Supplementary Information for

PKA facilitates relaxation of mouse ileum via phosphorylation of neuronal NO synthase

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This file includes:

1. Supplemental Table and Legend
2. Supplemental Figures and Legends

Supplemental Table 1

	WT					WT			
log(inhibitor) vs. normalized response -- Variable slope	Vehicle for kinase inhibitors	H-89	Rp-cAMPs	Myr-PKI	MK-2206	Vehicle for NO pathway inhibitors	cPTIO	ODQ	Rp-cGMPs
Replicates test for lack of fit									
SD replicates	12.84	19.35	11.66	10.9	12.7	13.93	23.01	16.8	11.49
SD lack of fit	16.31	10.8	11.14	12.56	9.425	15.93	7.674	5.905	13.05
Discrepancy (F)	1.614	0.3115	0.9128	1.329	0.5508	1.308	0.1112	0.1235	1.291
P value	0.1738	0.9043	0.484	0.2807	0.737	0.2685	0.9895	0.9867	0.2901
Evidence of wrong model?	No	No	No	No	No	No	No	No	No

				nNOS^{S1412A}			nNOS^{S1412A} + L-NAME		
log(inhibitor) vs. normalized response -- Variable slope	WT	nNOS^{S1412A}	nNOSα KO	Vehicle for kinase inhibitors	H-89	MK-2206	Vehicle for kinase inhibitors	H-89	MK-2206
Replicates test for lack of fit									
SD replicates	13.56	14.84	11.43	13.56	16.16	12.55	14.77	15.7	14.48
SD lack of fit	13.51	9.23	15.07	6.014	7.809	6.645	8.845	1.622	6.264
Discrepancy (F)	0.9916	0.3866	1.739	0.1966	0.2334	0.2803	0.3588	0.0107	0.1871
P value	0.459	0.9273	0.1279	0.9628	0.9462	0.9213	0.873	>0.9999	0.9659
Evidence of wrong model?	No	No	No	No	No	No	No	No	No

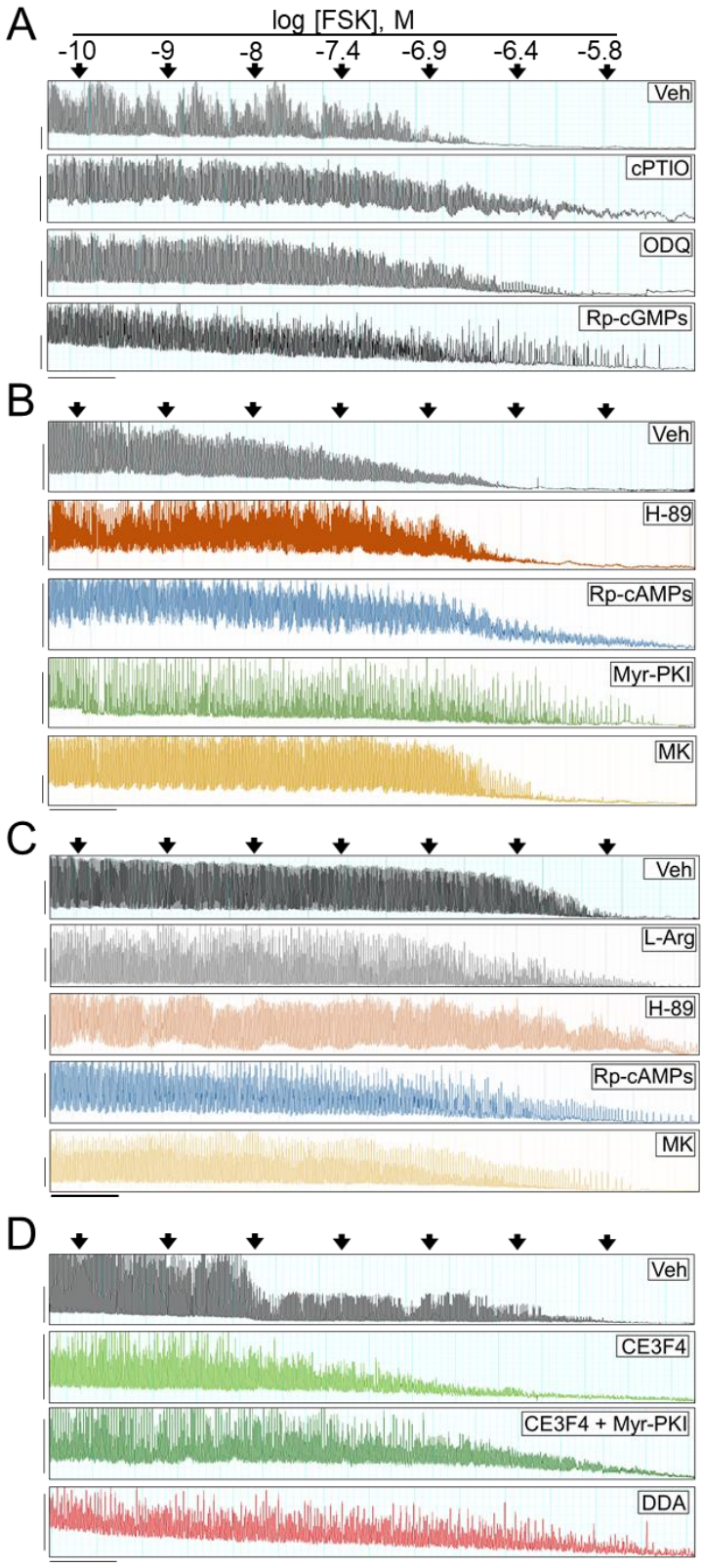
	eNOS KO						+ L-NAME		
log(inhibitor) vs. normalized response -- Variable slope	Vehicle for kinase and NOS inhibitors	H-89	MK	L-NAME	eNOS KO	eNOS KO nNOS^{S1412A}	WT	nNOS^{S1412A}	nNOSα KO
Replicates test for lack of fit									
SD replicates	11.57	16.02	15.94	13.49	10.36	11.22	15.72	13.51	15.83
SD lack of fit	8.746	7.543	7.923	10.57	8.636	10.74	14.72	9.163	14.82
Discrepancy (F)	0.5718	0.2216	0.2472	0.6138	0.6947	0.9161	0.8767	0.4604	0.8755
P value	0.7214	0.9519	0.9389	0.6897	0.6318	0.4722	0.5779	0.8828	0.5006
Evidence of wrong model?	No	No	No	No	No	No	No	No	No

	WT				WT + L-NAME				
log(inhibitor) vs. normalized response -- Variable slope	Vehicle for NOS inhibitors	NANT	1400W	TTX	Vehicle for kinase inhibitors	L-Arg	H-89	Rp-cAMPs	MK-2206
Replicates test for lack of fit									
SD replicates	10.02	19.12	15.6	17.53	12.19	10.6	15.53	16.66	15.58
SD lack of fit	10.8	10.56	12.3	14.89	8.856	13.27	10.17	10.77	7.993
Discrepancy (F)	1.162	0.3053	0.6213	0.7213	0.5279	1.568	0.4287	0.418	0.2632
P value	0.332	0.9077	0.6843	0.6088	0.7543	0.1868	0.8262	0.8335	0.9301
Evidence of wrong model?	No	No	No	No	No	No	No	No	No

	WT					nNOS^{S1412A}	L-NAME	
log(inhibitor) vs. normalized response -- Variable slope	Vehicle for EPAC inhibitors	Dideoxy-adenosine (DDA)	CE3F4	CE3F4 + Myr-PKI	Non-NANC	Non-NANC	eNOS KO nNOS^{S1412A}	WT
Replicates test for lack of fit								
SD replicates	12.75	12.57	9.332	6.55	12.07	8.271	9.964	8.96
SD lack of fit	13.93	5.142	8.326	8.984	7.193	7.093	7.314	11.2
Discrepancy (F)	1.193	0.1674	0.7961	1.881	0.355	0.7354	0.5388	1.56
P value	0.3288	0.9732	0.5555	0.1228	0.877	0.5997	0.7462	0.2
Evidence of wrong model?	No	No	No	No	No	No	No	No

Supplemental Table 1: Goodness-of-fit tests for sigmoidal regressions. A replicates test with Prism indicates that individual data points for FSK dose-dependent relaxation do not significantly deviate from regression curves.

Supplemental Figure S1



	Hill Slope (95% C.I.)	R^2
Veh	**-0.91** (-0.74, -1.16)	0.87
cPTIO	**-0.71** (-0.50, -1.02)	0.65
ODQ	**-0.89** (-0.70, -1.14)	0.81
Rp-cGMPs	**-0.89** (-0.69, -1.15)	0.90

	Hill Slope (95% C.I.)	R^2
Veh	**-0.78** (-0.61, -1.0)	0.88
H-89	**-0.80** (-0.59, -1.08)	0.75
Rp-cAMPs	**-0.77** (-0.61, -0.98)	0.89
Myr-PKI	**-0.64** (-0.53, -0.77)	0.87
MK	**-0.86** (-0.73, -1.03)	0.94

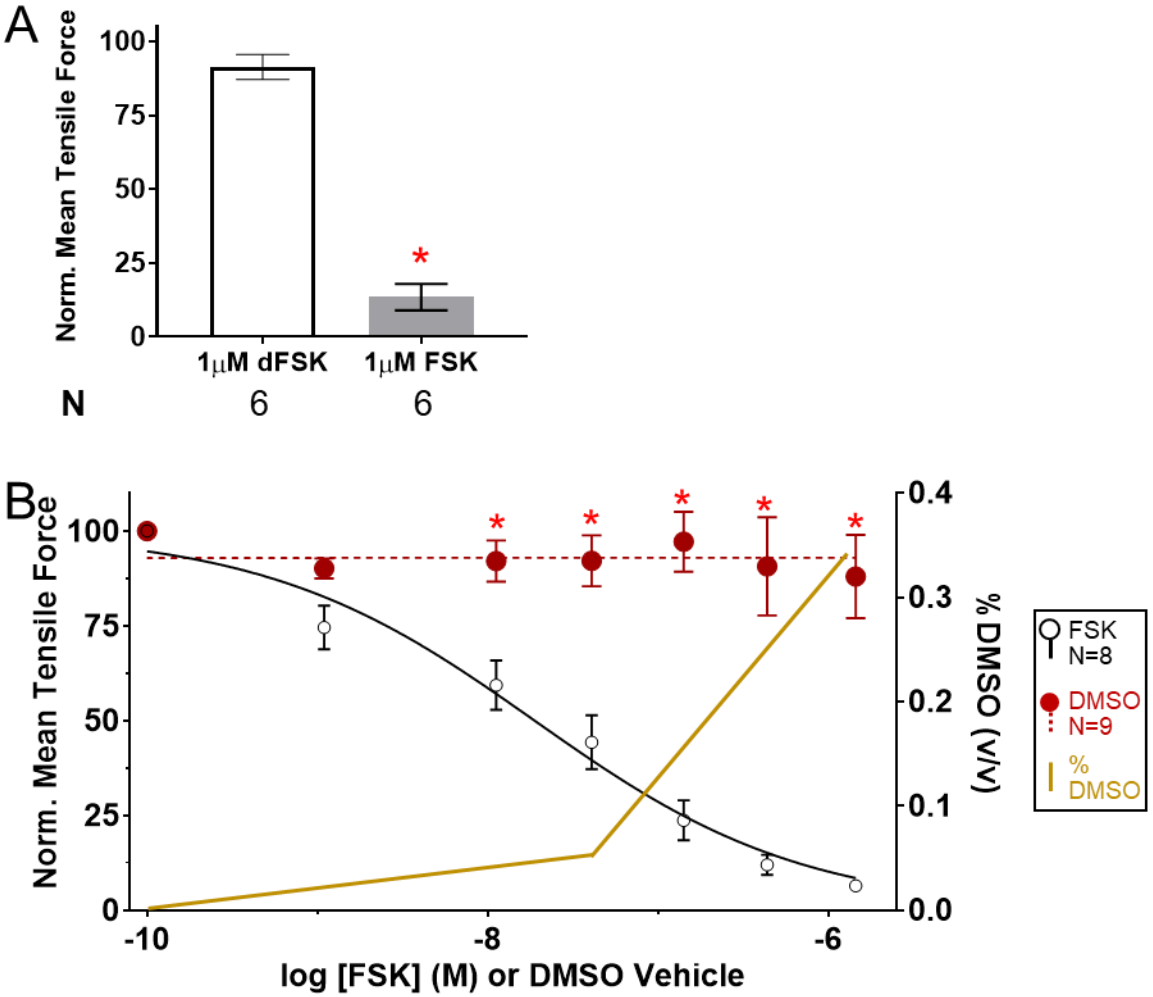
+ L-NAME

	Hill Slope (95% C.I.)	R^2
Veh	**-0.92** (-0.77, -1.11)	0.88
L-Arg	**-0.98** (-0.80, -1.2)	0.91
H-89	**-0.78** (-0.59, -1.03)	0.78
Rp-cAMPs	**-0.88** (-0.63, -1.24)	0.79
MK	**-1.13** (-0.82, -1.60)	0.84

	Hill Slope (95% C.I.)	R^2
Veh	**-0.55** (-0.45, -0.68)	0.86
CE3F4	**-0.82** (-0.72, -0.93)	0.92
CE3F4 + Myr-PKI	**-0.70** (-0.61, -0.81)	0.94
DDA	**-0.56** (-0.44, -0.72)	0.81

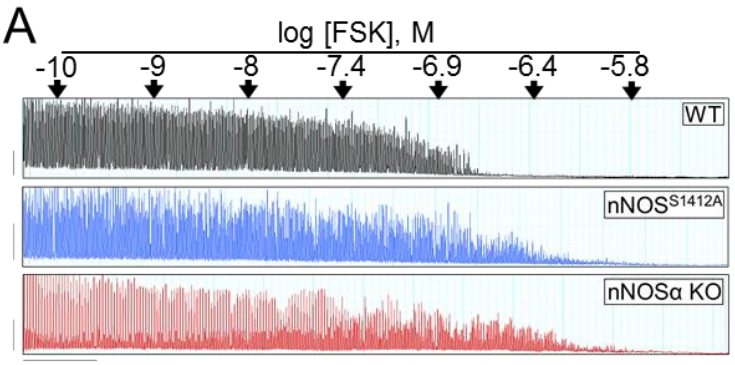
Supplemental Figure S1: PKA and Akt facilitate nitrergic FSK relaxation of ileum. *Left*: Representative force-time plots. *Right*: Summary regression statistics. Hill coefficients are bold, and 95% confidence intervals are in parentheses. A. cPTIO, ODQ and Rp-cGMPs (NO signaling inhibitors) decrease sensitivity to FSK-dependent relaxation. B. H-89, Myr-PKI, and Rp-cAMPs (PKA inhibitors) and MK-2206 (Akt inhibitor) decrease sensitivity to FSK-dependent relaxation. C. Under NOS blockade with L-NAME, Akt and PKA inhibitors do not affect FSK-dependent ileal relaxation. L-Arg (NOS substrate) partially rescues L-NAME attenuation of FSK relaxation. D. EPAC and PKA inhibition additively attenuate FSK relaxation, and adenylate cyclase inhibition attenuates relaxation as much as combined PKA and EPAC inhibition. Scale bars: 0.08g (A-C) and 0.12g (D) x 120 sec.

Supplemental Figure S2

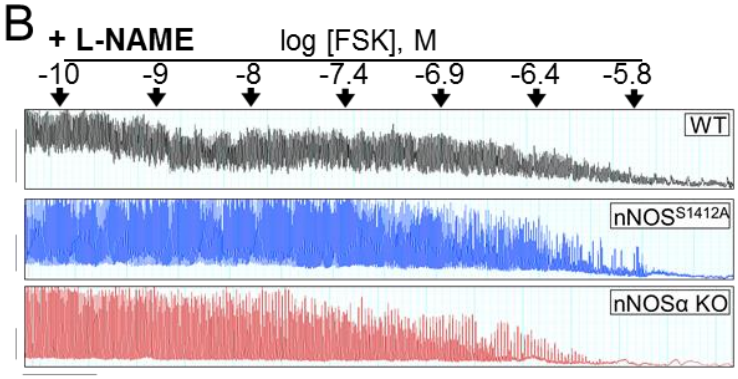


Supplemental Figure S2: FSK relaxes WT ileal rings. A. WT ileal rings relax when treated with 1μM FSK, but not with 1μM 1,9-dideoxyforskolin (dFSK). B. Cumulative FSK relaxes WT ileal rings, but cumulative DMSO vehicle does not. N: ileal rings. *: $p<0.05$ vs dFSK (A) or vs. DMSO vehicle at each [FSK] or [DMSO] (B) by Mann-Whitney tests.

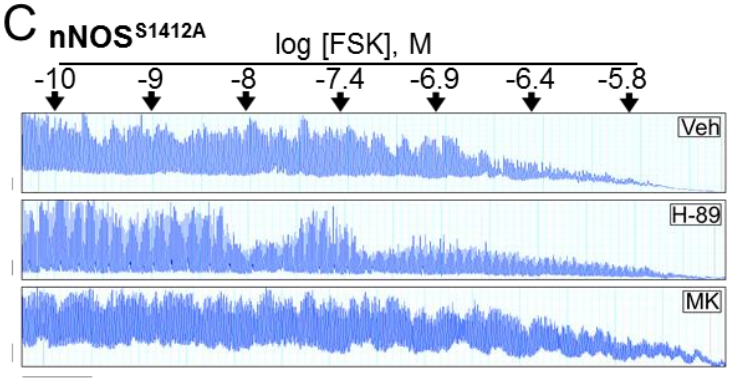
Supplemental Figure S3



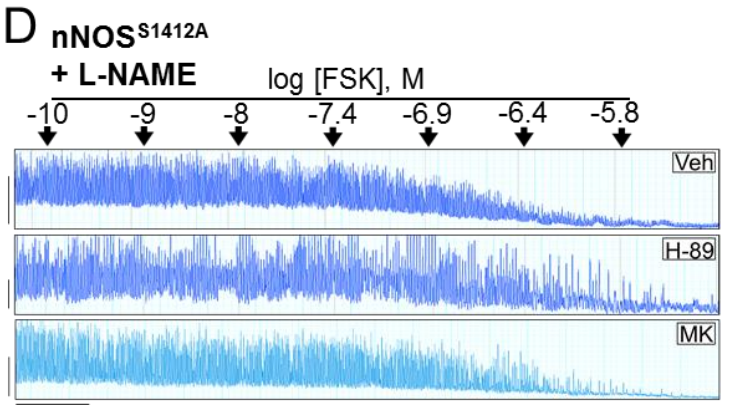
	Hill Slope (95% C.I.)	R^2
WT	**-0.82** (-0.75, -0.91)	0.88
nNOS^{S1412A}	**-0.85** (-0.76, -0.96)	0.85
nNOSα KO	**-0.78** (-0.70, -0.87)	0.89



	Hill Slope (95% C.I.)	R^2
WT	**-0.81** (-0.74, -0.90)	0.79
nNOS^{S1412A}	**-0.90** (-0.79, -1.04)	0.84
nNOSα KO	**-0.79** (-0.59, -0.90)	0.79



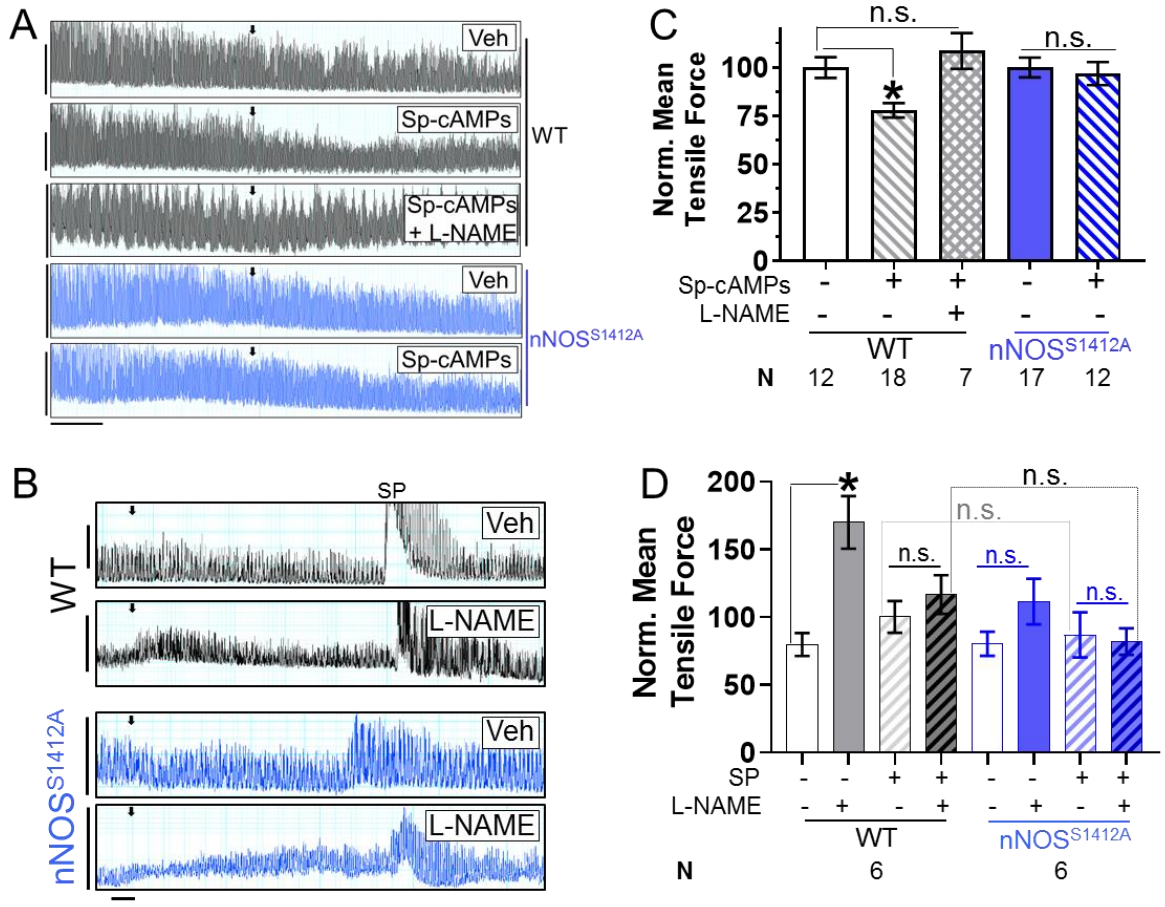
	Hill Slope (95% C.I.)	R^2
Veh	**-0.85** (-0.70, -1.04)	0.87
H-89	**-0.78** (-0.61, -1.00)	0.82
MK	**-1.02** (-0.81, -1.31)	0.89



	Hill Slope (95% C.I.)	R^2
Veh	**-0.75** (-0.55, -1.01)	0.82
H-89	**-0.95** (-0.73, -1.25)	0.83
MK	**-0.96** (-0.75, -1.25)	0.86

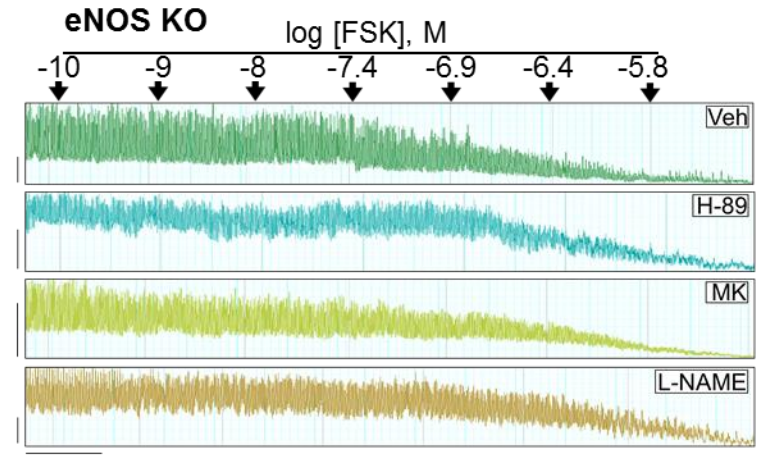
Supplemental Figure S3: **The nNOS^{S1412A} and nNOSα KO mutations partially block nitrergic FSK relaxation of ileum**. *Left*: Representative force-time plots. *Right*: Summary regression statistics. Hill coefficients are bold, and 95% confidence intervals are in parentheses. A. FSK relaxation is reduced for nNOS^{S1412A} and nNOSα KO ileal rings compared with WT ileal rings. B. In the presence of the NOS inhibitor L-NAME, WT, nNOS^{S1412A}, and nNOSα KO ilea are equally sensitive to FSK relaxation. C. FSK relaxation of nNOS^{S1412A} ilea is sensitive to MK (Akt inhibitor), but not to H-89 (PKA inhibitor). C. Under NOS blockade with L-NAME, Akt and PKA inhibitors do not affect FSK-dependent relaxation of nNOS^{S1412A} ilea. Scale bars: 0.15g x 120 sec.

Supplemental Figure S4

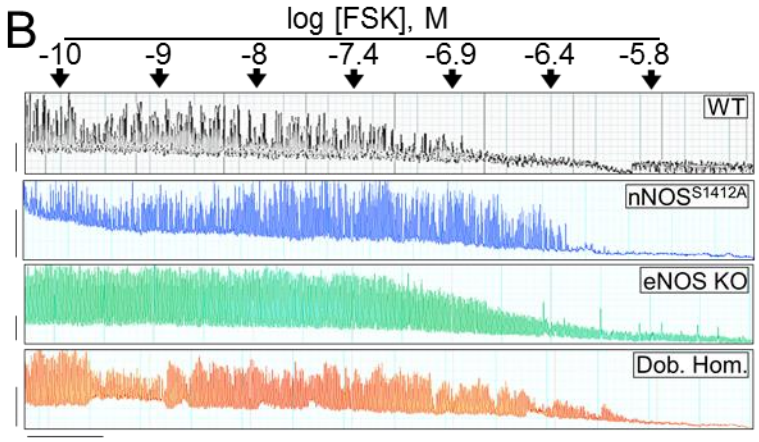


Supplemental Figure S4: NO synthesis and nNOS Serine-1412 facilitate ileal relaxation. A. L-NAME (1mM) and the nNOS^{S1412A} mutation curtail ileal relaxation induced by Sp-cAMPs (25µM). B. The nNOS^{S1412A} mutation attenuates L-NAME enhancement of basal ileal tone. Subsequent substance P (SP) treatment abolishes differences in tensile force caused by L-NAME. C, D. Quantification of A, B. *: $p<0.05$ by Dunn's post tests after Kruskal-Wallis. n.s.: not significant. Veh: water. Scale bars: 0.13g x 120 sec (A) and 0.15g x 60 sec (B).

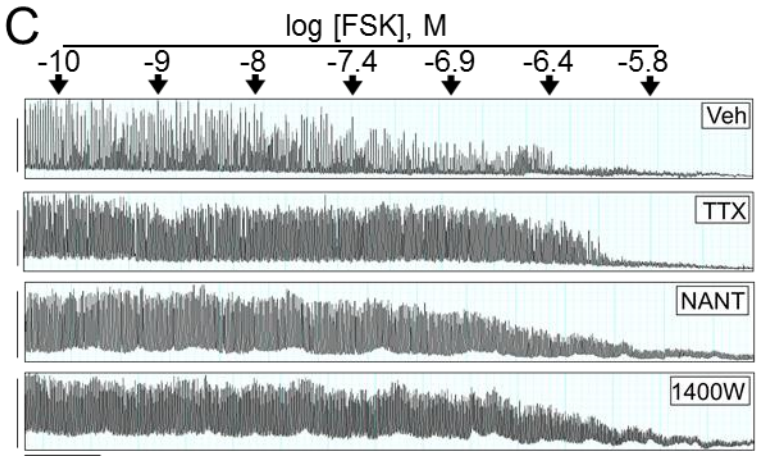
Supplemental Figure S5



	Hill Slope (95% C.I.)	R^2
Veh	**-0.84** (-0.59, -0.98)	0.89
H-89	**-0.74** (-0.59, -0.94)	0.89
MK	**-0.65** (-0.51, -0.83)	0.76
L-NAME	**-0.81** (-0.64, -1.04)	0.79



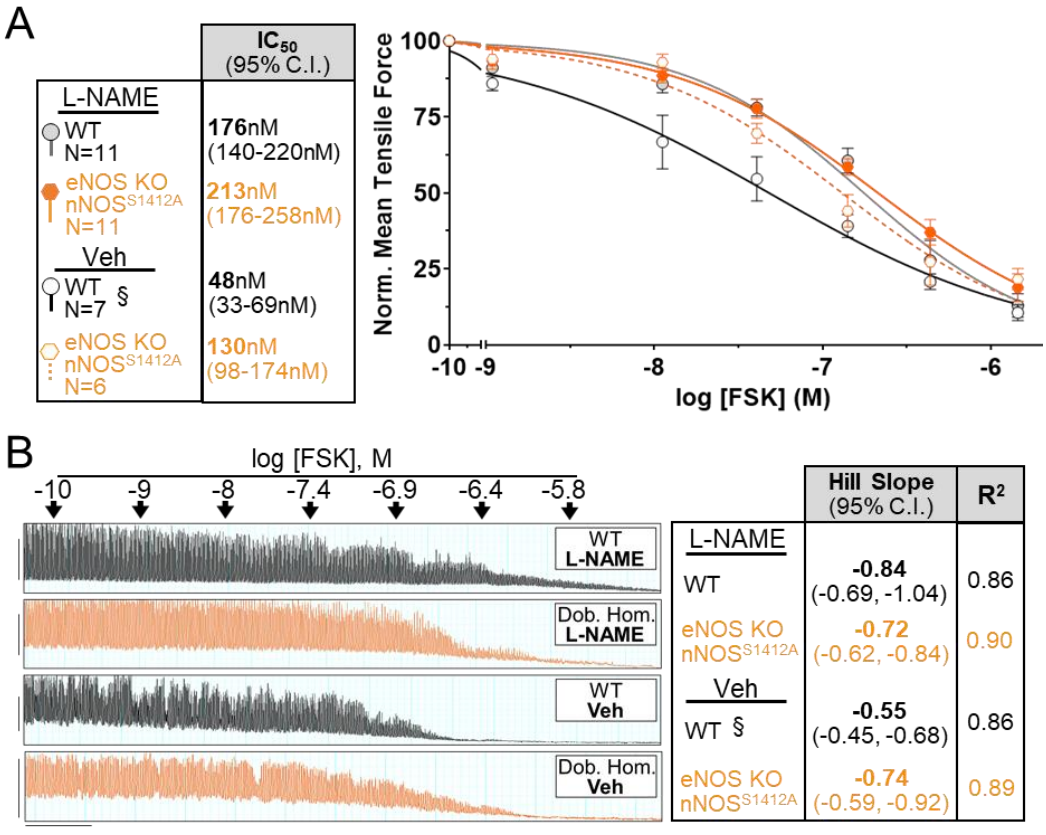
	Hill Slope (95% C.I.)	R^2
WT	**-0.82** (-0.75, -0.91)	0.88
nNOS^{S1412A}	**-0.85** (-0.76, -0.96)	0.85
eNOS KO	**-0.71** (-0.57, -0.90)	0.90
Dob. Hom.	**-0.88** (-0.78, -0.98)	0.90



	Hill Slope (95% C.I.)	R^2
Veh	**-0.89** (-0.76, -1.05)	0.93
TTX	**-0.90** (-0.73, -1.12)	0.79
NANT	**-0.76** (-0.55, -1.06)	0.74
1400W	**-0.63** (-0.48, -0.83)	0.80

Supplemental Figure S5: nNOS facilitates most nitrergic FSK relaxation. *Left*: Representative force-time plots. *Right*: Summary regression statistics. Hill coefficients are bold, and 95% confidence intervals are in parentheses. A. L-NAME (NOS inhibitor) and H-89 (PKA inhibitor) reduce eNOS KO ileal sensitivity to FSK relaxation, but MK (Akt inhibitor) has no effect on eNOS KO relaxation. B. Ilea from eNOS KO nNOS^{S1412A} double homozygotes (Dob. Hom.) are as sensitive to FSK relaxation as eNOS KO and nNOS^{S1412A} single mutants. C. TTX (neuronal depolarization inhibitor), NANT (specific nNOS inhibitor), and 1400W (selective iNOS/nNOS inhibitor) attenuate FSK relaxation of WT ileal rings. Scale bars: 0.08g (A-B) and 0.18g (C) x 120 sec.

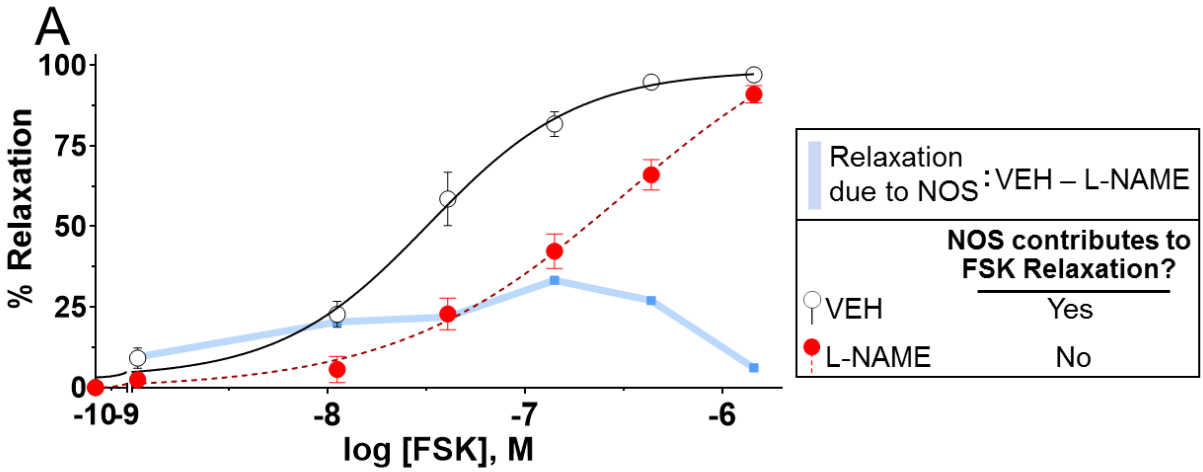
Supplemental Figure S6



Supplemental Figure S6: WT and eNOS KO nNOS^{S1412A} ilea exhibit similar non-nitrergic FSK relaxation. A. FSK IC_{50} values are the same for WT and eNOS KO and nNOS^{S1412A} double mutants treated with the NOS inhibitor L-NAME. B. Representative force-time plots and summary regression statistics. §: Dataset from Fig. S1D for comparison. Scale bars: 0.12g x 120 sec.

Supplemental Figure S7

A



B

% Relaxation

	WT				eNOS KO		nNOS^{S1412A}		
Log [FSK], M	VEH	L-NAME	NANT	H-89	VEH	L-NAME	VEH	L-NAME	H-89
-8.96	12.0	2.5	8.8	7.4	4.9	4.2	5.3	2.9	6.1
-7.95	26.0	5.6	15.6	13.5	12.3	4.9	13.9	6.1	16.0
-7.39	44.7	22.8	26.0	29.6	25.2	17.0	31.4	20.3	36.2
-6.85	75.6	42.3	49.1	59.8	52.3	24.0	60.0	45.5	54.6
-6.36	93.0	66.0	73.0	72.4	75.7	48.3	78.6	67.8	77.4
-5.84	97.0	90.9	87.0	90.4	86.5	73.8	90.2	88.6	92.5

C

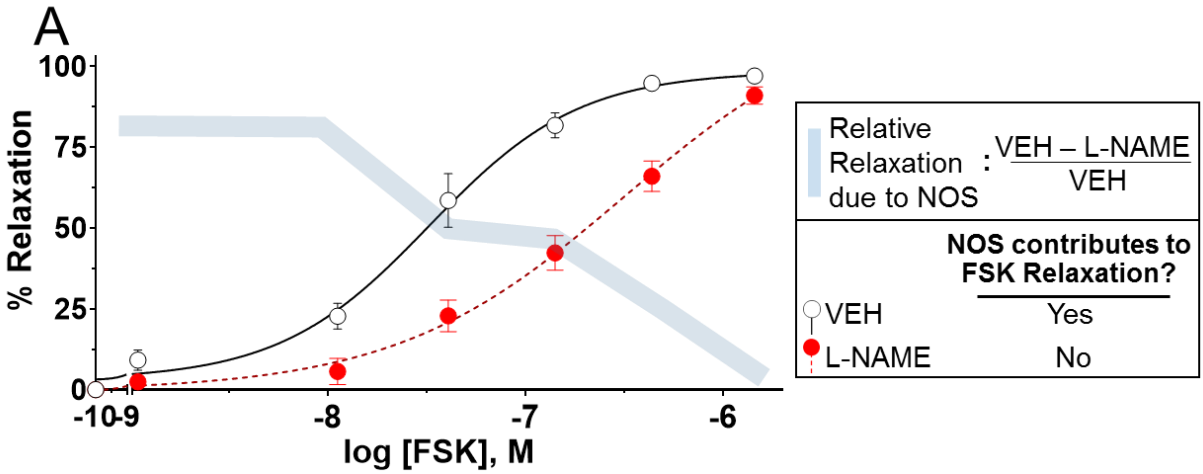
% Relaxation Due to NOS, nNOS, and PKA

	WT			eNOS KO	nNOS^{S1412A}	
Log [FSK], M	NOS VEH – L-NAME	nNOS VEH – NANT	PKA VEH – H-89	NOS VEH – L-NAME	NOS VEH – L-NAME	PKA VEH – H-89
-8.96	9.5	3.2	4.6	0.7	2.4	0.0
-7.95	20.4	10.4	12.5	7.4	7.8	0.0
-7.39	21.9	18.7	15.1	8.2	11.1	0.0
-6.85	33.3	26.5	15.8	28.3	14.5	5.4
-6.36	27.0	20.0	20.6	27.4	10.8	1.2
-5.84	6.1	10.0	6.6	12.7	1.6	0.0

Supplemental Figure S7: Contributions of NO synthesis and PKA to absolute (total) ileal relaxation. A. Graphical representation of percent relaxation due to NO synthesis: the absolute difference in percent relaxation with vehicle and L-NAME at each [FSK]. B. Percent relaxation for WT, eNOS KO, and $nNOS^{S1412A}$ ileal rings treated with vehicle, L-NAME (NO synthesis inhibitor), NANT (nNOS inhibitor), or H-89 (PKA inhibitor). The first two columns (WT treated with vehicle or L-NAME) are depicted in A. C. Total percent relaxation of WT, eNOS KO, or $nNOS^{S1412A}$ ileal rings due to NO synthesis, nNOS, or PKA. The first column (WT relaxation due to NO synthesis) is depicted in A. The first row of each column includes the formula to calculate percent relaxation.

Supplemental Figure S8

A



B

% Relaxation

	WT				eNOS KO		nNOS^{S1412A}		
Log [FSK], M	VEH	L-NAME	NANT	H-89	VEH	L-NAME	VEH	L-NAME	H-89
-8.96	12.0	2.5	8.8	7.4	4.9	4.2	5.3	2.9	6.1
-7.95	26.0	5.6	15.6	13.5	12.3	4.9	13.9	6.1	16.0
-7.39	44.7	22.8	26.0	29.6	25.2	17.0	31.4	20.3	36.2
-6.85	75.6	42.3	49.1	59.8	52.3	24.0	60.0	45.5	54.6
-6.36	93.0	66.0	73.0	72.4	75.7	48.3	78.6	67.8	77.4
-5.84	97.0	90.9	87.0	90.4	86.5	73.8	90.2	88.6	92.5

C

Relative Relaxation Due to NOS, nNOS, and PKA

	WT			eNOS KO	nNOS^{S1412A}	
Log [FSK], M	NOS (VEH – L-NAME)/VEH	nNOS (VEH – NANT)/VEH	PKA (VEH – H-89)/VEH	NOS (VEH – L-NAME)/VEH	NOS (VEH – L-NAME)/VEH	PKA (VEH – H-89)/VEH
-8.96	79.2	50.0	38.3	14.3	45.3	0.0
-7.95	78.5	36.2	48.1	60.2	56.1	0.0
-7.39	49.0	12.5	33.8	32.5	35.4	0.0
-6.85	44.0	41.3	20.9	54.1	24.2	9.0
-6.36	29.0	25.4	22.2	36.2	13.7	1.5
-5.84	6.3	11.8	6.8	14.7	1.8	0.0

Supplemental Figure S8: Relative ileal relaxation by FSK due to NO synthesis or PKA. This method calculates the proportion of relaxation at a particular [FSK] due to NO synthesis or PKA, relative to control relaxation at the same [FSK]. Thus, nitrergic relaxation is the difference in percent relaxation with vehicle and L-NAME, divided by percent relaxation with vehicle, at each [FSK]. A. Graphical depiction. B. Percent relaxation for WT, eNOS KO, and nNOS^{S1412A} ileal rings treated with vehicle, L-NAME, NANT, or H-89 during FSK relaxation. C. Relative percent relaxation of WT, eNOS KO, or nNOS^{S1412A} ileal rings due to NO synthesis, nNOS, or PKA. The first row of each column includes the formula used to calculate percent relaxation values for that column.