The regulation of catecholamine release from the adrenal medulla is altered in deer mice (*Peromyscus maniculatus*) native to high altitudes

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Running title: Adaptation of chromaffin cell function in high altitude deer mice

Keywords: Evolutionary physiology, oxygen chemosensitivity, catecholamines, chromaffin cells

Number of words: Abstract: 250 words; Introduction: 666 words; Discussion: 1804 words
Number of figures: 5
Number of tables: 2

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Acknowledgements: We sincerely thank Paras Patal for his excellent technical assistance. G. Scott, A. Scott, and C. Nurse are funded by Natural Sciences and Engineering Research Council operating grants. The authors declare no competing financial interests.

Abstract

High-altitude natives have evolved to overcome environmental hypoxia and provide a compelling system to understand physiological function during reductions in oxygen availability. The sympathoadrenal system plays a key role in responses to acute hypoxia, but prolonged activation of this system in chronic hypoxia may be maladaptive. Here, we examined how chronic hypoxia exposure alters adrenal catecholamine secretion, and how adrenal function is altered further in high-altitude natives. Populations of deer mice (*Peromyscus maniculatus*) native to low and high altitude were each born and raised in captivity at sea level, and adults from each population were exposed to normoxia or hypobaric hypoxia for 5 months. Using carbon-fiber amperometry on adrenal slices, catecholamine secretion evoked by low doses of nicotine (10 μM) or acute hypoxia (PO_2 ~15-20 mmHg) was reduced in lowlanders exposed to hypobaric hypoxia, attributable mainly to a decrease in quantal charge rather than event frequency. However, secretion evoked by high doses of nicotine (50 μM) was unaffected. Hypobaric hypoxia also reduced plasma adrenaline and protein expression of DOPA decarboxylase in the adrenal medulla of lowlanders. In contrast, highlanders were unresponsive to hypobaric hypoxia, exhibiting typically low adrenal catecholamine secretion, plasma adrenaline, and DOPA decarboxylase. Highlanders also had consistently lower catecholamine secretion evoked by high nicotine, smaller adrenal medullae with fewer chromaffin cells, and a larger adrenal cortex compared to lowlanders across both acclimation environments. Our results suggest that plastic responses to chronic hypoxia along with evolved changes in adrenal function attenuate catecholamine release in deer mice at high altitude.

Abbreviations list: AMC, adrenomedullary chromaffin cell; CHox, chronic hypoxia; Nox, normoxia; CAT, catecholamines; TH, tyrosine hydroxylase; NF, neurofilament; GAP-43, growth associated protein 43; DDC, DOPA decarboxylase; DβH, dopamine β hydroxylase; PMNT, phenylethanolamine N-methyltransferase.

Introduction

Reductions in environmental oxygen availability (hypoxia) pose a serious threat to homeostasis in most species. The coordinated efforts of the sympathetic nervous system (SNS) and the adrenal medulla, the two limbs of the sympathoadrenal system, provide an essential line of defense during acute exposure to low oxygen conditions. As a key effector in the hypoxic chemoreflex, the system works to increase cardiac output and modify blood flow distribution to safeguard the delivery of oxygen to vital tissues – particularly the heart and brain – at the expense of other less-sensitive tissues (20). The pre-ganglionic splanchnic nerve of the SNS supplies cholinergic innervation to the adrenal medulla and stimulates the adrenal medullary chromaffin cells (AMCs) to release catecholamines (e.g. noradrenaline, NA; adrenaline, A) into the circulation in response to stressors such as hypoxia (6, 35). This system is highly regulated and designed to ready the organism for coping with acute stress. Indeed, interference of catecholamine (CAT) release from AMCs reduces survival during acute exposure to extreme hypoxia (41). Although the benefits of sympathoadrenal activation during short-term exposures to hypoxia are well established, chronic activation of this system is maladaptive and can lead to long-term cardiovascular complications, such as increased arterial stiffness, systemic hypertension, and compromised exercise performance (12).

The regulation of CAT biosynthesis and secretion is tightly controlled by sympathetic nerve activity and relatively plastic in response to different stressors (reviewed in 43). Early investigation of sympathoadrenal responses to hypoxia showed that activation is highly dependent upon both the severity and duration of exposure (24). However, unlike the responses to acute hypoxia, chronic exposure to either moderate or severe hypoxia over days or weeks is accompanied by high levels of both SNS activity and catecholamine (CAT) release from the adrenal medulla into the blood (5, 24). Relatively little is known about the mechanisms underlying changes in sympathoadrenal activity during chronic exposures *in vivo*, but studies based on exposure of isolated AMCs to short-term (≤ 48 h) chronic hypoxia *in vitro* suggest that stabilization of hypoxia-inducible transcription factors in such conditions enhances low-threshold CAT secretion via increased T-type calcium channel expression (7), and enhances neurotrophin (BDNF)-induced CAT secretion via increased neurotrophin receptor (TrkB) expression

(38). Elevated levels of tyrosine hydroxylase and dopamine β hydroxylase, two enzymes involved in CAT production, have also been noted after chronic long-term hypoxia (19).

Honed by generations of natural selection, many high-altitude natives have evolved exquisite mechanisms for coping with chronic hypoxia and the rigors of everyday life at high altitude. High-altitude natives can thus provide insight into mechanisms that are truly adaptive and contribute to fitness at high altitude. Deer mice (*Peromyscus maniculatus)* are a valuable model organism for studying high-altitude adaptation as they occupy the greatest altitudinal range of all North American mammals, from sea level to ~4300m (33). They are exposed to extreme hypoxia at the summit of their altitudinal range, where O_2 partial pressures are just above half of those at sea level. Highland populations are genetically distinct from lowland populations, based on comparisons of α- and β-globins as well as neutral autosomal loci (32, 45), and they have evolved several key physiological specializations that improve hypoxia resistance and aerobic performance (31, 44). Therefore, contrasting the phenotypes of high- and low-altitude populations is a powerful approach for elucidating adaptive strategies for coping with hypoxia stress. In this study, we examine possible adaptive modifications to the sympathoadrenal system at the level of the adrenal medulla. Specifically, we compared the cellular physiology and molecular profiles of chromaffin cells, as well as the plasma catecholamine levels and structural features of the medulla, in both high- and low-altitude populations of deer mice. Comparisons between each of these populations were made after chronic exposure to both normoxic and hypoxic conditions, in order to distinguish the effects of high-altitude ancestry from acclimation environment. Our results suggest that the adrenal medulla is a site of considerable plasticity during exposure to chronic hypoxia in low-altitude mice and is also a target tissue for adaptive evolutionary change in high-altitude natives.

Materials and Methods

Ethical Approval. All procedures for animal handling and tissue isolation followed guidelines established by the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board at McMaster University.

Animal Procedures. Wild deer mice were live trapped using Sherman traps at each of two locations: 1) on the Great Plains in Nine Mile Prairie, Lancaster County, NE, USA (40°52'12"N, 96°48'20.3"W; 430 m above sea level); and 2) on the summit of Mount Evans in Clear Creek County, CO, USA (39°35'18"N, 105°38'38"W; 4,350 m above sea level). Mice were shipped to McMaster University by a commercial animal transporter (World Courier), in conventional rodent shipping containers with dividers for individual housing. These mice were then housed and bred within each population in common laboratory conditions to produce first generation (G1) progeny of either low-altitude or high-altitude ancestry (often referred to here as either 'lowland' or 'highland' populations). These G1 mice were maintained in normoxic laboratory conditions until approximately 6 months of age, after which mice from both populations were acclimated for 18-20 weeks to each of two different acclimation conditions: standard normobaric normoxia or hypobaric hypoxia. The hypobaric conditions recapitulated environmental pressures and low levels of oxygen experienced at the native altitude of our high-altitude population (barometric pressure of 60 kPa; O_2 partial pressure of 12.5 kPa) using specialized hypobaric chambers (described previously in 22, 26). Mice in hypobaric conditions were temporarily (~20 min/week) returned to normobaric conditions for cage cleaning.

Carbon Fibre Amperometry. Mice were placed into a small jar (~500 ml) containing high concentrations of volatile isoflurane (administered on a lightly soaked cotton ball), and as soon as they lost consciousness and reached a surgical plane of anesthesia (typically <15 s) they were euthanized by cervical dislocation. The intact adrenal glands were then isolated, dissected, and placed in cold L-15 plating medium (Gibco, Grand Island, NY, USA), and then promptly transferred to a glass petri dish with oxygenated, chilled Tyrodes Solution (115 mM NaCl, 10 mM glucose, 10 mM HEPES, 2 mM KCl, and 3 mM $MgCl_2$; pH 7.4). The glands were secured to the base stage of the vibrotome (VT1000, Leica Biosystems) by embedding in 3% agarose, and were then cut into 200 μm thick sections and incubated in bicarbonate buffer (24 mM sodium bicarbonate, 115 mM NaCl, 10 mM glucose, 12 mM sucrose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$) that was bubbled with 95% O_2 and 5%

CO_2 and maintained at 37°C. Both adrenal glands were isolated from each mouse, and 1-3 slices were obtained for amperometric measurements from each gland.

Measurements of vesicular catecholamine release from adrenal medullary cells were made for each population in each acclimation condition, using carbon fibre amperometry approaches that have been described elsewhere (38). Release was measured from freshly sectioned adrenal gland slices that were perfused with bicarbonate buffer and exposed to the following series of conditions: (i) bicarbonate buffer alone (5-10 min), (ii) nitrogen-purged bicarbonate buffer (hypoxia treatment, 1 min), (iii) 10 μM nicotine (low dose, 1 min), (iv) 50 μM nicotine (high dose, 1 min), and finally (v) 30 mM KCl (positive control, 30 s). The slice was re-perfused with bicarbonate buffer alone between each treatment, for a minimum of 5 min or the required time for the recording to return to the original baseline. For the hypoxia treatment, a high-purity nitrogen (95%) and carbon dioxide (5%) gas mixture was bubbled into buffer for at least 30 minutes prior to use, as done in previous studies (3, 13, 50, 51) and has been shown to elicit O_2 tensions of 15-20 Torr in the buffer solution (4). Measurements were made using a polarized (+800 mV) carbon fibre electrode (Dagan Corporation, Minneapolis, MN, USA) that was attached to a CV203BU headstage and was gently lowered to the surface of the slice near the center of the adrenal medullary region. The signal was amplified with Axopatch 200B (Molecular Devices, Sunnyvale, CA, USA), recorded at 10 KHz using Digidata 1322A Software, and analyzed with Clampex version 9.2. Any events recorded that measured greater than 2 standard deviations above baseline noise and greater than 0.5 ms in duration were included in the analysis.

For each recording, the integrated area of secretory events (in units fC; indicative of the number of oxidizable catecholamines) was measured to determine total secretion across the entire duration of the response. The end of the response was deemed to be the point at which the last significant event (2X standard deviations greater than baseline) occurred. Total secretion (fC), response duration (s), quantal frequency (events/min), secretion rate (fC/min), and quantal charge (integrated area of each event, fC) were determined for each recording in which there was a significant response to KCl. All of the recorded measurements obtained from the 3-4 slices isolated from each mouse were averaged together to reflect the phenotype of that individual animal (n). In total, adrenal glands were

isolated from n=10 animals of each population for the normoxia acclimation groups (which included 16 slices from lowlanders and 17 slices from highlanders), and n=9 animals of each population for the hypoxia acclimation groups (11 slices from lowlanders, 15 slices from highlanders).

Immunohistochemistry. Adrenal glands isolated from adult deer mice as above were immediately placed and incubated overnight at 4°C in fixative (4% paraformaldehyde in phosphate buffered saline, PBS: 150 mM NaCl, 15 mM NaH_2PO_4, pH 7.4), and then cryoprotected for 24 hours at 4°C in PBS containing 30% sucrose. The glands were then coated in embedding medium (Cryomatrix embedding resin, ThermoFisher Scientific, Mississauga, ON, Canada), flash frozen in liquid nitrogen, and stored at -80°C. Serial cryosections (10 μm) of the adrenal glands were obtained in a cryostat at -20°C, mounted onto glass slides, air dried, and returned to storage at -80°C. Sections were later thawed and washed in 0.1 M PBS (10 min), and then incubated for 1 h in blocking medium (1% bovine serum albumin [BSA] in PBS). Sections were then incubated overnight in PBS containing 10% BSA, 0.5% Triton-X and the following primary antibodies: anti-neurofilament (NF; 1:100, host rabbit; Millipore Cat# AB1989, RRID: AB_91202)(34), anti-growth-associated protein 43 (GAP-43; 1:2000, host rabbit; Millipore Cat# AB5220, RRID: AB_2107282) (47), and anti-tyrosine hydroxylase (TH; 1:2500, host mouse; Millipore Cat# 657010-100UG, RRID: AB_212601)(54). The next day, sections were washed three times in PBS (15 min each) and then incubated for 2 h in PBS containing secondary antibodies conjugated to either Alexa 488 or 594 (both used at 1:400; Molecular Probes Cat# A-11094, RRID:AB_221544; I-21413, RRID: AB_2313921). Sections were again washed in PBS as before, then incubated in PBS containing 0.2 μM DAPI for 25 min. Several droplets of Vectasheild (Vector Laboratories, Burlington, Canada) were applied to each slide for coverslipping.

Specimen epifluorescence was examined using an Olympus BX60 (20x air objective) and images of the entire adrenal gland cross-section were created by stitching together individual images using ImageJ and the stitching plugin (developed by 36). For each adrenal gland, 8 cross-sections (60 μm apart) were included in the analysis. Several measurements were made on the stitched images using ImageJ (version 1.46r), including

the maximal sectional area of the entire adrenal gland (determined by the outer edge of the Zona Glomerulosa) and the adrenal medulla alone (measured by the outer edge of TH^+ cells), the number of TH^+/DAPI cells in the medulla, and the integrated neuronal density in the adrenal medulla (densiometric analysis of NF florescence in a defined area). Adrenal medulla and adrenal cortex volumes are expressed here as a percentage of the entire gland volume. Volumetric values of the medullary and cortical regions were estimated from the relative areas of each layer across evenly spaced sections throughout the gland (10 μm sections). For TH^+ cell counts and neurofilament densitometry, the images were first set to a threshold (range 30-255) in order to eliminate background florescence. Automated cell counts within the section medullary boundary were determined in individual images via Analyze Particle function (settings: >50 pixel diameter and circularity of 0.5-1.00), and are expressed here as the number of cells in the total medulla volume.

ELISA Measurements of Plasma Catecholamines. Plasma catecholamine levels were measured during routine activity. Mice (n=8 normoxic lowlanders; n=6 hypoxic lowlanders; n=14 normoxic highlanders; n=8 hypoxic highlanders) were first anaesthetized deeply by quietly and carefully dropping an isoflurane-soaked cotton-ball into their cage, and mice were then immediately decapitated for blood collection. Blood samples were collected in heparinized tubes and were centrifuged for 6 min in a haematocrit centrifuge. Plasma samples were then flash frozen in liquid nitrogen and stored at -80°C. At the time of the catecholamine measurements, samples were thawed and kept cold 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.

Western Blotting. Both adrenal glands were dissected from each mouse and were placed in ice-cold L-15 Medium (Gibco, Grand Island, NY, USA). The glands were then further dissected to isolate the inner adrenal medulla relatively free from the surrounding cortical tissue. The dissected adrenal medullae were flash frozen in liquid nitrogen and stored at -80°C. Due to the required protein levels in each sample for this assay, each sample (n) contained the adrenal medulla from 4 adrenal glands pooled from 2 mice. Samples were

later mechanically dissociated in 50 µl of ice-cold RIPA buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholic Acid, 0.1% SDS, 50 mM Tris [pH 8.0], Roche ULTRA protease inhibitor tablet, Roche PhosSTOP phosphatase inhibitor tablet). Homogenates were then incubated on ice for 1 h and centrifuged at 16000 g for 15 min at 4°C. The total protein content within each sample was measured by a DC protein assay (Bio-Rad, Mississauga, ON, CA).

For each sample, 10 µg of protein was combined with 2X Laemmli Sample buffer (Bio-Rad), incubated in 95°C for 5 min, centrifuged briefly and then loaded onto a gradient precast polyacrylamide gel (4-15%, Bio-Rad) for electrophoretic separation. Separated proteins were transferred onto a polyvinyl-difluoride membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were washed and incubated for 1 h at room temperature in Tris-buffered saline (TBS) containing 5% non-fat milk powder and 1% Tween-20. The membranes were then incubated overnight at 4°C in TBS with Tween-20 alone (TBS-T) containing one of the following primary antibodies: anti-tyrosine hydroxylase (TH; 1:1000 dilution, host rabbit; Millipore Cat# AB59866, RRID: AB_92190)(8), anti-DOPA decarboxylase (DDC; 1:1000, host rabbit; Abcam Cat# 3905, RRID: AB_304145)(52), anti-dopamine β hydroxylase (DβH; 1:1000, host sheep; Abcam Cat# 19353, RRID: AB_731851)(28) and anti-phenylethanolamine N-methyltransferase (PMNT; 1:1000, host rabbit; Abcam Cat# ab167427)(28). Following the incubation in primary antibody, membranes were washed three times (3X10 min) in TBS-T at room temperature and were then incubated in TBS-T containing horseradish peroxidase-conjugated secondary antibody against primary antibodies from either rabbit (1:5000) or sheep (1:1500) (GE Healthcare Life Sciences, Mississauga, ON, CA) for 2 h at room temperature. Membranes were then washed again and developed using enhanced chemiluminescence developer (5 minutes; Bio-Rad) and imaged used a Chemidoc Imaging System (Bio-Rad). Densitometry measurements of band intensity were conducted using Image Lab Software 5.2 (Bio-Rad).

The membrane was then stripped using Blot Restore Solution (Millipore, Temecula, USA) as per the manufacturer's instructions. The membrane was incubated at room temperature with Solution A for 10 min, transferred to Solution B for 15 min and then rinsed well with TBS-T. Membranes were then incubated overnight at 4°C in TBS-T

containing a primary antibody against β-actin (1:2500, host mouse; Sigma-Aldrich Cat# A1978, RRID: AB_476692), which was used as a loading control. As above, the membrane was then washed and placed in TBS-T containing horseradish peroxidase conjugated secondary antibody against mouse primary antibody (1:2500; GE Healthcare Life Sciences) for 2 h. Imaging and densitometry measurements of band intensity for β-actin were then performed as above.

Normalized protein abundance of each protein of interest was determined as follows. We first carried out a within gel normalization by dividing the band intensity of the protein of interest to the band intensity of the load control (β-actin) for each sample, in order to control for variation in the amount of protein loaded into each lane. All samples for each protein of interest were processed at the same time, but they had to be run across multiple (2-3) gels, so we ran a cross gel control sample (a mixed collection of all other samples) on each gel. We then carried out a cross gel normalization by dividing the value for each sample from the within gel normalization to the value of the cross-gel control on each corresponding gel. These normalized values are all expressed as a percentage of the mean normalized value for normoxic lowland mice.

Statistics. Statistical analyses were performed using Prism (version 5, GraphPad Software, RRID: SCR_002798) and data compared using two-way ANOVA and Bonferroni multiple comparisons were used to determine effects of population or acclimation environment for each parameter. The tests used and P values are specified in the results section and figure legends for each figure presented. $P < 0.05$ was considered to be statistically significant.

Results

Catecholamine secretion via nicotinic acetylcholine receptor activation is blunted in highland deer mice

Application of a potent nAChR agonist, nicotine, was used to simulate sympathetic activation of chromaffin cells within the adrenal medulla slice preparation. High-resolution amperometric detection of real-time catecholamine secretion demonstrated that chromaffin cells from normoxic lowland mice had robust responses to both low (10 μM) and high (50

μM) concentrations of nicotine (Fig.1A, C). This high concentration of nicotine is ~2-fold the recorded EC50 for nicotine-mediated activation of human and rat chromaffin cells (4, 18), and is the lowest dose that produces maximal catecholamine release in bovine chromaffin cells (40). The low concentration is ~50% of the EC50 (21), but is great enough (>5 μM) to produce measurable responses without inducing desensitization of nicotinic receptors (18). Hypoxia acclimation reduced the secretory response of lowlanders to 10 μM nicotine, leading to a significant main effect of acclimation in two-way ANOVA (P=0.037). However, secretion in response to 50 μM of nicotine was unaffected by hypoxia acclimation (Fig. 1C). By contrast, CAT secretion was very low in highland mice during stimulation with both low (left panel) and high (right panel) concentrations of nicotine (Fig.1B, C) (main effects of *population*: P=0.039 at 10 μM; P=0.012 at 50 μM) and was unaffected by hypoxia acclimation.

Variation in the quantal charge per secretion event, a measurement that reflects vesicular loading and/or the concentration of CAT released per vesicle, was very similar to the variation in total CAT secretion. For 10 μM nicotine, quantal charge was significantly (P=0.034) greater for lowlanders than for highlanders in comparisons of normoxic mice (Fig. 1D). As was the case for total secretion, hypoxia acclimation reduced quantal secretion induced by 10 μM nicotine in lowlanders (left panel), leading to a significant main effect of hypoxia acclimation (P=0.009). However, there was not a significant effect of hypoxia acclimation on the response to higher nicotine concentrations (50 μM), as secretion in hypoxia-acclimated lowlanders was similar to normoxia-acclimated lowlanders (right panel). Highlanders maintained consistently low levels of quantal charge for both low and high concentrations of nicotine. Interestingly, other amperometric measurements suggested that the population differences in total CAT secretion appeared to be largely, if not exclusively, due to the variation in quantal charge described above. There were no differences in the frequency of nicotine-induced quantal events between populations, in mice acclimated to either normoxia or hypoxia (Table 1). As a result, the quantal secretion rate, measured as the total quanta released per minute (i.e., the product of quantal charge and event frequency), exhibited a similar pattern of variation to that for quantal charge (Table 1). The duration of nicotine-induced release of CAT from chromaffin cells was significantly lower (< 50%) in highlanders acclimated to hypoxia compared to lowlanders,

but there were no differences between lowlanders and highlanders when acclimated to normoxia (Table 1). Taken together, variation in total catecholamine secretion between highlanders and lowlanders or in lowlanders in response to hypoxia acclimation were primarily due to the differences in quantal charge, rather than the duration of the nicotinic response or the frequency of vesicular release.

Catecholamine secretion in response to acute hypoxia is altered in highland deer mice

A subpopulation of chromaffin cells in adrenal slices from adult deer mice retained direct O_2 sensitivity (Fig. 2), similar to other rodents (15, 41, 51). Hypoxia-induced CAT secretion in such cells was particularly robust in AMCs from normoxic lowlanders (Fig. 2A, C), but hypoxia acclimation reduced this response (Fig. 2B, C) and there was a corresponding significant main effect of hypoxia acclimation in two-way ANOVA (P=0.006). However, hypoxia-induced CAT secretion was lower in highlanders than in lowlanders in normoxia (Fig. 2B, C; significant main effect of population in two-way ANOVA, P=0.010). Furthermore, hypoxia acclimation did not change acute responsiveness of highlander AMCs to low oxygen treatment, which remained low in both acclimation groups (Fig. 2C). The response duration and frequency of vesicular release were not different between any of the groups (Table 2). Variation in quantal charge was the primary contributor to differences in total secretion, as was the case for nicotine-induced CAT release, and quantal charge was highest in normoxic lowlanders (Fig. 2D). We noted that the secretion of CAT in response to acute low oxygen was still appreciably lower than the secretion response to nicotine (e.g., ~3-fold lower than the response to 10 μM nicotine in normoxia-acclimated lowland mice).

Structural comparison between adrenal glands of highland and lowland deer mice

The adrenal gland contains two distinct regions, the outer cortex and the inner medulla, and the proportion of adrenal gland volume that was composed of medullary tissue was much smaller in highland mice than in lowland mice (Figure 3C). This difference was evident in mice that were acclimated to either normoxic or hypoxic conditions, and there was a significant main effect of population in two-way ANOVA (P=0.0002). The opposite relationship was observed for the cortical tissue (cortical layers

designated in Fig. 3A), with highland mice having significantly higher proportional volume of cortical tissue within the adrenal gland than lowland mice (Figure 3D; $P<0.0001$).

In order to determine if the smaller medullary volumes in the highland population were due to differences in chromaffin cell number or size, we performed tyrosine hydroxylase-immunoreactive (TH+) cell counts by quantifying the number of DAPI-stained nuclei that co-localized with TH+ staining. There was a main effect of population on TH+ cell counts, with highland mice having fewer chromaffin cells than lowland mice ($P=0.028$), and pairwise differences were most prominent in the hypoxia acclimation groups (Fig. 3E; $P<0.05$). In addition, we examined possible differences in the level of sympathetic innervation of the adrenal medulla, given the observed differences in the responsiveness of chromaffin cells to nicotine. We found that the density of neuronal processes, labelled with neurofilament and GAP-43, within a fixed area of medulla tissue sections was not significantly different between the populations in either acclimation condition (Fig. 3F; $P=0.219$). Taken together, these findings suggest that there has been an evolved reduction in AMC number and in the volume of medullary tissue within the adrenal gland of highland mice, but they have retained sympathetic innervation.

Circulating plasma adrenaline levels are relatively low in highlanders and are reduced by hypoxia acclimation in lowlanders

We examined whether the variation in CAT secretion and structure of the adrenal medulla was associated with variation in plasma levels of dopamine (DA), noradrenaline (NA) and adrenaline (NA) under routine conditions. These CATs are synthesized from L-tyrosine within the chromaffin cells by an intracellular biochemical pathway that is illustrated in Fig. 4A. The secreted products – dopamine (DA, ~5%), noradrenaline (NA, ~15%) and adrenaline (A, ~80%) – are released via vesicular exocytosis in response to nicotinic receptor activation (25). Chromaffin cells are the main source of adrenaline in the blood stream, in contrast to dopamine and noradrenaline, which can also enter the blood by spillover from peripheral SNS synapses. Hypoxia acclimation reduced plasma adrenaline in lowlanders, which led to a significant main effect of hypoxia acclimation ($P=0.0479$) (Fig. 4D). However, highlanders had 3-times lower plasma adrenaline levels

than lowlanders among normoxia-acclimated mice and highlanders did not respond to hypoxia acclimation, such that there was a significant difference in the hypoxia acclimation response between populations (i.e., significant population×acclimation interaction; P=0.0159). In contrast, there were no significant population differences in plasma levels of dopamine (Fig. 4B; lowland vs. highland, P=0.5896) or noradrenaline (Fig. 4C; P=0.5265), and no significant main effects of hypoxia acclimation for these two catecholamines (noradrenaline, P=0.4349; and dopamine, P=0.8299). Therefore, the patterns of variation in circulating adrenaline levels are consistent with the patterns of variation in CAT release evoked by low doses of nicotine or acute hypoxia.

Variation in expression of catecholamine biosynthetic enzymes

Hypoxia is known to alter the expression of enzymes involved in CAT synthesis (1) (see Fig. 4A), so we next sought to determine if variation CAT secretion could be explain by variation in enzyme expression. This was not the case for tyrosine hydroxylase (TH), which was more highly expressed in highlanders than in lowlanders among normoxia acclimation groups (P<0.05), but there were no differences between the populations after hypoxia acclimation (Fig. 5A). There was also a significant population×acclimation interaction for this enzyme in two-way ANOVA (P=0.0263).

In contrast, expression of DOPA decarboxylase (DDC) mirrored the variation in CAT release evoked by 10 μM nicotine or acute hypoxia (Fig. 5B). The antibody to DDC detected the enzyme dimer as a single or double band at the expected size of ~55kDa, which we have quantified here, as well as a known reduced form at ~27kDa. DDC expression was reduced after hypoxia acclimation in lowlanders (hypoxia, n=5; P=0.0252). DDC expression changed little with hypoxia acclimation in highlanders, in which expression was lower than lowlanders among normoxia-acclimated mice (P=0.0282), and there was a significant main effect of population in two-way ANOVA (P=0.0023).

The expression of dopamine β hydroxylase (DβH) exhibited a different pattern of variation (Fig. 5C). Hypoxia acclimation appeared to increase DβH in lowlanders, leading to a marginally significant acclimation effect in two-way ANOVA (P=0.050). However, hypoxia acclimation had no effect on DβH expression in highlanders, such that DβH was

lower in highlanders than in lowlanders after hypoxia acclimation (P<0.010) and there was a significant population effect in two-way ANOVA (P=0.0037).

The variation in expression of phenylethanolamine N-methyltransferase (PNMT) was similar to that for TH (Fig. 5D). There was no significant effect of acclimation environment on PMNT expression, but expression was greater in highlanders among normoxia-acclimated mice and the main effect of population in two-way ANOVA was significant (P=0.037).

Discussion

High-altitude natives of several species demonstrate various physiological adaptations to the challenges of living in hypoxic environments. Deer mice living in the highlands of North America are no exception. Previous work has demonstrated that highland populations of this species have evolved higher aerobic capacity in hypoxia, in association with evolved changes in control of breathing, haemoglobin-O_2 affinity, cardiac function, muscle capillarity and metabolic phenotype, and tissue gene expression (10, 22, 23, 26, 27, 39, 42, 46, 49, 53). In this study, we demonstrate that the function of the sympathoadrenal system has also evolved in high-altitude deer mice. Phenotypic plasticity of the adrenal medulla was evident in response to chronic hypoxia exposure in lowlanders; yet, in highlanders there was a relative lack of plasticity with adrenal function staying virtually fixed at low levels of catecholamine secretion across varying environmental conditions. This was reflected by the relatively low levels of circulating adrenaline in the plasma of highlanders when examined during routine activity in normoxia or after hypoxia acclimation. Our results suggest that genetically-based modifications in adrenal medulla function may play an important role in high-altitude adaptation in deer mice.

Chronic Hypoxia Alters Sympathoadrenal Function in Lowlanders

Sympathetic activation of chromaffin cells from the adrenal medulla results in fast secretion of catecholamines into the bloodstream. The medulla is innervated by sympathetic fibers of the splanchnic nerve, which release acetylcholine to activate neuronal-type nicotinic acetylcholine receptors (nAChRs) expressed by chromaffin cells. In

this study, we found that the responsiveness of AMCs to mild nicotinic ACh receptor stimulation was significantly diminished following chronic exposure (18-20 weeks) of lowland mice to hypoxia, in association with a decrease in circulating adrenaline levels in the plasma. After hypoxia acclimation, higher concentrations (50 μM) of nicotine were needed for CAT release from chromaffin cells to match that seen in normoxic lowlanders. Therefore, chromaffin cells maintained the capacity to respond to nicotinic stimulation, but their activation threshold was higher. These adjustments mimic the changes in chromaffin cell sensitivity that have been observed in rats exposed to hypoxemia or nicotine in early development. For example, adrenal CAT depletion and reduced chromaffin cell activation was noted in neonates born to mothers exposed to chronic hypoxemia (11). Similarly, prenatal exposure to chronic nicotine (14 days) blunted the CAT secretory responses to hypoxic stimulation of adrenal chromaffin cells from neonatal rat pups (3). Considering the potentially maladaptive consequences of having chronically elevated circulating CAT levels, decreasing the responsiveness of AMCs may be a beneficial cellular modification to long-term hypoxia in adults. However, in the current study, lowlanders were exposed to hypoxia for 5 months. Although we did not determine the amount of time needed for these adjustments of AMCs to long-term hypoxia to arise, lowlanders colonizing high altitude may be at a disadvantage until they have had sufficient time to fully acclimatize.

We also confirmed that the adrenal medulla of adult lowland mice retained some capacity for non-neurogenic CAT release in response to acute hypoxia, but this response was significantly reduced following long-term acclimation of the animal to chronic hypoxia. Hypoxia sensing and CAT release via chromaffin cells is required in the neonate for the essential priming of the cardiovascular system for birth. While much of this sensitivity is diminished in postnatal life following innervation of the adrenal medulla (50, 51), subsets of chromaffin cells seem to preserve this function into adulthood in certain species (15, 21, 48). The chronic hypoxia-induced reductions in this non-neurogenic stimulation of CAT secretion by low oxygen, as well as the sensitivity to nicotinic stimulation, were both primarily due to a decrease in quantal charge rather than the frequency of quantal events. These findings suggest that total vesicular CAT storage or the fractional release of vesicular CAT stores was reduced after hypoxia acclimation.

Regulation of Catecholamine Biosynthesis in Lowlanders Exposed to Long-Term Hypoxia

One of the primary determinants of vesicular CAT content is the cascade of enzymes that regulate CAT production within chromaffin cells. Previous studies have shown that expression of CAT biosynthetic enzymes are altered by chronic hypoxia, with much of the focus on tyrosine hydroxylase (TH) and dopamine β hydroxylase (DβH) due to their reliance on molecular oxygen for catalytic activity (9, 37). Similar to our findings here (Fig. 5), expression of both TH and DβH in the adrenal gland were elevated in rats after days or weeks of sustained hypoxia (10% O_2) (19). However, in the present study, these increases were not associated with increased CAT secretion by adrenal tissue slices nor with increased plasma CAT levels.

In contrast to TH and DβH, the expression of DOPA decarboxylase (DDC or aromatic L-amino acid decarboxylase) in the adrenal gland was significantly reduced following hypoxia acclimation in lowlanders. Because DDC expression was correlated with adrenal CAT secretion evoked by mild nicotine and with plasma adrenaline following hypoxia acclimation, this enzyme may have acted as a critical regulator of CAT biosynthesis. DDC expression is developmentally regulated by hypoxia inducible factor 2α (HIF-2α), a transcription factor that is elevated in chromaffin cells during hypoxia exposure (1). DDC is not normally considered to be a rate-limiting factor in neurotransmitter biosynthesis, but it can limit the production of CAT in situations when its substrates are in abundance. For example, production of dopamine and serotonin are directly determined by DDC activity in patients treated with either exogenous L-DOPA for Parkinson's disease (2) or 5-HTP for mild depression (30), respectively. While not directly tested, it is likely that CAT synthesis was not limited by L-DOPA production, particularly after exposure to chronic hypoxia when TH expression in chromaffin cells had increased in lowland mice, but instead that the reduced levels of DDC limited downstream CAT synthesis.

Evolutionary Adaptations to High Altitude in the Sympathoadrenal System

In amperometric studies on adrenal slices, we show that catecholamine secretion evoked by high doses of nicotine is dramatically reduced in highland deer mice when compared to their lowland counterparts. Furthermore, CAT secretion evoked by low

doses of nicotine or by acute hypoxia was not altered by acclimation to long-term hypoxia in highland mice, fixed at levels that were similar to hypoxia-acclimated lowlanders. This lack of plasticity within the adrenal medulla of highland deer mice is reminiscent of that observed in other highland species in the carotid body, a key peripheral chemoreceptor that initiates the hypoxic chemoreflex and regulates sympathetic activity in adult mammals. For example, in guinea pigs, a species that originates from the Andes, stimulus-evoked CAT secretion by the carotid body is unaffected by chronic hypoxia due to two factors: (i) dampened chemosensitivity, as demonstrated by reduced activation of chemoreceptor (glomus) cells in response to hypoxic stimuli; and (ii) diminished CAT content within the glomus cells (16). Similarly, diminished CAT content appears to contribute to the attenuation of CAT secretion from adrenal chromaffin cells of highland deer mice, because of the significant reduction of the quantal charge yet no change in the frequency or duration of vesicle release in response to nicotine or acute hypoxia. It is plausible that the low cellular CAT content results from a decline in CAT synthesis, given the correspondingly low expression of either DDC or DβH in the adrenal medullae of highlanders acclimated to normoxia or hypoxia, respectively.

Highland deer mice also had a much smaller volume of medullary tissue within the adrenal gland and had fewer TH+ chromaffin cells compared to their lowland counterparts. Interestingly, reduced numbers of TH+ cells in the adrenal medulla have also been reported in adult rats that had been previously exposed to chronic prenatal hypoxia (15 days, E5-E20) (29), suggesting that developmental plasticity of the tissue can occur and have persistent effects. In addition, recent studies on high-altitude species or populations have showed that the characteristic hypertrophy of the carotid body that occurs in many low-altitude natives (including mice and rats) exposed to chronic hypoxia is not observed in guinea pigs (16) or high-altitude deer mice (22). The evolved structural differences in the adrenal medulla likely contributed to the lower levels of plasma CAT found in highlanders compared to lowlanders in normoxia.

Surprisingly, very little is known about how evolutionary adaptation has shaped sympathoadrenal function in high-altitude populations and species. This study is one of the first to suggest that genetically-based variation in adrenal medulla physiology can

554 contribute to high-altitude adaptation. The unique differences in highlanders that persist
555 after exposure to chronic hypoxia – namely, the differences in adrenal gland structure and
556 the reduced catecholamine secretion in response to strong nicotinic stimulation – may be
557 particularly important for providing highlanders with an advantage in their native high-
558 altitude environment. The attenuation of the adrenal medulla's role in CAT secretion may
559 help avoid some potentially maladaptive effects of chronic hypoxia in adulthood, such as
560 systemic hypertension and/or increases in vascular resistance in many tissues (12). It may
561 also improve reproductive success by helping offset intrauterine growth restriction and
562 impairments in placental blood flow that are associated with chronically elevated CAT in
563 pregnant females (32). However, given that the sympathoadrenal system is critical in
564 lowlanders for responses involved in surviving acute stressors (e.g., the 'fight or flight'
565 response involved in escaping predation, attaining food, and coping with extreme
566 weather) and for regulating blood pressure after blood loss, there may be trade-offs
567 associated with evolved changes in the sympathoadrenal system in high-altitude
568 populations. These trade-offs could foreseeably lead to compensatory changes in other
569 systems to maintain these important homeostatic responses. The greater volume of the
570 adrenal cortex in highland deer mice may also have important consequences. This
571 difference was analogous to the cortical hyperplasia observed in rats following sustained
572 hypobaric hypoxia (17), and suggest that corticosteroid, aldosterone, and/or androgen
573 signaling pathways may play an altered role in high-altitude environments.

574 ***Perspectives and Significance***

575 Because high-altitude natives have evolved exquisite mechanisms for coping with
576 chronic hypoxia, comparisons between highland and lowland natives can provide unique
577 and valuable insight into mechanisms of hypoxia resistance that have been favoured by
578 natural selection. Our results here suggest that genetically-based modifications in adrenal
579 medulla function play an important role in high-altitude adaptation in deer mice. High-
580 altitude mice had small adrenal medullae due to an evolved reduction in chromaffin cell
581 number, and their chromaffin cells generally exhibited low rates of catecholamine
582 secretion in response to nicotinic stimulation. The mechanisms underlying these evolved
583 changes are not fully understood, but our findings suggest that reductions in

584 catecholamine synthesis and/or vesicle loading play an important role and have thus
585 provided insight into the molecular underpinnings governing chromaffin cell physiology.
586 This apparent outcome of natural selection in high-altitude deer mice provides significant
587 insight into aspects of adrenal medulla physiology that may become a liability during
588 chronic hypoxia, as well as how these liabilities can be avoided. This and future work on
589 high-altitude natives will continue to provide valuable insight into the genetic, molecular,
590 cellular, and physiological bases of adaptive mechanisms for coping with chronic
591 hypoxia in mammals.

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Figure Legends

Figure 1. Nicotine-induced catecholamine release measured *in situ* from the adrenal medulla of deer mice native to low altitude and high altitude, and acclimated to normoxic or hypoxic conditions. ***A*** and ***B***, Example amperometric recordings from chromaffin cells in adrenal slices from a lowland mouse (***A***) and a highland mouse (***B***), each acclimated to normoxia, in which the tissue was bathed in low (10 μM) then high (50 μM) concentrations of nicotine for 1 min, followed by 30 s exposure to 30 μM K^+ (positive control). ***C***, Cumulative catecholamine secretion in response to 10 μM nicotine was higher in lowlanders (black symbols; n=8) than in highlanders (grey symbols; n=10) among mice acclimated to normoxia, but both highlanders (n=8) and lowlanders (n=9) had low levels of catecholamine secretion after hypoxia acclimation (left panel). However, for lowlanders, total secretion in response to 50 μM nicotine was high in both acclimation groups, similar to secretion in response to 10 μM nicotine in normoxia-acclimated lowlanders, and higher than the consistently low levels of secretion in highlanders (***C***, right panel). ***D***, Patterns of variation for the quantal charge of secretory events was very similar to the pattern of variation for total catecholamine secretion. * Pairwise differences between populations within an acclimation environment; † significant main effect of population in two-way ANOVA. Error bars = +/- SEM.

Figure 2. Hypoxia-induced catecholamine release measured in tissue slices from the adrenal medulla of deer mice native to low altitude and high altitude and acclimated to normoxic or hypoxic conditions. ***A*** and ***B***, Examples of amperometric recordings from adrenal medullae isolated from lowland mice (***A***) and highland mice (***B***) acclimated to either normoxia (top panels) or hypoxia (bottom panels) following application of a physiological bath with low oxygen levels (PO_2 ~15-20 mmHg for 1 min). ***C***, Cumulative catecholamine secretion in response to low oxygen treatment was significantly higher in lowlanders (black symbols; n=10) than in highlanders (grey symbols; n=10) when acclimated to similar normoxic conditions ($p<0.01$), but both populations had low levels of catecholamine secretion after hypoxia acclimation. ***D***, Average quantal charge in response to low oxygen treatment was higher in normoxia-acclimated lowlanders compared to all other groups. * Pairwise differences between populations within an acclimation

environment; † significant main effect of population in two-way ANOVA. Error bars = +/- SEM.

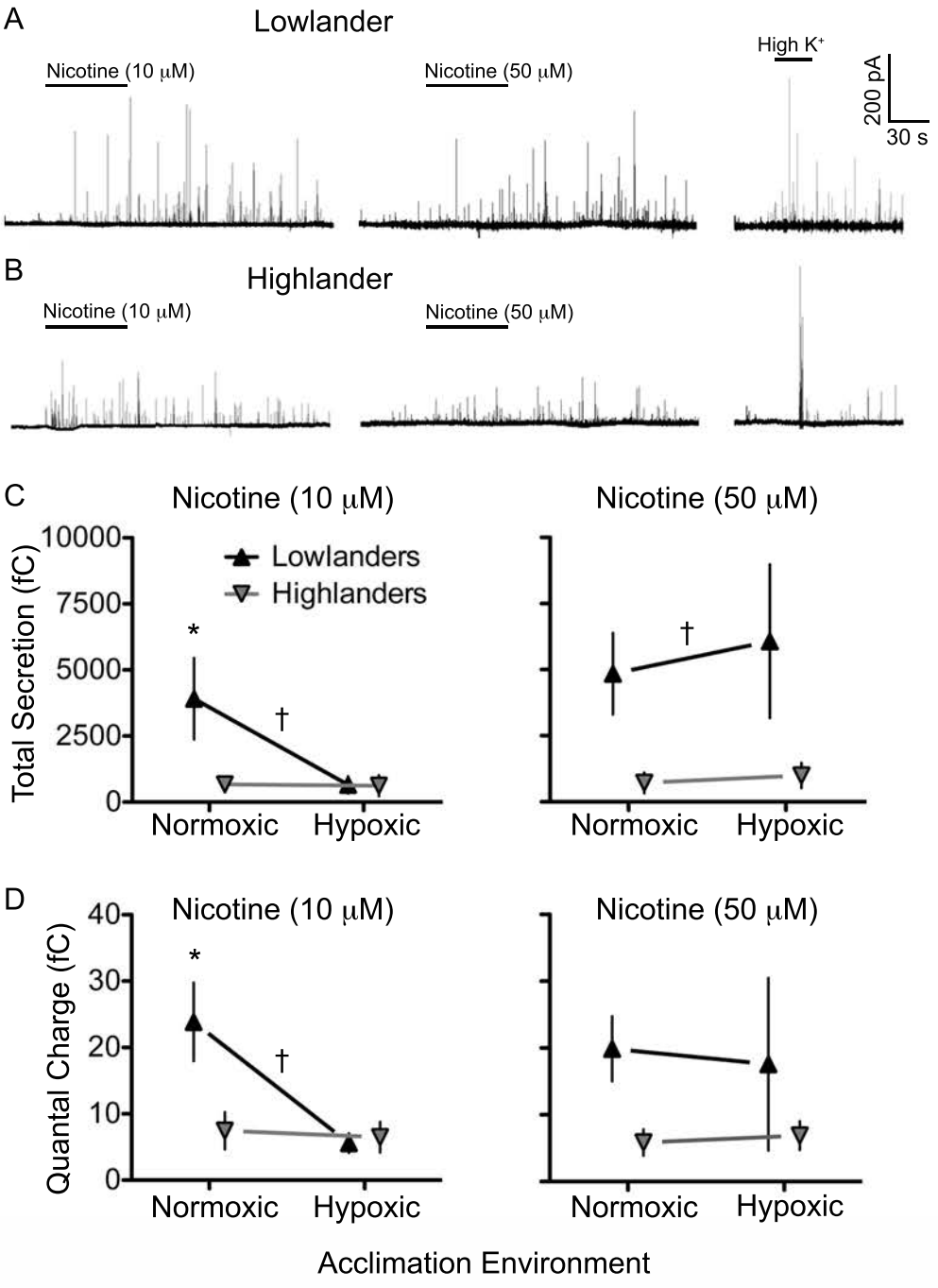
Figure 3. Anatomy of the adrenal gland from deer mice native to low altitude and high altitude, following acclimation to normoxia or hypoxia. ***A*** and ***B***, Immunohistochemical sections labelled with a nuclear marker (DAPI) to illustrate the adrenal medulla and cortex of the glands in lowlanders (***A***) and highlanders (***B***). Inset boxes (***A′*** and ***B′***) illustrate staining for tyrosine hydroxylase to identify chromaffin cells (TH^+ cells) and neurofilament (NF) and growth-associated protein 43 (GAP-43) to identify neural elements (nerve fibers). ***C***, The tissue volume of the adrenal medulla relative to the overall gland size was greater in lowlanders acclimated to either normoxia (n=4) or hypoxia (n=7) (black symbols) compared to normoxic (n=10) or hypoxic (n=8) highland mice (grey symbols; $p<0.001$). Correspondingly, the relative cortical tissue volume within the adrenal gland of lowlanders was lower than that of highlanders (***D***) ($p<0.001$). ***E***, Differences in the relative number of TH^+ cells ($p=0.028$) contribute to the population differences in adrenal medulla volume. ***F***, There were no significant differences in neural density within the medulla based on densitometric analysis of NF/GAP-43 (lower boxes in ***A′*** and ***B′***). * Pairwise differences between populations within an acclimation environment; † significant main effect of population in two-way ANOVA. Scale bars = 50 μm; error bars = +/- SEM.

Figure 4. Plasma catecholamine levels in deer mice native to low altitude and high altitude, following acclimation to normoxia or hypoxia. ***A***, Schematic shows the catecholamine biosynthesis pathway within chromaffin cells of the adrenal medulla leading up to vesicular release into the circulation (14). ***B-D***, In mice acclimated to normoxic conditions, the plasma concentrations of dopamine (***B***) and norephinephrine (***C***) were similar in lowland (black symbols) and highland (grey symbols) populations, but levels of epinephrine (***D***) were higher in lowlanders (n=8) compared to highlanders (n=14). All differences in catecholamine levels between populations were abolished after mice were acclimated to hypoxic conditions (hypoxic lowlanders, n=6; hypoxic highlanders, n=8). * Pairwise differences between populations within an acclimation environment; † significant

interaction between population and acclimation environment in two-way ANOVA. Error bars = +/- SEM.

Figure 5. Relative protein abundance of catecholamine biosynthetic enzymes in the adrenal medulla of deer mice native to low altitude (Low; black symbols) and high altitude (High; grey symbols), following acclimation to normoxia (N) or hypoxia (H). ***A***, Tyrosine hydroxylase (TH; single band at ~60 kDa) levels were lower in lowlanders than in highlanders when compared in normoxic conditions ($p < 0.05$) but were equivalent between lowland ($n = 5$) and highland ($n = 8$) populations after hypoxia acclimation. ***B,*** Relatively low levels of DOPA decarboxylase (DDC) were found in the highlanders in both acclimation conditions, and levels were lower than those in lowlanders among normoxia-acclimated mice exposures ($p<0.01$). The DDC antibody detected bands at ~55 and 27 kDa, and we quantified the 55 kDa band at the expected size of the enzyme. ***C,*** Dopamine β hydroxylase (DβH; single band at ~70 kDa) levels did not change with hypoxia acclimation in highland mice, but levels were elevated in lowlanders after hypoxia acclimation to higher levels than in highlanders ($p<0.01$). ***D,*** Phenylethanolamine N-methyltransferase (PNMT; single band at ~28 kDa) levels were highest in normoxic highlanders, but populations were equivalent after hypoxia acclimation. β-actin (~42 kDa) was used as a loading control, and the normalized abundance of each protein of interest is expressed as a percentage of the mean normalized value for normoxic lowland mice (see Materials and Methods). * Pairwise differences between populations within an acclimation environment; † significant main effect of population in two-way ANOVA. Error bars = +/- SEM.

Figure One


A
Lowlander
Nicotine (10 μM)
Nicotine (50 μM)
High K+
200 pA
30 s
B
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Nicotine (10 μM)
Nicotine (50 μM)
Lowlanders
Highlanders
Total Secretion (fC)
10000
7500
5000
2500
0
*
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Normoxic
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D
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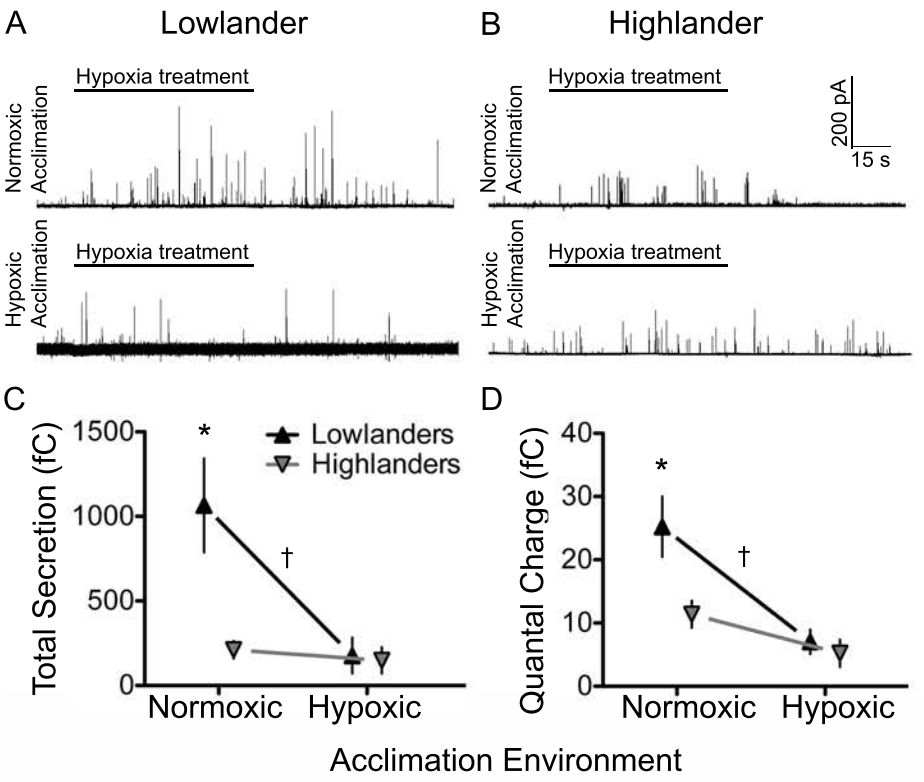
Nicotine (50 μM)
Quantal Charge (fC)
40
30
20
10
0
Acclimation Environment

Figure Two


A
Lowlander
B
Highlander
Hypoxia treatment
Normoxic Acclimation
Hypoxic Acclimation
200 pA
15 s
C
D
Total Secretion (fC)
Quantal Charge (fC)
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40
30
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*
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Lowlanders
Highlanders
Normoxic
Hypoxic
Acclimation Environment

Figure Three

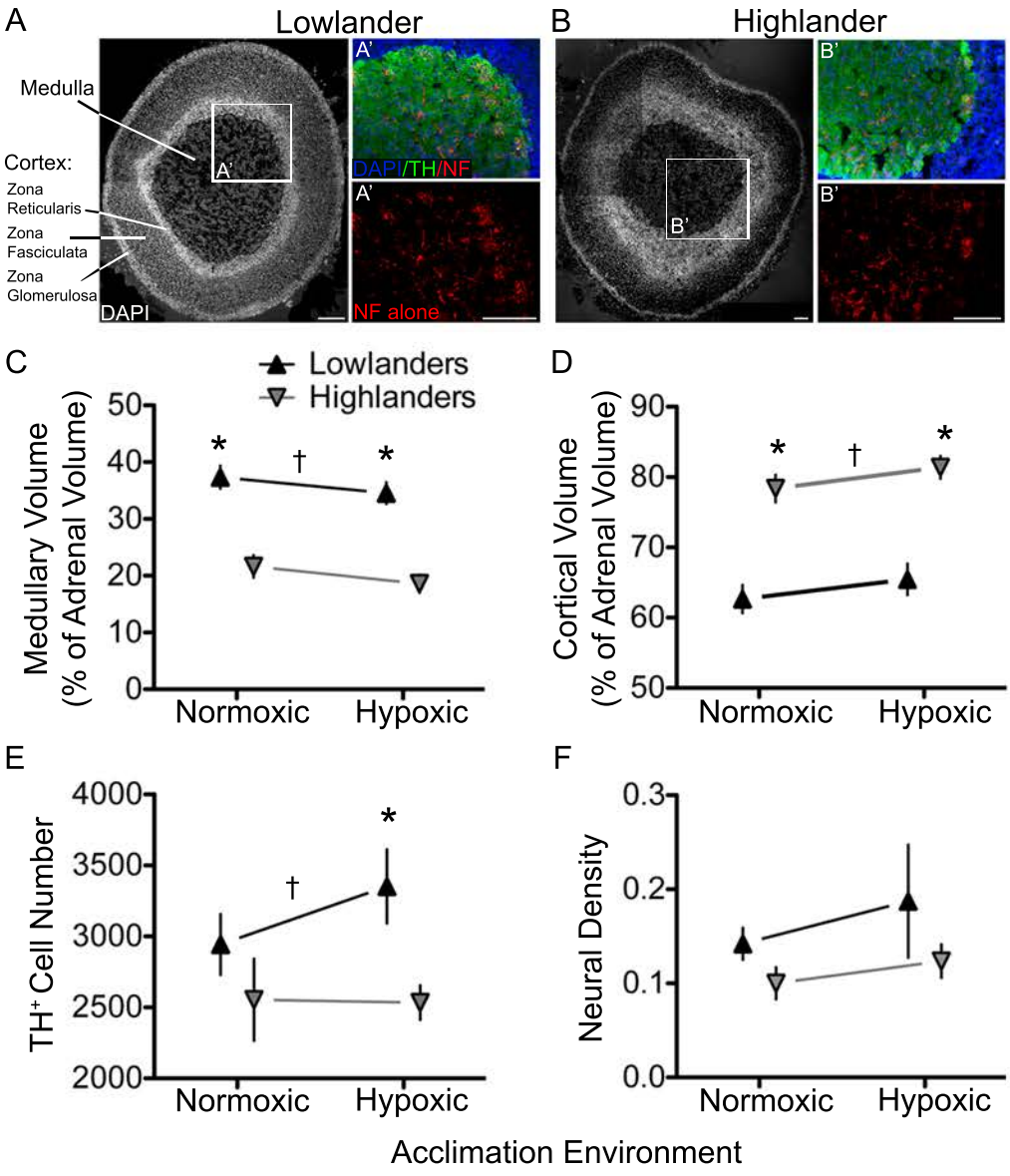

Figure Four

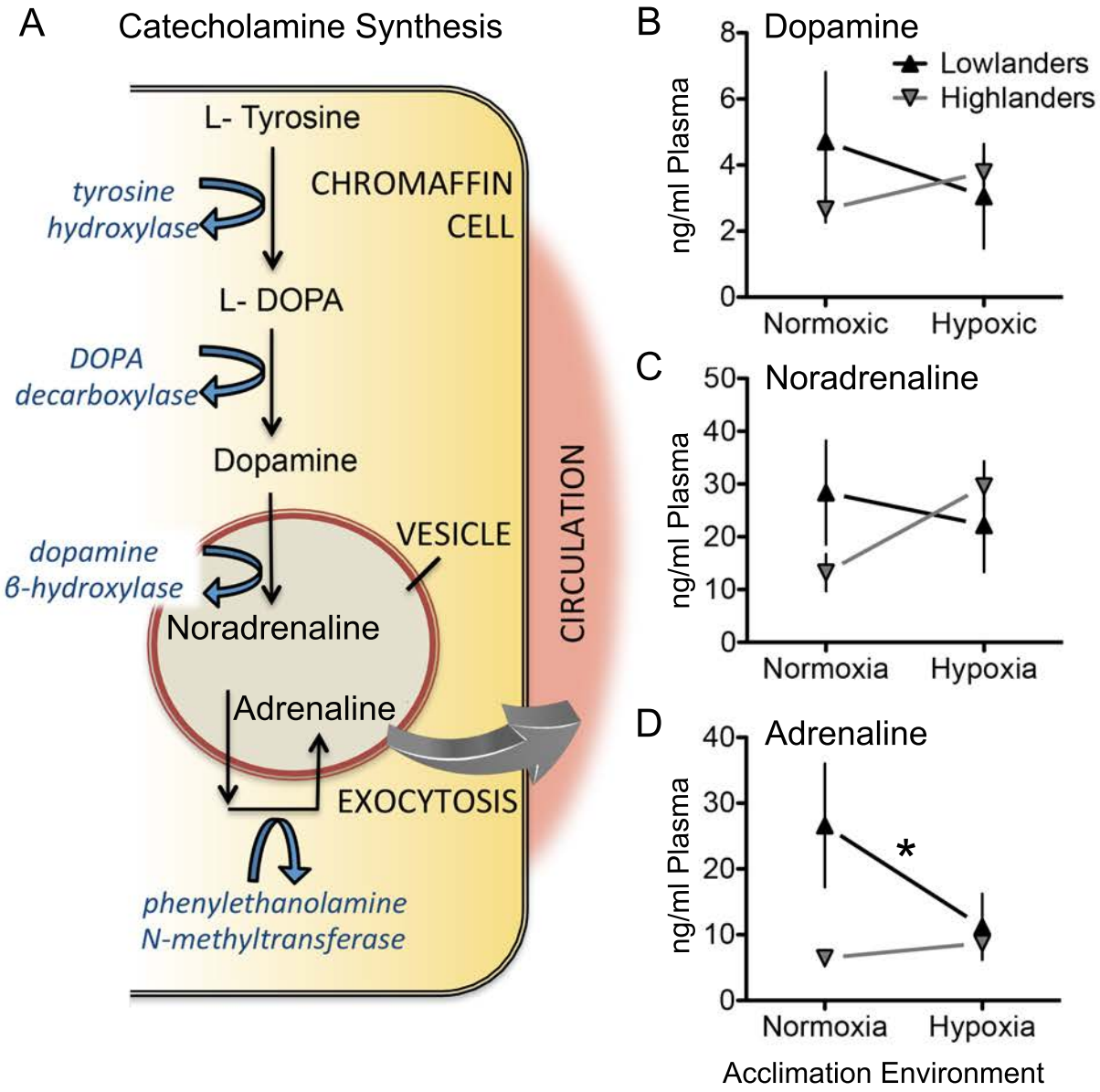
A
Catecholamine Synthesis
L- Tyrosine
tyrosine hydroxylase
CHROMAFFIN CELL
L- DOPA
DOPA decarboxylase
Dopamine
VESICLE
dopamine β-hydroxylase
Noradrenaline
Adrenaline
EXOCYTOSIS
phenylethanolamine N-methyltransferase
CIRCULATION
B
Dopamine
Lowlanders
Highlanders
ng/ml Plasma
0
2
4
6
8
Normoxic
Hypoxic
C
Noradrenaline
ng/ml Plasma
0
10
20
30
40
50
Normoxia
Hypoxia
D
Adrenaline
ng/ml Plasma
0
10
20
30
40
*
Normoxia
Hypoxia
Acclimation Environment

Figure 5

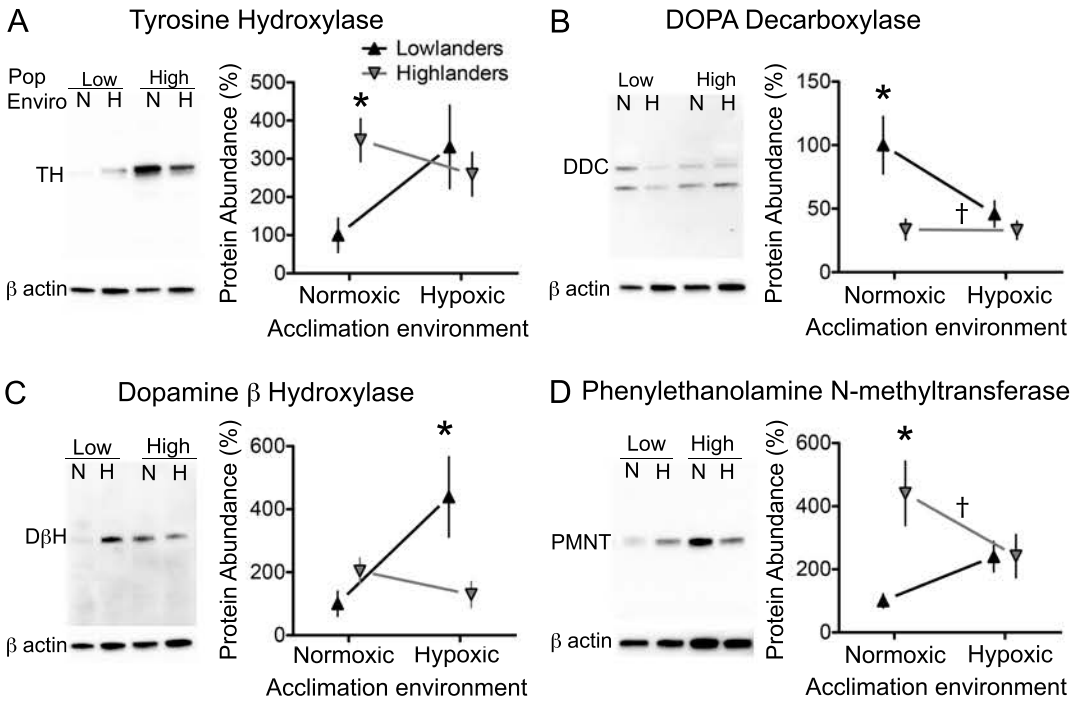
A
Tyrosine Hydroxylase
Lowlanders
Highlanders
Pop
Enviro
Low
High
N H N H
TH
β actin
Protein Abundance (%)
500
400
300
200
100
0
*
Normoxic
Hypoxic
Acclimation environment
B
DOPA Decarboxylase
Low
High
N H N H
DDC
β actin
Protein Abundance (%)
150
100
50
0
*
†
Normoxic
Hypoxic
Acclimation environment
C
Dopamine β Hydroxylase
Low
High
N H N H
DβH
β actin
Protein Abundance (%)
600
400
200
0
*
Normoxic
Hypoxic
Acclimation environment
D
Phenylethanolamine N-methyltransferase
Low
High
N H N H
PMNT
β actin
Protein Abundance (%)
600
400
200
0
*
†
Normoxic
Hypoxic
Acclimation environment

Table 1. Adrenal Catecholamine Secretion in Response to Nicotine Treatment.
* denotes significant differences between the populations within each group.

Secretion Variants	Lowlanders			
	Non-acclimated		Acclimated	
	10 µM	50 µM	10 µM	50 µM
Duration (sec)	150.5 ± 23.4	174.4 ± 42.4	173.9 ± 48.6	226.8 ± 52.3
Frequency (Hz)	6.9 ± 2.7	8.3 ± 1.7	6.8 ± 2.7	14.7 ± 8.6
Secretion Rate (fC/min)	238.6 ± 59.2*	198.7 ± 48.7*	55.9 ±14.6	175.8 ± 129.2*
	Highlanders			
	Non-acclimated		Acclimated	
	10 µM	50 µM	10 µM	50 µM
Duration (sec)	186.8 ± 23.5	203.9 ± 48.3	57.0 ± 14.2*	109.5 ± 17.5*
Frequency (Hz)	6.1 ± 0.8	5.9 ± 1.6	13.9 ± 4.1	10.1 ± 1.7
Secretion Rate (fC/min)	74.6 ± 28.1	82.3 ± 29.1	64.8 ± 22.9	68.9 ± 21.5

Table 2. Adrenal Catecholamine Secretion in Response to Hypoxia Treatment.
* denotes significant differences between populations within the group.

Secretion Variants	Lowlanders		Highlanders	
	Non-acclimated	Acclimated	Non-acclimated	Acclimated
Duration (sec)	174.4 ± 42.4	198.5 ± 53.4	203.9 ± 48.2	109.5 ± 17.6
Frequency (Hz)	14.4 ± 2.6	6.5 ± 1.5	8.7 ± 1.3	10.9 ± 3.0
Secretion (fC/min)	1065.1 ± 278.1*	176.0 ± 106.0	211.0 ± 51.7	148.6 ± 78.4