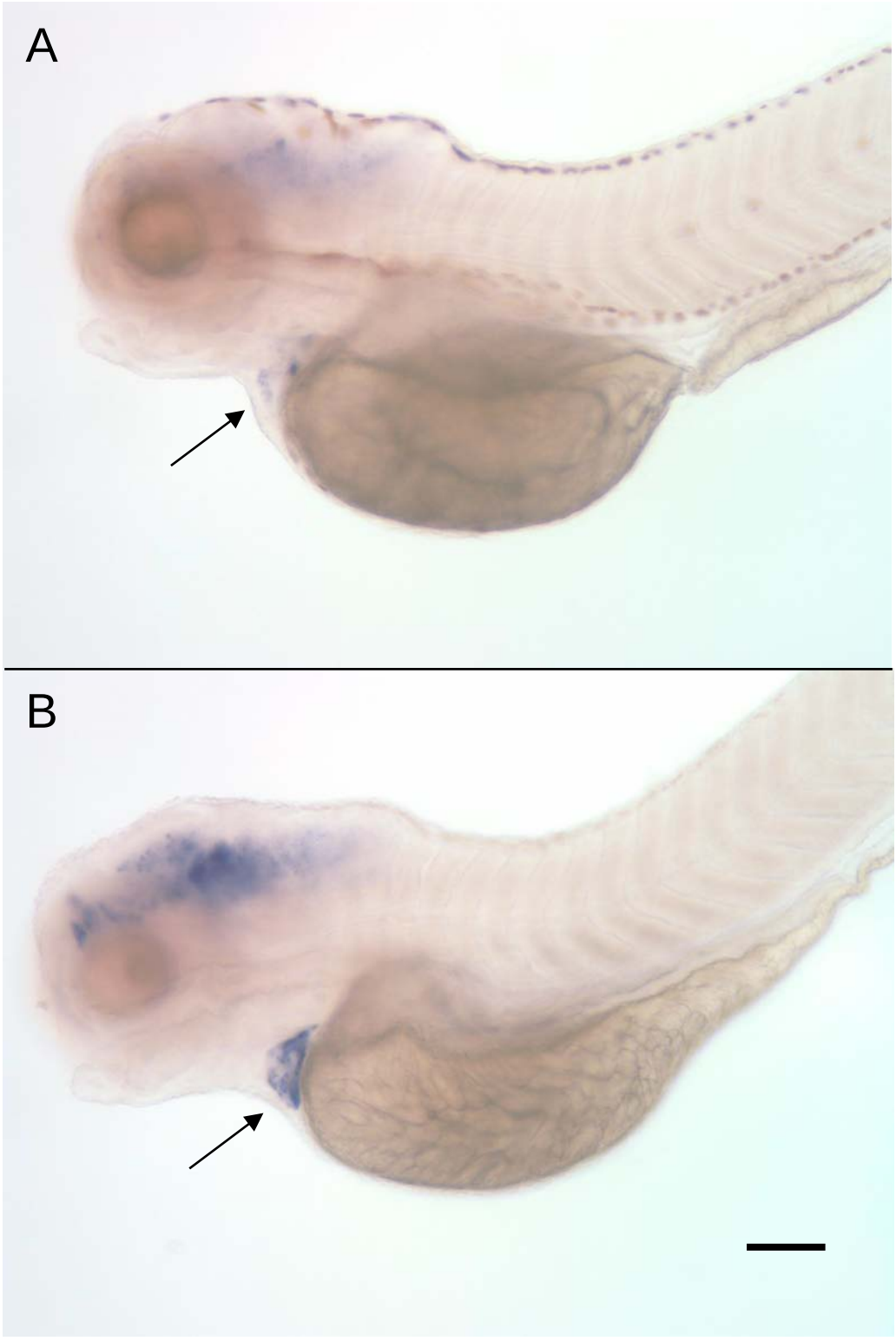


Figure 2.7

Figure 2.8

Fold change in mRNA expression of zebrafish β-adrenergic receptors (*β1AR, β2aAR* and *β2bAR*) and the M_2 muscarinic receptor in M_2 morphant zebrafish larvae relative to sham injected control fish. All samples are normalized to the expression of 18S ribosomal RNA. * indicates significant difference from 1, $p < 0.05$. Values are mean + SEM, N = 4.

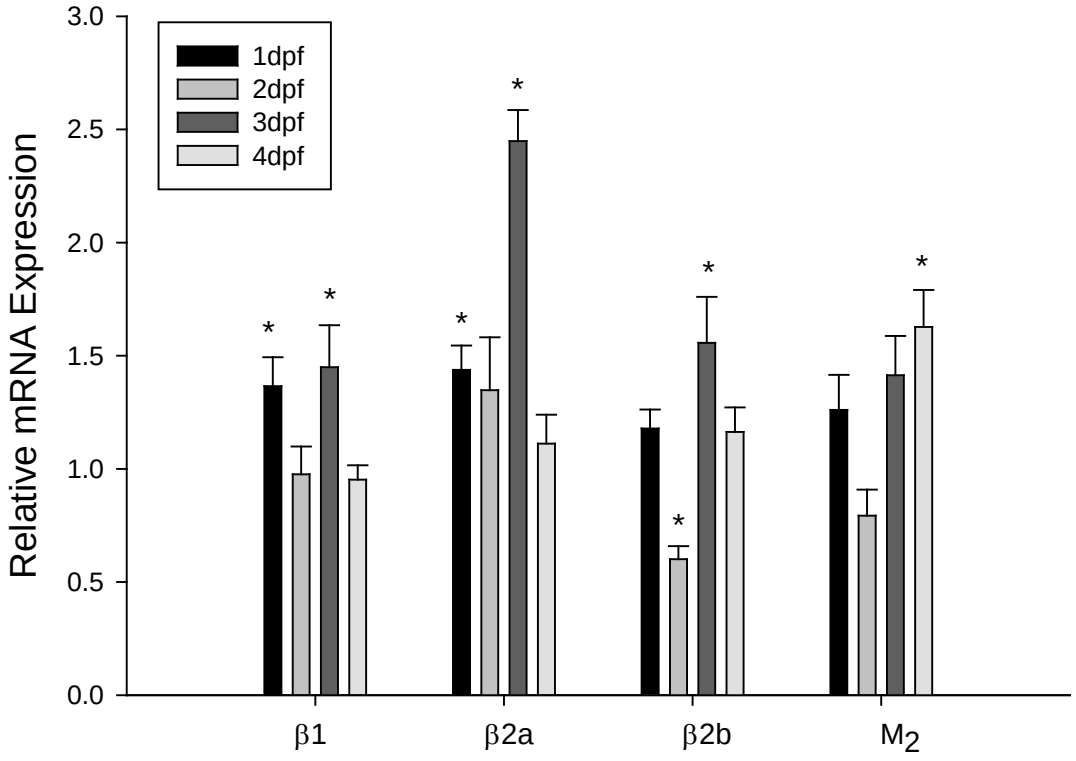

Relative mRNA Expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1dpf
2dpf
3dpf
4dpf
*
β1
β2a
β2b
M2

Figure 2.8

Figure 2.9

Change in heart rate (% of values in fish which were anaesthetized only) of 4 days post fertilization M_2 muscarinic receptor morphant zebrafish larvae (and sham injected controls) exposed to the general adrenergic receptor agonist norepinephrine (NE), the β-adrenergic receptor antagonist propranolol, and the β2-adrenergic receptor antagonist procaterol. Values are mean + SEM, N = 8-10. * indicates significant difference between absolute heart rates for each group before and after exposure to the drug (p <0.05). Absolute heart rate values (beats min^{-1}) for anaesthetized sham injected fish in the four treatment groups are 183, 183, 182, and 193, respectively in the order presented in the figure. In M_2 morphants, anesthetized heart rates are 184, 185, 178, and 196 respectively.

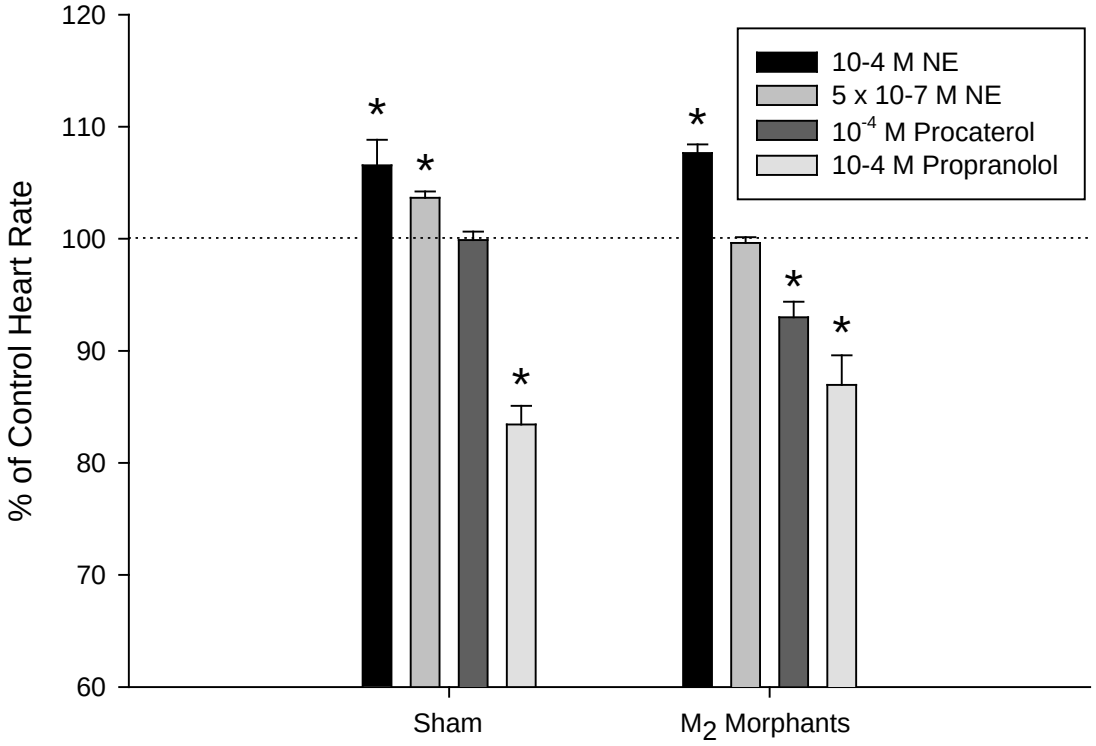

120
110
100
90
80
70
60
% of Control Heart Rate
10-4 M NE
5 x 10-7 M NE
10-4 M Procaterol
10-4 M Propranolol
*
*
*
*
*
*
Sham
M2 Morphants

Figure 2.9

Table 2.1

Accession numbers, primer sequences and expected product sizes for the genes examined in this study, including amplification efficiencies and R^2 values for standard curves generated for all real-time PCR reactions. M_2 RP refers to the M_2 receptor riboprobe.

	GenBank Accession #	Fwd Primer Sequence (5'- 3')	Rev Primer Sequence (5'-3')	Product Size (bp)	Amplification Efficiency (%)	R^2
β 1AR	XM_680208.2	GGGTTACTGGTGGTGCCATT	GCGTGACGCAAAGTACATC	110	96.2	0.989
β 2aAR	XR_029238.1	GCTTCCAGCGTCTTCAGAAC	CCGAAGGGAATCACTACCAA	91	89.4	0.989
β 2bAR	XM_695628.3	CTCGTTCCTACCCATCCACA	ATGACCAGCGGGATGTAGAA	150	103.0	0.991
M_2	NM_178301	GGTGCCAGAGAAGGAGTGCT	CGGGAAACCTGCCAGTAGAG	123	104.9	0.988
18S *	N/A	GGCGGCGTTATTCCCATGACC	GGTGGTGCCCTTCCGTCAATTC	117	98.3	0.997
M_2 RP	NM_178301	CGAAGATGGCAGGAATGATGAT	CCCTCGTCAGCAGCAGCCAATG	1049		

* Primer sequences for zebrafish 18S ribosomal subunit as per Esbaugh et al. (2009).

Discussion

The results of the present study show that, in zebrafish larvae, the development of bradycardia in response to chronic hypoxia is mediated by the cholinergic activation of the cardiac M_2 receptor subtype. It has been demonstrated previously that treating fish with atropine blocks the development of hypoxic bradycardia, suggesting that changes in vagal tone are critical in this response (see Introduction for references). The M_2 subtype is the predominant muscarinic receptor found in the hearts of mammals and presumably fish, suggesting that this particular subtype likely mediates cardiac vagal tone. It should be noted that the distribution of the other muscarinic receptor subtypes has not been determined in zebrafish. It would be interesting to learn if in zebrafish, as in rat (Myslivecek et al., 2008), other subtypes such as the M_1 receptor are expressed significantly in the heart. Loss of function studies for other muscarinic receptors, or adrenergic receptors, could be particularly useful in determining what causes variability in the response of the heart to different levels of hypoxia. In the present study, M_2 knockdown completely abolishes hypoxic bradycardia at PO_2 = 40 Torr, suggesting the change in heart rate is controlled exclusively by cardiac vagal tone. However, bradycardia is still present in M_2 morphants reared at PO_2 = 30 Torr, although it is significantly attenuated compared to hypoxic sham injected fish (Figure 2.5). It would be interesting to know what other receptors may be involved in regulating cardiac function at this lower level of environmental oxygen.

The use of morpholinos as a method of gene silencing is a powerful tool in determining gene function, and it is critical to use appropriate controls in these experiments (for review see Eisen and Smith, 2008). The M_2 morpholino used in the present study has been used previously by Hsieh and Liao (2002) who reported the function of this receptor in regulating heart rate in zebrafish larvae. A physiological assay was used here and by Hsieh

and Liao (2002) to demonstrate the effectiveness of the M_2 morpholino. In this assay, knockdown larvae do not experience bradycardia when exposed to the cholinergic antagonist carbachol. Thus, I am confident that the morpholino effectively causes loss of M_2 receptor function in the present experiments.

The mRNA expression of the β1- and β2-adrenergic receptor subtypes and the M_2 receptor was investigated in whole larvae reared at two different levels of hypoxia from 3 to 6 dpf. The only significant changes occurred at the lowest PO_2 studied (30 Torr), where the expression of *M_2*, *$\beta 1AR$*, *$\beta 2aAR$*, and *$\beta 2bAR$* were all significantly increased at 4 dpf relative to normoxic fish (Figure 2.6). Because the development of hypoxic bradycardia is mediated by the cardioinhibitory M_2 receptor, an increase of *M_2* mRNA expression and also receptor density would appear to be a physiologically appropriate response. In the rat, for example, muscarinic receptor density increases in the left ventricle with chronic hypoxic exposure (Kacimi et al., 1993). Why β-adrenergic receptor gene expression would be concurrently increased is unclear. Gamperl et al. (1998) suggest that fish myocardial β-adrenergic receptors may be more resistant to hypoxic downregulation to maintain adrenergic responsiveness in the event that cardiac output needs to be increased. Indeed, cardiac β-adrenergic receptor density is unchanged in rainbow trout exposed to 6 h of hypoxia (PO_2 ~ 55 Torr; Gamperl et al., 1998). Whether or not those results can be related to an increase in β-adrenergic receptor gene expression in order to help maintain high protein levels is unclear. Interestingly, the expression of *$\beta 1AR$* did not change in whole zebrafish embryos (2 dpf) exposed to either 12 h (Ton et al., 2002) or 24 h (Ton et al., 2003) of hypoxia (5% environmental O_2) as determined by microarray analysis. In this case, the duration of hypoxic exposure may not have been adequate to induce changes in mRNA expression, or the embryos were simply not old enough to elicit a response. Cultured neonatal rat myocytes exposed to 1% environmental O_2 for 72 h

show a significant increase in *β1AR* mRNA (Li et al., 1996). However, *β1AR* mRNA levels were significantly decreased in the left ventricles of hypoxic lambs (Bernstein et al., 1992). These discrepancies may be attributed to species differences, *in vivo* versus *in vitro* effects, as well as differences in the duration of hypoxia exposure. The relatively small fold change in expression of all genes seen at 4 dpf reared in PO_2 = 30 Torr should probably be viewed with some caution because only at this time period was there a slight decrease in the Ct for the reference gene, 18S.

Although bradycardia is the typical response seen in adult fish exposed to hypoxia, the situation is less certain in zebrafish larvae. Bagatto et al. (2005) demonstrated a significant and prolonged bradycardia (as observed in the present study) in zebrafish larvae reared at a low PO_2 (approximately 20 Torr). However, in a similar experiment using a milder degree of hypoxia (PO_2 = 75 Torr), Jacob et al. (2002) reported that zebrafish larvae experienced significant tachycardia. The level of hypoxic exposure may be the defining factor that determines the cardiovascular response of these animals. Indeed, the actual physiological benefit of hypoxic bradycardia is not entirely clear, although it is hypothesized that slowing the circulation of blood can increase time for gas diffusion at the gills and in the myocardium (for review see Farrell, 2007). In zebrafish larvae, this would not be expected to be an issue, since they do not require convective oxygen transport until approximately 15 dpf (Jacob et al, 2002). However, it has recently been demonstrated that oxygen delivery by circulating haemoglobin may be more important at a low PO_2 (from 20 – 50 Torr) in zebrafish larvae as young as 7 dpf (Rombough and Drader, 2009). This suggests that hypoxic bradycardia may be more likely to occur in zebrafish larvae reared in lower PO_2s, when oxygen is more limiting.

It is important to note that mRNA expression for the β-adrenergic and M_2 muscarinic receptors was determined in whole hypoxic larvae, not just the heart. *In situ* hybridization results for M_2 expression indicate that expression of this gene is increasing in the heart and, apparently, the brain at 4 dpf (Figure 2.7). The high expression of M_2 in the brain and heart of larvae mirrors that seen in the adult zebrafish (Figure 2.2). Even though expression of M_2 is relatively low in other tissues, these make up a larger proportion of the whole larva, and therefore may mask small increases in mRNA expression in the heart and brain in the animals exposed to the PO_2 of 40 Torr, for example. Because β-adrenergic receptors are expressed in a variety of tissues where hypoxia could be having effects on these receptors (Perry et al., 1996; Manzl et al., 2002), it is conceivable that changes in expression in the whole larva due to hypoxic exposure are not necessarily representative of changes in the heart.

It was my hypothesis that β-adrenergic activity and/or tone would be decreased in the hearts of M_2 knockdown zebrafish larvae. While the present data do not suggest any change in sympathetic tone, there were marked differences in the reactivity of the heart to adrenergic receptor agonists. The heart rate of both sham injected larvae and M_2 morphants increased in a similar manner when exposed to a high concentration of NE (10^{-4} M; Figure 2.9). However, only the sham injected fish experienced significant tachycardia when exposed to a lower concentration (5×10^{-7} M; Figure 2.9). These data suggest that the hearts of M_2 knockdown zebrafish are less sensitive to adrenergic agonists. While no similar data currently exist for fish, $M_2^{-/-}$ mice show the opposite reaction to acute doses of the general β-adrenergic agonist isoproterenol; the heart appears to be more sensitive to β-adrenergic stimulation (LaCroix et al., 2008). One must be cautious in comparing these data because there are distinct inter-specific and developmental differences in the contribution of vagal tone to cardiac function.

Indeed, while parasympathetic activity may be critical for maintaining baseline heart rate in fish (e.g. Hsieh and Liao, 2002), it may not be so in mice (Lacroix et al., 2008).

An unexpected finding of the present study was that M_2 receptor knockdown unmasked a possible cardioinhibitory role of one or both β2ARs. The evidence for this was that the β2AR agonist, procaterol, reduced heart rate only in the M_2 knockdown fish. While β1ARs appear to couple exclusively to stimulatory G proteins (G_s), β2ARs couple to both G_s and G_i (inhibitory) proteins (for review see Xiang et al., 2003). This phenomenon was demonstrated in cultured cardiac myocytes from β1AR knockout mice (Devic et al., 2001). In that study, stimulating the β2ARs with isoproterenol caused an initial increase in the rate of contraction followed by a sustained decrease, indicating that the receptor couples first to a stimulatory pathway but then to an inhibitory one. Additional *in vivo* or *in vitro* studies are required to determine which of the β2ARs exert a cardioinhibitory role.

It is intriguing that the knockdown of the M_2 receptor affects both hypoxic bradycardia (Figure 2.5) and cardiac β-adrenergic activity (Figure 2.9) at 4 dpf even though there is no detectable muscarinic tone at this stage based on exposure to 10^{-4} M atropine (data not shown). The presence of the receptor in cardiac tissue is readily evident in this species at 4 dpf. Indeed, Hsieh and Liao (2002) showed *M_2* mRNA expression in the heart of 30 hpf (and up to 3 dpf) larvae by *in situ* hybridization, and the present study shows strong *M_2* mRNA expression in the heart at 4 dpf by the same method (Figure 2.7). Gene expression analysis in whole larvae by RT- (Hsieh and Liao, 2002) and real-time RT-PCR (Figure 2.1) shows that *M_2* expression is faintly detectable at 12 hpf and increases significantly by 1 dpf. Finally, exposure of zebrafish larvae to muscarinic receptor agonists such as carbachol (Hsieh and Liao, 2002; Bagatto, 2005; present study, Figure 2.3) and acetylcholine (Schwerte et al., 2006) show that the heart is reactive to these compounds as early as 3 dpf (Hsieh and Liao, 2002) or

as late as 5 dpf (Schwerte et al., 2006). To date, development of the cardiac vagus in zebrafish larvae has not been examined. Vagal innervation of gut is evident in zebrafish larvae as early as 3 dpf (Olsson et al., 2008). Because the cardiovascular system is one of the first to develop in zebrafish, it stands to reason that the cardiac vagus may develop at least as early as it would in the gut. Indeed, Hsieh and Liao (2002), reported a significantly higher resting heart rates in M_2 morphants versus control morphants at 3 dpf, suggesting a disruption in parasympathetic tone due to the absence of this receptor. While an increased heart rate in M_2 morphants was not observed in the present study, the observation that the hypoxic bradycardia is abolished or significantly attenuated in low oxygen environments in fish experiencing to M_2 receptor knockdown strongly supports the presence of vagal tone at this early stage.

The results of the present study strongly suggest that the M_2 muscarinic receptor plays a crucial role in the development of hypoxic bradycardia in the zebrafish larva, and that both hypoxia and loss of M_2 function lead to changes in the β-adrenergic system. Future research can use similar loss of function methods to look at the potential role of other muscarinic receptor subtypes in hypoxic bradycardia, or that of the cardiac β-adrenergic receptors, particularly at very low PO_2s. The loss of M_2 function in this species revealed a potential cardioinhibitory role for the β2AR subtype, for which the zebrafish has two distinct genes. These fish likely produce two distinct functional gene products for the β2AR receptor, the cardiovascular function of which remain unexplored. Indeed, if the β2AR can mediate a decrease in heart rate in the absence of an M_2 receptor, when does this function present itself, and are both isoforms implicated? Future studies, therefore, should assess the cardiovascular responses of β2aAR and β2bAR loss of function zebrafish larvae to hypoxia as well as the effects of simultaneous M_2/β2AR loss of function.

Acknowledgements

Funding for this project was provided by a National Science and Engineering Research Council (NSERC) of Canada grant to S.F.P. and a NSERC post-graduate scholarship to S.L.S.

CHAPTER 3.

In vivo and *in vitro* assessment of cardiac β-adrenergic receptors in larval zebrafish (*Danio rerio*)

Notes on Chapter

The present chapter has been prepared as a manuscript that was most recently submitted to the American Journal of Physiology, Regulatory, Integrative and Comparative Physiology as per the following citation:

Steele SL, Yang X, Debiais-Thibaud M, Schwerte T, Pelster B, Ekker M, Tiberi M and Perry SF. *In vivo* and *in vitro* assessment of cardiac β-adrenergic receptors in larval zebrafish (*Danio rerio*). Submitted manuscript.

The contribution of M. Debiais-Thibaud is represented in Figure 3.2. T. Schwerte and B. Pelster are collaborators at the University of Innsbruck, where measurements of heart rate, cardiac output, and stroke volume were made by S.L. Steele. M. Tiberi and X. Yang are collaborators from the Ottawa Hospital Research Insitute. Their contribution is represented in Figures 3.8, 3.9, 3.10 and in Tables 3.2 and 3.3.

Abstract

β-adrenergic receptors (βARs) are critical for maintaining the rate and force of cardiac muscle contraction in vertebrates. Zebrafish (*Danio rerio*) have one β1AR gene (*β1AR*) and two β2AR genes (*β2aAR* and *β2bAR*). I examined the roles of these receptors in larval zebrafish *in vivo* by assessing the impact of translational gene knockdown on cardiac function. Zebrafish larvae lacking β1AR expression by morpholino knockdown displayed lower heart rates than control fish, whereas larvae deficient in both β2aAR and β2bAR expression exhibited significantly higher heart rates. These results suggested a potential inhibitory role for one or both β2AR genes. By using cultured HEK293 cells transfected with zebrafish βARs, it was demonstrated that stimulation with epinephrine or procaterol (a β2AR agonist), resulted in an increase in intracellular cAMP levels in cells expressing any of the three zebrafish βARs. In comparison to their human βAR counterparts, zfβ2aAR expressed in HEK293 cells, appeared to exhibit a unique binding affinity profile for adrenergic ligands. Specifically, zfβ2aAR had a high binding affinity for phenylephrine, a classical α-adrenergic receptor agonist. The zebrafish receptors also had distinct ligand binding affinities for adrenergic agonists when compared to human βARs in culture, with zfβ2aAR being the most distinct compared to human β2AR and zfβ2bAR. Overall, this study provides insight into the function and evolution of both fish and mammalian β-adrenergic receptors.

Introduction

Adrenergic receptors (adrenoreceptors, ARs) are G-protein coupled receptors that transduce the cellular effects of epinephrine and norepinephrine and are expressed ubiquitously in vertebrate tissues (e.g. Cavalli et al., 1997, Tanoue et al., 2002). The β1AR subtype is traditionally classified as the "cardiac" βAR because stimulation of β1AR *in vivo* stimulates heart rate and contractility (Lands et al., 1967b). β2ARs were originally thought to be restricted to the lungs and peripheral vasculature (Lands et al., 1967a); however later studies have demonstrated significant expression of β2ARs in the mammalian heart. Current models of heart function show that both of these βAR subtypes play critical roles in regulating the rate (chronotropy) and force (inotropy) of heart contraction (e.g. Bernstein, 2002; Brodde et al., 2008).

When considering heart rate, the β1AR appears to be exclusively stimulatory, whereas the role of the β2AR is less clear. Resting heart rate in $\beta 1AR^{-/-}$ and $\beta 1AR^{-/-}\beta 2AR^{-/-}$ mice was lower compared to wildtypes, while β2AR loss of function alone had no effect (Ecker et al., 2006). Also, cardiac myocytes isolated from $\beta 1AR^{-/-}$ mice and exposed to isoproterenol showed an initial increase followed by a sustained decrease in contraction rate compared to baseline levels (Devic et al., 2001), suggesting a dual stimulatory/inhibitory role for the remaining β2AR receptors in these cells. An inhibitory role for cardiac β2AR was recently proposed for larval zebrafish experiencing translational knockdown of M_2 muscarinic receptors because exposure to procaterol (a β2AR agonist) caused a lowering of heart rate (Steele et al., 2009). Because zebrafish have two distinct β2AR receptors (herein termed β2aAR and β2bAR; Wang et al., 2009), it is not clear as to whether one or both of the β2ARs is contributing an inhibitory influence on heart function.

Numerous studies have assessed the contribution of adrenergic tone in maintaining resting heart rate in adult fish (for references see Mendonça and Gamperl, 2009), however, considerably less is known about larval fish. Larval zebrafish begin to exhibit a chronotropic response to adrenergic agonists at 4 (Schwerte et al., 2006) or 6 days post fertilization (dpf; Bagatto, 2005), and first demonstrate adrenergic tone at 5 dpf (Schwerte et al., 2006). Beyond this, the role of specific βAR subtypes in regulating cardiac function and development beyond the measurement of cardiac frequency in zebrafish has yet to be explored. Both β1- and β2AR subtypes are linked to stimulatory G proteins (Gs), which increase adenylyl cyclase activity yielding higher levels of cAMP and thus increasing cardiac chronotropy and inotropy. Whereas the β1AR is exclusively linked to Gs proteins, a growing body of evidence suggests that the β2 subtype also associates with inhibitory Gi proteins and can thereby inhibit contraction of heart cells (e.g. Xiao et al., 1999; Bernstein, 2002). This dual coupling of the β2AR might help explain why this receptor is not equally involved in Gs mediated cAMP accumulation in heart cells in some species, compared to the β1AR (for references see Xiao et al., 1999; Xiao et al., 2001). Molecular and pharmacological experiments have shown that there is significant expression of βARs in the fish heart, and that fish hearts are responsive to classic βAR ligands (Nickerson et al., 2001; Kawasaki et al., 2008; Mendonça and Gamperl, 2009; Steele et al., 2009). Some studies on fish βARs have also shown that they can have unexpected affinity for (presumed) subtype specific agonists. Phenylephrine, a classic α-adrenergic agonist, had similar competitive binding characteristics as norepinephrine for β-adrenergic receptors in catfish liver (Fabbri et al., 1992). Also, β3bARs in the red blood cells of rainbow trout have distinct β2AR-like binding characteristics based on their affinity for classic β2AR ligands (Nickerson et al., 2003). To date, the ligand binding affinities of

zebrafish βARs, and their ability to initiate intracellular cAMP accumulation via agonist stimulation, have yet to be explored.

The first goal of this study was to determine the developmental pattern of the cardiac-type β-adrenergic receptor expression in zebrafish and distinguish their role in regulating heart function in early life. Developmental mRNA expression of the classic cardiac-type β-adrenergic receptors (*β1AR*, *β2aAR*, *β2bAR*) was determined using semi quantitative real-time PCR and qualitative *in situ* hybridization. Zebrafish larvae lacking expression of β1AR, β2aAR, and β2bAR either alone or in combination were generated by translational knockdown using antisense oligonucleotide morpholinos. Microscopic imaging techniques were used to determine heart rate, stroke volume and cardiac output in larvae at 4 dpf. Larvae were also exposed to a variety of adrenergic ligands to determine any heart rate and cardiac output changes related to agonist and antagonist exposure. A second goal was to characterize the affinity of each of the zebrafish cardiac-type β-adrenergic receptors for classic adrenergic ligands, as well as the ability of each receptor type to associate with Gs proteins within the cell. For this, the zebrafish β1AR and β2ARs were transiently expressed in HEK293 cells in culture to determine their affinity for common adrenergic agonists as well as their ability to initiate cAMP production within these cells.

Materials and Methods

Zebrafish Husbandry and Culture – University of Ottawa

Adult zebrafish were maintained in 10 l acrylic tanks in multi-rack aquatic housing systems (Aquatic Habitats, Apopka, FL USA). All tanks were supplied with well aerated dechloraminated City of Ottawa tap water at 28^{o}C (for ion composition refer to Perry and Vermette, 1987). Fish were maintained under a 14h:10h light: dark cycle and spawning occurred daily at the beginning of the light cycle. To obtain embryos, breeder tanks (1 l; Aquatic Habitats, Apopka, FL USA) were placed in each 10 l tank prior to spawning and collected after spawning was allowed to proceed for at least 15 min. All experiments were performed in accordance with University of Ottawa animal care guidelines in accordance with those of the Canadian Council of Animal Care (CCAC).

Zebrafish Husbandry and Culture – University of Innsbruck

Adult zebrafish (Tübingen line) were housed in small aquaria at 28°C (Schwarz Aquarium Systems, Germany). Fish were maintained under a 14h:10h light: dark cycle and spawning occurred daily at the beginning of the light cycle. To obtain embryos, 8 – 10 random pair wise crossings were established at the beginning of the spawning period in 2 l breeding tanks. Embryos were collected from each tank every 15 – 20 min and the clutches were pooled. These experiments were performed in accordance with the animal ethics permission GZ 66.008/4-BrGT/2004 of the Austrian Bundesministerium für Bildung, Wissenschaft und Kultur.

Injection of β-adrenergic Receptor Morpholinos

Antisense morpholino oligonucleotides (conjugated to the green fluorescent tag carboxyfluorescein) were designed to block translation of β1AR, β2aAR, or β2bAR and are described in Table 3.4. For all experiments, an injection volume of approximately 1 nl per embryo was used. All working stocks of morpholino were diluted prior to injection in 1x Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES (pH = 7.6)], and 0.05% phenol red (for visualization of the injection volume). Working concentrations of the morpholino solutions were 4 ng nl^{-1} for single knockdown of β1AR and β2aAR, and 3 ng nl^{-1} for β2bAR. For dual knockdowns, a working concentration of either 8 ng nl^{-1} or 7 ng nl^{-1} were created by combining these concentrations. Matching concentrations of a standard control morpholino (for sequence see Table 3.4; Gene Tools, LLC, Philomath, OR USA) were used in both the single and dual knockdown experiments. Injections were performed using either a Narishige IM 300 Microinjector system in Ottawa (Narishige International USA Inc, Long Island NY, USA) or a pneumatic picopump (World Precision Instruments, Berlin, Germany) in Innsbruck. After injection, embryos were placed in 30 ml petri dishes containing E3 medium with 0.03‰ ethylene blue and incubated at 28°C.

To test for the sequence binding specificity of the β1AR, β2aAR, and β2bAR morpholinos, *in vitro*-synthesized fusion constructs were made in which the β1AR, β2aAR, and β2bAR morpholino target sequences were separately introduced upstream of and in frame with the red fluorescent protein dTomato (Shaner et al., 2004) coding sequence. Each of these constructs was cloned in the forward direction into a pCS2+ expression vector. Constructs were then amplified from these plasmids by PCR using SP6 and T3 primers (IDT, Coralville, IA USA), run on a 0.8% native agarose gel, and purified by gel extraction (Sigma-Aldrich Inc., St. Louis MO, USA). Capped mRNAs were synthesized from each purified PCR

product using a mMESSAGE mMACHINE® RNA transcription kit (AM1340; Ambion Inc, Austin, TX USA) as per manufacturers protocol. Embryos were injected with each dTomato mRNA construct individually (100 pg nl^{-1}) or together with the corresponding morpholino (4 ng nl^{-1}). To test for cross-reactivity of the β2 morpholinos, co-injections were also performed with the β2aAR morpholino/β2bAR dTomato mRNA, and *vice versa*.

Heart Rate Measurements – University of Ottawa

For baseline heart rate measurements, 4 dpf larvae were placed individually in a small volume of 100 mg l^{-1} Tris buffered MS-222 (ethyl-3-aminobenzoate methanesulfonate salt, Sigma-Aldrich Inc., St. Louis MO, USA) at 28°C. After 3 min, the heart rate was measured by observing the embryo under a dissecting microscope and counting heart beats for 30 sec. Each larva was then placed in a fresh solution also containing 100 mg l^{-1} MS-222 and either 10^{-4} M epinephrine (general adrenergic receptor agonist), 10^{-4} M isoproterenol (βAR agonist), 10^{-4} M procaterol (β2AR agonist), or 10^{-4} M propranolol (βAR antagonist). These concentrations were chosen after trials with other concentrations to determine the dose required to produce the heart rate effects, and are in keeping with concentrations used in other studies on zebrafish larvae (e.g. Schwerte et al., 2006; Steele et al., 2009). After 10 min of exposure to these chemicals, heart rate was measured again. Heart rates in β1/β2aAR and β1/β2bAR morphants (Figure 3.6) was acquired this way, all other heart rates were measured as described below.

Heart Rate, Stroke Volume, and Cardiac Output Measurements – University of Innsbruck

Larvae (4 dpf) were individually anaesthetized in 100 mg l^{-1} Tris buffered MS-222 (ethyl-3-aminobenzoate methanesulfonate salt, Sigma-Aldrich Inc., St. Louis MO, USA) at

28°C. Once immobilized, larvae were embedded in a small volume of 2% low melting point agarose prepared in 100 mg l^{-1} MS-222. The animal was then covered in 1 ml of 100 mg l^{-1} MS-222 and placed in the temperature controlled stage (28°C) of an inverted microscope (Zeiss Axiovert 25, Zeiss Vienna, Austria). A digital high speed video camera (Basler A504k, Basler Ahrensburg, Germany) attached to the microscope and connected to a personal computer captured images of the larval ventricle (dimensions 240 x 240 pixels, 30 frames s^{-1}) under 40-fold magnification. Images from larvae were acquired for approximately 1 min prior to the addition of drugs to the surrounding media to obtain baseline (i.e. anaesthetized) values for heart rate, stroke volume (SV), and cardiac output (CO). 1 µl of a 10^{-1} M solution of either epinephrine, isoproterenol, procaterol, or propranolol was added to the 1 ml of solution bathing the fish and gently mixed to create a final concentration of 10^{-4} M for each treatment. After 10 min, images from each larval ventricle were acquired for 1 min to obtain the treatment values. Heart rate, SV, and CO were measured/calculated from the captured images as per Kopp et al. (2007).

Tissue Collection – Adults, Embryos and Larvae

All adult tissues and pooled samples of larvae were collected and stored prior to analysis as per Steele et al. (2009).

RNA Extraction, cDNA Synthesis, and Real-Time PCR

All RNA extraction and cDNA synthesis was performed as per Steele et al. (2009). For the current study, Brilliant II ™ SYBR Green Master Mix was used for all real-time PCR reactions. Otherwise, all real-time PCR (including standard curve validation and data analysis) were performed as per Steele et al. (2009).

Whole Mount in situ Hybridization

PCR products for *β1AR*, *β2aAR*, and *β2bAR* were amplified from adult heart cDNA using the primers listed in Table 3.4. From these PCR products, *in situ* RNA probes were developed as per Steele et al. (2009). All larvae were reared and fixed, and *in situ* hybridization was performed as per Steele et al. (2009).

Synthesis of Expression Constructs

Full length coding regions for *β1AR*, *β2aAR,* and *β2bAR* were amplified from adult zebrafish heart cDNA using primers listed in Table 3.4. All PCR products were run on a 0.8% native agarose gel and gel purified using a GenElute gel extraction kit (Sigma). Gel purified PCR products were ligated into pDrive cloning vector according to manufacturer's directions (231122; Qiagen Inc., Valencia CA USA). Several positive clones from each group were selected and sequenced to confirm orientation and correct reproduction of each sequence. One clone was then selected from each group and digested with Bam HI and Not I restriction enzymes according to manufacturers' protocols (Invitrogen Inc., Carlsbad CA USA). Gel purified restriction products were then ligated into pcDNA3 expression vector (Invitrogen Inc., Carlsbad CA USA) also digested with Bam HI and Not I to ensure proper ligation of the insert in the forward direction. Ligation was performed with T4 DNA Ligase according to manufacturer's directions (Fermentas International Inc., Burlington ON, Canada). Each ligation was transformed into Subcloning Efficiency DH5α cells (Invitrogen Inc., Carlsbad CA USA) and incubated on agar plates containing 50 μg ml^{-1} ampicillan. Positive colonies were grown overnight at 37^{o}C in 150 ml of LB media containing 50 μg ml^{-1} ampicillan. Plasmid DNA was purified from the resulting culture using a HiSpeed Plasmid Midi kit (12643; Qiagen Inc., Valencia CA USA) according to manufacturer's protocol. Human β1AR (EcoR

I) and β2AR (EcoR I and Sal I) in CMV-based expression vector pRK5 (Lattion et al., 1999) were generously provided by Dr. Susanna Cotecchia (University of Lausanne, Switzerland).

Cell Culture and Transfection

Human embryonic kidney 293 (HEK293) cells (CRL-1573; American Type Culture Collection, Manassas, VA) seeded in 100 mm dishes were grown in minimal essential medium (MEM; Invitrogen, Burlington, Ontario, Canada) with 10% heat-inactivated fetal bovine serum (FBS; PAA Laboratories Inc., Etobicoke, ON, Canada) and gentamicin (10 μg ml^{-1}; Invitrogen) at 37°C in a humidified 5% CO_2 environment. Cells (2.5×10^6 cells/dish) were transiently transfected with human (hβ1AR, hβ2AR) and zebrafish (zfβ1AR, zfβ2aAR, zfβ2bAR) receptors with a total of 5 μg DNA per 100-mm dish using a modified calcium phosphate procedure (Tumova et al., 2004). For radioligand binding studies, 5 μg of receptor plasmid DNA were employed per transfection dish. For whole cell cAMP studies, empty pCMV5 vector was added to normalize the total amount of DNA to 5 μg per 100-mm dish as the quantity of receptor DNA used was less than 5 μg to obtain submaximal receptor expression. HEK293 cells used in experiments were from 40 to 50 passages.

Crude Membrane Preparation

Transfected HEK293 cells were washed with phosphate-buffered saline (PBS), trypsinized, pooled into 150-mm dishes and incubated in 37°C in a 5% CO_2 environment for ~48 h prior to radioligand saturation studies. Crude membrane preparations from cells grown in 150-mm dishes were prepared by centrifugation washes as previously described (D'Aoust and Tiberi, 2010). Final pellets were homogenized using a Brinkman Polytron (17,000 rpm for 15 sec) in 3 ml of cold resuspension buffer (62.5 mM Tris-HCl, pH 7.4; 1.25 mM EDTA,

pH 8.0). A fraction of membrane preparations (0.6 ml) was used immediately for saturation studies and the remaining homogenates were frozen in liquid nitrogen, and stored at -80°C until used for competition studies.

Radioligand Binding Assays

Binding reactions were carried out with 100 µl of membrane preparations and 50 µl of [^{3}H]-dihydroalprenolol (DHA, 97-102 Ci mmol^{-1}; Perkin-Elmer, Boston, MA) in the absence or presence of "cold" competing drugs in a total volume of 500 µl of assay buffer (final in assays: 50 mM Tris-HCl, pH 7.4; 120 mM NaCl; 5 mM KCl; 4 mM $MgCl_2$; 1.5 mM $CaCl_2$; 1 mM EDTA, pH 8.0) at 20ºC for 1 h. For saturation studies, fresh membrane preparations were incubated with increasing concentrations of [^{3}H]-DHA (0.005-5 nM for hβ2AR and zfβ2bAR; 0.05-25 nM for hβ1AR, zfβ1AR and zfβ2aAR) in the absence or presence of 1 µM alprenolol hydrochloride (A8676) to delineate total and nonspecific binding, respectively. For competition studies, frozen membranes were thawed on ice and incubated with 50 µl of [^{3}H]-DHA (~0.5 nM for hβ2AR and zfβ2bAR; ~2.5 nM for hβ1AR, zfβ1AR and zfβ2AaR) and increasing concentrations of competing ligands dissolved in double distilled water (dobutamine hydrochloride (D0676), final in assays: 0.1 - 1000 µM; (-)-epinephrine (+)-bitartrate salt (E4395), final in assays: 0.1 - 1000 µM; (*R*)-(-)-isoproterenol (286303), final in assays: 0.1 - 100 µM; (±)-norepinephrine (+)-bitartrate salt (A0937), final in assays: 0.1 - 1000 µM; (*R*)-(-)-phenylephrine (P6126), final in assays: 0.1 - 1000 µM; procaterol hydrochloride (P9180); final in assays: 0.1 - 1000 µM). Drugs were from Sigma-Aldrich (Oakville, ON, Canada). Binding reactions were stopped by rapid filtration through glass fiber filters (GF/C, Whatman) and bound radioactivity was quantified by liquid scintillation counting (Beckman Counter, LS6500). Protein concentrations were measured using the Bio-Rad assay kit (Bio-

Rad Laboratories Inc, Mississauga, ON, Canada) with bovine serum albumin (BSA) as standard. Binding curves were analyzed using the non-linear curve-fitting program GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA) to calculate equilibrium dissociation constant (K_d, nM) and maximal binding capacity (Bmax, pmol mg^{-1} of membrane proteins) of [^{3}H]-DHA (saturation studies), and equilibrium dissociation constant of unlabelled adrenergic drugs (K_i, nM) at [^{3}H]-DHA-labeled receptors (competition studies). Affinity ratios were calculated by dividing the ligand affinity of zfβ1AR and zfβ2AR by that measured with the same ligand at hβ1AR and hβ2AR, respectively. Affinities measured with different ligands at hβ2AR and zfβ2AR were divided by the corresponding ligand affinity of hβ1AR and zfβ1AR, respectively. Representative saturation and competitive binding curves (for zfβ2bAR) are provided in Figure 3.8.

Whole Cell cAMP Assays

Transfected HEK293 cells were seeded in 12-well plates and cultured in MEM with 10% FBS (v/v) and gentamicin (10 μg ml^{-1}) for 24 h. Media was then removed and cells were cultured in labeling MEM containing 5% FBS (v/v), gentamicin (10 μg ml^{-1}) and [^{3}H]-adenine (1 μCi ml^{-1}) overnight. The next day, labeling medium was aspirated and cells were incubated with 1 ml of 20 mM HEPES-buffered MEM containing 1 mM isobutylmethylxanthine (phosphodiesterase inhibitor; Sigma-Aldrich) in the absence (0.1% (v/v) ethanol) or presence of adrenergic drugs dissolved in double distilled water (final in assays: 100 μM epinephrine, 1 μM procaterol) or ethanol (final in assays: 10 μM (*S*)-(-)-propranolol hydrochloride; Sigma-Aldrich) at 37ºC for 30 min. Following the incubation period, plates were put on ice, medium was aspirated and 1 ml of lysis solution (2.5% (v/v) perchloric acid, 0.1 mM cAMP and [^{14}C]-cAMP (~3.3 nCi, 9000-11,000 dpm)) added to each well. Cells lysed for 30 min at 4°C and

lysates transferred to tubes containing 0.1 ml of a neutralizing solution (4.2 M KOH), vortexed and clarified using low-speed centrifugation (1500 rpm, 15 min) at 4°C. [^{3}H]-cAMP in supernatants was purified by sequential chromatography columns using Dowex AG 50W-4X resin (Bio-Rad Laboratories Inc, Mississauga, ON, Canada) and alumina N Super I (MP Biomedicals Canada, Montréal, Québec, Canada) as described before (Johnson et al., 1994). [^{3}H]-cAMP levels (CA) over the total amount of intracellular [^{3}H]-adenine uptake (TU) was calculated and used as relative index of adenylyl cyclase activity (expressed as CA/TU × 1000). Receptor expression (Bmax) was determined using a saturating concentration of [^{3}H]-DHA on fresh membranes prepared from one 100-mm dish of cells as described above.

Statistical Analyses

All statistical analyses presented in Table 3.1 and Figures 3.1, 3.3, 3.4, 3.5, and 3.6 were performed using SigmaStat statistical analysis software (v. 3.5; Systat Software Inc., San Jose CA, USA). In Table 3.1 and Figures 3.5 and 3.6, all comparisons between control (MS-222) and drug treated (adrenergic ligand in MS-222 solution) fish within morphant groups were made using paired Student's *t* test. All comparisons between morphant groups within the MS-222 treatment (i.e. control) were made using unpaired Student's *t* test. Real time PCR data in Figs 3.1, 3.3, and 3.4 were compared using a one way ANOVA on Ranks (due to failure of normality and equal variance test) with a Tukey post-hoc test.

Statistics for Geometric (Kd and Ki) and arithmetic (Bmax) means with the 95% lower and upper confidence intervals are used to report binding values (Tables 3.2 and 3.3). Arithmetic means (± standard error) are calculated to describe all other data in Figures 3.8 and 3.9. One-sample *t* test, unpaired *t* test and one-way ANOVA (followed by Newman-Keuls post test) were used to perform statistical analysis presented in Tables 3.2 and 3.3 and Figures 3.8

and 3.9. Statistical tests were performed using GraphPad Prism version 5.03 for Windows. All statistical analyses were two-sided and performed with a level of significance established at $p < 0.05$.

Results

Developmental and adult tissue mRNA expression of β1AR, β2aAR, β2bAR

The mRNA expression of *β1AR* was detectable as early as 1 hpf, and increased approximately 200-fold by 12 hpf and was significantly higher than expression at 1 hpf at 6 and 8 dpf (Figure 3.1A). The expression of *β2aAR* was detectable beginning at 6 hpf and was significantly higher than expression at this early stage at 4, 8, and 10 dpf (Figure 3.1B). *β2bAR* transcripts were below detection levels at 6 hpf, but expression levels were significantly higher than those at 1 hpf at 8 dpf (Figure 3.1B). Using *in situ* hybridization, the expression of all three transcripts in 3 dpf larvae was compared. *β1AR, β2aAR, β2bAR* mRNA all appeared to be expressed in the heart region, and also in different regions of the brain (Figure 3.2). When comparing adult tissue mRNA, all three transcripts were expressed in the heart. *β1AR* expression was highest in the brain and heart (Figure 3.3A), *β2aAR* in the gill (Figure 3.3B), while *β2bAR* expression was not significantly different than in the liver in any tissue (Figure 3.3C). When comparing between genes, the expression of *β1AR* was higher than that of *β2bAR* in eye, gill, brain, heart, and kidney, while it was significantly lower in the muscle (Figure 3.4). *β2aAR* expression was lower than *β2bAR* expression in the liver, whereas both *β1AR* and *β2aAR* expression were lower in the gut (Fig. 3.4).

βAR Morphants – General observations

β1AR morphants showed no observable physical abnormalities when compared to control morphant fish. In general, β1AR knockdown larvae had significantly lower heart rates than control larvae (Figure 3.5). β2aAR morphants were also physically similar to control morphants in all experiments. In one experiment (epinephrine), cardiac output was

significantly lower in β2aAR morphants versus controls, whereas in another experiment (propranolol), cardiac output was significantly higher (Table 3.1).

Some percentage of every β2bAR morpholino injected clutch (10-50%) had a phenotype different from control morphants. This phenotype generally presented itself as a curled body often accompanied by an enlarged pericardial cavity. Because of the differences in body shape which could lead to changes in blood flow, and in particular the enlarged pericardial cavity which can affect the proper function of the heart, only β2bAR morphants which appeared physically similar to control morphants were used in these experiments. In two out of four experiments, β2bAR morphants had significantly higher heart rates than control morphants (Figure 3.5).

Dual β2AR morphant larvae had consistently higher heart rates than control morphants in every experiment conducted (Figure 3.6). These morphants also occasionally had the β2bAR morphant phenotype described above, although it was not as common in the dual β2AR morphants (and these fish were not used in subsequent experiments).

βAR Morphants – Drug Treatments

Treatment of 4 dpf control morphants with 10^{-4} M epinephrine caused a significant increase in heart rate in all experiments (Figures 3.5 and 3.6). Epinephrine also caused a significant increase in heart rate in β2bAR and dual β1/β2aAR morphants, but not in any of the other βAR morphant groups (Figures 3.5 and 3.6). Stroke volume and cardiac output were not affected by epinephrine exposure in any of the morphants tested (Table 3.1). Isoproterenol exposure caused a significant increase in heart rate in all control groups (Figures 3.5 and 3.6). It also caused a significant increase in cardiac output in the β2a/β2bAR morphants (Table 3.1). Procaterol exposure caused a significant decrease in heart rate in 2 out of 6 groups of control

morphants, and a significant decrease in β1/β2aAR and β1/β2bAR morphants (Figures 3.5 and 3.6). Procaterol had the greatest overall effect on β2aAR morphants, in which heart rate, stroke volume, and cardiac output were all significantly higher upon exposure to the drug (Figures 3.5 and 3.6; Table 3.1). Propranolol caused highly significant decreases in heart rate in all control morphants and every βAR morphant group examined (Figures 3.5 and 3.6). Two out of four groups of control morphants showed a significant increase in stroke volume as a result of propranolol exposure, and one control group showed a significant increase in cardiac output (Table 3.1). β1AR and β2aAR morphants also had significantly lower cardiac outputs during propranolol exposure (Table 3.1).

Morpholino Controls - dTomato Red Fluorescent Protein

Similar results were found in all experiments testing the efficacy of the β1AR, β2aAR, β2bAR morpholinos in blocking dTomato protein synthesis, therefore only the results of the β2bAR experiment are presented here, as an example (Figure 3.7). Injection of the morpholino sequence tagged dTomato capped mRNAs alone caused the 4 dpf larvae to express the red fluorescent protein in all three cases (e.g. Figure 3.7). Co-injection of each of the capped mRNAs with its corresponding morpholino consistently blocked the production of the dTomato protein in all three cases, demonstrated by the lack of red fluorescence in these fish (e.g. Figure 3.7), suggesting that the morpholinos were binding specifically to their antisense sequence tagged to the 5' end of the dTomato mRNA and thereby blocked the production of this protein (therefore presumably the production of their native adrenergic receptor proteins). To test for cross-reactivity of the two β2AR morpholinos, each morpholino was injected with the dTomato mRNA of the opposite β2AR. As seen in Figure 3.7, injecting the β2aAR morpholino with the β2bAR dTomato mRNA did not block the synthesis of the red

fluorescent protein (Figure 3.7D). The same result was seen for the β2aAR dTomato protein/β2bAR morpholino combination, suggesting both β2AR morpholinos are efficiently binding to their own gene targets.

Zebrafish βAR Ligand Binding Properties

To gain insight into the pharmacological properties of zebrafish adrenergic receptors, transfected HEK293, a common cellular model for G-protein-coupled receptors, were used (Thomas and Smart, 2005). Notably, HEK293 cells express very low levels of endogenous human β-adrenergic receptors (Figure 3.10). Equilibrium dissociation constant (K_d) and maximal binding capacity (Bmax) of the non-selective β-adrenergic radioligand [^{3}H]-DHA in HEK293 cell membranes expressing human and zebrafish β-adrenergic receptors are reported in Table 3.2. The hβ1AR and hβ2AR were expressed at Bmax values in HEK293 cells using transfection conditions leading to maximal expression in this cellular system (5 μg/dish). Interestingly, zfβ2aAR exhibited lower Bmax when compared to hβ2AR and zfβ2bAR values. Likewise, zfβ1AR was expressed at significantly lower levels than hβ1AR. Importantly, the lower Bmax of zfβ1AR and zfβ2AR is not explained by their lower K_d for [^{3}H]-DHA as these values are indistinguishable from K_d of hβ1AR, which expressed at a higher Bmax. Moreover, it is unlikely that Bmax values measured here are linked to differences in receptor transfection efficiency in HEK293. Indeed, it has been previously shown that transfection efficiency in HEK293 cells remains similar regardless of the receptor expression construct used (Tumova et al., 2003). Alternatively, these data potentially suggest that zfβ1AR and zfβ2aAR have distinct determinates regulating their protein expression in cells relative to human adrenergic receptors and zfβ2bAR. Additionally, these data demonstrate that zebrafish β-adrenergic receptors bound to [^{3}H]-DHA with high affinity. However, zfβ2aAR displayed a ~3-fold lower affinity

for [^{3}H]-DHA in comparison with hβ2AR. The selectivity ratio of hβ2AR over hβ1AR was ~ 3-fold, a value that was recapitulated when comparing zfβ2bAR and zfβ1AR. Interestingly, no [^{3}H]-DHA selectivity was observed between zfβ1AR and zfβ2bAR. Overall, differences in affinity and selectivity of K_d values for [^{3}H]-DHA potentially suggest differences between ligand binding properties of human and zebrafish adrenergic receptors. This idea was further tested using competition studies with a wider range of adrenergic compounds.

Inhibitory constants (K_i) for different ligands (epinephrine, norepinephrine, isoproterenol, procaterol, phenylephrine and dobutamine) are shown in Table 3.3 along with affinity and selectivity ratios for these compounds (Figure 3.9). While K_i values of epinephrine, norepinephrine and procaterol were essentially unchanged between zfβ1AR and hβ1AR, affinities of other synthetic adrenergic drugs (isoproterenol, phenylephrine and dobutamine) were significantly different between these two receptors (Table 3.3, Figure 3.9). Additionally, ligand affinities were all significantly different between zfβ2aAR and hβ2AR. With the exception of epinephrine and procaterol having similar K_i values for zfβ2bAR and hβ2AR, other tested drugs displayed significant differences in their affinity for human and zebrafish β2-adrenergic receptors. Notably, procaterol, a selective β2-adrenergic agonist, had a ~10-fold lower affinity for zfβ2aAR relative to hβ2AR (Figure 3.9). Altogether, affinity and selectivity of adrenergic drugs suggest that zfβ1AR and zfβ2bAR are zebrafish orthologues of hβ1AR and hβ2AR, respectively. Meanwhile, zfβ2aAR may represent another zebrafish β2-adrenergic receptor isoform with distinct pharmacological properties (Table 3.3, Figure 3.9E). Indeed, zfβ2aAR strikingly displayed higher affinity for the α1-adrenergic receptor agonist, phenylephrine when compared with hβ2AR and zfβ2bAR. Overall, while ligand K_d and K_i values suggest that zfβ1AR, zfβ2aAR and zfβ2bAR behave pharmacologically as β-adrenergic receptors, the distinct drug selectivity points to important functional differences in the binding

mechanisms and ligand discrimination of human and zebrafish β1 and β2-adrenergic receptors.

Zebrafish βAR Gs Coupling Properties

The ability of different zebrafish β-adrenergic receptors expressed at similar and moderate levels to stimulate adenylyl cyclase (AC) activity was tested using epinephrine and procaterol. Epinephrine (100 μM) robustly stimulated AC activity in HEK293 cells overexpressing human and zebrafish β1ARs and β2ARs (~10-fold over basal) in comparison to mock-transfected cells (~2-fold over basal). In contrast to cells transfected with hβ2AR, procaterol (1 μM) partially simulated AC activity in HEK293 cells expressing hβ1AR relative to epinephrine exposure. Interestingly, procaterol behaved as a full agonist in cells expressing zfβ1AR (Figure 3.10C). In agreement with the idea that zfβ2bAR is the zebrafish orthologue of hβ2AR, procaterol evoked a strong and weak stimulation of AC activity in HEK293 cells expressing zfβ2bAR and zfβ2aAR, respectively. The lower intrinsic activity of procaterol relative to epinephrine in cells transfected with zfβ2aAR may be explained by the lower procaterol affinity for zfβ2aAR in comparison to zfβ2bAR and hβ2AR. Propranolol did not produce detectable AC activation in cells expressing human or zebrafish adrenergic receptors (Figure 3.10). Collectively, these whole cell cAMP studies suggest that zebrafish β-adrenergic receptors exhibit differences in procaterol-mediated AC activation.

Figure 3.1

Relative mRNA Expression of *β1AR* (A) and *β2aAR* (black bars) and *β2bAR* (grey bars; B) in zebrafish larvae at 1, 6, and 12 h post fertilization and 1 through 10 days post fertilization. Each gene of interest expression is standardized to 18S ribosomal RNA expression and is shown relative to its own level of expression at 1 hpf (for β1AR and β2bAR) or 6 hpf (for β2aAR). ND = not detectable. * indicates significant difference within gene from its own expression level at 1 hpf (for β1AR and β2bAR) or 6 hpf (for β2aAR).. Values = Mean + SEM. N = 4.

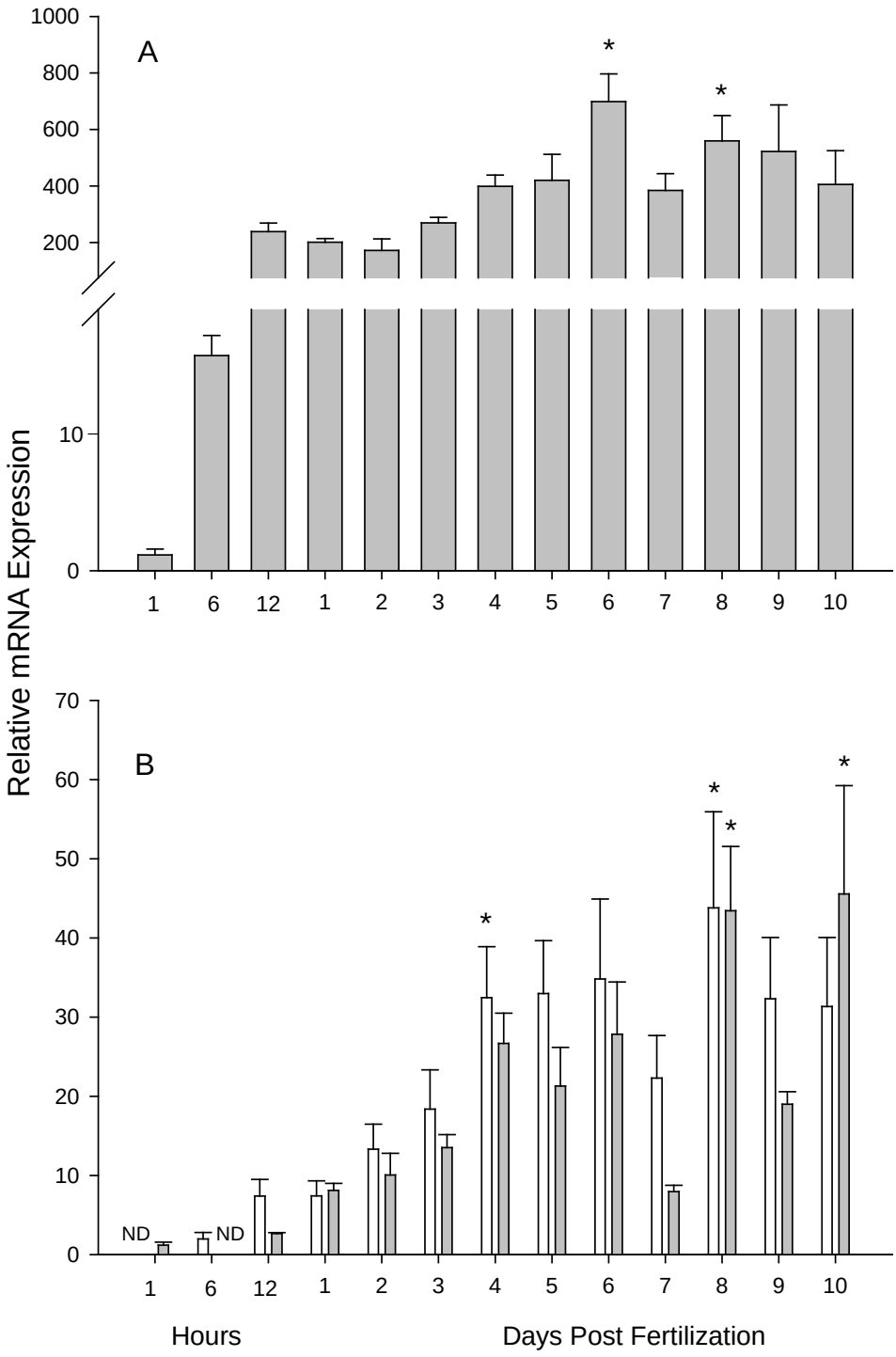

A
B
Relative mRNA Expression
1000
800
600
400
200
10
0
70
60
50
40
30
20
10
0
ND
ND
1
6
12
1
2
3
4
5
6
7
8
9
10
Hours
Days Post Fertilization

Figure 3.1

Figure 3.2

In situ hybridization of *β1AR* (A and B), *β2aAR* (C and D), and *β2bAR* (E and F) mRNA in 3 dpf larval zebrafish. Pictures on right (A, C, E) are lateral views and on the left (B, D, F) are ventral. Yolk sacs have been removed from larvae. Heart region is indicated by arrows. Bar = 250 μm.

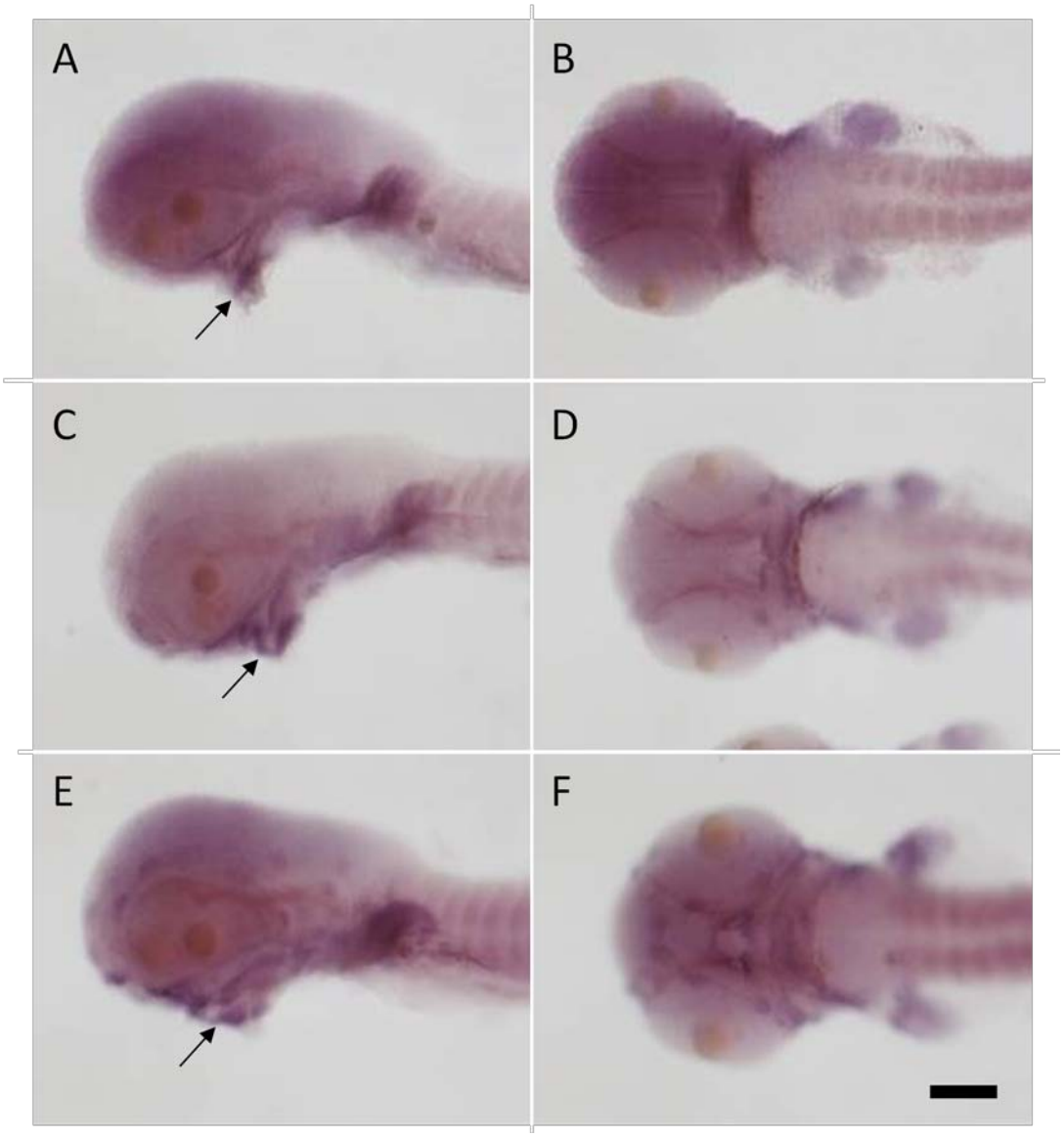


Figure 3.2

Figure 3.3

Relative mRNA Expression of *β1AR* (A), *β2aAR* (B), and *β2bAR* (C) in various adult zebrafish tissues. All values are standardized to 18S ribosomal RNA and the level in each tissue is expressed relative to mRNA expression in the liver within each gene of interest. * indicates significant difference from the level of expression in the liver within each gene of interest ($p < 0.05$). . Values = Mean + SE. N = 4.

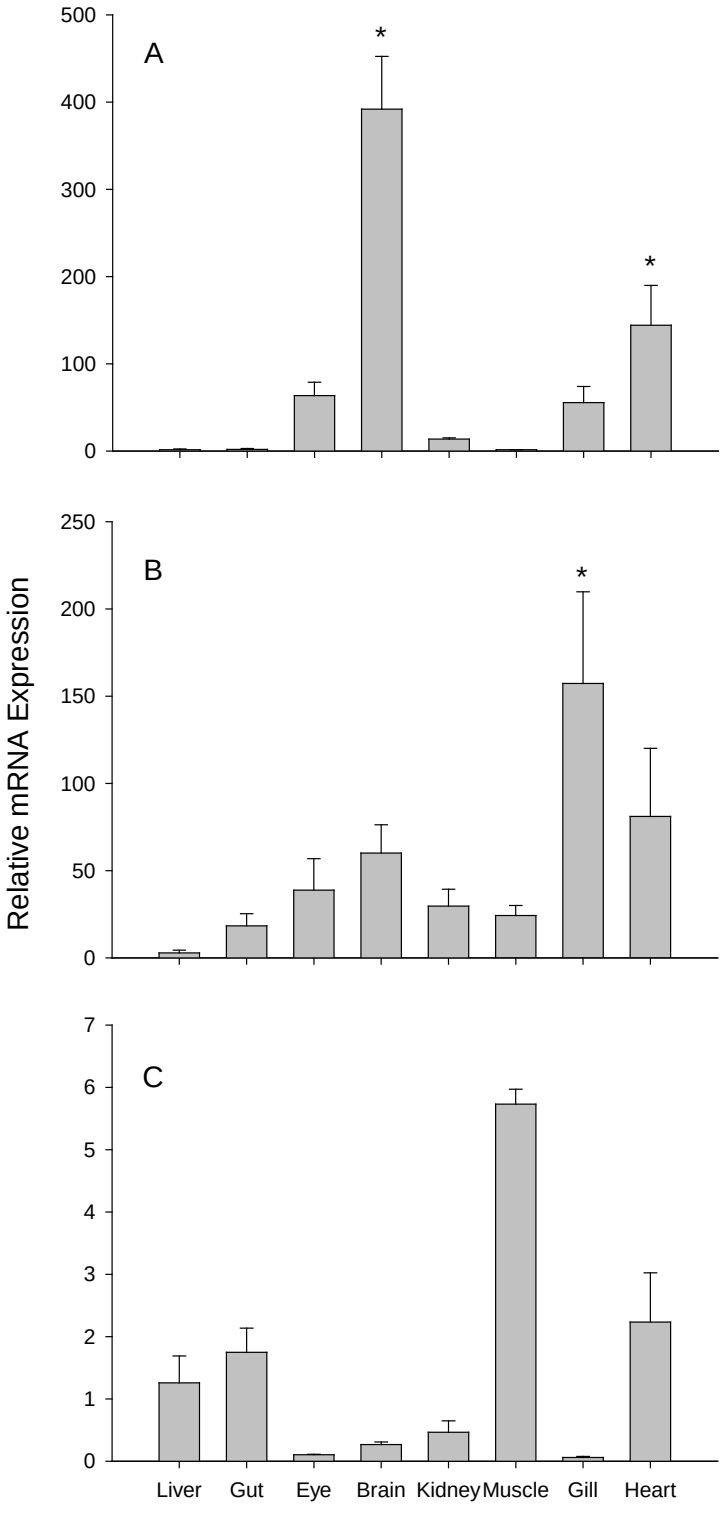

A
B
C
*
*
*
Relative mRNA Expression
500
400
300
200
100
0
250
200
150
100
50
0
7
6
5
4
3
2
1
0
Liver
Gut
Eye
Brain
Kidney
Muscle
Gill
Heart

Figure 3.3

Figure 3.4

Relative mRNA Expression of *β1AR, β2aAR,* and *β2bAR* in adult zebrafish eye, gill, brain (A), heart, muscle, liver, gut, kidney (B). All values are standardized to 18S ribosomal RNA and expressed relative to *β2bAR* mRNA expression in the specified tissue. * indicates significant difference from the level of expression of *β2bAR* within each tissue ($p < 0.05$). Values = Mean + SEM. N = 4.

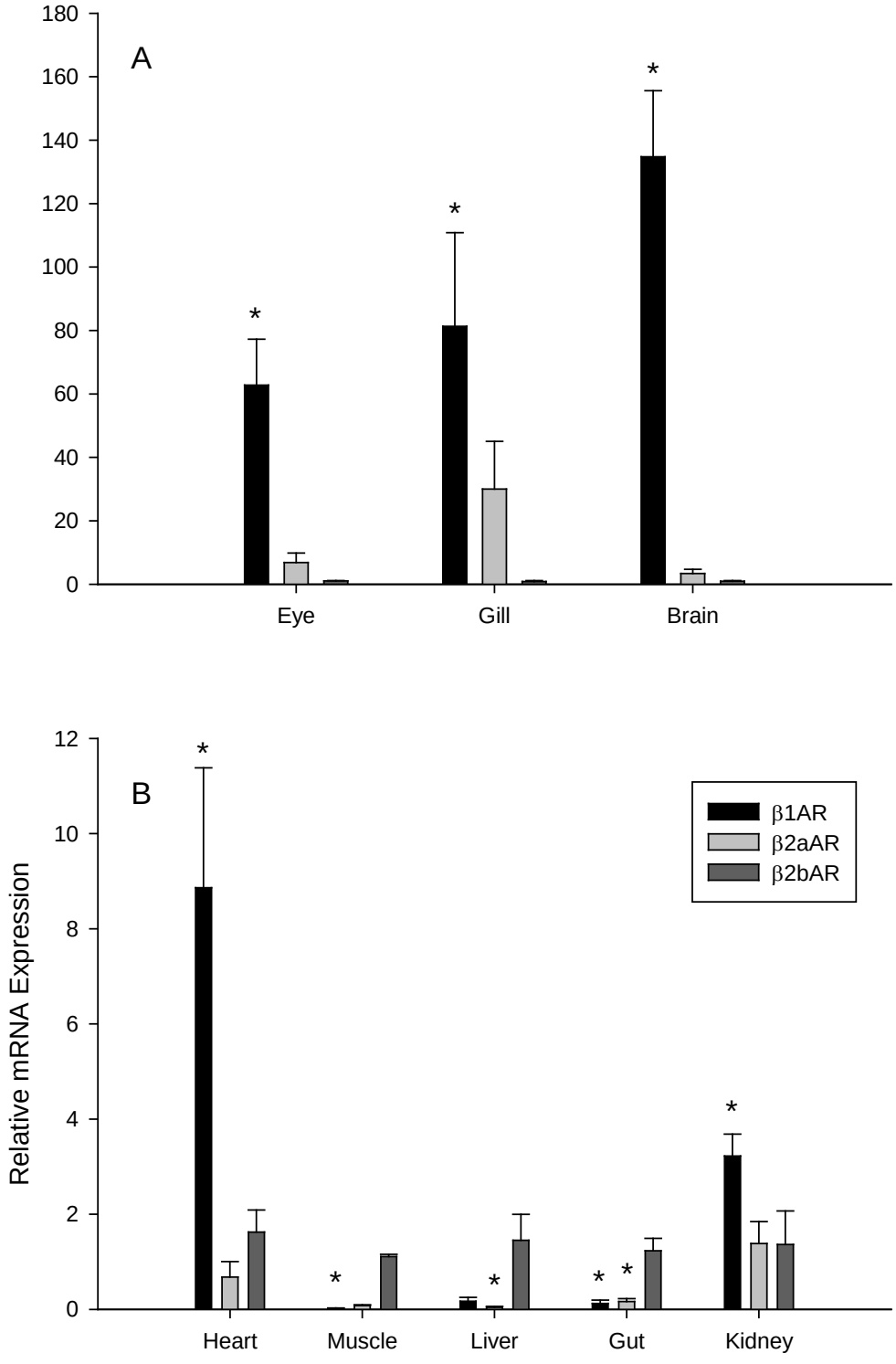

A
180
160
140
120
100
80
60
40
20
0
Eye
Gill
Brain
B
Relative mRNA Expression
12
10
8
6
4
2
0
β1AR
β2aAR
β2bAR
Heart
Muscle
Liver
Gut
Kidney

Figure 3.4

Figure 3.5

Heart rates of β1AR, β2aAR, and β2bAR knockdown larvae ("Morphants") exposed to 10^{-4} M epinephrine (A), isoproterenol (B), procaterol (C), and propranolol (D). "Control" fish were injected with a standard control morpholino. MS-222 indicates heart rate of larvae when anaesthetized only, prior to drug exposure. Values = Mean + SEM, N=8-10. * indicates significant difference between MS-222 and drug treated fish within morphant group. + indicates significant difference between control and morphant fish in MS-222 ($p < 0.05$).

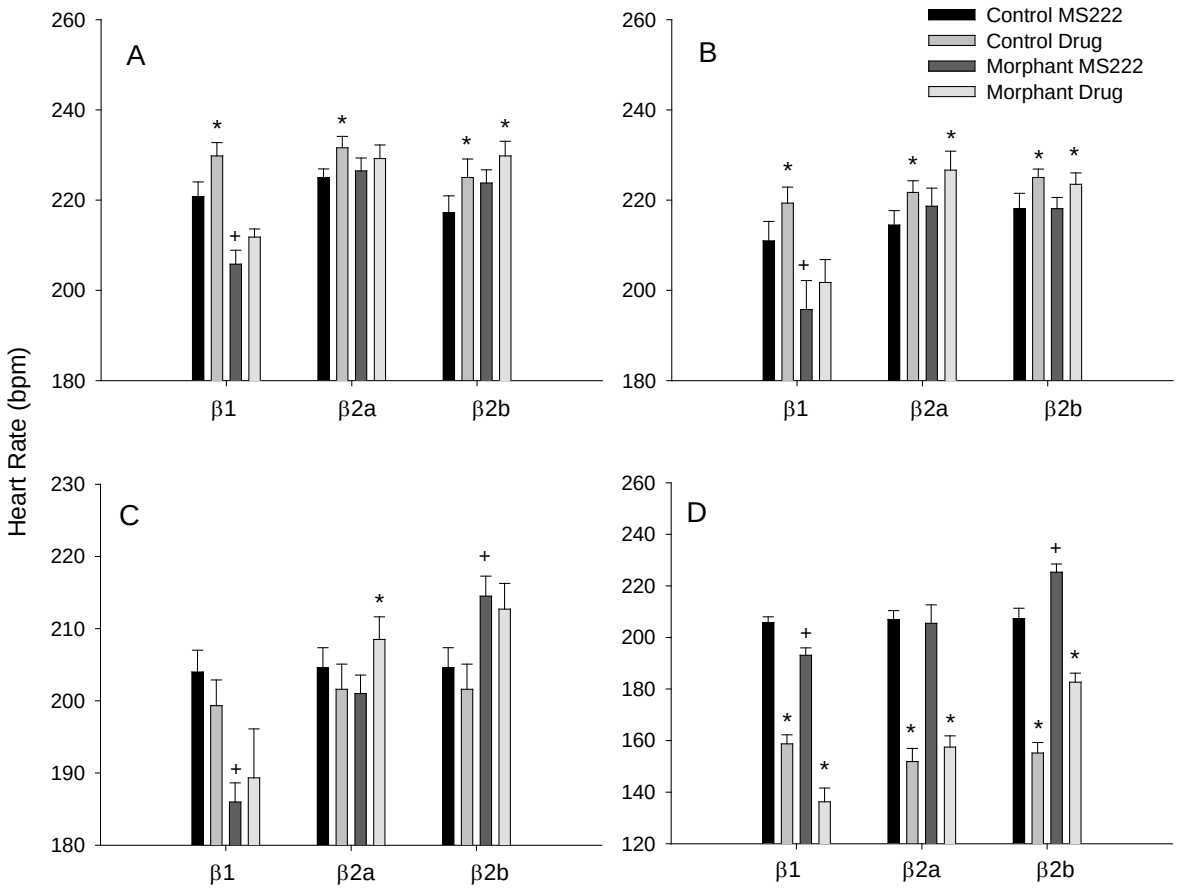

Heart Rate (bpm)
A
B
C
D
Control MS222
Control Drug
Morphant MS222
Morphant Drug
β1
β2a
β2b

Figure 3.5

Figure 3.6

Heart rates of dual β2a/β2bAR, β1/β2aAR, and β1/β2bAR knockdown larvae ("Morphants") exposed to 10^{-4} M epinephrine (A), isoproterenol (B), procaterol (C), and propranolol (D). "Control" fish were injected with a standard control morpholino. MS-222 indicates heart rate of larvae when anaesthetized only, prior to drug exposure. Values = Mean + SEM, N=8-10. * indicates significant difference between MS-222 and drug treated fish within morphant group. + indicates significant difference between control and morphant fish in MS-222 ($p < 0.05$).

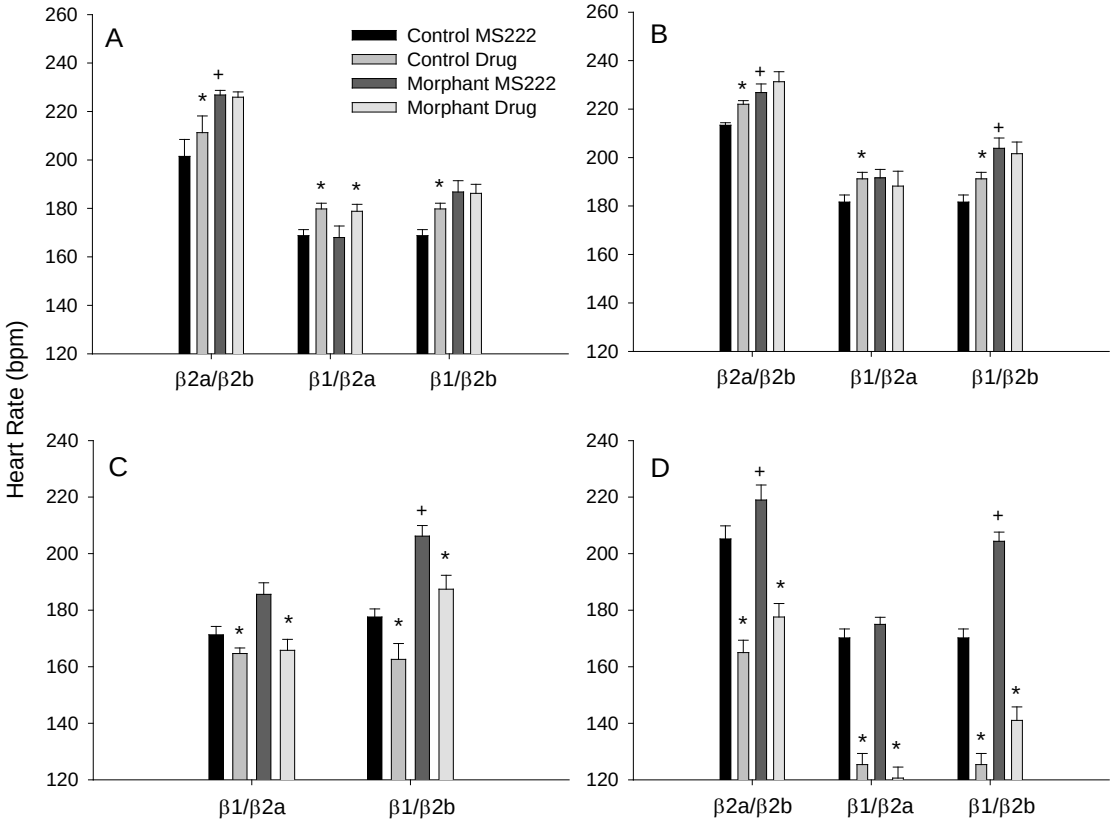

A
B
C
D
Control MS222
Control Drug
Morphant MS222
Morphant Drug
Heart Rate (bpm)
260
240
220
200
180
160
140
120
β2a/β2b
β1/β2a
β1/β2b
*
+

Figure 3.6

Figure 3.7

Lateral images of zebrafish larvae at 4 dpf after being injected with a red fluorescent dTomato capped mRNA (100 pg) tagged with the β2bAR morpholino sequence, alone or in conjunction with β2bAR or β2aAR morpholinos (4 ng, see Materials and Methods). (A) Representative bright field image of a 4 dpf injected larva (B) Red fluorescent image of a β2bAR dTomato injected larva (C) Red fluorescent image of β2bAR dTomato plus β2bAR morpholino injected larva (D) Red fluorescent image of a β2bAR dTomato plus β2aAR morpholino injected larva. Scale bar = 250 μm.

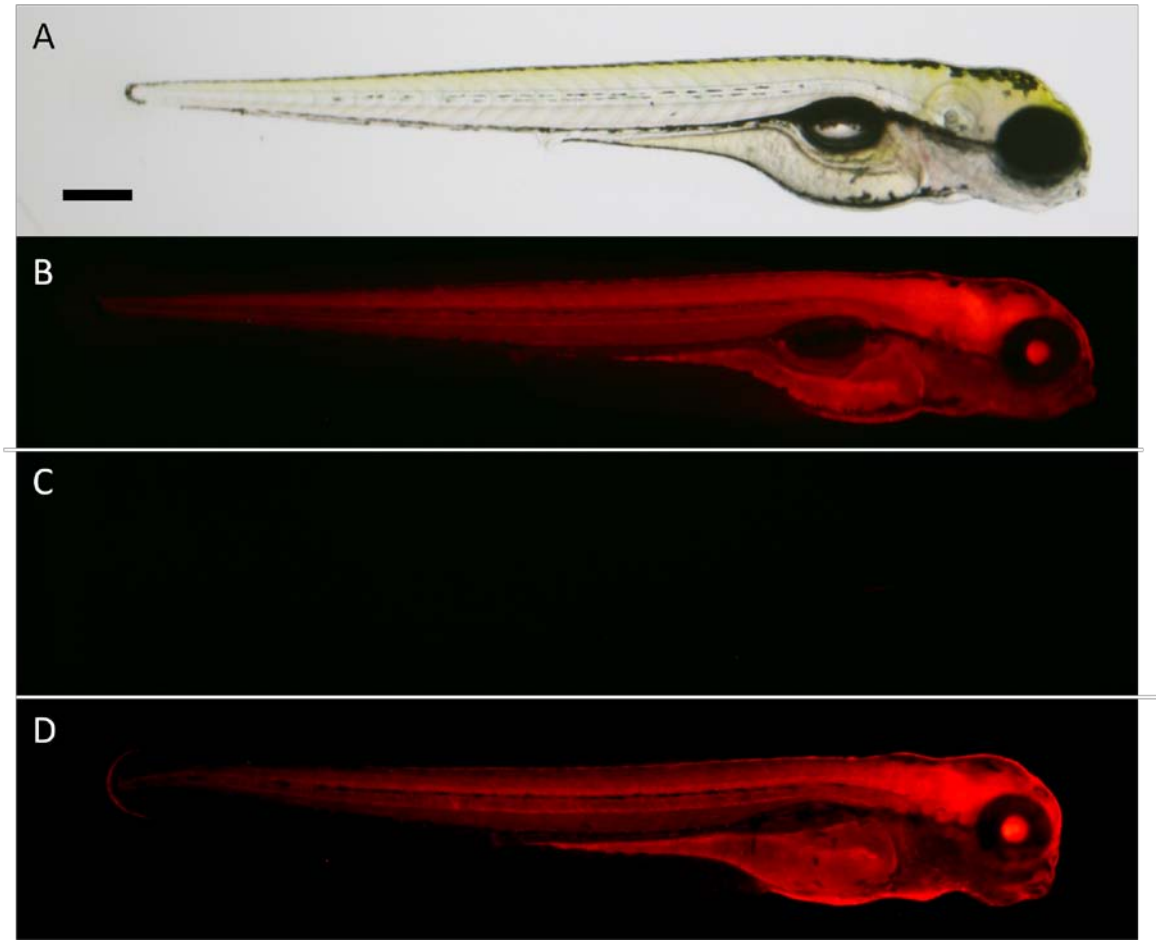
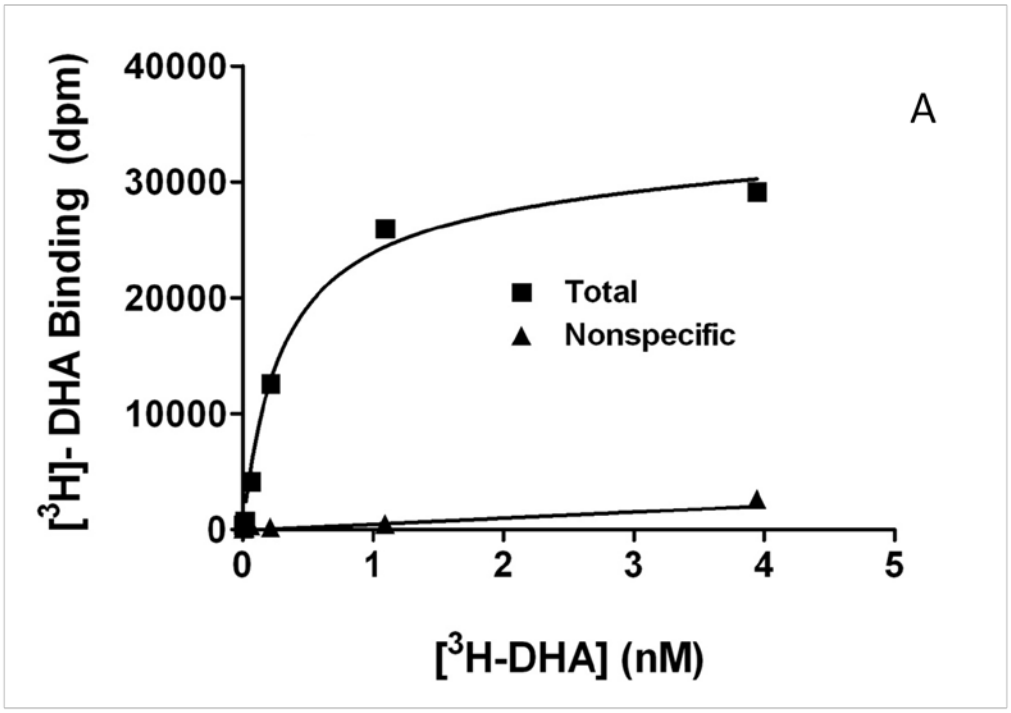


Figure 3.7

Figure 3.8

Saturation (A) and competitive (B) curves for HEK293 cells transfected with zfβ2bAR (chosen as representatives for curves generating data for Tables 3.2 and 3.3). Saturation curves of [^{3}H]-DHA were determined for in the presence of 1 μM alprenolol. Competitive curves for zfβ2bAR transfected cells were performed with 0.5 nM [^{3}H]-DHA (for others, see Results).


A
40000
30000
20000
10000
0
[3H]- DHA Binding (dpm)
Total
Nonspecific
0
1
2
3
4
5
[3H-DHA] (nM)

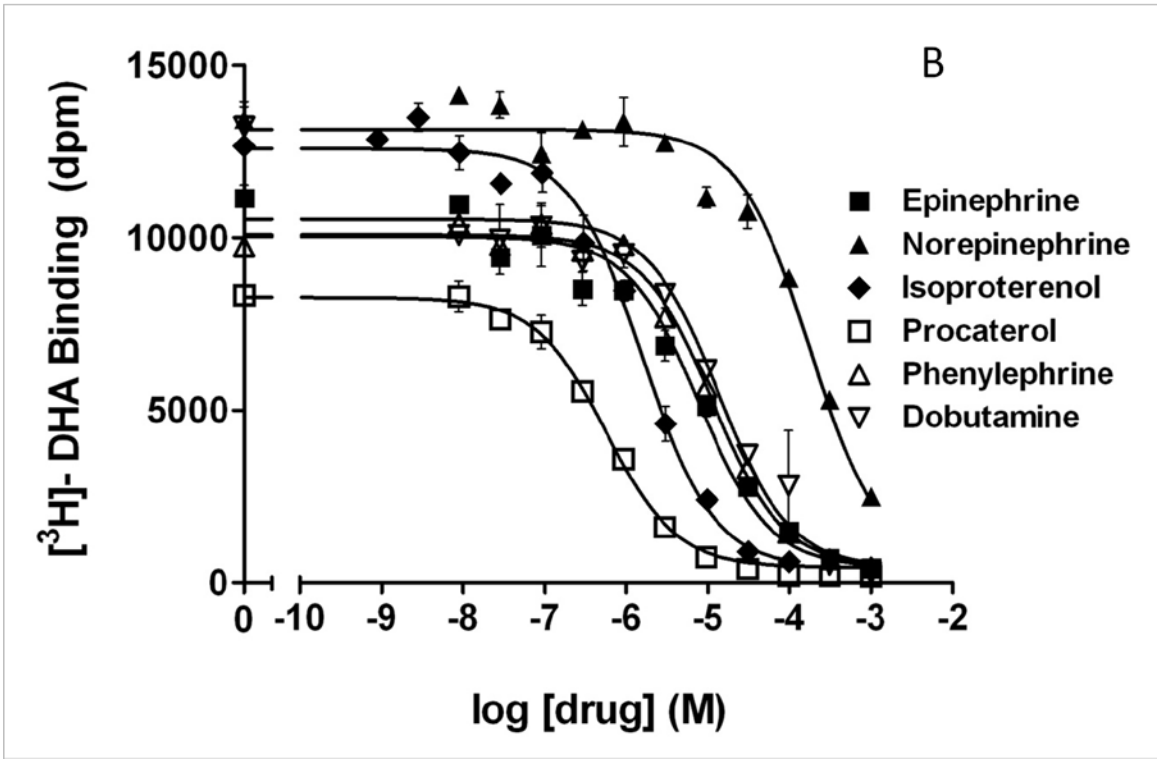

B
15000
10000
5000
0
[3H]- DHA Binding (dpm)
Epinephrine
Norepinephrine
Isoproterenol
Procaterol
Phenylephrine
Dobutamine
0
-10
-9
-8
-7
-6
-5
-4
-3
-2
log [drug] (M)

Figure 3.8

Figure 3.9

Ligand affinity (left) and selectivity (right) ratios for human and zebrafish β-adrenergic receptors expressed in HEK293 cells. (A), epinephrine; (B), norepinephrine; (C), isoproterenol; (D), procaterol; (E), phenylephrine; (F), dobutamine. Affinity ratios for zfβ1AR and zfβ2AR (a and b isoforms) were calculated relative to hβ1AR and hβ2AR, respectively. Selectivity ratios for hβ2AR and zfβ2AR (a and b isoforms) were computed relative to hβ1AR and zfβ1AR, respectively. $*p < 0.05$ when compared to value of 1 (affinity ratio). $^{\#}p < 0.05$ when compared to value of 1 (selectivity ratio).

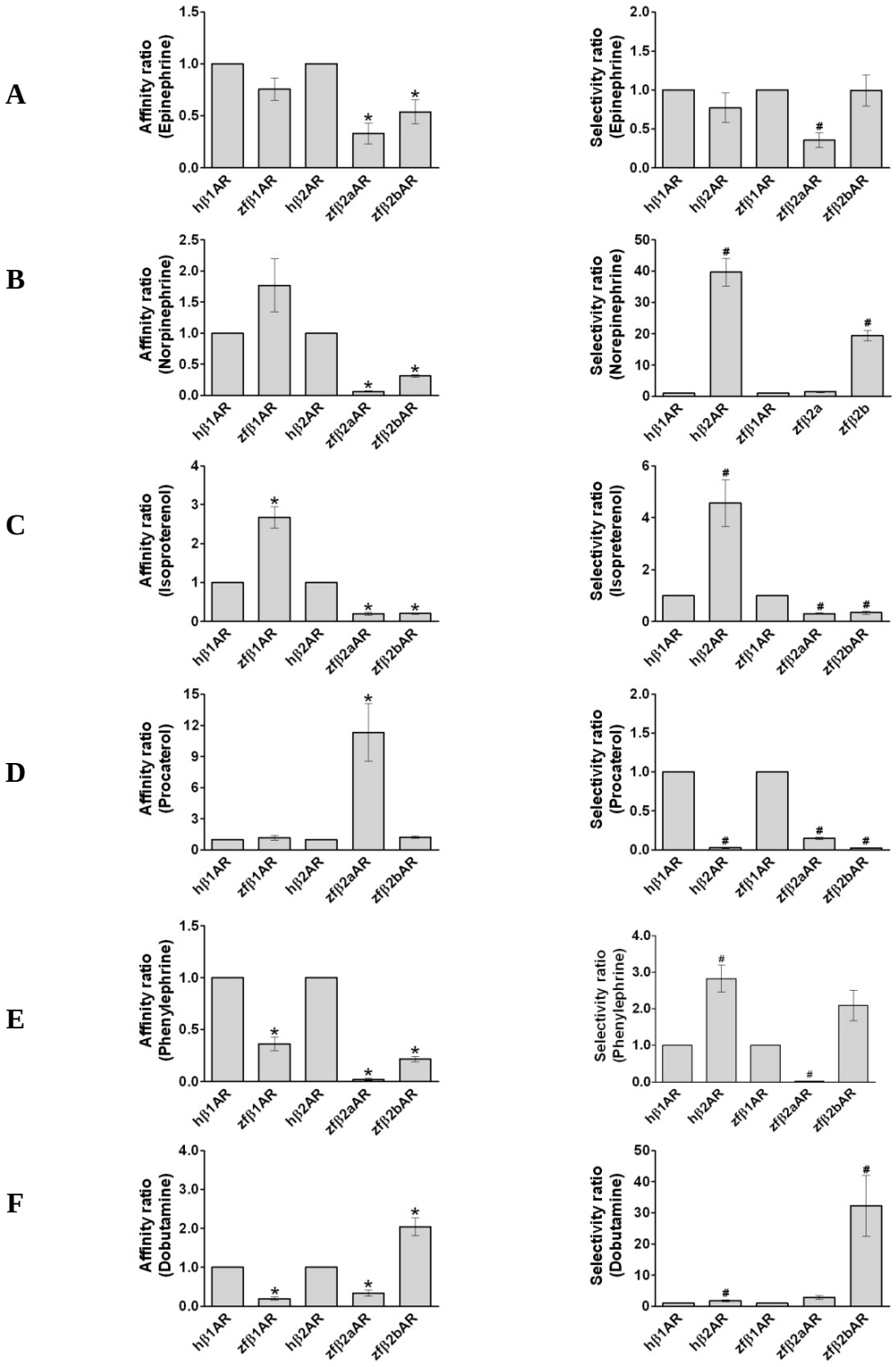

A
Affinity ratio (Epinephrine)
Selectivity ratio (Epinephrine)
B
Affinity ratio (Norpinephrine)
Selectivity ratio (Norepinephrine)
C
Affinity ratio (Isoproterenol)
Selectivity ratio (Isopreterenol)
D
Affinity ratio (Procaterol)
Selectivity ratio (Procaterol)
E
Affinity ratio (Phenylephrine)
Selectivity ratio (Phenylephrine)
F
Affinity ratio (Dobutamine)
Selectivity ratio (Dobutamine)
hβ1AR
zfβ1AR
hβ2AR
zfβ2aAR
zfβ2bAR
zfβ2a
zfβ2b

Figure 3.9

Figure 3.10

Drug-mediated AC activity in HEK293 cells expressing human and zebrafish β-adrenergic receptors. Arithmetic means ± standard error of raw data (N = 5-8) are reported. *$p < 0.05$ when compared with basal condition and $^{\#}p < 0.05$ when compared with epinephrine condition. (A), Mock-transfected cells; (B), hβ1AR; (C), zfβ1AR; (D), hβ2AR; (E), zfβ2aAR; (F), zfβ2bAR. Bmax values (in pmol mg^{-1} protein) were as follows: Mock (0.09 ± 0.03); hβ1AR (3.00 ± 1.01); zfβ1AR (4.63 ± 2.12); hβ2AR (3.49 ± 0.66); zfβ2aAR (2.94 ± 0.80); zfβ2bAR (2.95 ± 0.44). Drug concentrations were as follows: epinephrine (100 μM); procaterol (1 μM) and propanolol (10 μM). All significant differences were maintained when cAMP accumulation in βAR transfected cells was corrected for accumulation in mock transfected cells as shown in A (data not shown).

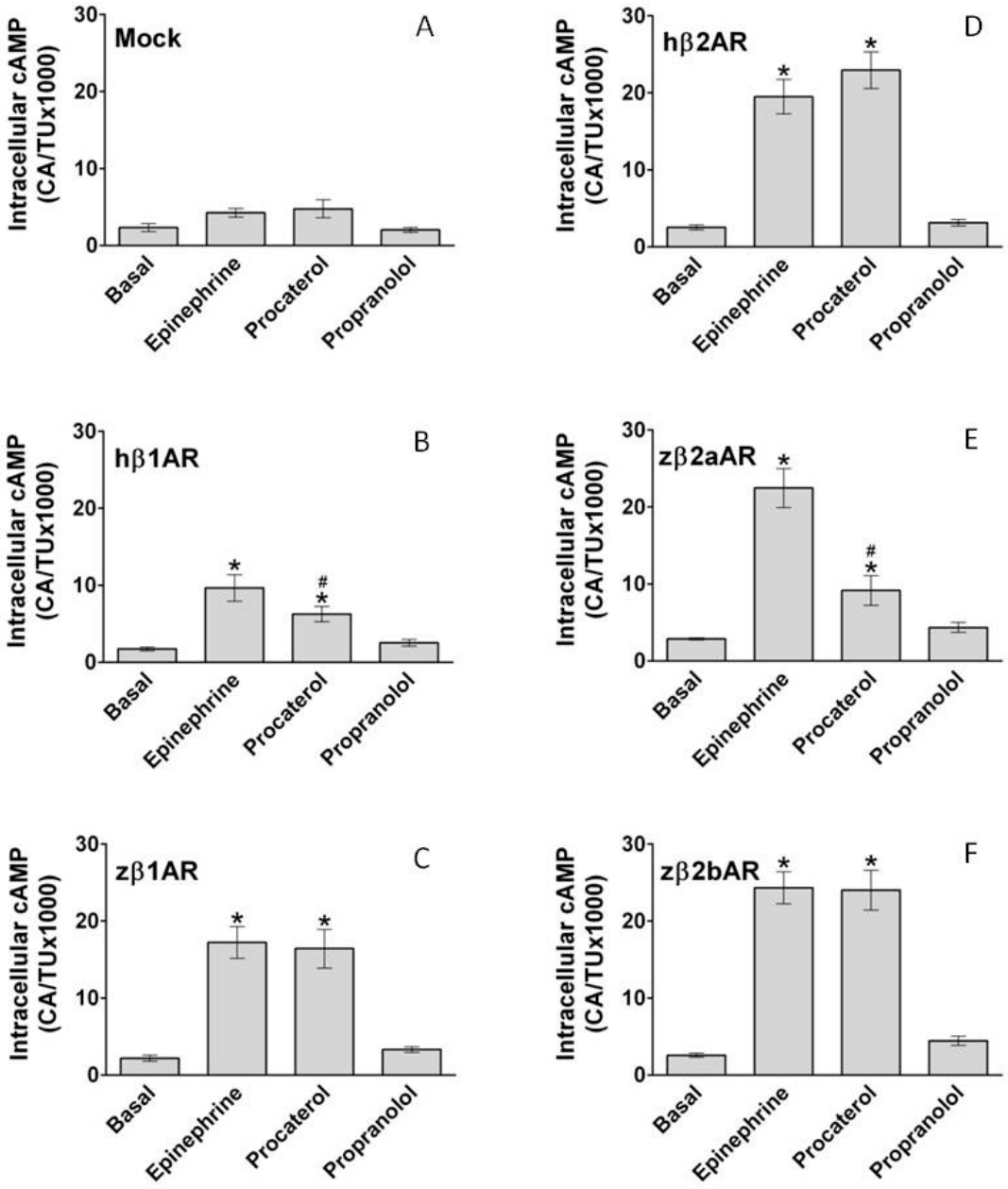

Mock
A
hβ2AR
D
hβ1AR
B
zβ2aAR
E
zβ1AR
C
zβ2bAR
F
Intracellular cAMP (CA/TUx1000)
0
10
20
30
Basal
Epinephrine
Procaterol
Propranolol
*
#

Figure 3.10

Table 3.1: Stroke volume (SV, nl beat^{-1}), and cardiac output (CO, nl min^{-1}) of 4 dpf zebrafish βAR or control morphants before (MS-222) and after exposure to adrenergic agonists epinephrine, isoproterenol and procaterol or the antagonist propranolol.

	Control	β1	Control	β2a	Control	β2b	Control	β2a/ β2b
				MS-222 – Epinephrine Experiment				
SV	0.23 ± 0.02	0.21 ± 0.03	0.28 ± 0.03	0.23 ± 0.02 †	0.21 ± 0.01	0.22 ± 0.01	0.18 ± 0.01	0.19 ± 0.02
CO	51.25 ± 3.31	42.84 ± 5.52	61.91 ± 6.28	49.30 ± 5.31 †	46.31 ± 2.88	48.11 ± 3.31	36.36 ± 2.35	44.04 ± 4.34 †
				Epinephrine				
SV	0.23 ± 0.02	0.21 ± 0.02	0.28 ± 0.03	0.23 ± 0.02	0.18 ± 0.02	0.23 ± 0.02	0.18 ± 0.01	0.23 ± 0.03
CO	51.77 ± 4.57	43.70 ± 4.65	65.65 ± 6.18	51.57 ± 4.27	41.69 ± 5.32	52.91 ± 5.58	37.26 ± 3.29	51.28 ± 7.71
				MS-222 – Isoproterenol Experiment				
SV	0.23 ± 0.01	0.19 ± 0.02	0.21 ± 0.02	0.20 ± 0.02	0.19 ± 0.02	0.21 ± 0.02	0.24 ± 0.02	0.26 ± 0.01
CO	49.11 ± 2.48	36.05 ± 2.59	44.61 ± 4.50	43.57 ± 4.00	40.77 ± 4.23	45.06 ± 3.79	50.64 ± 4.71	58.19 ± 3.86
				Isoproterenol				
SV	0.22 ± 0.02	0.19 ± 0.02	0.22 ± 0.02	0.21 ± 0.02	0.20 ± 0.01	0.24 ± 0.02	0.24 ± 0.02	0.28 ± 0.01
CO	47.27 ± 3.98	39.02 ± 4.23	47.81 ± 4.06	46.98 ± 4.47	44.32 ± 2.90	53.53 ± 4.05	52.38 ± 4.54	65.19 ± 3.24 *
				MS-222 – Procaterol Experiment				
SV	0.18 ± 0.01	0.19 ± 0.01	0.23 ± 0.01	0.25 ± 0.02	0.23 ± 0.01	0.26 ± 0.02		
CO	36.40 ± 1.71	34.75 ± 2.90	46.87 ± 2.94	50.81 ± 4.69	46.87 ± 2.94	55.20 ± 3.21		
				Procaterol				
SV	0.19 ± 0.02	0.22 ± 0.02	0.21 ± 0.02	0.30 ± 0.02 *	0.21 ± 0.02	0.26 ± 0.01		
CO	36.53 ± 3.23	41.83 ± 4.74	41.52 ± 3.36	61.60 ± 3.91 *	41.52 ± 3.36	54.64 ± 1.87		
				MS-222 – Propranolol Experiment				
SV	0.25 ± 0.02	0.21 ± 0.02	0.13 ± 0.02	0.24 ± 0.02 †	0.22 ± 0.02	0.21 ± 0.01	0.20 ± 0.02	0.20 ± 0.02
CO	50.91 ± 4.62	41.07 ± 4.24 †	26.28 ± 3.51	49.68 ± 3.88 †	46.42 ± 3.68	47.96 ± 3.45	40.36 ± 4.07	43.07 ± 4.77
				Propranolol				
SV	0.27 ± 0.03	0.25 ± 0.02	0.19 ± 0.02 *	0.23 ± 0.02	0.27 ± 0.02 *	0.23 ± 0.02	0.22 ± 0.02	0.20 ± 0.02
CO	43.12 ± 4.42 *	33.72 ± 3.00 *	29.80 ± 3.43	36.29 ± 3.25 *	42.26 ± 3.05	42.50 ± 4.29	35.57 ± 3.28	36.12 ± 3.65

Column headings indicate morphant type, row headings indicate treatment. All agonists were used at a concentration of 10^{-4} M and MS-222 concentration was 100 mg l^{-1}. * indicates significant difference between same measurement within morphant group due to chemical exposure. † indicates significant difference between control and corresponding βAR morphant (p<0.05, N = 8-10).

Table 3.2: Equilibrium dissociation constant (K_d) and Bmax values of [^{3}H]-DHA in membranes from HEK293 cells expressing human and zebrafish adrenergic receptors.

	hβ1AR	**zfβ1AR**	**hβ2AR**	**zfβ2aAR**	**zfβ2bAR**
K_d (nM)	1.24 (0.84-1.82)	1.05 (0.78-1.42)	0.49 (0.28-0.84)	1.25* (0.84-1.86)	0.47$^{\#}$ (0.26-0.85)
Bmax (pmol mg^{-1})	27.0 (23.9-30.1)	4.36$^{\&}$ (3.73-4.99)	20.0 (18.6-21.6)	1.64* (1.18-2.10)	16.1$^{\#}$ (14.4-17.8)

Saturation curves (N = 6) were individually analyzed using GraphPad Prism version 5.03. K_d and Bmax values for [^{3}H]-DHA are expressed as geometric and arithmetic means, respectively. The 95% lower and upper confidence intervals are shown in brackets. Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls post test. $^{\&}p < 0.05$ when compared with hβ1AR. $*p < 0.05$ when compared with hβ2AR and $^{\#}p<0.05$ when compared with zfβ2aAR.

Table 3.3*:* Equilibrium dissociation constant values of unlabelled drugs (Ki, nM) in membranes from HEK293 cells expressing human and zebrafish β-adrenergic receptors.

	Epinephrine	**Norepinephrine**	**Isoproterenol**	**Procaterol**	**Dobutamine**	**Phenylephrine**
hβ1AR	1924 (1303-2842)	1258 (744-2043)	74.9 (49.6-113)	7025 (4869-10137)	1296 (923-1821)	7580 (5060-11356)
zfβ1AR	1394 (1189-1634)	1977 (1492-2620)	207[&] (169-254)	9066 (6309-13026)	193[&] (110-338)	2562[&] (1813-3619)
hβ2AR	1376 (590-3210)	55911 (25691-121679)	478 (160-1424)	156 (93.4-257)	2172 (1183-3985)	20837 (8883-48882)
zfβ2aAR	481* (234-991)	2717* (1837-4020)	66.3* (49.1-89.4)	1214* (718-2053)	715* (470-1088)	115* (23.7-561)
zfβ2bAR	1076[#] (484-2396)	18277*[#] (9445-35369)	94.4* (30.9-289)	162[#] (101-260)	4299*[#] (2461-7510)	4081*[#] (2267-7346)

K_i for different ligands are expressed as geometric means with the 95% lower and upper confidence intervals (N = 4-6). Statistical analysis was performed using unpaired *t* test to compare ligand affinity between hβ1AR and zfβ1AR. One-way ANOVA followed by Newman-Keuls post test was used to compare hβ2AR, zfβ2aAR and zfβ2bAR. [&]$p < 0.05$ when compared with hβ1AR. *$p < 0.05$ when compared with hβ2AR and [#]$p < 0.05$ when compared with zfβ2aAR

Table 3.4: List of primer sets and morpholino sequences used in present study

	GenBank Accession Number	Forward Primer Sequence (5'- 3')	Reverse Primer Sequence (5'-3')	Product Size (bp)	Efficiency (%)	R^2
			*dTomato Plasmid**			
β 1AR	XM_680208.2	TGAGCAAGGGCGAGGAGG	TTACTTGTACAGCTCGTCCATG			
β 2aAR	XR_029238.1	GTGAGCAAGGGCGAG	Same			
β 2bAR	XM_695628.3	TGAGCAAGGGCGAGGAGG	Same			
			*Cell Culture Amplicon***			
β 1AR		ATGAACGCGCTTCTTTTCTC	GCGTAAAGTAAAACCCGAAGTG	1468		
β 2aAR		CTGTCAGGTCATGGGAAACA	TTGAGTGTGCTAGCCTTTTTGA	1454		
β 2bAR		AAGCTCATGGAGGGAGACAA	CGGTTGTAAGTTGGGACATTT	1551		
			In situ *Probe Synthesis*			
β 1AR		CAGAGGCTCCAGACGCTCAC	GACATCCTGCCGTTTCTCTC	983		
β 2aAR		CTAATGCCTCCACCAAAAGC	GAAGGCAGAGTTTGCGTACC	896		
β 2bAR		GGAGGGAGACAATACGCTGA	TTCCCATTTTTGTTTTTGGTG	1200		
			Real-Time PCR			
β 1AR		GGGTTACTGGTGGTGCCATT	GCGTGACGCAAAGTACATC	110	96.2	0.989
β 2aAR		GCTTCCAGCGTCTTCAGAAC	CCGAAGGGAATCACTACCAA	91	89.4	0.989
β 2bAR		CTCGTTCCTACCCATCCACA	ATGACCAGCGGGATGTAGAA	150	103.0	0.991
18S***	N/A	GGCGGCGTTATTCCCATGACC	GGTGGTGCCCTTCCGTCAATTC	117	98.3	0.997
			Morpholino Sequence (5' – 3')			
β 1AR		ACGGTAGCCCGTCTCCCATGATTTG				
β 2aAR		GTATTGAGGACCTTATGTTTCCCAT				
β 2bAR		GATCAGCGTATTGTCTCCCTCCATG				

***** corresponding morpholino sequence added to 5' end of the forward primer

** Bam HI restriction sequence added to 5' end of forward primers, Not I restriction sequence added to 5' end of reverse primer

*** 18S primer sequences as per Esbaugh et al. (2009)

Discussion

The results of the present study show that the β1AR has a stimulatory role in the zebrafish heart, and that the two β2AR subtypes have unique cardioinhibitory roles *in vivo*. Wang et al. (2009) also noted a similar trend in zebrafish β1AR morphants, reporting a significant reduction in heart rate at 3 and 4 dpf but not 2 and 5 dpf. Comparatively, in $\beta 1AR^{-/-}$ mice, heart rate was as much as 25% lower than in wildtypes (Ecker et al., 2006). These data conform to the widely accepted canon that β1ARs are stimulatory in *in vivo* systems. It is interesting to note that knocking down β1AR together with either β2aAR or β2bAR did not cause a significant decrease in heart rate (Figure 3.6). This is in contrast to mice, where $\beta 1AR^{-/-}\beta 2AR^{-/-}$ animals have significantly lower heart rates than wildtypes in anaesthetized (Rohrer et al., 1999) or waking (Ecker et al., 2006) animals. These differences could be attributed to species specific differences in β1AR signaling, or to the activity of other adrenergic receptor subtypes in the heart (see below).

Morpholino knock down of β2aAR had no effect on heart rate, however stroke volume and cardiac output both increased significantly in one experiment but decreased significantly in another, a phenomenon which is not explainable using the current data set (Table 3.1). β2bAR appears to play a more significant role in the regulation of heart rate *in vivo* in these larvae. Loss of function of β2bAR alone caused a significant increase in heart rate in 2 out of 3 experiments presented (Figure 3.5). When knocked down in conjunction with either β2aAR or β1AR, it caused an even more robust and reproducible increase in heart rate (Figure 3.6). This was despite the fact that β2aAR is more highly expressed in the heart than β2bAR in zebrafish (Wang et al., 2009; present study, Figure 3.3). One possible explanation for this phenomenon is that while β1- and β2ARs are generally the most plentiful β-adrenergic receptor found in the heart, they are not the only GPCR that can affect heart rate. The β3AR

subtype plays a minimal but not insignificant role in cardiovascular function throughout most species studied. Both isoproterenol and the β3AR specific agonist CL-316243 cause a brief decrease in rate of contraction in myocytes cultured from *β1AR/β2AR*$^{-/-}$ mice (Devic et al., 2001). Likewise, β3ARs in the heart of the freshwater eel exert negative inotropic effects by linking with pertussis toxin sensitive (presumably Gi/o) proteins (Imbrogno et al., 2006). β3ARs have also been found in the heart of the rainbow trout (Nickerson et al., 2003) and possibly the winter flounder (Mendonça et al., 2009). While the rainbow trout appears to express β3aAR mRNA in the heart (Nickerson et al., 2003), Wang et al. (2009) reported minimal to nonexistent expression of either β3aAR or β3bAR mRNA anywhere but in the blood of adult zebrafish. It is therefore unlikely that these β3ARs play a significant role in regulating heart rate in the β2AR morphants of the current study, however the present data cannot entirely rule out the possibility.

The present *in vivo* findings led us to hypothesize that one or both of the zebrafish β2ARs has a negative chronotropic role in the zebrafish heart, either due to associations with Gi proteins and/or differences in their association with Gs proteins as compared to other βARs. Therefore, cell culture experiments in which each of the zebrafish (and human) βARs were expressed in HEK293 cells were used to determine the effect of various agonists on intracellular cAMP accumulation. Data obtained in HEK293 cells are important as they are the first to demonstrate that the zebrafish β1- and β2-adrenergic receptor proteins behave as those previously described in other species, in that they associate with Gs proteins. However, these results by themselves do not help rationalize the increase in heart rate seen in dual β2AR zebrafish morphants. Many factors including subcellular localization (e.g. caveolae; Rybin et al., 2000), changes in conformation, dual coupling to Gs and Gi proteins, and agonist-

mediated internalization of the receptor can all play a role in how the β2AR affects different cell signaling pathways (for reviews see Xiao et al., 2003, Zheng et al., 2004).

Stimulation of mammalian β1- and β2ARs increases intracellular cAMP in cardiomyocytes (Freyss-Beguin et al., 1983; Kuschel et al., 1999). Despite this, β2AR activation does not appear to increase cAMP-dependent PKA activity in normal canine (Kuschel et al., 1999) or murine (Devic et al., 2001) cardiomyocytes, nor does it increase phosphorylation of proteins involved in the excitation-contraction pathway of these cells (Kuschel et al., 1999). Regardless of this disassociation within the classic Gs-cAMP/PKA pathway, it is obvious that β2AR signaling is involved in regulating chronotropic and inotropic activity of the heart, possibly mediated by its additional association with Gi proteins. For one, the present study shows that loss of β2AR function *in vivo* causes increased heart rate in zebrafish larvae (Figures 3.5 and 3.6). Also, the stimulation of β2ARs by isoproterenol causes an initial increase in contraction rate followed by a sustained decrease in murine cardiomyocytes (Devic et al., 2001, Wang et al., 2008), suggesting some cardioinhibitory role for the receptor. This is further supported by the observation that disruption of Gi activity by pertussis toxin (PTX) enhances the β2AR mediated contractile response of murine (for review see Xiao, 2001; Xiao et al., 2003) and canine (Kuschel et al., 1999) cardiomyocytes. Indeed, while the β2AR-Gi complex does not seem to directly inhibit global cAMP production, it does seem to affect downstream PKA activity and also the association of β2ARs with Gs proteins. For example, the β2AR-Gi complex activates phosphoinositide 3 kinases (PI3Ks) which provide a cell survival effect for cardiomyocytes. When PI3K activity is blocked in isolated rat myocytes, β2AR stimulation causes an increase in PKA mediated cell protein phosphorylation and positive contractile response without a concurrent increase in intracellular cAMP (Jo et al., 2002). Overall, therefore, the fact that cAMP levels are increased in HEK293

cells expressing β1AR and β2AR in the present study do not negate the possibility that β2AR-Gi associations limit the contractile response of the heart, as is suggested by the present *in vivo* data.

This is the first study to examine the ligand binding affinities and cellular activity of the zebrafish β1- and β2ARs. The first result of note is that the zebrafish β1AR had a similar binding affinity profile for the endogenous ligands epinephrine and norepinephrine when compared to the human β1AR. The zebrafish protein, however, had a lower binding affinity for isoproterenol and a higher affinity for dobutamine and phenylephrine (Table 3.3). This finding is in keeping with previous observations that fish receptors can have different receptor binding properties than are expected based on mammalian data (Janssens and Grigg, 1988; Fabbri et al., 1992). Perhaps the most interesting outcome of the competitive binding experiments are the obvious difference in binding affinities between the two zebrafish β2ARs, with β2aAR having the greatest divergence in binding profile from that of human β2AR (Table 3.3). Each of the zebrafish β2ARs had unique mRNA expression profiles in adult tissues (Figure 3.3B and 3.3C). Also, they had differential effects on heart rate when knocked down individually in zebrafish larvae, with the β2bAR subtype potentially playing the more critical role at this stage (Figures 3.5 and 3.6). The β2aAR has been shown to be involved in pigment formation in the larval zebrafish (Wang et al., 2009). It would be interesting to further investigate the potential sub-function of each of these β2ARs in zebrafish, both in the heart and other tissues where β2AR function is critical, such as the liver (e.g. Dugan et al., 2008).

Comparing the binding affinities of the different βAR agonists used in this study revealed some unexpected specificities of the β1- and β2ARs. Each of the β2ARs (including the human receptor) had a high affinity for phenylephrine, a classic α1AR agonist (Table 3.2).

Fabbri et al. (1992) showed that phenylephrine is equally potent at displacing [^{3}H]DHA binding in catfish liver membranes as are norepinephrine and epinephrine. Significant displacement of the βAR ligand [^{125}I]ICP by phenylephrine has also been demonstrated in liver membranes of *X. laevis*, the Australian lungfish (*Neoceratodus fosteri*), and the axolotl (*Ambystoma mexicanum*) (Janssens and Grigg, 1988). Thus, there is a growing body of evidence that mammalian and non-mammalian proteins do not always conform to the same functional paradigms. The present data for cAMP activation in human and zebrafish βAR transfected cells also highlight some of these differences. Epinephrine caused a robust increase in intracellular cAMP in whole HEK293 cells transfected with all five of the βARs (Figure 3.10), which supports similar findings in other studies which show that activation of both β1- and β2ARs with epinephrine causes cellular cAMP accumulation (e.g. Green et al., 1992). Procaterol, a classic β2AR agonist, also induced cAMP accumulation in both β1- and β2AR transfected cells, however cAMP levels were significantly lower in the zfβ2aAR transfected cells exposed to procaterol versus epinephrine (Figure 3.10). These data suggest that procaterol 1) behaves as a strong partial or full agonist to both human and zebrafish β1ARs, respectively and 2) is not as effective at increasing cAMP accumulation in zfβ2aAR transfected cells as the endogenous catecholamine epinephrine. The current *in vivo* data show that in two control morphant groups, procaterol exposure caused a significant decrease in heart rate (Figure 3.6), and a significant increase in zebrafish experiencing β2aAR knockdown (Figure 3.5). Zebrafish lacking M_2 muscarinic receptor function also show a negative chronotropic response to procaterol (Steele et al., 2009). Considering the activity of the receptors in the present HEK293 experiments, it is possible that these effects are mediated by the β1AR, the β2ARs, or both types. *In vitro* assessment of β2AR agonist effects on rat cardiomyocyte chronotropy suggest that some of these chemicals increase the rate of cell

contraction via a β1AR mediated pathway (Freyss-Beguin et al., 1983; Juberg et al., 1985). It would seem, therefore, that even mammalian βARs do not always interact with synthetic ligands in a predictable fashion.

In conclusion, it appears that the β-adrenergic receptors are necessary for regulating heart function during early life in zebrafish, with the β1- and β2bAR most implicated in controlling heart rate. It is also apparent that while the zebrafish β-adrenergic receptors are equally capable of instigating cAMP production as their human counterparts, they have distinct binding affinities for different ligands. While morpholino knockdown of β2a- and β2bAR suggest that one or both of these receptors may be cardioinhibitory, experiments expressing each of these receptors individually in HEK293 cells imply that stimulation of both β2AR subtypes increase intracellular cAMP levels. The β2ARs may cause inhibition by another indirect pathway, such as by interaction with other signaling cascades (e.g. PI3K) via an association with Gi proteins. Further research into how these receptors behave at the cellular level in native tissues would be key in clarifying how these unique paralogs function.

Acknowledgements

I would like to thank Dr. Dirk Meyer (Institute for Molecular Biology, Innsbruck, Austria) for the use of his zebrafish facilities. The study was supported by an operating grant (MOP-81341) from the Canadian Institutes of Health Research to M.T., an NSERC Discovery grant to S.F.P., and OGS and NSERC post-graduate scholarships to S.L.S.

Chapter 4.

Interactive effects of development and hypoxia on catecholamine synthesis in zebrafish (*Danio rerio*)

Abstract

The rate-limiting enzyme in the biosynthetic pathway of catecholamines is tyrosine hydroxylase (TH), the activity of which is dependent on molecular oxygen. Zebrafish (*Danio rerio*) possess two non-allelic TH coding genes, *TH1* and *TH2*. A principal goal of the present study was to determine if the expression of these genes, as well as that of the noradrenergic enzyme dopamine β hydroxylase (*DβH*), is sensitive to environmental hypoxia, both in larvae and adults. Additionally, I sought to determine if catecholamine content of larvae was changed by environmental hypoxia, and whether the hearts of hypoxic larvae were equally responsive to exogenous catecholamine (norepinephrine) exposure. After 2 days of exposure to hypoxia (from 5 to 7 days post fertilization (dpf); PO_2 = 30 Torr) *TH2* mRNA expression was significantly lower and *DβH* mRNA was significantly higher in whole larvae. TH protein expression in whole larvae was not affected after either 2 or 4 days of hypoxia exposure. Whole body catecholamine levels were unchanged until after 4 days of hypoxic exposure (5-9 dpf), at which time there was a significant increase in epinephrine and norepinephrine content. Norepinephrine content was significantly elevated in the hearts of adult fish after 2 and 4 days of hypoxic exposure, and *TH1* mRNA expression was increased in the kidney of both groups. After 2 or 4 days of exposure to hypoxia, larvae displayed significantly lower heart rates than normoxic fish. However, application of exogenous norepinephrine caused similar increases in heart rate in both groups. Overall, it can be concluded that the mRNA expression of *TH1* and *TH2* is differentially affected by hypoxia exposure in both larvae and adults. Also, in zebrafish larvae, the catecholamine biosynthetic pathway is fully functional in early life and although whole body catecholamine levels increase during hypoxia, there is no accompanying decrease in the cardiac response to adrenergic stimulation.

Introduction

The catecholamines epinephrine and norepinephrine are the endogenous ligands of the ubiquitous adrenergic receptors. These function both as neurotransmitters in the central and peripheral nervous systems, and as hormones when released into the bloodstream from neurosecretory chromaffin cells. In mammals, chromaffin cells are concentrated within the adrenal medulla whereas in most teleost fish, chromaffin cells are grouped diffusely in the head kidney within the walls of the posterior cardinal vein (Nandi, 1961). The enzymes required for catecholamine synthesis include tyrosine hydroxylase (TH), which catalyses the rate limiting step, the hydroxylation of L-tyrosine to L-DOPA, which is then converted to dopamine. Dopamine β hydroxylase (DβH) hydroxylates dopamine to norepinephrine. Both TH and DβH require molecular oxygen as a co-factor, therefore their activities may be affected by conditions of low oxygen either locally (e.g. originating at the tissue level) or environmentally (e.g. aquatic hypoxia in fish; for references see Reid et al., 1998).

Hypoxia and other acute stressors such as hypercapnia and exhaustive exercise increase circulating catecholamines in fish (e.g. Ristori and Laurent, 1989; Perry and Gilmour, 1996; McNeill and Perry, 2006; for review see Reid et al, 1998). When plasma catecholamines are increased, an agonist-mediated decrease in surface expression of βARs (termed receptor downregulation) has been reported in the red blood cells (RBCs) of rainbow trout (Perry al., 1996; Gilmour et al., 1994). An similar situation in cardiomyocytes could explain why isolated ventricle tissue of carp (Temma et al., 1985) and rat (Silva et al. 2001) pre-treated with isoproterenol (a β-adrenergic receptor agonist) showed reduced contractile response to repeated exposure. While surface expression of cardiac β-adrenergic receptors was not affected in rainbow trout exposed to 6 h of aquatic hypoxia (Gamperl et al., 1998), or in the heart of rainbow trout infused with bolus injections of catecholamines (Gamperl et al.,

1994), it was significantly decreased in the ventricle of chronically hypoxic rats (Guan et al. 2010).

The effects of hypoxia on circulating catecholamine levels have not been assessed in larval fish. The small size of zebrafish larvae presents a challenge for measuring circulating catecholamines, however changes in whole body catecholamines can be readily assessed by traditional HPLC methods. The hearts of zebrafish larvae reared in hypoxia remain responsive to adrenergic agonists (Bagatto, 2005) despite the fact that larvae reared in hypoxia (PO_2 = 20-30 Torr) from the point of fertilization show significantly reduced heart rates (Bagatto 2005; Steele et al., 2009). While the hypoxic bradycardia appears to be primarily related to the activity of the M_2 muscarinic receptor, there is also evidence that the mRNA expression of β-adrenergic receptors is increased in larval zebrafish reared from the time of fertilization in hypoxia (Steele et al., 2009). Additionally, in zebrafish larvae where M_2 function is reduced, heart rate was reduced in response to the presumed β2AR agonist procaterol, which had no effect on the hearts of control larvae (Steele et al., 2009). Therefore, exposure to hypoxia may affect cardiac βARs by two means in larval zebrafish. First, hypoxic exposure may cause an increase in catecholamines which could decrease adrenergic sensitivity of the heart owing to receptor downregulation. Secondly, an increase in inhibitory M_2 signaling within the heart to mediate hypoxic bradycardia can concurrently affect the βARs, as was previously demonstrated in larvae lacking M_2 receptor activity (Steele et al., 2009).

The zebrafish is among several species of teleosts which possess two distinct TH genes (*TH1* and *TH2*; Candy and Collet, 2005). In the barramundi (*Lates calcarifer*), these two gene transcripts are expressed differentially between tissues, with *TH1* being highly expressed in the brain and kidney and *TH2* being significantly expressed in brain only (Candy and Collet, 2005). Recently, the expression of the two TH genes was examined in zebrafish (Chen et al.

2009). Of four tissues examined in adults, *TH1* expression was highest in the brain, whereas *TH2* expression was highest in the kidney and liver (Chen et al. 2009). They also reported *TH1* and *TH2* expression in whole larvae from 12 hpf to 5 dpf, with expression of *TH1* being significantly higher than *TH2* at most stages. A similar developmental pattern of the norepinephrine-synthesizing enzyme DβH has yet to be established. The ability to synthesize catecholamines at early life stage may be critical for survival and development; both $TH^{-/-}$ (Kobayashi et al., 1995; Zhou et al., 1995) and $D\beta H^{-/-}$ (Thomas et al., 1995) mice die *in utero* or shortly after birth. Also, changes in *TH1* or *TH2* mRNA transcript levels could reveal differential regulation of these genes in response to hypoxia, which could relate to their relative contribution to the production of the total catecholamine pool in zebrafish.

The present study attempts to establish a link between the ability of zebrafish larvae to regulate catecholamines in environmental hypoxia and changes in βAR mRNA expression and changes in cardiac adrenergic sensitivity. Ultimately, this study aims to provide evidence that zebrafish are able to produce catecholamines in early life and that this process is sensitive to environmental O_2 availability at both the gene and protein level.

Materials and Methods

Animal Care and Breeding

Adult zebrafish used in the present study were acquired, housed, and bred as described in Steele et al. (2009). Once embryos were collected from the traps as described, they were kept in an incubator at 28^{o}C in E3 embryo media in 30 ml petri dishes until they were old enough to use in experiments (3 – 5 days). Once the larvae were placed in the experimental tanks, they were fed daily beginning at 5 dpf; the water in the tanks was changed every 2 days.

Hypoxia Exposure

Hypoxia was initiated in experimental tanks by bubbling with a metered mixture of N_2 and air to reach the desired PO_2 of 30 Torr. The PO_2 of the tanks was maintained and monitored continuously throughout the experimental period as described in Steele et al. (2009).

Larvae Sampling and Heart Rate Measurements

At the end of the hypoxic exposure (2 or 4 days after the onset of hypoxic conditions), groups of 30-40 larvae (N = 1 for statistical purposes) were collected randomly from the tanks and pooled into a pre-weighed RNAse and DNAse free 1.5 ml centrifuge tube. Water was removed using a pipet and the animals were flash frozen in liquid N_2 in the tube, which was subsequently weighed to determine the wet tissue mass; samples were stored at -80^{o}C until processed.

Larvae were removed from hypoxic and normoxic tanks for heart rate measurements after 2 and 4 days of exposure (from 5 dpf). Larvae were placed in a small volume of TRIS buffered MS-222 (100 mg l^{-1}) maintained at 28^{o}C and baseline heart rate was measured after

the animal became immobilized, after approximately 2 min. After the initial heart rate measurement, larvae were transferred to a second dish containing 10^{-4} M norepinephrine hydrochloride (Sigma) dissolved in TRIS buffered MS-222. This dose of norepinephrine has been used to effectively increase heart rate in previous studies (Schwerte et al., 2006; Steele et al., 2009). After 10 min, heart rate was re-measured.

Adult Tissue Sampling

After the hypoxic period, fish were removed individually and killed with an overdose of benzocaine and tissues were harvested as described in Steele et al. (2009).

Real-Time PCR Analysis

Total RNA was extracted from larvae and adult tissue samples as described in Steele et al. (2009). All real-time PCR analysis was performed in 12.5 µl reactions using Brilliant II™ SYBR Green Mastermix (Stratagene, La Jolla CA USA), otherwise as described in Steele et al. (2009). All primer sequences used for real-time PCR analysis are listed in Table 4.1.

Analysis of Catecholamines

To each sample of pooled larvae, 250-300 µl of ice cold 4% perchloric acid (PCA) containing 2 mg l^{-1} EDTA and 0.5 mg l^{-1} of sodium bisulphate was added. Samples were homogenized in the PCA and centrifuged at 16,000 x g for 1 min at room temperature. Supernatants were alumina-extracted for catecholamines as previously described (Woodward, 1982). 100 µl of extracted sample was injected via an HPLC autosampler; the HPLC consisted of a Varian ProStar 410 solvent delivery system (Varian Chromatograhy Systems, Walnut Creek, CA) connected to a a Decade II electrochemical detector containing a VT-03

electrochemical flow cell (both Antec Leyden, Zoeterwoude, The Netherlands). Epinephrine and norepinephrine concentrations (nmol g^{-1}) were calculated (taking dilutions into account) relative to standards of known concentration and 3,4-dihydroxybenzalamine hydrobromide (DHBA) was incorporated in all standards and unknowns as an internal standard.

Western Blot Analysis

Protein was extracted and quantified from pooled larvae samples as per Braun et al. (2009). All protein samples were diluted in Laemmli sample buffer (BioRad) with beta-mercaptoethanol and heated to 100^{o}C for 5 min, then cooled to room temperature before loading onto gels. Sixty μg of protein per sample was loaded onto a 4% stacking/10% running polyacrylamide gel and size fractionated by reducing SDS-PAGE. Fractionated proteins were transferred to a PVDF membrane (BioRad). To determine protein loading, membranes were stained with MemCode™ Reversible Protein Stain Kit for PVDF Membranes (Thermo Scientific). After blocking in 5% skim milk/TBS-T (1xTBS, 0.1% Tween 20) for 1 h at room temperature, membranes were incubated in 5% skim milk/TBS-T containing 1:2000 monoclonal anti-TH antibody (MAB318, Millipore) overnight at 4^{o}C. Membranes were rinsed 4 x 10 min in TBS-T at room temperature then incubated in 5% skim milk/TBS-T containing 1:50,000 horseradish peroxidase-conjugated IgG goat anti-mouse secondary antibody (170-6516; BioRad) for 1 h at room temperature. Membranes were washed 4 x 10 min in TBS-T at room temperature. To account for possible loading differences, each blot was stripped with Re-Blot Plus Mild antibody stripping solution (Millipore) and re–incubated with a rabbit anti-actin primary antibody (1:500; A2066, Sigma) in 5% skim milk/TBS-T for 1 h at room temperature with gentle shaking. After washing 4 x 10 min in TBS-T, membranes were incubated in 1:50,000 horseradish peroxidase-conjugated IgG goat anti-rabbit antibody

(170-6515; BioRad). All antibody conjugated proteins were visualized using Supersignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific). Band intensities of both TH and actin proteins as well as total protein from MemCode protein staining were quantified using ImageJ software (v 1.43, imagej.nih.gov/ij).

Statistical Analyses

All statistical analyses were performed using SigmaStat statistical analysis software (v. 3.5; Systat Software Inc., San Jose CA, USA). Data in Figures 4.1 and 4.2, were compared using one way ANOVA and a Tukey post-hoc test. Data in Figures 4.3, 4.7B and C, and 4.8 were compared using a Student *t* test. Data in Figure 4.7A were compared using a one way ANOVA on Ranks (due to failure of normality and equal variance test) with a Dunn's post-hoc test. For Figures 4.4 and 4.5, each treatment mean was compared using a one sample *t* test to individual controls that were set to a value of 1. For Figure 4.6A, normalized TH protein levels were compared by Rank Sums *t* test. In Figure 4.9, data from the 2 and 4 day exposed fish were compared separately using a two-way repeated measures ANOVA and a Tukey post-hoc test.

Results

mRNA Expression – Adult Tissues, Whole Larvae, and Changes in Hypoxia

TH1 and *DβH* expression were detectable from 1 hpf to 10 dpf, whereas TH2 expression was detectable beginning at 12 hpf. *TH1* and *DβH* expression was significantly higher than in 12 hpf embryos at 3 and 4 dpf, and *TH2* expression did not reach its highest level until approximately 5 dpf and remained significantly higher than 12 hpf from 7 to 9 dpf. *DβH* expression was significantly higher than in 12 hpf embryos at 3 and 8 dpf (Figure 4.1). When examining adult tissues, neither *TH1* nor *DβH* expression was detectable in the gut or liver, and *DβH* expression was not detectable in the gill. Comparing expression of each gene between tissues, expression of all three genes was highest in brain, with significant expression of *TH2* also occurring in the kidney (Figure 4.2). However, expression of *TH1* and *TH2* varied significantly within tissues, with *TH1* being more highly expressed in the brain and eye, and *TH2* being more highly expressed in the heart. *TH1* and *TH2* expression in the kidney was approximately equal (Figure 4.3).

When zebrafish larvae were reared from 5 – 7 dpf in hypoxia (PO_2 = 30 Torr), there was a significant 25% decrease in whole larvae *TH2* gene expression and a significant increase (~1.5-fold) in *DβH* expression. After a 4 day hypoxic exposure (5-9 dpf), only *β2aAR* gene expression was significantly altered being decreased by about ~60% from normoxic controls (Figure 4.4). In adult tissues, levels of *TH1*, *β1AR*, *β2aAR*, and *β2bAR* mRNA were significantly lower in the eye, and both *DβH* and *β2bAR* transcripts were significantly lower in the heart after 2 days of hypoxia. *TH1* mRNA expression in kidney was significantly elevated in these same fish (Figure 4.5A). After 4 days, *TH1* expression was still significantly higher in kidney of hypoxic fish. *DβH* expression was lower in brain and higher in the heart of hypoxic fish. *β2bAR* mRNA levels were significantly higher in eye and heart, but

significantly lower in kidney. Finally, *β2aAR* mRNA levels were significantly lower in the kidney of hypoxic versus normoxic fish (Figure 4.5B).

TH Protein Expression in Hypoxic Larvae

TH protein expression was not significantly different between normoxic and hypoxic whole larvae after either 2 or 4 days of exposure (Figure 4.6A). Although all membranes were assessed for equal loading/transfer using an anti-actin antibody, the mean intensity of the actin bands appeared to be significantly lower in the hypoxia treated larvae (2 day exposure only) suggesting a possible effect of treatment on actin expression. Therefore, TH band intensity was instead normalized to a selected band intensity from the MemCode ™ total protein stain described in the Materials and Methods to control for loading differences. Specifically, a the relative density of a dense group of proteins at ~20 kDa that appeared on every membrane was measured and used to normalize TH protein intensity (see Figure 4.6B)

Catecholamines – Adult Tissues, Whole Larvae, and Changes in Hypoxia

Epinephrine and norepinephrine were detectable in embryos at 1 hpf. Levels of each increased gradually from 1 hpf to 5 dpf (Figure 4.7A). When larvae were reared in hypoxia for 2 days starting at 3 or 5 dpf, there was no change in whole larva catecholamine levels (Figure 4.7B). The only significant change in catecholamine levels occurred in whole larvae exposed from 5 dpf for 4 days. Both epinephrine and norepinephrine levels were higher (~32% and 44% higher, respectively) in hypoxic larvae compared to normoxic controls. Consequently, total catecholamines were approximately 37% higher in the hypoxic group (Figure 4.7C).

Norepinephrine was detected in the brain, eye, heart and kidney of adult zebrafish. Epinephrine levels were low in 3 of 4 tissues examined, ranging from approximately 0.3% (in

normoxic brain) to 1.5% (in normoxic heart) of total catecholamines. In kidney however, levels of epinephrine and norepinephrine were approximately equal. In adult zebrafish exposed to hypoxia for either 2 or 4 days, levels of norepinephrine and total catecholamines in the heart were significantly higher compared to the hearts of normoxic fish. Brain epinephrine levels were significantly lower than controls in fish exposed to hypoxia for 4 days (Figure 4.8).

Larvae in Hypoxia – Cardiac Response to Norepinephrine

The results of the two way repeated measures ANOVA revealed a significant interaction term for the treatments within the 2 day hypoxia experiment. Post-hoc analysis showed that norepinephrine treatment caused a significant increase in heart rate in both normoxic and hypoxic groups. Also, the heart rates of norepinephrine-treated hypoxic larvae were significantly lower during hypoxia in both control (non NE-treated) and NE treated fish (Figure 4.9A).

Analysis of the 4 day hypoxia experiment by two way repeated measures ANOVA showed no significant interaction term between the two treatments (hypoxia and norepinephrine). Results of the ANOVA showed that there was a significant overall decrease in heart rate due to hypoxia within the control and norepinephrine treated groups, as well as a significant overall increase with norepinephrine treatment within normoxic and hypoxic groups (Figure 4.9B).

Figure 4.1

Relative mRNA **e**xpression of *TH1* (A), *TH2* (B) and *DβH* (C) in zebrafish larvae at 1, 6, and 12 h post fertilization and 1 through 10 days post fertilization. Each gene of interest expression was standardized to 18S ribosomal RNA expression and is shown relative to its own level of expression at 1 hpf, or 12 hpf in the case of *TH2*. * indicated significant difference from expression at 12 hpf (p <0.05). Values = mean + SEM, N = 4. ND indicates not detectable

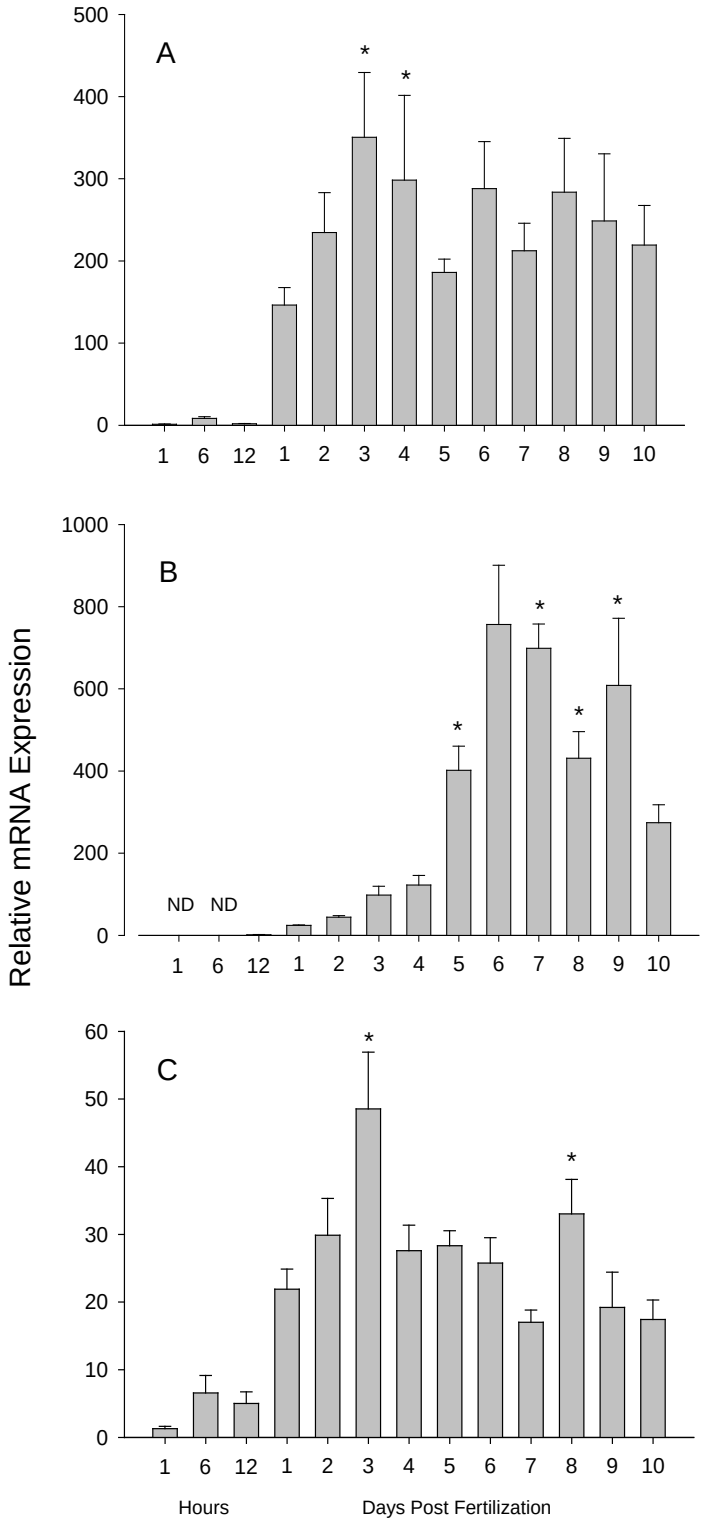

A
B
C
Relative mRNA Expression
ND ND
1 6 12 1 2 3 4 5 6 7 8 9 10
Hours
Days Post Fertilization

Figure 4.1

Figure 4.2

Relative mRNA expression of *TH1* (A), *TH2* (B) and *DβH* (C) in various tissues of adult zebrafish. All values were standardized to 18S ribosomal RNA and expressed relative to its own expression in muscle. * indicates significant difference from expression in muscle ($p < 0.05$). Values = mean + SEM, N = 4-5. ND indicates not detectable.

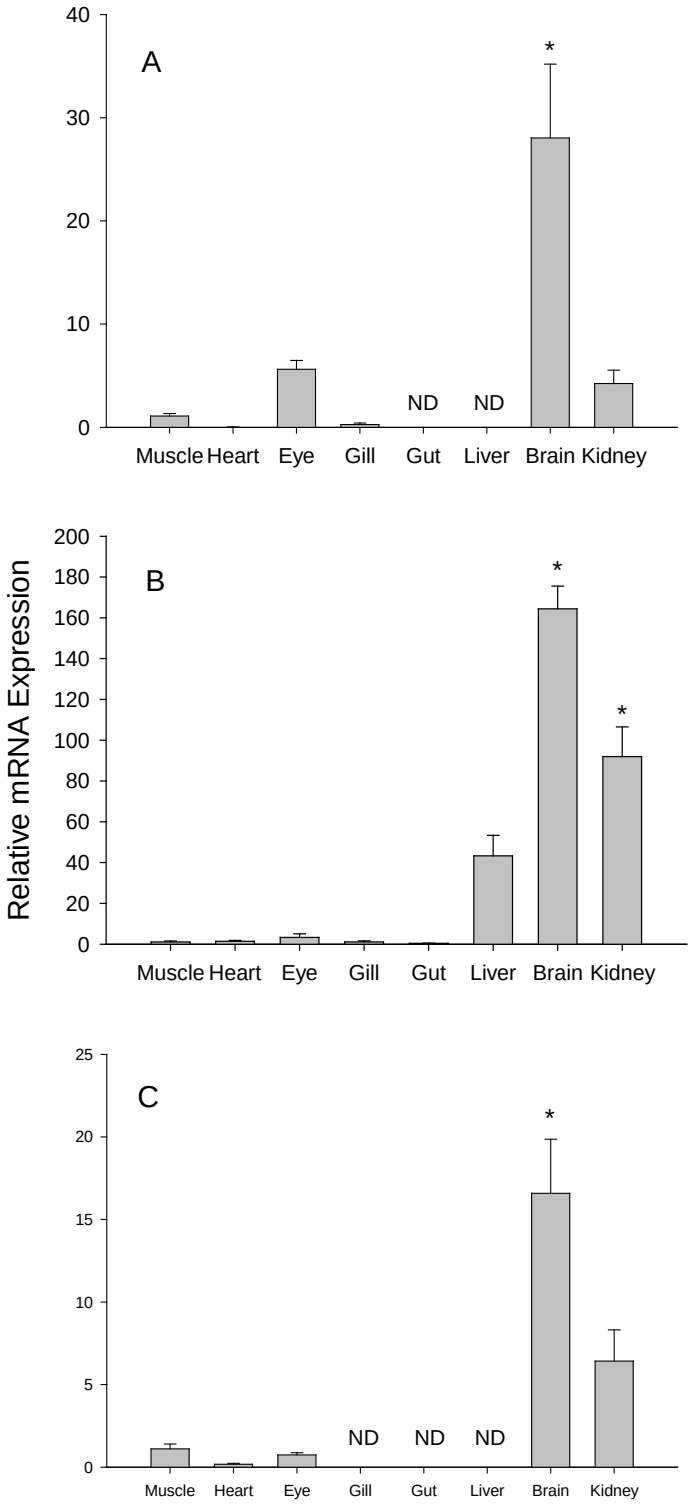

A
B
C
Relative mRNA Expression
Muscle
Heart
Eye
Gill
Gut
Liver
Brain
Kidney
ND
*

Figure 4.2

Figure 4.3

Relative mRNA expression of *TH1* (black bars) and *TH2* (grey bars) in eye, brain, heart, and kidney of adult zebrafish. All values were standardized to 18S ribosomal RNA and expressed relative to the expression of *TH1* in the same tissue. Values = mean + SEM, N = 5. * indicates significant difference from expression of *TH1* in the same tissue.

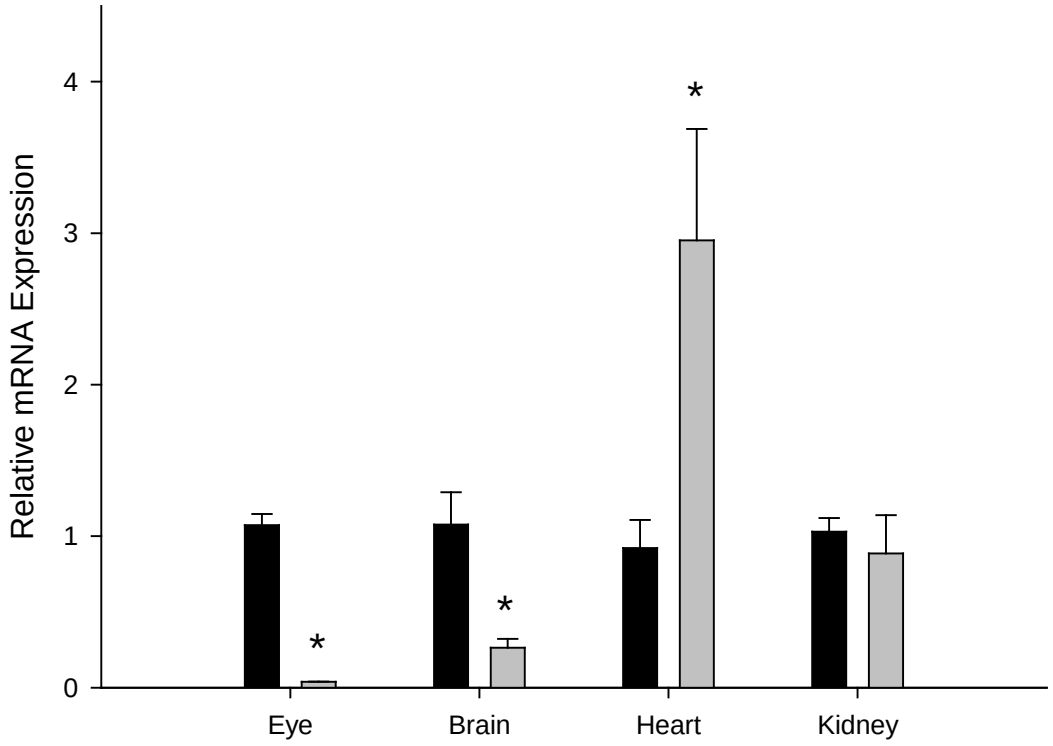


Figure 4.3

Figure 4.4

Relative mRNA expression of *TH1*, *TH2*, *DβH*, *β1AR*, *β2aAR* and *β2bAR* in whole zebrafish larvae exposed to hypoxia 2 days (black bars) or 4 days (grey bars) beginning at 5 dpf. All values were standardized to 18S ribosomal RNA and expressed relative to the expression of the same gene in control (normoxic) larvae. Values = mean + SEM, N = 5. * indicates significant difference from 1 (dashed line; $p < 0.05$).

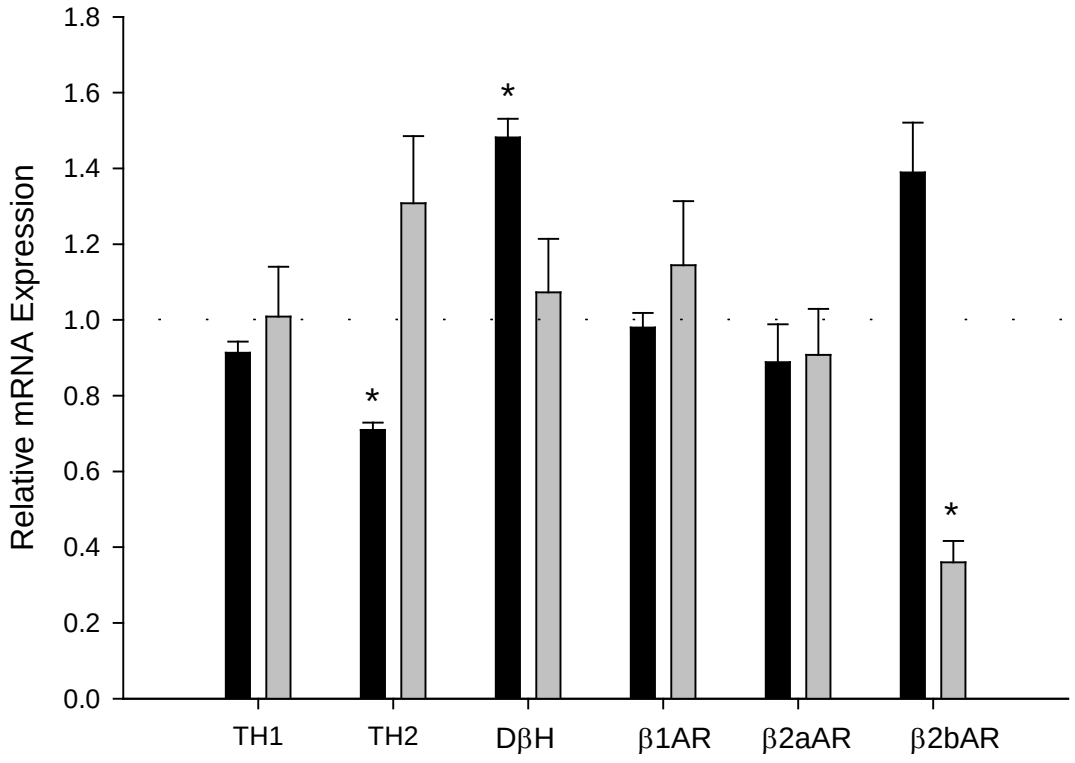

Relative mRNA Expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
TH1
TH2
DβH
β1AR
β2aAR
β2bAR

Figure 4.4

Figure 4.5

Relative mRNA expression of *TH1*, *TH2*, *DβH*, *β1AR*, *β2aAR* and *β2bAR* in eye, brain, heart, and kidney of adults exposed to hypoxia (PO_2 = 30 Torr) for (A) 2 days or (B) 4 days. All values were standardized to 18S ribosomal RNA and expressed relative to the expression of the same gene in control (normoxic) fish. Values = mean + SEM, N = 5. * indicates significant difference from 1 (dashed line; $p < 0.05$).

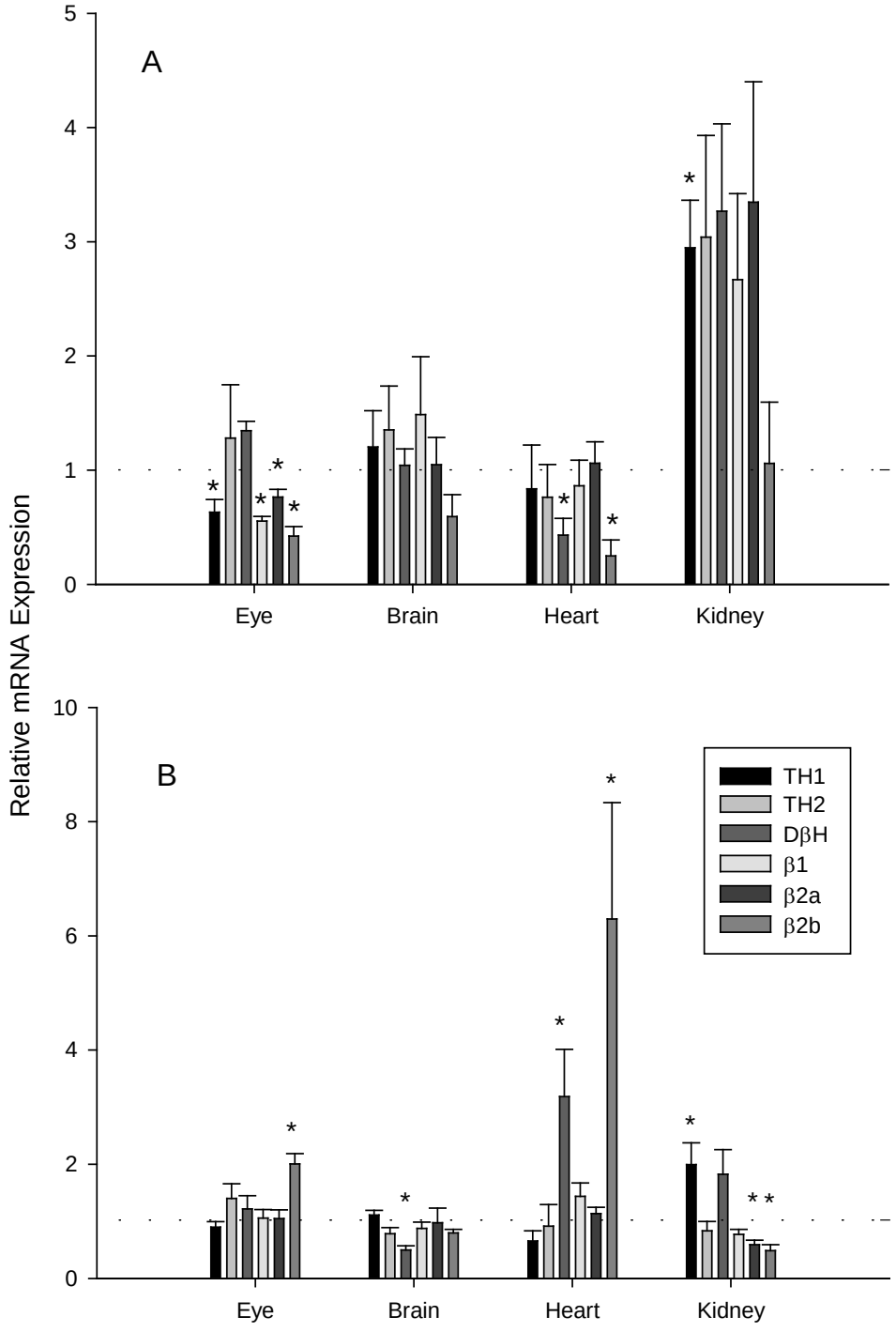

A
B
Relative mRNA Expression
Eye
Brain
Heart
Kidney
TH1
TH2
DβH
β1
β2a
β2b

Figure 4.5

Figure 4.6

Relative TH protein levels in whole larvae exposed to either normoxia (black bars) or aquatic hypoxia (PO_2 = 30 Torr; grey bars; A) for 2 days or 4 days beginning at 5 dpf, determined by Western blot analysis. (B) Representative duplicate protein samples analyzed by Western blot for protein expression of TH, actin, and total protein from "control" (normoxic) larvae and larvae exposed to hypoxia for 2 or 4 days. MemCode™ protein staining was used as a loading control for Western blots, as described in Materials and Methods. Values = mean + SEM, N = 5-7.

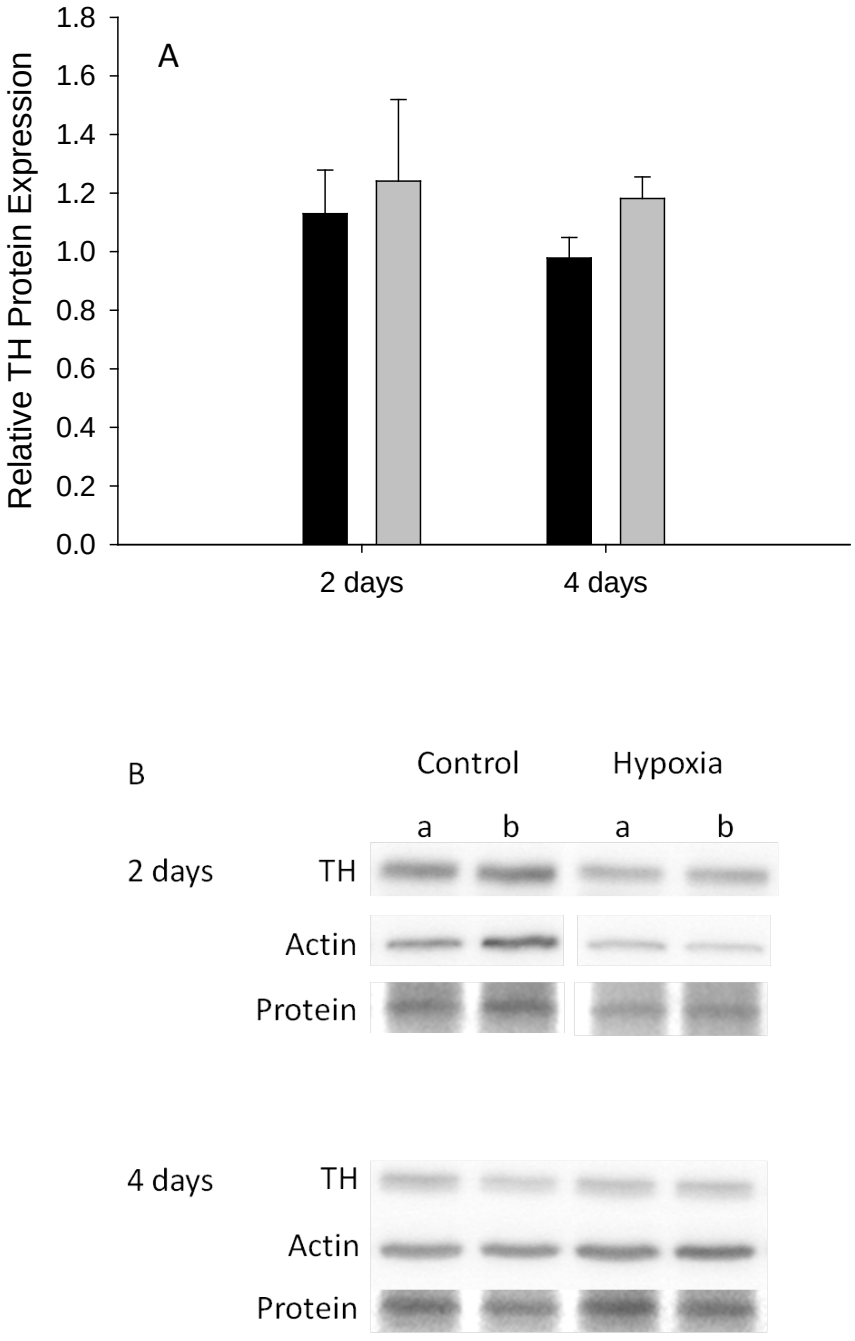

A
Relative TH Protein Expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2 days
4 days
B
Control
Hypoxia
a
b
a
b
2 days
TH
Actin
Protein
4 days
TH
Actin
Protein

Figure 4.6

Figure 4.7

Norepinephrine (NE; grey bars) and epinephrine (E; white bars) content of whole larvae at 1 hpf and from 1 to 5 dpf (A). Stacked plot of NE and E levels in whole larvae exposed to 2 days (B) or 4 days (C) of normoxia or aquatic hypoxia (PO_2 = 30 Torr) from either 3 dpf or 5 dpf. Total catecholamine content is represented by the height of the plot (NE + E). Values = mean + SEM, N = 8-11. Letters (a-d) indicate significant differences between catecholamine levels at various developmental stages (top letters for NE, bottom for E). * indicates significant difference from value in normoxic larvae (p <0.05).

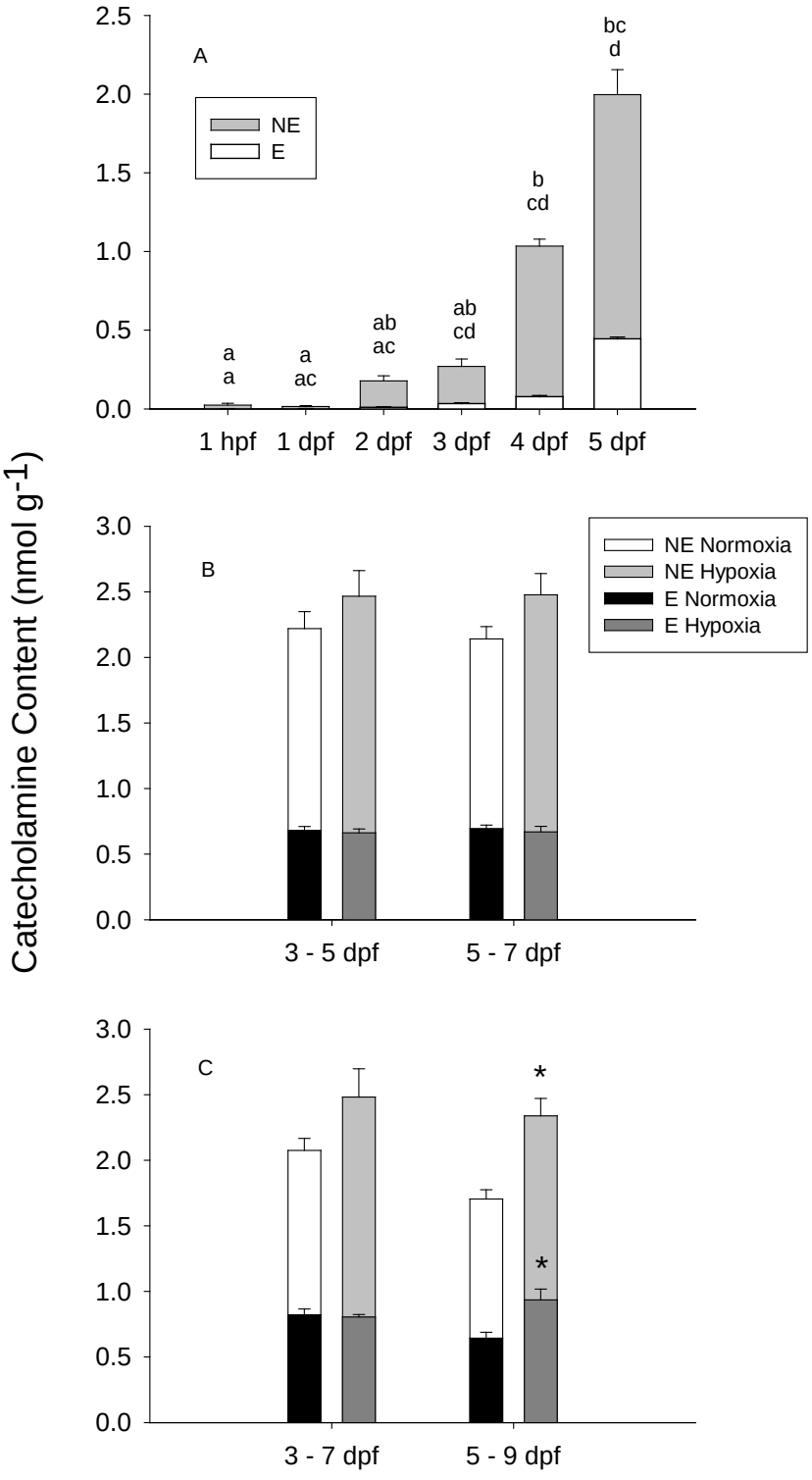

Catecholamine Content (nmol g-1)
A
NE
E
a
a
a
ac
ab
ac
ab
cd
b
cd
bc
d
1 hpf
1 dpf
2 dpf
3 dpf
4 dpf
5 dpf
B
NE Normoxia
NE Hypoxia
E Normoxia
E Hypoxia
3 - 5 dpf
5 - 7 dpf
C
*
*
3 - 7 dpf
5 - 9 dpf

Figure 4.7

Figure 4.8

Norepinephrine (NE) and epinephrine (E) content of (A) brain, (B) eye, (C) heart , and (D) kidney of adult zebrafish exposed to normoxia or hypoxia (PO_2 = 30 Torr) for 2 or 4 days Total catecholamine content is represented by the height of the plot (NE + E). Values = mean + SEM, N = 10 for brain and eye, 4-5 for heart and kidney. * indicates significant difference from value in normoxic fish (p <0.05).

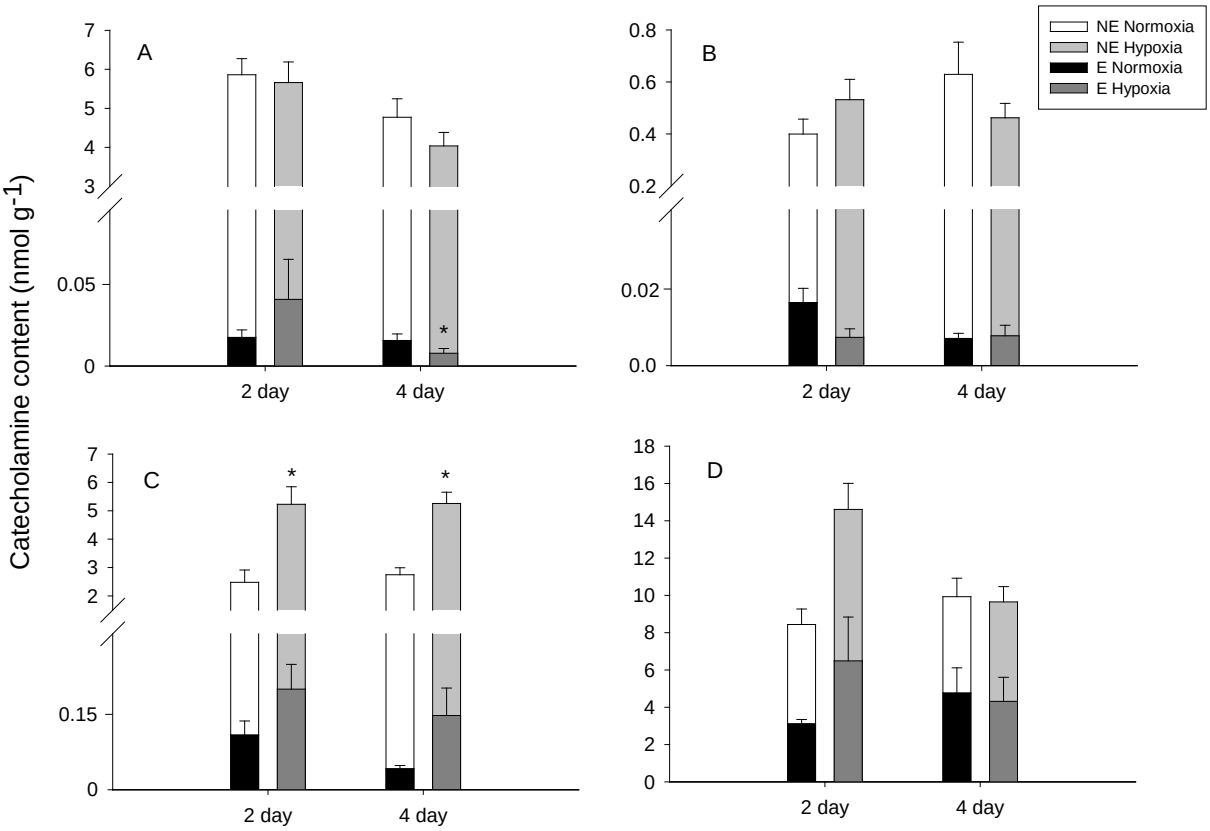

Catecholamine content (nmol g-1)
A
B
C
D
NE Normoxia
NE Hypoxia
E Normoxia
E Hypoxia
2 day
4 day
*

Figure 4.8

Figure 4.9

Heart rate of zebrafish larvae exposed to either normoxia or hypoxia (PO_2 = 30 Torr) from 5 dpf for 2 days (A) or 4 days (B) and anaesthetized in MS-222 (black bars) then treated with norepinephrine (10^{-4} M; grey bars) for 10 min.. Values = mean + SEM, N= 8-10. * indicates a significant effect of NE within either the normoxic or hypoxic groups. + indicates a significant effect of hypoxia in the control (no NE treatment) or NE treated groups. Significant difference was found by two-way repeated measures ANOVA within (B) between total normoxic and hypoxic values, as well as between total control and norepinephrine treated values (p <0.05).

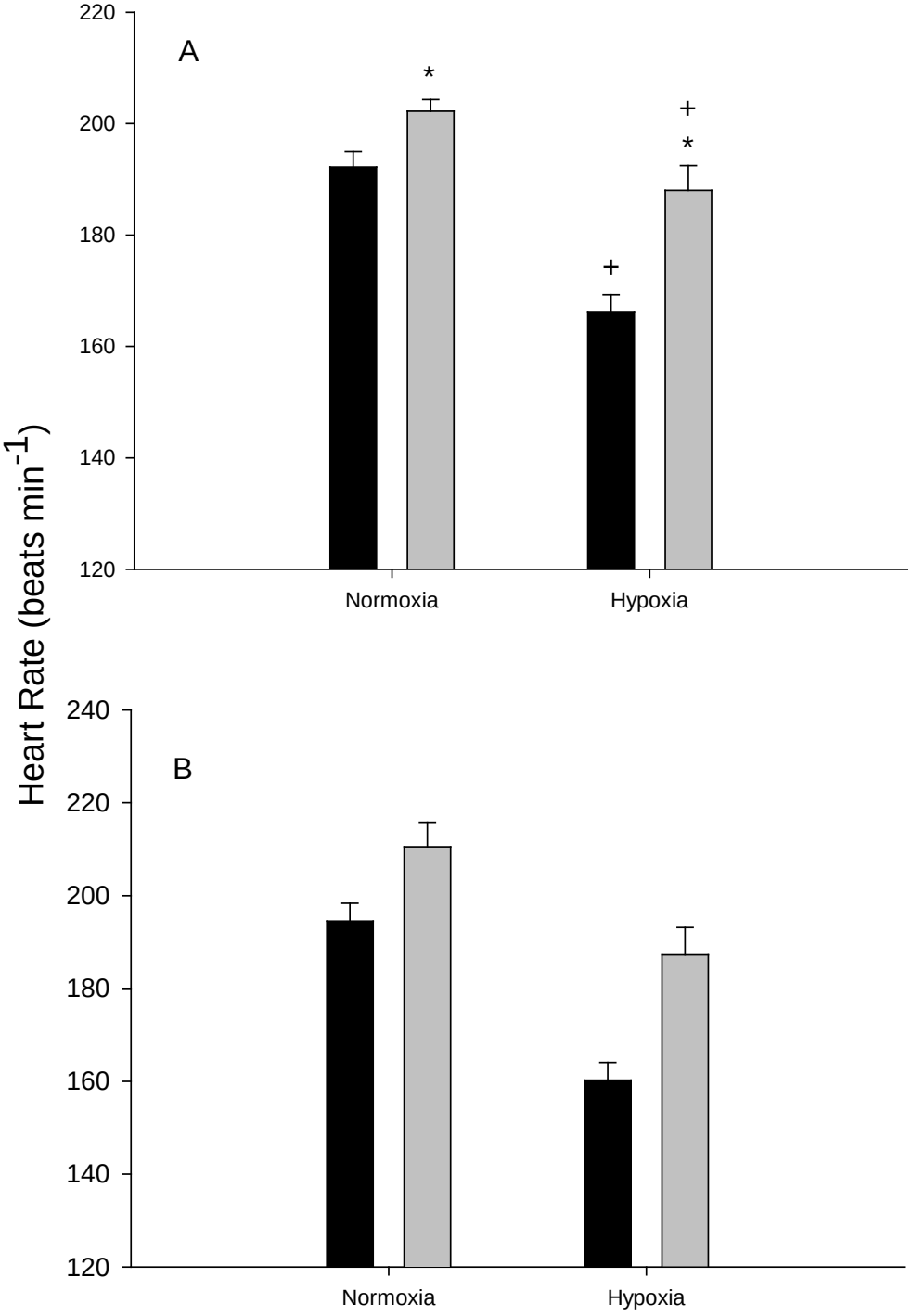

A
B
Heart Rate (beats min-1)
220
200
180
160
140
120
240
220
200
180
160
140
120
*
+
+
*
Normoxia
Hypoxia
Normoxia
Hypoxia

Figure 4.9

Table 4.1: Real-time PCR primer sets used in current study.

	GenBank Ref	Forward Primer (5'-3')	Reverse Primer (5'-3')	Length	Eff %	R^2
TH1	XM_682702	GATCGGAGACATTGCCTTCA	TCGGAGGGTGGAGTAGACCT	106	101.0	0.982
TH2	NM_001001829	GCTTCGTGTTTGAGGAGGAG	AAAGACTTTGCCAGCCTTGA	104	84.7	0.995
DβH	XM_682825.1	TGCCTATGGGAGGAGAAGGA	TAAACGAATGCCCGAGGAGT	104	117.7	1.000

Primers for β1AR., β2aAR, β2bAR, and 18S are as per Steele et al. (2009)

Discussion

The present study is the first to investigate the interactive effects of hypoxia and development on catecholamine synthetic pathways and cardiac function in larval zebrafish. Larvae showed a significant bradycardia after 2 and 4 days of exposure to hypoxic water despite the fact that whole body catecholamine content was significantly increased after 4 days, which would presumably be applying some additional adrenergic stimulation to the heart tissue of these young animals. Hypoxic bradycardia is a phenomenon that has been reported extensively in adult fish (for review see Farrell, 2007) and also in the larvae of zebrafish (Barrionuevo and Burggren, 1999; Bagatto et al., 2005; Steele et al., 2009; Barrionuevo et al., 2010). The principle origin of this hypoxic bradycardia was shown to be activation of the cardiac M_2 muscarinic receptor, although at a PO_2 of 30 Torr, bradycardia was not completely prevented by the loss of M_2 receptor function (Steele et al., 2009). Potentially, alterations to the cardiac adrenergic receptors and/or changing levels of circulating catecholamines could be influencing the net cardiac response to hypoxia, especially given the possibility of an inhibitory β2AR in the zebrafish heart (Chapter 3). Two lines of evidence suggest that the cardiac β2aAR may play an inhibitory role in zebrafish. First, in M_2 loss of function larvae, exposure to procaterol (a β2AR agonist) caused a decrease in heart rate implying negative chronotropic effects of the β2aAR in the absence of parasympathetic tone (Steele et al., 2009). Second, loss of function of the β2bAR receptor in conjunction with either β2aAR or β1AR also caused a significant increase in resting heart rate in larvae (Chapter 3). Therefore, if this receptor had some cardioinhibitory role to complement M_2 receptor mediated hypoxic bradycardia, mRNA expression of *β2bAR* could be expected to increase with hypoxia. However, *β2bAR* mRNA was significantly decreased in larvae after 4 days of hypoxia exposure (Figure 4B). In comparison, *β2bAR* mRNA expression was lower than controls in

the heart of adults after 2 days of hypoxia but higher after 4 days (Figure 4.5). Therefore, while *β2bAR* mRNA expression is affected by hypoxia in both larvae and adult hearts, no conclusion can be reached based on current data for an inhibitory role for this receptor in hypoxia.

Plasma membrane β-adrenergic receptors are prone to desensitization and internalization during sustained agonist stimulation (see Introduction). Despite a significant increase in whole body catecholamines, which would presumably affect circulating levels, larvae continued to respond to exogenous norepinephrine with increased heart rates after 2-4 days of hypoxia. It is possible that cardiac β-adrenergic surface expression was not affected by hypoxia in the present study, as shown previously in Chinook salmon *Oncorhynchus tshawytscha* (Gamperl et al., 1998). Alternatively, if the surface βARs were indeed down-regulated by hypoxia in larval heart in the present study, it is possible that the sensitivity of the remaining βARs to exogenous catecholamines was increased. Such a phenomenon was reported for rainbow trout red blood cells (RBCs) exposed to hypoxia *in vitro* (Reid et al., 1993) or collected from repeatedly chased fish (Perry et al., 1996). Here, catecholamines caused a greater increase in intracellular cAMP in hypoxic RBCs, or RBCs collected from stressed fish, *versus* their respective controls. It would therefore be informative to determine if the hearts of either larval or adult zebrafish not only had decreased surface expression of βARs in hypoxia, but if the sensitivity of hypoxic hearts to adrenergic stimulation was increased in hypoxia. Technical limitations due to minute tissue sizes, however, are currently limiting such experiments.

The present study shows that catecholamines are detectable in zebrafish embryos as early as 1 hpf and increase significantly up to 5 dpf (Figure 4.7A). The presence of catecholamines and the mRNA for catecholaminergic enzymes at 1 hpf precedes the onset of

zygotic transcription (Kane and Kimmel, 1993) and indicates a maternal source for both. The increase in norepinephrine and epinephrine levels across development, however, suggests an early onset of endogenous catecholamine synthesis in zebrafish larvae. A similar situation was reported in *Xenopus*, where both epinephrine and norepinephrine were detected in unfertilized oocytes but increased significantly up to embryonic stage 4-6 (Devic et al., 1997). Interestingly, *β1AR* mRNA and binding sites were also expressed in whole *Xenopus* oocytes prior to the onset of zygotic transcription (Devic et al., 1997). The simultaneous presence of both β1AR protein the adrenergic ligands at this pre-zygotic stage could signify that the function of this receptor is necessary for some as yet undefined developmental processes in the embryo. Indeed, *β1AR* mRNA expression has also been detected in 1 hpf zebrafish embryos (Chapter 3). Furthermore, in *Xenopus* larvae, the presence of catecholamines and chromaffin cells in the heart prior to the development of sympathetic innervation may be involved in early cardiac control in this species (Kloberg and Fritsche, 2000). Sympathetic tone in the larval zebrafish heart by treatment with propranolol has been revealed as early as 5 dpf (Schwerte et al., 2006; Mann et al., 2010). Changes in heart rate due to βAR loss of function, however, were detected in larvae as early as 4 dpf (Chapter 3). Therefore, the presence of catecholamines in embryos prior to the onset of zygotic transcription, as well as the early onset of catecholamine accumulation in whole larvae, may represent a means of cardiac control in larval zebrafish prior to the development of cardiac adrenergic innervation.

Total catecholamine levels in whole larvae at 5 dpf (Figure 4.7A) are comparable to those in adult tissues (Figure 4.8). However, without the ability to examine specific tissues, it is impossible to determine the relative contribution of each larval tissue to total body levels. In *Xenopus* late stage larvae (NF 57), levels of norepinephrine and epinephrine were highest in kidney (75 and 100 ng mg protein^{-1}) compared to heart (15 and 12 ng mg protein^{-1}) and whole

body without these organs (1.5 and 2 ng mg protein^{-1}; Kloberg and Fritsche, 2002). In adult zebrafish catecholamine levels were also highest in kidney, followed by brain and heart, respectively (Figure 4.8C and 4.8D). Therefore, if whole larva catecholamine content can be mostly attributed to these developing tissues, then the significant increase in whole larvae norepinephrine and epinephrine seen after 4 days of hypoxia exposure (Figure 4.7C) could be attributed to any of these tissues.

The overall increase in whole larva catecholamine content in hypoxia is intriguing; if the activity of TH and/or DβH is decreased by oxygen availability, one might expect a decrease in catecholamine synthesis. The K_m of TH for molecular oxygen has not, to my knowledge, been measured for any fish species. However, it has been repeatedly shown that the K_m of TH for molecular oxygen in mammals is very close to the oxygen content of the tissues in normoxic (i.e. normobaric) animals (for references and summary see Rostrup et al., 2008). TH enzyme activity does go down in certain brain regions of chronically hypoxic rats (Gozal et al., 2005), but up in others (Soulier et al, 1995; Gozal et al., 2005). Increases in TH activity could be related to an increase in protein levels (Pepin et al., 1996; Hui et al., 2003) or post-translational phosphorylation of the protein (Hui et al., 2003; Gozal et al., 2005). While no data appear to exist on TH enzyme activity in the tissues of hypoxic fish, the present study shows both *TH1* and *TH2* gene expression are sensitive to hypoxia in both adult and larval zebrafish, even though protein levels of TH in larvae are unaffected after either 2 or 4 days of hypoxic exposure (Figure 4.6). This apparent discrepancy between changes in TH mRNA and protein levels in hypoxia is not unique to the present study (e.g. Gozal et al. 2005). Comparatively, in mice overexpressing human TH, an increase of more than 50 fold in TH mRNA expression in the brain resulted in comparatively small changes in TH protein content (Kaneda et al. 1991). This suggests that the stability of the TH protein could be more tightly

linked to post-translational modifications than absolute changes in mRNA expression. Therefore, if an increase in catecholamine production is responsible for the significant increase in catecholamines observed in hypoxic larvae after 4 days (Figure 4.7C), it is likely due to overall changes in TH enzyme activity rather than changes in protein expression. However, it is worth mentioning that detecting changes in TH protein expression may be confounded by the presence of the two unique genes/proteins. Chen et al. (2009) correlated TH immunoreactivity (using a different commercial mouse monoclonal antibody) with the highly localized mRNA expression of *TH1* in the brain, with no apparent overlap with *TH2* expression. This could be due to the fact that zebrafish *TH1* has a higher amino acid sequence similarity to the mouse TH gene (83%) than zebrafish *TH2* (77%; Chen et al., 2009). The TH antibody used in the present study was previously validated for immunoreactivity in zebrafish larvae by immunohistochemistry (e.g. Thirumalai and Cline, 2008; Kojima et al., 2009), however, the specificity of this antibody for either zebrafish TH isoform was not established. The protein levels presented in Figure 4.6 can therefore represent a mix of TH1 and TH2 protein levels, or be biased towards one or the other.

Total norepinephrine content decreased in certain areas of the brain of rainbow trout exposed to chronic hypoxia (Pouliot et al., 1988), and also in the whole brain of chronically anoxic crucian carp (Nilsson et al., 1989). While no change in brain norepinephrine was observed in zebrafish in the present study, there was a significant decrease in epinephrine content. Considering that epinephrine contributes only a miniscule component of total catecholamines in the brain (<1%; Figure 4.8C), the physiological significance of this result is questionable. No change in catecholamine content was observed in the kidney of adult zebrafish exposed to hypoxia (Figure 4.8D), however Nilsson et al. (1989) observed a significant decrease in kidney norepinephrine levels in crucian carp exposed to 160 h of

anoxia. Catecholamine release from chromaffin cells in the head kidney of teleosts is stimulated by hypoxia (see Reid et al., 1998), therefore it is somewhat surprising a decrease in catecholamine content in this tissue was not seen in the present study. Perhaps a compensatory increase in catecholamine production had occurred by the time of first sampling in these fish (i.e. after 2 days of hypoxic exposure). Of the four tissues examined in adult zebrafish, the heart showed a robust increase in catecholamine content after 2 and 4 days of exposure to hypoxia. Interestingly, the hearts of normoxic zebrafish had relatively high levels of both catecholamines, with heart norepinephrine being comparable to brain levels. Heart norepinephrine levels were about 2.5 nmol g^{-1} (or 458 ng g^{-1}), and brain levels were about 5 nmol g^{-1} (or 916 ng g^{-1}) in control fish (Figure 4.8C). Comparatively, the eel (*Anguilla anguilla*) had a similar brain:heart norepinephrine ratio (~2:1) at its seasonal peak (0.282 versus 0.145 nmol g^{-1}; Le Bras, 1984) although absolute levels were much lower than in zebrafish. There appears to be high inter-specific variation in heart catecholamine content (e.g. Jarrott, 1970; Chang and Rand, 1971; Temma et al., 1990). For example, norepinephrine levels of 20 ng g^{-1} were reported in the carp heart (Temma et al., 1990), whereas the norepinephrine concentration in guinea pig (*Cavea porcellus*) atrial tissue is 3440 ng g^{-1} (Jarrott, 1970). Regardless of absolute levels, Jarrott et al. (1970) examined the uptake and metabolism or norepinephrine in the heart of several species, with the understanding that tissue uptake of catecholamines may be an important measure by which animals clear and metabolize locally released catecholamines (e.g. from sympathetic innervation). If this is the case, then the increase in catecholamines in the heart of zebrafish exposed to hypoxia in the present study may have been caused by uptake from increased circulating catecholamines. Alternatively (or in parallel), this accumulation of catecholamines in the heart could be a reduction in oxygen dependent clearance of catecholamines (e.g. by monoamine oxidase). A

similar mechanism might account for the accumulation of catecholamines in whole zebrafish larvae after 4 days of hypoxia exposure (Figure 4.7C).

The results of the present study show that *TH1* and *TH2* are differentially expressed in the eye, brain, heart, and kidney of adult zebrafish. *TH1* mRNA levels are much higher in eye and brain, which is in agreement with similar analysis by Chen et al. (2009). However, while the present study found no difference between *TH1* and *TH2* expression in the kidney, Chen et al. (2009) reported that *TH2* expression was much higher than *TH1* in kidney. Conversely, Candy and Collet (2005) showed only *TH1* expression in the kidney of the barramundi and no detectable expression of *TH2*. The present study also found that *TH2* expression was higher in heart than *TH1* (Figure 4.3), whereas Candy and Collet (2005) found no detectable expression of either of these transcripts in the barramundi heart. The diversity of expression patterns between these two genes in zebrafish is not unusual when considering the development of novel or tissue specific function in the duplicated genes of many teleostean species. Indeed, the fact that *TH1* is generally expressed at higher levels in neural tissues such as brain and eye (Chen et al., 2009; present study) may indicate that this isoform is more critical in synthesizing neurotransmitters, including dopamine, in the brain. Much of the work that has been done on TH expression in larval zebrafish is concerned with the development of aminergic neurons. Qualitatively, *TH1* mRNA expression was detected earlier (1 dpf) than *TH2* (4 dpf) in the brain of larval zebrafish on the basis of *in situ* hybridization (Chen et al., 2009). The distribution of these transcripts within the larval brain also appears to be highly localized with little overlap (Chen et al., 2009). A loss of function or transgenic over-expression technique for both of the TH genes in zebrafish would likely highlight the relative contribution of each of these proteins to catecholamine production, as well as the ability of

both larval and adult zebrafish to mount an adrenergic stress response to environmental stressors such as hypoxia.

In conclusion, this study is the first to demonstrate that catecholamine levels are modified by environmental hypoxia in the early life stages of zebrafish. mRNA expression of genes required for catecholamine synthesis, particularly *TH2* and *DβH*, were affected by hypoxia although the protein expression of the rate limiting TH enzyme was not altered. Finally, the hearts of hypoxic larvae remain sensitive to exogenous norepinephrine exposure, although the absolute heart rates achieved were not as high as in normoxic larvae. Thus, hypoxic larvae are clearly able to maintain a cardiac adrenergic response even after chronic exposure to increased tissue catecholamine levels.

Acknowledgements

Funding for this project was provided by a National Science and Engineering Research Council (NSERC) of Canada grant to S.F.P. and a NSERC post-graduate scholarship to S.L.S. I would especially like to thank Jen Jeffrey, as well as Yusuke Kumai, Paul Craig, and Chris Le Moine for all their help and advice with the Western blot analysis.

Chapter 5.

General Discussion

Hypoxic Bradycardia and Development

Hypoxic bradycardia in fish is a theme explored extensively within the present thesis, however its potential physiological benefits to zebrafish larvae in particular has not been discussed. Indeed, even in adult fish, the physiological benefit of hypoxic bradycardia is unclear. Several studies sought to test the hypothesis that bradycardia helps to enhance branchial gas transfer, thereby increasing arterial blood oxygen levels when environmental oxygen is limiting (Taylor and Barrett, 1985; Perry and Desforges, 1996). However, preventing bradycardia using pharmacological intervention (atropine) did not affect arterial blood oxygen tension in hypoxic fish when compared to non-atropinized rainbow trout (Perry and Desforges, 1996), although complications arising from non-target effects of the pharmaceuticals used may have confounded these results. Alternatively, a lower heart rate in hypoxia could afford several physiological advantages to the heart itself, such as increased blood retention time and increased stretching of the cardiac chambers which could enhance oxygen delivery to the myocardium (Farrell et al., 2007). Whether or not any of these beneficial effects are relevant to the adult fish, it is worth noting that none of these should apply to the larval zebrafish, particularly at the developmental stages examined in the current work. Neither routine metabolic rate nor heart rate are affected in zebrafish larvae up to 15 dpf when haemoglobin function is eliminated (Pelster and Burggren, 1996; Jacob et al., 2002), suggesting diffusive oxygen transport is sufficient for aerobic metabolism at these early stages. Therefore, it is particularly interesting from a developmental point of view that zebrafish larvae both decrease their heart rate in hypoxia (Chapter 2 and Chapter 3), and that it is induced by the same parasympathetic pathway as is seen in adult fish (Chapter 2). Several possibilities exist to explain these observations. First, bradycardia in larval fish can occur once cardiac parasympathetic tone is established, simply due to changes in signaling by the

developing vagus that are not related to any physiological benefit to the organism. However, this would not explain why M_2 receptor mRNA expression is concurrently increased in the heart of zebrafish larvae reared in hypoxia as seen at 4 dpf (Chapter 2), a change that is presumably linked to the development of bradycardia. Alternatively, the development of hypoxic bradycardia could be imparting some cardiorespiratory benefits to the larvae that have yet to be elucidated and that may (or may not) be applicable to the adult fish. Indeed, it would seem counterproductive for the larva to invest resources into an unnecessary process during a period where extensive growth and development is taking place. Therefore, when evaluating the observation that the M_2 receptor regulates hypoxic bradycardia in zebrafish larvae, it is important to consider this result in the context of development as well as how it may apply to the fish at all life stages.

The parasympathetically-mediated hypoxic bradycardia seen in larvae in Chapter 2 may not have the same cardiorespiratory function as it would in adult fish. In a review commenting on some of the frequent assumptions made when working with developing animals, Burggren (2005) observed that the developmental increase in heart rate and oxygen consumption seen in zebrafish up to 20 dpf is disconnected from typical allometric patterns. Specifically, heart rate and mass-specific oxygen consumption both increase as body mass increases; these changes are likely linked to rapid growth and organogenesis at the larval stage. This window of development encompasses the developmental period (up to 15 dpf) within which zebrafish appear to rely exclusively on diffusion for oxygen consumption (Jacob et al., 2002). Therefore, under normal (e.g. normoxic) conditions, it would seem that adequate oxygen is supplied to the developing zebrafish tissues even when oxygen demand is increasing. The same may not be true for fish exposed to hypoxic conditions. Indeed, the effects of environmental hypoxia may be magnified at the level of the chorion (in unhatched

embryos) or at the surface of larvae due to the presence of what is termed the "boundary layer". This is a semistagnant layer of water which exists at the surface of the chorion and at the skin surface in hatched larvae, within which the dissolved oxygen content decreases with increased proximity to the animal (e.g. Cihuandu et al., 2007; Miller et al., 2008). The levels of dissolved oxygen at the surface of the chorion and larvae become even lower in situations of both chronic and acute hypoxia (Cihuandu et al., 2007; Miller et al., 2008). It is plausible that hypoxic bradycardia in zebrafish larvae is a mechanism unique to these early developmental stages to help increase oxygen uptake when oxygen levels at the skin become limiting. It is important to relate such a hypothesis to the capacity of blood to carry oxygen in zebrafish larvae, because it has already been stipulated that blocking haemoglobin function (either by carbon monoxide or phenylhydrazine exposure) has no effect on routine metabolic rate under normoxic conditions (Pelster and Burggren, 1996; Jacob et al., 2002). However, haemoglobin in young zebrafish larvae may allow for more efficient extraction of oxygen from water, since residual oxygen tensions were higher in carbon monoxide-treated fish than in control fish when measuring routine metabolic rate (Rombough and Drader, 2009). Taken together, the combined effects of functional haemoglobin and hypoxic bradycardia that develop in early life may be a physiological adaptation of fish larvae to increase their oxygen uptake in extreme hypoxia. To test these combined effects, it would be necessary to compare oxygen consumption in zebrafish larvae lacking both functional haemoglobin (e.g. by carbon monoxide exposure) and hypoxic bradycardia (e.g. by M_2 morpholino).

Gene Duplication and Sub-functionalization

Gene duplication, either following whole genome duplication events or localized gene duplications, has been critical for the evolution of new species and of gene function within the

plant and animal kingdoms. Occurrence of three major genome duplication events within the evolution of the chordates is a widely accepted concept (the "3R" hypothesis; see Meyer and Schartl, 1999; Taylor et al., 2001; Prince and Pickett, 2002). The most recent of these events occurred after the divergence of the teleosts from the tetrapods, and has been credited for the enormous amount of speciation that has occurred within this lineage (Taylor et al., 2001; Aris-Brosou et al., 2009). Persistent gene duplicates in fish include the *Hox* gene clusters (Amores et al., 1998) and α_2-adrenergic receptors (Bylund, 2005; Aris-Brosou et al., 2009). The fate of duplicated genes can be jeopardized due to the potential for functional overlap between paralogs. Therefore the redundant copy can accumulate deletions and mutations that render it non-functional. Alternatively, two scenarios for the retention of gene function exist. One of the duplicated genes can acquire and entirely new function within the organism (i.e. neo-functionalization), or the sum of the two gene products become necessary to maintain the same function as the parent gene (i.e. sub-functionalization; see Prince and Pickett, 2002).

The present thesis examines the expression and function of two sets of gene paralogs in zebrafish, *TH1* and *TH2*, *β2aAR* and *β2bAR*. The selective pressures that have caused zebrafish to retain these two functional sets of paralogs are unknown. Interestingly, zebrafish have single functional genes for most of the components of the hypothalamus-pituitary-interrenal (HPI) axis, having lost function of the paralogs that most other teleosts have retained (see Alsop and Vijayan, 2009). Again, the exact nature of this phenomenon is unknown, although Alsop and Vijayan (2009) postulated that this streamlining of gene products for the zebrafish HPI stress axis may reflect evolutionary pressures based on their life history. Zebrafish belong to the subfamily Rasborinae, which is made up of relatively small species and therefore may have adapted to different stressful conditions than larger teleosts (Alsop and Vijayan, 2009) such as intense predation (for example). In this context it is

difficult to rationalize the function of two TH and β2AR genes in zebrafish, since the "fight-or-flight" adrenergic stress response is (arguably) equally important in situations such as predator avoidance than the HPI stress response. The retention of these duplicated genes and their products could, instead, be related to functional compartmentalization between distinct tissue types. TH, for example, is required for catecholamine synthesis in both neuronal and non-neuronal (i.e. chromaffin) cell types. The β2ARs are expressed in a wide variety of tissues and, as has been implied in the present thesis, the β2bAR may be more critical for cardiac control in zebrafish than the β2aAR.

In Chapter 4, it was shown that the pattern of *TH1* and *TH2* mRNA expression is different across development and across tissue types. *TH1* mRNA levels peaked in developing larvae before *TH2* levels, and *TH1* mRNA expression was higher in the brain and eye than *TH2*. This pattern confirms that seen by Chen et al. (2009), who also demonstrated that *TH1* mRNA is detectable in the brain before that of *TH2*. Discrete areas of non-overlapping *TH1* and *TH2* mRNA expression in the brain of developing zebrafish have also been identified (Chen et al., 2009). Therefore, sub-functionalization of the different TH genes may not only be related to expression in different tissue types, but also to different specific regions of the brain. The present study does not attempt to characterize the function of each of the TH genes in catecholamine synthesis within zebrafish larvae. As previously mentioned, targeted gene deletion of TH is embryonically lethal in mice (Kobayashi et al., 1995; Zhou et al., 1995). The ability to individually repress TH1 and TH2 gene function by morpholino in zebrafish larvae would demonstrate if one or the other is individually necessary for maintaining life early in development. Alternatively, zebrafish retaining function of only one TH paralog may not be able to increase overall catecholamine tissue levels as seen in Chapter 4 (Figure 4.7). Finally, the ability of TH1 or TH2 morphants to withstand environmental hypoxia may be

altered. These experiments present a fascinating avenue for future research into the function of each of these genes in catecholamine production in zebrafish larvae.

The sub-functionalization of the zebrafish β2aAR and β2bAR has been previously postulated by Wang et al. (2009), who investigated the role of the β2AR in the formation of skin pigmentation. They showed that zebrafish larvae lacking function of the β2aAR, but not the β2bAR, were hypo-pigmented (Wang et al., 2009). This was consistent with the observation that the mRNA expression of *β2aAR* was higher in the skin of adult zebrafish than β2bAR. It is interesting to note that Wang et al. (2009) did not observe any morphants in which both β2aAR and β2bAR function was removed. The combined loss of function of the β2ARs may have caused an even more pronounced effect on pigmentation. No such phenomenon was observed in the present studies, however, it was not the focus of attention in the present experiments.

Present data suggest that the two β2ARs in zebrafish have unique function in this animal, at the level of the heart but likely also in other tissues. First, the two β2ARs have much different ligand binding affinity profiles as well as unique ability to initiate adenylyl cyclase activity in response to procaterol exposure (Chapter 3). Secondly, sub-functionalization within the heart could explain why the β2bAR has a predominating effect on the negative chronotropy seen in β2AR knockout fish. In Chapter 3, only larvae lacking both β2a- and β2bAR function together had significantly higher heart rates than controls. Interestingly, a similar response was seen when β2bAR and β1AR function are removed together, suggesting that the removal of cardioinhibition by removing β2bAR function overpowers the effect of β1AR loss of function alone. The cultured cell line used in Chapter 3 to determine adenylyl cyclase activity related to βAR activation was ideal for this purpose, based on their low background activity of adenylyl cyclase and endogenous βARs. In order to

make a concrete link between inhibitory β2bAR signaling and chronotropy in the zebrafish cardiomyocyte, it would be more helpful to obtain cultured heart cells from zebrafish lacking expression of or overexpressing this receptor. In this medium, the sensitivity of contraction rate to PTX inhibition would be a clear indication of whether or not zebrafish β2ARs are functionally linked to inhibitory G proteins.

Alternative Effects of Loss of Gene Function

The methodology used to cause loss of gene function in both mammals and fish presents a situation at the cellular and tissue level that is not entirely comparable to pharmacological methods where receptor function may be temporarily blocked. Not only is the function of the receptor inhibited, but the protein itself is not present. This is an important distinction, first because even unactivated β1- and β2ARs (i.e. receptors not bound to an agonist) can display spontaneous activity in the cardiomyocytes of mice (see Port and Bristow, 2001). Therefore, changes in heart function seen in βAR loss of function animal models could also reflect a change in the balance of basal intracellular 3333ing properties between adrenergic and cholinergic receptors.

Another interesting factor which potentially affects surface expression of adrenergic receptors is the formation of receptor homo- or heterodimers. Evidence of functional β2AR homodimerization has been described in cultured cells expressing human β2AR, such that the chemical disruption of these homodimers inhibited the intracellular accumulation of cAMP caused by β2AR stimulation (Hebert et al., 1996). Therefore, the disruption of β2AR dimerization with other βARs could be an explanation for the increased heart rate seen in β2a/β2bAR and β1/β2bAR morphants in Chapter 3. β2AR interactions also enhance the surface expression and signaling properties of both the α_{2C}- (Prinster et al., 2006) and α_{1D}-

(Uberti et al., 2005) adrenergic receptors by the formation of β2AR/αAR heterodimers. This concept has not been explored using fish receptors, but when considering the potential for cross-talk between receptor types *in vivo*, this would be particularly interesting because the α_1 adrenergic receptors are also implicated in regulating cardiovascular function. The α_{1D} receptor, for example, may be key in the development and maintenance of hypertension, at least in mammals (for review see García-Sáinz and Villalobos-Molina, 2004). If the loss of function of one or both of the zebrafish β2ARs affected α_1AR expression and signaling, α1AR mediated changes in heart rate and/or peripheral resistance could also be contributing to the increase in heart rate observed in the β2a/β2bAR morphants in Chapter 3. Revealing the structural and possibly functional dimerization between zebrafish adrenergic receptors would be a worthwhile avenue for future research.

Conclusion

This thesis provides some of the first data on the role that specific muscarinic and adrenergic receptors in regulating cardiac function in larval zebrafish, as well as the developmental dynamics of catecholamine production in these animals. Importantly, these studies have highlighted the importance of the M_2 muscarinic receptor in the development of hypoxic bradycardia, as well as the different functions of the zebrafish β2ARs in the heart. The discovery of an apparent cardioinhibitory role for the β2bAR is one that is unique among fish species. Altogether, these present thesis provides an exciting new perspective on cardiac control in fish, from both an evolutionary and developmental perspective.

LIST OF REFERENCES

Abrahamsson T. Phenylethanolamine-*N*-Methyl Transferase (PNMT) activity and catecholamine storage and release from chromaffin tissue of the spiny dogfish, *Squalus acanthias*. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 64: 169-172, 1979.

Abrahamsson T, Holmgren S, Nilsson S and Pettersson K. Adrenergic and cholinergic effects on the heart, the lung and the spleen of the African lungfish, *Protopterus aethiopicus*. *Acta Physiol Scand* 107: 141-147, 1979.

Ahlquist RP. A study of the adrenotropic receptors. *Am J Physiol* 153: 586-600, 1948.

Alsop D and Vijayan M. The zebrafish stress axis: molecular fallout from the teleost-specific genome duplication event. *Gen Comp Endocrinol* 161: 62-66, 2009.

Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M and Postlethwait JH. Zebrafish *hox* clusters and vertebrate genome evolution. *Science* 282: 1711-1714, 1998.

An M, Luo R and Henion PD. Differentiation and maturation of zebrafish dorsal root and sympathetic ganglion neurons. *J Comp Neurol* 446: 267-275, 2002.

Aris-Brosou S, Chen X, Perry SF and Moon TW. Timing of the functional diversification of α- and β-adrenoceptors in fish and other vertebrates. *Ann N Y Acad Sci* 1163: 343-347, 2009.

Ask JA. Comparative aspects of adrenergic receptors in the hearts of lower vertebrates. *Comp Biochem Physiol A Comp Physiol* 76: 543-552, 1983.

Axelsson M, Farrell AP and Nilsson S. Effects of hypoxia and drugs on the cardiovascular dynamics of the Atlantic hagfish *Myxine glutinosa*. *J Exp Biol* 151: 297-316, 1990.

Bagatto B. Ontogeny of cardiovascular control in zebrafish (*Danio rerio*): effects of developmental environment. *Comp Biochem Physiol A Mol Integr Physiol* 141: 391-400, 2005.

Barrionuevo WR and Burggren WW. O_2 consumption and heart rate in developing zebrafish (*Danio rerio*): influence of temperature and ambient O_2. *Am J Physiol* 276: R505-R513, 1999.

Barrionuevo WR, Fernandes MN and Rocha O. Aerobic and anaerobic metabolism for the zebrafish, *Danio rerio*, reared under normoxic and hypoxic conditions and exposed to acute hypoxia during development. *Braz J Biol* 70: 425-434, 2010.

Beavo JA, Bechtel PJ and Krebs EG. Activation of protein kinase by physiological concentrations of cyclic AMP. *Proc Natl Acad Sci USA* 71: 3580-3583, 1974.

Bernier N and Perry S. Control of catecholamine and serotonin release from the chromaffin tissue of the Atlantic hagfish. *J Exp Biol* 199: 2485-2497, 1996.

Bernstein D. Cardiovascular and metabolic alterations in mice lacking β1- and β2- adrenergic receptors. *Trends Cardiovasc Med* 12: 287-294, 2002.

Bernstein D, Doshi R, Huang S, Strandness E and Jasper JR. Transcriptional regulation of left ventricular beta-adrenergic receptors during chronic hypoxia. *Circ Res* 71: 1465-1471, 1992.

Braun MH, Steele SL, Ekker M and Perry SF. Nitrogen excretion in developing zebrafish (*Danio rerio*): a role for Rh proteins and urea transporters. *Am J Physiol Renal Physiol* 296: F994-F1005, 2009.

Bristow MR, Hershberger RE, Port JD, Minobe W and Rasmussen R. β1- and β2-adrenergic receptor-mediated adenylate cyclase stimulation in nonfailing and failing human ventricular myocardium. *Mol Pharmacol* 35: 295-303, 1989.

Brodde OE. β1- and β2-adrenoceptors in the human heart: properties, function, and alterations in chronic heart failure. *Pharmacol Rev* 43: 203-242, 1991.

Brodde OE. β1- and β2-adrenoceptor polymorphisms: Functional importance, impact on cardiovascular diseases and drug responses. *Pharmacol Ther* 117: 1-29, 2008.

Brodde OE and Michel MC. Adrenergic and muscarinic receptors in the human heart. *Pharmacol Rev* 51: 651-690, 1999.

Bruck H, Ulrich A, Gerlach S, Radke J and Brodde OE. Effects of atropine on human cardiac β1- and/or β2-adrenoceptor stimulation. *Naunyn Schmiedebergs Arch Pharmacol* 367: 572-577, 2003.

Burggren WW. Developing animals flout prominent assumptions of ecological physiology. *Comp Biochem Physiol A Mol Integr Physiol* 141: 430-439, 2005.

Bylund DB. Alpha-2 adrenoceptor subtypes: are more better? *Br J Pharmacol* 144: 159-160, 2005.

Candy J and Collet C. Two tyrosine hydroxylase genes in teleosts. *Biochim Biophys Acta* 1727: 35-44, 2005.

Caulfield MP. Muscarinic receptors--characterization, coupling and function. *Pharmacol Ther* 58: 319-379, 1993.

Cavalli A, Lattion A-L, Hummler E, Nenniger M, Pedrazzini T, Aubert J-F, Michel MC, Yang M, Lembo G, Vecchione C, Mostardini M, Schmidt A, Beermann F and Cotecchia S. Decreased blood pressure response in mice deficient of the α_{1b}-adrenergic receptor. *Proc Natl Acad Sci USA* 94: 11589-11594, 1997.

Chai C, Liu YW and Chan WK. *ff1b* is required for the development of steroidogenic component of the zebrafish interrenal organ. *Dev Biol* 260: 226-244, 2003.

Chang P and Rand MJ. Temperature dependence of catecholamine depletion by reserpine in the heart of the toad (*Bufo marinus*). *Br J Pharmacol* 42: 371-374, 1971.

Chen YC, Priyadarshini M and Panula P. Complementary developmental expression of the two tyrosine hydroxylase transcripts in zebrafish. *Histochem Cell Biol* 132: 375-381, 2009.

Chruscinski AJ, Rohrer DK, Schauble E, Desai KH, Bernstein D and Kobilka BK. Targeted disruption of the β2 adrenergic receptor gene. *J Biol Chem* 274: 16694-16700, 1999.

Ciuhandu CS, Wright PA, Goldberg JI and Stevens ED. Parameters influencing the dissolved oxygen in the boundary layer of rainbow trout (*Oncorhynchus mykiss*) embryos and larvae. *J Exp Biol* 210: 1435-1445, 2007.

D'Aoust JP and Tiberi M. Role of the extracellular amino terminus and first membrane-spanning helix of dopamine D1 and D5 receptors in shaping ligand selectivity and efficacy. *Cell Signal* 22: 106-116, 2010.

Dale HH. The action of certain esters and ethers of choline, and their relation to muscarine. *J Pharmacol Exp Ther* 6: 147-190, 1914.

Denvir MA, Tucker CS and Mullins JJ. Systolic and diastolic ventricular function in zebrafish embryos: influence of norepenephrine, MS-222 and temperature. *BMC Biotechnol* 8: 21, 2008.

Devic E, Paquereau L, Steinberg R, Caput D and Audigier Y. Early expression of a β1-adrenergic receptor and catecholamines in *Xenopus* oocytes and embryos. *FEBS Lett* 417: 184-190, 1997.

Devic E, Xiang Y, Gould D and Kobilka B. β-adrenergic receptor subtype-specific signaling in cardiac myocytes from β_1 and β_2 adrenoceptor knockout mice. *Mol Pharmacol* 60: 577-583, 2001.

Dhein S, van Koppen CJ and Brodde OE. Muscarinic receptors in the mammalian heart. *Pharmacol Res* 44: 161-182, 2001.

Dixon RA, Kobilka BK, Strader DJ, Benovic JL, Dohlman HG, Frielle T, Bolanowski MA, Bennett CD, Rands E, Diehl RE, Mumford RA, Slater EE, Sigal IS, Caron MG, Lefkowitz RJ and Strader CD. Cloning of the gene and cDNA for mammalian β-adrenergic receptor and homology with rhodopsin. *Nature* 321: 75-79, 1986.

Donald J and Campbell G. A comparative study of the adrenergic innervation of the teleost heart. *J Comp Physiol* 147: 85-91, 1982.

Dugan SG, Chen X, Nickerson JG, Montpetit CJ and Moon TW. Regulation of the black bullhead hepatic beta-adrenoceptors. *Comp Biochem Physiol B Biochem Mol Biol* 149: 265-274, 2008.

Ecker PM, Lin CC, Powers J, Kobilka BK, Dubin AM and Bernstein D. Effect of targeted deletions of β_1- and β_2-adrenergic-receptor subtypes on heart rate variability. *Am J Physiol Heart Circ Physiol* 290: H192-H199, 2006.

Eisen JS and Smith JC. Controlling morpholino experiments: don't stop making antisense. *Development* 135: 1735-1743, 2008.

Esbaugh AJ, Perry SF and Gilmour KM. Hypoxia-inducible carbonic anhydrase IX expression is insufficient to alleviate intracellular metabolic acidosis in the muscle of zebrafish, Danio rerio. *Am J Physiol Regul Integr Comp Physiol* 296: R150-R160, 2009.

Fabbri E, Brighenti L, Ottolenghi C, Puviani AC and Capuzzo A. β-adrenergic receptors in catfish liver membranes: characterization and coupling to adenylate cyclase. *Gen Comp Endocrinol* 85: 254-260, 1992.

Farrell AP. Tribute to P. L. Lutz: a message from the heart--why hypoxic bradycardia in fishes? *J Exp Biol* 210: 1715-1725, 2007.

Fishman MC and Chien KR. Fashioning the vertebrate heart: earliest embryonic decisions. *Development* 124: 2099-2117, 1997.

Forster ME, Davison W, Axelsson M and Farrell AP. Cardiovascular responses to hypoxia in the hagfish, *Eptatretus cirrhatus*. *Respir Physiol* 88: 373-386, 1992.

Freyss-Beguin M, Griffaton G, Lechat P, Picken D, Quennedey MC, Rouot B and Schwartz J. Comparison of the chronotropic effect and the cyclic AMP accumulation induced by β_2-agonists in rat heart cell culture. *Br J Pharmacol* 78: 717-723, 1983.

Frielle T, Collins S, Daniel KW, Caron MG, Lefkowitz RJ and Kobilka BK. Cloning of the cDNA for the human β1-adrenergic receptor. *Proc Natl Acad Sci USA* 84: 7920-7924, 1987.

Fritsche R, Axelsson M, Franklin CE, Grigg GG, Holmgren S and Nilsson S. Respiratory and cardiovascular responses to hypoxia in the Australian lungfish. *Respir Physiol* 94: 173-187, 1993.

Gamperl AK, Vijayan MM, Pereira C and Farrell AP. β-receptors and stress protein 70 expression in hypoxic myocardium of rainbow trout and chinook salmon. *Am J Physiol* 274: R428-R436, 1998.

Gamperl AK, Wilkinson M and Boutilier RG. Beta-adrenoreceptors in the trout (*Oncorhynchus mykiss*) heart: characterization, quantification, and effects of repeated catecholamine exposure. *Gen Comp Endocrinol* 95: 259-272, 1994.

Gannon BJ and Burnstock G. Excitatory adrenergic innervation of fish heart. *Comp Biochem Physiol* 29: 765-&, 1969.

Garcia-Sáinz JA and Villalobos-Molina R. The elusive α_{1D}-adrenoceptor: molecular and cellular characteristics and integrative roles. *Eur J Pharmacol* 500: 113-120, 2004.

Gilmour KM, Didyk NE, Reid SG and Perry SF. Down-regulation of red blood cell β-adrenoreceptors in response to chronic elevation of plasma catecholamine levels in the rainbow trout. *J Exp Biol* 186: 309-314, 1994.

Gomeza J, Shannon H, Kostenis E, Felder C, Zhang L, Brodkin J, Grinberg A, Sheng H and Wess J. Pronounced pharmacologic deficits in M_2 muscarinic acetylcholine receptor knockout mice. *Proc Natl Acad Sci USA* 96: 1692-1697, 1999.

Gozal E, Shah ZA, Pequignot JM, Pequignot J, Sachleben LR, Czyzyk-Krzeska MF, Li RC, Guo SZ and Gozal D. Tyrosine hydroxylase expression and activity in the rat brain: differential regulation after long-term intermittent or sustained hypoxia. *J Appl Physiol* 99: 642-649, 2005.

Green SA, Holt BD and Liggett SB. β1- and β2-adrenergic receptors display subtype-selective coupling to Gs. *Mol Pharmacol* 41: 889-893, 1992.

Grunwald DJ and Eisen JS. Headwaters of the zebrafish -- emergence of a new model vertebrate. *Nat Rev Genet* 3: 717-724, 2002.

Guan Y, Gao L, Ma HJ, Li Q, Zhang H, Yuan F, Zhou ZN and Zhang Y. Chronic intermittent hypobaric hypoxia decreases β-adrenoceptor activity in right ventricular papillary muscle. *Am J Physiol Heart Circ Physiol* 298: H1267-H1272, 2010.

Hammer R and Giachetti A. Muscarinic receptor subtypes: M_1 and M_2 biochemical and functional characterization. *Life Sci* 31: 2991-2998, 1982.

Hebert TE, Moffett S, Morello JP, Loisel TP, Bichet DG, Barret C and Bouvier M. A peptide derived from a β2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J Biol Chem* 271: 16384-16392, 1996.

Henning RJ, Khalil IR and Levy MN. Vagal stimulation attenuates sympathetic enhancement of left ventricular function. *Am J Physiol* 258: H1470-H1475, 1990.

Holeton GF. Respiratory and circulatory responses of rainbow trout larvae to carbon monoxide and to hypoxia. *J Exp Biol* 55: 683-694, 1971.

Hsieh DJ and Liao CF. Zebrafish M_2 muscarinic acetylcholine receptor: cloning, pharmacological characterization, expression patterns and roles in embryonic bradycardia. *Br J Pharmacol* 137: 782-792, 2002.

Hui AS, Striet JB, Gudelsky G, Soukhova GK, Gozal E, Beitner-Johnson D, Guo SZ, Sachleben LR, Jr., Haycock JW, Gozal D and Czyzyk-Krzeska MF. Regulation of catecholamines by sustained and intermittent hypoxia in neuroendocrine cells and sympathetic neurons. *Hypertension* 42: 1130-1136, 2003.

Imbrogno S, Angelone T, Adamo C, Pulera E, Tota B and Cerra MC. Beta3-adrenoceptor in the eel (*Anguilla anguilla*) heart: negative inotropy and NO-cGMP-dependent mechanism. *J Exp Biol* 209: 4966-4973, 2006.

Ishii M and Kurachi Y. Muscarinic acetylcholine receptors. *Curr Pharm Des* 12: 3573-3581, 2006.

Iversen NK, McKenzie DJ, Malte H and Wang T. Reflex bradycardia does not influence oxygen consumption during hypoxia in the European eel (Anguilla anguilla). *J Comp Physiol B* 180: 495-502, 2010.

Jacob E, Drexel M, Schwerte T and Pelster B. Influence of hypoxia and of hypoxemia on the development of cardiac activity in zebrafish larvae. *Am J Physiol Regul Integr Comp Physiol* 283: R911-R917, 2002.

Janssen GJ, Jerrett AR, Black SE and Forster ME. The effects of progressive hypoxia and re-oxygenation on cardiac function, white muscle perfusion and haemoglobin saturation in anaesthetised snapper (*Pagrus auratus*). *J Comp Physiol B* 180: 503-510, 2010.

Janssens PA and Grigg JA. Binding of adrenergic ligands to liver plasma membrane preparations from the axolotl, *Ambystoma mexicanum*; the toad, *Xenopus laevis*; and the Australian lungfish, *Neoceratodus forsteri*. *Gen Comp Endocrinol* 71: 524-530, 1988.

Jarrott B. Uptake and metabolism of catecholamines in perfused hearts of different species. *Br J Pharmac* 38: 810-821, 1970.

Jo SH, Leblais V, Wang PH, Crow MT and Xiao RP. Phosphatidylinositol 3-kinase functionally compartmentalizes the concurrent Gs signaling during β2-adrenergic stimulation. *Circ Res* 91: 46-53, 2002.

Johnson RA, Alvarez R and Salomon Y. Determination of adenylyl cyclase catalytic activity using single and double column procedures. *Methods Enzymol* 238: 31-56, 1994.

Johnsson M and Axelsson M. Control of the systemic heart and the portal heart of *Myxine glutinosa*. *J Exp Biol* 199: 1429-1434, 1996.

Juberg EN, Minneman KP and Abel PW. β1- and β2-adrenoceptor binding and functional response in right and left atria of rat heart. *Naunyn Schmiedebergs Arch Pharmacol* 330: 193-202, 1985.

Kacimi R, Richalet JP and Crozatier B. Hypoxia-induced differential modulation of adenosinergic and muscarinic receptors in rat heart. *J Appl Physiol* 75: 1123-1128, 1993.

Kane DA and Kimmel CB. The zebrafish midblastula transition. *Development* 119: 447-456, 1993.

Kaneda N, Toshikuni S, Kobayashi K, Kazutoshi K, Nagatsu I, Kurosawa Y, Fujita K, Yokoyama M, Nomura T, Katsuki M, Nagatsu T. Tissue-specific and high-level expression of the human tyrysine hydroxylase gene in transgenic mice. *Neuron* 6: 583-594, 1991.

Kawasaki T, Saito K, Deguchi T, Fujimori K, Tadokoro M, Yuba S, Ohgushi H and Kawarabayasi Y. Pharmacological characterization of isoproterenol-treated medaka fish. *Pharmacol Res* 58: 348-355, 2008.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B and Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn* 203: 253-310, 1995.

Kloberg AJ and Fritsche R. Catecholamines are present in larval Xenopus laevis: a potential source for cardiac control. *J Exp Zool* 292: 293-303, 2002.

Kobayashi K, Morita S, Sawada H, Mizuguchi T, Yamada K, Nagatsu I, Hata T, Watanabe Y, Fujita K and Nagatsu T. Targeted disruption of the tyrosine hydroxylase locus results in severe catecholamine depletion and perinatal lethality in mice. *J Biol Chem* 270: 27235-27243, 1995.

Kobilka BK, Dixon RA, Frielle T, Dohlman HG, Bolanowski MA, Sigal IS, Yang-Feng TL, Francke U, Caron MG and Lefkowitz RJ. cDNA for the human β2-adrenergic receptor: a protein with multiple membrane-spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet-derived growth factor. *Proc Natl Acad Sci USA* 84: 46-50, 1987.

Kojima D, Torii M, Fukada Y and Dowling JE. Differential expression of duplicated VAL-opsin genes in the developing zebrafish. *J Neurochem* 104: 1364-1371, 2008.

Kopp R, Pelster B and Schwerte T. How does blood cell concentration modulate cardiovascular parameters in developing zebrafish (*Danio rerio*)? *Comp Biochem Physiol A Mol Integr Physiol* 146: 400-407, 2007.

Kuschel M, Zhou YY, Spurgeon HA, Bartel S, Karczewski P, Zhang SJ, Krause EG, Lakatta EG and Xiao RP. β2-adrenergic cAMP signaling is uncoupled from phosphorylation of cytoplasmic proteins in canine heart. *Circulation* 99: 2458-2465, 1999.

LaCroix C, Freeling J, Giles A, Wess J and Li YF. Deficiency of M2 muscarinic acetylcholine receptors increases susceptibility of ventricular function to chronic adrenergic stress. *Am J Physiol Heart Circ Physiol* 294: H810-H820, 2008.

Landman MJ, Van Den Heuvel MR and Ling N. Relative sensitivities of common freshwater fish and invertebrates to acute hypoxia. *New Zealand Journal of Marine and Freshwater Research* 39: 1061-1067, 2005.

Lands AM, Arnold A, McAuliff JP, Luduena FP and Brown TG, Jr. Differentiation of receptor systems activated by sympathomimetic amines. *Nature* 214: 597-598, 1967a.

Lands AM, Luduena FP and Buzzo HJ. Differentiation of receptors responsive to isoproterenol. *Life Sci* 6: 2241-2249, 1967b.

Langley K and Grant NJ. Molecular markers of sympathoadrenal cells. *Cell Tissue Res* 298: 185-206, 1999.

Lattion A, Abuin L, Nenniger-Tosato M and Cotecchia S. Constitutively active mutants of the β1-adrenergic receptor. *FEBS Lett* 457: 302-306, 1999.

Lazar-Wesley E, Hadcock JR, Malbon CC, Kunos G and Ishac EJ. Tissue-specific regulation of α1b, β1, and β2-adrenergic receptor mRNAs by thyroid state in the rat. *Endocrinology* 129: 1116-1118, 1991.

Le Bras YM. Circadian variations of catecholamine levels in brain, heart, and plasma in the eel, *Anguilla anguilla* L., at three different times of year. *Gen Comp Endocrinol* 55: 472-479, 1984.

Leite CA, Taylor EW, Guerra CD, Florindo LH, Belao T and Rantin FT. The role of the vagus nerve in the generation of cardiorespiratory interactions in a neotropical fish, the pacu, *Piaractus mesopotamicus. J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 195: 721-731, 2009.

Levine MA and Leenen FH. Role of β1-receptors and vagal tone in cardiac inotropic and chronotropic responses to a β2-agonist in humans. *Circulation* 79: 107-115, 1989.

Levy FO, Zhu X, Kaumann AJ and Birnbaumer L. Efficacy of β1-adrenergic receptors is lower than that of β2-adrenergic receptors. *Proc Natl Acad Sci USA* 90: 10798-10802, 1993.

Levy MN. Cardiac sympathetic-parasympathetic interactions. *Fed Proc* 43: 2598-2602, 1984.

Li HT, Honbo NY and Karliner JS. Chronic hypoxia increases β1-adrenergic receptor mRNA and density but not signaling in neonatal rat cardiac myocytes. *Circulation* 94: 3303-3310, 1996.

MacCormack TJ and Driedzic WR. The impact of hypoxia on in vivo glucose uptake in a hypoglycemic fish, *Myoxocephalus scorpius*. *Am J Physiol Regul Integr Comp Physiol* 292: R1033-R1042, 2007.

Mann KD, Hoyt C, Feldman S, Blunt L, Raymond A and Page-McCaw PS. Cardiac response to startle stimuli in larval zebrafish: sympathetic and parasympathetic components. *Am J Physiol Regul Integr Comp Physiol* 298: R1288-R1297, 2010.

Manzl C, Schubert M, Schwarzbaum PJ and Krumschnabel G . Effects of chemical anoxia on adrenergic responses of goldfish hepatocytes and the contribution of α- and β-adrenoceptors. *J Exp Zool* 292: 468-476, 2002.

McCurley AT and Callard GV. Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. *BMC Mol Biol* 9: 102, 2008.

McDonald MD, Gilmour KM, Walsh PJ and Perry SF. Cardiovascular and respiratory reflexes of the gulf toadfish (*Opsanus beta*) during acute hypoxia. *Respir Physiol Neurobiol* 170: 59-66, 2010.

McDonald DG and McMahon BR. Respiratory development in Arctic char *Salvelinus alpinus* under conditions of normoxia and chronic hypoxia. *Can J Zool* 55: 1461-1467, 1977.

McGehee DS and Role LW. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu Rev Physiol* 57: 521-546, 1995.

McKenzie DJ, Skov PV, Taylor EW, Wang T and Steffensen JF. Abolition of reflex bradycardia by cardiac vagotomy has no effect on the regulation of oxygen uptake by Atlantic cod in progressive hypoxia. *Comp Biochem Physiol A Mol Integr Physiol* 153: 332-338, 2009.

McNeill B and Perry SF. The interactive effects of hypoxia and nitric oxide on catecholamine secretion in rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 209: 4214-4223, 2006.

Mendonça PC and Gamperl AK. Nervous and humoral control of cardiac performance in the winter flounder (*Pleuronectes americanus*). *J Exp Biol* 212: 934-944, 2009.

Meyer A and Schartl M. Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr Opin Cell Biol* 11: 699-704, 1999.

Miller SC, Reeb SE, Wright PA and Gillis TE. Oxygen concentration in the water boundary layer next to rainbow trout (*Oncorhynchus mykiss*) embryos is influenced by hypoxia exposure time, metabolic rate, and water flow. *Can J Fish Aquat Sci* 65: 2170-2177, 2008.

Minneman KP, Hegstrand LR and Molinoff PB. Simultaneous determination of β1 and β2-adrenergic receptors in tissues containing both receptor subtypes. *Mol Pharmacol* 16: 34-46, 1979.

Mitchelson F. Muscarinic receptor differentiation. *Pharmacol Ther* 37: 357-423, 1988.

Moens CB, Donn TM, Wolf-Saxon ER and Ma TP. Reverse genetics in zebrafish by TILLING. *Brief Funct Genomic Proteomic* 7: 454-459, 2008.

Myslivecek J, Klein M, Novakova M and Ricny J. The detection of the non-M2 muscarinic receptor subtype in the rat heart atria and ventricles. *Naunyn Schmiedebergs Arch Pharmacol* 378: 103-116, 2008.

Nandi J. New arrangement of interrenal and chromaffin tissues of teleost fishes. *Science* 134: 389-390, 1961.

Nasevicius A and Ekker SC. Effective targeted gene 'knockdown' in zebrafish. *Nat Genet* 26: 216-220, 2000.

Nickerson JG, Dugan SG, Drouin G and Moon TW. A putative β2- adrenoreceptor from the rainbow trout (*Oncorhynchus mykiss*); Molecular characterization and pharmacology. *Eur J Biochem* 268: 6465-6472, 2001.

Nickerson JG, Dugan SG, Drouin G, Perry SF and Moon TW . Activity of the unique β-adrenergic Na^+/H^+ exchanger in trout erythrocytes is controlled by a novel β3-AR subtype. *Am J Physiol Regul Integr Comp Physiol* 285: R526-R535, 2003.

Nikolaev VO, Bunemann M, Schmitteckert E, Lohse MJ and Engelhardt S. Cyclic AMP imaging in adult cardiac myocytes reveals far-reaching beta1-adrenergic but locally confined β2-adrenergic receptor-mediated signaling. *Circ Res* 99: 1084-1091, 2006.

Nikolaev VO, Moshkov A, Lyon AR, Miragoli M, Novak P, Paur H, Lohse MJ, Korchev YE, Harding SE and Gorelik J. β2-adrenergic receptor redistribution in heart failure changes cAMP compartmentation. *Science* 327: 1653-1657, 2010.

Nilsson GE. Effects of anoxia on catecholamine levels in brain and kidney of the crucian carp. *Am J Physiol* 257: R10-R14, 1989.

Nilsson S. Comparative anatomy of the autonomic nervous system. *Auton Neurosci* 2010.

Olsson C, Holmberg A and Holmgren S. Development of enteric and vagal innervation of the zebrafish (*Danio rerio*) gut. *J Comp Neurol* 508: 756-770, 2008.

Panula P, Chen YC, Priyadarshini M, Kudo H, Semenova S, Sundvik M and Sallinen V. The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases. *Neurobiol Dis* 40: 46-57, 2010.

Pelster B and Burggren WW. Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of zebra fish (*Danio rerio*). *Circ Res* 79: 358-362, 1996.

Pepin JL, Levy P, Garcin A, Feuerstein C and Savasta M . Effects of long-term hypoxia on tyrosine hydroxylase protein content in catecholaminergic rat brainstem areas: a quantitative autoradiographic study. *Brain Res* 733: 1-8, 1996.

Perry SF and Capaldo A. The autonomic nervous system and chromaffin tissue: Neuroendocrine regulation of catecholamine secretion in non-mammalian vertebrates. *Auton Neurosci* 2010.

Perry SF and Desforges PR. Does bradycardia or hypertension enhance gas transfer in rainbow trout (*Oncorhynchus mykiss*)? *Comp Biochem Physiol A Mol Integr Physiol* 144: 163-172, 2006.

Perry S and Gilmour K. Consequences of catecholamine release on ventilation and blood oxygen transport during hypoxia and hypercapnia in an elasmobranch *Squalus acanthias* and a teleost *Oncorhynchus mykiss*. *J Exp Biol* 199: 2105-2118, 1996.

Perry SF, Gilmour KM, Vulesevic B, McNeill B, Chew SF and Ip YK. Circulating catecholamines and cardiorespiratory responses in hypoxic lungfish (*Protopterus dolloi*): a comparison of aquatic and aerial hypoxia. *Physiol Biochem Zool* 78: 325-334, 2005.

Perry SF, Reid S and Salama A. The effects of repeated physical stress on the β-adrenergic response of the rainbow trout red blood cell. *J Exp Biol* 199: 549-562, 1996.

Perry SF and Vermette MG. The effects of prolonged epinephrine infusion on the physiology of the rainbow trout, *Salmo gairdneri*. I. Blood respiratory, acid-base and ionic states. *J Exp Biol* 128: 235-253, 1987.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45, 2001.

Phatarpekar PV, Durdan SF, Copeland CM, Crittenden EL, Neece JD and Garcia DM. Molecular and pharmacological characterization of muscarinic receptors in retinal pigment epithelium: role in light-adaptive pigment movements. *J Neurochem* 95: 1504-1520, 2005.

Pierce KL, Premont RT and Lefkowitz RJ. Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 3: 639-650, 2002.

Port JD and Bristow MR. β-Adrenergic receptors, transgenic mice, and pharmacological model systems. *Mol Pharmacol* 60: 629-631, 2001.

Pouliot T, de la Noue J and Roberge AG. Influence of diet and hypoxia on brain serotonin and catecholamines in rainbow trout (*Salmo gairdneri*). *Comp Biochem Physiol C* 89: 57-64, 1988.

Prince VE and Pickett FB. Splitting pairs: the diverging fates of duplicated genes. *Nat Rev Genet* 3: 827-837, 2002.

Prinster SC, Holmqvist TG and Hall RA. α_{2C}-adrenergic receptors exhibit enhanced surface expression and signaling upon association with β2-adrenergic receptors. *J Pharmacol Exp Ther* 318: 974-981, 2006.

Randall DJ and Perry SF. Catecholamines. In: *Fish Physiology Vol XIIB: The Cardiovascular System*, edited by Randall DJ and Hoar WS. New York: Academic Press, 1992.

Reid SD, Lebras Y and Perry SF. The *in vitro* effect of hypoxia on the trout erythrocyte β-adrenergic signal transduction system. *J Exp Biol* 176: 103-116, 1993.

Reid SG, Bernier NJ and Perry SF. The adrenergic stress response in fish: control of catecholamine storage and release. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 120: 1-27, 1998.

Ristori MT and Laurent P. Plasma catecholamines in rainbow trout (*Salmo gairdneri*) during hypoxia. *Exp Biol* 48: 285-290, 1989.

Rohrer DK, Desai KH, Jasper JR, Stevens ME, Regula DP, Jr., Barsh GS, Bernstein D and Kobilka BK. Targeted disruption of the mouse beta1-adrenergic receptor gene: developmental and cardiovascular effects. *Proc Natl Acad Sci USA* 93: 7375-7380, 1996.

Rohrer DK, Schauble EH, Desai KH, Kobilka BK and Bernstein D. Alterations in dynamic heart rate control in the β1-adrenergic receptor knockout mouse. *Am J Physiol* 274: H1184-H1193, 1998.

Rohrer DK, Chruscinski A, Schauble EH, Bernstein D and Kobilka BK. Cardiovascular and metabolic alterations in mice lacking both β1- and β2-adrenergic receptors. *J Biol Chem* 274: 16701-16708, 1999.

Roman BL and Weinstein BM. Building the vertebrate vasculature: research is going swimmingly. *Bioessays* 22: 882-893, 2000.

Rombough P and Drader H. Hemoglobin enhances oxygen uptake in larval zebrafish (*Danio rerio*) but only under conditions of extreme hypoxia. *J Exp Biol* 212: 778-784, 2009.

Rostrup M, Fossbakk A, Hauge A, Kleppe R, Gnaiger E and Haavik J. Oxygen dependence of tyrosine hydroxylase. *Amino Acids* 34: 455-464, 2008.

Rybin VO, Xu X, Lisanti MP and Steinberg SF. Differential targeting of β-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *J Biol Chem* 275: 41447-41457, 2000.

Sallinen V, Torkko V, Sundvik M, Reenila I, Khrustalyov D, Kaslin J and Panula P. MPTP and MPP+ target specific aminergic cell populations in larval zebrafish. *J Neurochem* 108: 719-731, 2009.

Sanchez A, Soncini R, Wang T, Koldkjaer P, Taylor EW and Glass ML. The differential cardio-respiratory responses to ambient hypoxia and systemic hypoxaemia in the South American lungfish, *Lepidosiren paradoxa*. *Comp Biochem Physiol A Mol Integr Physiol* 130: 677-687, 2001.

Sandblom E, Grans A, Seth H and Axelsson M. Cholinergic and adrenergic influences on the heart of the African lungfish *Protopterus annectens*. *J Fish Biol* 76: 1046-1054, 2010.

Schäfers RF, Poller U, Pönicke K, Geissler M, Daul AE, Michel MC and Brodde OE. Influence of adrenoceptor and muscarinic receptor blockade on the cardiovascular effects of exogenous noradrenaline and of endogenous noradrenaline released by infused tyramine. *Naunyn Schmiedebergs Arch Pharmacol* 355: 239-249, 1997.

Schwerte T, Prem C, Mairosl A and Pelster B. Development of the sympatho-vagal balance in the cardiovascular system in zebrafish (*Danio rerio*) characterized by power spectrum and classical signal analysis. *J Exp Biol* 209: 1093-1100, 2006.

Seo JS, Kim MS, Park EM, Ahn SJ, Kim NY, Jung SH, Kim JW, Lee HH and Chung JK. Cloning and characterization of muscarinic receptor genes from the nile tilapia (*Oreochromis niloticus*). *Mol Cells* 27: 383-390, 2009.

Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE and Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Biotechnol* 22: 1567-1572, 2004.

Shoubridge EA and Hochachka PW. Ethanol: novel end product of vertebrate anaerobic metabolism. *Science* 209: 308-309, 1980.

Silva CO, Monteiro-Filho WO, Duarte GP and Lahlou S. Effects of long-term pretreatment with isoproterenol on inotropic responsiveness to α-adrenoceptor stimulation: study in isolated perfused rat hearts. *J Pharm Pharmacol* 53: 233-242, 2001.

Soulier V, Dalmaz Y, Cottet-Emard JM, Kitahama K and Pequignot JM. Delayed increase of tyrosine hydroxylation in the rat A2 medullary neurons upon long-term hypoxia. *Brain Res* 674: 188-195, 1995.

Speers-Roesch B, Sandblom E, Lau GY, Farrell AP and Richards JG. Effects of environmental hypoxia on cardiac energy metabolism and performance in tilapia. *Am J Physiol Regul Integr Comp Physiol* 298: R104-R119, 2010.

Sprague J, Bayraktaroglu L, Bradford Y, Conlin T, Dunn N, Fashena D, Frazer K, Haendel M, Howe DG, Knight J, Mani P, Moxon SA, Pich C, Ramachandran S, Schaper K, Segerdell E, Shao X, Singer A, Song P, Sprunger B, Van Slyke CE and Westerfield M. The Zebrafish Information Network: the zebrafish model organism database provides expanded support for genotypes and phenotypes. *Nucleic Acids Res* 36: D768-D772, 2008.

Stainier DYR and Fishman MC. The zebrafish as a model system to study cardiovascular development. *Trends Cardiovasc Med* 4: 207-212, 1994.

Stecyk JA and Farrell AP. Regulation of the cardiorespiratory system of common carp (*Cyprinus carpio*) during severe hypoxia at three seasonal acclimation temperatures. *Physiol Biochem Zool* 79: 614-627, 2006.

Steele SL, Lo KH, Li VW, Cheng SH, Ekker M and Perry SF. Loss of M_2 muscarinic receptor function inhibits development of hypoxic bradycardia and alters cardiac β-adrenergic sensitivity in larval zebrafish (*Danio rerio*). *Am J Physiol Regul Integr Comp Physiol* 297: R412-R420, 2009.

Stengel PW, Gomeza J, Wess J and Cohen ML. M_2 and M_4 receptor knockout mice: muscarinic receptor function in cardiac and smooth muscle *in vitro*. *J Pharmacol Exp Ther* 292: 877-885, 2000.

Stengel PW, Yamada M, Wess J and Cohen ML. M_3-receptor knockout mice: muscarinic receptor function in atria, stomach fundus, urinary bladder, and trachea. *Am J Physiol Regul Integr Comp Physiol* 282: R1443-R1449, 2002.

Sutherland EW and Rall TW. Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. *J Biol Chem* 232: 1077-1091, 1958.

Sutherland EW, Rall TW and Menon T. Adenyl cylase. I. Distribution, preparation, and properties. *J Biol Chem* 237: 1220-1227, 1962.

Sutherland EW and Robison GA. The role of cyclic-3',5'-AMP in responses to catecholamines and other hormones. *Pharmacol Rev* 18: 145-161, 1966.

Sylvén C, Arner P, Hellstrom L, Jansson E, Sotonyi P, Somogyi A and Bronnegard M. Left ventricular β1 and β2 adrenoceptor mRNA expression in normal and volume overloaded human heart. *Cardiovasc Res* 25: 737-741, 1991.

Tang R, Dodd A, Lai D, McNabb WC and Love DR. Validation of zebrafish (*Danio rerio*) reference genes for quantitative real-time RT-PCR normalization. *Acta Biochim Biophys Sin (Shanghai)* 39: 384-390, 2007.

Tanoue A, Koba M, Miyawaki S, Koshimizu T, Hosada C, Oshikawa S and Tsujimoto G. Role of the α_{1D}-adrenergic receptor in the development of salt-induced hypertension. *Hypertension* 40: 101-106, 2002.

Taylor EW and Barrett DJ. Evidence of a respiratory role for the hypoxic bradycardia in the dogfish *Scyliorhinus canicula* L. *Comp Biochem Physiol A Comp Physiol* 80: 99-102, 1985.

Taylor EW and Butler PJ. Nervous control of heart rate: activity in the cardiac vagus of the dogfish. *J Appl Physiol* 53: 1330-1335, 1982.

Taylor EW, Leite CA and Skovgaard N. Autonomic control of cardiorespiratory interactions in fish, amphibians and reptiles. *Braz J Med Biol Res* 43: 600-610, 2010.

Taylor EW, Short S and Butler PJ. The role of the cardiac vagus in the response of the dogfish *Scyliorhinus canicula* to hypoxia. *J Exp Biol* 70: 57-75, 1977.

Taylor JS, Van de Peer Y, Braasch I and Meyer A. Comparative genomics provides evidence for an ancient genome duplication event in fish. *Philos Trans R Soc Lond B Biol Sci* 356: 1661-1679, 2001.

Temma K, Hirata T, Kitazawa T and Kondo H. Isoproterenol-induced desensitization to the positive inotropic effect of isoproterenol in ventricular strips isolated from carp heart (*Cyprinus carpio*). *Comp Biochem Physiol C* 82: 403-408, 1985.

Temma K, Hirata T, Kitazawa T, Kondo H and Katano Y. Are β-adrenergic receptors in ventricular muscles of carp heart (*Cyprinus carpio*) mostly the β2 type? *Comp Biochem Physiol C* 83: 261-263, 1986a.

Temma K, Iwata M, Kondo H and Ohta T. Seasonal variations in the content of catecholamines in carp heart (*Cyprinus carpio*). *Comp Biochem Physiol C* 97: 107-110, 1990.

Temma K, Kishi H, Kitazawa T, Kondo H, Ohta T and Katano Y. β2 adrenergic receptors are not only for circulating catecholamines in ventricular muscles of carp heart (*Cyprinus carpio*). *Comp Biochem Physiol C* 83: 265-269, 1986b.

Thirumalai V and Cline HT. Endogenous dopamine suppresses initiation of swimming in prefeeding zebrafish larvae. *J Neurophysiol* 100: 1635-1648, 2008.

Thomas P and Smart TG. HEK293 cell line: a vehicle for the expression of recombinant proteins. *J Pharmacol Toxicol Methods* 51: 187-200, 2005.

Thomas SA, Matsumoto AM and Palmiter RD. Noradrenaline is essential for mouse fetal development. *Nature* 374: 643-646, 1995.

To TT, Hahner S, Nica G, Rohr KB, Hammerschmidt M, Winkler C and Allolio B. Pituitary-interrenal interaction in zebrafish interrenal organ development. *Mol Endocrinol* 21: 472-485, 2007.

Ton C, Stamatiou D, Dzau VJ and Liew CC. Construction of a zebrafish cDNA microarray: gene expression profiling of the zebrafish during development. *Biochem Biophys Res Commun* 296: 1134-1142, 2002.

Ton C, Stamatiou D and Liew CC. Gene expression profile of zebrafish exposed to hypoxia during development. *Physiol Genomics* 13: 97-106, 2003.

Triposkiadis F, Karayannis G, Giamouzis G, Skoularigis J, Louridas G and Butler J. The sympathetic nervous system in heart failure physiology, pathophysiology, and clinical implications. *J Am Coll Cardiol* 54: 1747-1762, 2009.

Tumova K, Iwasiow RM and Tiberi M. Insight into the mechanism of dopamine D1-like receptor activation. Evidence for a molecular interplay between the third extracellular loop and the cytoplasmic tail. *J Biol Chem* 278: 8146-8153, 2003.

Tumova K, Zhang D and Tiberi M. Role of the fourth intracellular loop of D1-like dopaminergic receptors in conferring subtype-specific signaling properties. *FEBS Lett* 576: 461-467, 2004.

Uberti MA, Hague C, Oller H, Minneman KP and Hall RA. Heterodimerization with β2-adrenergic receptors promotes surface expression and functional activity of α_{1D}-adrenergic receptors. *J Pharmacol Exp Ther* 313: 16-23, 2005.

Vogel AM and Weinstein BM. Studying vascular development in the zebrafish. *Trends Cardiovasc Med* 10: 352-360, 2000.

Vornanen M and Tuomennoro J. Effects of acute anoxia on heart function in crucian carp: importance of cholinergic and purinergic control. *Am J Physiol* 277: R465-R475, 1999.

Wang Y, De A, V, Gao X, Ramani B, Jung YS and Xiang Y. Norepinephrine- and epinephrine-induced distinct β2-adrenoceptor signaling is dictated by GRK2 phosphorylation in cardiomyocytes. *J Biol Chem* 283: 1799-1807, 2008.

Wang Z, Nishimura Y, Shimada Y, Umemoto N, Hirano M, Zang L, Oka T, Sakamoto C, Kuroyanagi J and Tanaka T. Zebrafish β-adrenergic receptor mRNA expression and control of pigmentation. *Gene* 446: 18-27, 2009.

Woodward JJ. Plasma catecholamines in resting rainbow trout, *Salmo gairdneri* Richardson, by high pressure liquid chromatography. *J Fish Biol* 21: 429-432, 1982.

Xiang Y, Rybin VO, Steinberg SF and Kobilka B. Caveolar localization dictates physiologic signaling of β2-adrenoceptors in neonatal cardiac myocytes. *J Biol Chem* 277: 34280-34286, 2002.

Xiang Y and Kobilka BK. Myocyte adrenoceptor signaling pathways. *Science* 300: 1530-1532, 2003.

Xiao RP, Avdonin P, Zhou YY, Cheng H, Akhter SA, Eschenhagen T, Lefkowitz RJ, Koch WJ and Lakatta EG. Coupling of β2-adrenoceptor to Gi proteins and its physiological relevance in murine cardiac myocytes. *Circ Res* 84: 43-52, 1999.

Xiao RP. β-adrenergic signaling in the heart: dual coupling of the β2-adrenergic receptor to Gs and Gi proteins. *Sci STKE* 2001: RE15, 2001.

Xiao RP, Zhang SJ, Chakir K, Avdonin P, Zhu W, Bond RA, Balke CW, Lakatta EG and Cheng H. Enhanced Gi signaling selectively negates β2-adrenergic receptor (AR)--but not β1-AR-mediated positive inotropic effect in myocytes from failing rat hearts. *Circulation* 108: 1633-1639, 2003.

Zheng M, Han QD and Xiao RP. Distinct β-adrenergic receptor subtype signaling in the heart and their pathophysiological relevance. *Sheng Li Xue Bao* 56: 1-15, 2004.

Zhou QY, Quaife CJ and Palmiter RD. Targeted disruption of the tyrosine hydroxylase gene reveals that catecholamines are required for mouse fetal development. *Nature* 374: 640-643, 1995.