Displacement-encoded and manganese-enhanced cardiac MRI reveal that nNOS, and not eNOS, plays the dominant role in modulating calcium cycling in the mammalian heart

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Introduction Within healthy cardiomyocytes, L-type calcium (Ca^{2+}) channel (LTCC) flux and cardiac contractility are linked through the process of calcium-induced calcium release (CICR), whereby a small influx of Ca^{2+} through the LTCC ilicits a proportionally larger release of sarcoplasmic reticular (SR) Ca^{2+} and causes cardiomyocyte contraction. Two isoforms of nitric oxide synthase (NOS): endothelial NOS (eNOS) and neuronal NOS (nNOS), are believed to play different roles in modulating CICR in the healthy heart, and have been shown to be cardioprotective in the failing heart[1]. The prominent model of NO signaling, based mostly on *in vitro* studies of isolated myocytes, suggests that eNOS inhibits LTCC function while nNOS modulates contractile function via SR Ca^{2+} cycling[2]. However, most *in vitro* studies have failed to clearly demonstrate a role for eNOS in modulating LTCC function, and have also produced divergent results with respect to modulation of contractile function by nNOS. In contrast to *in vitro* methods, cardiac magnetic resonance (CMR) imaging enables noninvasive, *in vivo* phenotyping of the mouse heart. In addition, dynamic manganese (Mn)-enhanced CMR can be used to probe *in vivo* LTCC function[3], and cine displacement-encoded (DENSE)[4,5] CMR can be used to quantify myocardial strain and strain rate. In this study, we sought to elucidate the *in vivo* roles of both eNOS and nNOS in modulating LTCC function and contractile function. **Methods** Ten wild type (WT), 10 $eNOS^{-/-}$, and 10 $nNOS^{-/-}$ male mice were studied at age 10±3 weeks. Measurement of LTCC function using Mn-enhanced CMR was performed on a 4.7T MR system (Varian, Palo Alto CA), and has been previously described in detail[6]. Briefly, Mn^{2+} uptake was probed in two mid-ventricular short axis slices using an ECG-gated saturation recovery pulse sequence with a constant saturation time of 200ms. Images were acquired every 2-3 minutes for 90 minutes prior to, during, and after a 30 minute intraperitoneal (IP) infusion of $MnCl_2$ (0.42 mg/kg·min). **LTCCI**, an index of Mn^{2+} flux through the LTCC, and an *in vivo* analogue of LTCC current density, was quantified as shown in Fig 1. In WT mice, LTCCI was measured at baseline (Bsl), during β-adrenergic stimulation (βAS) with IP infusion of dobutamine (Dob, 5μg/kg·min), during concomitant βAS and muscarinic cholinergic stimulation with Dob and carbamylcholine chloride (CCh, 3 mg/kg·min) (Dob+CCh), in response to the partial LTCC inhibitor nifedipine (10mg/kg, n=3), and in response to increased heart rate (HR) with the A_{2A} adenosine receptor agonist ATL313 (12.5μg/kg, n=4). LTCCI was assessed in $eNOS^{-/-}$ and $nNOS^{-/-}$ mice at Bsl, Dob, and Dob+CCh, with one week between imaging sessions. Contractile function was assessed using a spiral cine DENSE method[4,5] in 2 mid-ventricular short-axis slices. Specifically, peak systolic circumferential shortening ($\mathbf{E_{cc}}$), representing maximal contraction, was measured sequentially at Bsl, Dob, and Dob+CCh, during the same imaging session in all mice. DENSE was performed on a 7T ClinScan MR system (Bruker, Ettlingen, Germany). Finally, systolic blood pressure was measured using tail plethysmography.

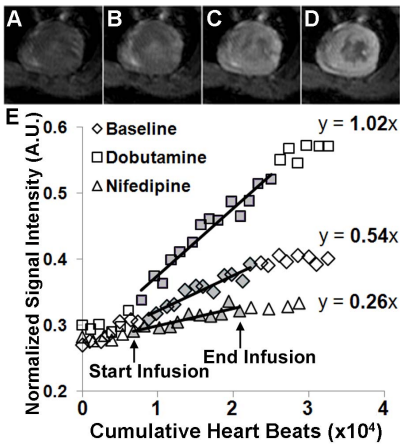

Figure 1. (A-D) Mid-ventricular short-axis T_1 weighted images from a WT mouse demonstrate increasing SNR during IP infusion of $MnCl_2$. The specific times for A-D are 15min, 30min, 50min, and 80min. (E) Accumulation of Mn^{2+} in cardiomyocytes results in a linear increase in SNR during infusion. The rate of increase in SNR, measured as the slope of a linear fit (solid black line) over the points corresponding to $MnCl_2$ infusion (filled gray symbols between the start and end of infusion), provides an index of integrated Mn^{2+} flux through the LTCC (LTCCI, bold numbers). Sample data from one WT mouse demonstrate an increase in LTCCI over Bsl with Dob, and a decrease from Bsl with nifedipine.

Results At Bsl, LTCCI was highest in $nNOS^{-/-}$ mice (Fig 2). In response to Dob, LTCCI increased in WT and $eNOS^{-/-}$ mice over Bsl, but decreased in $nNOS^{-/-}$ mice (Fig 2). With Dob+CCh, LTCCI returned to Bsl levels in WT and $eNOS^{-/-}$ mice and dropped significantly in $nNOS^{-/-}$ mice (Fig 2). Additionally in WT mice, LTCCI decreased from Bsl with both ATL313 (0.45±0.07 A.U./10^5 Heart Beats (H.B.), P<0.05 vs. Bsl) and nifedipine (0.35±0.04 A.U./10^5 H.B., P<0.05 vs. Bsl). At Bsl all 3 groups demonstrated similar E_{cc} (-12.6±0.4% WT, -11.6±0.4% $eNOS^{-/-}$, -11.8±0.4% $nNOS^{-/-}$, P=NS). However, in response to Dob, only WT and $eNOS^{-/-}$ mice demonstrated an increase in E_{cc} (Fig 3). With Dob+CCh, E_{cc} was similar to Bsl in all 3 groups (Fig 3). Systolic blood pressure was significantly higher in $eNOS^{-/-}$ mice (110±3 mmHg WT, 131±2 mmHg $eNOS^{-/-}$, 110±2 mmHg $nNOS^{-/-}$, P<0.05 $eNOS^{-/-}$ vs. WT & $nNOS^{-/-}$). **Conclusions** Our LTCCI results in WT mice parallel measurements of LTCC current ($I_{Ca,L}$) density in isolated myocytes under all tested conditions, showing that LTCCI can be used to probe *in vivo* LTCC function. In WT mice, LTCCI decreased significantly with ATL313 while HR increased on average 20%. As ATL313 increases HR without increasing $I_{Ca,L}$, the observed negative LTCCI-frequency response resembles a negative $I_{Ca,L}$-frequency effect seen in isolated myocytes[7]. When applied to elucidating the roles of each NOS isoform, our LTCCI results indicate that nNOS, and not eNOS, plays a dominant role in modulating basal LTCC function. In $nNOS^{-/-}$ mice LTCCI was maximal at Bsl, and given CICR principles, one would expect basal contractile function to also be elevated in $nNOS^{-/-}$ mice. However, basal E_{cc} was normal in $nNOS^{-/-}$ mice, and did not change with Dob or Dob+CCh (Fig 3), despite significant drops in LTCCI (Fig 2). This suggests that nNOS differentially regulates Ca^{2+} cycling by acting to inhibit LTCC function at Bsl while promoting SR Ca^{2+} cycling during βAS. In contrast, no significant differences were seen between $eNOS^{-/-}$ and WT mice in terms of LTCC or contractile function at any level of physiological stimulation. While basal LTCCI was slightly elevated in $eNOS^{-/-}$ mice, this more likely reflects the results of hypertension. Thus, our results demonstrate that *in vivo*, it is nNOS, and not eNOS, that plays a dominant role in modulating calcium cycling in the mammalian heart.

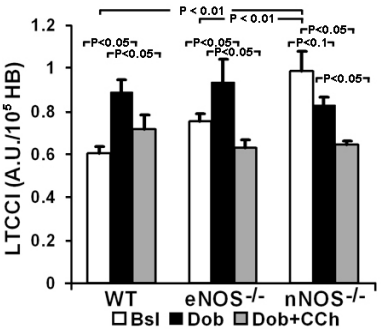

Figure 2. LTCCI assessed at Bsl, Dob, and Dob+CCh in WT, $eNOS^{-/-}$ & $nNOS^{-/-}$ mice.

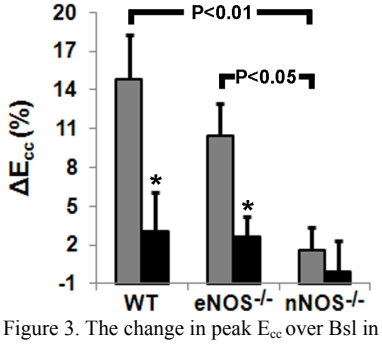

Figure 3. The change in peak E_{cc} over Bsl in response to Dob, and Dob+CCh in WT, $eNOS^{-/-}$ & $nNOS^{-/-}$ mice. *P<0.05 vs. Dob.

References
(1) Belge et al. *Ann NY Acad Sci*. 2005; 1047:173-182. (2) Hare et al. *J. Mol Cell Cardiol*. 2003; 35(7):719-729 (3) Hu et al. MRM (4) Aletras et al. JMR 1999;137:247-252 (5) Zhong et al. *Proc ISMRM*. 2008; 16:580 (6) Vandsburger et al. *Proc ISMRM*. 2009; 17:807. (7) Antoons et al. *Journal of Physiology*. 2002; 543(3):889-898. Support: AHA pre-doctoral grant AHA0815242E to MHV, and AHA Established Investigator Award 0540060N and NIH R01EB001763 to FHE.