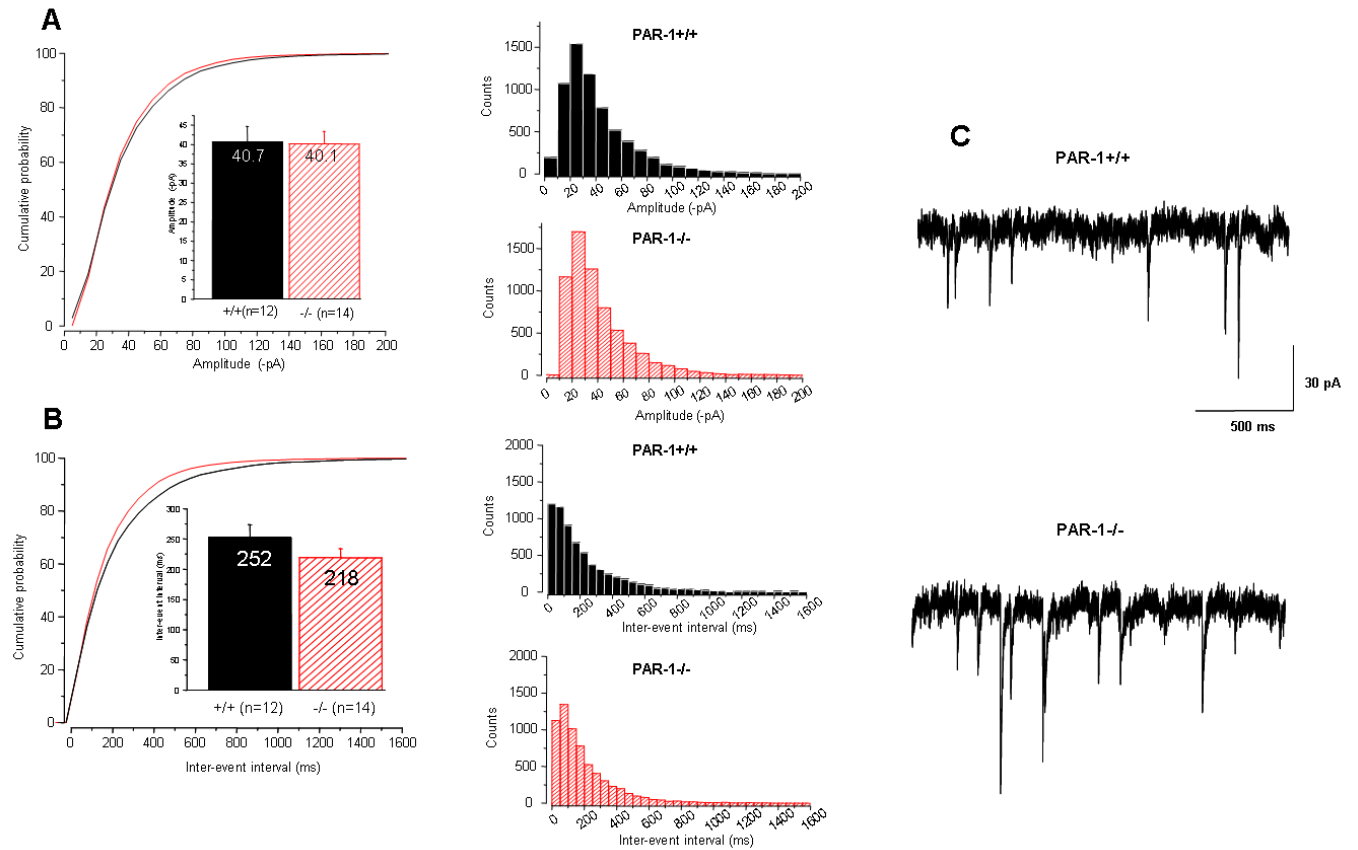
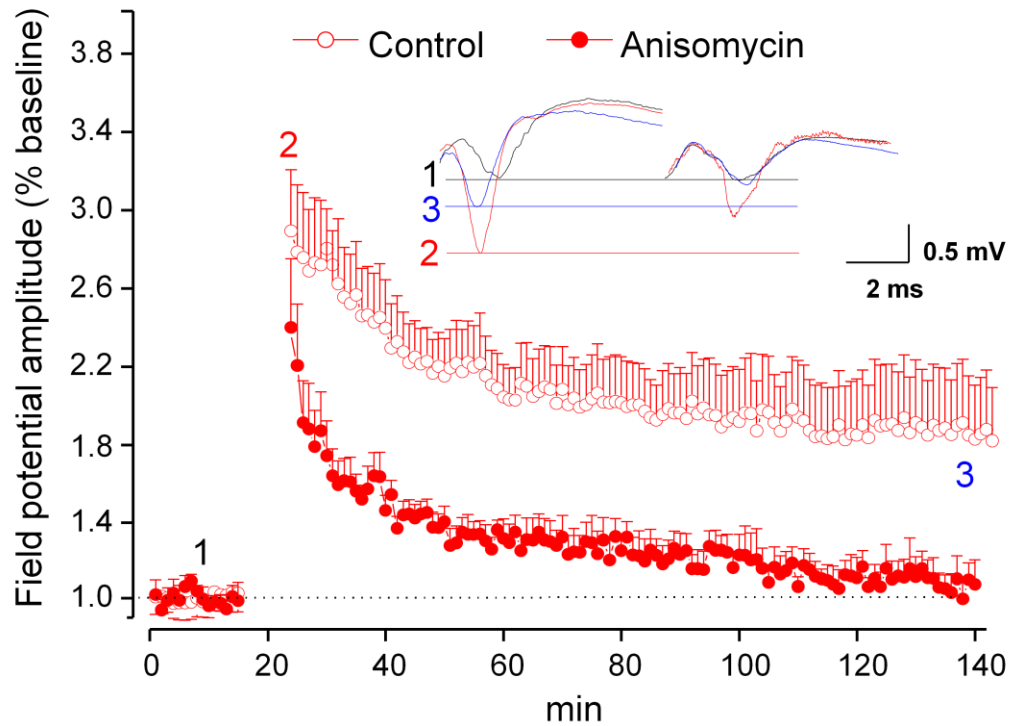


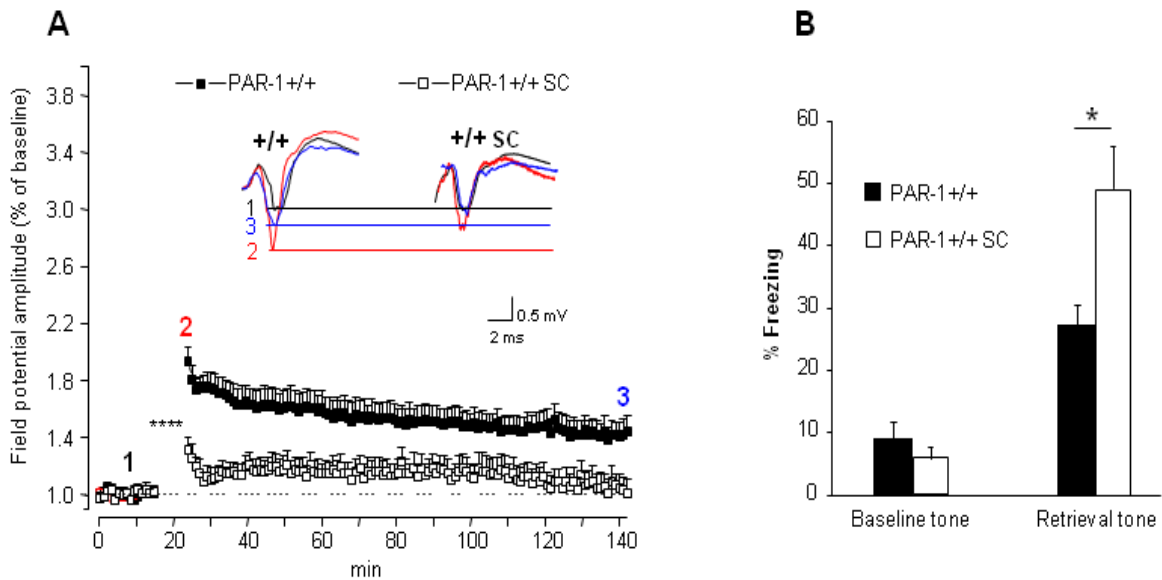
**Figure S1. Spontaneous miniature inhibitory post-synaptic currents (mIPSCs) in the basal nucleus of the amygdala of naïve mice are not affected by the deletion of the PAR-1 gene.** Whole-cell recording of mIPSCs in principal neurons of the basal nucleus of the amygdala revealed a similar amplitude (**A**) and frequency (**B**) in  $PAR-1^{+/+}$  and  $PAR-1^{-/-}$  mice (mean  $\pm$  SEM and cumulative distributions shown for each parameter). Representative recordings in **C**. n = 12 cells per group. Data shown as mean  $\pm$  SEM.



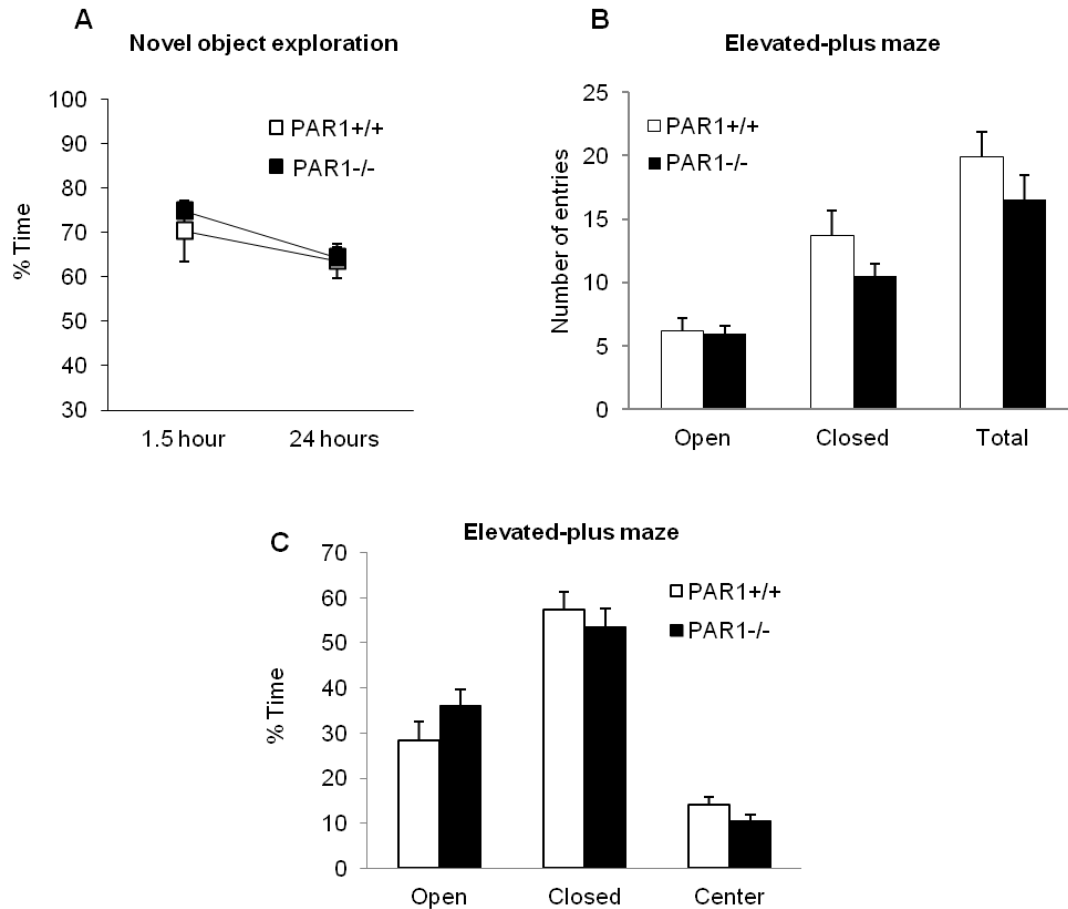
**Figure S2. Spontaneous miniature inhibitory post-synaptic currents (mIPSCs) in the basal nucleus of the amygdala of fear conditioned mice are not affected by the deletion of the *PAR-1* gene.** mIPSCs recorded in principal neurons of the basal nucleus of the amygdala two days after fear conditioning revealed a similar amplitude (**A**) and frequency (**B**) in  $PAR-1^{+/+}$  and  $PAR-1^{-/-}$  mice (mean  $\pm$  SEM and cumulative distributions shown for each parameter). Representative recordings in **C**. n = 12 cells per group. Data shown as mean  $\pm$  SEM.



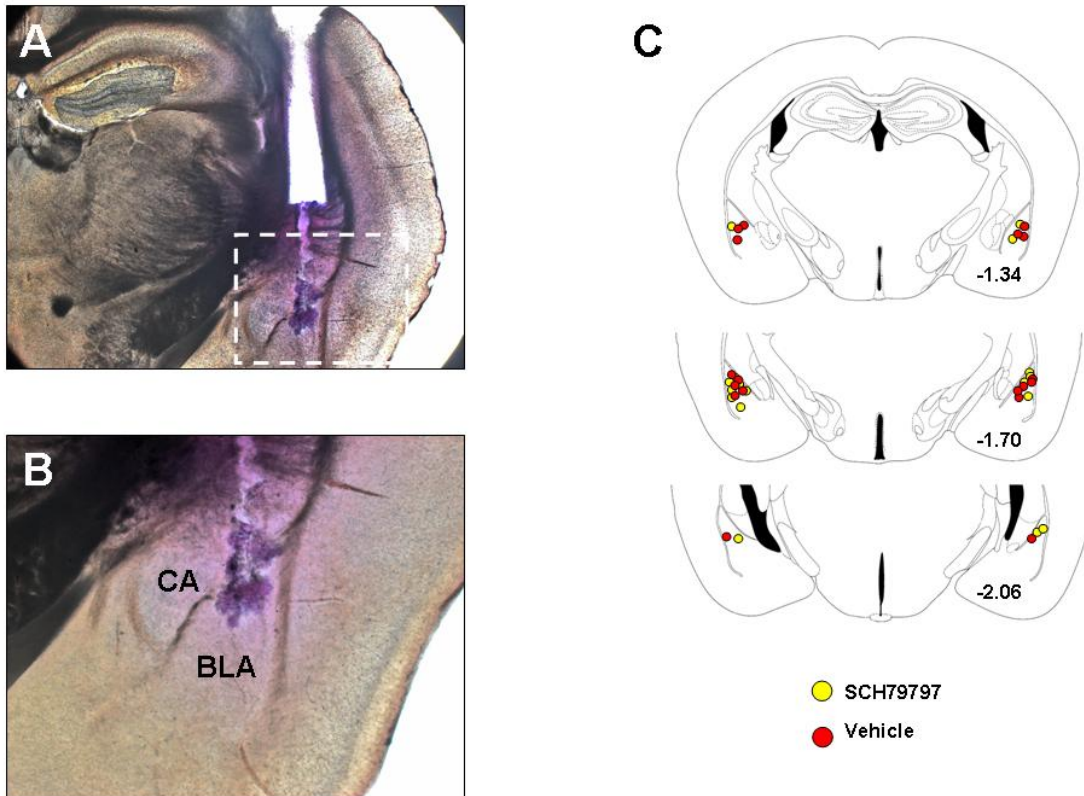
**Figure S3. LTP in *PAR-1*<sup>-/-</sup> mice is abolished by inhibition of protein synthesis.** Mice were subjected to fear conditioning, amygdala slices prepared 48 hours later and LTP induced as described in Material and Methods with or without incubation with anisomycin (20  $\mu$ M present during the whole recording period).  $n = 8$  per group. Data shown as mean  $\pm$  SEM.



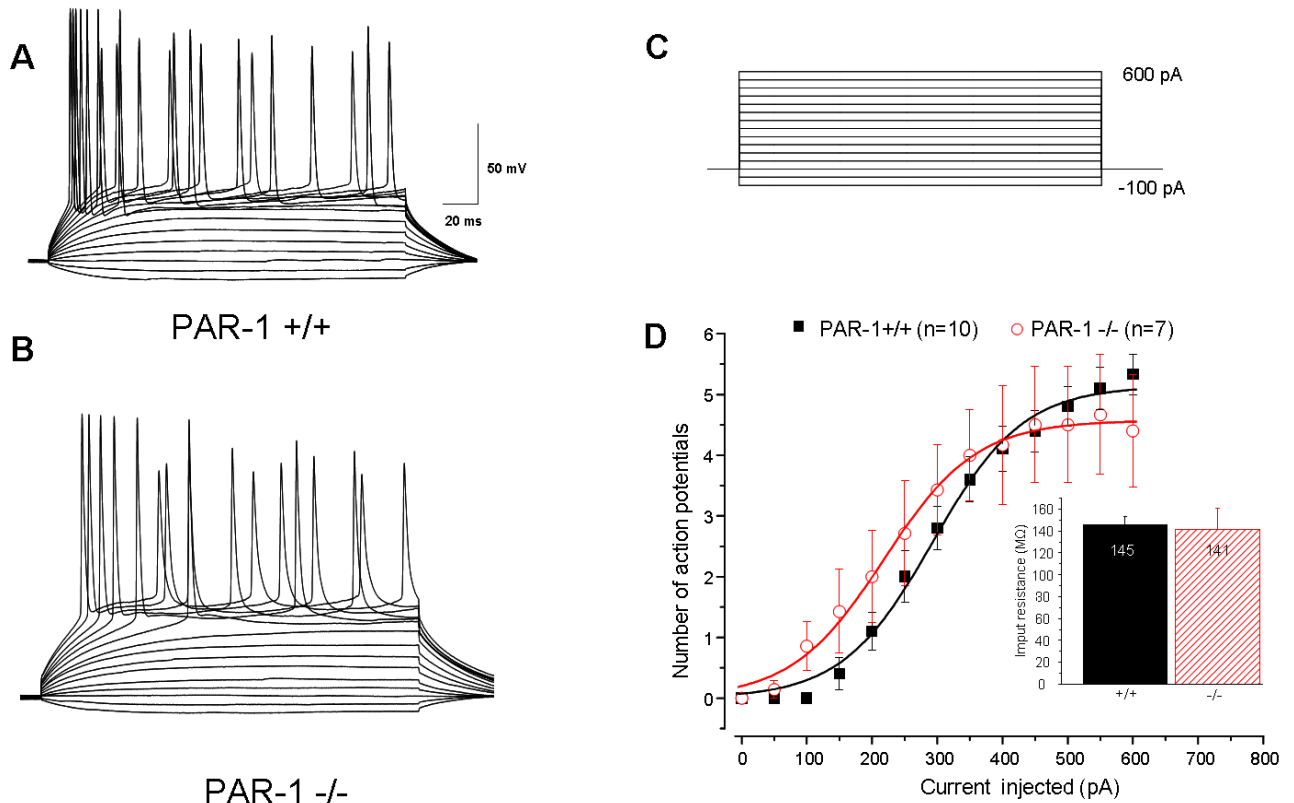
**Figure S4. Occlusion of electrically evoked LTP by strong fear conditioning protocol in PAR1+/+ mice.** Mice were subjected to either mild (three CS-US pairings at 0.4 mA; n = 11) or strong conditioning (SC; five CS-US pairings at 0.5 mA; n = 4) and after 48 hours either LTP was evoked (**A**) or fear memory retrieved (**B**). Electrically evoked LTP (N = 4; n=7) was occluded in the presence of strong fear memories (N = 4; n = 4). \*p<0.05.



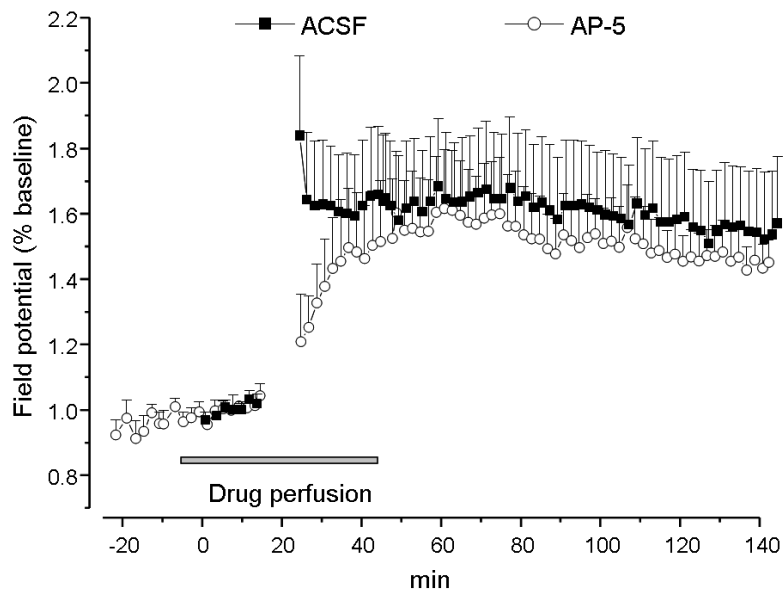
**Figure S5. Basal hippocampus-dependent functions, anxiety and motor activity are intact in PAR1<sup>-/-</sup> mice.** In the novel object recognition task (**A**) the mice displayed similar exploratory behaviour towards the novel object introduced either at 1.5 or 24 hours after the training session, indicating that hippocampal functions are intact in PAR1<sup>-/-</sup> mice (n = 10 per genotype). Anxiety (measured by the number of entries into open arms or % time spent in open arms of the elevated-plus maze) was indistinguishable between the genotypes (**B** and **C**). General motor activity (total number of entries) was similar between PAR1<sup>-/-</sup> and PAR1<sup>+/+</sup> mice (n = 7 per genotype).



**Figure S6. Histological verification of stereotaxic amygdala injections.** (A) Representative picture of the guide cannula penetrating towards the amygdala and the tip of the injection cannula (marked with bromophenol blue) inside the lateral-basal complex. Higher magnification in (B). The positions of the injection cannulae tips are shown on consecutive coronal brain atlas planes (C). CA - central amygdala, BLA - basolateral amygdala.

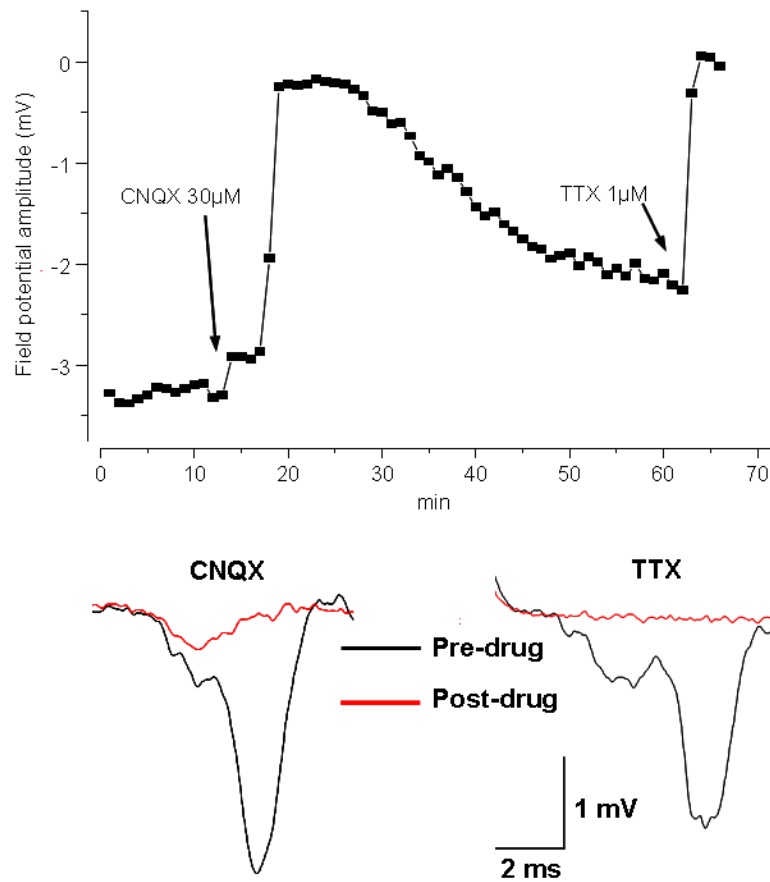


**Figure S7. Deletion of the PAR-1 gene does not affect neuronal firing rate in the basal nucleus of the amygdala of naïve mice.** Current-clamp experiments revealed that neuronal firing rate is comparable in PAR-1<sup>-/-</sup> and PAR-1<sup>+/+</sup> animals. Voltage responses (representative traces in **A**, **B**) were recorded by currents steps from -100 to +600 pA in 50 pA increments (**C**) from principal neurons of the basal nucleus. The number of action potential spikes was counted as a function of depolarizing current injection (**D**). Insert in **D** shows a similar mean input resistance in PAR-1<sup>-/-</sup> mice compared to PAR-1<sup>+/+</sup> animals. n = 7-10 cells per group. Data shown as mean ± SEM.

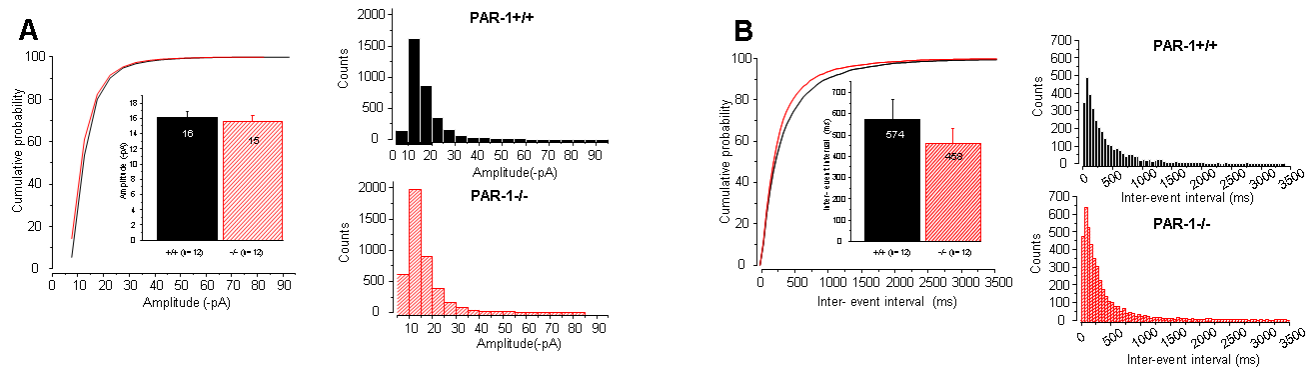


**Figure S8. L-LTP in the lateral-basal amygdala pathway is NMDA-receptor independent.** LTP was evoked by 4 trains of tetanic stimulation as described in Material and Methods. The contribution of NMDA receptors to the early and late phase of LTP was investigated by blocking them with a specific antagonist (50  $\mu$ M AP-5; the period of drug perfusion shown as a horizontal bar). AP-5 (open circles) reduced the early phase of LTP while the late phase was intact.  $n = 5$  per group. Data shown as mean  $\pm$  SEM.





**Figure S9. The lateral-basal amygdala field potentials require AMPA-receptors.** The time course of changes in the field potential amplitude was recorded in the basal amygdala. After 15 min of a stable baseline (upper panel and left lower panel, black line) the slice was perfused with CNQX (30 μM), which completely abolished the responses (lower left panel, red line). The amplitude recovered partially after 40 min of wash out (lower right panel, black line). Pre-volley fibre responses were not affected by CNQX, but were completely abolished by the addition of the sodium channel blocker TTX (1 μM; lower right panel, red line).  $n = 3$ .



**Figure S10. Spontaneous mEPSCs in the basal nucleus of the amygdala of naïve mice are not affected by the deletion of the PAR-1 gene.** Continuous whole-cell voltage clamp recording of mEPSCs in principal neurons of the basal nucleus of the amygdala revealed a similar amplitude (**A**) and frequency (**B**) of mEPSCs in PAR-1<sup>+/+</sup> and PAR-1<sup>-/-</sup> mice (mean ± SEM and cumulative distributions shown for each parameter). n = 12 cells per group analyzed over 2 minutes. Data shown as mean ± SEM.

## Supplementary Material and Methods

### Animals

Experiments were performed on adult wild-type (C57/BL6) or PAR1<sup>-/-</sup> mice backcrossed to C57/BL6 for 10 generations. Animals were housed three to five per cage in a colony room with a 12 h light/dark cycle (lights on at 7AM) with *ad libitum* access to commercial chow and tap water. The experiments were approved by the UK Home Office and the UoL Ethical Committee.

For genotyping DNA was extracted from wild-type and PAR1<sup>-/-</sup> mice ear samples with the GenElute™ Mammalian Genomic DNA Miniprep kit (Sigma). Mice were genotyped as previously described (1).

### Fear Conditioning

PAR1<sup>+/+</sup> or PAR1<sup>-/-</sup> mice were individually placed in the conditioning chamber for 2 minutes before they received three conditioned stimulus-unconditioned stimulus (CS-US) pairings. The last 2 sec of the tone (CS, 30 sec, 2.8 kHz, 85dB) were paired with the footshock (US, 2 sec, 0.4 mA) delivered through a grid floor. In unpaired mice the tone and footshock were delivered in a random (unpaired) manner. After training was completed mice remained in the conditioning chamber for one more minute and were then moved to their home cage. Cued-conditioning was evaluated 48 h after training. The mouse was placed in a novel context (chamber with flat plastic floor and walls) for 2 min, after which the CS was delivered (2 min, 2.8 kHz, 85 dB) and freezing was monitored. Data were analyzed using FreezeView software (Coulbourn Instruments).

### Pain threshold

Each behavioural experiment was performed on a separate cohort of mice. PAR1<sup>+/+</sup> and <sup>-/-</sup> mice were subjected to a series of mild footshocks of increasing intensities (in 0.05 mA increments) and behavioral reaction was measured as previously described (2).

### Novel object recognition

Mice were placed in a 50x50x50 cm plexiglas box, left free to explore two objects for 5 min. The time of exploration of each object was recorded (head facing the object within 3 cm). At the end of the session mice were put back to their home cage. 1.5 hours and 24 hours later one of the objects

was replaced with an unfamiliar object and mice were retested and time spent exploring each object recorded.

### **Elevated-plus maze**

The elevated-plus maze test was performed as previously described (3). The apparatus consisted of four non-transparent white Plexiglas arms: two enclosed arms (50 × 10 × 30 cm) that formed a cross shape with the two open arms (50 × 10 cm) opposite each other. The maze was 55 cm above the floor and dimly illuminated. Mice were placed individually on the central platform, facing an open arm, and allowed to explore the apparatus for 5 min. Behavior was recorded by an overhead camera. The number of entries of the animal from the central platform to closed or open arms as well as % time spent in open/closed arms was counted.

### **Western blotting**

Mice were anaesthetized with pentobarbital and transcardially perfused with ice-cold PBS. Their brains were removed and four brain regions (cortex, amygdala, hippocampus and thalamus) were dissected from a coronal slice approx -0.58 to -2.3 mm relative to Bregma. Samples from the hypothalamus medulla and cerebellar cortex were taken from the remaining tissue. To measure fear conditioning-induced changes in PAR1 levels amygdala fragments (slice -0.58 to -2.3 mm relative to Bregma) containing the basolateral complex were collected using a vibratome and a dissecting microscope. Samples were homogenized in 0.1 M Tris, 0.1% Triton X-100, pH 7.4, containing phosphatase (10 mM NaF, 1 mM Na orthovanadate) and protease inhibitors (Complete, Roche) and the protein concentration was adjusted to 2 mg/mL using the Bradford method. Samples (40 µg per lane) were reduced, denatured (100°C for 5 min) and subjected to SDS-PAGE electrophoresis. After transferring onto nitrocellulose membrane and blocking in TBST-milk (TBS, 0.1% Tween 20, 5% skim milk) for 1 h at RT, the membrane was probed with a rabbit anti-PAR1 (gift of Dr. M. Runge, 1:1000, 4°C, overnight), followed by a peroxidase-labeled anti-rabbit secondary (Vector Labs, 1:2000, 1 h at RT). To normalize the results the membrane was stripped and re-blotted using mouse anti-β-actin antibody (Sigma, 1:1000 in TBST-5% milk, 1 h, RT; Sigma) followed by an anti-mouse HRP-conjugated secondary (Vector Labs, 1:1000, 1 h, RT). Luminescence was detected using Western Blot luminal reagent and photographic film. Band intensities were measured using Scion Image.

For G protein levels control or fear conditioned PAR1<sup>+/+</sup> mice were sacrificed 48 h after training. Brains were removed and coronally sliced (-0.58 to -2.3 mm relative to Bregma) in ice-cold

Ringer's solution (25 mM glucose, 115 mM NaCl, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 3.3 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 25.5 mM NaHCO<sub>3</sub>, equilibrated with 95% O<sub>2</sub>:5% CO<sub>2</sub> for 15 min before use) (4) using a vibratome and the basolateral complex of the amygdala was dissected bilaterally under the microscope. Samples were prepared for Western blot as described above. Membranes were incubated at 4°C overnight with either rabbit anti-G $\alpha_{q/11}$  antiserum (1:500 in TBST 5% milk)(5) or mouse anti-G $\alpha_o$  antibody (Santa Cruz Biotechnology, 1:500 in TBST 5% milk) followed by HRP-labeled anti-rabbit or anti-mouse antibodies (both at 1:1000, 1 h at RT).

### **Immunohistochemistry**

To investigate the expression pattern of PAR1 and to reveal the phenotype of PAR1-positive cells, quadruple staining was performed. Mice were anesthetized with pentobarbital, perfused transcardially with ice-cold 4% paraformaldehyde (PFA) in PBS and their brains removed. The brains were fixed in 4% PFA overnight. 70  $\mu$ m coronal brain sections were then cut using a vibrating microtome and stored in 4°C in PBS until analyzed. Free-floating sections were blocked with goat serum in PBS (1:500 for 4 h, RT) and incubated with rabbit anti-PAR1 (1:500, 4°C for 4 h), followed by the addition of mouse anti-NeuN (Chemicon, 1:200, 4°C, overnight) and chicken anti-GFAP (Abcam, 1:1000, 4°C, overnight) to the same wells. To examine the level of c-Fos and P-CREB separate sections were incubated with anti-c-Fos (Cell Signaling, 1:200) or anti-phospho-CREB antibodies (Cell Signaling, 1:200), respectively. After several washes, the sections were incubated with the appropriate AlexaFluor 488, 546, 647 or Cy3-labeled secondary antibodies (Invitrogen or Abcam, all at 1:500, 4°C, overnight). DAPI (pseudocolored grey) was used to visualize cell nuclei in conjunction with PAR1, NeuN and GFAP labeling. Sections in which the primary antibodies were omitted served as controls. The images were collected using confocal microscope.

### **G protein coupling**

#### **Membrane preparation**

Mouse amygdalae (collected before or 48 h after conditioning) were dissected from coronal vibratome slices under a microscope and homogenized using a syringe with 0.8 x 40 mm needle in 10 volumes of 10 mM HEPES, 1 mM EGTA, 1 mM dithiothreitol and 1 mM MgCl<sub>2</sub>, pH 7.4, containing complete protease inhibitor cocktail and centrifuged at 20,000 x g for 20 min. The resulting pellet was re-suspended in assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, pH 7.4) to give protein concentration of 10  $\mu$ g per 60  $\mu$ L.

### **[<sup>35</sup>S]GTPγS binding and Gα-specific immunoprecipitation assay**

[<sup>35</sup>S]GTPγS-Gα<sub>q/11</sub> immuno-specific binding was performed using a method previously described in detail (5, 6) with minor modifications. Briefly, membranes were incubated on ice for 5 min with GDP (10 and 0.1 μM respectively for Gα<sub>o</sub> and Gα<sub>q/11</sub> coupling). Assays were carried out in a final volume of 100 μL in Eppendorf tubes. Buffer (20 μL) containing the PAR1 agonist TRag (Neosystem Laboratoire, final concentration 1 μM) was added to each tube followed by the membrane/GDP mix (60 μL) and incubated at room temperature for 20 min. After this time [<sup>35</sup>S]GTPγS (20 μL; 0.4 nM final concentration) was added to each tube and incubations continued for a further 60 min at 30°C. Reactions were terminated by the addition of 10 μL of ice-cold 0.27% Igepal CA-630, and membranes allowed to solubilize at 4°C for 30 min. For immunoprecipitation of [<sup>35</sup>S]GTPγS-Gα proteins, Gα<sub>o</sub> or Gα<sub>q/11</sub> antisera (10 μL; final antibody dilution 1:300) were added to each tube. Samples were vortex-mixed and rotated for 60 min at 4°C. A protein A-sepharose bead suspension (3% (w/v), 70 μL) was next added to each tube, and the samples mixed and rotated for 90 min at 4°C. Protein A-sepharose bead-associated material was pelleted (20,000 x g, 6 min, 4°C) and the supernatant was removed by aspiration. Beads were washed three times with 500 μL of wash-buffer (100 mM Tris/HCl, 200 mM NaCl, 1 mM EDTA, 1.25% Igepal CA-630, pH 8.0), and following the final wash, beads were mixed with scintillation cocktail and counted. Non-specific binding was determined in the presence of unlabelled GTPγS (10 μM). Basal [<sup>35</sup>S]GTPγS binding to Gα<sub>o</sub>: naïve, 3108 ± 508; unpaired, 2816 ± 377; fear conditioned, 2929 ± 501 d.p.m.; binding to Gα<sub>q/11</sub>: naïve, 1118 ± 185; unpaired, 1411 ± 242; fear conditioned, 1097 ± 69 d.p.m.

### **Stereotaxic injections**

Mice were intraperitoneally anaesthetized with ketamine/xylazine (100 and 10 mg/kg, respectively) placed in a stereotaxic apparatus and bilaterally implanted with stainless steel guide cannulae (26-gauge; Plastics One, Roanoke, VA) aimed above the basolateral complex of the amygdala (1.5 mm posterior to bregma, 3.5 lateral and 4.0 ventral). The cannulae were secured in place with dental cement. Dummy cannulae were inserted into all implanted cannulae to maintain patency. After one week dummy cannulae were replaced with the injection cannulae (33-gauge, projecting 0.75 mm from the tip of the guide cannulae to reach the basolateral complex of the amygdala) and the mice were injected with SCH79797 (1 μM, 0.5 μL) or vehicle followed by fear conditioning. After the experiment a small amount of bromophenol blue was injected to visualize the guide and injection cannulae tracks, the brains were sectioned and the cannulae placement was determined

histologically.

## **Electrophysiology**

### **Slice preparation**

Coronal slices containing the amygdala (400  $\mu\text{m}$  for extracellular recordings or 300  $\mu\text{m}$  for whole cell recordings) were prepared from 8-12 weeks-old PAR1<sup>-/-</sup> or PAR<sup>+/+</sup> mice. The mice were either left undisturbed (naïve), subjected to the unpaired protocol or fear conditioning. The animals had been anaesthetized with ketamine/xylazine (2:1) injected at the concentration of 2.4  $\mu\text{L/g}$ . The whole brain was rapidly isolated and slices were prepared using a vibroslicer in ice-cold, low sodium-Artificial Cerebral Spinal Fluid (ACSF) containing (in mM): sucrose (249); KCl (2.5); NaH<sub>2</sub>PO<sub>4</sub> (1.25); D-glucose (10); NaHCO<sub>3</sub> (26); CaCl<sub>2</sub> (0.1); MgSO<sub>4</sub> (2.9); ascorbic acid (0.5), bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> mixture and with a final pH 7.3. Slices were placed in a holding chamber for 30 min at 35°C and then for at least 30 min (for whole-cell recording) or 2 h (for extracellular recording) at room temperature (25°C) in ACSF. ACSF composition was the following (in mM): for extracellular recordings: NaCl (124.0); KCl (5.0); NaH<sub>2</sub>PO<sub>4</sub> (1.25); D-glucose (10.0); NaHCO<sub>3</sub> (26.0); CaCl<sub>2</sub> (2.4); MgSO<sub>4</sub> (1.3); for whole-cell recordings: NaCl (124.0); KCl (2.5); NaH<sub>2</sub>PO<sub>4</sub> (1.25); D-glucose (10.0); NaHCO<sub>3</sub> (26.0); CaCl<sub>2</sub> (2.0); MgSO<sub>4</sub> (1.0).

### **Extracellular recording**

Recordings were made using a bipolar tungsten electrode (WPI). For recordings glass microelectrodes (1-2 m $\Omega$ ) filled with ACSF were used. To record field potentials in the lateral–basal amygdala pathway stimulating electrode was placed in the lateral amygdaloid nucleus close to the external capsule and the recording electrode in the basal nucleus. The stimulus intensity was adjusted to evoke a field potential (FP) of 60-70% (0.2 ms pulse duration) of the maximal amplitude. The test stimuli for basal synaptic response was injected every 30 sec in order to record a stable baseline at least for 15 min. These stimuli elicited negative field potentials that had a mean latency of 3 msec and mean duration of 6 msec. Several FP traces were averaged to create a template and only the responses matching the template were analyzed. Long-term potentiation (LTP) was elicited by two trains of high-frequency tetanic stimulation (100 Hz, 1 sec, 10 sec interval) repeated 4 times at 3 min interval. The recordings were amplified, filtered (10 kHz) and digitized (50 kHz).

### **Whole-cell recordings**

Recordings were made from somata of principal neurons of the basal nucleus of the amygdala. Neurons were visualized by infrared videomicroscopy (CCD camera). Principal neurons and interneurons were distinguished by their morphological and electrophysiological properties (7). After whole-cell configuration the series resistance was regularly monitored and a maximum series resistance of 10-15 M $\Omega$  was tolerated. Only neurons with a resting membrane potential below -50 mV were used. In current-clamp recordings the membrane potential was kept at -80 mV (or -40mV where indicated). Input resistance and instantaneous spike frequency were derived from traces in which cells were injected with 200 msec current pulses (-100 to +600 pA; 50 pA increments). In order to record mEPSCs TTX (1  $\mu$ M; Latoxan) and picrotoxin (100  $\mu$ M; Sigma) were routinely included in the extracellular solution. The cell membrane was clamped at -70 mV and at the end of each experiment mEPSCs were blocked with CNQX (10  $\mu$ M) to confirm they were mediated by AMPA receptors. To study mIPSCs cell membrane was clamped at -70 mV. TTX (1  $\mu$ M), AP-V (50  $\mu$ M) and CNQX (10  $\mu$ M; Tocris Bioscience) were routinely added to the extracellular solution. At the end of each experiment mIPSCs were blocked with picrotoxin (100  $\mu$ M) to confirm they were GABA<sub>A</sub>-mediated.

Drugs were applied through the perfusion solution reservoir (perfusion rate 1.5 mL/min using a peristaltic pump). The recording electrodes were glass pipettes (2-3 m $\Omega$ ) pulled from borosilicate glass tubing. For the pipette was filled with the following solutions (in mM): For mEPSCs: Cs-methyl sulfonate (130.0), KCl (8.0), EGTA (0.5), Mg-ATP (2.0), HEPES (10.0), D-glucose (5.0). For mIPSC: KCl (135.0), Mg-ATP (2.0) EGTA (0.5), HEPES (10.0), D-glucose (5.0). For current-clamp recordings: K-D-gluconate (130.0), KCl (4.0), EGTA (0.5), Mg-ATP (2.0), HEPES (10.0), D-glucose (5.0) Na<sub>3</sub>-GTP (0.5). Voltage-clamp experiments were performed at 32°C. Current clamp experiments were performed at 25°C.

### Statistical analysis

All values are expressed as means  $\pm$  SEM. Student t-test (for two groups) or analysis of variance (ANOVA) for multiple comparisons followed by Tukey's post test were used as appropriate. P values of less than 0.05 were considered significant.

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