SUPPLEMENTARY INFORMATION

Adaptive remodeling of rat adrenomedullary stimulus-secretion coupling in a chronic hypertensive environment

Vincent Paillé, Joohee Park, Bertrand Toutain, Jennifer Bourreau, Pierre Fontanaud, Frédéric De Nardi, Claudie Gabillard-Lefort, Dimitri Bréard, David Guilet, Daniel Henrion, Christian Legros and Nathalie C. Guérineau

1- Supplementary Methods

Determination of $Na_V1.3$ and $Na_V1.7$ expression by western blot. Adrenal glands from WKY rats (n = 6) and SHRs (n = 6) were macrodissected to isolate the medulla. The adrenomedullary tissues were crushed using a pestle in frozen state and lysed in the extraction buffer (10 mM Tris-HCl, 5 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail (Thermo Fisher Scientific). After homogenization for 30 min at 4°C, the samples were centrifuged at 2,000 g at 8°C for 5 min. Protein quantification were achieved using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific). 15 µg of proteins were separated by SDS-PAGE (8% polyacrylamide gel) and transferred to a PVDF membrane. To block the non-specific binding sites, the membranes were placed in Tris-buffered saline solution containing 0.1% Tween and 5% BSA for 90 min at room temperature. Next, the membranes were incubated with the mouse anti-$Na_V1.3$ (1:500, Monoclonal Anti-SCN3A antibody, Sigma-Aldrich Merck) or the mouse anti-$Na_V1.7$ (1:1,000, N68/6, NeuroMab) primary antibodies over the weekend at 4°C. The mouse anti-HSC70 (heat shock 70 kDa protein 8) primary antibodies (1:10,000, A3854, SC-7298, Santa Cruz Biotechnology) were used as a loading control. After washing, the membranes were incubated with the anti-mouse secondary antibodies conjugated to horseradish peroxidase (1:5,000, Thermo Fisher Scientific) for 90 min at room temperature. The membranes were placed in SuperSignal West Femto Maximum Sensitivity Substrate solution (Thermo Fisher Scientific) for 5 min in the dark. Then the proteins were revealed by LAS-3000 imager (Fuji, Tokyo, Japan). The image acquisition was performed with Image Labs (Bio- Rad, California, USA).

2- Supplementary Tables

Table S1: Passive membrane properties of WKY and SHR chromaffin cells. Cells were recorded in the whole-cell configuration of the patch-clamp technique. The number of recorded cells is indicated in parentheses.

	WKY	**SHR**	
Passive membrane properties	Mean ± SD	Mean ± SD	p value (unpaired t test)
Resting membrane potential, mV	-64.8 ± 6.4 (67)	-64.0 ± 7.4 (66)	0.4951, ns
Input resistance, GΩ	1.19 ± 0.36 (68)	1.53 ± 0.42 (66)	<0.0001, ****
Membrane capacitance, pF	8.94 ± 0.67 (104)	8.74 ± 0.31 (96)	0.009, **

Table S2: Action potential properties of WKY and SHR chromaffin cells. Depolarization-triggered and spontaneous action potentials were recorded in cells current-clamped at their resting potential. Evoked action potentials were triggered by a depolarizing step (+30 pA above the rheobase and 500 ms duration). For spontaneous APs, the analysis was performed in 30-50 serial APs/cell. All parameters were analyzed by Mini Analysis. The number of recorded cells is indicated in parentheses. *, p<0.05; **, p<0.01; ***, p<0.005, ****, p<0.001.

	WKY (n = 25 cells)	**SHR** (n = 23 cells)	
PROPERTIES OF EVOKED APs	mean ± SD	mean ± SD	p value unpaired t test
rise time (ms)	8.25 ± 0.17	8.42 ± 0.25	0.0086**
half-rise time	1.18 ± 0.24	1.74 ± 0.66	0.0003***
10%-90% rise time	3.41 ± 0.54	4.09 ± 0.83	0.0016**
10%-90% slope (mV/ms)	16.98 ± 4.67	13.44 ± 5.28	0.0175*
amplitude (mv)	70.03 ± 7.38	62.56 ± 8.48	0.0021**
half width (ms)	3.86 ± 1.01	5.73 ± 1.95	0.001****
decay time (ms)	5.18 ± 1.74	7.99 ± 2.64	<0.001****

	WKY (n = 10 cells)	**SHR** (n = 8 cells)	
PROPERTIES OF SPONTANEOUS APs	mean ± SEM	mean ± SEM	p value Mann-Whitney test
rise time (ms)	7.80 ± 0.32	8.23 ± 0.23	0.0085**
half-rise time	0.64 ± 0.10	0.80 ± 0.10	0.0062**
10%-90% rise time	1.80 ± 0.33	2.50 ± 0.55	0.0014**
10%-90% slope (mV/ms)	41.95 ± 9.86	27.41 ± 6.61	0.0044**
amplitude (mv)	89.51 ± 6.53	79.98 ± 7.78	0.0124*
half width (ms)	3.32 ± 0.67	5.41 ± 2.16	0.0008***
decay time (ms)	6.00 ± 2.30	10.37 ± 6.60	0.0085**

Table S3: Primer sequences used for real-time RT-PCR

gene name	GenBank accession number	protein name	forward primer (5'-3')	reverse primer (5'-3')
Scn1a	NM_030875	$Na_V1.1$	gttccgacatcgccagtt	catctcagtttcagtagttgttcca
Scn2a	NM_012647	$Na_V1.2$	tggtgtccctggttggag	ccttatttctgtctcagtagttgtgc
Scn3a	NM_013119	$Na_V1.3$	gcaccgtccattctaaccat	tttagcttcttgcataagaattgc
Scn4a	NM_013178	$Na_V1.4$	ggcactgtctcgatttgagg	ttcatgatggaggggatagc
Scn5a	NM_013125	$Na_V1.5$	tgccaccaatgccttgta	catgatgagcatgctaaagagc
Scn8a	NM_019266	$Na_V1.6$	ggaagttttccatcatgaatcag	gctgttatgtcgggagagga
Scn9a	NM_133289	$Na_V1.7$	cagcagatgttagaccgactca	actcgtgaactcagcagcag
Scn1b	NM_017288	Na_V1b	gcggagatggtgtactgctac	tcggaagtaatggccaggtat
Scn2b	NM_012877	Na_V2b	tggacttaccaggagtgtagca	ccagcttcaggttgatgatct
Scn3b	NM_139097	Na_V3b	ctgataccttgcgagtcactg	catcatgatttccgagaccac
Scn4b	NM_001008880	$Na_{VV}4b$	catcctgaagaagaccagagaga	agcagggcctcacactttt
Cacna1c	NM_012517	$Ca_V1.2$	tggctcacagaagtgcaaga	agcatttctgccgtgaaaag
Cacna1d	NM_017298	$Ca_V1.3$	ccatgctcactgtgttccag	ctcccatcctatcgcatcat
Cacna1a	NM_012918	$Ca_V2.1$	ctgctttgaagaggggacag	ctgacatttggtcccgttg
Cacna1b	NM_147141	$Ca_V2.2$	taagcgcatcaccgaatg	ggccaggacaatacagttgg
Cacna1e	NM_019294	$Ca_V2.3$	taccgcgcctggatagac	gctgatgttcccgagttttt
Cacna1g	NM_031601	$Ca_V3.1$	catctacttcattcttctcatcatcg	aactgcgtggcaatcacc
Cacna1h	NM_153814	$Ca_V3.2$	acggatactctgcagacagga	gctctgtgtagtctgggatgc
Cacna1i	NM_020084	$Ca_V3.3$	tctgcctcaatgttgtcacc	aagggctgtctctagggatgt
Kcnma1	NM_031828	KCa1.1	aagtaattccatcaagctggtga	ggtcccctgaattctccac
Kcnn1	NM_019313	KCa2.1	tcggaaacaccagcgtaagt	cttcacagtccggagcttct
Kcnn2	NM_019314	KCa2.2	tgcttgcttaccggaataatg	tagcttccttgccactacgg
Kcnn3	NM_019315	KCa2.3	cacgccaaagtcaggaaac	ccatcttgacacccctcagt
Kcnn4	NM_023021	KCa3.1	gcaagattgtctgcttgtgc	gccaccacagccaatagtaga
Kcnt1	NM_021853	KCa4.1	ggtcaagaaccgaatgaagc	tggctgttaaatttgctacgtc

Kcnt2	NM_198762	KCa4.2	ccagcaaatatgagattcatgc	acctcgttctcgctctttctt
Chrna3	NM_052805	α3nAChR	tccatgctgatgctggtg	tcttcgaacaggtactggaaca
Chrna4	NM_024354	α4nAChR	ccagtacattgcagaccacct	gccacgtatttccagtcctc
Chrna5	NM_017078	α5nAChR	cacgtcgtgaaagagaacga	ccacaaaaacatccgatcaa
Chrna7	NM_012832	α7nAChR	ggcaaaatgcctaagtggac	cttcatgcgcagaaaccat
Chrnb2	NM_019297	ß2nAChR	caactcaatggcgctgttc	cactagccgctcctctgtgt
Chrnb4	NM_052806	ß4nAChR	tcctctcagctcatctccatc	tgatctgttctcgctcattca
Gjd2	NM_019281	Cx36	cggtactgcccagtctttgt	caccaccacagtcaacagga
Gja1	NM_012567	Cx43	cccgacgacaaccagaatg	tggctaatggctggagttc
Tjp1	NM_001106266	ZO-1	tcaacacatgaagatgggattc	cgcaaacccacactatctcc
Gusb	NM_017015	Gus	ctctggtggccttacctgat	cagactcaggtgttgtcatcg
Hprt	NM_012583	Hprt	gaccggttctgtcatgtcg	acctggttcatcatcactaatcac
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3- Supplementary figures

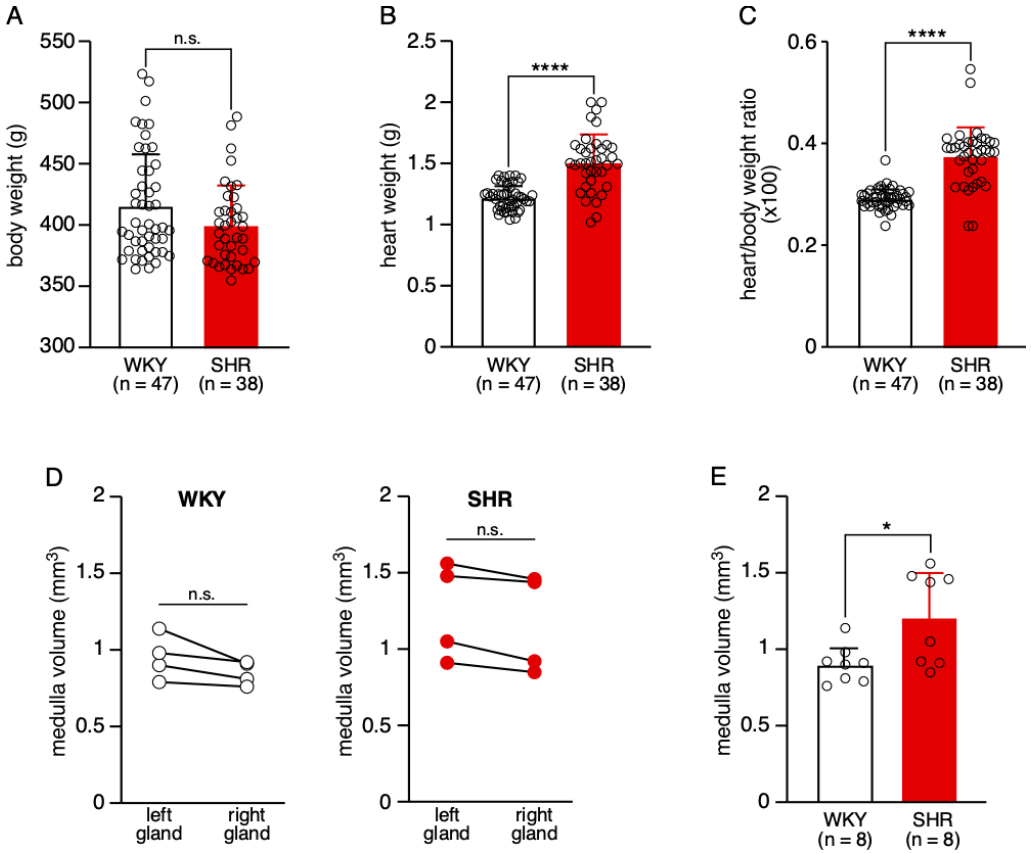

FIGURE S1

Figure S1: Validation of phenotypic traits of SHRs compared to WKY rats. A. No overt difference in body weight in the two rat strains (401.4 ± 33.3 g, n = 38) and WKY rats (417.2 ± 43.6 g, n = 47, p = 0.0689, unpaired t test). **B.** Significant increase in heart weight in hypertensive animals compared to normotensive animals (1.51 ± 0.23 g, n = 38 *versus* 1.22 ± 0.10 g, n = 47 in WKY rats, p<0.0001, unpaired t test). **C.** Enhanced heart/body weight ratio in SHRs (0.38 ± 0.06, n = 38 *versus* 0.29 ± 0.02, n = 47 in WKY rats, p<0.0001, unpaired t test). **D-E.** Comparison of the adrenal medulla volume between WKY rats and SHRs. The medulla volume was calculated from acute slices (see Material and Methods). **D.** No difference between the left and the right adrenals (0.85 ± 0.08 mm^3, n = 4 and 0.95 ± 0.15 mm^3, n = 4 for the right and left WKY glands, respectively, p = 0.125, and 1.17 ± 0.33 mm^3, n = 4 and 1.25 ± 0.32 mm^3, n = 4 for the right and left SHR glands, respectively, p = 0.125, Wilcoxon matched-pairs signed-rank test) **E.** Significantly greater medulla volume in SHRs *versus* WKY rats. (1.21 ± 0.30 mm^3, n = 8 glands for SHRs *versus* 0.90 ± 0.12 mm^3, n = 8 glands for WKY rats, p = 0.0351, Mann-Whitney test). For each rat, the volume represents the average volume of the two glands. Values and error bars represent mean and SD. *, p<0.05 and ****, p<0.0001.

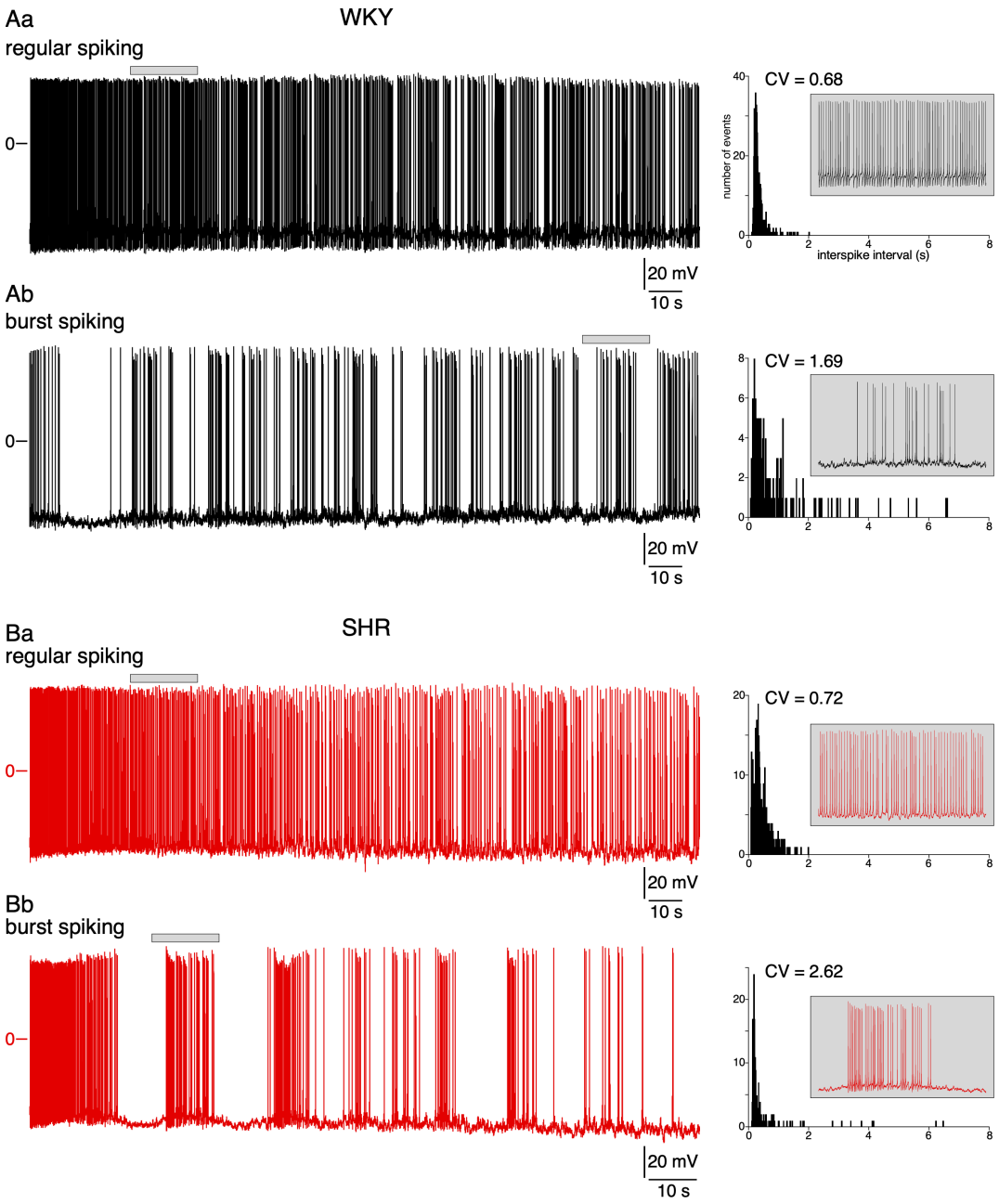

FIGURE S2

Figure S2: Whole-cell recordings of spontaneously spiking chromaffin cells in adrenal acute slices: occurrence of both regular and burst firing. Action potentials were recorded at resting membrane potential (no current injection), in normotensive (**A**) and hypertensive (**B**) rats. **A.** Representative chart recordings of two cells exhibiting a regular firing (**Aa**) or a burst firing (**Ab**). The histograms on the right illustrate the distribution of the inter-spike intervals (10 ms bin), from which the coefficients of variation (CV) were calculated. Insets: expanded time scale illustrating a 20-s spiking period. **B.** Same recording conditions and analysis performed in two SHR chromaffin cells.

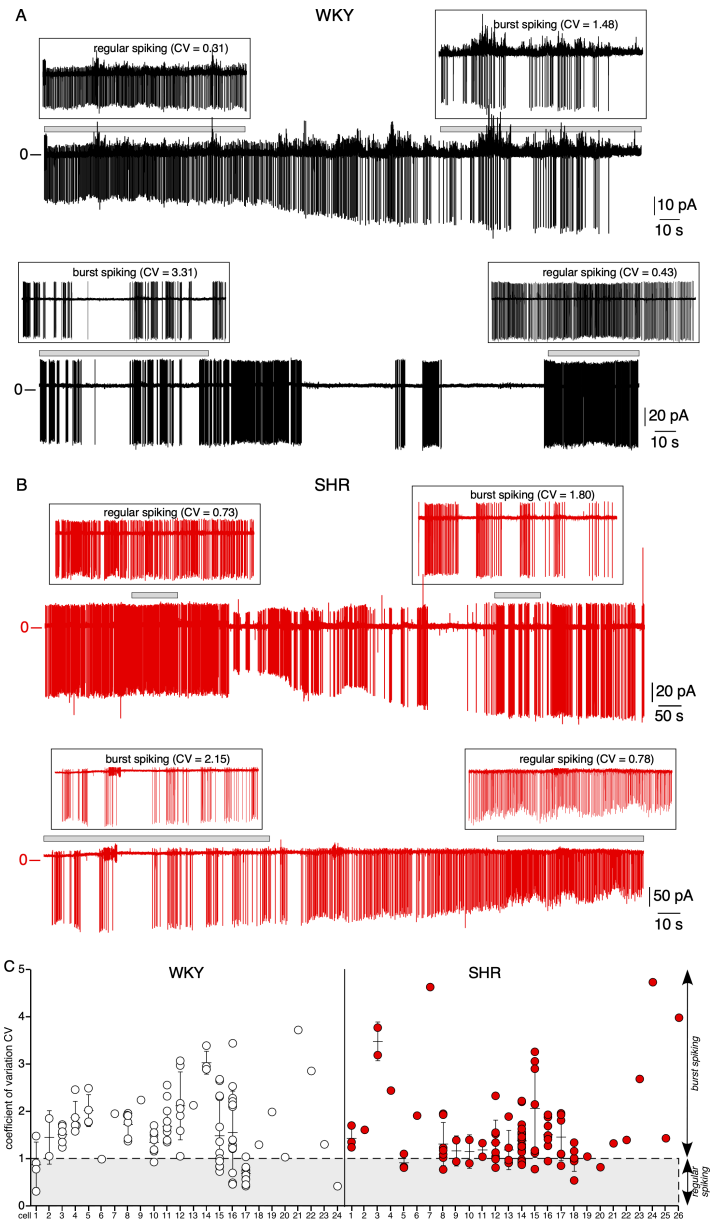

FIGURE S3

Figure S3: Dynamic switch between regular and bursting spiking patterns in a same chromaffin cell, in WKY rats and SHRs. A. Example of two WKY cells whose spontaneous electrical activity was recorded for 4-5 minutes and in which the spiking pattern switched from a regular to a bursting mode or vice versa. **B.** Same recordings in two SHR chromaffin cells, in which spontaneous action potentials were monitored for 4-20 min. Highlighted sections in A and B: expanded time scale (100 s) showing representative periods during which cells fired either regularly or in bursts. For each 100 s recording period, the distributions of inter-spike intervals were plotted and the associated CV calculated, as indicated in Figure S2. **C.** Distribution of CV values for each individual cell. All CV values were calculated from a 100 s recording (1-15 runs/cell in WKY rats and 1-21 runs/cell in SHRs). Note the wide distribution, both in normotensive and hypertensive animals, not only between cells but also between series of a same cell. Values and error bars represent mean and SD.

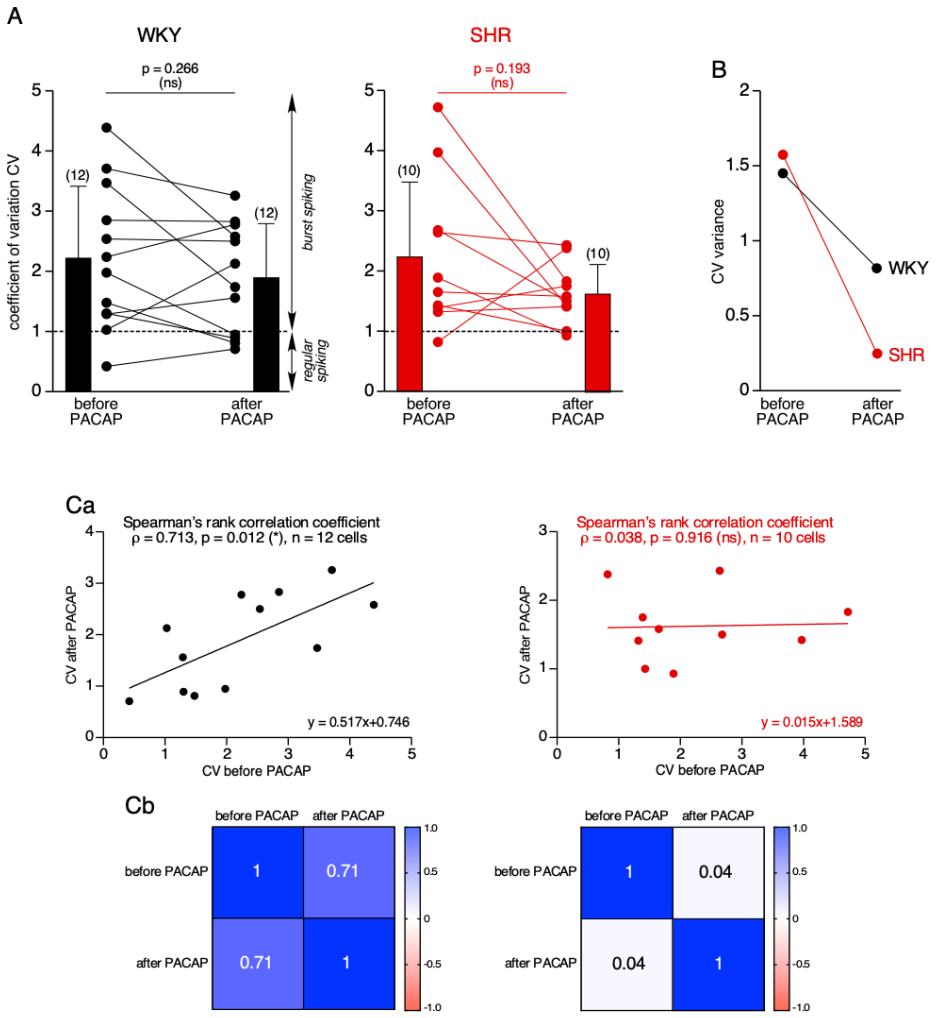

FIGURE S4

Figure S4: Analysis of CV changes in response to PACAP. Chromaffin cells from WKY rats and SHRs were exposed to 0.1-10 µM PACAP. Basal and PACAP-stimulated electrical activity were recorded in the loose cell-attached configuration (clamped at 0 mV). **A.** For each cell (12 WKY cells and 10 SHR cells), a CV was calculated for the basal condition (before PACAP) and for PACAP-exposed condition (after PACAP). Although CV tends to decrease in response to PACAP, the result is not significant, neither in normotensive or hypertensive animals (2.22 ± 1.20 before PACAP and 1.89 ± 0.91 after PACAP, n = 12 cells for WKY rats, p = 0.2661, Wilcoxon matched-pairs signed-rank test and 2.25 ± 1.26 before PACAP and 1.62 ± 0.50 after PACAP, n = 10 cells, p = 0.1934, Wilcoxon matched-pairs signed-rank test). **B.** Reduced CV variance after PACAP exposure, in particular in SHRs, indicating that PACAP acts by homogenizing the firing pattern. **C.** Loss of the correlation between CV after PACAP and CV before PACAP in SHRs (**Ca**) (ρ = 0.713 in WKY rats, p = 0.0012, n = 12 cells, Spearman's rank correlation coefficient *versus* ρ = 0.038 in SHRs, p = 0.916, n = 10 cells, Spearman's rank correlation coefficient) and associated table of correlation (**Cb**). Values and error bars represent mean and SD. *, p<0.05.

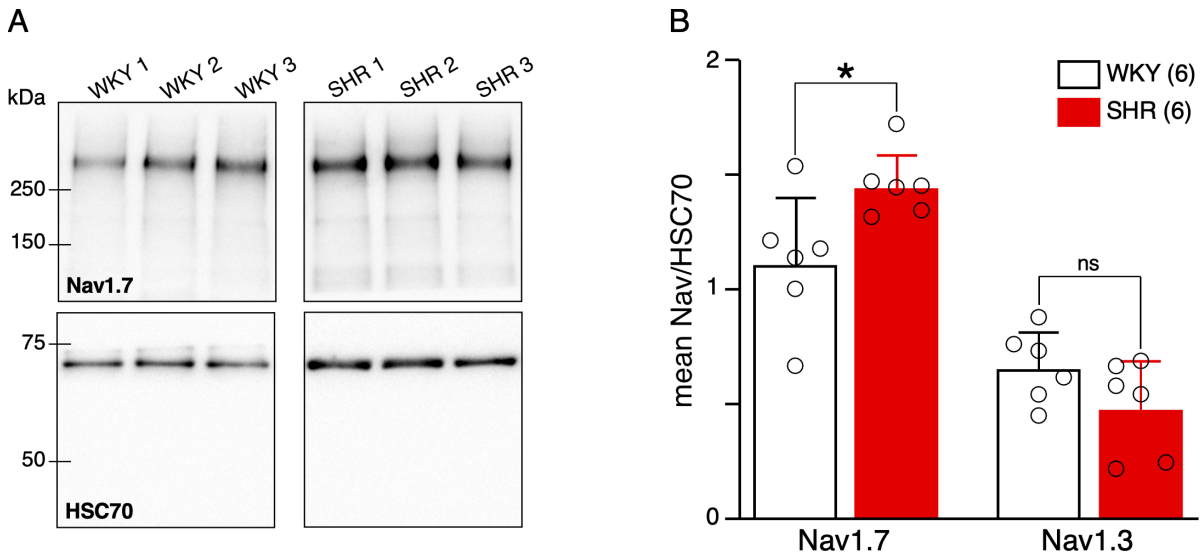

FIGURE S5

Figure S5: Increased expression of Na$_V$1.7 protein in SHRs. The adrenomedullary tissue was macrodissected in 6 WKY rats and 6 SHRs. **A.** Western blot illustration of Na$_V$1.7 in 3 WKY rats and 3 SHRs. Intensities of Na$_V$1.7 bands were normalized to those of HSC70. **B.** Pooled data showing that Na$_V$1.7 protein expression is significantly upregulated in SHRs (Na$_V$1.7/HSC70 ratio: 1.11 ± 0.28 for WKY rats, n = 6 and 1.45 ± 0.14 for SHRs, n = 6, p = 0.0411, Mann-Whitney test). By contrast, the expression of Na$_V$1.3 protein, the more expressed protein of the Nav family in rat chromaffin cells, is not modified in hypertensive rats compared to normotensive rats. Values and error bars represent mean and SD. *, $p<0.05$.

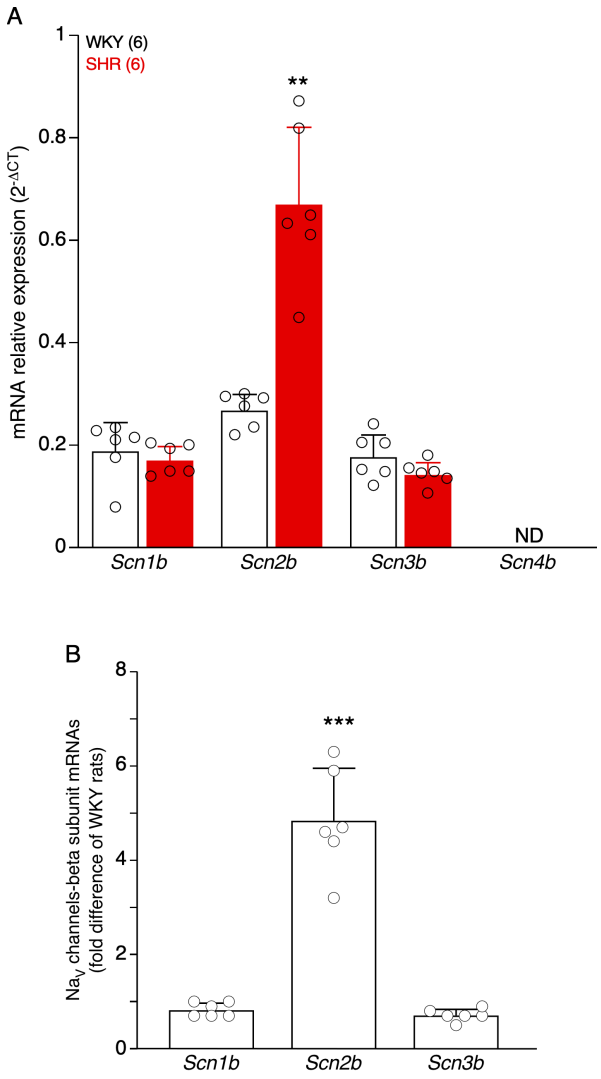

FIGURE S6

Figure S6: Expression changes in transcripts encoding auxiliary β subunits of voltage-gated Na^+ channels in SHRs. Changes in mRNA expression levels were assessed by real-time RT-PCR in macrodissected adrenal medullary tissues from 6 WKY rats and 6 SHRs. **A.** Of the four existing β subunits, only transcripts encoding β1 (*Scn1b*), β2 (*Scn2b*) and β3 (*Scn3b*) were detected, with a significant increase for *Scn2b* (relative expression of 0.27 ± 0.03 for WKY rats, n = 6 and 0.67 ± 015 for SHRs, n = 6, p = 0.0022, Mann-Whitney test). The *Scn4b* mRNA encoding β4 subunit was not detected (ND). **B.** Fold difference in SHRs, as compared to WKY rat. A significant difference occurs for the gene encoding Na_Vβ2 subunit (4.8-fold). Fold difference values were determined according to Livak's method (see Material and Methods). The Shapiro-Wilk test was used to analyze the normality of data distribution, and parametric or non-parametric unpaired tests were used when appropriate. Fold difference between x0.5 and x1.5 (grey area) are considered irrelevant. Values and error bars represent mean and SD. **, $p<0.01$and ***, $p<0.001$.

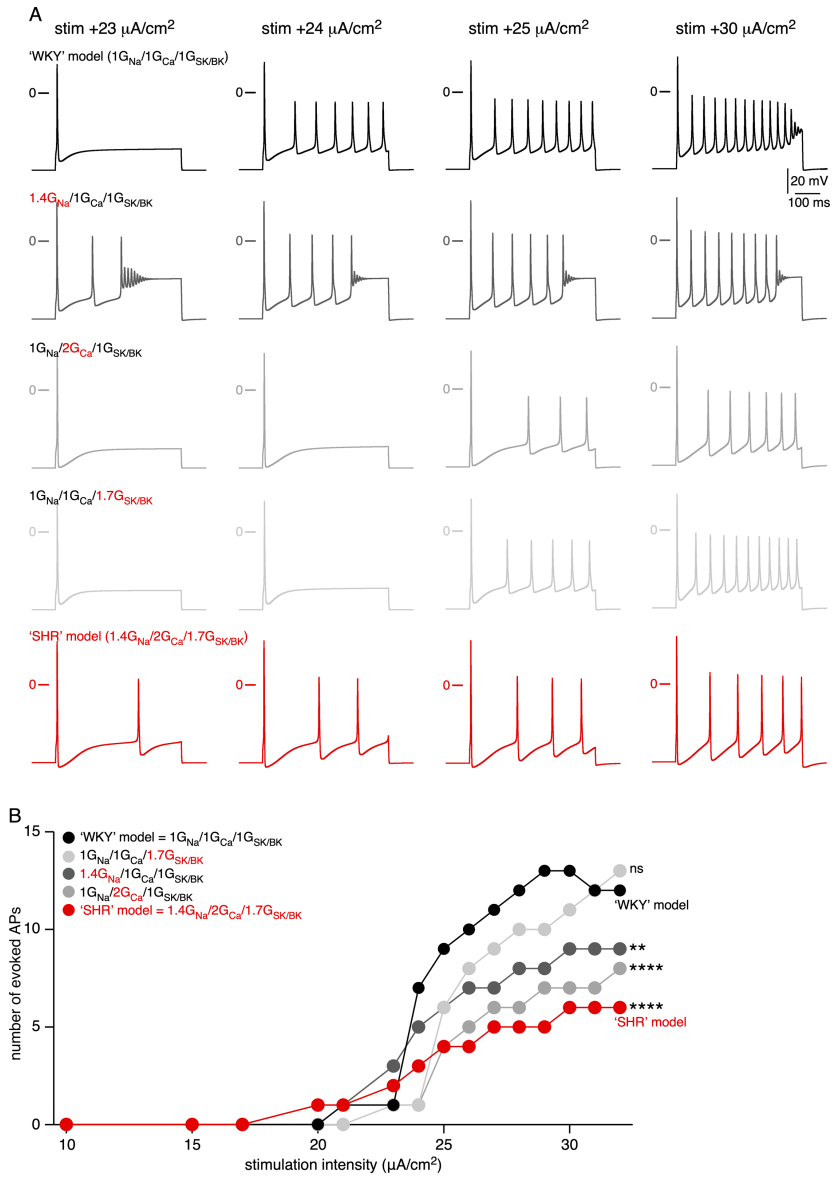

FIGURE S7

Figure S7: Impact of changes in G_{Na}, G_{Ca} and G_{SK} conductances on the electrical firing of computed rat chromaffin cells. 'WKY' and 'SHR' models were built from the rat chromaffin cell numerical simulation developed by Warashina and Ogura. **A.** Electrical firing evoked at four stimulation intensities (from 23 to 30 µA/cm^2, 500 ms duration). The 'SHR'-like condition (red traces) corresponds to 1.4G_{Na}, 2G_{Ca} and 1.7$G_{SK/BK}$ 'WKY' model (see Material and Methods). In the intermediate conditions (light, medium and dark grey traces), either G_{Na}, G_{Ca} or $G_{SK/BK}$ were modified by their respective multiplication coefficients, leaving the two other conductance unchanged. **B.** Pooled data showing that the most pronounced reduction in AP frequency is found when G_{Na}, G_{Ca} and $G_{SK/BK}$ are modified simultaneously ($p<0.0001$), in particular at high stimulation intensities. Statistically significant differences were also observed for modified G_{Na} alone ($p = 0.0044$), or G_{Ca} ($p<0.0001$), but not for $G_{SK/BK}$ ($p = 0.0582$, ns) (two-way ANOVA followed by a Bonferroni's post-hoc test, **, $p<0.01$ and ****, $p<0.0001$).