ONLINE SUPPLEMENT

TRANSCRIPTIONAL UPREGULATION OF $\alpha_2\delta$-1 ELEVATES ARTERIAL SMOOTH MUSCLE CELL $CA_V1.2$ CHANNEL SURFACE EXPRESSION AND CEREBROVASCULAR CONSTRICTION IN GENETIC HYPERTENSION

by

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Short Title: $\alpha_2\delta$-induced vasoconstriction in hypertension

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Supplemental Methods

Cell isolation and tissue preparation

All animal protocols used were reviewed and approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center. Male 6 or 12 week old SHR and Wistar Kyoto (WKY) rats were euthanized by intraperitoneal injection of sodium pentobarbital (150 mg/Kg body weight). The brain was removed and placed in oxygenated (21 % O_2/5 % CO_2) physiological saline solution (PSS) containing (in mmol/L): KCl 6, NaCl 112, $NaHCO_3$ 24, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.8, and glucose 10. Middle cerebral, posterior cerebral and cerebellar arteries (~100-200 μm diameter) were dissected from the brain.

Blood pressure measurements

Diastolic, systolic, and mean arterial blood pressures were measured in 6 and 12 week old WKY and SHR rats using a CODA 2 tail cuff system (Kent Scientific, Torrington, Conn). Rats were acclimatized to the chamber and tail cuff on a heated platform (37°C) for 3 days prior to acquisition of blood pressure data. On the day of data acquisition, rats were again acclimatized for 30 min before blood pressure measurements were acquired. For each rat, 10-15 acclimatization cycles were run and data taken as the mean of 5-9 measurements.

RT-PCR

Total RNA was isolated from brain and cerebral arteries using Trizol (Invitrogen) and from isolated smooth muscle cells using the Absolutely RNA nanoprep kit (Strategene). RT-PCR was performed using $\alpha_2\delta$ isoform-specific primers, as previously described.[1] Standard PCR reactions consisting of a 2 min heat shock (94˚C) and 40 cycles of denaturation (94˚C for 30 s), annealing (55˚C for 30 s) and extension (72˚C for 1 min), were used for all reactions.

Quantitative real time PCR

Quantitative Taqman PCR reactions were performed using an LC480 light cycler (Roche Applied Science). Reaction conditions were an initial denaturation step at 95˚C for 5 min followed by 40 cycles of 95˚C for 10 s, 60˚C for 30 s and 72˚C for 10 s. Negative control without cDNA was run for each reaction. Standard curves using four 10-fold dilutions of cDNA were run for all probe and primer pairs to determine PCR efficiency. $\alpha_2\delta$-1 or $Ca_V1.2\alpha_1$ mRNA expression was calculated from the difference between fluorescence (Ct) values (ΔCt) of $\alpha_2\delta$-1 or $Ca_V1.2\alpha_1$ and Rps5. $\Delta\Delta$Ct was calculated from the difference between the ΔCt values for SHR and WKY. $\alpha_2\delta$-1 or $Ca_V1.2\alpha_1$ mRNA levels in SHR compared to WKY were calculated using the formula $100 \times 2^{(-\Delta\Delta Ct)}$.[2] All PCR reactions including standard curves were performed in triplicate. Gene specific primers and probes were designed using the Universal Probe Library (UPL). The overall efficiency of each primer pair and probe for PCR reactions was calculated. Primer sequences, probes, gene accession numbers, amplicon length, and real-time PCR reaction efficiencies are provided in Table S1.

The following reference genes were screened: beta (β) actin, hypoxanthine-guanine phosphoribosyltransferase (HGPRT), TATA box binding protein (TBP), ribosomal RNA 18s (18S rRNA), ribosomal subunit protein 16 (Rps16), platelet/endothelial cell adhesion molecule 1 (Pecam1), cyclophilin B and Rps5. Rps5 was identified as the most suitable reference gene for this study after comparing both the fold change in mRNA levels in SHR compared to WKY and the coefficient of variation (expressed as %) in Ct values of WKY and SHR samples (Table S2). For each reference gene, coefficient of variation of the Ct values in all (SHR and WKY) samples was calculated using the following equation:

$$\text{Coefficient of variation} = (\text{Standard Deviation}/\text{Mean}) \times 100$$

Protein analysis and biochemistry
Arteries were homogenized in 1x Laemmli buffer supplemented with 2 % β-mercaptoethanol. Cellular debris was removed by centrifugation. The method of Henkel et al.[3] was used to determine protein concentration. Proteins were separated on 7.5 % SDS-PAGE gels and analyzed by Western blotting. Blots were cut at the 75 kDa marker to allow simultaneous probing of higher molecular mass (>75 kDa) proteins for $\alpha_2\delta$-1 and lower mass proteins (<75 kDa) for actin. The higher molecular mass blot was stripped and re-probed for $Ca_V1.2\alpha_1$. Antibodies used were anti-$\alpha_2\delta$-1 (Aviva Systems Biology), anti-$Ca_V1.2\alpha_1$ (Neuromab) and anti-actin (Millipore). Bands on Western blots were visualized using HRP conjugated secondary antibodies and a West Pico Chemiluminescence kit (Pierce), using a Kodak Image F-Pro system. Protein band intensities were determined using Quantity One (BioRad) software. For quantification, protein band intensities were first normalized to actin and then to appropriate control samples.

Artery surface biotinylation
Intact, cleaned arteries were incubated for 1 h in a mixture of EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG_2-Biotin reagents (Pierce, 1 mg/ml each) in phosphate-buffered saline (PBS, Invitrogen). Unbound biotin was removed by quenching with PBS supplemented with 100 mmol/L glycine and washing with PBS. For determination of protein concentration, arteries were homogenized in RIPA buffer (Sigma) and cellular debris removed by centrifugation. Protein concentration was determined using the method of Henkel et al.[3] Equal amounts of biotinylated arterial lysate for each sample were then used for Avidin (Monomeric Avidin, Pierce) pulldown of biotinylated proteins. Following pulldown, the supernatant comprised the intracellular proteins and biotinylated surface proteins remained bound to Avidin. Following removal of the supernatant (intracellular fraction), biotinylated surface proteins were eluted from Avidin beads by boiling in 1x Laemmli buffer supplemented with 2 % β-mercaptoethanol. The entire supernatant comprising the intracellular and the entire eluate comprising the surface protein fraction from the pulldown, were separated on SDS-PAGE gels and analyzed by Western blotting. Band intensities were determined using Quantity One software (BioRad). Total protein was calculated as the sum of surface and intracellular band intensities. To determine the proportion of a protein located at the plasma membrane, surface band intensity was divided by total. Alternatively, the surface:intracellular ratio of a protein was calculated by dividing surface band intensity by intracellular band intensity. Pregabalin-induced changes in protein cellular distribution were calculated by normalizing to control protein distribution. The ratio of surface $\alpha_2\delta$-1 to $Ca_V1.2\alpha_1$ was calculated by dividing surface $\alpha_2\delta$-1 by surface $Ca_V1.2\alpha_1$ band intensity from the same blots.

Patch-clamp electrophysiology
$Ca_V1.2$ currents were recorded in isolated smooth muscle cells using the whole cell patch-clamp configuration at room temperature using an Axopatch 200B amplifier (Axon Instruments). Borosilicate glass electrodes (4-5 MΩ) were filled with pipette solution containing (in mmol/L): $CsMeSO_4$ 135, CsCl 5, EGTA 5, MgATP 4, Na_2GTP 0.25, HEPES 10 and glucose 10 (pH 7.2 adjusted using CsOH). Extracellular bath solution contained (in mmol/L): $BaCl_2$ 10, NMDG 130, $MgCl_2$ 1, HEPES 10 and glucose 10 (pH 7.4 adjusted using L-Aspartic acid). Cell capacitance was measured by applying a 5 mV test pulse and correcting transients with series resistance compensation. Pregabalin (100 μmol/L) was present in the bath solution during recordings for chronically-treated cells, and applied acutely to the bath otherwise.

To measure whole-cell $Ca_V1.2$ currents and steady-state inactivation, 1 s conditioning pulses to between -80 and +60 mV were applied in 10 mV increments prior to a 200 ms test pulse to 0 mV. Current-voltage (IV) relationships were generated from the peak current obtained during the 1 s conditioning pulses. The rate of current inactivation was calculated from the current decay during each 1 s conditioning pulse. Steady state inactivation was determined from the current generated during the 200

ms test pulse to 0 mV. To measure steady state activation, tail currents were elicited by repolarization to -80 mV from 20 ms test pulses from -60 to +60 mV in 10 mV increments. To measure the time course and magnitude of acute pregabalin (100 μmol/L) inhibition, $Ca_V1.2$ currents were elicited by repetitive 300 ms steps to 10 mV from -80 mV every 30 s. Whole cell currents were filtered at 1 or 5 kHz and digitized at 5 or 20 kHz for the inactivation and activation protocols, respectively. P/-4 protocols were used to subtract leak and capacitive transients.

Scatter plot data points were fit with a linear function: $y = mx$, where m is the slope, r is the strength of the linear relationship between the x and y variables, and p is the probability of the correlation co-efficient, with $P<0.05$ significant. Steady state inactivation and activation curves were fit with a single power Boltzmann function: $I/I_{MAX} = R_{in} + (R_{MAX} - R_{in})/(1 + \exp((V - V_{1/2})/k))$, where I/I_{MAX} is the normalized peak current, V is the conditioning pre-pulse voltage, $V_{1/2}$ is the voltage for half-inactivation or half-activation, *k* is the slope factor, R_{in} is the proportion of non-inactivating current and R_{Max} is the maximal current amplitude. Inactivation kinetics data were fit with a single exponential function: $I_t = (A \times e^{(-t/\tau)}) + I_0$, where I_t is the inward current at time *t,* A the amplitude and I_0 the residual current.

Pressurized artery myography

The endothelium was denuded by introduction of an air bubble into the artery lumen for ~1 min followed by PSS. Cerebral artery segments were cannulated at each end in a perfusion chamber (Living Systems Instrumentation) that was maintained at 37ºC and continuously perfused with PSS. Intravascular pressure was monitored using a pressure transducer and altered using an attached reservoir. Wall diameter was measured using a charge-coupled device camera and the edge detection function of IonWizard (Ionoptix), at 1 Hz. Arteries treated with pregabalin for 24 h were maintained in pregabalin throughout these experiments to inhibit $Ca_V1.2$ subunit membrane re-insertion. Myogenic tone (%) was calculated as: $100 \times (1 - D_{active}/D_{passive})$, where D_{active} is active arterial diameter and $D_{passive}$ is the passive arterial diameter determined by applying Ca^{2+}-free PSS supplemented with 5 mM EGTA.

Chemicals

Pregabalin was a gift from Pfizer Inc (Groton, Conn) or was purchased from Sigma Aldrich (Milwaukee, WI).

References

1 Bannister JP, Adebiyi A, Zhao G, Narayanan D, Thomas CM, Feng JY, Jaggar JH. Smooth muscle cell $\alpha_2\delta$-1 subunits are essential for vasoregulation by $Ca_V1.2$ channels. *Circ Res.* 2009;105:948-955.

2 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta Ct)}$ Method. *Methods.* 2001;25:402-408.

3 Henkel AW, Bieger SC. Quantification of proteins dissolved in an electrophoresis sample buffer. *Anal Biochem.* 1994;223:329-331.

Gene	Primer sequence		UPL probe #	Amplicon length (nt)	Amplification efficiency
$Ca_V1.2\alpha_1$	Forward Reverse	5'-GAGAGCTTTCCGTGTGCTTC-3' 5'-GTTCAGGACCACCTGGAGAC-3'	109	70	1.991
$\alpha_2\delta$-1	Forward Reverse	5'-CATACTCCAGATTGGCTGGTG-3' 5'-AGTAGCTGCTGGAGAATAGACCA-3'	74	60	1.997
18S rRNA	Forward Reverse	5'-AATCAGTTATGGTTCCTTTGTCG-3' 5'-GCTCTAGAATTACCACAGTTATCCAA-3'	55	65	1.94
β-actin	Forward Reverse	5'-CCCGCGAGTACAACCTTCT-3' 5'-CGTCATCCATGGCGAACT-3'	17	72	2.01
TBP	Forward Reverse	5'-CCCTATCACTCCTGCCACA-3' 5'-GGTCAAGTTTACAGCCAAGATTC-3'	110	98	1.883
HGPRT	Forward Reverse	5'-TCAACGGGGGACATAAAAGT-3' 5'-AGTGTCAATTATATCCAAACCCAAT-3'	22	93	2.087
Pecam1	Forward Reverse	5'-CTCAGTCGGCTGACAAGATG-3' 5'-AGGCTTGCATAGAGCAGCAT-3'	21	61	2.038
Rps16	Forward Reverse	5'-GCAGTCCGTTCAAGTCTTCG-3' 5'-GATGAGCCCATTTCCTCGT-3'	5	76	2.028
Rps5	Forward Reverse	5'-GACTGAGAAGCCCGGTTTG-3' 5'-CTTGATGTCCGGGGTCTCT-3'	5	83	1.949
Cyclophilin B	Forward Reverse	5'-ACGTGGTTTTCGGCAAAGT-3' 5'-CTTGGTGTTCTCCACCTTCC-3'	97	62	2.105

Table S1
Gene specific oligonucleotide sequences for primers, probes, amplicon length, and reaction efficiencies of quantitative PCR experiments.

Reference Gene	Fold difference in mRNA levels (SHR/WKY)	Coefficient of variation in Ct values (%)
18S rRNA	8.51 ± 0.37 (3)	6.02
β-actin	3.77 ± 0.27 (3)	3.35
TBP	4.70 ± 3.2 (3)	4.15
HGPRT	2.96 ± 0.26 (3)	2.61
Pecam1	2.27 ± 0.19 (3)	2.24
Rps16	4.39 ± 0.07 (3)	4.41
Rps5	1.14 ± 0.10 (3)	0.37
Cyclophilin B	1.88 ± 0.22 (3)	1.88

Table S2
Comparison of mRNA levels for eight potential reference genes in 12 week old WKY and SHR cerebral arteries. Experimental number is given in parentheses.

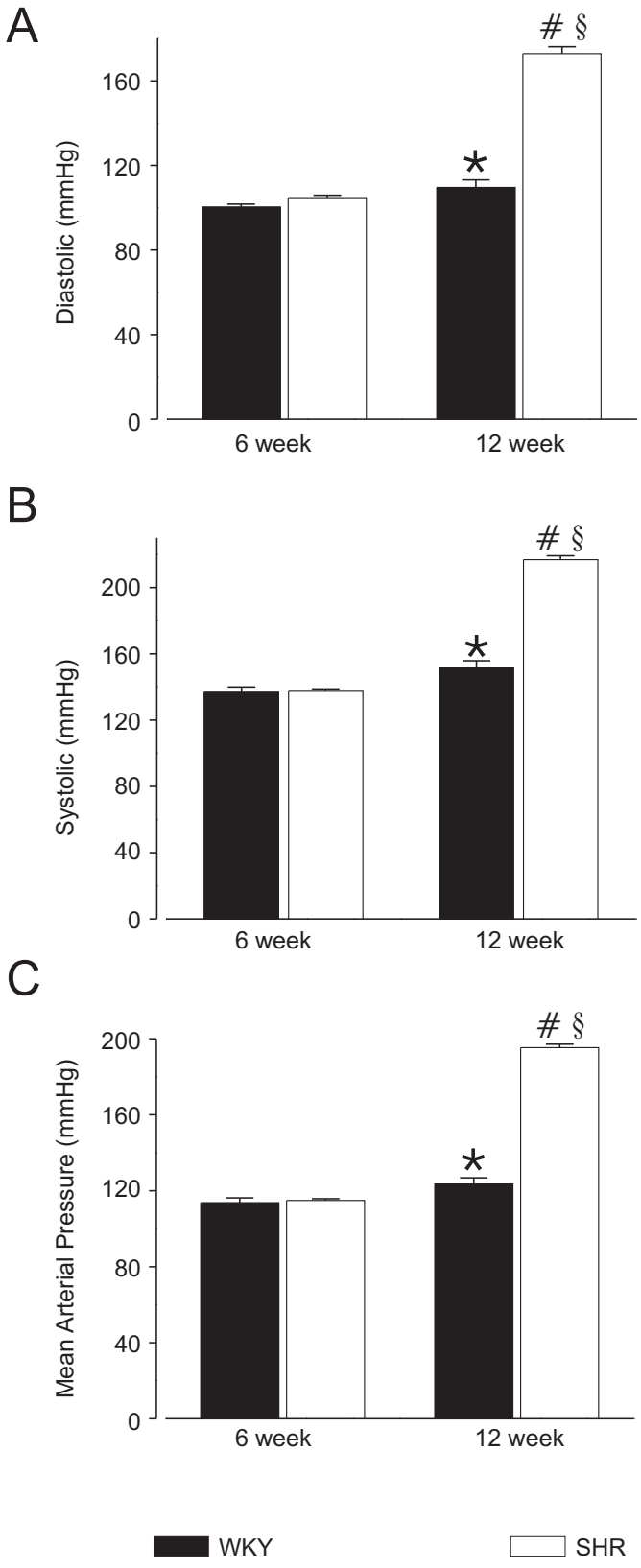

Figure S1. Diastolic, systolic, and mean arterial blood pressures are elevated more so in 12 week hypertensive SHR than age-matched WKY rats. A-C, Bar graphs showing mean diastolic pressures (A), mean systolic pressures (B), and mean arterial pressures (C) obtained from 6 week WKY, 6 week SHR, 12 week WKY and 12 week SHR rats by tail cuff. N numbers were as follows: 6 week WKY (n=10), 6 week SHR (n=6), 12 week WKY (n=10), 12 week SHR (n=11). * indicates $P<0.05$ when compared to 6 week WKY, # indicates $P<0.05$ when compared to 6 week SHR and § indicates $P<0.05$ when compared to 12 week WKY.

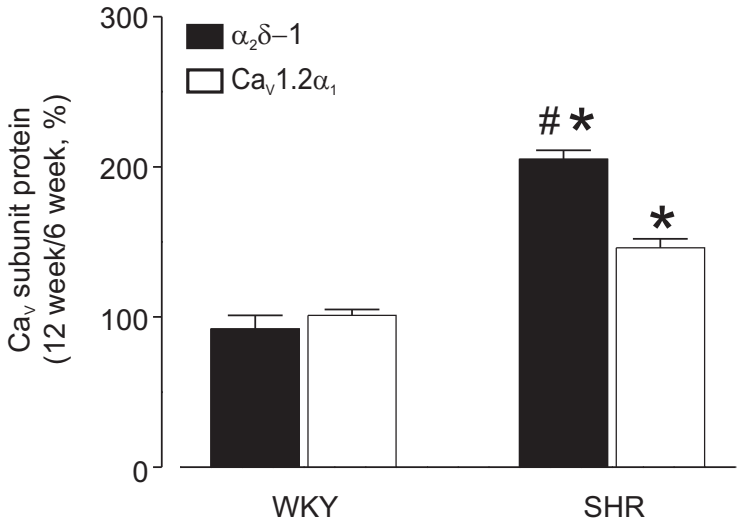

Figure S2. $\alpha_2\delta$-1 and $Ca_V1.2\alpha_1$ subunit protein are elevated during development of hypertension. Mean data illustrating that $\alpha_2\delta$-1 and $Ca_V1.2\alpha_1$ subunit protein increase with age in SHR but not in WKY cerebral arteries. * indicates $P<0.05$ when compared with WKY. # indicates $P<0.05$ when compared with $Ca_V1.2\alpha_1$ in SHR (n=8 for each protein at each age).

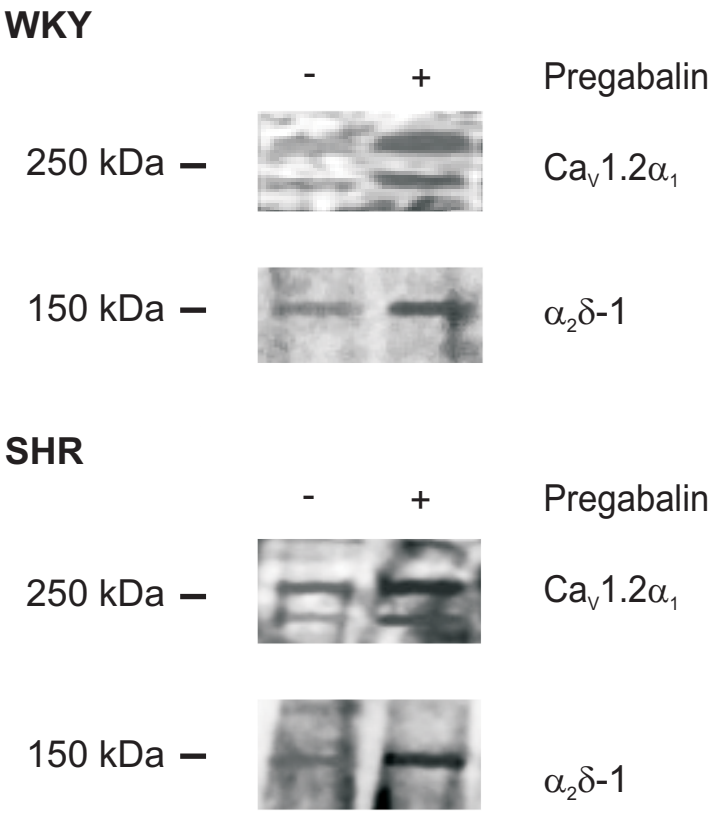

Figure S3. A longer time exposure of the intracellular lanes of the blot in Figure 3B illustrating that pregabalin increased intracellular $\alpha_2\delta$-1 and $Ca_V1.2\alpha_1$ in WKY and SHR cerebral arteries.

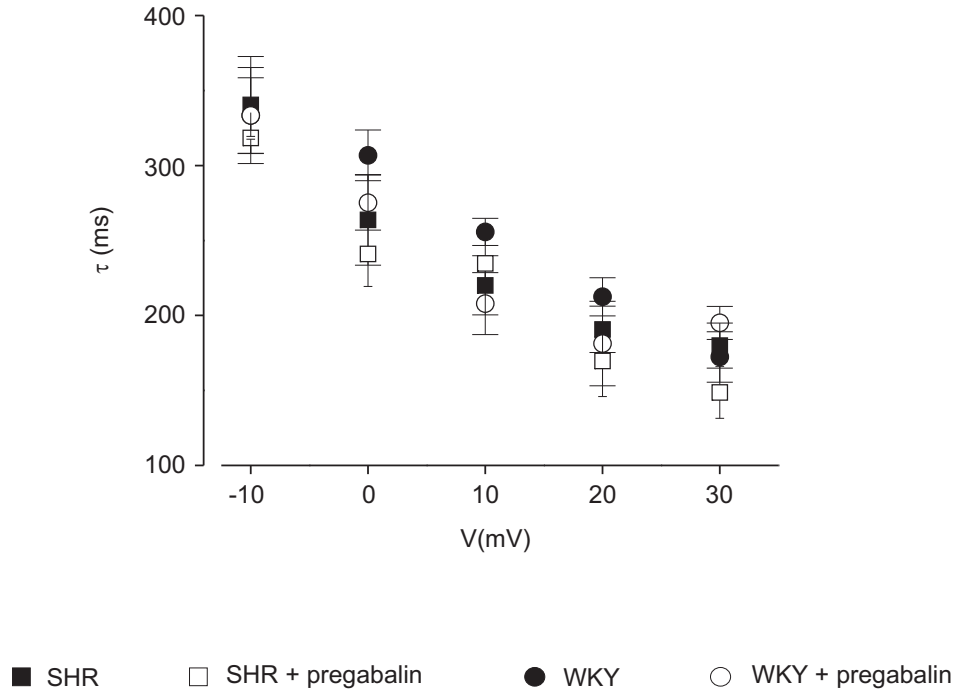

Figure S4. Pregabalin (100 μmol/L) does not alter the rate of $Ca_v1.2$ current inactivation in WKY or SHR cells. Graph showing that the rate of current inactivation (τ) was similar in untreated WKY (n=17), pregabalin-treated WKY (n=13), untreated SHR (n=16) and pregabalin-treated SHR (n=18) cells.