Supplementary Information

Biocompatibility of a Genetically Encoded Calcium Indicator in a Transgenic Mouse Model

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Supplementary Figures S1-S10
Supplementary Table S1
Supplementary Methods
Supplementary Figures

Figure S1 | Expression pattern of TN-XXL in cryosections in various tissues of transgenic mice.

Fluorescence images of cryosections of organs were recorded with a laser scanning confocal microscope. The top left picture shows the expression in the hippocampus (Scale bar, 250 µm). The image next to it shows an enlarged picture of the region indicated by the black box. The other pictures display the expression of TN-XXL in other brain regions (cortex, cerebellum), in skeletal muscle, the atrial (upper part) and ventricular (lower part) region of heart, the liver, the kidney and the pancreas (Scale bars, 50 µm).
Figure S2 I No expression of TN-XXL in the hematopoietic system.

(A) Lymphoid organ sections from a homozygous TN-XXL transgenic mouse. White circles mark where the lymph follicles are situated in the spleen (Scale bars, 500 µm). (B) Fluorescence activated cell sorting (FACS) analysis of single cell suspensions from lymph node, thymus and bone marrow revealed no expression of TN-XXL. (C) mRNA analysis of TN-XXL pre-mRNA and mature mRNA yielded a 300-fold higher number of copies in kidney compared to spleen. Note that for mRNA analysis the spleen was meshed through a 40 µm gaze, thus eliminating structural cells expressing TN-XXL. Therefore, low mRNA levels are detected, in contrast to the Western Blot results in Figure 1 for which whole spleen was used (including structural cells that are the predominant source of TN-XXL expression in spleen). Values are means ± s.e.m. with triplicates each.
Figure S3 | Distribution of TN-XXL expression.

(A) Dilution of purified TN-XXL and corresponding fluorescence images showing the brightness recorded with a laser scanning confocal microscope. (B-F) Fluorescence images of various acute organ slices (400 µm thickness) from homozygous transgenic mice and histograms illustrating the distribution of TN-XXL expression within the given organ. B, muscle; C, heart; D, kidney; E, liver; F, hippocampus (Scale bars, 150 µm).
Figure S4 | Long-term expression of TN-XXL in transgenic mice under the control of the CAG promoter does not yield altered subcellular sensor distribution in neurons.

(A) Laser scanning confocal imaging sections through the CA1 and CA3 region of the hippocampus of P13, P36, 2 month and 6 month old homozygous mice (scale bar, 150 µm). (B) Fluorescence distribution through a cross section of the cell soma. Even though the expression in CA3 neurons is lower than in CA1 cells, in both regions fluorescence is restricted to the cytosol (cyto) and is not present in the nuclear region (nucl) (mean value in red and standard deviation in grey). (C) Statistical analyses show that even expression over 6 month does not change the cytosol to nucleus ratio significantly. Values are means ± s.d. with triplicates each.
Figure S5 | *Ex vivo* calcium imaging in the developing heart.

Imaging sequence of calcium dependent changes in ΔR/R during a cardiac cycle in a cultured E8.5 embryonic heart at 30°C. Scale bar, 150 µm.
Figure S6 I Microarray analyses comparing gene expression in hippocampus, heart and skeletal muscle for homozygous transgenic (TN-XXL) and wild-type control mice (WT).

(A) Principal component analysis (PCA) illustrates the difference in gene regulation in tissues of TN-XXL mice and controls (heart (H), yellow; hippocampus (B), blue; skeletal muscle (M), red). (B) Venn diagram showing the distribution and intersection of up- and down-regulated genes in TN-XXL compared to WT of the three tissues (cut-off value regulation of at least 2-fold).
Figure S7 I Heart rate histogram of TN-XXL mice (black bars, n = 4) and wild-type mice (white bars, n = 5)

Bin width was 25 beats per minute. Values are means ± s.e.m.
Supplementary Figure S8 | Heart rate variability in TN-XXL mice is reduced.

Representative traces from wild-type control mice (WT) and TN-XXL mice in the time domain (A, B) and in the frequency domain (C) recorded from conscious freely moving mice. (A) ECG traces obtained from WT (upper panel) and TN-XXL mice (lower panel) recorded during a phase of low heart rate (average heart rate < 480 bpm). (B) Raw tachogram of R-R interval recorded over 60 seconds during a phase of low heart rate (average heart rate < 480 bpm) from control (upper panel) and TN-XXL mice (lower panel). (C) Representative frequency domain power spectral density plots obtained from WT (upper panel) and TN-XXL mice (lower panel). LF: low frequency band 0.4-1.5 Hz; HF: high frequency band 1.5-4 Hz.
Supplementary Figure S9 I Spectral analysis of heart rate variability.

Results are illustrated for total power spectra (0 to 4 Hz, TP) and specific frequency bands, i.e. low frequency (LF) (0.4-1.5 Hz) and high frequency (1.5-4 Hz) bands determined during a phase of low heart rate (average heart rate < 480 bpm) (A) and high heart rate (average heart rate >500 bpm) (B). White bars: wild-type control mice (n = 5), black bars: TN-XXL mice (n = 4). Values are means ± s.e.m.
Figure S10 I TN-XXL mice display biatrial enlargement.

Micrograph of wild-type (WT, A) and TN-XXL transgenic mice (B). (C, D) Cell cross-sectional diameter was measured in sections following wheat-germ agglutinin staining showing a significant increase in atrial cell size of TN-XXL mice (D) as compared to wild-type mice (C). Scale bar 15 µm. (E, F) Sirius red fast green staining of atrial sections obtained from TN-XXL transgenic (E) and wild-type mice (F) did not reveal increased fibrotic changes (collagen stained pink; scale bar 50 µm). (G) Population data for atrial cell diameter indicate an increase of 17% in TN-XXL mice. (n = 4 mice, 315 cells, **p < 0.01). White bars: wild-type control mice, black bars: TN-XXL mice. Values are means ± s.e.m. Statistical test was t-test.
Table S1 | Comparison of heart, atrial and body weights of wild-type and TN-XXL mice.

<table>
<thead>
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<th>WT</th>
<th>TN-XXL</th>
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<tr>
<td>n</td>
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<td>7</td>
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<tr>
<td>body weight (g)</td>
<td>23.0 ± 0.38</td>
<td>24.06 ± 0.98</td>
</tr>
<tr>
<td>heart weight (mg)</td>
<td>122.0 ± 2.44</td>
<td>139.0 ± 8.75</td>
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<tr>
<td>right atrial weight (mg)</td>
<td>2.89 ± 0.07</td>
<td>3.83 ± 0.22**</td>
</tr>
<tr>
<td>left atrial weight (mg)</td>
<td>2.34 ± 0.06</td>
<td>3.14 ± 0.17**</td>
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<td>right atrial weight/body weight ratio (mg/g)</td>
<td>0.126 ± 0.004</td>
<td>0.159 ± 0.008**</td>
</tr>
<tr>
<td>left atrial weight/body weight ratio (mg/g)</td>
<td>0.102 ± 0.004</td>
<td>0.131 ± 0.006**</td>
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<tr>
<td>heart weight/body weight ratio (mg/g)</td>
<td>5.31 ± 0.10</td>
<td>5.76 ± 0.20</td>
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Significant differences between wild-type and TN-XXL mice: Values are means ± SEM, **: p≤ 0.01.
Supplementary Methods

Primary cell culture

For cell culture experiments 6 - 8 week old wild-type or transgenic mice were sacrificed by cervical dislocation. Murine kidney fibroblasts (MKF) were obtained from kidneys which were removed and the surface membrane was peeled off. Smooth muscle cells (SMC) were gained from bladder. Kidneys and bladder were transferred to a PBS containing dish and cut into small pieces. The pieces were incubated for 30 min at 37°C in DMEM (Gibco, Wetzlar, Germany) containing 1% Pen/Strep and 2 mg/ml collagenase II. The digested tissue was placed into a cell culture dish containing DMEM + 10% FCS + 1% Pen/Strep. MKF culture was left untouched for one week until the first change of medium and splitting. For SMC the medium was changed the day after preparation.

For the preparation of astrocyte cultures 2 day old pups were sacrificed. The skull was removed and the brain was dissected. The cortices were separated from the brain and placed into HEPES (15 mM) buffered saline solution (HBSS) (Gibco, Wetzlar, Germany). The meninges were removed and the cortices were homogenized. Trypsin-EDTA (2 mg/ml) was added and incubated for 10 min at 37°C. DMEM containing 10% FCS and 1% Pen/Strep was added and the sample was centrifuged. The pellet was resuspended and filtered through a 70 µm cell strainer (BD Falcon, Franklin Lakes, NJ, USA) and plated in T75 cell culture flasks. The medium was changed every 2 - 3 days. After 10 days of incubation the flasks were shaken at 300 rpm for 6 hours at 37°C to separate oligodendrocytes from astrocytes. After changing the medium the shaking process was repeated for additional 24 hours. For all experiments the medium was changed to HBSS
containing 1.8 mM calcium and 1 mM magnesium and either ATP or Carbachol was applied at a final concentration of 10 µM and 100 µM, respectively.

**Determining TN-XXL concentrations inside living cells**

A calibration standard using recombinant ÄKTA-purified TN-XXL of known concentration was prepared and compared to fluorescence intensities of living cells under the confocal microscope (Leica TPC SP5 II, Leica Microsystems, Wetzlar, Germany). Acute slices from hippocampus, hind limb muscle, heart, kidney and liver were imaged with the same imaging settings to evaluate TN-XXL concentration using the calcium insensitive directly excited acceptor fluorescence intensity as a measure (Fig. 2B). To minimize tissue scattering artifacts z-stacks of images were taken from the first two cell layers of the sample (20 µm in z). The mean values of the actual measured brightness for chosen regions of interest (ROI) were converted to concentrations determined by the calibration standard. Chosen ROIs were the cell bodies without the nucleus.

**Preparation of E8.5 hearts**

Pregnant transgenic mice were sacrificed by cervical dislocation. The abdomen was opened, the uterus was cut out and put in a dish containing chilled PBS. The embryos were removed and the hearts were dissected. The hearts were kept in DMEM containing 10% FCS and 1% Pen/Strep and incubated overnight at 37°C with 5% CO₂. The experiments were done the day after dissection. For imaging experiments hearts were transferred into glass bottom dishes (MatTek Corp., MA, USA) containing Tyrode III (140 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂,
5 mM HEPES, 5.5 mM Glucose). The used imaging setup was an inverted fluorescence microscope (iMic, Till Photonics, Germany).

**Preparation and imaging of mesenterial arteries**

Resistance arteries were prepared from the mesenterium from sacrificed mice in MOPS buffer. To avoid radial shrinking with accompanying curling of the vessel wall, vessels were either pulled on two glass capillaries, tied with yarn and pressurized by buffer perfusion, or two metal wires were pushed through the length of the vessel and fastened in a wire myograph system (Danish Myo Technology, Aarhus, Denmark). Calcium increase in endothelial cells was induced by addition of acetylcholine to a final concentration of 3 µM. Microscopy was performed with an inverted Leica Confocal SP5 (Leica Microsystems, Wetzlar, Germany) equipped with a 63x/1.2 NA water immersion objective for use with coverslips. Imaging was performed with the 458 nm laser line of an argon laser. Recording channels were 465 – 505 nm for CFP and 525 – 600 nm for cpCitrine.

**SAN whole mount dissection**

The hearts of 6 - 8 weeks old transgenic mice were put in a dish and the sinoatrial node (SAN) region was cut out along the superior vena cava and the crista terminalis. The dissected SAN were pinned on a custom built stage equipped with a temperature controlled perfusion system and rinsed with Tyrode III solution.

**Immunocytochemistry of tissue sections and SAN explants**

Organs of transgenic mice were fixed with 4% PFA for 2 hours and afterwards treated with 30% sucrose solution until settled to bottom. After sectioning (25 µm) the
tissue slices were stained with DAPI (1:1000). SAN whole mounts were fixed in PBS containing 4% PFA, permeabilized in 0.25% Triton X-100 and incubated in blocking buffer (PBS, 1% Triton X-100, 10% fetal calf serum). Tissues were stained with rat monoclonal antibody to HCN4 (1:300; Santa Cruz Biotechnology, CA, USA) and Alexa 568-conjugated goat anti-rat (1:400; Invitrogen, Karlsruhe, Germany) secondary antibody. All images were acquired using a Leica TPC SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany). For atrial cell size analysis atrial sections were stained with wheat germ agglutinin-tetrarhodamine isothiocyanate (TRITC; 20 µg/ml; Sigma). For characterization of atrial fibrosis, sections were stained with 0.01% Sirius red (Sigma) and 0.1% Fast Green (Sigma).

**Behavioral Testing**

To assess general motor activity, transgenic and wild type (WT) mice were tested in an hole board (L26×W26×H38cm³) contained 16 equidistant holes (5.5 cm apart, 2.5 cm diameter, 3 cm depth) placed in the central zone of the apparatus equipped with infrared beams (TrueScan; Coulbourn Instruments, Allentown, PA) at 300 lux for 30 min. Three infrared sensor rings (sensor spacing, 1.52 cm) around the boxes allowed the measurement of horizontal and vertical (up or down) activity. All sensor rings were connected via interface to a computer equipped with TruScan Software Version 99 (Coulbourn Instruments). Boxes and sensor rings were surrounded by an additional box made of opaque plexiglas side walls (L47 x W47 x H38 cm) without roof and floor. Horizontal locomotion (total distance moved), vertical movements (exploratory rearing), and time spent at rest, frequency of head dipping (the mouse
introduced its nose in a hole) were analyzed during the 30 min monitoring period. After each session, the apparatus was cleaned with a solution containing neutral soap.

The rotarod test, using an accelerating rotarod, was performed by placing mice on rotating drums (3 cm diameter), which is accelerated from 4 to 20 rpm over the course of 5 min. The time at which each animal fell from the drum was recorded automatically when it contacted a plate which stopped the timer. Each animal received three consecutive trials for three consecutive days. Data of the three trials per day were averaged to obtain a single score.

The elevated plus maze (EPM) apparatus consisted of two opposite open arms, (L30 x W5 cm²) and two arms with walls (L30 x W5 x H14 cm³) that were attached to a central platform (L5 x W5 cm²) to form a cross. The maze was elevated 50 cm from the floor. Illumination measured at the center of the maze was 300 lux. The animal was placed at the center of the maze with its nose in the direction of one of the closed arms, and observed for 5 min, according to the following parameters: number of entries in the open and closed arms, and time of permanence in each of them (i.e., the time spent by the animal in the open and closed arms). An entry was defined as all four paws having crossed the line between an arm and the central area. It is accepted that the anxiolytic effect of a drug treatment is illustrated by increased parameters in open arms (time and/or number of entries). The augmented percentage of entries in open arms over the total number of entries in both arms is a good indicator of anxiolytic-like behavior as well, although entries in closed arms and total entries reflect the motor component of the exploratory activity. On removal of each mouse, the maze floor was carefully wiped with a wet towel. The behavior of all
animals was recorded on a tape using a video-camera and then scored in monitor display by an experienced observer by means of a video/computer system.

The setup for the fear conditioning test has been described and displayed in detail before (Kamprath, K. & Wotjak, C. T. Nonassociative learning processes determine expression and extinction of conditioned fear in mice. *Learning & Memory* 11, 770-786 (2004). Briefly, the experiments were performed in two contexts: (1) the neutral test context, which was cylindrically shaped and made of transparent plexiglas, with wood shavings as bedding and (2) the shock context was a cubic-shaped box with a metal grid for shock application. All two contexts were cleaned thoroughly after each trial with differently smelling detergents, and bedding was changed. For conditioning (d0), mice were placed into the shock chamber. After 180 s a tone was presented (9 kHz, 80 dB, 20 s), which co-terminated with a single scrambled electric foot shock (2 s, 0.7 mA). Animals remained in the shock chamber for another 60 s before they were returned to their home cages. On day 1 and day 2 after conditioning, mice were exposed either to the tone (200 s) in the neutral test context (d1) or to the conditioning context (180 s). The behavioral performance was videotaped by small CCD cameras (Conrad Electronics, Hirschau, Germany). Animals' behavior was rated off-line by a trained observer. Freezing behavior was defined as immobility except for respiration movements and served as a measure of fear memory.

Social interaction tests were performed in a novel environment (new cage with fresh bedding) at 300 lux that was placed into a soundproof isolation cubicle. The lid of the new cage was removed and the walls elongated by 12.5 cm of semi transparent plastic. Pairs of unfamiliar mice (n=7 pairs of TN-XXL transgenic or WT) were placed into the cage per 5 min. The time spent in social interaction (active contact such as sniffing, licking, close following, grooming) was recorded for each pair of mice.
To test for hyperarousal, acoustic startle responses were measured in the following way: mice were placed into one out of seven identical startle set-ups, consisting of a non-restrictive Plexiglas cylinder (inner diameter 4 cm, length 8 cm) mounted onto a plastic platform, each housed in a sound attenuated chamber (SRLAB, San Diego Instruments SDI, San Diego, CA, USA). The cylinder movement was detected by a piezoelectric element mounted under each platform and the voltage output of the piezo was amplified and then digitized (sampling rate 1 kHz) by a computer interface (I/O-board provided by SDI). The startle amplitude was defined as the peak voltage output within the first 50 ms after stimulus onset and quantified by means of SRLAB software. Before startle measurements, we calibrated response sensitivities for each chamber in order to assure identical output levels. Startle stimuli and background noise were delivered through a high-frequency speaker placed 20 cm above each cage. The 3 different startle stimuli consisted of white noise bursts of 20 ms duration and 75, 90, 105 and 115 dB(A), respectively, and were presented in a constant background noise of 50 dB(A) provided by the fan. Noise intensity was measured using an audiometer (Radio Shack, 33-2055, RadioShack, Fort Worth, TX, USA). On control trials only background noise was present. After an acclimation period of 5 min duration, 10 control trials and 20 startle stimuli of each intensity were presented in pseudorandom order in each test session. The interstimulus interval was 15 s averaged (13-17 s, pseudorandomized). The startle set-ups were localized in a different building and startle measurements were performed by a scientist unfamiliar to the animals in order to avoid context reminders, thus minimizing confounding influences by context generalization. Plexiglas cylinders were cleaned thoroughly with soap water after each trial.
For the tail Suspension test (FST), mice were individually suspended by the tail from a metal rod (35 cm above the floor) using adhesive tape. Mice demonstrated several escape-oriented behaviors interspersed with temporally increasing bouts of immobility. The duration of immobility was recorded during the last 4-min of the 6-min testing period, after a 2-min habituation period from videotapes.

**Analysis of heart rate variability**

Heart rate variability (HRV) was determined using an analysis based on previous reports (Mitchell, G.F., Jeron, A., & Koren, G. Measurement of heart rate and Q-T interval in the conscious mouse. *American Journal of Physiology-Heart and Circulatory Physiology* **274**, H747-H751 (1998); Wickman, K., Nemec, J., Gendler, S.J., & Clapham, D.E. Abnormal heart rate regulation in GIRK4 knockout mice. *Neuron* **20**, 103-114 (1998); Ecker, P.M., Lin, C.C., Powers, J., Kobilka, B.K., Dubin, A.M., & Bernstein, D. Effect of targeted deletions of beta(1)- and beta(2)-adrenergic-receptor subtypes on heart rate variability. *American Journal of Physiology-Heart and Circulatory Physiology* **290**, H192-H199 (2006)). We compared HRV of wild-type and TN-XXL mice during a phase of relatively low heart rate (average heart rate < 480 bpm) corresponding to a RR-interval of 125 ms and during a phase of relatively high heart rate (average heart rate > 600 bpm) corresponding to an average RR interval of 100 ms. Mean HR was determined over 24 h from the mean heart rate calculated for 60 s every 30 min. The raw ECG strip was manually inspected to confirm stable sinus rhythm and ectopic beats were excluded, no averaged or interpolated beats were used to replace them. Subsequently, Dataquest A.R.T. 4.0 data analysis software was used to detect the R peaks of the ECG signal and to calculate the RR intervals. Resulting RR data was
used to calculate HRV data in both frequency and time domains using standard HRV parameters: In the time domain, the 60 s time series of RR intervals were plotted as tachograms. For frequency domain analysis these tachograms were interpolated by cubic spline interpolation at 50 ms intervals to create equidistant points suitable for Fast Fourier Transform (FFT). Detrending was used to remove potential trends. Subsequently, FFT using 1024 spectral points and a half overlap within a Hanning window was performed and power spectral density plots were determined. For each time segment, the total power (TP) (s²/Hz) was calculated as the integral sum of total variability after FFT over the frequency range recorded (0–4.0 Hz). In addition, for each time segment, the cutoff frequencies previously determined to be accurate for mice were used to divide signal into three major components, very low frequency (VLF:0.015-0.4 Hz), low frequency (LF 0.4–1.5 Hz), and high frequency (HF 1.5–4.0 Hz). The data obtained for each time segment were averaged. In TN-XXL mice adaptive changes in heart rate between minimal heart rate and maximal heart rate were significantly slower than in wild type controls. These differences were more pronounced during phases of slow heart rate. Spectral analysis demonstrated reduced total power, low (LF; 0.4-1.5 Hz) and high frequency (HF; 1.5-4 Hz) components in TN-XXL mice as compared to wild-type controls during phases of slow heart rate (Supplementary Fig. S8C and Fig S9A) and fast heart rate (Supplementary Fig. S9B). Analysis of the heart rate variability indicates that autonomic regulation of dynamic heart rate oscillations is significantly reduced in TN-XXL mice. Since the increase in pacemaker potential is dependent on intracellular Ca²⁺ it is well possible that an increase of intracellular Ca²⁺ buffering significantly reduces the dynamic range of heart rate variability in TN-XXL mice by damping and filtering oscillations in RR interval and stabilizing the heart rate. Besides increased
intracellular Ca\textsuperscript{2+} buffering in the SA node, other possibilities for the reduced dynamic heart rate oscillations in TN-XXL mice such as more generalized atrial conduction changes or central or peripheral nervous system alterations, even though unlikely, cannot completely be ruled out.