SUPPLEMENTAL MATERIAL

Controlled and Cardiac-Restricted Overexpression of the Arginine Vasopressin V1A Receptor Causes Reversible Left Ventricular Dysfunction Through $G\alpha_q$-Mediated Cell Signaling

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Key words

Vasopressin receptor, V1A receptor, signal transduction, heart failure, hypertrophic cardiomyopathy

Methods:

V1A-R Transgenic Mouse Generation. The human AVP V1A receptor cDNA was cloned into a cardiac-specific and inducible controlled vector (TREMHC) composed of a modified mouse α-myosin heavy chain minimal promoter fused with nucleotide binding sites for tetracycline transactivating factor (tTA)[1]. Both our laboratory[2] and Sanbe and colleagues[1] have shown that this inducible system has no effect on cardiac function, most likely due to the fortuitous use of a bacterially derived tTA construct in which the less than ideal usage of bacterial codon in a mammalian system resulted in multiple aberrant splice sites and less than 1% of the bacterial V1A cDNA encoded the full-length protein. As a result, the very low level expression of bacterial tTA does not affect mouse cardiac functions, but is sufficient to induce robust expression of transgenes in double transgenic mice expressing both bacterial tTA transgene and various responder genes, including GSK3, adenosine A_1 receptor, adenosine A_{2A} receptor, $G\alpha_q$ and NCX[1-5]. To prevent phenotype selection due to line propagation, the strains were propagated in the absence of inducer transgene, tTA. To induce transgene expression, V1A receptor transgenic (V1A-TG) mice in FVB background were crossed with mice that expressed tTA in the heart (MHC-tTA) (Fig. 1A in the main text). In this "tet-off" inducible system, the stable tetracycline analog, doxycycline, inhibits tTA transactivation. To inhibit transgene expression, mouse diets containing 300mg DOX/kg (Bio-Serv, NJ) were administered for the time periods indicated in the experiments. Since crossings of double or triple transgenes were required for gene expression, we used multiple control mice for our experiments. Where possible, these consisted of non-transgenic littermates. In the case of multiple crosses using homozygous parental lines (eg. tTA+/+ crossing V1A+/+), we assessed the phenotype in age matched and co-habitating mice expressing the parental single transgene as seen in Table 1 and Table 3 of the main text.

Generation and maintenance of tTA/V1A-R transgenic mouse lines that co-expresses $G\alpha_q$ inhibitor, Gq-I. Transgenic mice that overexpressed a peptide derived from a carboxyl-terminal peptide of the α subunit $G\alpha_q$ (Gq-I TG) were described previously[6]. Since tTA and V1A-R transgenic lines were in FVB background, but Gq-I transgenic mice were in C57B6 background, we first crossed V1A-TG with Gq-I TG to obtain double transgenic (V1A/Gq-I) with 50% FVB/50% C57B6 strain background. The V1A/Gq-I double transgenic mice were then crossed with MHC-tTA mice in FVB background to induce V1A transgene expression. The tTA/V1A/Gq-I triple transgenic mice and their littermates in 75%FVB/25%C57/B6 strain background were used for analysis. In addition, our crossing strategy ensured that tTA/V1A double transgenic mice and tTA/V1A/GqI triple transgenic mice were all hemizygous. All protocols were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Echocardiography Echocardiography studies were performed using an ultrasonographic system (ACUSON Sequoia C256) as described [2]. Briefly, mice were anesthetized with 2.5% Avertin (10ul/g body weight, IP) and placed in the supine position. A14-MHz transducer was applied to the left hemithorax. Two –dimensional targeted M-mode images were obtained from the short-axis view at the level of the greatest left ventricular dimension at baseline. M-mode measurements of left ventricular end-diastolic and end-systolic diameter and left ventricular anterior- and posterior-wall thickness were made using the leading-edge convention of the American Society of Echocardiography. End-diastole was determined at the maximal left ventricular diastolic dimension, and end-systole was taken at the peak of posterior-wall motion.

Real-Time Quantitative PCR Real-time quantitative polymerase chain reaction analysis determined gene expression. Briefly, reverse-transcribed cDNA from myocardial RNA was used to determine gene expression. Real time PCR was performed in a 50 μl reaction (5 μl cDNA or 40ng of genomic DNA;

250 nM each primer; 1X SYBRE Green Master Mix). Each experimental group was performed in triplicate. The ΔCT method using GAPDH as reference gene was used to quantify the results and perform statistic analysis as suggested [7, 8]. Since small sample sizes were used in real-time PCR experiments, non-parametric alternative to the t-test (Mann-Whitney test) was used to test statistical significance. ΔΔCT values were then converted to fold changes in gene expression relative to WT samples as described [7]. Sequences of primers used:

Human V1A receptor (F –5'-TCC ATG ATC GAG GTG AAC AA-3', R- 5'-TGT TGT AGC AGA TGA AGC CG-3')

ANP (F-5' CGT GCC CCG ACC CAC GCC AGC ATG G 3', R-5' GCC TCC GAG GGC CAG CGA GCA GAG C 3')

BNP (F-5' CTG AAG GTG CTG TCC CAG AT 3', R-5' CCT TGG TCC TTC AAG AGC TG 3')

SERCA2 (F-5' TGA GAC GCT CAA GTT TGT GG 3', R -5' ATG CAG AGG GCT GGT AGA TG 3')

B-Myosin heavy chain (F-5' ACT GTC AAC ACT AAG AGG GTC A - 3', R-5' TTG GAT GAT TTG ATC TTC CAG GG 3')

NCX (F-5' CCC AAT GTT TCA ATG GGA TT 3', R-5' AGA TGG GTC TTG GGG TTC 3')

GAPDH (F-5' AAC GAC CCC TTC ATT GAC 3', R -5' TCC ACG ACA TAC TCA GCA C 3')

Histopathology of Myocardium. 5μm sections of fixed tissue were stained with Picrosirius Red for assessment of fibrosis (Research Animal Diagnostic Laboratory, University of Missouri). To determine fibrosis, independent high-power fields of stained images were digitally photographed using identical exposure times and incident light intensities and analyzed using Image J system (NIH 1.42 Version software) as described [9, 10]. The data were reported as arbitrary units.

Hemodynamic Analysis of Cardiac Function. For in vivo hemodynamic measurements, a 1.4 French micromanometer-tipped catheter (SPR-671; Millar Instruments Inc.) was inserted into the right carotid artery and advanced into the LV of mice that were lightly anesthetized (i.e., maintained

spontaneous respirations) with tribromoethanol/amylene hydrate (2.5% wt/vol, 8 μl/g injected intraperitoneal; Avertin). Hemodynamic parameters, including heart rate, LV end-diastolic pressure, and +dP/dt and –dP/dt, were recorded in closed-chest mode, both at baseline and in response to isoproterenol (10 ng), administered via cannulation of the right internal jugular vein [11].

Isolation of Adult Murine Cardiac Myocytes. Cardiac myocytes were isolated from the septum and LV free wall of WT and V1A-TG mice as previously described [12, 13]. Briefly, excised hearts were mounted on a steel cannula and retrograde perfused (100 cm H_2O, 37°C) with Ca^{2+}-free bicarbonate buffer followed by enzymatic digestion (collagenases B and D, protease XIV). Isolated myocytes were cultured on laminin-coated glass cover slips and the Ca^{2+} concentration of the buffer was incrementally increased from 0.05 to 0.5 mM (0.05, 0.125, 0.25, 0.5 mM) with 10-min of exposure at each Ca^{2+} concentration. The 0.5 mM Ca^{2+} buffer was then aspirated and replaced with minimal essential medium (MEM; Sigma M 1018) containing 1.2 mM Ca^{2+}, Insulin-Transferrin-Selenium supplement (Invitrogen Corporation), 1% penicillin-streptomycin and 2.5% FCS.

Contraction and $[Ca^{2+}]_i$ Transient Measurements. Myocytes from V1A-TG and WT mice were exposed to 0.67 μM of fura-2 AM for 15 minutes at 37°C. Fura-2 loaded myocytes were field-stimulated to contract (1 Hz, 37°C) in medium 199 containing 1.8 mM $[Ca^{2+}]_o$. $[Ca^{2+}]_i$ transient measurements were performed as previously described [12, 13].

Action Potential Measurements. Action potentials were recorded using current-clamp configuration at 1.5x threshold stimulus and 4-ms duration [14, 15]. Pipette solution consisted of (in mM): 125 KCl, 4 $MgCl_2$, 0.06 $CaCl_2$, 10 HEPES, 5 K^+-EGTA, 3 Na^{2+}ATP, and 5 Na^{2+}–creatine phosphate (pH 7.2). External solution consisted of (in mM): 132 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.8 $MgCl_2$, 0.6 NaH_2PO_4, 7.5 HEPES, 7.5 Na^+-HEPES, and 5 glucose, pH 7.4.

Immunoblots. Immunoblotting of ventricular protein extracts was digitally detected as described [3]. Briefly, frozen ventricular tissues were homogenized on ice using a non-ionic detergent-based lysis buffer (25mM Tris-HCl pH 7.6, 137mM NaCl, 10% glycerol, 1% NP40 or IGEPAL CA-630, 10mM NaF) freshly supplemented with 1mM sodium pyrophosphate, 5µg/ml leupeptin, 5µg/ml aprotinin, 1mM EDTA, 10mM PMSF, and 1mM NaVO4. Proteins were separated by SDS-PAGE (4-12%) and transferred onto nitrocellulose membranes. The following antibodies were used: rabbit anti-phospho-Erk1/2, rabbit anti-phospho-Akt Thr308 (Cell Signaling Tech., Beverly, MA) and anti-GAPDH (Fitzgerald Industries International, Inc., Concord, MA). Secondary antibodies used were: goat anti-mouse Alexa Fluor 680 (Invitrogen, Carlsbad, CA) and IRDye 800 goat anti-rabbit (Rockland, Gilbertsville, PA).

D-myo-inositol 1,4,5 trisphosphate (IP3) surrogate, IP1, in adult myocytes.

Stable accumulation of IP1 in cells in the presence of LiCl is a surrogate measure of IP3 induction by $G\alpha_q$ coupled receptors, including vasopressin V1A and V1B receptors [16, 17]. IP1 measurements were performed by ELISA (IP-One-ELISA, Cisbio) according to the manufacturer's instructions. Briefly, 100,000 isolated adult cardiac myocytes were plated onto a 35mm plate and incubated in serum-free minimal essential medium containing 1.2 mM Ca^{2+}, Insulin-Transferrin-Selenium supplement (Invitrogen Corporation) and 1% penicillin-streptomycin for 2-3 hrs. Then myocytes were incubated in Stimulation Buffer (10mM Hepes, pH 7.4. 1mM CaCl2, 0.5mM MgCl2, 4.2mM KCl, 146mM NaCl, 5.5 mM glucose and 50 mM LiCl) for 1hr before stimulation with 1x Insulin-Transferrin-Selenium supplement, 0.5 uM vasopressin or 1 uM isoproterenol for 30 minutes. Myocytes were lysed in 300ul of lysis buffer and 50µl were used for IP1 measurement and quantified by optical density at 450nm.

Supplemental Results:

Effects of induced V_{1A} receptor overexpression on myocyte contraction, $[Ca^{2+}]_i$ transients and action potential

In both WT and V1A myocytes, elevating extracellular calcium ($[Ca^{2+}]_o$) resulted in the expected increase in contraction amplitudes (Sup. Fig. 2 and Sup. Table 1; $[Ca^{2+}]_o$ group effect, $P<0.05$). Maximal shortening amplitude of V1A myocytes was significantly less than that of WT myocytes at all 3 $[Ca^{2+}]_o$ examined (group effect, $P<0.05$). There was no significant group x $[Ca^{2+}]_o$ interaction effect ($P<0.3$), indicating that the magnitude and/or direction of the effects of $[Ca^{2+}]_o$ on cell shortening were similar between WT and V1A myocytes. $[Ca^{2+}]_i$ transient amplitudes (% increase in fura-2 fluorescence intensity ratio) were significantly lower in V1A myocytes when compared to WT myocytes (Sup. Table 1; group effect, $P<0.05$). As expected, raising $[Ca^{2+}]_o$ resulted in larger $[Ca^{2+}]_i$ transient amplitudes in both groups of myocytes ($[Ca^{2+}]_o$ effect; $P<0.05$). Interestingly, the group x $[Ca^{2+}]_o$ interaction effect was significant ($P<0.05$), suggesting that raising $[Ca^{2+}]_o$ resulted in larger $[Ca^{2+}]_i$ transients in WT myocytes. The $t_{1/2}$ of $[Ca^{2+}]_i$ transient decline is an estimate of SR Ca^{2+} uptake activity. Compared to WT myocytes, SR Ca^{2+} uptake appears to be slower in V1A-TG myocytes (Sup. Table 1, $P<0.05$).

Resting membrane potential and action potential duration at 50% repolarization (APD_{50}) were similar between WT and V1A myocytes (Sup. Fig. 3 and Sup. Table 2). But action potential amplitude ($P<0.037$) was significantly lower in V1A-TG myocytes. The most dramatic finding is a 3.5-fold prolongation of action potential duration at 90% repolarization (APD_{90}) in V1A myocytes ($P<0.0001$).

Supplemental Discussion:

Comparison to signaling of angiotensin II type 1 receptor

The angiotensin II type 1 receptor is another G protein-coupled cell surface receptor that mediates cardiac hypertrophy through $G\alpha_q$; however, unlike the V1A receptor, the angiotensin type 1

receptor also couples to $G\alpha_i$ thereby regulating adenylyl cyclase [18, 19]. The angiotensin type 1 receptor also activates unconventional signaling mechanisms including a $G\alpha_q/G\alpha_i$ independent and/or heterotrimeric G protein-independent signaling mechanism [20, 21]. We found that inhibition of $G\alpha_{q/11}$ alone could attenuate the development of hypertrophy, dilatation and failure in the myocardium of mice over-expressing the V1A receptor, suggesting that the myocardial effects of V1A signaling are mediated through $G\alpha_{q/11}$ alone. Our finding that V1A signaling through $G\alpha_{q/11}$ differs from signaling with other $G\alpha_q$-coupled seven transmembrane-spanning receptors supports the presence of what Liggett has referred to as "signaling diversification." That is, cells are able to respond differently to signals from a wide variety of seven-transmembrane spanning G protein-coupled receptors that couple to effectors through a relatively few G proteins.

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Supplemental Tables and Figures

Sup. Table 1

$[Ca^{2+}]_i$ transients and SR Ca^{2+} uptake in 24 Week-Old WT and V1A-TG Mice

$[Ca^{2+}]_o$	0.6mM		1.8mM		5.0mM	
	WT	**V1A-TG**	**WT**	**V1A-TG**	**WT**	**V1A-TG**
Contraction Amplitude[#]	3.18±0.28 (16)	2.64±0.2 (12)	6.56±0.68 (14)	5.18±0.42[†] (27)	9.75±0.5 (18)	7.58±0.57*[†] (14)
$[Ca^{2+}]_i$ transient amplitude (%)[#]	8.5±0.8 (16)	5.7±0.8 (12)	17.0±1.0 (26)	13.9±1.3[†] (19)	28.0±1.2 (27)	20.1±1.2*[†] (17)
$t_{1/2}$ of $[Ca^{2+}]_i$ transient decline, ms[#]	229±8 (16)	277±10[†] (17)	213±13 (26)	248±16[†] (19)	183±6 (27)	194±6 (17)

Calcium transient and contraction amplitude measurements were measured in isolated myocytes in the indicated fixed outside calcium concentrations ($[Ca^{2+}]_o$). Numbers in parentheses are myocytes pooled from 3 mice/group. Values are means ± SE. *p≤0.05, compared to WT, following adjustment for multiple comparisons. [†]Unadjusted p≤0.05, compared to WT. [#]Combined experimental group effect (0.6mM, 1.8mM and 5.0mM) p≤0.05, V1A-TG compared to WT.

Sup. Table 2

Action Potential in 24 Weeks-Old WT and V1A-TG Mice

	WT (N=15)		**V1A (n=10)**		
	median	**(25th%ile, 75%ile)**	**median**	**(25th%ile, 75%ile)**	**p-value**
Resting E_m, mV	-69.0	(-74.0, -64.0)	-71.5	(-77.0, -69.0)	0.2104
AP amplitude, mV	114.0	(101.0, 120.0)	103.0	(97.0, 109.0)	0.0368
APD_{50}, ms	4.3	(1.9, 7.5)	4.8	(3.0, 7.1)	0.5036
APD_{90}, ms	24.2	(19.1, 27.0)	77.8	(52.2, 97.3)	<0.0001

For action potential measurements, numbers in parentheses are myocytes pooled from 3-mice/treatment group. E_m, membrane potential; AP, action potential; APD_{50} and APD_{90}, action potential duration at 50 and 90% repolarization, respectively. Cells were paced at 1 Hz.

Supplemental Figure Legends

Sup. Figure A. V1A receptor transgene expression in aorta and heart. Total ventricular mRNA extracts from 24-week-old male mice were used to detect V1A receptor and GAPDH. Signals were normalized to GAPDH expression in WT. Samples were analyzed by exact measures method and shown in the table.

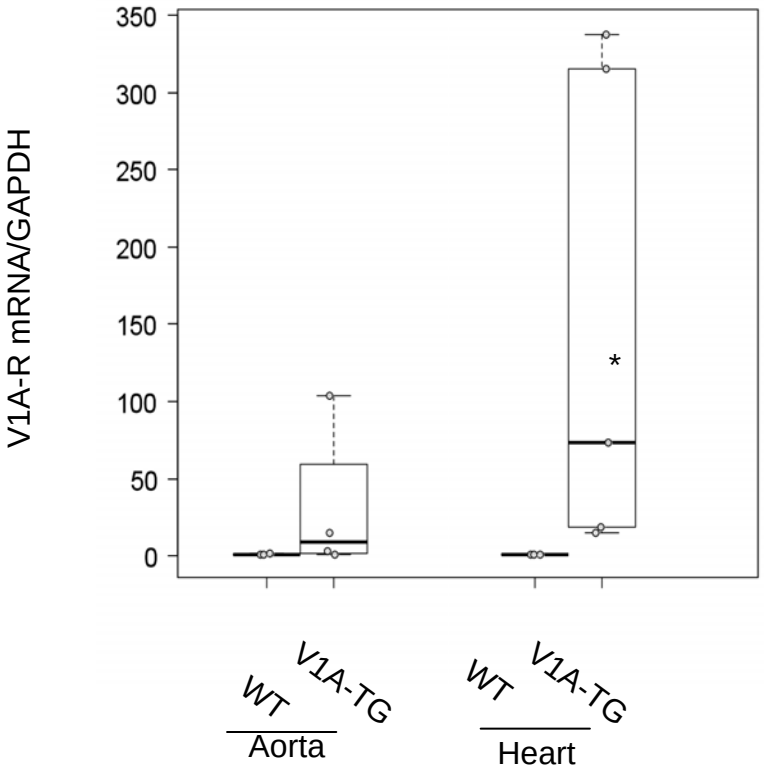
Sup. Figure B. Overexpression V1A receptor transgene altered myocyte contractility and prolongs action potential duration. (A) Myocytes from isolated from LV of WT, V1A-TG mice were paced (1Hz) to contract at 37℃ and $[Ca^{2+}]_o$ of 0.6 (top), 1.8 (middle) and 5.0 mM (bottom). Steady-state twitches are shown. Samples were analyzed by exact Wilcoxon test and shown in the table.

Sup. Figure C. Action potentials were measured in WT and V1A-TG myocytes at 3 and 1.8 mM $[Ca^{2+}]_o$ Composite data are presented in Table 3B. (3 mice/group).

Sup. Figure D. IP1 production in WT and V1A-TG myocytes. (A) Adult myocytes were isolated from wild-type FVB mouse and cultured for 2-3 hrs. Then, in the presence of 50mM LiCl, cells were acutely mock stimulated (Con) or stimulated with Insulin-Transferrin-Selenium supplement (containing 10ug/ml insulin), 1 uM isoproterenol or 0.5 uM vasopressin for 30 minutes before lysis. The graph shows IP1 expression normalized to unstimulated WT myocytes. $^{*}p<0.001$ vs control. Table of median, IQR and P values were included.

Sup. Figure E. Adult myocytes were isolated from WT and V1A-TG mice. In the presence of 50mM LiCl, myocytes were acutely stimulated with 0.5 uM vasopressin for 30 minutes before lysis. Data were pooled from experiments using age and sex matched 21-week-old wild-type FVB (WT) and V1A TG mice, 3 mice/group. 100,000 myocytes were plated onto each well for each experiment. The graph shows IP1 expression normalized to unstimulated WT myocytes. $^{*}p<0.001$ vs WT control, $^{\#}p<0.001$ vs WT-vasopressin. Table of median, IQR and P values were included.

Sup. Fig. 1


V1A-R mRNA/GAPDH
350
300
250
200
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100
50
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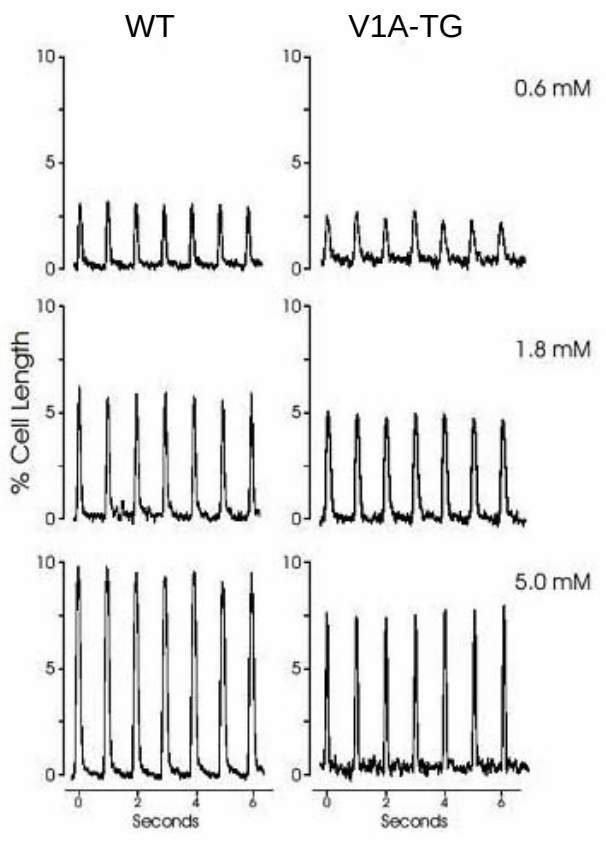
WT
V1A-TG
WT
V1A-TG
Aorta
Heart

Sup. Fig. 2

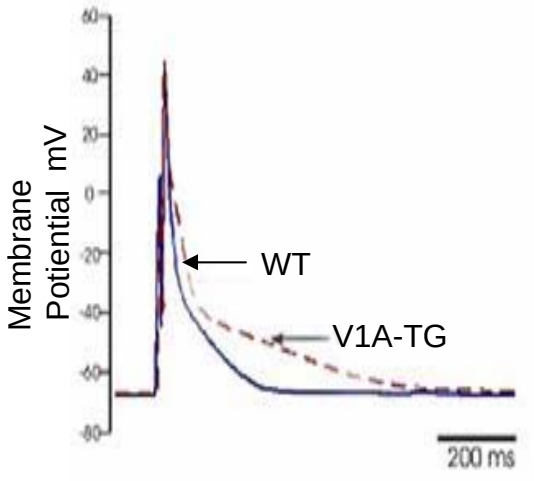


	n	Mean	SEM	p-value
WT	16	3.18	±0.28	NS
V1A	12	2.64	±0.2	

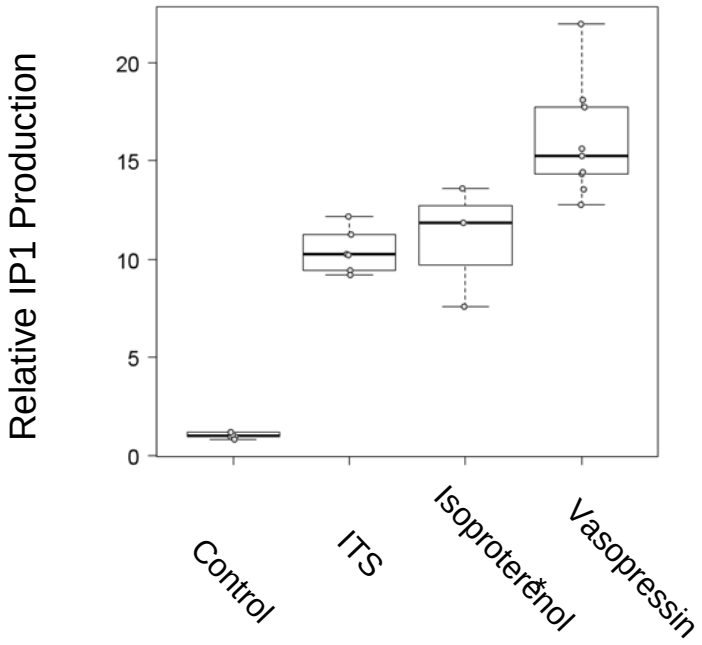
	n	Mean	SEdM	p-value
WT	14	6.56	±0.68	<0.05
V1A	27	5.18	±0.42	

	n	median	SEM	p-value
WT	18	9.75	±0.5	<0.05
V1A	14	7.58	±0.57	

Sup. Fig. 3

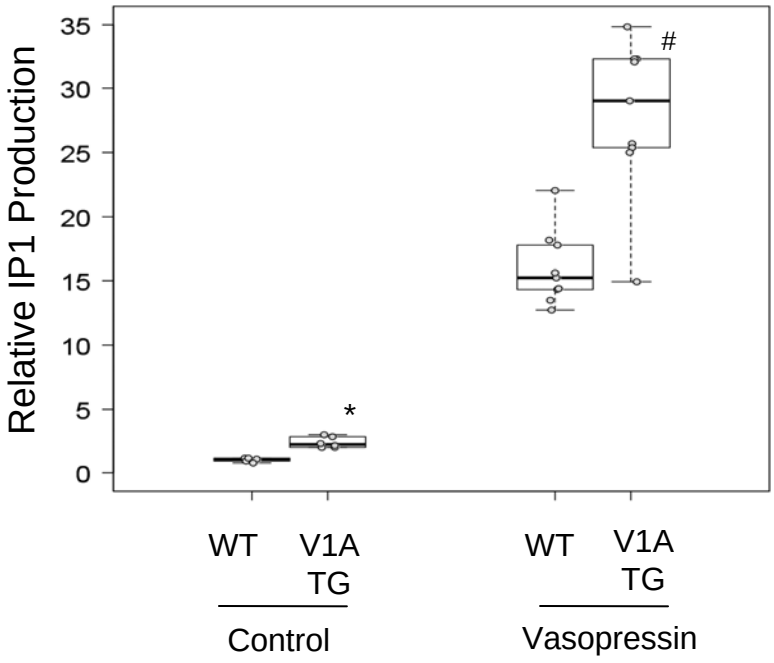


Sup. Fig. 4



	n	median	(25th%ile, 75%ile)	Control vs Vasopressin p-value
Control	6	1.01	(0.96, 1.16)	0.0004
ITS	6	10.26	(9.42, 11.24)	
Isoproterenol	3	11.84	(7.57, 13.58)	
Vasopressin	9	15.25	(14.31, 17.73)	

Sup. Fig. 5



		n	median	(25th%ile, 75%ile)	p-value
Control	WT	6	1.01	(0.96, 1.16)	0.0022
	V1A	6	2.24	(2.03, 2.82)	
Vasopressin	WT	9	15.25	(14.31, 17.73)	0.0008
	V1A	9	29.03	(25.34, 32.30)	

Exact Values for Main Text Figures

Exact Values for Fig. 1D

	n	median	(25th%ile-75th%ile)	p
WT	10	0.896	(0.490,1.000)	0.0079
V1A	8	4.468	(1.004,20.019)	

Exact Values for Fig. 2

		n	median	(25th%ile, 75%ile)	WT vs. V1Acon p-value	WT vs. V1Aind p-value
24wk	WT	8	47.47	(45.13,52.84)	<0.0001	0.1949
	V1A con	8	31.52	(29.66,32.31)		
	V1A ind	8	40.92	(37.24,52.36)		
	tTA	8	46.21	(44.33,50.59)		
35-45wk	WT	7	44.40	(39.88,52.47)	<0.0001	0.0221
	V1A con	7	28.90	(22.09,33.88)		
	V1A ind	6	35.30	(25.57,39.09)		
	tTA	7	44.00	(38.14,47.12)		

Exact Values for Fig. 3A

		n	median	(25th%ile, 75th%ile)	p-value
systolic	wt	8	87.89	(80.18,94.81)	0.4892
	v1a	8	90.6	(86.49,96.00)	
diastolic	wt	8	61.48	(49.47,68.30)	0.9796
	v1a	8	59.74	(54.12,67.37)	

Exact Values for Fig. 3B

		n	median	(25th%ile, 75th%ile)	p-value
base	wt	11	4.1	(1.77,4.89)	0.0004
	v1a	13	6.8	(5.80,12.04)	
iso	wt	11	2.11	(1.25,3.50)	0.0002
	v1a	15	8.35	(5.68,13.50)	

Exact Values for Fig. 3C

		n	median	(25th%ile, 75th%ile)	p-value
base	wt	11	7439.85	(6792.09,8035.71)	0.3766
	v1a	15	7189.29	(6377.55,7544.39)	
ISO	wt	11	13065.05	(12468.11,13488.52)	<0.0001
	v1a	15	9757.65	(7684.95,11096.94)	

Exact Values for Fig. 5B

	n	median	(25th%ile, 75%ile)	V1A vs. V1A DOX adjusted p-value	WT vs. V1A DOX adjusted p-value
WT	7	41.28	(40.43,45.11)	0.0046	0.5303
V1A	7	28.38	(27.16,35.15)		
V1A DOX	7	41.28	(40.61,46.15)		

Exact Values for Fig. 5C

	n	median	(25th%ile, 75%ile)	V1A-TG vs V1A-TGDOX adjusted p value	V1A-TG vs V1A-TGDOX adjusted p value
WT	7	3.928	(3.599,4.019)		
V1A-TG	7	5.592	(5.064,5.941)		
V1A-TG DOX	7	4.139	(3.957,4.395)	0.0012	0.0973

Exact Values for Fig. 5E

		n	median	(25%ile, 75%ile)	V1A vs WT p-value	V1A 4wk vs WT p-value	V1A 4wk vs V1A p-value
none	WT	8	46.85	(41.74,51.58)			
	V1A-TG	7	33.94	(26.64,35.15)	0.006		
DOX 4wk	WT	9	45.57	(43.70,47.69)			
	V1A-TG	8	48.86	(43.97,54.61)		0.1672	0.0006

Exact Values for Fig. 5F

		n	median	(25th%ile, 75%ile)	V1A vs WT p-value	V1A 4wk vs WT p-value	V1A 4wk vs V1A p-value
none	WT	6	3.79	(3.58,3.95)			
	V1A	6	5.41	(5.33,5.45)	0.0036		
DOX 4wk	WT	6	3.75	(3.63,4.06)			
	V1A	7	3.64	(3.63,3.91)		0.7308	0.0036

Exact Values for Fig. 6A

		n	median	(25th%ile, 75th%ile)	p
WT	none	18	1.02	(0.99,1.11)	0.0029
	insulin	7	1.47	(1.38,2.35)	
V1A	none	13	1.75	(1.25,2.44)	<0.0001
	insulin	9	5.06	(3.41,6.76)	

Exact Values for Fig. 7B

	n	median	(25th%ile, 75%ile)	Overall p-value
tTa/V1A	8	4.31	(3.19,6.75)	0.0206
tTa/V1A/GqI	9	2.65	(1.54,3.15)	

Exact Values for Fig. 7C

	n	median	(25th%ile, 75%ile)	tTaV1A vs. tTa/V1a/GqI
WT	9	44.40	(44.08,47.41)	<0.0001
tTa/V1A	7	30.18	(27.15,34.56)	
tTa/V1a/GqI	8	45.58	(40.83,48.16)	

Exact Values for Fig. 7D

	n	median	(25th%ile, 75%ile)	tTa/V1A vs. tTa/V1a/GqI
WT	8	3.814	(3.661,3.958)	<0.0001
tTa/V1A	7	5.460	(5.412,5.752)	
tTa/V1a/GqI	7	4.215	(4.053,4.305)	