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Altered atrial neurotransmitter release in transgenic p75$^{-/-}$ and gp130 KO mice

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HIGHLIGHTS

- Simultaneous quantification of acetylcholine and norepinephrine released from atrial nerve fibers.
- Norepinephrine release is impaired in p75$^{-/-}$ atria.
- Atrial acetylcholine content and release are augmented in neuronal gp130 KO mice.

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ABSTRACT

Heart rate is controlled by stimulatory sympathetic and inhibitory parasympathetic nerves innervating the sino-atrial node and cardiac conduction system. Sympathetic release of norepinephrine (NE) and parasympathetic release of acetylcholine (ACh) are controlled by the central nervous system, and by pre-synaptic inhibition of transmitter release within the atria. An increase in cardiac sympathetic transmission relative to parasympathetic transmission is pathological as it can lead to disturbances in heart rhythm, catecholaminergic toxicity and development of arrhythmias or fibrillation. Mice lacking the p75 neurotrophin receptor (p75$^{-/-}$) have elevated atrial NE but a low heart rate suggesting autonomic dysregulation. Similarly, mice whose sympathetic neurons lack the gp130 cytokine receptor (gp130 KO) have a normal heart rate but enhanced bradycardia after vagal nerve stimulation. What is unclear is whether cardiac autonomic disturbances in these animals reflect systemic alterations in nerve activity or whether localized defects in neurotransmitter stores or release are involved. To examine local stimulus-evoked release of neurotransmitters, we have developed a novel method for simultaneous quantification of both NE and ACh after *ex vivo* atrial field stimulation. Using HPLC with electrochemical detection for NE, and HPLC with mass spectrometry for ACh, we found that following field stimulation NE release was impaired in p75$^{-/-}$ atria while ACh content and release was elevated in gp130 KO atria. Thus, alterations in localized transmitter release from atrial explants are consistent with *in vivo* deficits in heart rate control, suggesting peripheral alterations in autonomic transmission in these mice.

1. Introduction

Heart rate is controlled by sympathetic and parasympathetic innervation projecting to the atria (including the sino-atrial node) and cardiac conduction system. Norepinephrine (NE) released from sympathetic nerves stimulates heart rate and cardiac conduction, while acetylcholine (ACh) released from parasympathetic nerves slows heart rate and cardiac conduction. Activation of autonomic nerves by the central nervous system stimulates transmitter release, but NE and ACh release from cardiac nerves is additionally regulated in the atria by activation of inhibitory presynaptic receptors [12,24]. Neurotransmitter release from cardiac terminals can also be altered by a number of factors derived from cardiac and inflammatory cells [16,20,7,19]. An increase in cardiac sympathetic transmission relative to parasympathetic transmission is pathological [10,21], so it is important to understand local mechanisms that regulate NE and ACh release in the atria.

Cardiac autonomic disturbances are present in transgenic mice that lack the p75 neurotrophic receptor (p75$^{-/-}$) and in mice whose noradrenergic neurons lack the gp130 cytokine receptor (gp130 KO). Nerve growth factor (NGF) stimulates sympathetic neuron survival, axon outgrowth, and NE production through activation of TrkA [1,9], and Trk signaling is enhanced in the absence of p75 [4].

Abbreviations: HPLC–MS, high performance liquid chromatography–mass spectrometry; HPLC–ED, high performance liquid chromatography–electrochemical detection; ACh, acetylcholine; NE, norepinephrine; P75$^{-/-}$, p75 neurotrophic receptor knockout mice; GP130 KO, mice lacking gp130 in noradrenergic neurons; DHBA, dihydroxybenzylamine; PCA, perchloric acid; NET, norepinephrine transporter.

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Thus, NE content is elevated in p75$^{-/-}$ cardiac sympathetic nerves, but heart rate is low [2]. Although the p75 receptor is also present on cardiac parasympathetic neurons [6], parasympathetic innervation is normal in these animals [2], suggesting the low heart rate is due to impaired NE release. Inflammatory cytokines regulate neurochemistry and function of autonomic sympathetic neurons through activation of the gp130 receptor [1,23,3]. The absence of gp130 in neurons expressing the enzyme dopamine beta hydroxylase (gp130 KO) [17] results in mice that display cardiac autonomic disturbances including increased reperfusion arrhythmias and altered adrenergic and cholinergic transmission [17]. Therefore, autonomic dysregulation is present in both of these transgenic populations, but it is unclear whether local neurotransmitter stores and/or stimulus-evoked neurotransmitter release are compromised.

Since alterations in atrial neurotransmitter release will also impact co-projecting nerves, it is crucial to determine storage and release in both cardiac sympathetic and parasympathetic populations simultaneously. In order to verify the site of the autonomic defect in these animals, we have developed a novel method for simultaneous detection of autonomic neurotransmitters from the isolated right atrium following *ex vivo* nerve stimulations. We found that the alterations in transmitter stores and release detected in atrial explants were consistent with *in vivo* generalized deficits in heart rate control.

2. Materials and methods

2.1. Animals

gp130$^{DBH\text{-}Cre/lox}$ mice lack gp130 in neurons expressing dopamine beta hydroxylase including peripheral sympathetic and a subpopulation of parasympathetic neurons. They were obtained from Dr. Hermann Rohrer and maintained as previously described [17]. p75$^{-/-}$ (B6.129S4-Ngfr^{tm1Jae}/J) mice, which lack functional p75 receptor [11] were obtained from Jackson Labs. Wild type C57BL/6J (WT) mice were obtained from Jackson Labs West (Sacramento, CA). All mice were kept on a 12 h:12 h light–dark cycle with *ad libitum* access to food and water. Male and female mice 12–18 weeks old were used for all experiments. All procedures were approved by the Institutional Animal Care and Use Committee and complied with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996).

2.2. Atrial explants and stimulation

The right atrium was removed (n = 4–6 per group) under isofluorane anesthesia and pinned to a thin layer of Sylgard (Dow Corning, Midland, MI) in a preheated (37 °C), continuously oxygenated, water-jacketed organ bath containing 2 mL of Ringer's solution (120 mM NaCl, 1.2 mM KH_2PO_4, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 11 mM glucose, pH 7.4). The atrial tissue was placed between platinum stimulating electrodes, and after 60 min of equilibration, atria were stimulated using an S88X Stimulator (Grass Technologies, West Warwick, RI) in constant-voltage mode. Tissue was "primed" for 10 min with brief bouts of stimulation (10 s stimulation, 20 s rest; 15 V, 5 Hz, 0.1 ms pulse duration). The Ringer's solution was replaced every 5 min. After the priming phase atria remained unstimulated during a 20 min rest phase. Fresh Ringer's solution (1 mL) was then added, and replaced every 5 min for the remainder of the experiment. The initial 5 min period was used as a baseline for transmitter release. During the second 5 min period, the atrial nerves were stimulated (15 V, 5 Hz, 0.1 ms pulse duration) for 1 min to trigger neurotransmitter release. Two additional 5 min samples were collected without further stimulation. Each 1 mL sample was split into 0.5 mL samples for analysis of ACh and NE. Baseline levels of transmitter were subtracted from the amounts recovered during the stimulation and recovery periods. At the end of the experiment, atria were incubated in 1 mL 0.1 M perchloric acid (PCA) for 24 h to extract total remaining ACh and NE, which were quantified by HPLC coupled with mass spectrometry (HPLC–MS) or electrochemical detection (HPLC–ED), respectively.

2.3. HPLC–MS for ACh quantification

For each atria, 0.5 mL aliquots of the collected samples, and a 0.5 mL aliquot of the PCA extract of the atrial tissue, were spiked with deuterated ACh (d4 ACh; 0.1 pg/μL, CDN Isotopes, Quebec, Canada). Deuterated ACh was also added to standards of known concentrations of ACh (10 fg/μL-100 pg/μL, Sigma–Aldrich, St. Louis, MO). All samples and standards were dried by speed-vac and re-constituted in 100 μL of acetonitrile + 6% formic acid. Quantification of d4ACh indicated that 49% of ACh was recovered following sample preparation. Samples were chromatographically separated on a hydrophilic interaction chromatography mode column (HILIC; PolyLC, Columbia, MD; mobile phase A: ammonium formate, B: acetonitrile) with a flow rate of 300 μL/min and detected and quantified by a linear ion trap mass spectrometer (Applied Biosystems MDS SCIEX 4000 QTrap mass spectrometer, Carlsbad, CA) [26]. Multiple reaction monitoring (MRM) was in positive ionization mode and electrospray ionization (ESI) source parameters were as follows: nebulizing and curtain gas (N_2) 50 psi, temperature 600 °C, ion spray voltage 3000 V. MRM was used to quantify d4 ACh (mass-to-charge ratio (m/z) 150 → 91.1, declustering potential 46 V, entrance potential 10 V, collision cell exit potential 16 V) and ACh (m/z 146 → 87, declustering potential 46 V, entrance potential 10 V, collision cell exit potential 16 V). Injections of 2.05 fmol (300 fg after 30 μl injection) ACh consistently gave signal-to-noise ratios above 3 indicating the detection limit for ACh in our system (lower limit mean for 21 HPLC–MS runs = 2.4 ± 0.04 fmol). There was excellent correlation for standards between individual HPLC–MS experimental runs (r^2 = 0.999, 21 runs), and between the predicted concentration for standards within a run and actual experimental values (r^2 mean = 0.999, 21 runs).

2.4. HPLC–ED for NE quantification

A 0.5 mL aliquot of each sample, and a 0.5 mL aliquot of the PCA atrial extract were spiked with an internal standard dihydroxybenzylamine (DHBA, 9 nM; Sigma–Aldrich, St. Louis, MO). A similar amount of DHBA was also added to standards of known amounts of NE (4–40 nM) and catecholamines were precipitated from samples and standards with alumina (15 mg, 30 min). The alumina was twice washed with ddH_2O, and the catecholamines, NE and DHBA, were desorbed with 0.1 M PCA [25]. Samples were chromatographically separated by reverse-phase HPLC (C18 column; 15 cm × 0.46 cm, 5 μm particle size; Varian, Lake Forest, CA) using a mobile phase containing 75 mM NaH_2PO_4 (pH 3.0), 1.7 mM sodium octane sulfonate, and 4% (v/v) acetonitrile. A coulometric detector (ESA, Bedford, MA) was used to detect and quantify NE (electrode potential 180 mV, 50 nA) with area under curve normalized to DHBA area. Detection limits were ~50 fmol with recoveries >60% [14].

2.5. Data analysis

The amount of ACh was calculated by comparing the ratio of ACh to d4 ACh in samples to those ratios from known standards for ACh and d4 ACh run in parallel. Similarly, NE concentration was