SUPPLEMENTAL MATERIAL

Reversibility of Adverse, Calcineurin-Dependent Cardiac Remodeling

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Detailed Methods

Animal Care

We engineered double transgenic mice (C57BL/6 background) harboring a truncated form of the calcineurin A subunit lacking the C-terminal autoinhibitory domain (CnA*)[1]. Temporal control of expression was accomplished using the tetracycline transactivator off promoter (TetO) system[2]. Multiple lines of TetO-CnA* transgenic mice were generated. These animals were crossed with mice harboring the tetracycline-inhibited transactivator (tTA) under control of the α-myosin heavy chain promoter (αMHC-tTA)[3] to yield double transgenic mice (tTA/CnA*) for use in experiments. Both male and female animals aged 5 to 8 weeks were studied using protocols approved by the animal care and use committee of UT Southwestern Medical Center.

Echocardiography

Echocardiograms were performed on conscious, gently restrained mice using a Sonos 5500 system and 15 MHz linear probe. A short axis view of the left ventricle at the level of the papillary muscles was obtained, and M-mode recordings were obtained from this view. Measurements of interventricular septum thickness (IVS), left ventricular internal diameter (LVID), and left ventricular posterior wall thickness (LVPW) were made from 2D parasternal short axis views in diastole. Left ventricular mass was calculated by the cubed method as 1.05 x $((IVS + LVID + LVPW)^3 - LVID^3)$ $(mg)^4$. Left ventricular internal diameter at end-diastole (LVIDd) and end-systole (LVIDs) were measured from M-mode recordings. Fractional shortening was calculated as (LVIDd - LVIDs) / LVIDd (%).

Immunoblot analyses

Hearts were harvested and snap frozen in liquid nitrogen. Protein lysates were prepared from left ventricular tissue homogenized in lysis buffer (0.1% Triton X-100, 2% glycerol, 10mM Tris, 1mM Na bisulfite, 1mM NaF, protease inhibitors, pH=7.0) and centrifuged (9,000 rpm, 10 min). For extraction of myosin protein, a different lysis buffer was used (0.3M KCl, 0.1M KH_2PO_4, 50mM K_2HPO_4, 10mM EDTA, protease inhibitors). Protein concentration of lysates was estimated using the Bradford method, and equal quantities of protein were loaded per gel lane and separated by electrophoresis. Proteins were transferred to a PVDF membrane and equivalence of protein loading and transfer confirmed by Ponceau stain. Proteins of interest were detected using primary antibody and HRP-conjugated secondary antibody.

Antibodies used were as follows: anti-calcineurin Aβ (AB1697, Chemicon International, Temecula, CA); anti-myosin (skeletal, slow; M8421, Sigma-Aldrich, St. Louis, MO), anti-RCAN (custom-made, Gilead, Bromfield, CO); goat anti-mouse HRP-conjugated antibody (1721011, BioRad, Hercules, CA); goat anti-rabbit HRP-conjugated antibody (1721019, BioRad, Hercules, CA).

Quantitative real-time PCR

RNA was extracted from snap frozen left ventricular tissue using Trizol reagent according to the manufacturer's instructions. RNA concentration was estimated from each sample using a Nanodrop ND-1000 spectrophotomer. Two μg RNA of each sample was used to make cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on an ABI 7000 system in triplicate for each sample. Relative quantities of each transcript were determined by normalizing to cyclophilin A.

Primers for real-time PCR reactions were as follows: Cyclophilin A: CAGACGCCACTGTCGCTTT / TGTCTTTGGAACTTTGTCTGCAA; βMHC:CTACAGGCCTGGGCTTACCT / TCTCCTTCTCAGACTTCCGC; BNP: CACCGCTGGGAGGTCACT / GTGAGGCCTTGGTCCTTCAA; ANF: CATCACCCTGGGCTTCTTCCT / TGGGCTCCAATCCTGTCAATC; SERCA 2a: CCATCTGCTTGTCCATGTCACT / CAAATGGTTTAGGAAGCGGTTACT

Histology and cardiomyocyte cross-sectional area

Selected hearts were perfusion fixed in paraformaldehyde and subjected to cutting and staining as described previously[5]. Hematoxylin and eosin-stained tissue sections from 4 hearts in each group were studied at 200x magnification. Six randomly selected fields were studied per heart, and all myocytes cut in short axis with a visible nucleus were measured. Cell borders were planimetered using ImageJ software by an operator who was blinded to treatment group. This experiment was repeated using WGA-stained images from 4 hearts.

Picrosirius red stained tissue sections were studied to assess fibrosis. Six randomly selected fields were studied per heart. The area of tissue staining positive for fibrosis was measured using ImageJ software by an operator who was blinded to treatment group. Results are reported as the percent area of tissue staining positive for fibrosis.

Hydroxyproline analysis was performed based on previously described methods[6]. Briefly, hearts were harvested, the left ventricle dissected free, and the tissue lyophilized. Equal quantities of lyophilized material were suspended in 6 N HCl and hydrolyzed at 120°C followed by neutralization with 4 N NaOH. Samples and standards (L-hydroxyproline, Sigma) were incubated for 20 minutes (RT) with chloramine T, followed by addition of Erlich reagent (3.75g of p-dimethylaminobenzaldehyde, 15 mL of l-propanol, 6.5 mL of perchloric acid (60%) in 25 mL for 20 min (60°C). Absorbances were read at 558 nm, and values were calculated from a standard curve generated for each analysis. Results are expressed as microgram of hydroxyproline per milligram of dried tissue sample.

In vivo electrophysiology studies

In vivo electrophysiology studies were performed as previously described[7]. Mice were sedated by intraperitoneal administration of pentobarbital (0.050 mg/gm). The right jugular vein was exposed, and a 1.1 F octapolar electrophysiology catheter (Millar EPR-800) was inserted transvenously into the right jugular vein and advanced to the right atrium or right ventricle using electrogram tracings for guidance. The EP catheter included eight electrodes with 1.0 mm electrode spacing, and the distal electrode pair was used to stimulate while recording from all other electrodes. A standard 6 lead surface ECG was recorded during the study. Programmed ventricular stimulation was performed with one, two, and three extrastimuli following an eight beat drive train. Each extrastimulus coupling interval was decreased by 10 msec intervals until refractory. The same protocol was performed on all mice while observing for ventricular tachyarrhythmia of 4 beats or more.

Statistical methods and data handling

All values are presented as mean ± SD. Comparison of data between groups was performed using the Mann-Whitney U test. Comparison of fractional shortening over time between groups was performed using two-way repeated measures ANOVA. Holm-Sidak *post hoc* testing was used to correct for multiple comparisons. Using the Bland-Altman analysis method, the agreement between LV mass determined echocardiographically and necropsy heart weight was calculated as the mean (bias) ± error (2 standard deviations). For statistical comparisons, significance was taken as $p < 0.05$. All statistical analyses were performed using SigmaStat v3.1 software.

The authors had full access to, and take full responsibility for, the integrity of these data. All authors have read and agree to the manuscript as written.

Supplemental Data

During the reverse remodeling experiment, there was variability in the degree of reverse remodeling when doxycycline was restarted (**Online Figure VI**). In an effort to gain insight into mechanisms, we examined echocardiographic characteristics of mice just prior to reinitiating doxycycline to identify potential markers of reversibility. Mice demonstrating significant reverse remodeling (%FS>50% at study completion) had a slightly higher %FS after 8 weeks of calcineurin activation (%FS: responders 40.2±6.0%, non-responders 36.4±2.8%, p=0.13). No other specific parameters of ventricular hypertrophy or chamber dimension were predictive of the extent of reverse remodeling (LVIDd: responders 3.9±0.5 mm, non-responders 4.1±0.3 mm, p=NS; LV posterior wall thickness: responders 1.1±0.2 mm, non-responders 1.2±0.2 mm, p=NS; ratio of wall thickness-to-LVIDd: responders 0.28±0.07, non-responders 0.28±0.06, p=NS) (**Online Figure VII**).

Given the presence of significant interstitial fibrosis with prolonged calcineurin activation, we hypothesized that these mice would be susceptible to ventricular arrhythmia. We have shown previously that mice subjected to severe transverse aortic constriction (sTAC) develop dilated cardiomyopathy that is associated with propensity to arrhythmia upon ventricular extra-stimulation[7]. Surprisingly, we were unable to induce ventricular arrhythmia in mice after 16 weeks of calcineurin activation; in contrast, sTAC, which elicited a similar cardiomyopathic phenotype, was associated with readily triggered ventricular arrhythmia (**Online Figure VIII**). These data suggest that aspects of the electrophysiological remodeling response elicited by calcineurin activation differ from that provoked by elevated afterload.

Supplemental Table

Left ventricular hypertrophy precedes systolic dysfunction with calcineurin activation. Echocardiographic measurements of left ventricular size and function. Left ventricular hypertrophy develops after two weeks of calcineurin activation (age 10 weeks). A decline in fractional shortening is not apparent until age 12 weeks. Left ventricular chamber dilation is just apparent at age 16 weeks.

Age (weeks)	8		10		12		14		16	
Mice	ON	OFF	ON	OFF	ON	OFF	ON	OFF	ON	OFF
N	9	9	9	9	9	9	9	9	9	9
LVIDd (mm)	3.31	3.30	3.47	3.63	3.81	3.75	3.78	3.92	4.04*	3.62
LVIDs (mm)	1.26	1.27	1.38	1.50	1.86*	1.54	2.10*	1.70	2.44*	1.51
FS (%)	62.0	61.7	60.1	58.8	51.2*	59.1	44.5*	56.7	39.6*	58.2
LV mass (g)	132	116	158*	127	186*	144	199*	155	299*	183
IVSd (mm)	1.03	0.93	1.10*	0.89	1.11*	0.94	1.16*	0.97	1.21*	0.94
LVPWd (mm)	1.10	1.03	1.20*	0.98	1.23*	1.03	1.30*	1.00	1.31*	1.00

LVIDd, left ventricular internal diameter at end-diastole; LVIDs, left ventricular internal diameter at end-systole; FS, fractional shortening; LV mass, left ventricular mass calculated from 2D echo images using cubed method; IVSd, interventricular septal thickness at end-diastole; LVPWd, left ventricular posterior wall thickness at end-diastole; * $p < 0.05$.

Supplemental Figure Legends

Online Figure I: Removal of doxycycline triggers increased RCAN1.4 and βMHC protein in tTA/CnA* mice. A. Representative immunoblots of LV lysates prepared from hearts harvested 7 days after removal of doxycycline and probed for calcineurin (CnA), RCAN, and tubulin. B. Representative immunoblots of LV lysates from tTA/CnA* hearts harvested 4 or 5 days after removal of doxycycline and probed for CnA, RCAN, and tubulin. C. Representative immunoblot of LV lysates prepared 8 weeks after removal of doxycycline and probed for βMHC.

Online Figure II: Calcineurin activation in adult heart triggers cardiomyocyte hypertrophy. A. Representative histological images of wheat germ agglutinin (WGA)-stained left ventricular tissue sections revealing cardiomyocyte size at study completion. Bar = 20 μm. B. Mean cardiomyocyte cross-sectional area measured from WGA-stained tissue sections. 60 cells were measured from 4 hearts. ON, calcineurin activated for 16 weeks; OFF, calcineurin suppressed for 16 weeks. * $p<0.01$.

Online Figure III: Expression of tTA protein triggers increased cardiac mass and no change in function. A. Mean heart weight to body weight ratio of mice sacrificed at age 16 weeks. B. Mean fractional shortening measured from M-mode tracings obtained at age 16 weeks. WT, wild-type (n=7), tTA, single transgenic for tTA (n=7), tTA/CnA*, double transgenic for both tTA and CnA* transgenes (n=10). *$p<0.05$.

Online Figure IV: Left ventricular mass calculated from 2D echocardiographic images accurately predicts heart weight. A: LV mass was determined by echocardiography on the same day that hearts were harvested and weighed. Line of equality is shown. B: Bland-Altman plot showing the difference in measurements as a function of the mean of measurements for each heart. The bias and error for echocardiographically determined LV mass compared to total heart weight was -3.1 ± 49.4 mg. This is consistent with previously published data in which the cubed formula slightly over-estimated LV weight measured at necropsy[4]. (Collins et al. *Am J Physiol Heart Circ Physiol* 2001; 280: H1954-62.)

Online Figure V: Average heart rate of mice during serial echocardiograms was not different for control (OFF) and experimental (ON) mice.

Online Figure VI: Mice demonstrate variable reverse remodeling when calcineurin activity is extinguished. Graph showing fractional shortening for individual mice during 8 weeks of calcineurin activation followed by 8 weeks of recovery. Those mice with fractional shortening greater than 50% at study completion were categorized as responders.

Online Figure VII: Echocardiographic data in responders and nonresponders prior to reverse remodeling. A: Mean fractional shortening measured from M-mode tracings obtained at 8 weeks after removing doxycycline and at 16 weeks (8 weeks after restarting doxycycline. B: Mean left ventricular internal diameter in diastole (LVIDd), left ventricular posterior wall thickness in diastole (LVPWd), and LVPWd-to-LVIDd ratio measured 8 weeks after removing doxycycline in responders and nonresponders.

Online Figure VIII: Calcineurin-induced cardiomyopathy was not associated with ventricular arrhythmia. A. Representative *in vivo* electrophysiology study showing inducible ventricular tachyarrhythmia with extra-stimulation in a mouse subjected to severe transverse aortic constriction (sTAC). Lead I, surface electrocardiogram lead; RV EGM, right ventricular electrogram, RA EGM, right atrial electrogram, S1/S2/S3, ventricular pacing stimuli. B. Frequency of inducible ventricular arrhythmia in mice during *in vivo* electrophysiology studies.

Supplemental References

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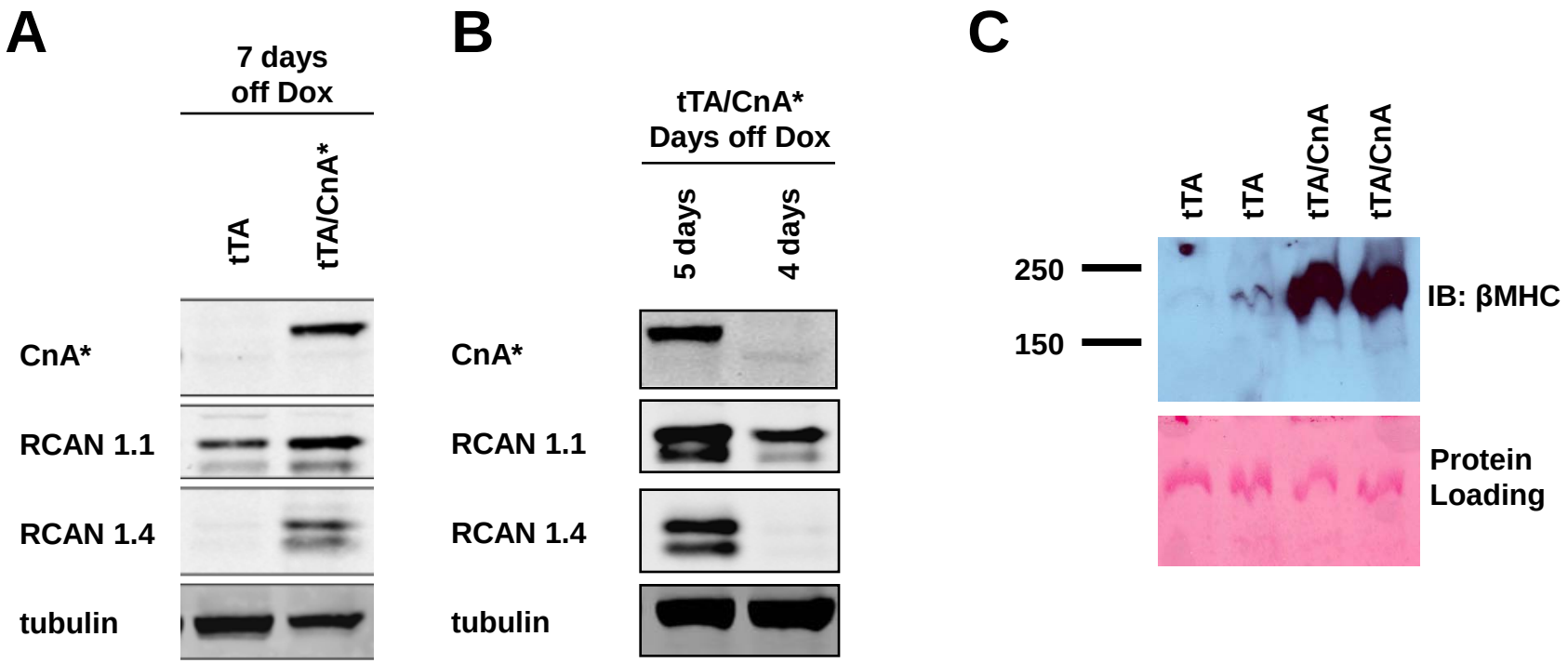
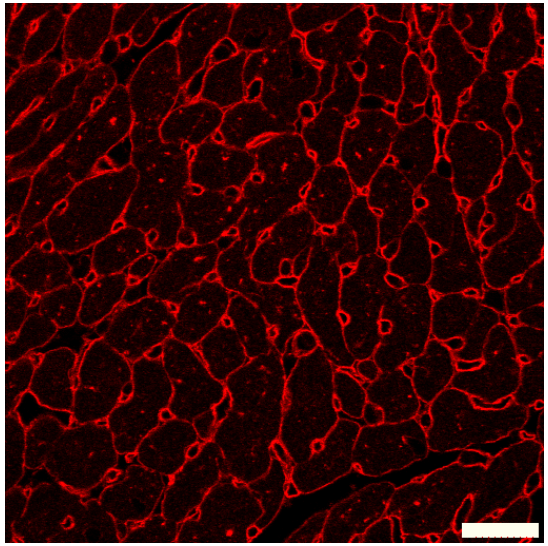


Online Figure I

A



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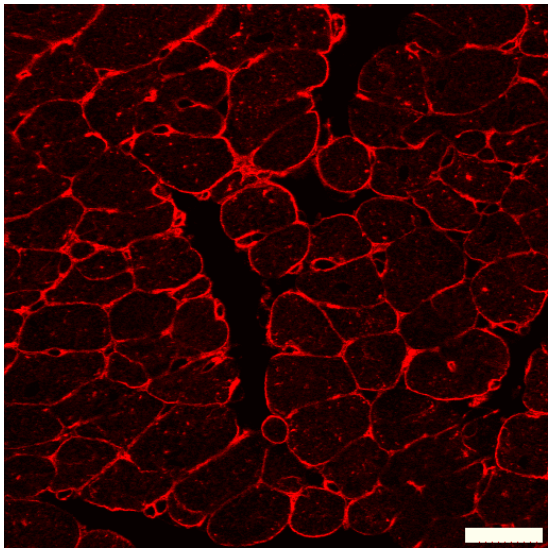

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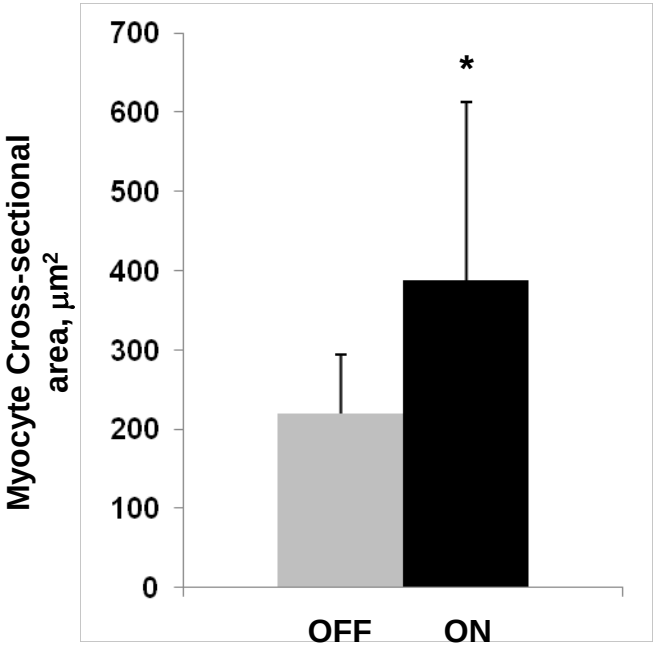

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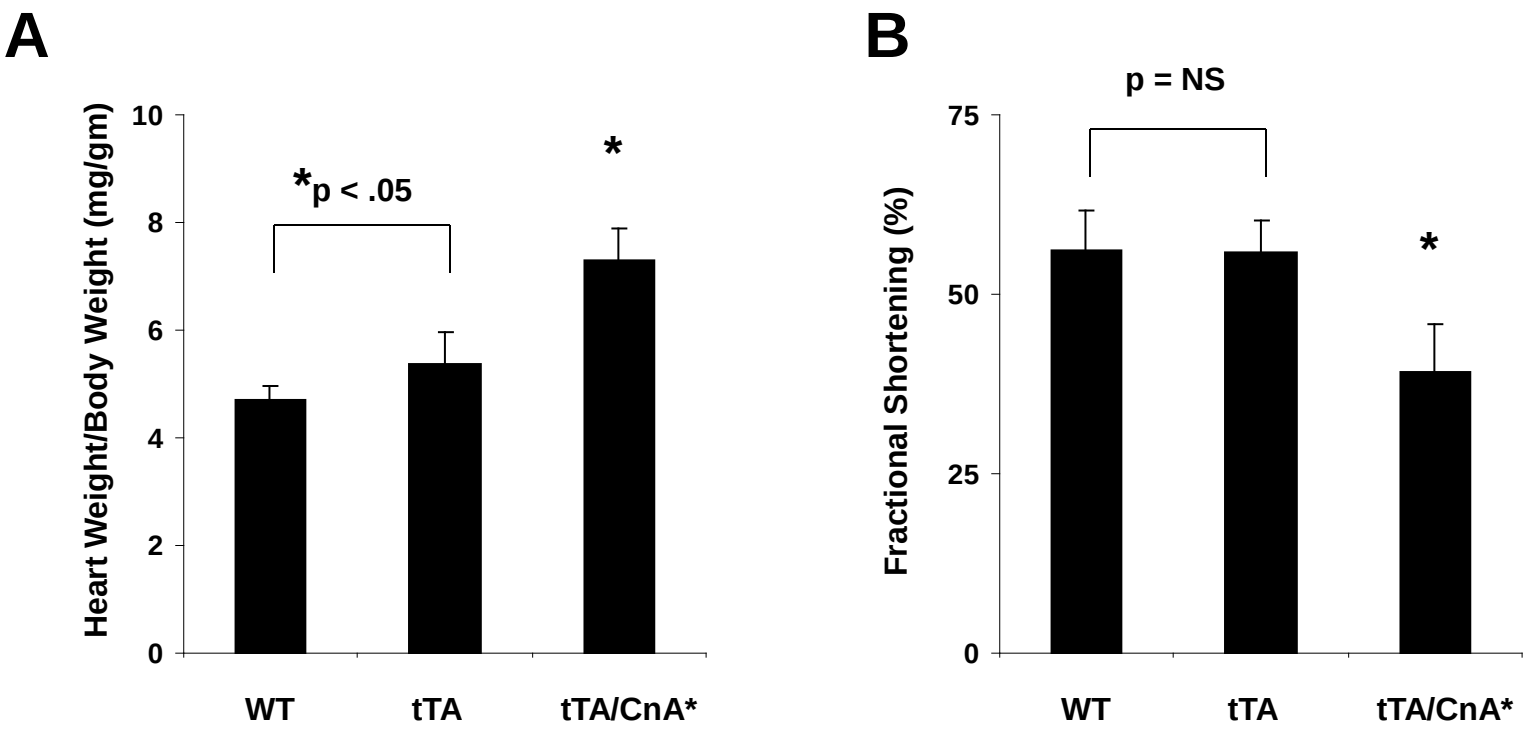

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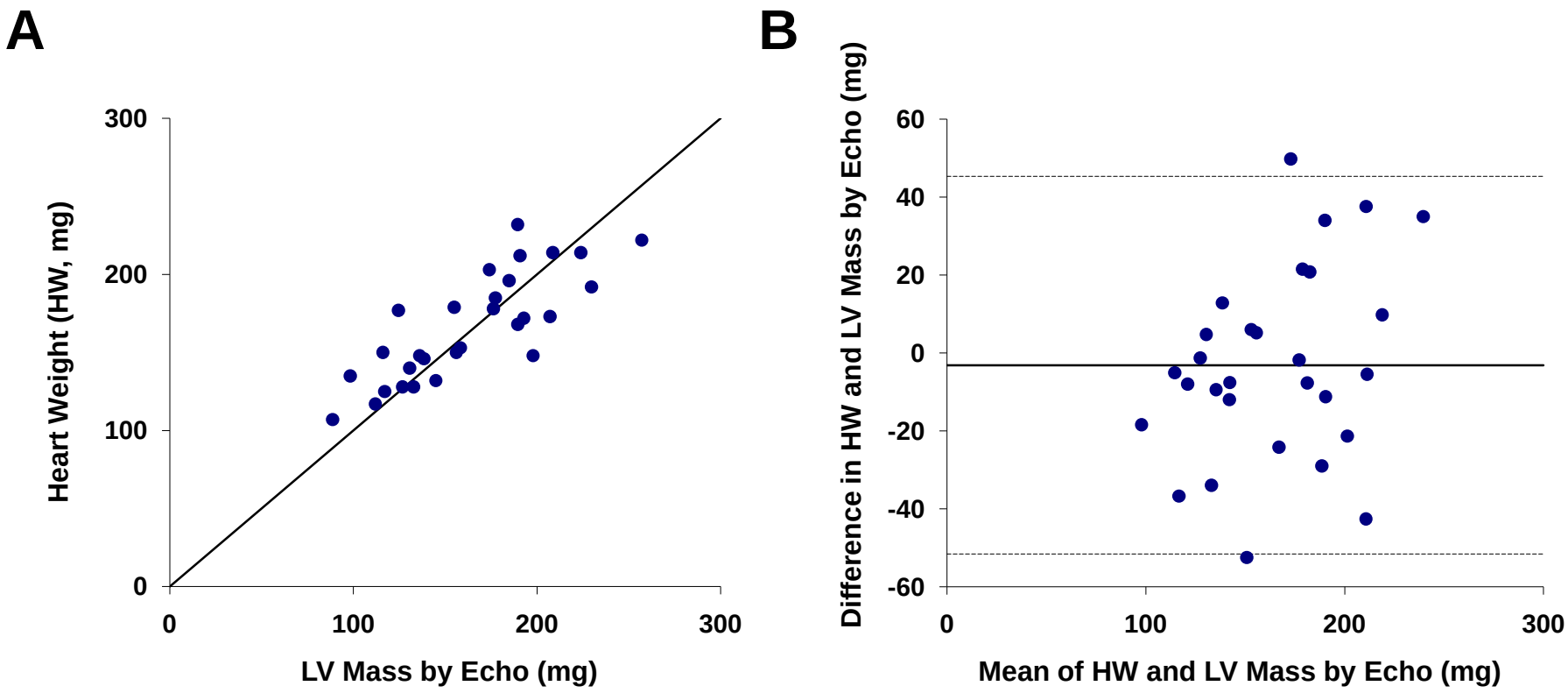

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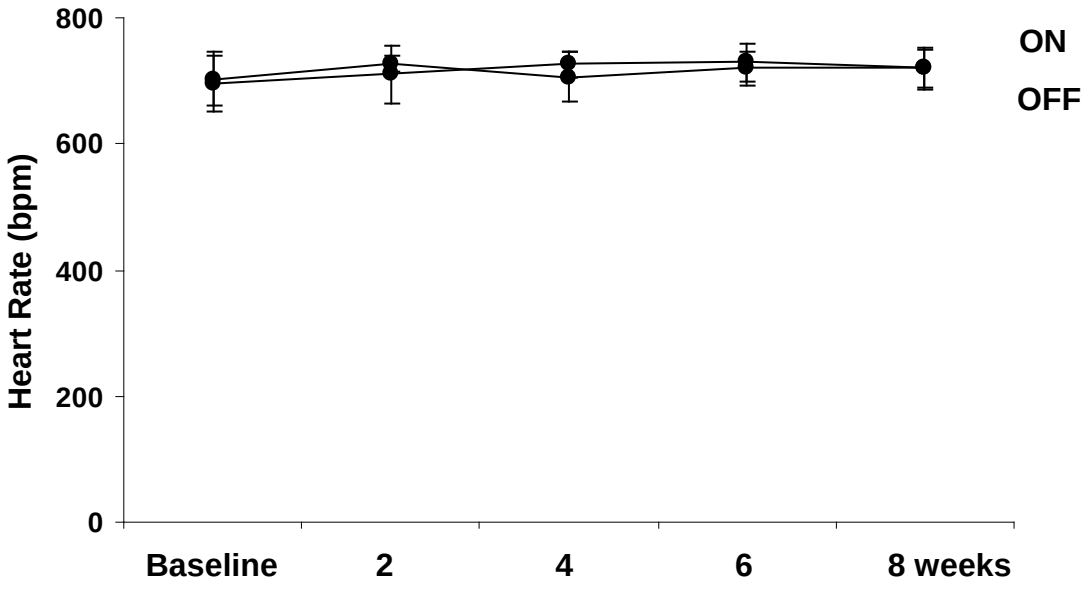

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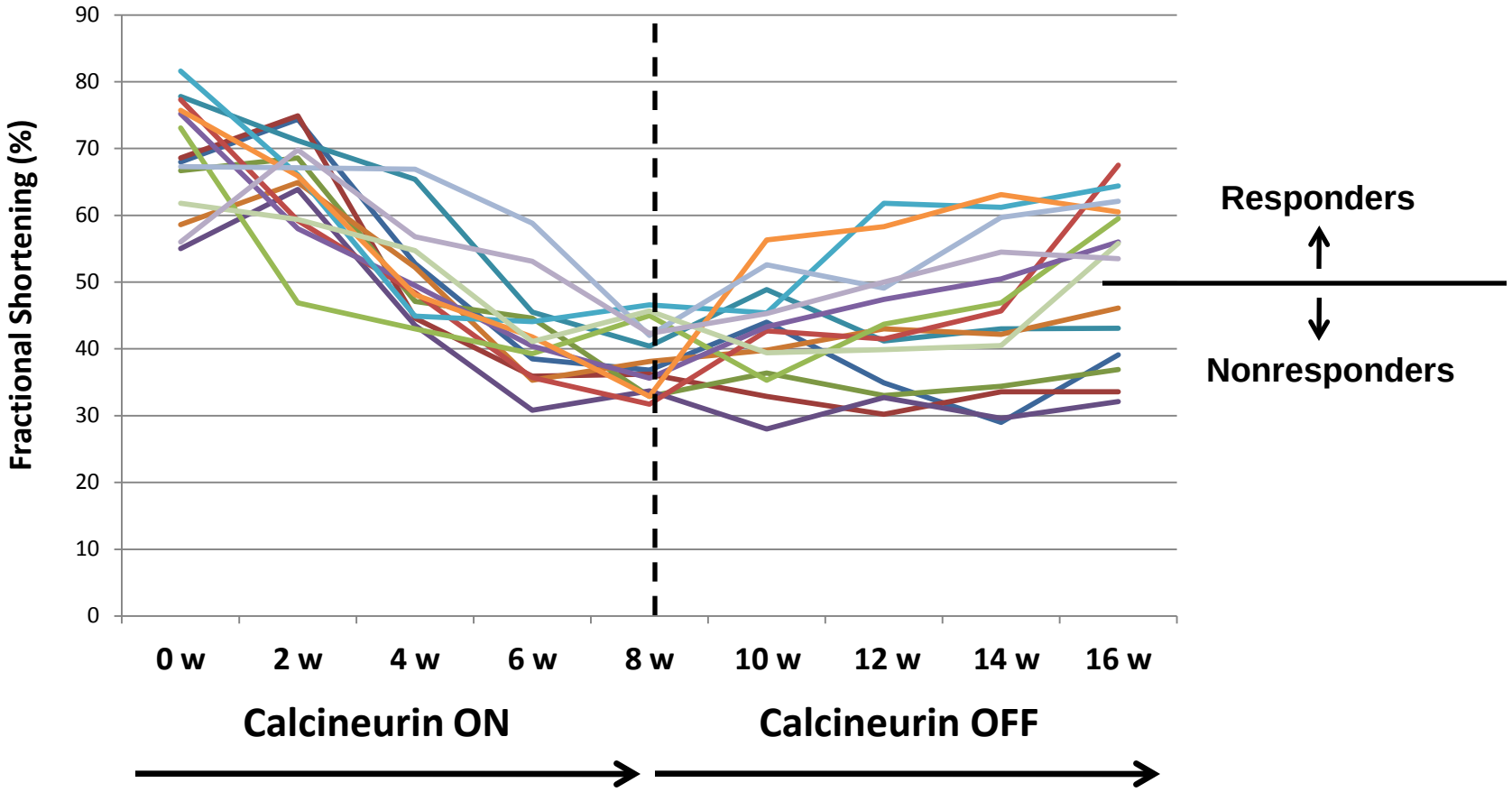

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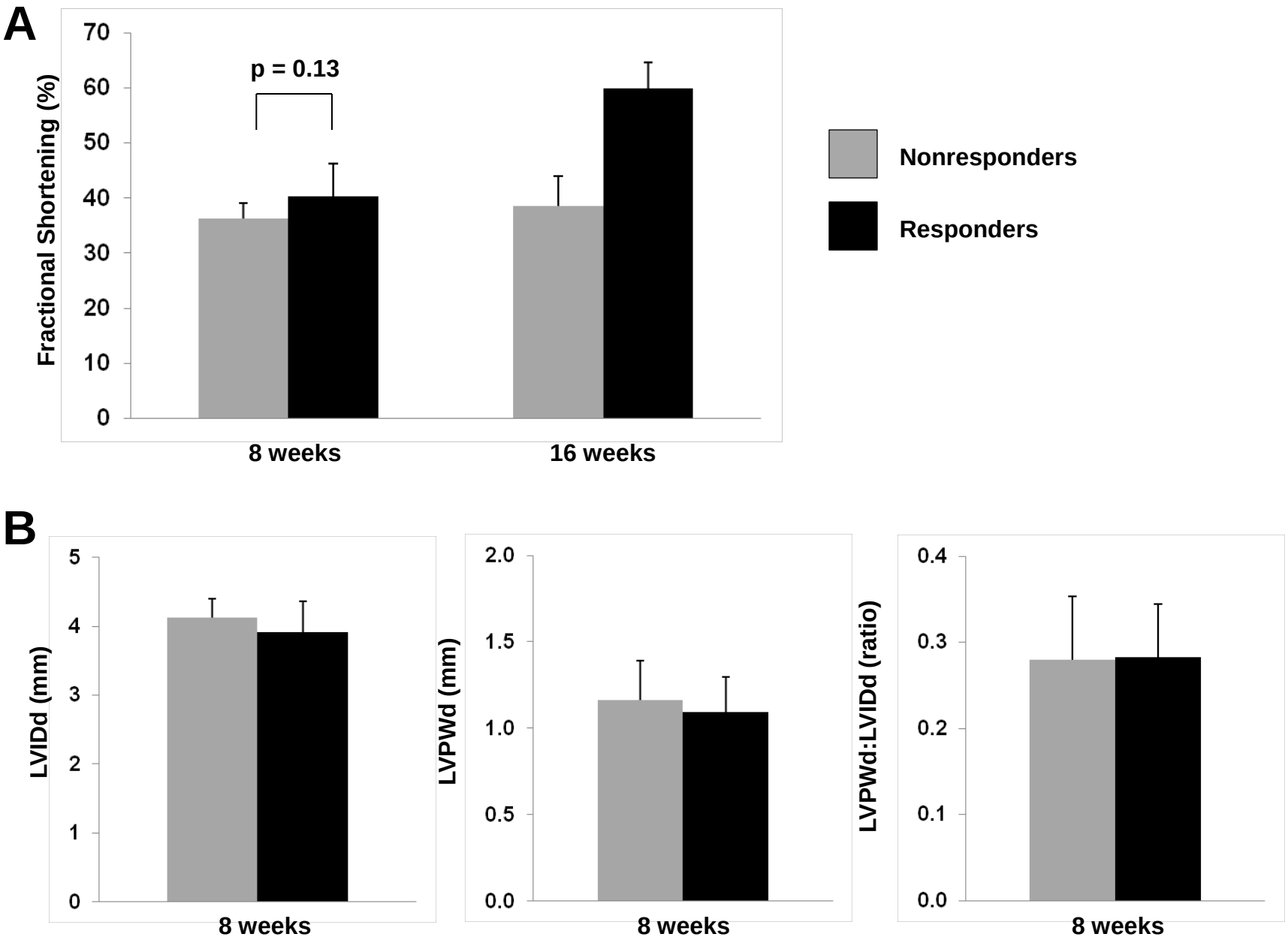

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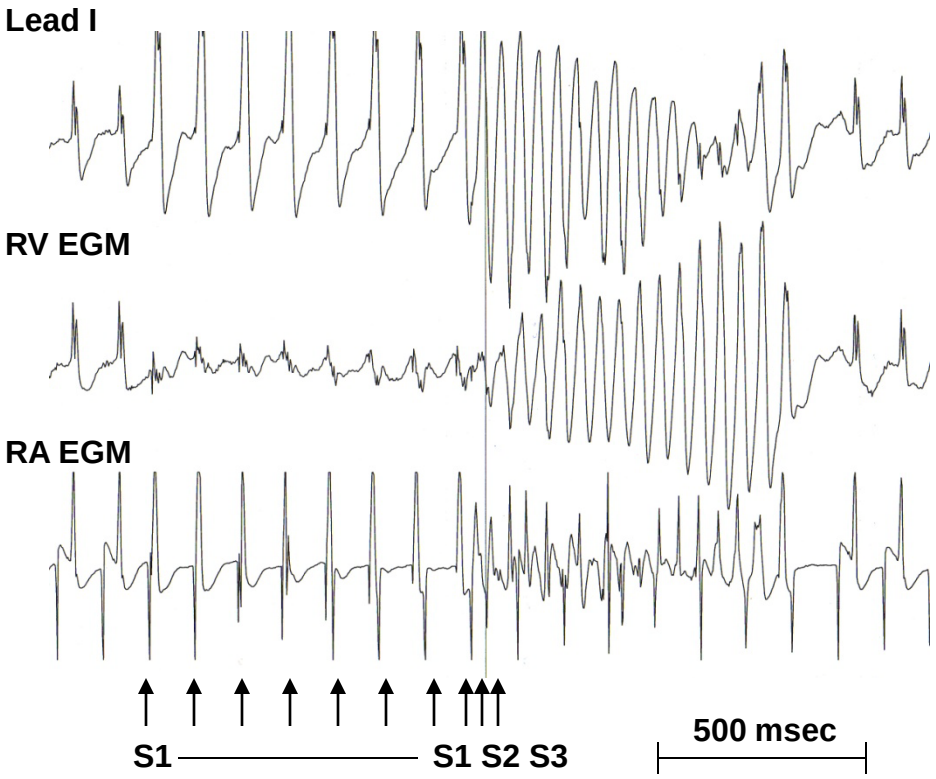

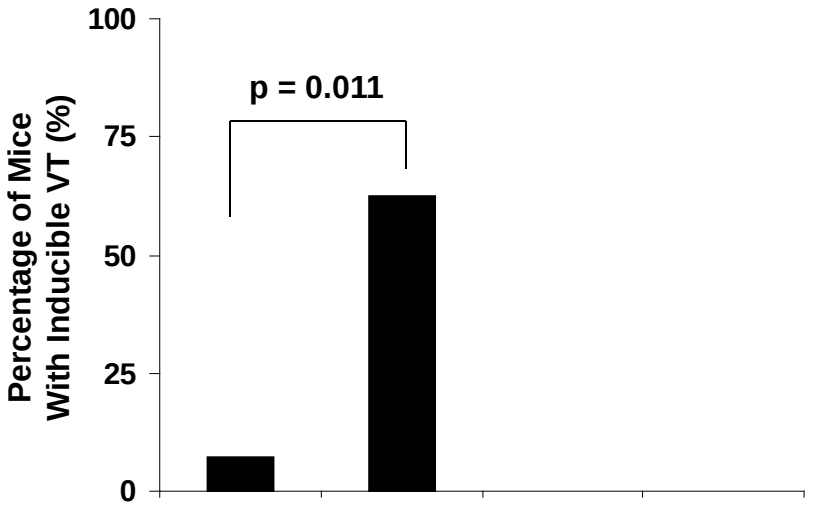

Sham	sTAC	CnA OFF	CnA ON
1 of 14	5 of 8	0 of 5	0 of 5

Online Figure VIII