Supplemental files

Serum Response Factor (SRF) ablation interferes with acute stress associated immediate and long-term coping mechanisms

Annemarie Zimprich, Gabi Mroz, Christopher Meyer zu Reckendorf, Sofia Anastasiadou, Philip Förstner, Lillian Garrett, Sabine M. Hölte, Lore Becker, Jan Rozman, Cornelia Prehn, Birgit Rathkolb, Kristin Moreth, Wolfgang Wurst, Thomas Klopstock, Martin Klingenspor, Jerzy Adamski, Eckhard Wolf, Raffi Bekeredjian, Helmut Fuchs, Valerie Gailus-Durner, Martin Hrabe de Angelis, Bernd Knöll

SUPPLEMENTAL FIGURES

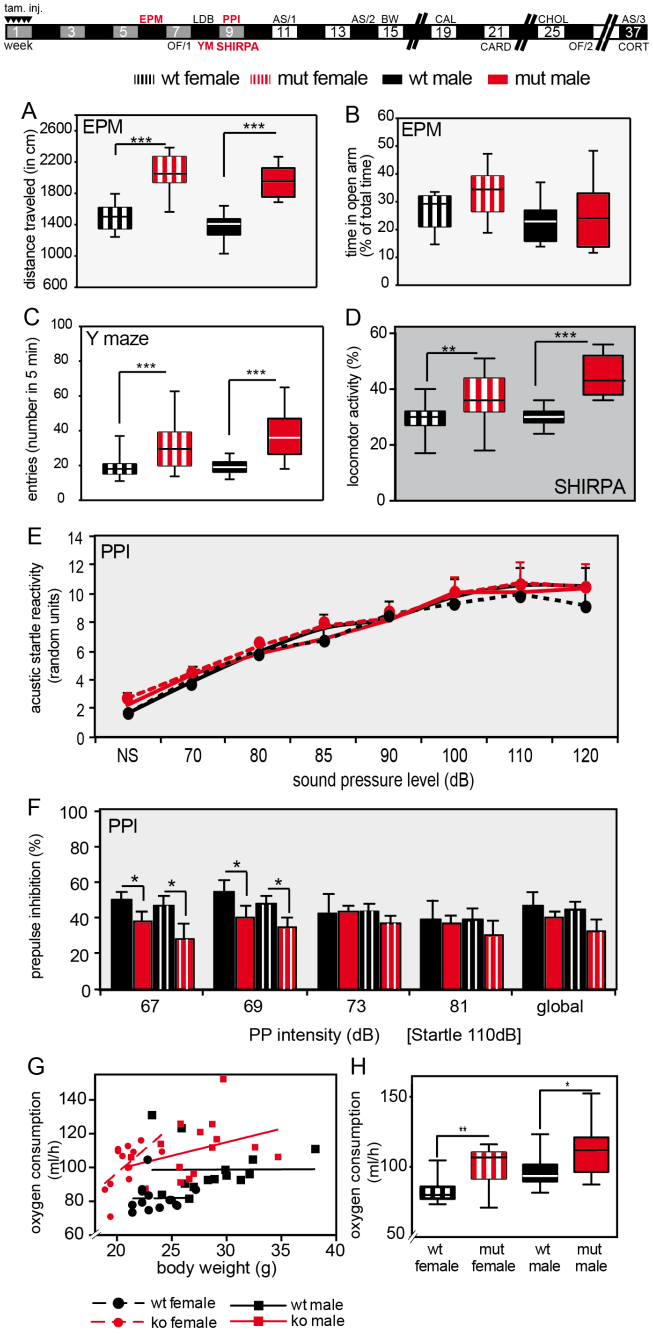

Figure S1

***Srf* mutant mice show enhanced locomotion, reduced PPI and elevated oxygen consumption**

(A, B) In the elevated plus maze (EPM), overall distance traveled was elevated for both female and male *Srf* mutants (A). Time spent in the open arm was slightly enhanced for mutant females compared to wt females, but no genotype effect was observed for males (B).

(C) In the Y maze, *Srf* mutant females and males had more entries indicating elevated locomotor activity.

(D) In the SHIRPA test, *Srf* mutant animals regardless of sex showed significantly increased locomotor activity.

(E, F) In the PPI, acoustic startle reactivity at several sound pressure levels was comparable between genotypes. PPI was significantly reduced in male and female *Srf* mutant mice at PPI intensities of 67 and 69 dB.

(G) Scatterplot of oxygen consumption vs. body weight of wt (black) and *Srf* mutant (red) animals. Red or black circles depict females, whereas red or black squares indicate males. *Srf* mutant females had elevated oxygen consumption compared to wt females. Also, body weight of female mutant animals was slightly decreased compared to wt females. In male animals, similar observations were made, however differences were less pronounced than in females (see also (B)).

(H) Box plot showing significantly elevated oxygen consumption in mutant females and males compared to wt.

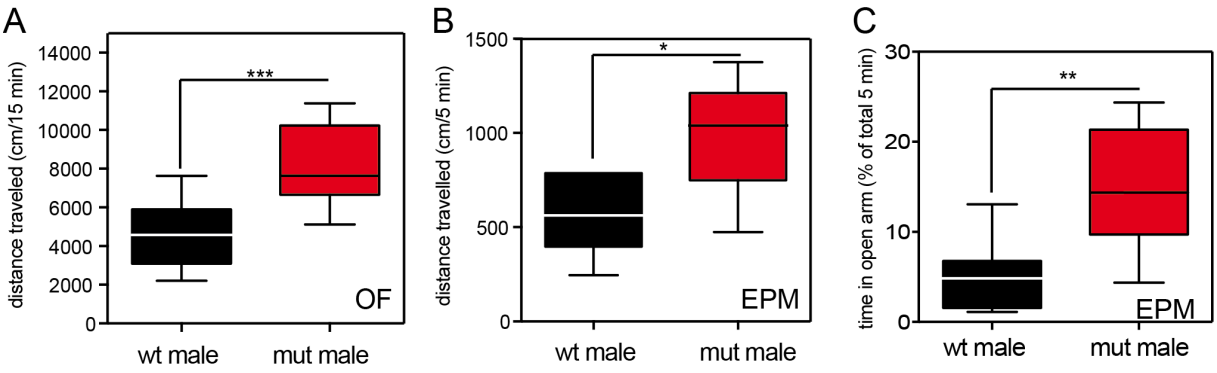

Figure S2

SRF deficient mice show hyperactivity and decreased anxiety independent of the test battery

We tested an independent cohort of male mice (N>7 each genotype) in the OF (A) and EPM (B, C) in the behavioral facility at Ulm University.

(A) In the OF, *Srf* mutant mice were hyperactive compared to wt animals.

(B) Hyperactivity of *Srf* mutant mice was also observed in the EPM.

(C) SRF deficient animals had decreased anxiety related behavior as revealed by more time spent in the open arms of the EPM.

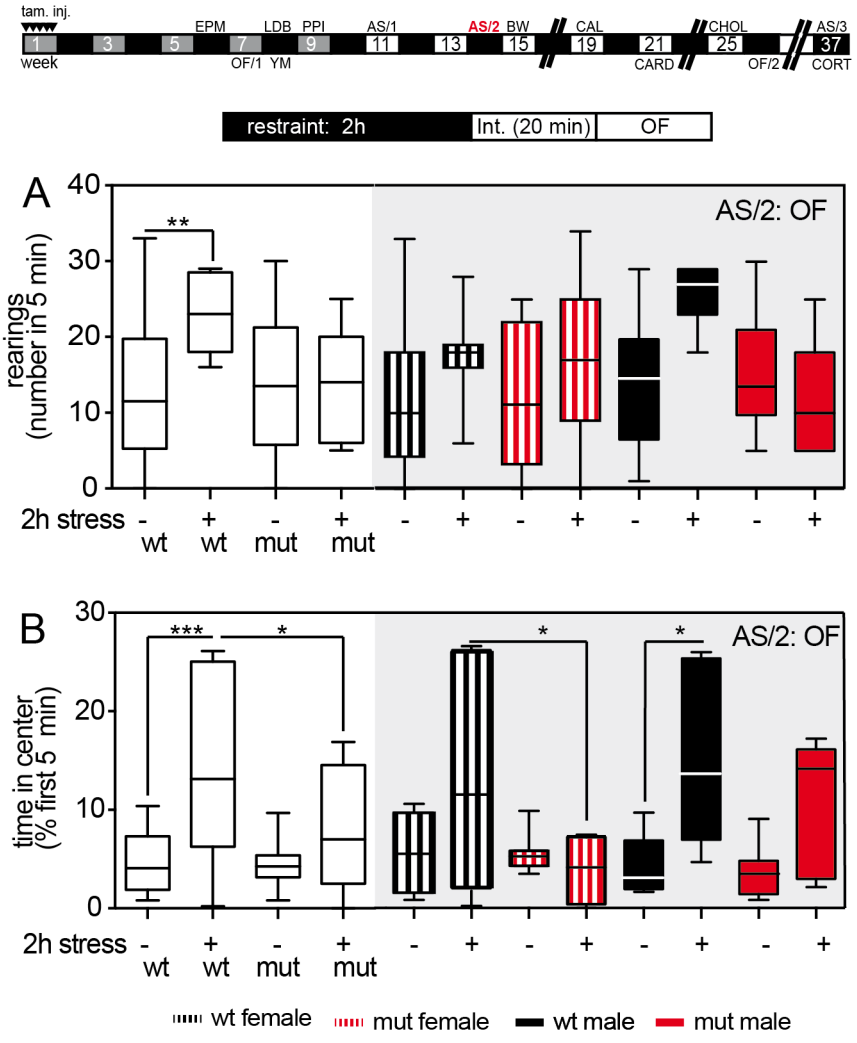

Figure S3

Acute stress enhances exploratory behavior in wt but not *Srf* mutant animals

After 2h of restraint stress (AS/2), animals were analyzed in the OF for number of rearings (A) and time spent in the OF center (B). White bars show pooled data of females and males, whereas patterned and solid boxes show results from female and male animals, respectively.

(A) In wt mice, the number of rearings doubled after stress exposure, irrespective of gender. In contrast, *Srf* mutant mice failed to enhance rearing frequency.

(B) Similar to rearings, wt mice responded to 2h AS with elevated time in the OF center, whereas this was not observed as clearly in *Srf* mutant animals.

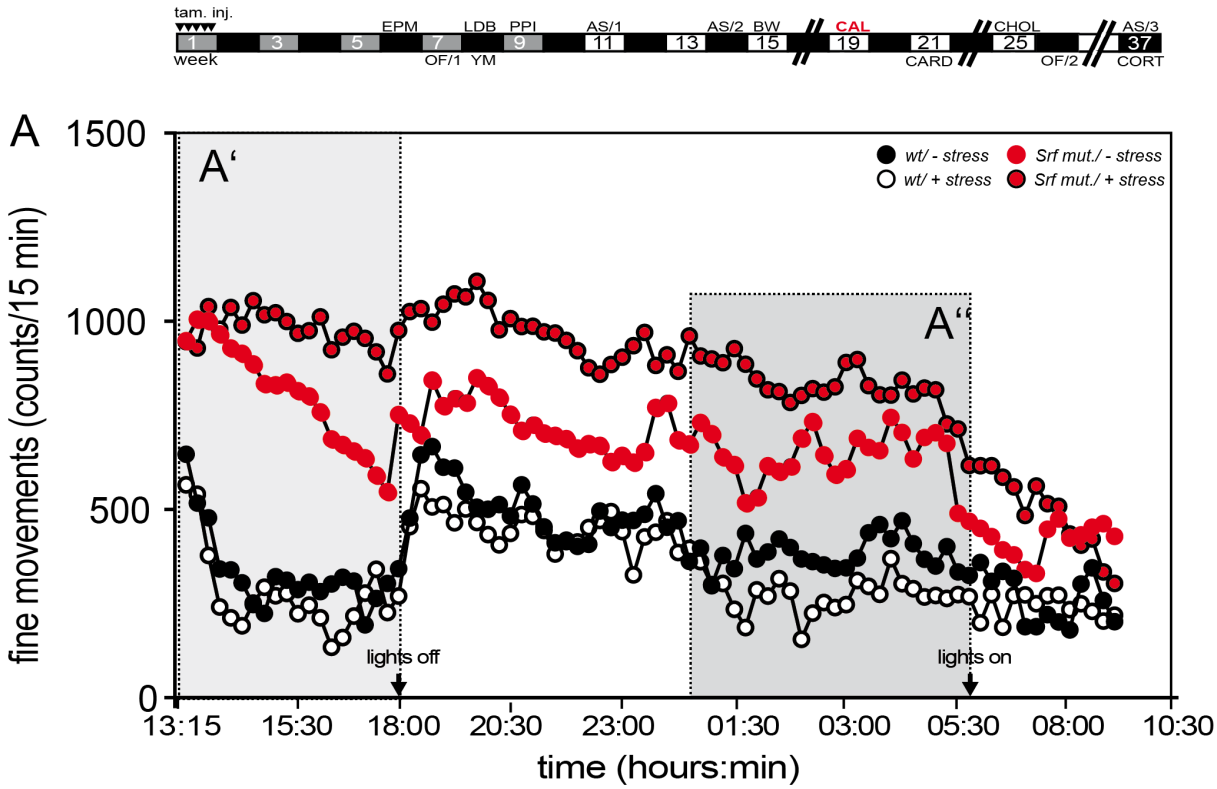

Figure S4

***Srf* deficiency modulates locomotor behavior in the absence and presence of a previous AS**

Five weeks after AS exposure (AS/1 and AS/2), mice were analyzed for 21h in calorimetric cages (CAL) regarding locomotor behavior. Recordings were taken every 15 minutes over the 21h period. Data points (circles) show average values of both female and male animals.

(A) The number of fine movements (a combination of distance traveled and the number of rearings per time interval) was elevated over the entire 21h period in *Srf* mutant mice compared to wt mice regardless of stress exposure. In wt mice, previous AS resulted in decreased locomotor activity in two time intervals: in the first three hours until lights were shut off (grey area A’ in A) and from approximately 24.00 until lights were switched on (grey area A’’). In both time intervals, stressed wt mice decreased fine movements compared to unstressed wt animals. Interestingly, in *Srf* mutant mice, the opposite result was observed. Here, previous AS experience elevated the locomotor activity in stressed mutant compared to unstressed mutant mice. This was observed not only in the two time intervals, but also throughout the 21h observation period. Identical results were obtained when only the total distance travelled was recorded (data not shown).

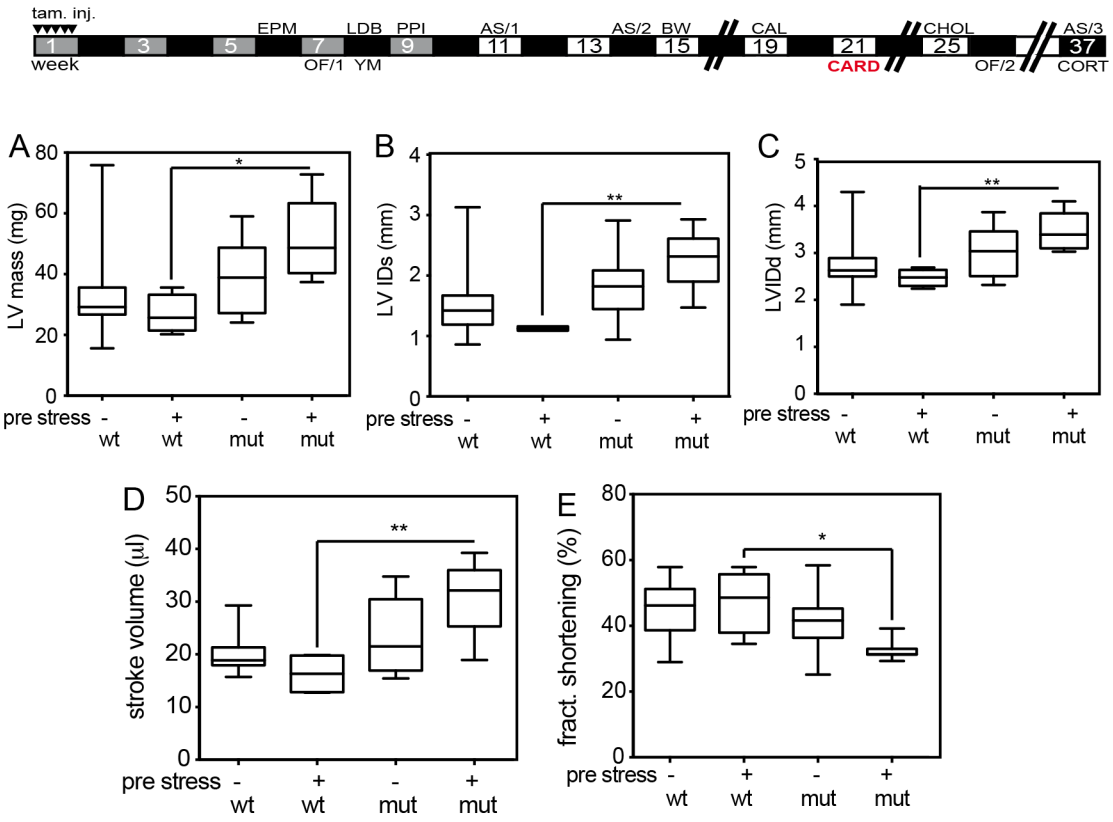

Figure S5

Previous acute stress modulates cardiovascular parameters in *Srf* mutant mice

Seven weeks after AS, animals were analyzed by echo-cardiography (see timeline). Data were compiled from female and male animals (wt -stress: N = 14 ; wt +stress: N = 4; mut -stress: N = 12; mut +stress: N = 8).

(A-C) In unstressed *Srf* mutant animals, left ventricular (LV) mass was elevated compared to unstressed wt animals (A). Previous stress exposure decreased LV mass slightly in wt animals compared to unstressed wt animals (A). In contrast, *Srf* mutant animals with previous stress experience responded with elevated LV mass compared to unstressed mutant animals (A). Similar alterations observed for LV mass (A) were made for the cardiovascular parameters Left ventricular internal diameter systole (LVIDs; B) and diastole (LVIDd; C).

(D) The stroke volume was slightly decreased in wt animals with previous stress exposure in relation to unstressed wt animals. On the contrary, in *Srf* mutant animals, previous AS exposure induced a trend towards an enhanced stroke volume compared to unstressed mutant animals.

(E) In wt mice, fractional shortening was indistinguishable between unstressed and stressed mice. In contrast, AS reduced fractional shortening in stressed mutant animals indicating impaired heart function.

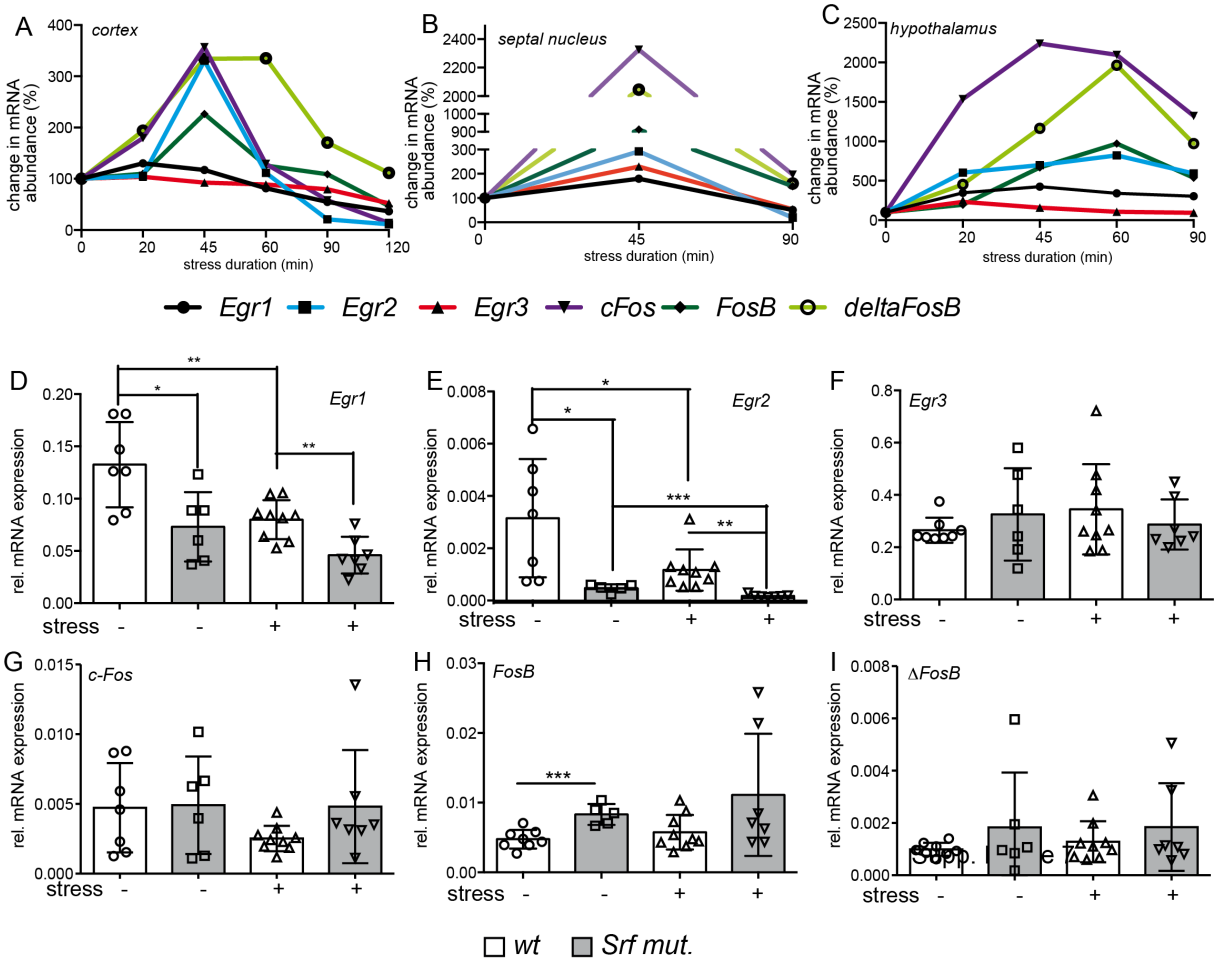

Figure S6

Temporal resolution of stress related gene induction in different brain regions of wt mice

Adult wt mice were subjected to AS for time points indicated. Subsequently, mRNA was prepared from the cortex (A), septal nucleus (B) or hypothalamus (C) and qPCR analysis for stress related genes was performed.

(A) All genes inspected, with the exception of *Egr3*, were induced upon 20 min and, more pronounced, after 45 min of AS. Beginning with 60 min and more clearly after 90 and 120 min of AS, all stress related genes with the exception of *ΔFosB* were down-regulated below levels observed in unstressed wt mice.

(B) Similar to cortex (A), in the septal nucleus, a peak in stress related gene expression was observed at 45 min. After 90 minutes of stress exposure, a decrease in gene expression was observed.

(C) In the hypothalamus, we observed a constant increase in stress related gene expression from 20 min to 60 min after AS. At 90 min, gene expression was declining for the most genes.

(D-I) Wildtype (white bars) and SRF deficient (grey bars) animals were stressed for 2h, followed by qPCR analysis of cortical tissue for genes indicated.

(D, E) *Egr1* (D) and *Egr2* (E) mRNA levels were down-regulated after 2h of restraint stress in wt animals. In unstressed and stressed *Srf* mutant animals, mRNA levels for *Egr1* and *Egr2* were reduced compared to wt.

(F) *Egr3* is unaltered by 2h stress and not affected by SRF deficiency in the cortex.

(G) *c-Fos* mRNA was down-regulated in wt but not *Srf* mutant animals after stress.

(H, I) *FosB* (H) and *ΔFosB* (I) mRNA levels were not modulated by 2h stress in wt cortex. In *Srf* mutant animals, both genes were elevated in non-stressed and stressed condition.

Each circle, square and triangle represents one animal.

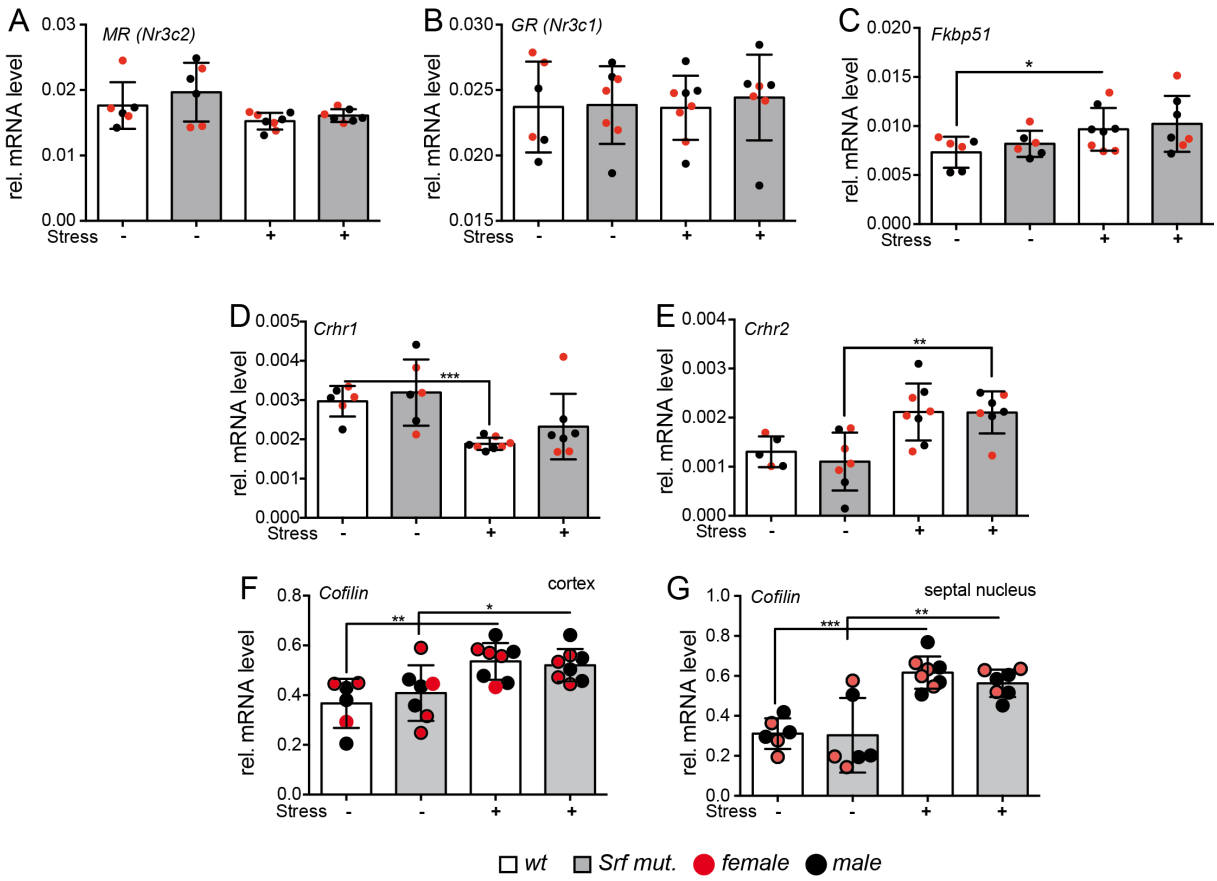

Figure S7

mRNA levels of HPA axis encoding proteins are not affected by SRF deletion

(A-E) Wt (white bars) and *Srf* mutant (grey bars) mice were subjected to 45 min AS followed by preparation of cDNA from the hypothalamic region (A-E), cortex (F) or septal nucleus (G). Subsequently, qPCR was performed for genes encoding the mineralcorticoid receptor (*MR*; A), the glucocorticoid receptor (*GR*; B), the FK506-binding protein 51 (*Fkbp51*; C), the CRH releasing hormone receptor 1 (*Crhr1*; D) and 2 *(Crhr2*; E) and *cofilin* (F, G). Red circles indicate female and black circles male mice.

(A) *MR* mRNA levels were slightly decreased in stressed wt and mutant animals compared to unstressed littermates.

(B) *GR* levels were not altered by stress exposure or SRF deficiency.

(C) *Fkbp51* was slightly induced by the stress challenge, however comparably in wt and mutant animals.

(D, E) *Crhr1* (D) and *Crhr2* (E) were down- and up-regulated by the stress exposure, respectively. However, no genotype dependent effect was observed.

(F, G) *Cofilin* mRNA was up-regulated by AS in cortex (F) and septal nucleus (G) irrespective of genotype.

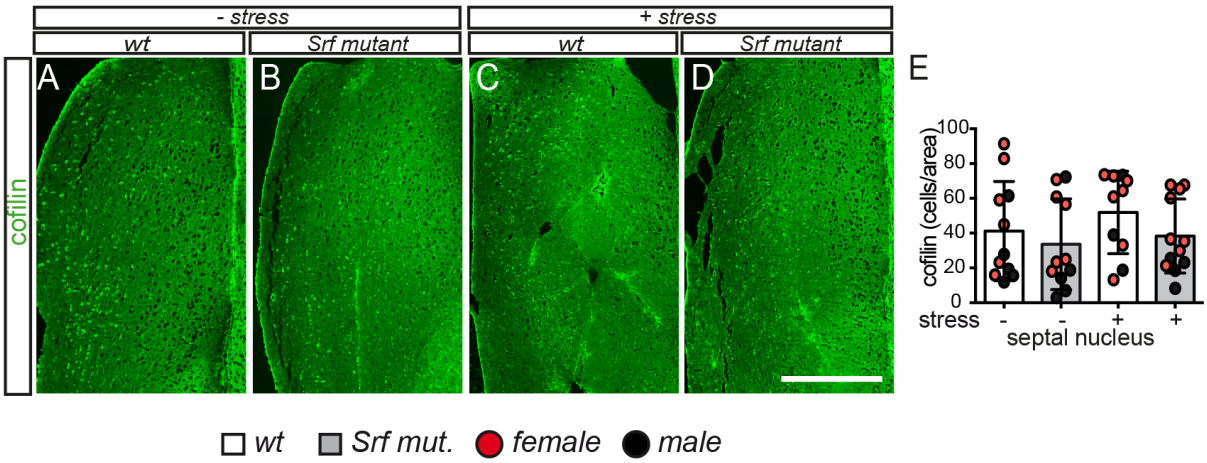

Figure S8
Total cofilin levels are unaltered by AS
(A-D) Septal nuclei of unstressed wt (A) and *Srf* mutant (B) as well as 45 minutes stressed wt (C) and *Srf* mutant (D) animals were stained for total cofilin abundance. No obvious differences in total number of cofilin positive cells were observed.
(E) Quantification of cofilin positive cells per area in the septal nucleus.
Red circles indicate female and black circles male mice.
Scale-bar (A-D) = 0.5 mm

SUPPLEMENTAL MATERIALS & METHODS

Housing

Mice were housed in GM500 cages in individually ventilated caging (IVC) systems (IVC System Green Line, Tecniplast, Italy) on wood fiber (Altromin, Lage, Germany). The IVCs operate with positive pressure. Mice were transferred in weekly intervals to new cages in Laminar Flow Class II changing stations. Mice were fed with irradiated standard rodent breeding diet (Altromin 1314) and given semi-demineralized filtered (0.2 µm) water *ad libitum*. Light was adjusted to a 12h/12h light/dark cycle; temperature and relative humidity are regulated to 22 ± 1 °C and 55 ± 5 %, respectively.

Behavioral tests

Open Field (OF)

The Open Field analysis was carried out as described previously [1-3]. It consisted of a transparent and infra-red light permeable acrylic test arena with a smooth floor (internal measurements: 45.5 × 45.5 × 39.5 cm). Illumination levels were set at approx. 150 lux in the corners and 200 lux in the middle of the test arena. Data were recorded and analyzed using the ActiMot system (TSE, Bad Homburg, Germany). At the beginning of the experiment, all animals were transported to the test room and left undisturbed for at least 30 minutes. Then each animal was placed individually into the middle of one side of the arena facing the wall and allowed to explore it freely for 20 min. For data analysis, the arena was divided by the computer in two areas, the periphery defined as a corridor of 8 cm width along the walls and the remaining area representing the center of the arena (42% of the total arena).

The following parameters were recorded: distance traveled, resting and permanence time as well as speed of movement for the whole arena, the periphery and the center. Additionally, rearing frequency, percentage distance traveled and percentage time spent in the center as well as the latency to first entry in center and center entry frequency were calculated. The time courses of distance traveled, rearing frequencies as well as percentage distance traveled and percentage time spent in the center were additionally analyzed in 5 min intervals.

Light/Dark Box (LDB)

The test box was made of plexiglas and divided into two compartments, connected by a small tunnel (4.5 × 5.6 × 13 cm high). The lit compartment (26.1 × 22.6 × 26 cm high) was made of transparent plexiglas and was illuminated by cold light with an intensity of 650 lux in the middle; the dark compartment (14 × 22.6 × 26 cm high) was opaque, with a lid and not directly illuminated (approx. 5 lux in the center). The mouse was placed in the center of the dark compartment facing the hind wall and allowed to freely explore the apparatus for 5 min. Data were recorded and analyzed using the ActiMot infrared beam break system (TSE, Bad Homburg, Germany).

Y Maze (YM)

Spontaneous alternations (SA) were assessed using the Y Maze, which was made of opaque light gray PVC and had three identical arms (29.5 × 8 × 15 cm) placed at 120° from each other; illumination in the center of the maze was 100 lux [4]. Each mouse was placed at the end of one arm and allowed to move freely through the maze during a 5 min session. SAs (defined as consecutive entries into all three arms without repetitions in overlapping triplet sets) were scored. Total numbers of arm entries were accumulated over the 5 min session. The latency to leave the starting arm is noted as well as the sequence of arm entries. After each trial the arena is cleaned and dried. The amount of spontaneous alternations (e.g. ABC), alternate arm returns (e.g. ABA) and same arm returns (e.g. AAB) are analyzed and calculated as percentage of the total number of triplets. When placed in the Y Maze, normal mice prefer to explore the least recently visited arm, and thus tend to alternate visits between the three arms. To explore the three arms successively, the mouse must maintain an ongoing record of the most recently visited arms, and continuously update such records. Therefore alternation behavior is a measure of spatial working memory.

Elevated Plus Maze (EPM)

The GMC EPM arena consists of four connected arms arranged in a plus shape elevated above the floor (70 cm). Two opposing arms are enclosed by walls (15 cm in height), whereas the other two are only surrounded by a small rim (0.5 cm in height). The open arms are lit with 300 lux at the ends, the light intensity in the enclosed arms measures 35 lux. The dimensions of the maze are 66 cm in its total length and 6 cm in the width of the arms. Above the arena a camera is mounted which detects the mouse via video tracking (EthoVision system, Version 3.1.16, Noldus, Wageningen, The Netherlands). Animals were put on the arena into an enclosed arm facing the back wall and left to explore the arena for 5 minutes. A number of parameters were recorded, including time spent in each compartment, distance travelled, entries and speed. After the trial the arena was cleaned and dried before the next animal was started. The time an animal spent in the open arms is taken as an indicator of anxiety-related behaviors.

Entry into an arm of the EPM was defined using the base of the tail (Fig. S1) or head (Fig. S2) as the tracking position. The percentage spent exploring the open arms was calculated by dividing the time spent in the open arms by the combined time spent in open and closed arms.

Prepulse inhibition (PPI)
PPI was assessed using a startle apparatus setup (Med Associates Inc., VT, USA) including eight identical sound-attenuating cubicles. The protocols were written using the Med Associates "Advanced Startle" software. Experiments were carried out between 08:30h and 17:00h. Background noise (NS = no stimulus) was 65 db and trial types for Acoustic Startle Response (ASR) included 7 different stimulus intensities (70, 80, 85, 90, 100, 110, 120 db). Trial types for PPI included 4 different prepulse intensities (67, 69, 73, 81 db), and each prepulse (12 kHz tone, 10 msec duration) preceded the startle pulse (110 db, white noise, 40 msec duration) by a 50 milliseconds inter-stimulus interval. Each trial type was presented 10 times in random order, organized in 10 blocks, each trial type occurring once per block. Inter-trial intervals varied from 20-30 sec. A session was initiated with a 5 min acclimation period followed by five presentations of leader startle pulses (110 dB) that were excluded from statistical analysis. This protocol is based on the Eumorphia protocol (see www.eumorphia.org), adapted to the specifications of our startle equipment.

SHIRPA
We examined the mice using 23 designed test parameters (See web page: http://www.har.mrc.ac.uk/services/phenotyping/neurology/shirpa.html) to detect phenotypic differences between mutant and control mice. Each test parameter contributes to an overall assessment in muscle, lower motor neuron, spinocerebellar, sensory and autonomic function and is scored qualitatively after a defined rating scale. Assessment of each animal began with observation of undisturbed behavior (Viewing Jar Behavior) in a glass cylinder (11 cm in diameter). The mice were then transferred to an arena consisting of a clear Perspex box (420 x 260 x 180 mm) in which a Perspex sheet on the floor is marked with 15 squares. Locomotor activity and motor behavior within this area was observed (Behavior recorded in the Arena). This was followed by a sequence of manipulations testing reflexes (behavior recorded on or above the arena). Measurements were completed with the recording of body weight. The last part of the primary screen also involved the analysis of contact righting reflex. Throughout the entire procedure, abnormal behavior, biting, defecation, and vocalization were recorded.

Acute stress (AS)
Mice were transferred to the behavioral testing room at least 30 min before the first test to acclimatize. Mice were assigned to one of two groups: either control or stress group [3]. Animals of the stress group were restrained in well-ventilated 50 ml falcon tubes and left undisturbed under an opaque box (25 × 12 × 8.5 cm) in a separate room from the control group for the duration of the stress (i.e., 15 min or 2 h). After the restraint period, the mouse was transferred into a clean animal housing cage for a 20 min interval and thereafter went through a 10 min OF testing. Control animals were taken directly from their housing cage into the OF test arena. For molecular analyses, animals were stressed for 45 min and immediately sacrificed thereafter.

Beam walk (BW)
Beams with different diameters (20 mm square and 22 mm, 12 mm 15 mm round) were used. The traversing time and numbers of falls, foot slips and stops were recorded. The mouse performed three trials each consecutively and the average time of these three trials were calculated.

Cholesterol (CHOL) and CORT levels
Blood samples were taken from isoflurane-anesthetized mice by puncturing the retro-bulbar sinus. Blood samples collected from *ad libitum* fed mice were divided into two portions. The major portion was collected in a heparinized tube (Li-heparin, KABE; Nümbrecht, Germany; Art.No. 078028). The smaller portion was collected in an EDTA-coated tube (KABE, Art.No 078035). Heparinized blood collected from ad libitum fed mice was left in a rack at room temperature for one to two hours. Afterwards, cells and plasma were separated by a centrifugation step (10 min, 5000 × g; 8 °C, Biofuge fresco, Heraeus; Hanau, Germany). Plasma aliquots for clinical chemistry analyses were transferred into Eppendorf tubes and diluted 1:2 with deionized water. The solution was mixed for a few seconds to prevent clotting and then centrifuged again for 10 min at 5000 x g at 8 °C. Cholesterol levels were measured using an Beckman-Coulter AU480 chemistry analyzer as described previously [5]. For quantification of plasma corticosterone (CORT), trunk blood was taken after decapitation for the last time point. The blood was collected into EDTA-Microvettes (Sarstedt, Germany) and left to coagulate. After centrifugation, the supernatant was collected and stored at -20°C until further processing. CORT-concentrations were measured out of 20 µL of the EDTA plasma using a validated high-throughput LC-MS/MS method (HPLC: Shimadzu Prominence, Shimadzu Deutschland GmbH, Duisburg, Germany ; MS/MS: 4000 QTRAP, Sciex Deutschland GmbH, Darmstadt, Germany). Sample preparation and LC-MS/MS measurements have been described in detail [5]. Concentrations are reported in nmol/L and have been calculated using stable

isotope-labeled internal standards and reference standard curves. The sensitivity of the tests was 2.8 nmol/L for CORT.

Indirect calorimetry (CAL)

For energy expenditure monitoring, mice were individually housed with *ad libitum* access to food and water. Using an open respirometer system for indirect calorimetry (PhenoMaster System; TSE Systems, Bad Homburg, Germany), oxygen consumed (VO_2) and carbon dioxide produced (VCO_2) were determined every 15 min. High precision CO_2 and O_2 sensors measure the difference in CO_2 and O_2 concentrations in air volumes flowing through control or animal cages [6]. The RER is calculated as the ratio VCO_2/VO_2. Heat production (HP) is calculated from VO_2 and RER using the formula: $HP[mW] = (4.44 + 1.43 \cdot RER) \cdot VO_2 [ml \cdot h^{-1}]$

The test is performed at regular room temperature (23 °C) with a 12:12 hours light/dark cycle in the room (lights on 06:00 CET, lights off 18:00 CET). Paper tissue is provided as bedding material. Each mouse is placed individually in the chamber for a period of 21 hours (from 13:00 CET to 10:00 CET next day following the IMPRESS standard operating protocol) with free access to food and water. Metabolic cuvettes are set up in a ventilated cabinet continuously supplied with an overflow of fresh air from outside.

In addition, body mass before and after gas exchange measurements are taken. Before returning the mice to their home cage, rectal body temperature is also determined. Food and water uptake was monitored by continuously weighing containers that are attached to electronic scales. Physical activity is measured by infrared light beam frames set up around the cages as x- and y- axes (Actimot, TSE Systems, Bad Homburg, Germany). This system allows the measurement of distance traveled and the number of rearings per time interval. In addition, fine movements were quantified as follows: two consecutive breaks of a single beam of light at the lower level X or Y. The counter records the interruption of a light barrier that has been interrupted for the second time in succession without a different light barrier having first been interrupted.

Cardiology (CARD)

Left ventricular function was evaluated with transthoracic echocardiography using a Vevo 2100 Imaging System (Visual Sonics) with a 30MHz probe. Examinations were performed on conscious animals. Left ventricular parasternal short and long-axis views were obtained in B-mode imaging and left ventricular parasternal short-axis views were obtained in M-mode imaging at the papillary muscle level. The short axis M-mode images were used to measure left ventricular end-diastolic internal diameter (LVEDD), left ventricular end-systolic internal diameter (LVESD), diastolic and systolic septal wall thickness (IVS) and diastolic and systolic posterior wall thickness (LVPW) in three consecutive beats according to the American Society of Echocardiography leading edge method. Fractional shortening (FS) was calculated as FS%=[(LVEDD-LVESD)/LVEDD] x 100. The corrected left ventricular mass (LV MassCor) was calculated as LV MassCor = 0.8 (1.053 x ((LVIDD + LVPWD + IVSD)3 - LVIDD3)). The stroke volume (SV) is the volume of blood pumped from one ventricle of the heart with each beat. The stroke volume of the left ventricle was obtained by subtracting end-systolic volume (ESV) from end-diastolic volume (EDV).

Chromatin immunoprecipitation (ChIP)

ChIP was performed according to the protocol of Nelson et al. [7]. Tissue pieces prepared from tissue chopper slices were fixed for 15 min at room temperature with 1% formaldehyde (FA) in PBS. 5 µg/ml anti-SRF (Santa Cruz; SC-335, rabbit polyclonal) or IgG antibody were used for each IP. After purification of DNA (PCR purification kit, Qiagen), 2 µl of each input or IP were subjected to qPCR. Ct values obtained with SRF or IgG ChIP were normalized to the respective input values.

The following primer sequences were used:

Egr1 fwd	ccc acc act ctt gga tgg gag ggc ttc ac
Egr1 rev	tcg gcc tct att tca agg gtc tgg aac agc
Egr2 fwd	gtg aag gag ggg ttt gac tg
Egr2 rev	att ccg gtt ctc cga gac tt
Egr3 fwd	gag gag ctt cca ttg tga cg
Egr3 rev	cca ggg aac tcc acc agt c
FosB fwd	ccc tct gac gta att gct agg
FosB rev	cca agg ctg cgt tct gag
cFos fwd	tcc ctc cct cct tta cac ag
cFos rev	gtg tag gat ttc gga gat ggt c

Quantitative real-time PCR (qPCR)

For RNA isolation from brain regions (septal nucleus, cortex and hypothalamus), we employed the ISOLATE II RNA/DNA/Protein kit (Bioline) according to manufacturer's instructions. Reverse transcription was performed with 500 ng (hypothalamus, septal nucleus) or 1 µg (cortex) RNA using reverse transcriptase (Promega) and random hexamers. We performed qPCR on the Light Cycler 480II (Roche) with the Power PCR SYBR green PCR master mix (Takara). Typically, 2 ml of cDNA were used in a 10 ml reaction volume/well of a 96-well

plate. Doublets were performed for each sample. At a certain point, the PCR product starts to accumulate and the detected fluorescence begins to increase exponentially. The LC480 II Software detects this threshold cycle value (Ct value) for each sample. The higher the Ct value, the lower the original cDNA amount for a certain gene in the sample. In order to neutralize potential variations in total mRNA amounts used for the cDNA synthesis, the Ct values of the house-keeping gene *Gapdh* (glycerinaldehyd-3-phosphat-dehydrogenase) were used for normalization according to the following equation: relative mRNA expression = $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{target\ gene} - Ct_{Gapdh}$). Primer sequences can be obtained upon request.

Immunohistochemistry
Brains were dissected in ice-cold PBS and fixed in 4% PFA for 2 days at 4 °C. Then, the tissue was dehydrated (2 x 70% EtOH, 2 x 90% EtOH, 2 x 100% EtOH, 3 x 100% Xylene, 3 x Paraffin; each 90 min), embedded in paraffin in plastic holders, followed by preparation of 5 µm paraffin microtome slices. After blocking with 2% BSA/PBS, sections were labeled with primary antibodies overnight at RT: rat anti-SRF (1:250; kindly provided by A. Nordheim, Tübingen University, Germany), rabbit anti-c-Fos (1:500; Santa Cruz; Cat. No. sc-52), rabbit anti-Egr-1 (1:500; Santa Cruz; Cat. No. sc-110), rabbit anti-Cofilin (1:500; Santa Cruz) and rabbit anti-P-Cofilin (Serine 3; 1:500; Santa Cruz). Detection of primary antibodies was performed using Alexa488 (1:500; molecular probes) as well as goat anti mouse or rabbit Biotin conjugated secondary antibodies (1:500; Vector Laboratories) and peroxidase-based detection systems using the ABC complex (Vector Laboratories) and DAB as substrate. The area per section used for quantification of cell numbers was 1 mm^2.

Biochemistry
Protein lysates of selected brain regions were prepared with the ISOLATE II RNA/DNA/protein kit (Bioline) according to manufactures instructions. 1x PhosStop (Roche) was added to the protein lysates. Samples were resolved on 8-10% SDS-PAGE, followed by transfer on PVDF membranes (Amersham). After 1 h of blocking, first antibodies were applied overnight at 4 °C: mouse anti-GAPDH (1:50000; Thermo; Cat. No. OTI2D9), rabbit anti-Cofilin (1:1000; Cell Signaling; Cat. No. 3312), rabbit anti-P-Cofilin (Serine 3; 1:1000; Cell Signaling; Cat. No. 3311). Detection of first antibodies involved horseradish-peroxidase conjugated secondary antibodies (1:2000; Santa Cruz Biotechnology) and the ECL Western Blotting Substrate (Pierce or Millipore). Membranes were exposed on X-ray films (Fujifilm) in a dark room and developed in an Agfa film processor (x-ray developer).

Statistics
If not stated otherwise, data was analyzed using R, a language and environment for statistical computing. Tests for genotype effects were made by using One-way ANOVA with Tukey's multiple comparisons test or unpaired t test depending on the assumed distribution of the parameter and the questions addressed to the data. A p-value < 0.05 has been used as level of significance. Statistical significance is depicted with *, **, *** indicating $p \leq 0.05$, 0.01 and 0.001, respectively. Standard deviation (s.d.) is provided if not mentioned otherwise. All figures except for Fig. 6 show all significant changes that were obtained between experimental groups relevant for comparison. Thus, if no significance is indicated between two experimental groups by an asterisk, no significant differences were observed.
The numbers of animals analyzed in the different tests are indicated in Table 1.

SUPPLEMENTAL REFERENCES

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