

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 Nav1.3 and Nav1.7 expression by western blot. Adrenal glands from WKY rats (n = 6) and SHR (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-Nav1.3 (1:500, Monoclonal Anti-SCN3A antibody, Sigma-Aldrich Merck) or the mouse anti-Nav1.7 (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 $\pm$ SD	Mean $\pm$ SD	p value (unpaired t test)
Resting membrane potential, mV	-64.8 $\pm$ 6.4 (67)	-64.0 $\pm$ 7.4 (66)	0.4951, ns
Input resistance, G Ω	1.19 $\pm$ 0.36 (68)	1.53 $\pm$ 0.42 (66)	<0.0001, ****
Membrane capacitance, pF	8.94 $\pm$ 0.67 (104)	8.74 $\pm$ 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 $\pm$ SD	mean $\pm$ SD	p value unpaired t test
rise time (ms)	8.25 $\pm$ 0.17	8.42 $\pm$ 0.25	0.0086**
half-rise time	1.18 $\pm$ 0.24	1.74 $\pm$ 0.66	0.0003****
10%-90% rise time	3.41 $\pm$ 0.54	4.09 $\pm$ 0.83	0.0016**
10%-90% slope (mV/ms)	16.98 $\pm$ 4.67	13.44 $\pm$ 5.28	0.0175*
amplitude (mv)	70.03 $\pm$ 7.38	62.56 $\pm$ 8.48	0.0021**
half width (ms)	3.86 $\pm$ 1.01	5.73 $\pm$ 1.95	0.001****
decay time (ms)	5.18 $\pm$ 1.74	7.99 $\pm$ 2.64	<0.001****

	WKY (n = 10 cells)	SHR (n = 8 cells)	
PROPERTIES OF SPONTANEOUS APs	mean $\pm$ SEM	mean $\pm$ SEM	p value Mann-Whitney test
rise time (ms)	7.80 $\pm$ 0.32	8.23 $\pm$ 0.23	0.0085**
half-rise time	0.64 $\pm$ 0.10	0.80 $\pm$ 0.10	0.0062**
10%-90% rise time	1.80 $\pm$ 0.33	2.50 $\pm$ 0.55	0.0014**
10%-90% slope (mV/ms)	41.95 $\pm$ 9.86	27.41 $\pm$ 6.61	0.0044**
amplitude (mv)	89.51 $\pm$ 6.53	79.98 $\pm$ 7.78	0.0124*
half width (ms)	3.32 $\pm$ 0.67	5.41 $\pm$ 2.16	0.0008***
decay time (ms)	6.00 $\pm$ 2.30	10.37 $\pm$ 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')
<i>Scn1a</i>	NM_030875	Nav1.1	gttccgacatcgccagtt	catctcagtttcagtagttgttcca
<i>Scn2a</i>	NM_012647	Nav1.2	tggtgtccctgggttgag	ccttatttctgtctcagtagttgtgc
<i>Scn3a</i>	NM_013119	Nav1.3	gcaccgtccattctaaccat	tttagcttcttgcataagaattgc
<i>Scn4a</i>	NM_013178	Nav1.4	ggcactgtctcgatttgagg	ttcatgatggaggggatagc
<i>Scn5a</i>	NM_013125	Nav1.5	tgccaccaatgccttgta	catgatgagcatgctaaagagc
<i>Scn8a</i>	NM_019266	Nav1.6	ggaagttttccatcatgaatcag	gctgttatgtcgggagagga
<i>Scn9a</i>	NM_133289	Nav1.7	cagcagatgtagaccgactca	actcgtgaactcagcagcag
<i>Scn1b</i>	NM_017288	Nav1b	gcggagatggtgtactgctac	tcggaagtaatggccaggtat
<i>Scn2b</i>	NM_012877	Nav2b	tggacttaccaggagtgtagca	ccagcttcaggttgatgatct
<i>Scn3b</i>	NM_139097	Nav3b	ctgatacctttgcgagtcactg	catcatgattccgagaccac
<i>Scn4b</i>	NM_001008880	Nav4b	catcctgaagaagaccagagaga	agcagggcctcacactttt
<i>Cacna1c</i>	NM_012517	Cav1.2	tggctcacagaagtgaaga	agcatttctgccgtgaaaag
<i>Cacna1d</i>	NM_017298	Cav1.3	ccatgctcactgtgttcag	ctcccatcctatcgcatcat
<i>Cacna1a</i>	NM_012918	Cav2.1	ctgctttgaagaggggacag	ctgacatttggtcccgttg
<i>Cacna1b</i>	NM_147141	Cav2.2	taagcgcataccgaatg	ggccaggacaatacagttgg
<i>Cacna1e</i>	NM_019294	Cav2.3	taccgcgcctggatagac	gctgatgttcccaggtttt
<i>Cacna1g</i>	NM_031601	Cav3.1	catctacttcattcttctcatcatcg	aactgcgtggcaatcacc
<i>Cacna1h</i>	NM_153814	Cav3.2	acggatactctgcagacagga	gctctgtgtagtctgggatgc
<i>Cacna1i</i>	NM_020084	Cav3.3	tctgcctcaatgttgcacc	aagggtgtctctagggatgt
<i>Kcnma1</i>	NM_031828	KCa1.1	aagtaattccatcaagctggtga	ggccccctgaattctccac
<i>Kcnn1</i>	NM_019313	KCa2.1	tcggaaacaccagcgtaagt	cttcacagtccggagcttct
<i>Kcnn2</i>	NM_019314	KCa2.2	tgttgccttaccggaataatg	tagcttccttgccactacgg
<i>Kcnn3</i>	NM_019315	KCa2.3	cacgccaaagttaggaac	ccatcttgacaccctcagt
<i>Kcnn4</i>	NM_023021	KCa3.1	gcaagattgtctgctgtgc	gccaccacagccaatagtaga
<i>Kcnt1</i>	NM_021853	KCa4.1	ggtcaagaaccgaatgaagc	tggctgttaaatttgctacgtc

<i>Kcnt2</i>	NM_198762	KCa4.2	ccagcaaatatgagattcatgc	acctcgttctcgtcttttctt
<i>Chrna3</i>	NM_052805	α 3nAChR	tccatgctgatgctgggtg	tcttcgaacaggtactggaaca
<i>Chrna4</i>	NM_024354	α 4nAChR	ccagtacattgcagaccacct	gccacgtatttcagtcctc
<i>Chrna5</i>	NM_017078	α 5nAChR	cacgtcgtgaaagagaacga	ccacaaaaacatccgatcaa
<i>Chrna7</i>	NM_012832	α 7nAChR	ggcaaaatgcctaagtggac	cttcatgcgcagaaacct
<i>Chrnb2</i>	NM_019297	β 2nAChR	caactcaatggcgctgttc	cactagccgctcctctgtgt
<i>Chrnb4</i>	NM_052806	β 4nAChR	tcctctcagctcatctccatc	tgatctgttctcgtctattca
<i>Gjd2</i>	NM_019281	Cx36	cggctactgcccagcttttgt	caccaccacagtcaacagga
<i>Gjal</i>	NM_012567	Cx43	cccgacgacaaccagaatg	tggtctaatggctggagttc
<i>Tjp1</i>	NM_001106266	ZO-1	tcaacacatgaagatgggattc	cgcaaaccacactatctcc
<i>Gusb</i>	NM_017015	Gus	ctctgggtggccttacctgat	cagactcaggtgttgcacg
<i>Hprt</i>	NM_012583	Hprt	gaccgggtctgtcatgtcg	acctgggtcatcatcactaatcac
<i>Gapdh</i>	NM_017008	Gapdh	tgggaagctggtcataaac	gcatcacccatttgatgtt

3- Supplementary figures

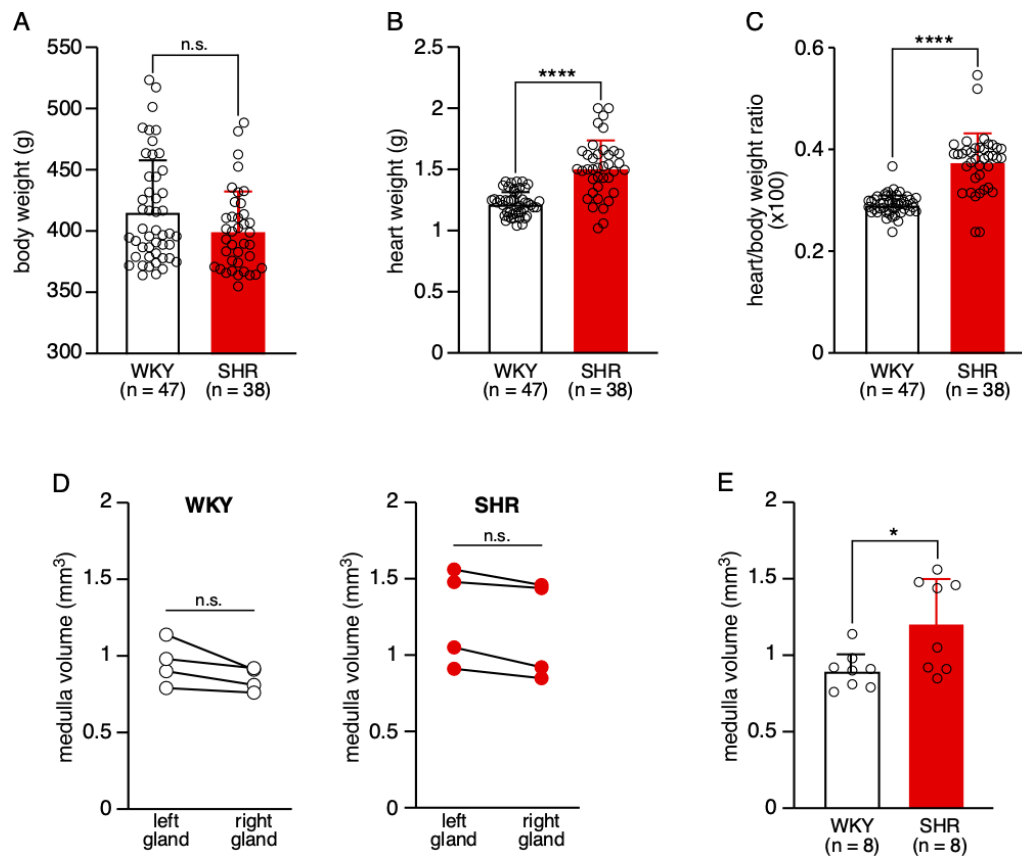


FIGURE S1

Figure S1: Validation of phenotypic traits of SHR rats 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 SHR rats (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 SHR rats. 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 mm³, $n = 4$ and 0.95 ± 0.15 mm³, $n = 4$ for the right and left WKY glands, respectively, $p = 0.125$, and 1.17 ± 0.33 mm³, $n = 4$ and 1.25 ± 0.32 mm³, $n = 4$ for the right and left SHR glands, respectively, $p = 0.125$, Wilcoxon matched-pairs signed-rank test). **E.** Significantly greater medulla volume in SHR rats versus WKY rats. (1.21 ± 0.30 mm³, $n = 8$ glands for SHR rats versus 0.90 ± 0.12 mm³, $n = 8$ 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 ****, $p < 0.0001$.

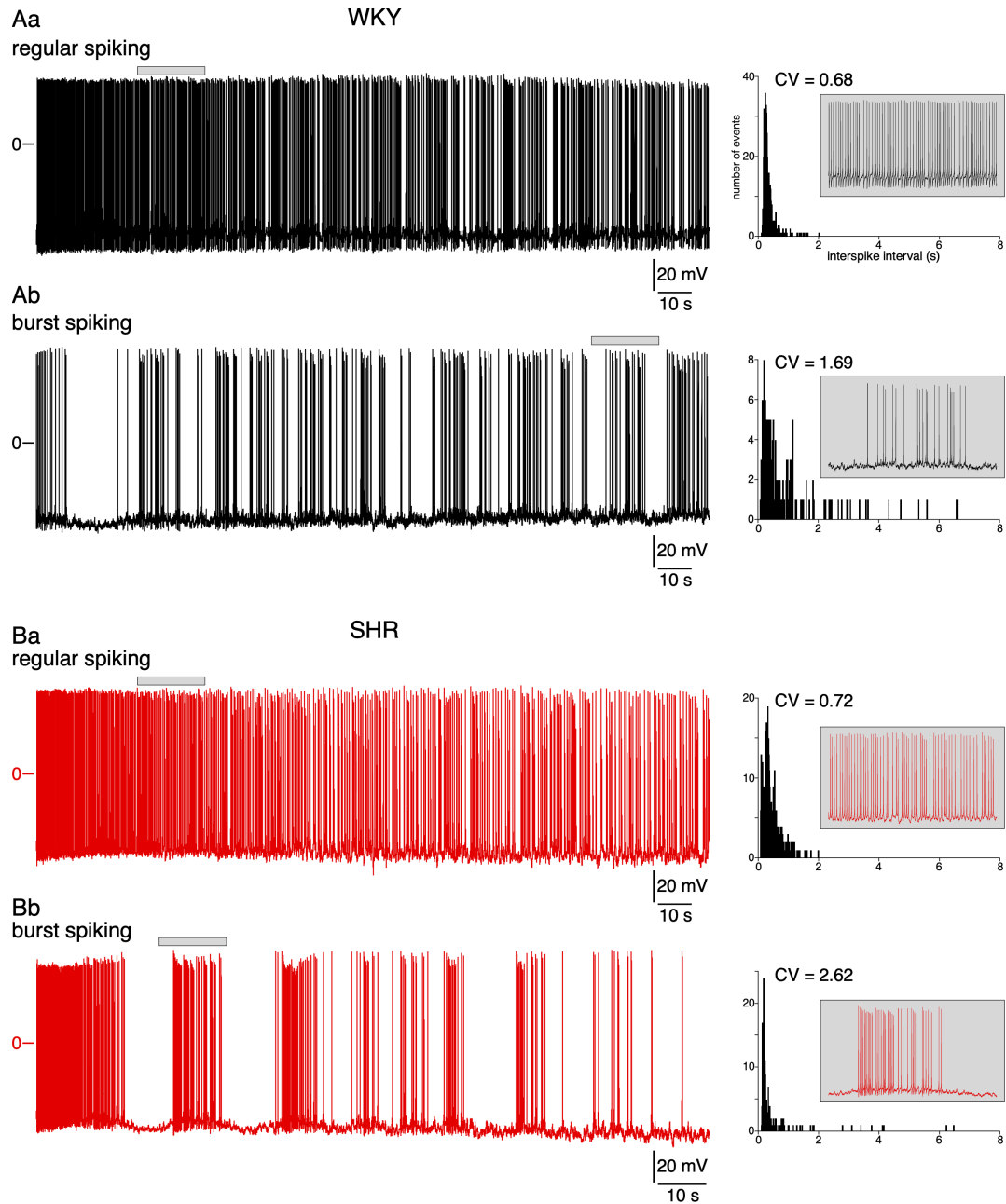


FIGURE S2

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.

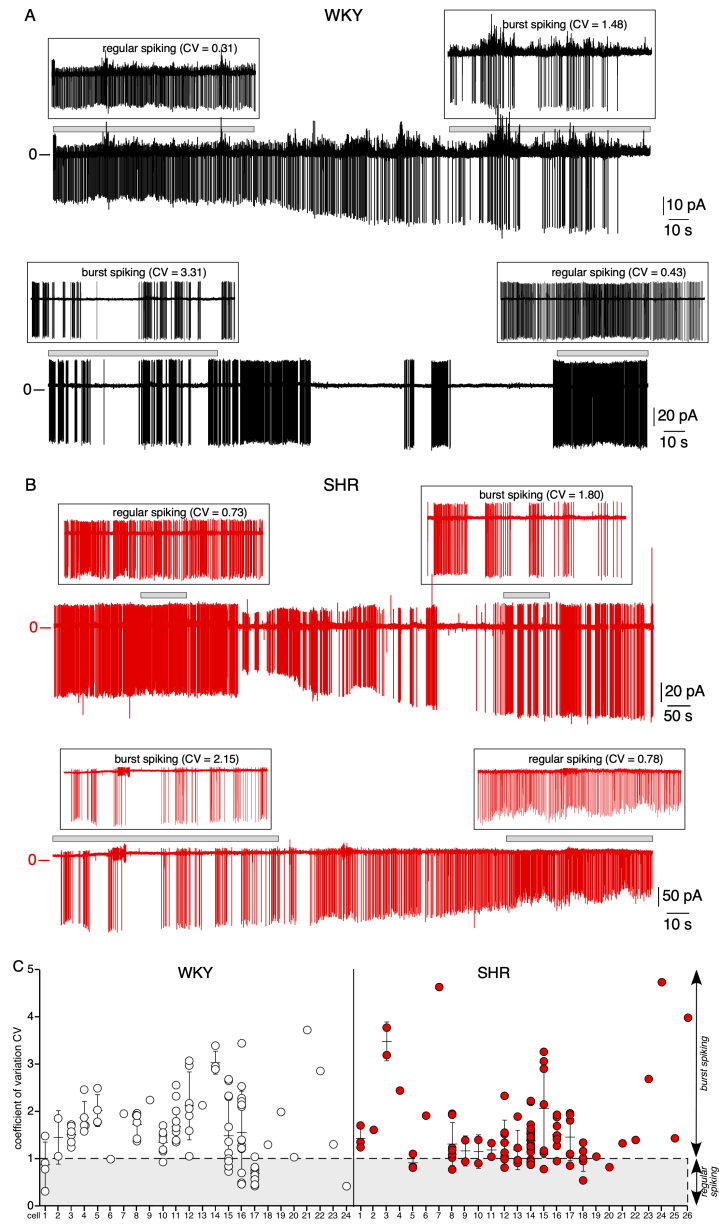


FIGURE S3

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.

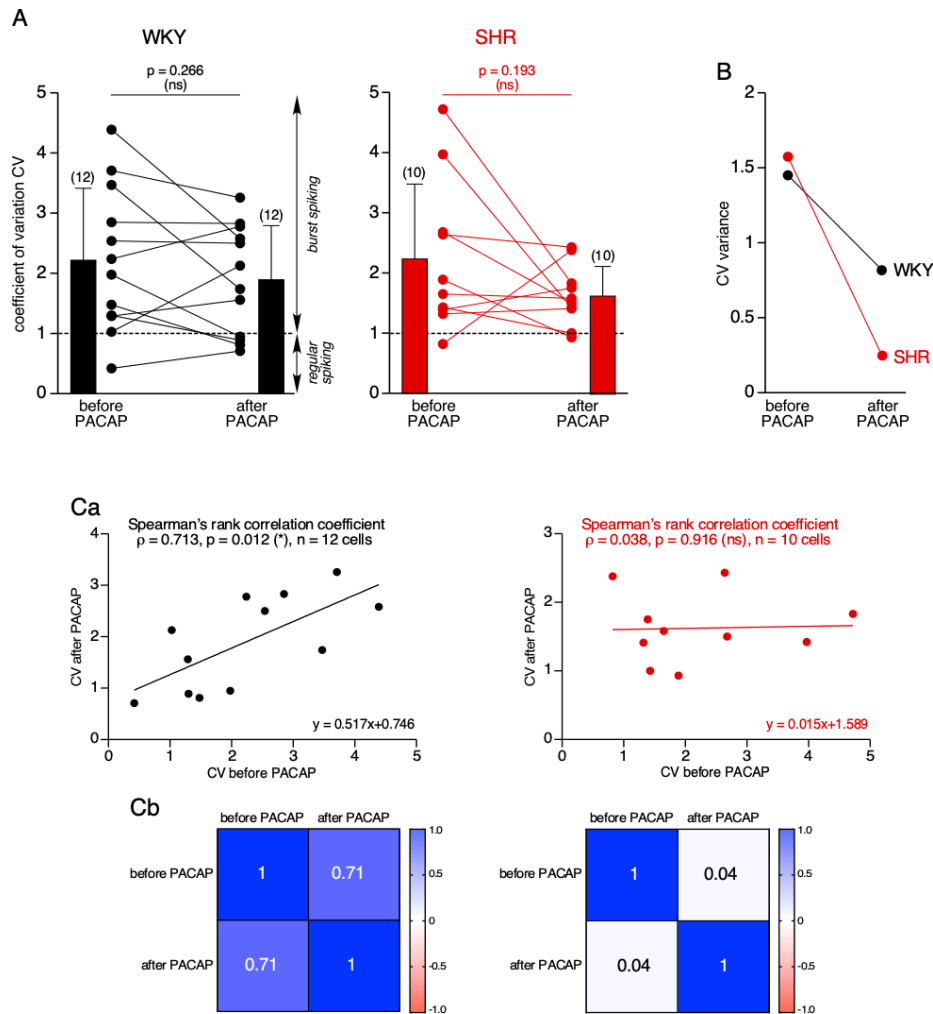


FIGURE S4

Figure S4: Analysis of CV changes in response to PACAP. Chromaffin cells from WKY rats and SHRs were exposed to 0.1-10 μ M 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**) ($\rho = 0.713$ in WKY rats, $p = 0.0012$, $n = 12$ cells, Spearman's rank correlation coefficient *versus* $\rho = 0.038$ 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. *, $p < 0.05$.

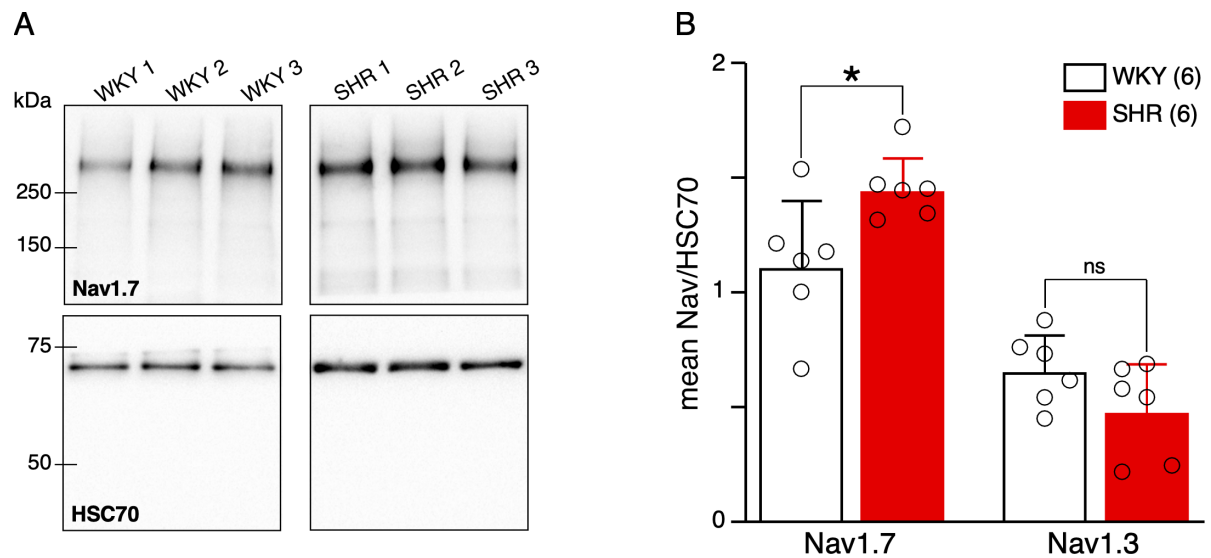


FIGURE S5

Figure S5: Increased expression of Nav1.7 protein in SHRs. The adrenomedullary tissue was macrodissected in 6 WKY rats and 6 SHRs. **A.** Western blot illustration of Nav1.7 in 3 WKY rats and 3 SHRs. Intensities of Nav1.7 bands were normalized to those of HSC70. **B.** Pooled data showing that Nav1.7 protein expression is significantly upregulated in SHRs (Nav1.7/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 Nav1.3 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. *, $p < 0.05$.

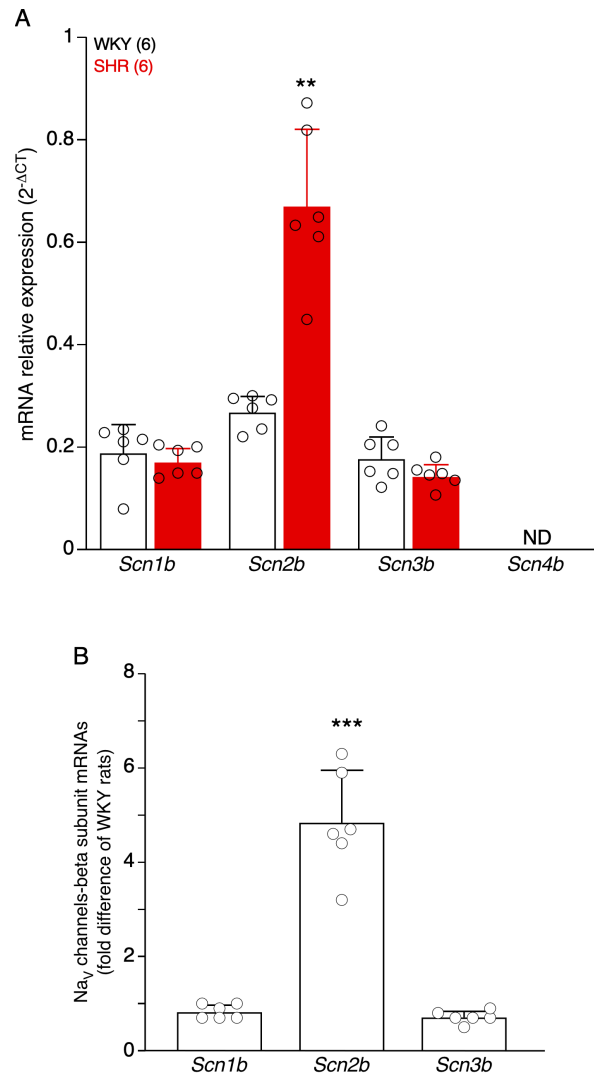


FIGURE S6

Figure S6: Expression changes in transcripts encoding auxiliary β subunits of voltage-gated Na^+ channels in SHR rats. Changes in mRNA expression levels were assessed by real-time RT-PCR in macrodissected adrenal medullary tissues from 6 WKY rats and 6 SHR rats. **A.** Of the four existing β subunits, only transcripts encoding $\beta 1$ (*Scn1b*), $\beta 2$ (*Scn2b*) and $\beta 3$ (*Scn3b*) were detected, with a significant increase for *Scn2b* (relative expression of 0.27 ± 0.03 for WKY rats, $n = 6$ and 0.67 ± 0.15 for SHR rats, $n = 6$, $p = 0.0022$, Mann-Whitney test). The *Scn4b* mRNA encoding $\beta 4$ subunit was not detected (ND). **B.** Fold difference in SHR rats, as compared to WKY rat. A significant difference occurs for the gene encoding Nav $\beta 2$ 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 $\times 0.5$ and $\times 1.5$ (grey area) are considered irrelevant. Values and error bars represent mean and SD. **, $p < 0.01$ and ***, $p < 0.001$.

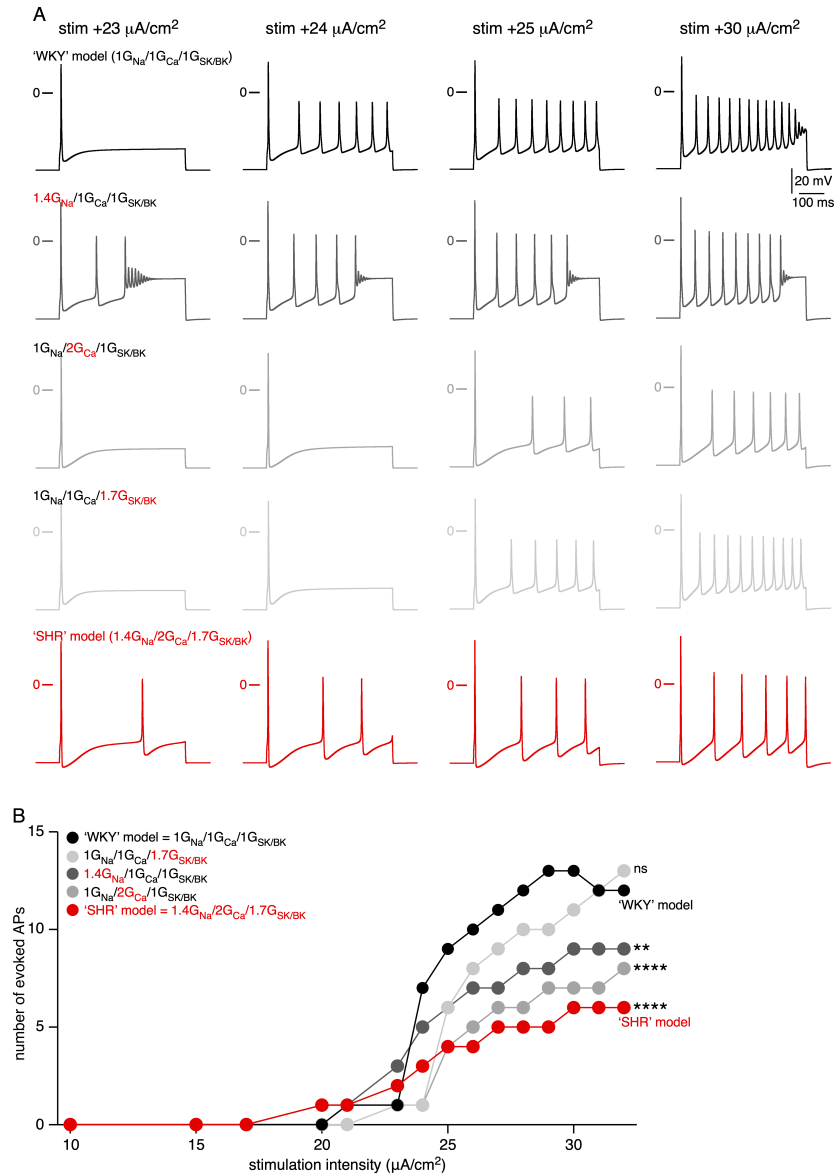


FIGURE S7

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 $\mu\text{A}/\text{cm}^2$, 500 ms duration). The 'SHR'-like condition (red traces) corresponds to $1.4G_{\text{Na}}$, $2G_{\text{Ca}}$ and $1.7G_{\text{SK/BK}}$ 'WKY' model (see Material and Methods). In the intermediate conditions (light, medium and dark grey traces), either G_{Na} , G_{Ca} or $G_{\text{SK/BK}}$ 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 $G_{\text{SK/BK}}$ 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 $G_{\text{SK/BK}}$ ($p = 0.0582$, ns) (two-way ANOVA followed by a Bonferroni's post-hoc test, **, $p < 0.01$ and ****, $p < 0.0001$).