Adrenal chromaffin cell function in high-altitude deer mice (*Peromyscus maniculatus*)

By

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ABSTRACT

The deer mouse (*Peromyscus maniculatus*) inhabits a broad altitudinal range from sea level to over 4300m, where they experience continuous hypoxia. Typically hypoxia activates the sympathetic nervous system; however this could become maladaptive in high-altitude residents if it is maintained over chronic periods. We hypothesized that high-altitude deer mice might have altered the physiology of adrenaomedullary chromaffin cells (AMC) in the adrenal gland to avoid chronic activation of the sympathetic response. Highland mice had lower plasma adrenaline levels compared to lowland populations of *Peromyscus* mice, both before and after acclimation to hypobaric hypoxia. This did not correspond to any apparent changes in AMC Ca^{2+}-signalling dynamics. Instead a profound blunting of catecholamine storage was found in highland AMCs that appeared to underlie the reduction in adrenaline release.

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THESIS ORGANIZATION AND FORMAT

This thesis is organized in a "sandwich" format and consists of three main chapters. Chapter one provides a general introduction and outlines the objectives of my thesis research. Chapter two is a manuscript that is prepared for submission to a peer-reviewed scientific journal. Chapter three discusses these findings and their implications in high-altitude adaptations.

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: Adrenal chromaffin cell function in high-altitude deer mice (*Peromyscus maniculatus*)

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Comments: NAP conducted the study under the supervision of GRS, CAN and ALS, and wrote the manuscript. GRS, CAN, ALS and NAP designed the experiments.

CHAPTER 3: GENERAL DISCUSSION

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LIST OF ABBREVIATIONS

AMC- Adrenomedullary chromaffin cell

AD- Adrenaline

$[Ca^{2+}]_i$- Intracellular Ca^{2+} concentration

CICR- Ca^{2+} induced Ca^{2+} release

DβH- Dopamine β-hydroxylase

DDC- Dopa decarboxylase

Hex- Hexamethonium

HIF- Hypoxia Inducible Factor

K_{ATP}- ATP dependent K^+ channels

nAChR- Nicotinic acetylcholine receptors

Mt- Mitochondria

NA-Noradrenaline

Nic- Nicotine

PMNT- Phenylethanolamine n-methyltransferase

VGCC- Voltage-gated Ca^{2+} Channels

ROS- Reactive oxygen species

RyR- Ryanodine receptors

V_m- Membrane potential

CHAPTER ONE: GENERAL INTRODUCTION

1.1 The Sympathetic Response at High-Altitude

The hypoxic chemoreflex is important for maintaining O_2 supply to tissues during acute hypoxic stress. Activation of the chemoreflex mounts a sympathetic response to increase heart rate, and to prioritize O_2 delivery to the most O_2 sensitive organs. This sympathetic response is a result of the activation of the hypoxic chemoreflex (Figure1.1). Low arterial O_2 saturation is sensed by chemoreceptors in the carotid bodies, which send afferent signals to the cardiorespiratory centers of the brain (Marshall, 1994; Prabhakar, 2000; Reis et al., 1994). This causes a coordinated sympathetic response, and results in: the activation of the hypoxic ventilatory response to increase arterial O_2 saturation (Powell, et al., 1998; Ivy & Scott, 2015, 2017); the increase in systemic vascular resistance and blood pressure (Ivy & Scott, 2015; Hainsworth & Drinkhill, 2007); and α-adrenergic mediated vasoconstriction, which together causes blood to be redistributed towards the most hypoxia-sensitive organs (i.e. brain and heart) (Ivy & Scott, 2015; Hainsworth & Drinkhill, 2007). α-Adrenergic mediated vasoconstriction results from elevated plasma levels of the 'flight or fight' hormones, adrenaline and noradrenaline. These hormones are released, in part, from chromaffin cells of the adrenal medulla after stimulation by the splanchnic nerve as a consequence of the hypoxic chemoreflex. The sympathetic activation and the adrenomedullary response to hypoxia is key for maintaining O_2 supply during acute hypoxic challenge.

Chronic exposure to hypoxia may alter the hypoxic chemoreflex; this is observed in both lowland and highland taxa at high-altitude (Powell et al., 1998; Kusakabe et al.,

1993; Pardal et al., 2007; Wang et al., 2008; Ivy & Scott, 2017; Schwenke et al .2007; Wu & Kayser, 2006). In lowland taxa, O_2 chemosensitivity can increase as a result of hypertrophy and neovascularization of the carotid body (Powell et al., 1997; Kusakabe et al., 1993; Pardal et al., 2007; Wang et al., 2008). Elevated sympathetic activation persists with chronic hypoxia. This can increase both systemic vascular resistance and blood pressure through continued α-adrenergic-mediated vasoconstriction. However, this unrelenting redistribution of blood to the most O_2 sensitive tissues in chronic hypoxia may negatively impact the function of less O_2- sensitive tissues (gastrointestinal tract, reproductive organs, etc.), thereby reducing fitness. An example of this can be observed in the decline in birth weight with increasing altitude (McCollough et, al. 1977; Jensen and Moore, 1997; Moore, 2003). This is thought to be due, in part, to reduced blood flow and vessel remodelling in the third trimester in high altitude populations (Moore et al., 2011). Interestingly, in multigenerational high-altitude populations (ie. Andeans, Tibetans), there is higher uterine blood flow than in less established high altitude populations (Moore, 2003). There is evidence that some highlander populations may have evolved protective mechanisms to reduce sympathetic activation or the magnitude of the sympathetic response, however this is not consistent across highland taxa (Bernardi, 1998; León-Velarde et al., 1996; Pichon et al., 2013). Interestingly, while some reports show decreased sympathoexcitation in highlanders (Bernardi et al., 1998), others indicate that there may be both increased sympathoexcitation and a decline in α-adrenergic mediated vasoconstriction in highlanders at high- altitude (Rostrup et al., 1998). This may suggest that the sympathetic activation and response is fine tuned in

highland taxa. This may be to maintain the potential benefits of increased sympathetic activation at high altitudes, while offsetting those effects that can be deleterious to fitness (i.e α-adrenergic mediated vasoconstriction, increased systemic resistance).

The primary goal of this thesis is to examine the adaptive and plastic changes in adrenergic signalling at high-altitudes. The activity of the adrenomedullary chromaffin cells (AMCs) is key in the sympathetic response and distribution of blood flow. In high-altitude adapted taxa, little is known regarding the role of AMCs in regulating the sympathetic response, however there is evidence for the reduction in catecholamine levels circulating in the blood of highland adapted humans (Van Hall, et al., 2009; Antezana et al., 1995). In lowland humans, adrenaline levels increase shortly after high-altitude ascent, and can decline after high-altitude acclimation; yet these values remain elevated compared to values at sea level (Mazzeo et al., 1994; Rostrup et al., 1998). This suggests that some lowland populations may also protectively reduce α-adrenergic vasoconstriction. While alterations in AMC signalling have been observed in a number of animals with chronic hypoxia, these have been shown to both increase (Del Toro et al., 2003; Carabelli et al., 2007; Evinger, et al., 2002; Lam et al., 2008) and decrease (Sala et al., 2008; Buttigieg et al., 2009) catecholamine release. Therefore the mechanism underlying plastic responses and adaptations to high- altitude remains unclear. Below I outline the general morphology of the adrenal medulla and AMCs, the signalling pathway involved in adrenaline and noradrenaline release, and finally changes in AMC signalling with chronic hypoxia.

1.2 Adrenal Chromaffin Cells

1.21 Adrenal Morphology

The adrenal medulla receives cholinergic innervation primarily from the greater thoracic splanchnic nerve. Minor innervation of the medulla can also stem from the lesser thoracic splanchnic nerve fibres or the first or second lumbar ganglia (Maycok, 1939; Young, 1939). Nerve fibres pass through the cortex in parallel with major blood vessels (Coupland, 1965), and branch to form fine nerve fibres that directly synapses with AMCs. AMC aggregate as groups and often associate with vascular sinusoids. The adrenal gland is perfused through a vast network of arteries that include: the adrenolumbar arteries, the phrenic artery, the coeliac axis and superior mesenteric artery, the renal arteries, and the abdominal aorta. This dense network of arteries can vary between species and even individuals, and is critical for the uptake and circulation of catecholamines (Douglas & Rubin, 1961). Additionally, the proximity of AMCs to the vascular bed can be important in determining their phenotype, in particular whether they secrete primarily adrenaline or noradrenaline (Wurtman & Axelrod, 1965, Wurtman, 1966).

The main catecholamine secreting cells of the adrenal medulla can be divided into adrenaline-secreting (A cells) and noradrenaline-secreting cells (NA cells). The distribution of A and NA cells is influenced by interactions with the adrenal cortex. Glucocorticoids released from the cortex are transported to the medulla via the intra-adrenal portal vascular system. Glucocorticoids are thought to stimulate a shift towards A

cells by inducing the synthesis of phenylethanolamine-N-methyl transferase (PNMT), which converts noradrenaline to adrenaline (Cole et al., 1995; Wurtman & Axelrod, 1965, Wurtman, 1966). As such, AMCs in closer proximity to arteries downstream of the cortex are predominately A cells, while those further away tend to be NA cells (Pohorecky & Wurtman, 1971). Therefore, there is a shift during development from predominately NA cells to ~90% A cells induced by increased cortex activity (Verhofstad et al., 1985; Margolis et at. 1966).

Morphological evidence suggests that chromaffin cells have a polar distribution of organelles (Coupland, 1989). *In vivo* chromaffin cells have a columnar shape, one end synapses with splanchnic nerve endings, while the other end lays in proximity to vascular sinusoids. Organelles are organized so that the nucleus is located at the "neural end", while the secretary vesicles are densely packed at the "sinusoid end" **(**Carmichael, 1989; Aunis & Garcia, 1981). While it is thought that the polarization may be due to the release of trophic factors, including nitric oxide from the endothelial cells (Furchgott & Zawadzki**,** 1980), polarity has also been shown to be maintained in isolated bovine chromaffin cells **(**Cuchillo-Ibanez**,** 1999). Together, this indicates that spatial separation may facilitate rapid transmission of signals leading to exocytosis and transport of catecholamines in the blood.

1.22 Stimulus-Secretion Coupling in AMCs

In 1932, Feldberd et al., found that the primary neurotransmitter at the splanchnic nerve-AMC synapse was acetylcholine. More recently, it has been found that other neruotransmitters (NTs) released from the splanchnic nerve, in addition to acetylcholine, can to a lesser extent activate (histamine, pituitary adenylate cyclase activating peptide-PCAP) or inhibit (opioid peptides) the secretion of catecholamines (Garcia et al. 2006). Acetylcholine activates nicotinic acetylcholine receptors (nAChRs), and causes a downstream release in catecholamines (Fig 1.1). Douglas & Rubin (1961) found that catecholamine release was dependent on an influx of Ca^{2+} from the extracellular environment, as the secretary response from the adrenal medulla was suppressed when perfused with Ca^{2+} free solution and catecholamine release was coupled with increased intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Douglas et al., 1965; Baker & Knight, 1978). Therefore Ca^{2+} influx plays a vital role in linking the acetylcholine- stimulus to catecholamine secretion

Since this initial discovery, it has been found that there are three sources for the increase in $[Ca^{2+}]_i$, the first being through nAChRs (Garcia et al. 2006). Stimulation with acetylcholine causes isotropic nAChRs to open, allowing for an influx of Na^+ and Ca^{2+}. The cation influx can raise the membrane potential (V_m), which activates voltage gated Ca^{2+} channels (VGCC). VGCCs are a main source of the increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ can in turn cause Ca^{2+} induced Ca^{2+} release (CICR) from the endoplasmic reticulum (ER), via the activation of ryanodine receptors (RyR) by free intracellular Ca^{2+}.

VGCCs in the plasma membrane, RyRs of the ER, and secretory vesicles are spatially linked to created functional units that tightly control Ca^{2+} microdomains and facilitate vesicle secretion. To temper the Ca^{2+} response, mitochondria (Mt) often co-localize. While not all functional units have Mt in close association, they are important in their ability to reduce the $[Ca^{2+}]_i$ by active transport of Ca^{2+} into the interior of the Mt (Montero, et al., 2000). If Mt function and/or its ability to take up Ca^{2+} is inhibited (ie. via CCCP, rotenone, oligomycin, etc.) secretion can be potentiated more than 7-fold (Montero et al., 2000). Blocking of the Mt Ca^{2+} uptake, in fact, may allow for larger Ca^{2+} waves and recruitment of reserve vesicles stored deeper in the cytosol (von Ruden et al., 1993). Mt of bovine AMCs have also been shown to have a unexpectedly large mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) maximal capacity and can cope with Ca^{2+} concentrations usually associated with Ca^{2+} overload and apoptosis (Montero et al., 2000), highlighting the unique characteristics of AMCs and their control of Ca^{2+} signalling.

Increases in $[Ca^{2+}]_i$ are ultimately required to complete the fusion of vesicles to the cellular membrane. While the mechanism is not fully understood, synaptotagmin (Syt), a protein of the vesicle fusion complex, is thought to act as the primary Ca^{2+} sensor (Schonn et al. 2008). This is due to its ability to bind to the plasma membrane, bind to Ca^{2+}, and colocalize to the site of Ca^{2+} influx. In the absence of Ca^{2+}, synaptotagmin acts as a clamp, preventing vesicle fusion but remaining in a fusion ready state (Jahn & Fasshauer, 2012). Therefore, Ca^{2+} is necessary for the final release of vesicle contents.

1.23 Catecholamine synthesis and Vesicle release

Catecholamine biosynthesis is initiated by the uptake of tyrosine into AMCs. Tyrosine is an essential amino acid; therefore uptake through dietary consumption is required. The conversion of tyrosine to DOPA by tyrosine hydroxylase is considered the rate limiting step in catecholamine synthesis. DOPA is further converted to dopamine by dopa decarboxylase (DDC), which in turn is converted to noradrenaline by dopamine β-hydroxylase. In A-cells, phenylethanolamine n-methyltransferase (PMNT) converts noradrenaline to adrenaline (Wan & Livett, 1989). While adrenaline and noradrenaline are primarily released from AMC vesicles, other autocrine/paracrine signalling molecules are released to provide feedback and control vesicle release. Opioids and ATP provide negative feedback, reducing the increase in $[Ca^{2+}]_i$ that stimulates catecholamine release (Diverse-Pierlussi et al., 1991; Gandia et al., 1993; Doupnik& Pun, 1994; Albillos et al., 1996; Currie & Fox, 1996). ATP binds to purinergic P_{2y} receptors, while opioids act on μ- and δ-opioid receptors, and through g-protein mediated pathways they cause VGCC activation to slow in acetylcholine- stimulated AMCs and reduce the amplitude of Ca^{2+} influx. This tempers the Ca^{2+} response and is thought to protect from over-activation of sympathetic activity.

1.3 AMCs and Chronic Hypoxia

Chronic hypoxia alters the cellular mechanisms that contribute to catecholamine release from the adrenal medulla. Hypoxia inducible factor (HIF) signalling activates during chronic hypoxia and can affect both catecholamine production and Ca^{2+} signalling machinery. Additionally, reactive oxygen species (ROS) increase under hypoxic stress in many species and have been shown to regulate Ca^{2+} signalling dynamics (Krishnaswamy & Cooper, 2012). These changes to AMC function and their implications for animals adapted to live in low-O_2 conditions are outlined below.

1.31 HIF Signalling and Catecholamine Synthesis

The expression of HIF is critical in regulating the synthesis and release of catecholamines from chromaffin cells of the adrenal medulla during acute and chronic hypoxia. HIF is composed of both α- and β-subunits; the dimerization of these subunits is necessary to induce expression of HIFs targets. During normoxia, HIFα is hydroxylated by O_2-dependent HIF prolyl-hydroxylase (PHD), which promotes the binding of von Hippel-Lindau protein (VHL), and leads to ubiquitination and proteasomal degradation (Ivan et al., 2001). During hypoxia, PHD can no longer use O_2 as a co-substrate, this allows for HIFα to accumulate and dimerize with the β-subunit. The stable, dimerized form of HIF mounts a broad response to promote survival in hypoxia in a cell specific manner.

Three HIFα isoforms exists, HIF-1α, HIF-2α, and HIF-3α. HIF-1α is the most broadly expressed HIF-α isoform and it plays a general role in signalling for hypoxia (Semenza, 2004). In contrast, HIF-2α has a restricted expression pattern, and is the predominate isoform expressed in AMCs. In studies of immortalized rat adrenomedullary chromaffin (MAH) cell lines, HIF-2α expression is induced after 15 minutes in hypoxia (2% O_2) and reaches its peak after 1-2 hours (Brown & Nurse, 2008). However, evidence suggests that HIF-2α expression can be relatively high under basal conditions (Scott et al, in prep). This is due, impart, to its critical role in inducing catecholamine synthesis in hypoxia. AMCs with HIF-2α knocked-down have severely blunted levels of cellular dopamine and noradrenaline due to a reduction in DβH and DDC expression (Brown and Nurse, 2008). Similarly, EPAS1 knock-down mice have substantially reduced catecholamine levels, which results in mid-gestational mortality due to severe bradycardia (Tian et al., 1998). These studies suggest that HIF-2α signalling regulates basal catecholamine synthesis in adrenal chromaffin cells.

Little is known regarding the expression of HIF-2α, DβH, and DDC in chronic hypoxic and their regulation of dopamine and noradrenaline. However HIF-2α levels in the carotid body have been shown to remain elevated after almost a month in chronic hypoxia (Lam et al., 2008). PNMT expression, on the other hand, is induced by hypoxia in AMCs (Evinger et al., 2002) and in PC12 pheochromocytoma cells (Tai et al., 2010). This is mediated via HIF-1α; it has been proposed that HIF-1α acts indirectly by inducing the expression of transcription factors Erg-1 and Sp-1, which in turn increase expression of PNMT (Wong et al., 2010).

1.32 Voltage-gated Ca^{2+} Channels

All six VGCC subtypes have been found in AMCs. High voltage activated (HVA) channels (L-type, Q-type/P-type,N-type) and the intermediate voltage activated (IVA) channel (R-type) largely predominate, however the relative expression of these subtypes is greatly variable between species and subject to plasticity (Garcia, et al., 2006). While the full implications of these species differences are not fully understood, it is thought that either: (i) this allows for fine control of the differential exocytotic release of adrenaline and noradrenaline to different stressors; or (ii) presuming that these VGCCs and the chromaffin cells have the same function across species, there may be a lack of evolutionary pressure to conserve patterns of expression of VGCCs (Garcia, et al., 2006).

The expression of low voltage activated (LVA), T-type Ca^{2+} channels, on the other hand can increase with chronic hypoxia. Typically only very low levels of T-type Ca^{2+} channel expression have been found in AMCs of adult bovine (Garcia-Palomero et al., 2000; Diverse-Pierluissi et al., 1990) and rats (Bournaud et al., 2001; Hollins et al., 1996). However, these levels increase with chronic hypoxia in a time and dose dependent manner (Del Toro et al., 2003; Carabelli et al., 2007). This HIF-2α dependent response lowers the threshold for channel activation, allowing Ca^{2+} influx with less stimulation via acetylcholine (Del Toro at al., 2003). T-type Ca^{2+} channels, however, are inactivated more quickly, and allow for smaller increases in $[Ca^{2+}]_i$. This could indicate a transition to small, more frequent exocytotic events with chronic hypoxia.

1.33 Nicotinic Acetylcholine Receptors

The Ca^{2+} signalling dynamics of AMCs can also be altered in hypoxia due to changes in the nAChR isoforms. NAChRs are formed by a variety of homo- or-heteromeric combinations of α and β subunits in a cell and species dependent manner (Sala et al., 2008). During basal conditions, α3, α5 and β4 -containing receptors are predominately responsible for acetylcholine-evoked Ca^{2+} release from AMCs, while the expression of alternate isoforms (ie. α7 and α9) are key in long term regulation in response to stress (Sala et al., 2008; Guérineau, et al., 2012). The expression of α7 and α9 can be upregulated during chronic activation of AMCs, this can cause AMCs to require a greater stimulus to elicit downstream catecholamine release.

As mentioned previously, stimulation of nAChRs leads to a rise in cytosolic Ca^{2+} that acts as a key signal for catecholamine release. Chronic hypoxia has been shown to dampen the magnitude of this cytosolic Ca^{2+}rise by changing nAChR isoform expression (Sala et al., 2008). This occurs because α3-containing nAChR subtypes, the primary subtypes involved in initiating catecholamine release in chromaffin cells (Sala et al., 2008; Guérineau, et al., 2012), are susceptible to inactivation during hypoxia by increases in reactive oxygen species (ROS). ROS inhibits α3-containing subtypes by acting on a cysteine residue in the receptor pore (Krishnaswamy & Cooper, 2012). Furthermore, the expression of homomeric α7 receptors, which are not sensitive to ROS, increases after chronic activation of chromaffin cells (e.g., by chronic hypoxia or nicotine) (Sala et al.,

2008). α7 receptors have a much higher permeability to Ca^{2+}, and are not thought to contribute to catecholamine release, and may instead contribute to an inhibitory reduction in membrane potential via an upregulation of ATP-dependent K^+ channels (K_{ATP}) (Buttigieg et al., 2009; Ducsay et al., 2007). As such, with AMC stimulation, it requires a greater magnitude of depolarization to cause the same activation of VGCCs. Therefore with similar stimuli, the increase in $[Ca^{2+}]_i$ is reduced and can ultimately cause a reduction in catecholamine release from the AMCs.

1.34 Evolutionary Adaptations to Hypoxia

Very little is known about evolutionary adaptations in AMC function in high-altitude populations. However, single nucleotide polymorphisms (SNPs) in the genes encoding for HIF-2α and EPAS1, have been found in a number of high altitude taxa (Simonson, et al., 2010; Zhang et al., 2014; Song et al., 2016). These SNPs highly correlate to 'adaptive' high-altitude traits, including avoiding an excessive increase in haemoglobin concentrations in high-altitude populations which protects against chronic mountain sickness (Beall et al., .2010; Xi et al., 2010). As mentioned previously HIF-2α controls many key aspects of catecholamine synthesis and Ca^{2+} signalling leading to vesicle secretion. Therefore, these adaptations may influence the regulator actions of HIF-2α in AMCs, and drive a reduction in adrenergic signalling.

1.4 Deer mice

Deer mice (*Peromyscus maniculatus)* were used as a model organism for studying high-altitude adaptation as they occupy the greatest altitudinal range of all North American mammals, from sea level to ~4300m. At the summit they are exposed to extreme hypoxia, with O_2 partial pressures that are half of that of sea level. Their large range of habitats allows us to elucidate both genetic and plastic contributions to coping with hypoxic stress by contrasting the phenotype of both high- and low-altitude populations. Studying these high-altitude natives is important as it provides insight into the responses to hypoxia that are truly adaptive and contribute to fitness at high altitude, as these are often shaped by natural selection over many generations.

While many current studies are examining adaptations in the oxygen cascade of highland deer mice, little is known regarding the hypoxic stress response, and whether it might differ from that of lowland *Peromyscus* mice due to plasticity (acclimatization, developmental plasticity, etc.) or evolved genetically-based adaptations. Previous morphometric data from our lab indicates that highland mice have a smaller medulla tissue area, compared to lowlanders (Scott et al, in prep). Additionally, amperometry studies on adrenal medulla tissue slices have shown profound blunting of catecholamine release in highland deer mice compared to lowland deer mice (Scott et al. In prep). Taken together this indicates that *in vivo* circulating catecholamine levels may be reduced in highland mice. Emerging work from the Cheviron lab (In prep.), suggests that high altitude mice contain single nucleotide differences in the EPAS1 gene similar to those

observed in Tibetan high-altitude human populations (Simonson, et al., 2010). Taken together, this data suggests that high-altitude populations may have genetic adaptations to reduce sympathetic activation.

1.5 Main Techniques

1.51 Ratiometric Ca^{2+} Imaging

Ratiometric Ca^{2+} imaging was used to examine the Ca^{2+} signalling dynamics in AMCs of both highland and lowland *Peromyscus* populations. Ratiometric Ca^{2+} imaging is an effective Ca^{2+} imaging technique as it reduces the effects of uneven dye loading, variable cell thickness, loss/leakage of the dye, and photobleaching (Grynkeiwicz G. 1985; Tsien 1989). We used fluorescent probe, Fura-2, as a Ca^{2+} indicator. When bound to Ca^{2+}, the maximal absorption spectrum of Fura-2 changes from 380 to 340nm, while maximal fluorescence emission remains at approximately 510 nm (Tsien 1989). Thus measures of intracellular Ca^{2+} can be obtained from the ratio of fluorescence at 340/380nm, measured at the maximum fluorescence. For this technique, cells are incubated with Fura-2 linked to acetoxymethyl (AM) ester (Fura-2 AM). This allows the Fura-2 to permeate across the plasma membrane, and allows it to remain inactive until it is taken up into the cytosol. Inside the cell, the AM ester is cleaved via nonspecific cytoplasmic esterases, which activates the Ca^{2+} probe. The removal of the AM ester also causes Fura-2 to become negatively charged; this prevents Fura-2 from leaking across the membrane and contributes to the reliability of ratiometric Ca^{2+} imaging (Grynkeiwicz G. 1985; Tsien 1989).

1.6 Main Objectives

The aims of this thesis were two-fold. The first and primary aim was to determine if there is a reduction in catecholamine levels circulating in the blood in high-altitude native deer mice, and to identify the alterations in the signalling pathway which underlie this reduction. The second aim was to identify the effect of acclimation to hypobaric hypoxia, in both lowland and highland *Peromyscus* populations, on catecholamine release from AMCs. This was intended to distinguish between the mechanisms underlying adaptation and/or the plastic changes in catecholamine secretion of AMCs. We approached this by:

1) Assessing the differences in resting plasma catecholamine levels between populations: in normoxia, after 24 h hypoxia exposure, and after prolonged 6-8 week acclimation to hypoxia.

2) Examining the factors which may underlie observed population differences and acclimation effects in catecholamine secretion by:

i) Assessing the Ca^{2+} signalling dynamics in cultured AMCs

ii) Examining the relative contribution of α7 nAChRs to the Ca^{2+} response

iii) Assessing cellular stores of adrenaline, noradrenaline and dopamine

Figure 1.1 Representation of the hypoxic chemoreflex pathway, as it relates to adrenal medulla function. 1) Low arterial O_2 saturation is sensed by chemoreceptors of the carotid body and medulla 2) cardiorespiratory centres of the brain (medulla and pons) are activated 3) this causes downstream excitation of the sympathetic chain 4) a number of tissues are activated, including the adrenal medulla 5) the adrenal medulla chromaffin cells release adrenergic catecholamines into circulation.

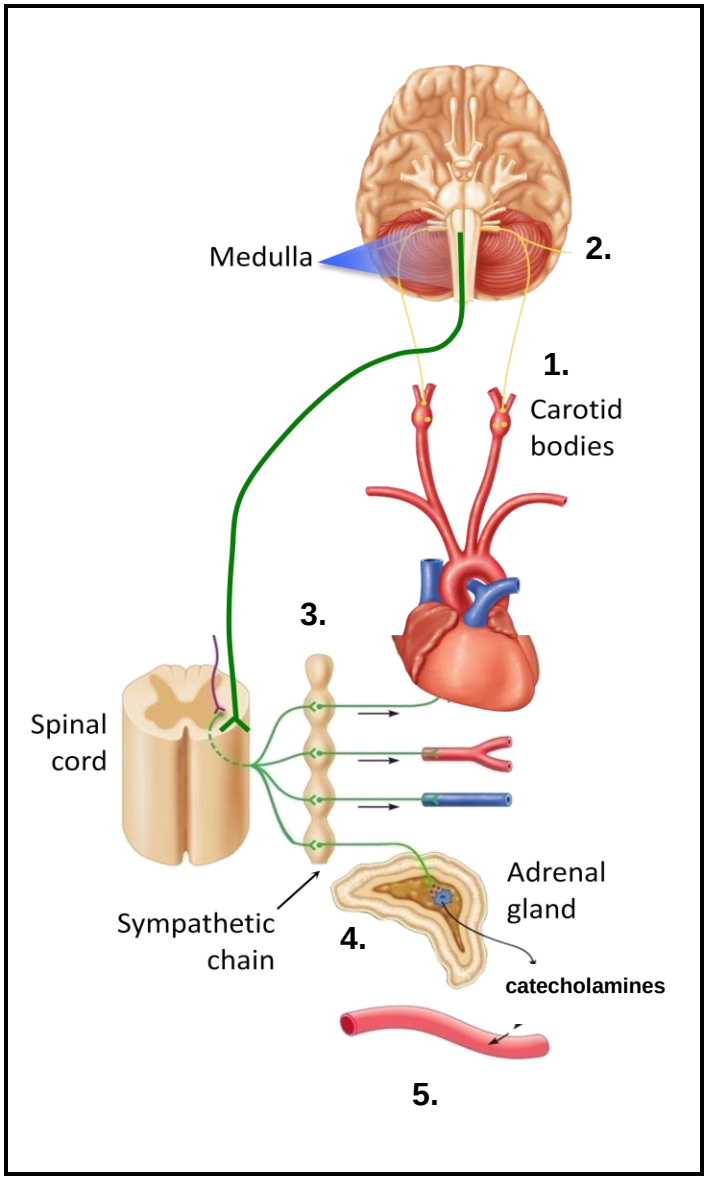
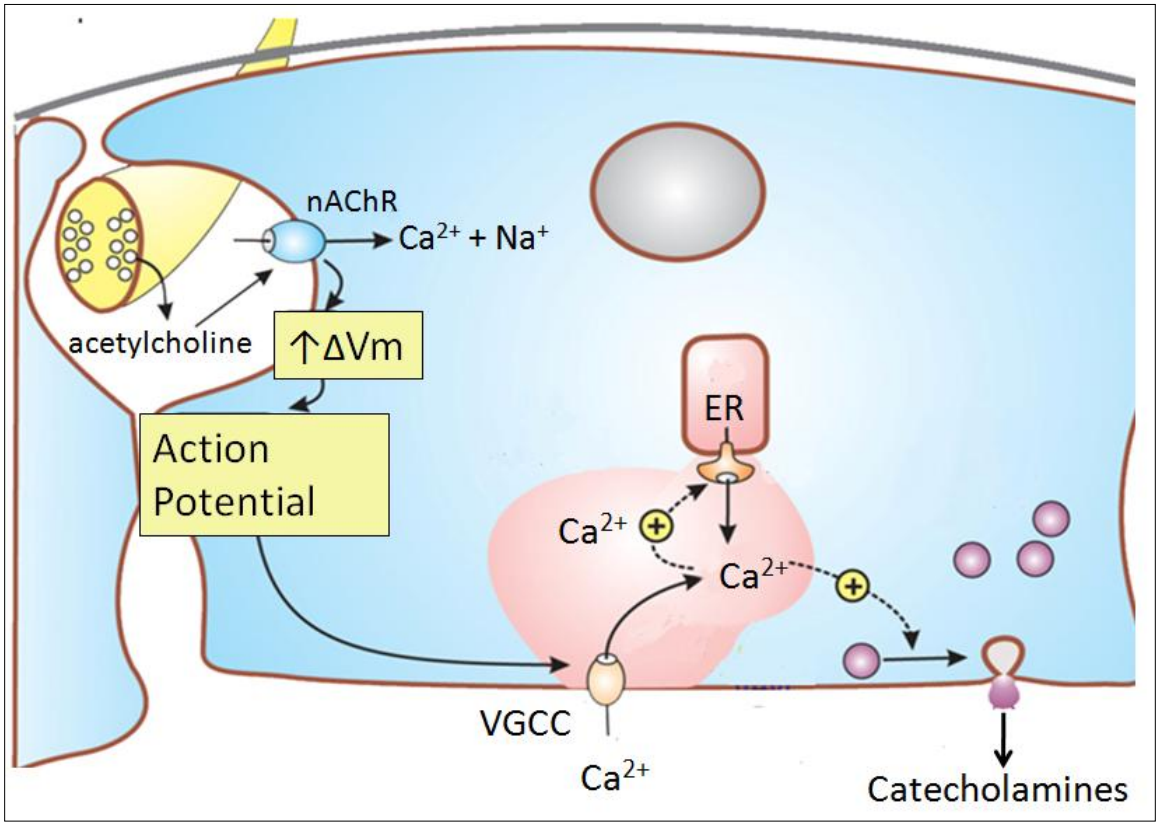

Medulla
2.
1.
Carotid
bodies
3.
Spinal
cord
Sympathetic
chain
4.
Adrenal
gland
catecholamines
5.

Figure 2.2 AMC signalling pathway leading to catecholamine release. The splanchnic nerve releases acetylcholine onto AMCs, this causes nAChRs to open and allows for Ca^{2+} and Na^{+} to influx into the cell. In turn, the cell depolarizes, and VGCC are activated. This initiates CICR from the ER. Increased $[Ca^{2+}]_i$ acts as a trigger for the fusion of vesicles to the plasma membrane, which allows the vesicular contents to be released.


nAChR
Ca^{2+} + Na^{+}
acetylcholine
↑ΔVm
Action Potential
ER
Ca^{2+}
Ca^{2+}
VGCC
Ca^{2+}
Catecholamines

CHAPTER TWO: BLUNTED CATECHOLAMINE RELEASE FROM ADRENAL CHROMAFFIN CELLS IN HIGHLAND DEER MICE (*PEROMYSCUS MANICULATUS*)

2.1 Abstract

Hypoxia at high altitudes can activate the sympathetic nervous system, which may become maladaptive in high-altitude natives if it is maintained over chronic periods. The deer mouse (*Peromyscus maniculatus*) has the largest altitudinal range of all North American mammals, with populations living above 4300m elevation. We hypothesized that high-altitude deer mice might have altered the physiology of chromaffin cells in the adrenal gland to avoid chronic activation of the sympathetic response. We used captive breeding colonies derived from wild populations at high and low altitudes, and compared them in normoxia and after 6-8 weeks of acclimation to hypoxia (12 kPa O_2, simulating the PO_2 at ~4300m). We measured plasma catecholamines as a marker of sympathoadrenal activity in mice at rest in normoxia, after acute (24 hr) exposure to hypoxia, and after hypoxia acclimation. It was found that high-altitude mice had lower levels of plasma adrenaline across treatments compare to lowland white-footed mouse populations (*Peromyscus Leucopus*). Using ratiometric Ca^{2+} imaging with cultured adrenomedullary chromaffin cells (AMCs), we found that the rise in $[Ca^{2+}]_i$ in response to nicotine (1, 5, 25, 125 μM) did not vary between populations and was not affected by hypoxia acclimation. We also examined the relative contributing of α7 nicotinic acetylcholine receptors (nAChRs) to the total Ca^{2+} response by using hexamethonium to block other all other nAChR subtypes. Although there was a greater Ca^{2+} signal in highland populations in response to α7 stimulation, it contributed a small proportion of the total Ca^{2+} signal, suggesting it is not a main contributing factor to the reduction in catecholamine secretion levels. In contrast, levels of cellular catecholamines were

drastically blunted in highland AMCs, both before and after hypoxia acclimation. Taken together this data indicates that chronic sympathetic response may be reduced in highland deer mice via a reduction in catecholamine storage in AMCs.

2.2 Introduction

Activation of the sympathetic nervous system in response to acute hypoxia is key in regulating the cardiovascular system and maintaining O_2 supply to the body's most hypoxia-sensitive tissues. One component of sympathetic activation involves the stimulation of adrenal medulla chromaffin cells (AMCs). These cells release catecholamines, adrenaline and noradrenaline, into the circulation, promoting α-adrenergic dependent vasoconstriction and counteracting the effect of local vasodilatory factors in tissues that are less sensitive to hypoxia. This redistributes blood flow to the most hypoxia-sensitive organs (brain, heart, etc) and is critical for survival in acute hypoxia (Ivy & Scott, 2015; Hainsworth & Drinkhill, 2007). However, the effects of sympathetic activation could become counterproductive if they are maintained with prolonged exposure to hypoxia, because blood flow risks being chronically insufficient to tissues that are less hypoxia-sensitive but are nevertheless important for long-term fitness (gastrointestinal tract, reproductive organs, etc). Despite this, neural sympathoexcitation typically remains high with hypoxia acclimation (Cunningham et al., 1965; Bärtsch et al., 1991; Reeves et al., 1992; Mazzeo et al., 1994; Antezana et al., 1994; Kanstrup et al., 1999; Hansen & Sander, 2003).

Chronic hypoxia alters the cellular mechanisms that contribute to catecholamine release from the adrenal medulla, suggesting that it may fine-tune the sympathetic response. Stimulation of nicotinic acetylcholine receptors (nAChR) on AMCs normally leads to rise in cytosolic Ca^{2+} that acts as a key signal for catecholamine release. Chronic hypoxia has been shown to reduce the magnitude of this cytosolic Ca^{2+} rise by changing nAChR isoform expression (Sala et al., 2008). This occurs because α3-containing nAChR subtypes, which are the primary subtypes involved in initiating catecholamine release in chromaffin cells (Sala et al., 2008; Guérineau, et al., 2012), are susceptible to inactivation during hypoxia by increases in reactive oxygen species (ROS). ROS inactivates α3-containing subtypes by acting on a cysteine residue in the receptor pore (Krishnaswamy, & Cooper, 2012). Furthermore, the expression of homomeric α7 receptors, which are not sensitive to ROS, increases after chronic activation of chromaffin cells (e.g., by chronic hypoxia or nicotine) (Sala et al., 2008). α7 receptors are not believed to contribute to catecholamine release, and may instead contribute to an inhibitory reduction in membrane potential via an upregulation of K_{ATP} channels (Buttigieg et al., 2009; Ducsay et al., 2007). Despite these changes, blood noradrenaline and adrenaline levels typically remain elevated after hypoxia acclimation (Van Hall, et al., 2009), suggesting that the adjustments in AMC physiology do not completely eliminate the rise in catecholamine release relative to baseline levels.

High-altitude natives have often evolved exquisite mechanisms for coping with chronic hypobaric hypoxia and the other rigors of life at high altitudes, often honed by many generations of natural selection. High-altitude natives can thus provide insight into

those responses to hypoxia that are truly adaptive and contribute to fitness at high altitude. High-altitude natives may also be expected to have evolved to blunt those responses to chronic hypoxia that may be detrimental to long-term fitness. While little is currently known regarding adaptations to adrenergic signalling, there is some emerging evidence for changes in adrenergic receptor expression that could reduce the sympathetic response. An example of this is seen in highland Pika (*Ochotona curzoniae*), which have reduced basal expression of β_1-adrenergic receptors in the heart to maintain a lower heart rate at high-altitude (Pichon, et al., 2003). In other highland taxa, including highland Tibetans, there is evidence that blood flow to less O_2-sensitive tissues is increased compared to their lowland counterparts (Moore et al., 2001b; Chen et al., 2002). While there are a number of associated genetic adaptations that could putatively effect adrenergic signalling, much remains unknown regarding the mechanism by which highland taxa alter their sympathetic response (Simonson, 2010; Moore, et al., 2003). Further insight into the control α-adrenergic signalling in both high- and low-altitude populations may illuminate the importance of circulatory changes for adaptation to high-altitude environments.

The purpose of this study was to examine whether circulating catecholamine levels are reduced in high-altitude native deer mice *(Peromyscus maniculatus*) compared to low-altitude *Peromyscus* mice, and to uncover the cellular mechanisms that are involved. Deer mice are a powerful model for studying high-altitude physiology. They inhabit the greatest altitudinal range of all North American mammals, from sea level to over 4300m in elevation (Hock, 1964; Natarajan et al., 2015; Snyder et al., 1982). At the

peak of their elevation range, where O_2 partial pressures are roughly half of those at sea level, deer mice are abundant. Previous studies suggest that high-altitude mice are subject to strong directional selection on a high aerobic capacity to support thermogenesis and exercise, and that high-altitude populations have responded to selection with evolved increases in aerobic capacity and changes in several traits that dictate oxygen uptake and circulation (Hayes & O'Connor, 1999; Lui et al., 2015; Cheviron et al., 2012; Ivy & Scott, 2017; Tate et al., 2017). Some recent evidence also suggests that catecholamine release from the adrenal medulla might be blunted in high-altitude deer mice, in association with a reduction in medulla size and a reduction in the expression of enzymes responsible for catecholamine synthesis (Scott et al., in prep). This study aims to elucidate the cellular mechanisms responsible for these observations by examining cytosolic Ca^{2+} signalling and catecholamine synthesis and storage.

2.3 Methods

2.31 Deer mouse populations

Adult mice were live-trapped both at the summit of Mount Evans (Clear Creek County, CO, USA at 39″35′18″N, 105″38′38″W; 4350 m above sea level) (*P. m. rufinus*; North American deer mice) and at low altitude on the Great Plains (Nine Mile Prairie, Lancaster County, NE, USA at 40″52′12″N, 96″48′20.3″W; 430 m above sea level) (*P. leucopus*, White-Footed mice; and *P. m. nebracensis*, North American deer mice) and subsequently transported to McMaster University (elevation 50 m above sea level). Mice were bred within their respective populations in common-garden conditions to obtain F_1

offspring. The F_1 generation was raised under normoxic conditions until at least 6 months of age before experiments were conducted. All mice were housed under standard conditions (24–25 °C, 12-h: 12-h light–dark photoperiod) and had access to unlimited food and water. All animal protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

2.32 Environmental Hypoxia Exposures

To disentangle the influence of population altitude and acclimation environment, mice from each population were acclimated to (i) standard holding conditions with normobaric normoxia) or (ii) hypobaric hypoxia simulating the pressure at an elevation of 4300 m (barometric pressure of 60 kPa, an O_2 partial pressure of ~12.5 kPa). Specially designed hypobaric chambers were used for hypoxia acclimations, as previously described (McClelland et al., 1998; Lui et al., 2015; Ivy & Scott, 2017b). Mice were acclimated for a minimum of 6 weeks, during which time they were briefly returned to normoxic conditions twice per week for <20 min to replenish food and clean cages.

To obtain plasma catecholamine measurements (more details below) before and after acute hypoxia exposure, highland deer mice and lowland white footed mice were exposed to isobaric hypoxia for 24 h (O_2 partial pressure of 12.5 kPa) using a O_2 Control InVitro Glove Box (Coy Lab Products; Grass Lake, MI, USA). Plasma measurements

were obtained from the same mice both before and after acute hypoxia exposure, with at least 6 weeks separating the blood sampling to allow for the mice to recover. A separate cohort of mice was used to obtain plasma catecholamine levels of hypoxia acclimated mice (acclimated as described above).

Summary of the acclimation/treatment groups used for each experiment:

Four main treatment groups that were used for all experiments: normoxia-acclimated white-footed lowlanders, normoxia-acclimated highlander deer mice, hypoxia-acclimated lowlander white-footed mice, and hypoxia-acclimated highland deer mice.

In addition to these four main groups, a group of hypoxia-acclimated lowland deer mice were used in the Ca^{2+} imaging dose-response experiment only (described below).

Plasma catecholamine measurements were made on the four main treatment groups as well as 24 h exposed hypoxic white-footed lowlanders and 24 h exposed highlander deer mice.

2.33 Plasma Catecholamine Sampling

Plasma catecholamine levels were obtained from the submandibular vein in resting mice. Mice were considered to be at 'rest' when they were sleeping, or showed no signs of activity. They were anesthetised with isoflurane to cause a rapid loss of consciousness. Samples were only used if mice showed no sign of activity during this process. Blood samples were collected in heparinized tubes, and spun down for 6 min to separate plasma from the red blood cells. The plasma was flash frozen and stored at -80°C for future measurements. At the time of catecholamine measurement, samples were thawed on ice. Adrenaline, noradrenaline, and dopamine concentrations were measured using 3-CAT Research ELISA (Rocky Mountain Diagnostics; Colorado Springs, CO, USA) as per the manufacturer's protocol.

2.34 Intracellular Ca^{2+} measurements from Cultured AMCs

Primary cultures AMC cultures were prepared from adult mice by combined enzymatic and mechanical dissociation as described in detail previously (Thompson et al., 1997; Thompson and Nurse, 1998). Briefly, most of the surrounding cortical tissue was trimmed and discarded. The remaining central medulla was incubated in an enzymatic solution, containing 0.1% trypsin and 0-1 % collagenase (Gibco) for 40 m at 37 °C. Following incubation, most of the enzyme was inactivated with supplemented of F-12 nutrient medium (Gibco) (Thompson et al., 1997), followed by mechanical dissociation and trituration. The dispersed cell suspension was allowed to adhere to the central wells of modified tissue culture dishes; the wells were pre-coated with a thin layer

of Matrigel (BD Biosciences, Mississauga, Ontario, Canada). The cells were cultured in supplemented F-12 nutrient medium and incubated in either 21% or 2% O_2 (each mixed with 5% $CO_2$and the balance N_2). By using both normoxic and hypoxic culture conditions we were able to asses if culture PO_2 effected cell signalling.

To perform ratiometric Ca^{2+} imaging cells were loaded with the fluorescent Ca^{2+} indicator fura-2 AM, by incubating the cells in 2.5 μM fura-2 in standard bicarbonate-buffered solution (BBS: NaHCO3, 24 mM; NaCl, 115 mM; glucose, 5 mM; KCl, 5 mM; CaCl 2mM and MgCl 2mM) for 20-40 min at 37 °C. Excess free fura-2 was subsequently washed off with BBS.

Ratiometric Ca^{2+} imaging was performed as described by Murali, et al., 2014, using a Nikon Eclipse TE2000-U inverted microscope (Nikon, Mississauga, ON, Canada) equipped with a Lambda DG-4 ultra high-speed wavelength changer (Sutter Instrument CO., Novato, CA, USA), a Hamamatsu OCRCAT-ET digital CCD camera (Hamamatsu, Sewickley, PA, USA), and a Nikon S-Fluor 40× oil-immersion objective lens(numerical aperture of 1.3).

Dual images (340 and 380 nm excitation; 510 nm emission) were collected. Simple PCI software, version 5.3 (Compix) was used to collect the pseudocolor ratiometric. The imaging system was calibrated using 11 buffers of known Ca^{2+} concentrations, ranging from a Ca^{2+}-free solution to a saturating Ca^{2+} solution (39 μM) (Molecular Probes, Cat. No. F-6774). These values were used to calculate the ratios as follows: ‘R’ is the 510 nm emission intensity at 340 nm excitation to 510 nm emission

intensity at 380 nm excitation; R_{min}, the ratio at zero free Ca^{2+}; R_{max} the ratio at saturating Ca^{2+}; and β, the fluorescence intensity with excitation at 380 nm for zero free Ca^{2+} (F380max), to the fluorescence intensity at saturating free Ca^{2+} (F380min). The intracellular free [Ca^{2+}] was obtained after substituting the ratios into the Grynkiewicz equation (Grynkiewicz et al., 1985) as follows: where R_{min} = 0.18, R_{max} = 7.81, β = 12.29, K_d = 225 nM and R is the ratio obtained during the experiment for a given cell.

Dishes were continuously perfused to maintain ion concentrations. The switch between control and test solutions was facilitated by the use of a double-barreled perfusion system (Zhang et al., 2000). Nicotine, at doses of 1 μM, 5 μM, 25 μM, and125 μM, was used as a nAChR agonist to examine its dose-response relationship with Ca^{2+} in AMCs. Hexamethonium (100 μM), a general blocker of all nAChRs, with the exception of α7 receptors, was used with nicotine to examine the Ca^{2+} response to α7 stimulation. All solutions containing drugs were made fresh on the day of the experiment. Drugs were obtained from Sigma-Aldrich.

2.35 Cellular Catecholamines

To obtain intracellular catecholamine measurements from AMCs, the adrenal chromaffin cells were cultured (as described above) and incubated for 48 h in normoxic conditions (21% O_2, 5% CO_2). To weaken their adhesion to the dish, the supplemented F-12 media was removed and replaced with 0.25% Tyrpsin/EDTA to weaken their

adhesion to the culture dishes. This was followed by triturating cells on the dish. Suspended cells were collected and spun down for 3 min (1200 rpm). The supernatant (0.25 % Trypsin/EDTA solution) was removed and cells were resuspended in supplemented F12 growth media and subsequently flash frozen in liquid nitrogen and stored at -80 °C for later use.

To measure cellular catecholamine content cells were thawed on ice and spun down for 3 min at 1200 rpm. Any remaining supernatant was removed and cells were resuspended in homogenization buffer (0.01 M HCl, 1 mM EDTA, 4 mM sodium metabisulfite) to optimize catecholamine solubility. Cells were sonicated as described in Brown et al, 2009 with two 10 s sonicating pulses. Adrenaline, noradrenaline, and dopamine content were measured using 3-CAT Research ELISA (Rocky Mountain Diagnostics; Colorado Springs, CO, USA) as per the manufacturer's protocol. For cellular catecholamines measurements, catecholamine concentration was normalized to total cellular protein content, quantified though a Bradford Assay.

2.36 Cellular Catecholamine Secretion

Following cell culture and incubation for 48 h in normoxic conditions (21% O_2, 5% CO_2) 10 μM nicotine (diluted in BBS to mimic physiological ion concentrations) was applied to AMCs. After 3.5 min the total volume of BBS was measured, and flash frozen for subsequent catecholamine measurements, and stored at -80 °C. Adrenaline,

noradrenaline, and dopamine concentrations were measured using 3-CAT Research ELISA (Rocky Mountain Diagnostics; Colorado Springs, CO, USA) as per the manufacturer's protocol. Catecholamine concentrations were normalized to the total BBS volume to obtain the total catecholamine secretion.

2.37 Statistical Analysis

Resting plasma measurements before and after acute hypoxia were analyzed using a two-way matched ANOVA; however, comparisons between normoxic *vs* acclimated mice, 24 h hypoxic *vs* acclimated mice, and comparisons across all three treatments are made with unmatched two-way ANOVAs. For the remainder of the experiments two-way ANOVAs were used exclusively for statistical comparisons, and bonferroni post-hoc tests were used throughout.

2.4 Results

2.41 Plasma Catecholamines

High-altitude deer mice had lower resting plasma adrenaline levels than low-altitude white-footed mice (Figure 1; main effect of population in two-factor ANOVA: $F_{1,26}$=8.606, P<0.01). Plasma adrenaline levels increased after 24 hours of hypoxia exposure compared to normoxic controls (effect of acclimation in two-factor subject-

matched ANOVA: $F_{1,8}$=12.55, P<0.01). After hypoxia acclimation plasma adrenaline decreased (24 h hypoxia *vs* hypoxia acclimated, two-factor ANOVA: $F_{1,18}$=10.84, P<0.01) to return to levels comparable to those in normoxic mice ($F_{1,18}$=3.718, P=0.0698). However, the hypoxia-induced changes in adrenaline levels were more pronounced in lowland mice, which increased 14% (P<0.05) after 24 hours hypoxia to levels that were 20% higher than those in the highland mice. Patterns of variation in noradrenaline and dopamine levels were less pronounced, and there were no differences in plasma noradrenaline or dopamine between populations (noradrenaline, $F_{1,25}$=0.018, P=0.8946; dopamine,$F_{1,27}$=1.140, P=0.2951). Noradrenaline levels did not change after 24 h in hypoxia in either population (effect of acclimation in two-factor subject-matched ANOVA: $F_{1,8}$=1.074, P=0.3303), but increased 213% in lowland and 393% in highland mice after hypoxia acclimation (effect of acclimation in two-factor ANOVA, $F_{1,19}$=10.69, P<0.01). Dopamine levels did not change with any exposure to hypoxia (two-factor ANOVA; $F_{1,27}$=0.2849, P=0.7543).

2.42 Ca^{2+} Response

There was no significant variation in the cytosolic Ca^{2+} response to nicotine between populations or in response to hypoxia acclimation. Hypoxia acclimation had no effect on the total Ca^{2+} response (normoxic incubation: two-factor ANOVA, $F_{1,14}$=1.729, P=0.2097; hypoxic incubation: $F_{1,18}$=2.015, P=0.1728), peak [Ca^{2+}] (normoxic incubation: $F_{1,14}$=2.566, P=0.1315; hypoxic incubation: $F_{1,18}$=0.00036, P=0.9850), or the duration of the Ca^{2+} response (normoxic incubation: $F_{1,14}$=0.7443, P=0.4028; hypoxic

incubation: $F_{1,18}$=2.858, P=0.1082) in either population. There were also no main effects of population altitude on the total response (normoxic incubation: $F_{1,14}$=6.573, P= 0.0225; hypoxic incubation: $F_{1,18}$=0.04788, P= 0.8293), peak [Ca^{2+}] (normoxic incubation: $F_{1,14}$= 5.539, P=0.0337; hypoxic incubation: $F_{1,18}$= 0.3061, P=0.5869), or response duration (normoxic incubation: $F_{1,14}$= 0.2336, P= 0.6363; hypoxic incubation: $F_{1,18}$=1.161, P=0.2955).

The total Ca^{2+} response to nicotine+hexamethonium, a treatment that was meant to only stimulate nAChRs containing α7 receptors (because hexamethonium inhibits all other subtypes), was greater in highland mice than in lowland mice (Figure 4). In normoxia and hypoxia acclimated mice, the total Ca^{2+} response was 2.3- and 3.7-fold greater, respectively, in highlanders than in lowlanders (effect of population in two-factor ANOVA, $F_{1,38}$=6.422, P<0.05). Similarly, the peak Ca^{2+} response was 1.4- and 2.6-fold greater, in normoxia and hypoxia respectively, in highlanders compared to lowlanders ($F_{1,38}$=5.331, P<0.05). Furthermore, there were fewer cells per dish whose Ca^{2+} response to nicotine was completely blocked by hexamethoniumin in highlanders than in lowlanders ($F_{1,14}$=7.543,P<0.05). This was particularly pronounced after hypoxia acclimation, at which the percent of completely blocked cells decreased by 86% in highlanders (P<0.05) and was 88% lower (P<0.05) than that in lowlanders.

2.43 Cellular Catecholamine Content

Chromaffin cells of highland deer mice contained fewer catecholamines than those of lowland white-footed mice. Total catecholamine contents were 62% and 80% lower in highlanders than in lowlanders, in normoxia and hypoxia acclimation, respectively (Fig.5: main effect of population in two-factor ANOVA: $F_{1,19}$=10.13, P<0.01). This was a result of highlanders having lower levels of each individual catecholamine, adrenaline (population effect: $F_{1,19}$=12.97, p<0.01), noradrenaline ($F_{1,19}$=15.50, P<0.001), and dopamine ($F_{1,19}$= 7.451, P<0.05) (Fig. 5). The lower levels of adrenaline and noradrenaline in highland mice were particularly pronounced after hypoxia acclimation, when levels were 80% and 90% lower in highlanders than in lowlanders (main effects of acclimation in two-factor ANOVA: adrenaline, $F_{1,19}$= 0.2776, P=0.6043; noradrenaline, $F_{1,19}$= 0.5729, P=0.4584). Dopamine levels were 62% lower in highlanders than in lowlanders in normoxia, and decreased in both populations after hypoxia acclimation (main effect of acclimation: $F_{1,19}$= 4.426, P=0.049).

2.44 Catecholamine Secretion

Catecholamine secretion was measured in cells from normoxic mice that were incubated in normoxic conditions. It was found that, across the three catecholamines, adrenaline, noradrenaline and dopamine cultured highlander chromaffin cells released a lower amount of catecholamines (Figure 2: effect of population in a two-way ANOVA $F_{1,12}$=5.407, P<0.05).

2.5 Discussion

Sympathetic control of the cardiovascular system has important implications for tissue blood flow and O_2 supply during high-altitude hypoxia. Here, we show that high-altitude deer mice have evolved in such a way to reduce the circulating levels of catecholamines in the plasma. This arises from a reduction in catecholamine storage in chromaffin cells of the adrenal medulla that lowers catecholamine secretion, without any differences in the global cytosolic Ca^{2+} response to stimulation by nicotine between populations. However, chromaffin cells of highlanders are more responsive to nicotinic stimulation via nAChRs that contain the α7 subunit compared to cells of lowlanders. Although this may have important implications for chromaffin cell function, the relatively low contribution of α7-nAChRs to the global Ca^{2+} response to stimulation suggests that this observation may not be a primary contributor to the evolved reductions in plasma catecholamines in highland deer mice.

2.51 Hypoxia Acclimation Decreased Circulating Adrenaline Levels

Hypoxia acclimation was observed to reduce plasma adrenaline levels compared to the elevated levels that were observed after 24 h exposure to hypoxia (Fig. 1A). This is consistent with previous observations in low-altitude populations of deer mice, in which hypoxia acclimation reduced stress-induced plasma adrenaline levels and reduced catecholamine secretion from adrenal medulla slices in response to sub-maximal nicotine

stimulation (Scott et al., in prep). Plasma noradrenaline levels, on the other hand, increased only after long term acclimation (Fig. 1B). This aligns with well established human data, which show no or little change in noradrenaline with acute hypoxia (Rostrup, 1998; Cunningham et al., 1965; Reeves et al., 1992; Mazzeo et al., 1994; Kanstrup et al., 1999; Bärtsch et al., 1991), but a robust long term increase with high- altitude acclimatization (Cunningham et al., 1965; Bärtsch et al., 1991; Reeves et al., 1992; Mazzeo et al., 1994; Antezana *et al.*1994; Ponchia et al., 1994). Noradrenaline can enter the plasma from spillover at synapses as well as from secretion by the adrenal medulla, so it is possible that the increase in plasma noradrenaline that we observed after hypoxia acclimation was caused by increases in neural sympathoexcitation (Anand et al., 1993; Antezana et al., 1995; Hansen & Sander, 2003). Adrenaline, unlike noradrenaline, is only released from the adrenal medulla and is a stronger indicator of AMC activity, so our adrenaline results suggest that adrenaline release from AMCs is reduced after hypoxia acclimation in *Permyscus* mice.

Cellular adrenaline storage and the global Ca^{2+} response to nicotine stimulation were unaltered by hypoxia acclimation, suggesting that other mechanisms contribute to the reduction in catecholamine release that we observed previously (Scott et al., in prep). For example, it is possible that hypoxia acclimation decreased the stimulation of the adrenal medulla *in vivo*. Hypoxia acclimation can partially recover arterial O_2 levels in lowland *Peromyscus* mice (Ivy and Scott, 2017) and other low-altitude natives (Slessarev et al., 2010; Yilmaz et al., 2005; Ivy & Scott, 2017b; Pamenter et al., 2017), which likely increases tissue O_2 levels of the adrenal medulla. Hypoxia acclimation may also blunt the

hypoxic chemoreflex that leads to sympathoexcitation. However, the maintenance of sympathoexcitation in chronic hypoxia has been well documented in lowland human populations (Cunningham et al., 1965; Bärtsch et al., 1991; Reeves et al., 1992; Mazzeo et al., 1994; Antezana et al., 1994; Kanstrup et al., 1999; Hansen & Sander, 2003), and carotid body size along with several efferent responses of the hypoxic chemoreflex (e.g., heart rate, hypoxic ventilatory response) increase after hypoxia acclimation in *Peromyscus* mice native to low-altitude (Ivy & Scott, 2017). It is also possible that the lack of any observed changes in the global cytosolic Ca^{2+} response after hypoxia acclimation resulted from effects on cellular Ca^{2+} signalling during isolation and culture of the cells, the use of a flow-through system, or the removal from the influences of *in vivo* innervation and autocrine and paracrine agents (García, et al., 2006). For example, opioid signalling occurs between AMCs and neurons, and the removal of stimulation of opioid receptors on AMCs by the neurons may reduce the inhibition of voltage-gated Ca^{2+} channels through a g-protein mediated pathway. In fact, opioid signalling from neurons is implicated in the loss in the ability of chromaffin cells to respond directly to hypoxia in postnatal sheep (Keating et al., 2004). Furthermore, opioids are also known to act as an autocrine and paracine agent, therefore the use of a flow through system may reduce the negative feedback on Ca^{2+} signalling observed *in vivo* (Doupnik & Pun, 1994; Albillos, et al., 1996; Currie & Fox, 1996).

2.52 Evolved Reduction of Catecholamine Release in Highland Deer Mice

High-altitude deer mice had lower levels of plasma adrenaline compared to low-altitude mice. This is consistent with lower plasma catecholamine measurements found in Bolivian high-altitude natives (Van Hall, et al., 2009), and observations that lower catecholamine levels correlate with a more adaptive highland phenotype (i.e. lower haemoglobin levels, decreased risk of chronic mountain sickness) (Antezana et al., 1995; Gamboa, et al., 2006). Previous studies of tissue slices from the adrenal medulla have shown that catecholamine secretion in response to nicotine stimulation is reduced in deer mice native to high altitudes, compared to their lowland counterparts (Scott et al., in prep), a finding that we confirm here for isolated AMCs (Fig. 2). Furthermore, the reduction in plasma catecholamines may be exacerbated *in vivo* due to a lower number of AMCs in the adrenal medulla of highlanders compared to lowlanders (Scott et al., in prep). These observations in deer mice suggest that decreases in circulating adrenaline in high-altitude natives result from evolved reductions in catecholamine secretion from the adrenal medulla.

There were no differences in the global Ca^{2+} response to nicotine stimulation that could account for the differences in catecholamine secretion between the populations. We had hypothesized that the increase in cytosolic [Ca^{2+}] in response to nicotine stimulation might be blunted in highlanders, possibly resulting from an increased expression of α7-nAChRs, and that this reduces the intracellular signal for catecholamine release. Previous

work has shown that chronic exposure of neonatal rats to nicotine reduces the Ca^{2+} response to acute hypoxia stimulation in AMCs (Buttigieg et al., 2008). This was attributed to an observed shift in the expression of nicotinic acetylcholine receptor subtypes from α3 to α7, because α7 receptors are believed to oppose catecholamine release by reducing the membrane potential via an upregulation of K_{ATP} channels (Buttigieg et al., 2009; Ducsay et al., 2007; Krishnaswamy, & Cooper, 2012). However, in contrast to our hypothesis, the increase in cytosolic [Ca^{2+}] in response to nicotine stimulation was similar between populations. Furthermore, although highland deer mice had greater response to α7 stimulation than white-footed mice, it was still very minor in proportion to the total Ca^{2+} response. These results raise the question of why a robust, and presumably energetically expensive, Ca^{2+} response is maintained in highland deer mice, even though it is associated with less catecholamine release. It is possible that intracellular Ca^{2+} signalling is preserved to maintain the release of other important paracrine or endocrine signalling molecules, like opioids and chromogranins (Tota, et al., 2010), a possibility that warrants further study.

The low circulating adrenaline and blunted catecholamine release of high-altitude deer mice appeared to be caused by evolved reductions in catecholamine synthesis and/or storage in chromaffin cells. We have previously shown that the chromaffin cells of highland deer mice express less dopamine decarboxylase (DDC) (Scott *et al*, in prep.), which (as the enzyme responsible for the synthesis of dopamine) may act as a bottleneck that reduces the production of subsequent catecholamines (adrenaline and noradrenaline) in these biosynthetic pathway. Interestingly, the expression of several key enzymes

involved in catecholamine synthesis, including DDC, is controlled by hypoxia inducible factor 2α (HIF-2α) (Brown et al., 2009), which is less abundant in the adrenal medulla of highland deer mice after hypoxia acclimation (Scott *et al*, in prep.), and whose gene (*Epas1*) has been a target of natural selection in high-altitude populations of deer mice (Cheviron, in prep.) and in several other highland taxa (Simpson, et al., 2010; Zhang et al., 2010; Song et al., 2016). Therefore, adjustments in adrenal catecholamine release, possibly mediated by adaptive changes in HIF-2α signalling and in association with other physiological changes, may be key adaptations to life at high altitude.

Figure 2.1 Lowland white-footed mice have great resting plasma adrenaline levels compared to highland deer mice. Plasma catecholamine levels increased with acute hypoxia exposure, but lowered with acclimation to hypoxia. *Significant pairwise difference between populations within each acclimation environment using bonferroni post-tests. †Significant pairwise difference with exposure to 24 hours of hypoxia, within each population using bonferroni post-tests. #Significant difference between 24 hour hypoxia exposure and hypoxia acclimation within each population. Adrenaline n =6 lowland white-footed mice before and after 24 hours of hypoxia, and n =4 highland deer mice before and after 24 hours of hypoxia, n =5 lowland mice after hypoxia acclimation, n =7 highland mice after hypoxia acclimation. Noradrenaline n =5 lowland mice before and after 24 hours of hypoxia, and n =4 highland mice before and after 24 hours of hypoxia, n =6 lowland mice after hypoxia acclimation, n =7 highland mice after hypoxia acclimation. Dopamine n =6 lowland mice before and after 24 hours of hypoxia, and n =4 highland mice before and after 24 hours of hypoxia, n =6 lowland mice after hypoxia acclimation, n =7 highland mice after hypoxia acclimation.

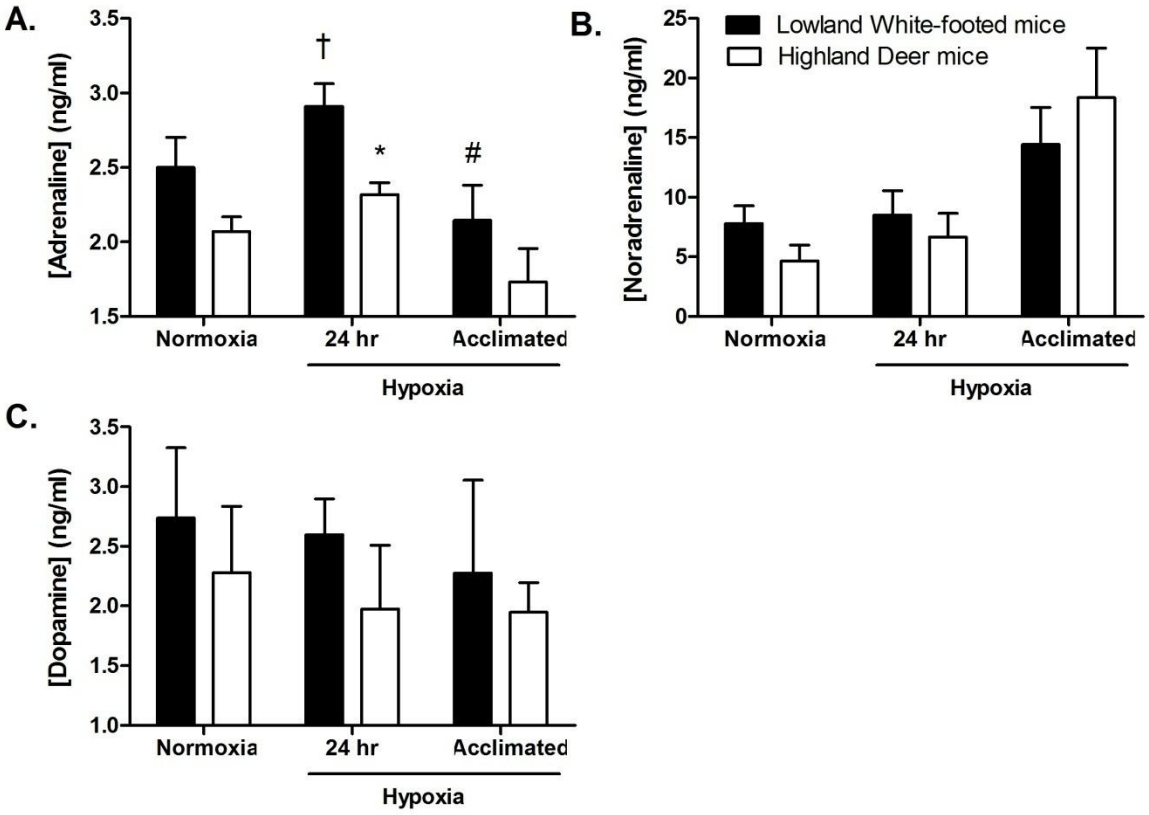

A.
[Adrenaline] (ng/ml)
3.5
3.0
2.5
2.0
1.5
†
*
#
Normoxia
24 hr
Acclimated
Hypoxia
B.
[Noradrenaline] (ng/ml)
25
20
15
10
5
0
Lowland White-footed mice
Highland Deer mice
Normoxia
24 hr
Acclimated
Hypoxia
C.
[Dopamine] (ng/ml)
3.5
3.0
2.5
2.0
1.5
1.0
Normoxia
24 hr
Acclimated
Hypoxia

Figure 2.2. High-altitude deer have a blunted secretion of catecholamines with application of 10μM nicotine. *Significant main effect of populations using two-way ANOVA. *n* =3 lowland white-footed mice and *n* =3 for highland deer mice.

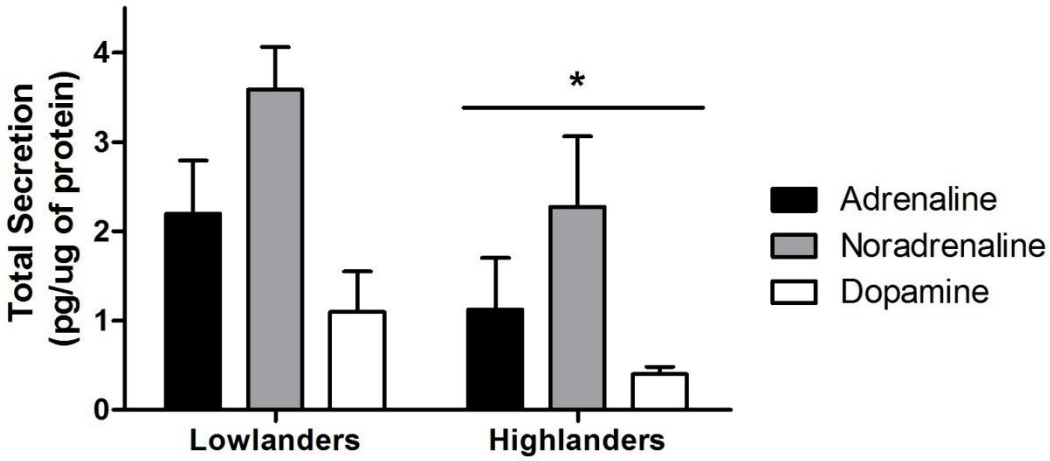

Total Secretion
(pg/ug of protein)
4
3
2
1
0
*
Lowlanders
Highlanders
Adrenaline
Noradrenaline
Dopamine

Figure 2.3. No differences with population or acclimation were observed in the dose-response relationship of AMCs when incubated in either normoxia (A-C), or hypoxia (G-I). There were no significant differences between populations or with acclimation using bonferroni post-hoc tests in the (D) maximal total Ca^{2+} response, (E) the peak response, and (F) the duration of response (*n*= 5 normoxic and hypoxia acclimated lowland white footed mice, *n*=4 normoxic and hypoxia acclimated highland deer mice, *n*=3 hypoxia acclimated lowland deer mice). Similarly, in hypoxia incubated cells no differences were observed in (J) maximal total Ca^{2+} response, (K) the peak response, and (L) the duration of response (*n*= 8 normoxic white footed lowlanders, *n*=4 hypoxia acclimated lowland white-footed mice, *n*= 6 normoxic highland deer mice, *n*=4 hypoxia acclimated highland deer mice, *n*=3 hypoxia acclimated lowland deer mice).

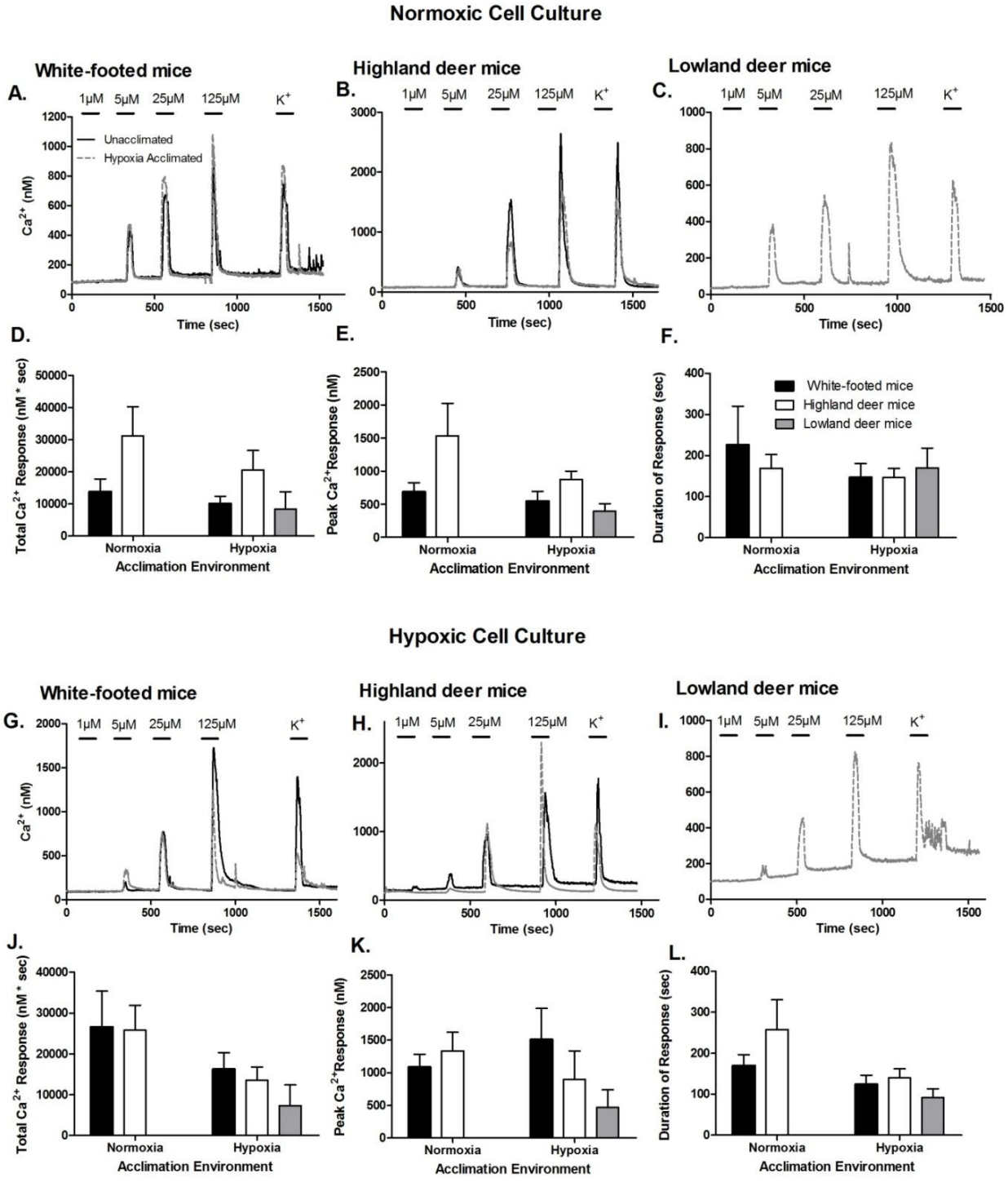

Normoxic Cell Culture
White-footed mice
Highland deer mice
Lowland deer mice
A.
B.
C.
1μM 5μM 25μM 125μM K+
Unacclimated
Hypoxia Acclimated
Ca2+ (nM)
Time (sec)
D.
E.
F.
Total Ca2+ Response (nM * sec)
Peak Ca2+Response (nM)
Duration of Response (sec)
White-footed mice
Highland deer mice
Lowland deer mice
Normoxia
Hypoxia
Acclimation Environment
Hypoxic Cell Culture
G.
H.
I.
J.
K.
L.

Figure 2.4. (A-C) High-altitude deer mice have a greater Ca^{2+} response to direct activation of α7 nAChRs (main effect of population two way ANOVA, $p<0.05$); α7 subtypes were activated via the blockade of other subtypes, with hexamethonium, and stimulation with nicotine (*n*= 9 normoxic lowlanders, *n*=7 hypoxic lowlanders, *n*= 10 normoxic highlanders, *n*=16 hypoxic highlanders). Additionally, the number of cells completely blocked by hexamethonium, is reduced with hypoxia acclimation in highland mice. Therefore the proportion of cells expressing α7 nAChRs may increase in hypoxia-acclimated highland mice (*n*= 4 normoxic lowlanders, *n*=3 hypoxic lowlanders, *n*= 5 normoxic highlanders, *n*=5 hypoxic highlanders).*Significant differences between populations within each acclimation environment using bonferroni post-tests. †Significant differences with acclimation environment within each population using bonferroni post-tests.

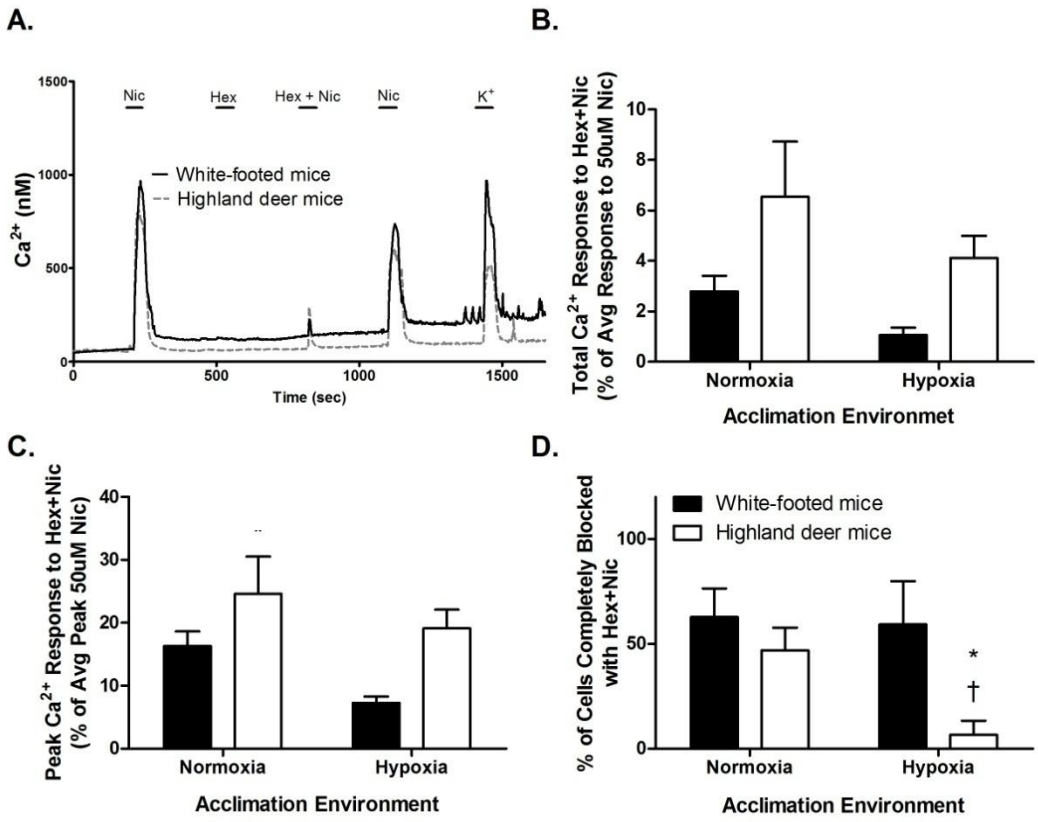
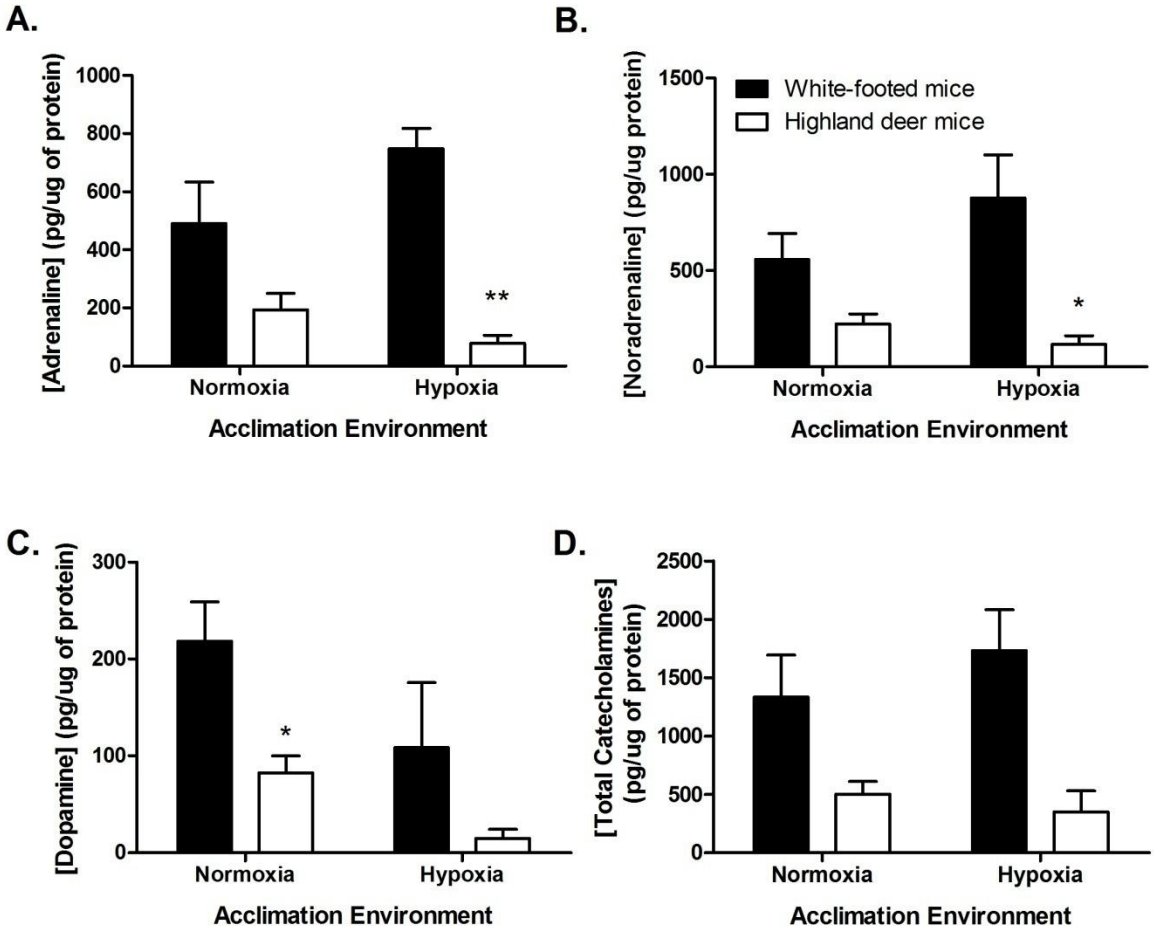

A.
B.
C.
D.
Nic
Hex
Hex + Nic
Nic
K+
White-footed mice
Highland deer mice
Ca2+ (nM)
Time (sec)
Total Ca2+ Response to Hex+Nic (% of Avg Response to 50uM Nic)
Normoxia
Hypoxia
Acclimation Environmet
Peak Ca2+ Response to Hex+Nic (% of Avg Peak 50uM Nic)
Acclimation Environment
% of Cells Completely Blocked with Hex+Nic
*
†

Figure 2.5. High-altitude deer mice AMCs exhibited blunted catecholamine storage relative to those of the white-footed mice (*n* = 9 normoxic lowlanders, *n* = 3 hypoxic lowlanders, *n* = 7 normoxic highlanders, and *n* = 4 hypoxic highlanders). *Significant differences between populations within each acclimation environment using bonferroni post-tests.


A.
[Adrenaline] (pg/ug of protein)
1000
800
600
400
200
0
**
Normoxia
Hypoxia
Acclimation Environment
B.
[Noradrenaline] (pg/ug protein)
1500
1000
500
0
White-footed mice
Highland deer mice
*
Normoxia
Hypoxia
Acclimation Environment
C.
[Dopamine] (pg/ug of protein)
300
200
100
0
*
Normoxia
Hypoxia
Acclimation Environment
D.
[Total Catecholamines] (pg/ug of protein)
2500
2000
1500
1000
500
0
Normoxia
Hypoxia
Acclimation Environment

CHAPTER 3: GENERAL DISCUSSION AND SUMMARY

3.1 Sympathoadrenal Signalling in Highland Deer Mice

In this study I have provided evidence for a blunting of the adrenomedullary response in highland deer mice, as well as the traits which underlie this reduction. Catecholamine storage in highland AMCs was dramatically lower compared to levels in lowland white footed mice (Fig 2.5). This is likely the primary factor underlying the reduction in catecholamine secretion from the isolated AMCs (Fig 2.2), and likely plays an important role in the reduced levels of plasma adrenaline levels *in vivo* (Fig 2.1.). This was supported by the finding of Scott et al., (in prep) that highland mice have low levels of DDC, the enzyme responsible for the synthesis of dopamine, in the adrenal medulla. As dopamine synthesis precedes that of adrenaline and noradrenaline, this may be an important bottleneck for catecholamine synthesis. As mentioned previously, DDC expression is controlled by HIF2α, therefore it is possible that the evolved specializations in the *EPAS*1 gene (Chevron, in prep) may cause a reduction in α-mediated vasoconstriction.

While this work focused on highlander AMC physiology and its role in the sympathetic response to hypoxia, many factors may contribute to the sympathoadrenal activity and the downstream sympathetic response. Morphological measurements of the adrenal medulla of highland and lowland deer mice found that highland mice had a lower number of AMCs (Scott et al., in prep). This likely compounds the effects of lower cellular catecholamine secretion *in vivo*. Other factors, such as neural sympathetic activation, and adrenergic receptor populations can also affect the sympathetic response

in vivo. Although we know little about the neural sympathetic activation of highland deer mice (Ivy & Scott, 2017), evidence suggests that while there are few differences in the adrenergic receptors between populations β_1 receptors had a greater capacity to increase heart rate in highland mice (Wearing et al., in prep). Taken together, these data suggests the highland mice have robust adaptations fine tune adrenergic-mediated responses.

3.2 Sympathoadrenal Signalling in Lowland White-Footed Mice

This study has shown that the lowland white-footed mice are able to plastically reduce their catecholamine release after acclimation to hypoxia (Fig 1); however, the underlying mechanism remains unclear. In contrast to highland deer mice, lowland white-footed mice showed no changes in catecholamine storage (Fig 5); this suggests that mechanisms involved in triggering vesicle fusion are likely altered after hypoxia acclimation. This may allow for more flexibility in the catecholamine response, such that greater stimuli to AMCs may increase catecholamine secretion, as was seen previously in lowland populations of deer mice (Scott el al. in prep).

3.3 Implications for High-Altitude Species

This research provides evidence for directional selection to reduce the α-adrenergic mediated vasoconstriction that occurs during sympathetic activation, in animals living at high-altitude. There has been little research on the AMC activity and

high-altitude adaptations, however this may be an important factor underlying adaptive traits in highland natives, including the reduction in of intrauterine growth restriction, which has negative long term effects on offspring fitness, and the reduction in the incidence of chronic mountain sickness (Moore et al., 2001a). Therefore, studying adrenergic signalling may be an important avenue in understanding highland adaptations in other taxa.

At high-altitudes the 'fight or flight' response may still remain critical in surviving acute stressors (ie. escaping predation, attaining food, and coping with extreme weather). Therefore, a decline in adrenergic signalling with high-altitude adaptation may result in the loss or reduction of the acute 'fight or flight' response, which can be critical for survival. While highland deer mice exposed to an acute 'startle' stress experienced an elevation in plasma catecholamines, it was much lower than that of lowland deer mice (Scott et al, in prep). While it is unclear if these levels are sufficient to mount an effective 'fight or flight' response in the wild, the blunted catecholamine release may present a trade-off required for high-altitude adaptation. This may result in greater risk of mortality with predation or attacks. Therefore, directional selection favouring reduced adrenergic activity may be more predominant in environments with less predation or competition, and in animals with life-styles that favour less burst activity.

3.4 Conclusions

These results illustrate that highland mice dramatically blunt adrenergic signalling through a reduction in catecholamine stores. The consistent drop in catecholamine storage across acclimations, as well as the putative genetic adaptations involved in controlling catecholamine synthesis, suggests that the reduction in sympathetic activity is highly selected for in these high-altitude mice. This was in stark contrast to the white-footed lowlander population. These results are in strong support of the hypothesis that α-adrenergic-mediated vasoconstriction is deleterious to animals living at high-altitude.

3.5 Future Directions

The work presented in this thesis has raised several questions that require further investigation.

1. What changes in AMC signalling cause the reduction in plasma catecholamine levels in hypoxia-acclimated lowland white-footed mice?

The reduction in plasma catecholamine release with acclimation, in white-footed mice, did not appear to be due to either a reduction in catecholamine synthesis or a reduction in Ca^{2+} signalling. While the *in vivo* decline of plasma adrenaline may be due to lower adrenal excitation by the splanchnic nerve, this would be at odds with our plasma noradrenaline measurements, which suggest that sympathetic activation may remain high in White-footed mice after acclimation. Therefore it is likely that changes in

AMC signalling account for the differences in catecholamine release. It is possible that Ca^{2+} signalling changes were masked in this data set due to either i) the loss of paracrine signalling with cell isolation ii) limitations in the Ca^{2+} imaging techniques used for this study. To explore this issue, catecholamine secretion from cells with paracrine signalling (from medullary slices) and without paracrine signalling (from isolated cells) can be compared. Furthermore, the use of stop-flow, as opposed to flow-through perfusion, during Ca^{2+} imaging can also be used to examine the effect of inhibitory paracrine signalling on changes in $[Ca^{2+}]_i$ (Doupnik & Pun, 1994; Albillos et al., 1996; Currie & Fox, 1996).

2. Why is robust Ca^{2+} signalling maintained in highland deer mice despite reductions in catecholamine release?

Our study presented evidence that Ca^{2+} signalling was maintained despite low catecholamine release. This aligns with the findings by Scott et al (in prep), which demonstrated that the reduced catecholamine secretion from the adrenal medulla in highland deer mice was due to a decline in quantal secretion and not secretion frequency. This suggests that there is a sustained Ca^{2+} response in highland mice. However, increases in intracellular Ca^{2+} are tightly regulated; this is an energetically expensive process, as it requires active transport to pump Ca^{2+} out of the cell or sequester it in the ER or mitochondria. As such, maintaining an ATP-demanding process in O_2 limiting environments appears counterintuitive. One possible explanation for this discrepancy is

that vesicle secretion may be maintained to facilitate the continued release of non-catecholamine vesicle contents (opioids, chromogranins, etc.). For example, chromogranin derived neuro-peptides (catestatin, vasostatin) are a main secretary component in of AMCs; they both negatively feedback on AMCs to reduce catecholamine release, and act as an 'anti-adrenergic' agent by promoting vasodilation and a reduction in heart rate (Tota, et al., 2010). Therefore Ca^{2+} signalling may be sustained to support the release of agents besides catecholamines, and examining vesicle contents between species may be an easy method to test this.

3. Does neural sympathetic activation change with hypoxia acclimation or adaptation in *Peromyscus* mice?

The changes in the hypoxic chemoreflex with high-altitude acclimation and adaptation in *Peromyscus* mice are being thoroughly mapped (Scott, et al., in prep; Ivy & Scott, 2017; Wearing, at al. in prep). However, there is a noticeable gap in knowledge regarding the afferent activation from carotid bodies and the subsequent neural stimulation. Investigating action potential amplitudes and their patterns of activation with varying degrees in hypoxia may be important in comprehensively understanding how the hypoxic chemoreflex is regulated in these highland and lowland mice.

4. Is there an impact on AMC signalling in other highland taxa with SNPs in the EPAS1 gene?

A number of other high altitude taxa, including Tibetan humans, have been found to have directional selection, favoring SNPs in the gene encoding for HIF2α (Simpson, et al., 2010; Zhang et al., 2010; Song et al., 2016). This may be advantageous as it helps maintain lower hemoglobin levels, which maintains blood flow, in animals at high-altitude. However, the link between SNPs and α-adrenergic activation has not been explored in other highland taxa. Examining AMC activity in a number of highland populations could shed light on i) how evolutionarily conserved the reduction in α-adrenergic signalling is at high-altitude, and ii) the factors that drive directional selection for the EPAS1 gene.

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