Supplement

It takes two: Bilateral bed nuclei of the stria terminalis mediate the expression of contextual fear, but not of moderate cued fear

Kelly Luyck, Lutgarde Arckens, Bart Nuttin, Laura Luyten

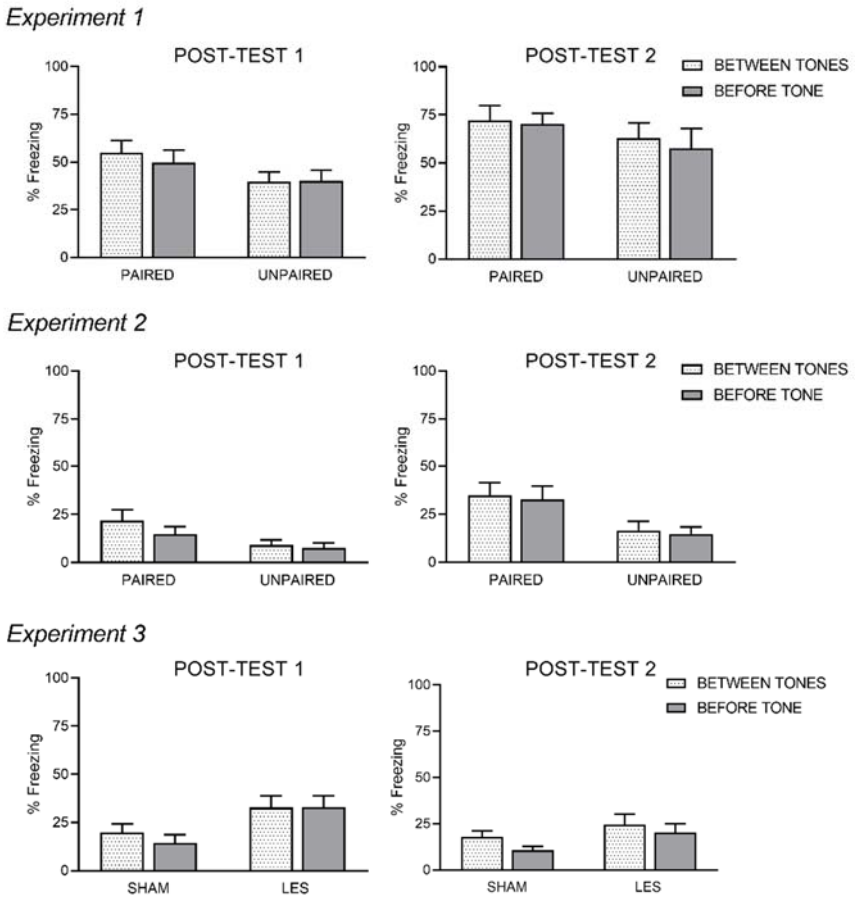
Experiments 1-3

Supplementary results

In the main article, we described two contextual freezing measurements: one during the 5-min acclimation periods and one during the 10-s periods preceding each CS ('before tone'). For the sake of completeness and since it was part of our preregistered analyses, we here present an additional contextual fear measurement, i.e. freezing 'between tones', as measured during 10-s periods at predetermined time points in the middle of the interval between CSs ('between tones', cf. Luyck et al., 2018). We compared these 'between tones' measurements with freezing during the 10-s periods preceding each CS ('before tone'), the metric that was reported in the main article for the comparison with freezing *during* the CS (*Suppl. Fig. 1*).

In the first two experiments, which were designed to optimize the behavioral protocol, RM ANOVAs did not find statistically significant differences between both measurements. We therefore decided to use the 'before tone' metric for all statistical analyses in the main article, given that this measurement, because of its maximal temporal distance from the preceding tone, is presumably a purer measurement of contextual fear that is less confounded by any post-tone freezing.

Note that in Experiment 3, on Post-test 2, contextual freezing 'before tone' was in fact significantly lower than 'between tones' in both SHAM and LES groups ($F_{(1,17)}$ = 14.5, p<.01). However, the conclusions as described in the main article, i.e., no differences in contextual and cued fear between SHAM and LES groups (*Fig. 5*, right panel), were the same when using 'before tone' or 'between tones' freezing (RM ANOVA with 'between tones' versus 'tone': main effect of Trial ($F_{(1, 18)}$ = 75.11; p < 0.0001), no effect of Group ($F_{(1, 18)}$ = 0.57; p = 0.46), no interaction ($F_{(1, 18)}$ = 0.16; p = 0.69)).



Suppl. Fig. 1: Comparison of measurements of contextual fear *before* and *between* CSs in Experiments 1-3.

Experiment 4

Supplementary methods

Tissue preparation

Two hours after initiation of the Post-test, rats were sacrificed with an intraperitoneal injection of pentobarbital (2.5 ml, Nembutal, CEVA Santé Animale, Brussels, Belgium). Next, the animals were perfused with saline and subsequently with a 4% paraformaldehyde dilution in phosphate-buffered saline (PBS). Brains were removed and post-fixed for 24h in 4% paraformaldehyde. Subsequently, samples were rinsed in water during 24h and stored at 4°C in PBS. Fifty-µm thick free-floating serial sections were prepared on a Vibratome (Microm HM 650 V, Thermo Scientific, Walldorf, Germany) and collected in 24-well plates. Cresyl violet-stained slices for this and the previous experiment are shown in *Suppl. Fig. 2*.

Immunofluorescent staining: c-Fos and NeuN

Next, six representative animals per group were selected for immunofluorescent staining. These animals were chosen according to freezing values that were the closest around the median freezing during acclimation (Post- minus Pre-test) in their respective groups. When tissue quality of one or more of these animals was of insufficient quality (e.g. due to problems with the vibratome), we selected a replacement animal with freezing values closest to the group median. For the selected twenty-four animals, we stained slides representative for each structure of interest, based on Paxinos coordinates: +2.40 (IL, PL), +1.20 (NAc, core and shell) and -2.40 (BLA). Note that for each structure, all slices were stained simultaneously. This approach decreased variability in c-Fos intensities and allowed us to directly compare neural activity between groups or hemispheres within the same structure.

Sections were rinsed in PBS-T (0.3% Triton) and incubated in normal goat serum (Chemicon International, Temecula, CA, USA) for 1h (Tris-NaCl blocking buffer (TNB); 1:5). Next, they were incubated overnight with primary antibody diluted in PBS-T (0.5% Triton). Following antibodies were used: polyclonal rabbit anti-cFos developed and characterized in the lab (Van Der Gucht et al., 2000) (1:2000) and monoclonal mouse anti-NeuN IgG1, clone 60 (MAB377, Millipore, Darmstadt, Germany; 1:300). The next day, sections were rinsed and first incubated with biotinylated polyclonal goat anti-rabbit IgG secondary antibody (E0433, Dako, Glostrup, Denmark; 1:250) for 2h. After rinsing in PBS-T, the sections were incubated with a mixture of Streptavidine-Cy5 conjugate (A1011, Life Technologies, Ghent, Belgium; 1:250) and AlexaFluor 488 pre-adsorbed polyclonal goat anti-mouse IgG (ab150117, Abcam, Cambridge, UK; 1:250), diluted in TNB for 2h. Next, slices were mounted, incubated with DAPI (2 µl/ 100 ml PBS, 32670, Sigma-Aldrich, St-Louis, MO, USA) for 30 min and coverslipped with Mowiol (4-88 Mowiol, Sigma-Aldrich).

Image acquisition

Projection images of the IL/PL, NAc and BLA were acquired through an inverted C2+ confocal microscope (Eclipse Ti2, Nikon, Tokyo, Japan) using a 20X objective at a resolution of 512x512. Upper and lower limits of the slice were determined in the Z-plane. Next, the middle Z-value was selected and pictures were acquired as 6-µm sections comprising the middle of the slice. For each structure of interest, 3 pictures were taken for each hemisphere for every animal. Image acquisition was done with NIS-Elements software (Nikon, Japan) and comprised c-Fos and NeuN channels at predetermined intensity settings that remained fixed throughout image acquisition.

Image analysis

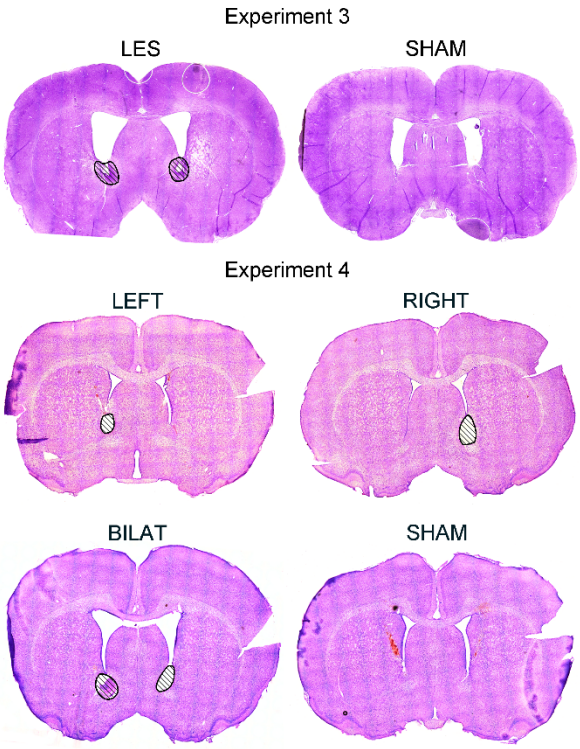
We analyzed neuronal c-Fos expression through intensity measurements in the IL, PL, BLA and NAc (core and shell). The images were imported in ImageJ (Rasband, ImageJ, National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/, 1997–2014) and regions of interests (ROI) were delineated as

200 x 200 µm squares situated in the relevant brain area (*Fig. 7*). For each ROI, a neuronal cell mask was obtained by auto-thresholding the NeuN image and detecting cells with circularity levels exceeding 20%. These cells were then automatically delineated and transferred to the c-Fos image, where intensity levels were detected and corrected for total neuronal cell size. Intensity levels had an arbitrary scaling between 0 (black) and 4095 (white, highest activity) (*Suppl. Fig. 3*). For each structure, all slices were stained simultaneously and imaged at fixed intensity settings, allowing for direct between-group comparison of c-Fos intensities within each ROI. This analysis provided information about the degree of c-Fos expression, rather than the number of positive neurons, and it was chosen to avoid a ceiling effect, since prior data (Luyck et al., in preparation) indicated that a high percentage of BST neurons expressed c-Fos under basal conditions (i.e., in non-conditioned animals).

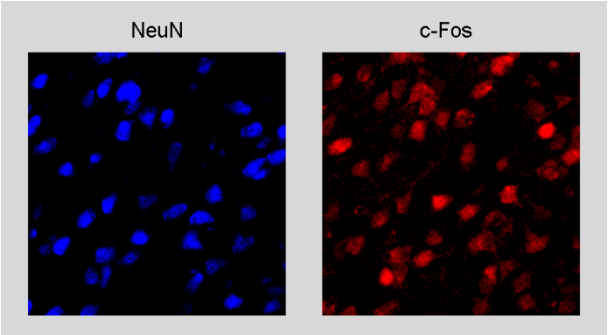
Supplementary results

We evaluated c-Fos intensities in five regions of interest, as described in our main article. We did not observe any significant group or hemisphere differences. The table below provides an overview of the statistical outcomes for every region of interest (2-way repeated measures ANOVAs with factors 'Group' (SHAM, BILAT, LEFT, RIGHT) and 'Hemisphere' (left, right)). Neuronal c-Fos intensities for each ROI, hemisphere and group are shown in *Suppl. Fig. 4-6*.

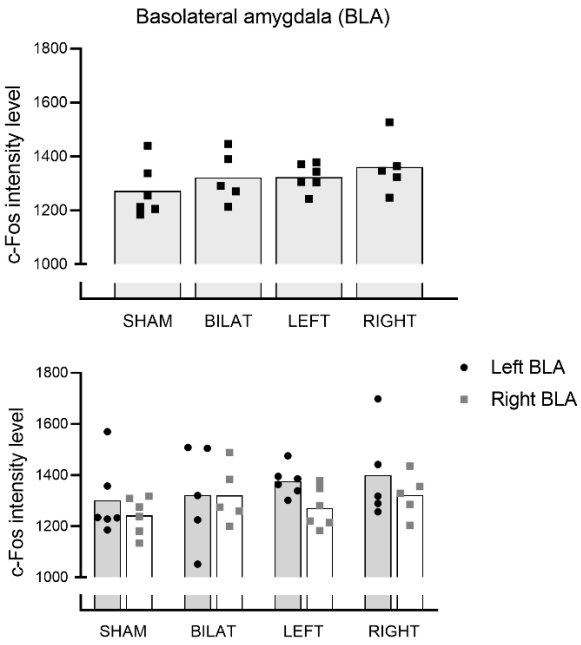
	Statistics		
ROI	Group	Hemisphere	Interaction
Basolateral amygdala	$F_{(3,18)} = 1.0$; p = 0.43	$F_{(1,18)} = 2.8$; p = 0.11	$F_{(3,18)} = 0.4$; p = 0.78
Infralimbic cortex	$F_{(3,18)} = 0.6$; p = 0.65	$F_{(1,18)} = 1.0$; p = 0.33	$F_{(3,18)} = 2.7$; p = 0.08
Prelimbic cortex	$F_{(3,17)} = 0.9$; p = 0.46	$F_{(1,17)} = 3.5$; p = 0.08	$F_{(3,17)} = 0.7$; p = 0.6
Nucleus accumbens: core	$F_{(3,20)} = 1.0$; p = 0.4	$F_{(1,20)} = 0.1$; p = 0.73	$F_{(3,20)} = 0.9$; p = 0.48
Nucleus accumbens: shell	$F_{(3,20)} = 1.1$; p = 0.35	$F_{(1,20)} = 2.5$; p = 0.13	$F_{(3,20)} = 1.9$; p = 0.16



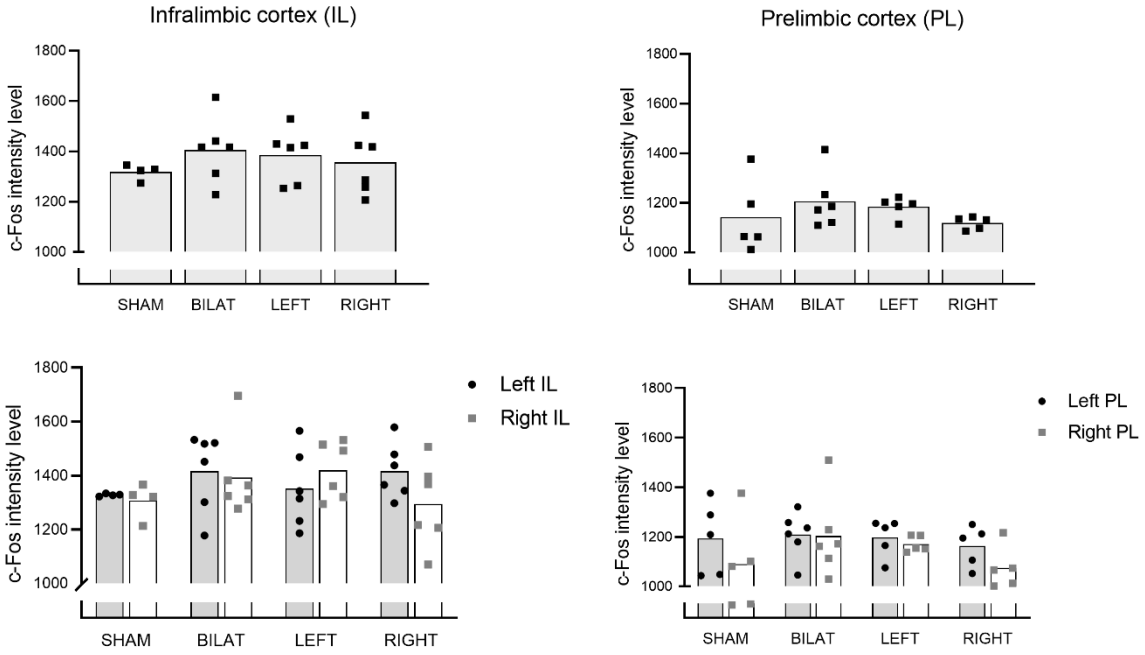
***Suppl. Fig. 2**: Examples of photomicrographs (cresyl violet stain) for Experiments 3 and 4. Coronal slices (5 µm thickness in Exp3 and 50 µm in Exp4) with the maximal lesion diameter are shown for uni- or bilaterally lesioned groups, corresponding slices near bregma are shown for the sham groups. For illustrative purposes, lesions were manually delineated (in black). Animals were perfused ±9 days after lesion induction in Exp3 and 1 day after lesion induction in Exp4.*



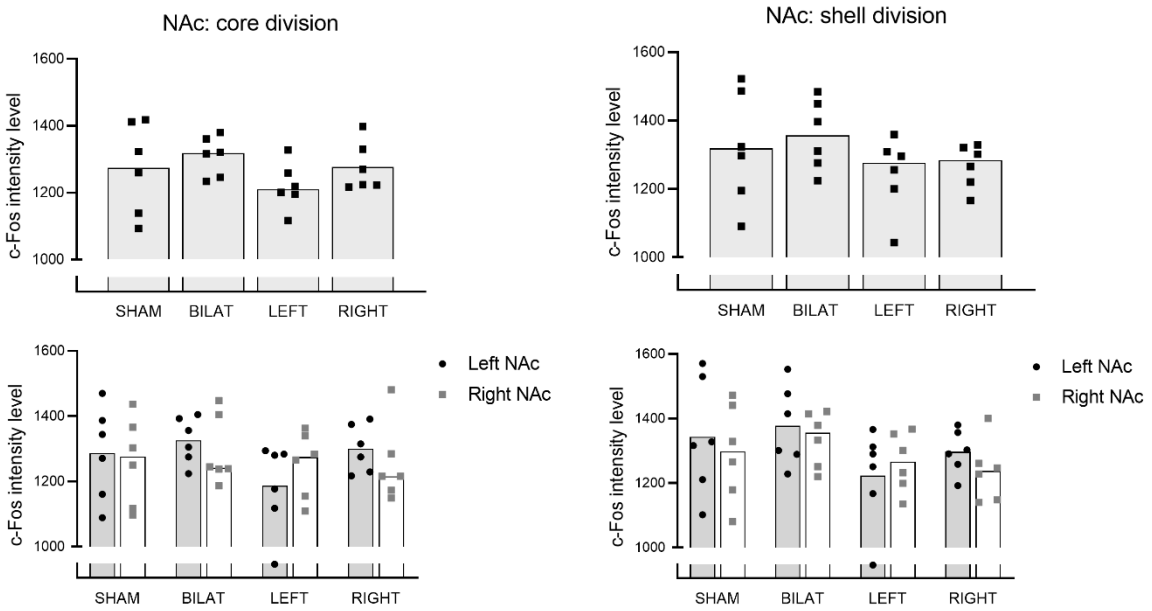
***Suppl. Fig. 3**: Representative NeuN and c-Fos staining from Experiment 4. Immunohistochemical staining of the basolateral amygdala (200 x 200 µm ROI) is shown, illustrating that c-Fos is primarily expressed by neurons. NeuN is shown in blue (left panel) and c-Fos in red (right panel).*



***Suppl. Fig. 4**: Experiment 4, neuronal c-Fos intensities (shown as individual data points with means) in the basolateral amygdala.*



***Suppl. Fig. 5**: Experiment 4, neuronal c-Fos intensities (shown as individual data points with means) in the infralimbic and prelimbic cortex.*



Suppl. Fig. 6*: Experiment 4, neuronal c-Fos intensities (shown as individual data points with means) in the nucleus accumbens (NAc), core and shell.*

Supplementary references

Luyck K, Nuttin B, Luyten L. Electrolytic post-training lesions of the bed nucleus of the stria terminalis block startle potentiation in a cued fear conditioning procedure. *Brain Struct Funct*. 2018;223(4):1839-48.

Luyck K, Scheyltjens S, Nuttin B, Arckens L, Luyten L. Asymmetry in the limbic system: c-Fos expression following context conditioning and deep brain stimulation in the bed nucleus of the stria terminalis in rats. *In preparation.*

Van Der Gucht E, Vandenbussche E, Orban GA, Vandesande F, Arckens L. A new cat Fos antibody to localize the immediate early gene c-fos in mammalian visual cortex after sensory stimulation. *J Histochem Cytochem*. 2000;48(5):671-84.