Supplementary Information for
The mouse brain after foot-shock in 4D: temporal dynamics at a single cell resolution.

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- R package, scripts, data and interactive visualizations are available at: https://osf.io/8muvw/.
- The DOI of this project is: 10.17605/OSF.IO/8MUUVW
Supplementary Notes

Supplementary Note 1: c-fos
c-fos is a proto-oncogene of the Fos family, which acts as a transcription factor upon heterodimerization with a member of the Jun family (1, 2). With the exception of a few constitutively active brain areas, c-fos is not expressed under baseline, i.e. non-stressed, circumstances (3) but transiently induced by mild-to-severe acute stimuli, with activity-dependent intensity (4).
Supplementary Methods

Animals and husbandry. 8 to 10 weeks old male C57Bl/6J OlaHsd mice were purchased from Envigo (Harlan, France) in 3 separate batches. The animals were habituated to the animal facility for at least two weeks, then tested at the age of 10 to 14 weeks. Until the experimental day, the animals were housed in groups of five in type II L cages (365x207x140mm, Tecniplast®) on a 12h dark/light cycle (light phase: 9.00AM–9.00PM), 22±2 °C, humidity at ± 64%, with ad libitum access to standard chow (Special Diet Services, UK, sdsdiets.com) and tap water. Experimental cages were placed on racks without a specific order and left undisturbed in the same experimental room, except for weekly cleaning by animal caretakers unfamiliar with the study design. A copy of the work-protocol (license: 527/16/644/01/06, 527/18/4806/01/01) as well as a step-by-step protocol can be found on the Open Science Framework page of the project (osf.io/8muvw). All animal procedures were approved by the Animal Ethical Committee at Utrecht University (license: AVD1150020184806), the Netherlands. Every effort was taken to minimize animal suffering in accordance with the FELASA guidelines and the Dutch regulation for housing and care of laboratory animals. For the main analysis, a total of 9 male mice per experimental group ($n_{block} = 9$, $n_{time\ point} = 4$, $n_{animals} = 36$) were used.

Experimental design. The primary aim of this study was to develop a methodology to be able to analyze brain-wide activation over time, specifically of the stress system. The experiments were designed to address batch effects, missing values and normalization. An overview of the experimental procedure, data cleaning, preprocessing, and analysis can be found in Figure 1.

The main experiment used a uniform block design, where each block corresponded to a separate cage of animals. Each cage contained an animal of each experimental group (being ‘time after foot-shock’ and control; $n_{time\ point} = 4$). The animals were killed at three different time points after receiving a foot-shock ($n_{time\ point\ foot-shock} = 3$). A control group underwent the same identical procedure but did not receive the foot-shock. This group is considered time point 0 min ($t_0$). Of note, this control group did experience a novel environment, and its activation should therefore be considered not as ‘baseline’ but as mildly stressed. The time points refer to the moment of transcardial perfusion, and were chosen to comprehensively model all phases of the stress response. They cover the initiation ($t = 30\ min, t_{30}$), maintenance ($t = 90\ min, t_{90}$) and termination ($t = 180\ min, t_{180}$) of the HPA axis response, while considering the required time to synthesize c-fos (4) as well as the delay of 30 minutes of increase of corticosteroid in brain tissue compared to blood (5). Randomization of the
experimental groups occurred within the cage, and samples of the same block were processed together both pre- and post-mortem. In other words, each experimental group was represented in every four animals (block), but the order of experimental groups differed across blocks. This type of design is important for batch effects’ correction.

The following control experiments were performed. A 15 min time point ($n_{\text{animals}} = 3$) to validate that indeed 30 min is the earliest time point to detect an increase in c-fos protein expression. A 300 min time point ($n_{\text{animals}} = 2$) to validate that c-fos expression eventually decreases. A no primary antibody staining ($n_{\text{animals}} = 3$) to estimate the extent of unspecific secondary antibody binding. A home-cage control group ($n_{\text{animals}} = 3$) was used to validate that the c-fos expression of the $t_0$ group was due to the experimental procedure (i.e., placement into a novel cage). To minimize the sample size of animals used, the animals of these control experiments were processed together with those of the main experiments, so that the comparison groups could be reused. As a consequence, these control experiments are only qualitatively assessed, and no formal statistical analysis is performed to avoid unnecessary multiple testing. Data of the control experiments is presented paired to the $t_0$ group of the same batch.

Initially, we aimed to include males as well as females (see work protocol at osf.io/8muvw) in the experiment. However, the percentage of animals lost due to unforeseen circumstances (due to the antibody) was much larger than anticipated (30% instead of 10%). That is, the secondary antibody was less stable than expected; as a consequence, the quality of the scans was often insufficient for a brain-wide quantification. We therefore simplified the experimental design and used only 9 male mice per experimental group ($n_{\text{block}} = 9$, $n_{\text{time point}} = 4$, $n_{\text{animals}} = 36$). These were processed in three different batches of 3, 4, and 2 blocks, respectively. We chose males instead of females because more male samples had sufficient quality after the first two batches. This qualitative assessment took place before c-fos quantification. The sample size of 9 animals per group was in line with or larger than manuscripts previously published using whole-brain microscopy (6–8). Furthermore, it was sufficiently large to identify, study and mitigate batch effects. No experimental animal was excluded from the study for reasons other than insufficient staining quality. For a full description of the missing animals, see Supplementary Table 2.

**Foot-shock induction.** At any given experimental day, a cage was brought to the experimental room. Each animal of the cage was placed in a separate foot-shock box (floor area: 250x300mm) at the
same time. Since the animals were not earmarked, selection bias was limited by randomizing the order of the shock boxes when placing the animals. In this way, we aimed to limit a "shock box" specific effect. After 60 seconds, the experimental animals received a single foot-shock (0.8 mA, 2 sec). The to animal of each block was always placed in a "sham shock box". This box was identical to the others, but it did not give a shock. After another 30 seconds, all animals were removed from the shock boxes. The to animals were euthanized immediately, whereas animals of the foot-shock groups were single-housed in new cages waiting for transcardial perfusion according to their specified time point. The new cages were enriched with ad libitum chow and water, as well as bedding material of the home cage to limit arousal due to a novel environment.

To acquire meaningful, yet above threshold c-fos expression, the optimal to condition (home-cage vs novel environment) and foot-shock intensity (0.4 mA vs 0.8 mA, assessed at t = 90 min) were established in a pilot study. The results of the pilot study were only qualitatively assessed.

The investigator (RD) performing the foot-shock procedure and the perfusion was not blinded to condition, since she needed to confirm that the foot-shock was successfully applied and that animals were perfused at the correct time point.

Perfusion and tissue preparation. Euthanasia was performed with an intraperitoneal injection of 0.1 mL pentobarbital (Euthanimal 20%, 200 mg/mL) ~10 minutes prior to transcardial perfusion. The animals were perfused with ice-cold 1x PBS until blood clearance, followed by perfusion with ice-cold 4% PFA/1x PBS. Brains were extracted from the skull of perfused animals and stored in 4% PFA/1x PBS overnight for post-fixation.

Brains were cleared and stained following the iDisco+ protocol (7). Briefly, 24h post-fixation, samples were washed with 1x PBS. The olfactory bulbs and cerebellum were trimmed. A methanol/H₂O gradient was applied to dehydrate the tissue, followed by decolorization with 5% hydrogen-peroxide. After rehydration with a methanol/H₂O gradient, brains were permeabilized and remained in a blocking buffer for two days. Samples were then incubated in the primary antibody for seven days (rabbit anti c-fos, Synaptic Systems, cat. 226003, 1/1000 in 92% PTwH / 3% Donkey Serum / 5% DMSO). Following washing with 1x PBS / 0.2%Tween-20 / 0.1%Heparin (PTwH), brains were incubated for another seven days in the secondary antibody (Donkey anti-Rabbit IgG (H+L) Alexa 647™, Thermo Fisher Scientific, cat. A31573, 1/1000 in 97% PTwH / 3% Donkey Serum). Lastly, samples were washed with PTwH, dehydrated with a methanol/H₂O gradient, incubated in
66% DCM / 33% Methanol, washed with 100% DCM, and then cleared and stored in the clearing agent dibenzyl ether (DBE), protected from the light. The total time required to complete the protocol is 25 days. The investigators processing the tissue samples were blind to the experimental groups.

**Imaging with Light-Sheet microscopy.** Starting three days after clearing, samples were imaged with a light-sheet microscope (UltraMicroscope II, LaVision BioTec), equipped with an MVPLAPO 2x/0.5NA objective (Olympus), an MVX-10 Zoom Body (Olympus) and a 10mm working distance dipping cap. The images were recorded with an sCMOS camera (Neo 5.5 sCMOS, Andor Technology Ltd; image size: 2560x2160 pixels; pixel size: 6.5x6.5μm²) using the software ImpectorPro (v.5.0285.0; LaVision BioTec). The samples were scanned in horizontal slices (step size: 3μm; effective magnification: zoom_body objective + dipping lens 2x0.63x=1.26x; sheet width: 60%) with two-sided illumination using the built-in blending algorithm. Two image stacks per sample were taken consecutively, without moving the sample in between recordings. This is essential for the later correct annotation of c-fos positive (c-fos+) cells to brain areas. To record the fluorescence of c-fos+ cells, we used a Coherent OBIS 647-120 LX laser (emission filter: 676/29). The images were recorded at 70% laser power, sheet NA of 0.076 (results in a 10μm thick sheet), and an exposure time of 100 msec, as well as with horizontal focus to reduce z-plane distortion (steps: 20). To highlight the intrinsic fluorescence of the tissue for registration of the sample to a template, we imaged with a Coherent OBIS 488-50 LX Laser (filter: 525/50nm). The images were recorded at 80% laser power, sheet NA at 0.109 (results in a 7μm thick sheet), and exposure time of 100 msec, without horizontal focus.

The investigators (VB, HS) conducting imaging and image processing were blind to condition. Samples were imaged in ascending order, with sample numbers being randomly assigned during perfusion.

**Image processing: cell detection.** c-fos+ cells were detected from the 647nm image stack using Imaris (v9.2.0, Bitplane). A spots object was created and parameterized to detect cells with estimated xy-diameter of 10μm and an estimated z-diameter of 35μm (to avoid overcounting cells due to z-plane distortion). Thereafter, detected cells were filtered by quality (>18) and required to cross a threshold of minimum intensity (>225). The quality filter verifies that the shape of the detected cells aligns to the spots object within a threshold specified automatically by the algorithm. These parameters were optimized on pilot imagined brains to achieve optimal signal-to-noise ratio while avoiding ceiling
effects of the cell intensity parameter. The xyz-position of each cell was exported for later annotation to a reference atlas.

**Image processing: alignment and annotation.** Each cleared brain was registered to the Allen Mouse Brain 25 µm reference Atlas (9) by using ClearMap (7) as an interface to the open-source software Elastix v5.0 (10). Registration was performed using the autofluorescent images (488nm image stack). As per our setting, the images were first rotated, sheared and scaled with affine transformation, then translated onto the reference atlas with b-spline transformation.

The transformation matrix as calculated by Elastix was applied to the xyz coordinates of the Imaris detected cells using Transformix. In this way, detected cells were transformed into a template space, and each c-fos+ cell was assigned to a brain region. This was possible because each sample remained in the same position for both image stacks.

To estimate the error in the approach, we drew in ImageJ (Fiji) artificial spots ($n_{spots} = 72$) in both hemispheres ($n_{animals} = 12$) in three different locations (i.e., within dentate gyrus, mammillothalamic tract, amygdalar capsule). To estimate the error of the alignment, we calculated the distance between the expected position of the artificial spots and the position resulting from the alignment procedure.

The output for further data analysis is the xyz-position of every cell with their corresponding area code. The code was then translated to the respective brain area according to the brain region table provided by Renier and colleagues (7), which follows the hierarchical organization of the Allen Brain Atlas (ABA).

At this point, images have been transformed in machine-readable numbers. Other tools can be used until here. To continue with the following steps, one is only required to have files with xyz coordinates for each cell, together with their annotated brain area.

**Quality control and data pre-processing.** Quality control, data pre-processing and analysis were planned on a subset of the data ($n_{blocks} = 3$), and then later extended to the full dataset. The experimenter coding the analyses (VB) was blinded to the experimental condition.

To mitigate technical noise, a series of quality control steps were performed on the xyz annotated coordinates of c-fos+ cells. We removed (false positive) cells from brain areas in which no counts were expected, either because these areas contain no brain tissue (background, ventricular
system) or because they were trimmed from the sample (olfactory bulb, cerebellum, hindbrain). Next, we grouped the highest resolution areas of the ABA in line with the ABA hierarchical organization. The aim was to preserve as much spatial specificity as allowed by alignment inaccuracies in areas likely to be stress or c-fos sensitive, while minimizing the total number of brain areas to ease interpretation and to avoid unnecessary subsequent multiple testing. Accordingly, the categorization considered the region-specific distribution of glucocorticoid receptors as well as the region-specific c-fos expression after acute stress (11, 12). The hierarchical relationship of ABA areas is not complete, meaning not all larger brain areas can be fully subdivided into smaller brain areas. These “left over” spaces were removed from the analysis since they were deemed not interpretable.

An illumination artifact was present in all samples on the outside borders of the brain and the ventricles, presumably due to unspecific antibody binding. Initially, we aimed to use the no primary antibody control group to correct for this artifact; however, this was not possible as the number of c-fos+ cells in the no primary antibody control group was minimal. As an alternative solution, a mask of 75µm thickness was modeled along the inside border of the brain and the ventricles of the aligned samples (Supplementary Figure 3a), and cells that fell within the mask coordinates were removed from further analysis. The size of the mask (25 through 175µm) was piloted in 3 samples. Ultimately, 89 brain areas were included in the analysis (Supplementary Table 1).

Lastly, we removed xyz coordinates with extremely high c-fos intensity. We qualitatively assessed histograms of the maximum intensity of c-fos+ xyz coordinates per brain area, and compared them across samples to identify potential unspecific binding of the protein (“spots”). The potential candidates had 2- to 10-fold higher intensity than others within the same brain area. These were checked against the raw scans and removed if they did not appear as “cells” during a qualitative evaluation.

**Outlying values.** The selection of parameters for cell identification, the removal of the illumination artefact, the managing of areas with small volumes, and the removal of mis-labelled spots are procedures to limit as much as possible the presence of outliers. Despite these efforts, residual biological / technical outliers could be expected, either at the single cell level (e.g. unspecific antibody binding) or at the brain area level (e.g. disproportionate activation). Due to the limited sample size and the batch effects, the identification of outlying values was not trivial. We therefore chose to not use any rule (e.g. 3SD away from the mean) or statistical test to detect / exclude / replace outliers. Rather,
we assumed that they may occur uniformly across samples, thereby giving rise to increased variation. To mitigate their effects, we used medians and quantiles rather than means to summarize the data.

**Missing values.** The main source of missing value was the loss of animals due to insufficient staining quality (see ‘Experimental Design’ in Methods).

A second source of missing values was due to damaged brain areas during the experimental procedure. c-fos+ cells were counted per brain area across the whole brain. Damaged areas were manually detected in the 488nm image stack independently by at least two of three researchers (VB, HS, RD). The researchers were blinded to the experimental condition, and discrepancies were resolved with discussion. c-fos+ cells of damaged areas were removed, and then imputed by mirroring the xyz cells’ coordinates of the same brain area of the opposite hemisphere. Although this approach inherently assumes no differences between hemispheres, we preferred it to a multiple imputation approach because it did not require batch effects’ mitigation and it could be performed at a single cell rather than at brain area level.

A third source of missing values was linked to cell detection. The cell detection algorithm requires the definition of a minimum c-fos+ intensity. This parameter was optimized in pilot experiments, and it was kept identical throughout all experimental brains. In principle this is not a problem, since by rigorous standardization it is possible to mitigate batch effects and obtain comparable relative statistics. However, when a brain area has no active cells at $t_0$, it needs to be further evaluated to conduct a proper standardization. In our experiment, two brain areas (FRP and AHN) had no c-fos+ cells in one $t_0$ sample. Since this occurred only in one sample, we considered these brain areas as missing, not as zeros for analyses that required standardization with ratios to baseline.

**Preprocessing for region-based statistics.** Additional pre-processing is required for region-based analyses. In Figure 1, we summarize which pre-processing steps were required for which type of analysis.

In region-based analyses, the total number of c-fos+ cells (i.e. absolute counts) was calculated per brain area. However, cell counts are by definition not normally distributed; rather, they follow a Poisson or (negative) binomial distribution. We therefore applied a Box-Cox transformation
per block (i.e. each set for four different timepoints), so that our data would resemble a normal distribution.

Different brain areas have different sizes; therefore, absolute counts of c-fos+ cells are not indicative of how active a certain brain area is. In analysis where different brain areas are compared, absolute counts need to be normalized to the size of the brain area. We therefore calculated the number of c-fos+ cells per thousand of the total cells in each brain area, by adapting the atlas by Erö and colleagues (13). We used the total cell count estimation rather than that of only neurons because several publications have reported c-fos+ glia and astrocytes (for a review, see (14)), and it is in agreement with the presence of c-fos+ cells in the fiber tracts of our own data.

The number of c-fos+ cells differed across batches, although the relationship across time points was consistent within batches. Therefore, a normalization step was required to make the data more comparable across batches. Z-score normalization was performed per block, i.e. a unit of one sample per experimental group. With z-score normalization, the data is scaled with a mean of 0 and a standard deviation of 1, according to the formula \( \frac{x - \mu}{\sigma} \), where \( x \) is the observed value, \( \mu \) is the mean of the sample, and \( \sigma \) corresponds to the standard deviation of the sample.

**Region-based analyses: active brain regions.** With the exception of the single-cell strategy analysis, the analyses were planned on a subset of data (n=3), and later extended to the full dataset. The experimenter coding the analyses (VB) was blinded to the experimental groups.

We tested which brain areas had a significant increase from baseline in c-fos+ cell count per thousand of total cells (\( n_{\text{c-fos+}}/\text{tot} \)). The dependent variable was scaled and normalized as explained in the data pre-processing section. We performed pairwise comparisons (Welch t-test, one-sided, alpha = 0.05) for each time point (t30, t90, t180) against t0. P-values were adjusted with the Benjamini-Hochberg (BH) procedure. For visualization only, we transformed the p-values with a \(-\log_{10}\) transformation, and we grouped the brain areas according to the ABA embryological origin.

Next, we tested which brain areas were most active. The analysis was independently performed per block; therefore, no other batch-effect correction step was taken. For each block, we calculated the top 5% of \( n_{\text{c-fos+}}/\text{tot} \) independently of time point, and thereby identified per block the most active brain areas. Next, we counted how often a specific brain area was categorized as most active. We considered a brain area to be consistent across samples if it was present in at least 5 out
of 9 blocks in a particular time point. If we consider the process to be random under the null hypothesis, the probability of a brain area to be present in 5 out of 9 blocks would be 0.1%. This probability was estimated with a simulation study. We simulated 1000 independent experiments, and each experiment consisted of 4 time points with 9 independent iterations (i.e., n_blocks = 9). For each iteration, we selected 18 brain areas, meaning the 5% of 90 (n_brain_areas) * 4 (n_time_points). Each brain area had an equal probability of being selected (i.e., uniform distribution), and a brain area could be picked multiple times within each iteration (i.e., block), up to 4 (n_time_point = 4). Then, we calculated across 1000 experiments the probability of a brain area to be in the top 5% of the distribution. This simulation gave information about how likely it is that the representation in the top 5% was chance.

Since c-fos is not uniformly distributed across the brain, we performed a simulation study to assess whether the pattern obtained was due to the baseline spatial distribution of c-fos. We downloaded via the ABA’s API the mRNA c-fos expression levels of 3 experiments that passed the ABA quality check (id: 80342219; 79912554; 68442895). We calculated the mean and standard deviation for each of the brain areas available (n_brain_areas = 8). Since the resolution available for c-fos expression is lower than the resolution in our dataset, we assumed that the c-fos expression available corresponded to the location and scale parameters of a normal distribution defined by all the sub-areas. In other words, for each sub-area we sampled values from a normal distribution with location and scale parameters equal to those derived from the ABA atlas. This was performed for 9 independent samples (n_samples = 4) for each time point (n_time_point = 4). Since the selection may not be linked only to baseline c-fos expression, but also to a natural increase due to the foot-shock, we multiplied the baseline expression levels with the overall increase in c-fos across time points in our experiment. For each brain area, we calculated the median of the ratio between each foot-shock time point (t_{30}, t_{90}, t_{180}) and t_0. This ratio was then multiplied by the estimated c-fos expression values. Of note, the ratio at t_0 was always 1, meaning that the expression levels were estimated only from the ABA. We performed this simulation 1000 times, thereby simulating 1000 independent experiments. For each simulated experiment, we considered 9 blocks and 4 time points, as in our actual experiment. Then, for each block in each experiment, we selected the brain areas whose expression was in the top 5% of the distribution. Across the 1000 experiments, we then calculated the mean and standard deviation.
**Region-based analyses: order of activation.** Since brain areas displayed a temporal dynamic pattern, we aimed to order the brain areas based on their c-fos+ expression. Ordering brain areas based on the time of their activation is not trivial, especially since in 3D microscopy time is discrete \((t_{\text{time point}} = 4)\) rather than continuous (as, for example, in fMRI). Additionally, c-fos protein is not transient, but it peaks ~90 min and decays ~180 min after a stimulus. This dynamic may even be different depending on the brain area \((4)\). With these challenges in mind, we aimed to analytically create a pseudo-time to increase the temporal resolution, which would in turn allow to order brain areas.

Among the approaches considered (Supplementary Table 3), we ultimately ordered areas based on the estimated time of mid-activation across blocks. c-fos+ cell counts were Z-transformed. Then, for each brain area we calculated the median across blocks of each time point. We interpolated a linear model between each two consecutive timepoints: this line is the "continuum" of pseudo-time. To order the brain areas, we then considered at which pseudo-time point, c-fos activation reached its mid-activation level. Since the data was Z transformed, this corresponded to reaching the value 0. To limit the sensitivity of the pseudo-time, we binned the pseudo-time variable in bouts of 10 minutes. Each brain area was therefore grouped to the closest bout (binning). This approach has the advantage to create a criterion on which to categorize brain areas, but it does not consider the range (error) among which it could happen. The approach is ideal for areas that have one point of activation; it is biased for brain areas with a biphasic activation (e.g., at the beginning and at the end of our time curve).

For interpretation and visualization purposes, we classified the brain areas (Supplementary Table 4) according to functional networks relevant to stress. We followed Henckens and colleagues’ \((15)\) results, to which the an amygdalar group was added.

**Combining voxel based and single cell analysis: sub-brain areas.** We questioned whether c-fos+ cells are uniform within a brain area, or whether there are locations in which c-fos+ cells are most dense, i.e., are in closer proximity to each other. For this, we selected a brain area important for the stress response, i.e. the basolateral amygdala (BLA). All other brain areas can be visualized on the \textit{abc4d} app.
For each sample separately, we estimated the probability density of the BLA at each cell, by using a kernel density estimation with Gaussian function. Kernel densities are routinely used to smooth data from a finite sample to make inferences about a population. Here, they were used to estimate the cell density within a brain area. Next, we filtered the cells with highest density per sample.

The BLA was divided into voxels of 30µm per side. Considering that there could be an alignment error of (maximum) ~23µm (Supplementary Figure 2b), we considered 30µm the minimum, interpretable size. To look for consistency across samples, we calculated how many samples had at least one cell in each voxel, and considered 3 the minimum for consistency. In each xyz direction, we calculated per time point the median and interquartile of the voxels’ position. Of note, due to the batch effects, calculating number of cells (or other measures of activation) across samples would have had little value, and would need to be standardized. We therefore opted for this more straightforward approach.

To determine whether our observations were due to a chance process, we randomly attributed each sample to a time point, and perform the exact same analysis.

**Combining region-based and single cell analysis: strategy.** We hypothesized that brain areas can show activation with different strategies. With the “count strategy”, a brain area increases the number of c-fos+ cells with a low c-fos expression (intensity); with an “intensity strategy”, c-fos+ cells increase in intensity rather than number.

To test this hypothesis, we analyzed 10 samples, where we calculated the n_{cfos+} of each brain area, as well as the mean intensity of the cells in that area (intensity refers to the maximum intensity as reported by Imaris). Here, the mean rather than the median was intentionally used to be able to observe the increase in intensity due to a subgroup of cells within a brain area. For this analysis, we did not perform a batch effects correction; rather we took advantage of the differences across blocks. In our cell detection methodology (‘Image processing; cell detection’ in Supplementary Methods), cells are identified as c-fos+ depending on intensity. This relationship should always be the same, irrespective of batch effects or time points. To quantify the relationship analytically, we therefore interpolated a linear model between the raw c-fos+ cell count and median intensity, of all brain areas of all samples.
The linear model was used as a discriminant criterion to classify whether a brain area had a strategy more towards intensity (above the regression line) or towards count (below the regression line). This categorization was performed for each brain area of each to sample (n\text{animal} = 9) independently. We then calculated the probability of a brain area to belong to a certain categorization. The resulting variable was continuous between the values of 0 (i.e., all samples had an intensity strategy) and 1 (i.e., all samples had a count strategy).

If the categorization of brain areas would be a random process, the probability of brain areas to belong to a certain categorization would be normally distributed around $\mu = 0.5$ under the null hypothesis (i.e. brain areas do not have a strategy). To validate that the null hypothesis would indeed follow a normal distribution, we performed a simulation study. In this study, we used the exact same analysis, but the values for intensity and c-fos+ cell count were drawn independently from a Poisson distribution $P(\lambda = 3.5)$. The value for lambda $\lambda$ was selected by qualitatively comparing the distribution of intensity and count in the data with computer generated Poisson distributions with different lambdas. However, the interpretation would not change if different values of lambda would be selected.

Next, we questioned whether brain areas may change strategy after stress, relatively to $t_0$. Our experiment was not powered sufficiently to answer this particular research question, and therefore results should be interpreted as exploratory. From the categorization probability, we selected those brain areas that were consistent across samples, i.e. that had a specific categorization in at least 6 out of 9 samples. Of these, we selected those with a consistent change (increase or decrease) in count and / or intensity in at least one of the foot-shock time points ($t_{30}, t_{90}, t_{180}$). We calculated the pairwise difference between $t_0$ and each foot-shock time point for count as well as intensity. From this, we calculated the rate of change (count over intensity) per block for each time point. To compare data across brain areas, we converted the rates across samples to standardized mean differences (Hedge's g). We then classified brain areas as having changed strategy after stress if the effect size was below 0.5 or higher than 2, meaning that the relative increase in activity must have doubled towards intensity or towards count after stress compared to baseline.

Software. We developed the R package *abc4d* ("Analysis Brain Cellular activation in 4 Dimensions") to ease the implementation of data pre-processing and analysis. Furthermore, we developed a web
tool (https://vbonapersona.shinyapps.io/brain_after_footshock/) to interactively visualize the effects of acute stress on the brain area of choice.

All analyses were conducted with R (version 4.0.0) in the R studio environment on a macOS Mojave (version 10.14.6). The following R packages were core to this study: 1) tidyverse (version 1.3.0) for general data handling and visualization; shiny (v 1.6.0) for the generation of the web interface; ComplexHeatmap (v 2.4.3) for heatmap visualization.
Fig. S1. Cell detection and alignment validation. a) Representative example of staining of c-fos+ cells (bright red). White squares represent objects identified by the Imaris algorithm as cells. b) Validation of alignment. Error of the alignment represented as distance between real and aligned objects along the horizontal and sagittal axis. Error was calculated in three separate brain areas (horizontal facets) for n = 3 samples per time point.
The number of cells removed does not differ across groups

Fig. S2. Data cleaning. Number of ‘cells’ removed during the data cleaning procedure across all groups. Each dot corresponds to one sample. Data presented as median and IQR. Of note, t15 and t300 were only investigated in control experiments, and not across all batches (Supplementary Figure 3). Sample sizes (n): nhome cage = 3; n0 = 9; n15 = 3; n30 = 0; n90 = 9; n180 = 9; n300 = 6; nno primary = 3.
Fig. S3. Total number of c-fos+ cells of control experiments. 15 min after foot-shock is insufficient to detect an increase in c-fos expression. At 300 minutes, c-fos+ cell count is comparable to t₀. Home cage group has lower c-fos+ cell count than the respective t₀ group. No primary antibody group has nearly no counts. Each dot represents a sample, with the bar indicating the median, and the errorbar the interquartiles (IQR). The control experiments are represented separately with t₀ groups of the same batch. Sample sizes (n): n₀ = 9 (3 + 4 + 2 in each batch); n₅₁₅ = 3; n₃₀₀ = 6; n_no primary = 3; n_home cage = 3.
Fig. S4. Simulation of most active brain areas based on c-fos ABA mRNA expression and increase of c-fos+ cells over time. a) Regional distribution of c-fos as displayed by the Allen Brain Atlas (ABA). Red: hypothalamic areas. b) Results of the simulation study. An in silico dataset was created by using a sampling approach. c-fos mRNA expression values were downloaded from all the experiments available at the ABA API (nexperiment = 3). These values were used to sample weights to mimic what one would expect if the data were only due to c-fos expression and increase of c-fos over time. The procedure was repeated 1000 times. Each dot corresponds to a brain area that was present in the top 5% of the c-fos+ cell counts (per thousand of total) in one block. The vertical line of each dot represents the 95% confidence interval across the 1000 simulated experiments. None of the brain areas that were consistent in more than 5 samples was present in the actual experimental data.
Fig. S5. a) c-fos+ cells in highly dense areas across scrambled time point. a1, a2, a3 refer to the different 2D views of the xyz coordinates. Time points were randomly allocated for each sample, so that each block (i.e. a unit of 4 time points) had still one sample per time point. b) Strategy probability of brain areas according to our hypothesis, i.e. the relationship between count and intensity is due to the technical set-up. c) Relationship between c-fos+ count and median intensity. Contrary to expectations, count of c-fos+ cells and mean intensity are not correlated to each other.
**Fig. S6.** Cheat-sheet of abc4d package.
Supplementary Tables

**Table S1.** List of brain areas included in the analysis. The categorization follows the structure and acronym of the Allen Brain Reference atlas (25µm). ID is the code used by the ABA. Of note, we excluded 6 brain areas because we deemed that their size was too small for a confident alignment, namely: subfornical organ, vascular organ of the lamina terminalis, bed nucleus of the anterior commissure, bed nucleus of the accessory olfactory tract, fasciola chierea, induseum griseum.

| ID | Name brain area                              | Acronym |
|----|---------------------------------------------|---------|
| 23 | Anterior amygdalar area                     | AAA     |
| 31 | Anterior cingulate area                     | ACA     |
| 56 | Nucleus accumbens                           | ACB     |
| 88 | Anterior hypothalamic nucleus               | AHN     |
| 95 | Agranular insular area                      | AI      |
| 223| Arcuate hypothalamic nucleus                | ARH     |
| 239| Anterior group of the dorsal thalamus       | ATN     |
| 247| Auditory areas                              | AUD     |
| 295| Basolateral amygdalar nucleus               | BLA     |
| 319| Basomedial amygdalar nucleus                | BMA     |
| 351| Bed nuclei of the stria terminalis          | BST     |
| 382| Field CA1                                   | CA1     |
| 423| Field CA2                                   | CA2     |
| 463| Field CA3                                   | CA3     |
| 776| corpus callosum                             | cc      |
| 536| Central amygdalar nucleus                   | CEA     |
| 583| Claustrum                                   | CLA     |
| 631| Cortical amygdalar area                     | COA     |
| 672| Caudoputamen                                | CP      |
| 784| corticospinal tract                         | cst     |
| 726| Dentate gyrus                               | DG      |
| 830| Dorsomedial nucleus of the hypothalamus     | DMH     |
| 856| Thalamus polymodal association cortex related| DORrpm |
| 864| Thalamus sensory-motor cortex related       | DORsm   |
| 814| Dorsal peduncular area                      | DP      |
| 895| Ectorhinal area                             | ECT     |
| 909| Entorhinal area                             | ENT     |
| 942| Endopiriform nucleus                        | EP      |
| 958| Epithalamus                                 | EPI     |
| 1000| extrapyramidal fiber systems                | eps     |
| 184| Frontal pole cerebral cortex                | FRP     |
| 998| Fundus of striatum                          | FS      |
| 1057| Gustatory areas                             | GU      |
| 1105| Intercalated amygdalar nucleus              | IA      |
|   | Term                                      | Abbreviation |
|---|------------------------------------------|--------------|
| 44 | Infra-limbic area                        | ILA          |
| 51 | Intralaminar nuclei of the dorsal thalamus | ILM          |
| 59 | Intermediodorsal nucleus of the thalamus | IMD          |
| 131 | Lateral amygdalar nucleus                | LA           |
| 138 | Lateral group of the dorsal thalamus      | LAT          |
| 896 | Thalamus related                         | thalst       |
| 194 | Lateral hypothalamic area                | LHA          |
| 226 | Lateral preoptic area                    | LPO          |
| 275 | Lateral septal complex                   | LSX          |
| 290 | Hypothalamic lateral zone                | LZ           |
| 323 | Midbrain motor related                   | MBmot        |
| 331 | Mammillary body                          | MBO          |
| 339 | Midbrain sensory related                 | MBsen        |
| 348 | Midbrain behavioral state related        | MBsta        |
| 362 | Mediodorsal nucleus of thalamus          | MD           |
| 403 | Medial amygdalar nucleus                 | MEA          |
| 991 | Medial forebrain bundle system           | mfbs         |
| 500 | Somatomotor areas                        | MO           |
| 515 | Medial preoptic nucleus                  | MPN          |
| 904 | Medial septal complex                    | MSC          |
| 619 | Nucleus of the lateral olfactory tract   | NLOT         |
| 698 | Olfactory areas                          | OLF          |
| 714 | Orbital area                             | ORB          |
| 754 | Olfactory tubercle                       | OT           |
| 780 | Posterior amygdalar nucleus              | PA           |
| 788 | Piriform-amygdalar area                  | PAA          |
| 818 | Pallidum dorsal region                   | PALd         |
| 826 | Pallidum medial region                   | PALm         |
| 835 | Pallidum ventral region                  | PALv         |
| 843 | Parasubiculum                            | PAR          |
| 922 | Perirhinal area                          | PERI         |
| 946 | Posterior hypothalamic nucleus           | PH           |
| 972 | Prelimbic area                           | PL           |
| 1037 | Postsubiculum                            | POST         |
| 1084 | Presubiculum                             | PRE          |
| 1109 | Parastrial nucleus                       | PS           |
| 63  | Paraventricular hypothalamic nucleus      | PVHd         |
| 141 | Periventricular region                   | PVR          |
| 149 | Paraventricular nucleus of the thalamus  | PVT          |
Table S2. Missing values. a) List of missing animals with reasons. b) Damaged brain areas. These were removed from the analysis and re-imputed.

### a) Missing animals

| Batch | Animals missing                     | Reason                                                                 |
|-------|-------------------------------------|------------------------------------------------------------------------|
| 2     | 11 male animals, 40 female animals  | Staining faded.                                                        |
| 3     | 3 male animals                      | Staining faded (2 animals), scanning mistake (1 animal). The 3 animals belonged to two separate blocks. To not exclude both blocks completely, we merged the remaining sample into one block by selecting the best quality stainings. |

### Missing brain areas

| Sample | Damaged brain areas                   |
|--------|---------------------------------------|
| 106    | SI right, FS right, SUBv-sp right     |
| 107    | ACB left, OT left, PIR left, CP left, SI left |

### b) Damaged areas

| Sample ID | Damaged areas                                      |
|-----------|----------------------------------------------------|
| 13        | AAA left, CP left, OT right, AAA right, CP right, ENTl right |
| 14        | CP left, AAA left, VISC right, Alp right           |
| 15        | MOp right, SSp right                              |
| 16        | PIR left, ENTI left, PL right, MOs right           |
| 18        | CTX left                                           |
| 19        | MOp left, Ald left, GU left, Alv left              |
| 21        | SSs left, CP left, PAR left, HPF left, AAA right   |
| 22        | RSPd left, ViSp left, PRE left, AAA right          |
| 23 | RSPd left, RSPagl left, VISpm left, RSPv left |
| 24 | SI left, FS left, CP left, AAA right, CP right |
| 25 | PTLP left |
| 26 | PAG left, ICe left, SCS left |
| 27 | SSS left, PAA left, PIR left, COApl left, COAa left |
| 28 | AAA right, CP right, SSS right, MEAav right, PERI right, ECT right |
| 29 | AAA right, CP right, CEAm right, AUDd right, VISI right, TEa right, PRE right, ec right, dhc right, PRE right, SUBv right |
| 30 | PERI left, Alp right, CP right, AAA right |
| 31 | PERI left, ENTI left |
| 32 | VISpl left, VISp left, ec left, dhc left, PRE left, MOs, MOp, TEa, ECT, ENTm, PAR, ec, dhc |
| 33 | VISC left, Alp left, CP left, ECT right, ec right, dhc right, PAR right, ENTI right |
| 34 | OT left, PIR left, CP left, OT right, PIR right, AAA right, FS right, CP right, Alp right |
| 35 | MOs left, RSPv left, RSPd left, NA left, MOs right, TEa right, ECT right, PERI right, SUBv-sp right, ENT right, PAR right |
| 36 | OT left, SI left, FS left, CP left, PTLP right, TEa right |
| 37 | ALd left, MOp left, ORBl left, CP left, OT left, FS left, CP left, VISpl left, ec left, dhc left, POST left, OT right, FS right, CP right |
| 38 | OT left, FS left, CP left, ECT left, OT right, FS right, CP right |
| 39 | OT left, FS left, CP left, CP right, OT right, FS right, AUDd right, AUDDp right, ECT right |
| 40 | TTd left, AON left, AAA left, CP left, IA left, ENTI left, ECT left, CP right, AAA right, IA right, ENTI right, ECT right |
| 41 | OT left, SI left, ACB left, CP left, Alp left, OT right, SI right, FS right, CP right, ENTI right |
| 42 | VISSL left, ECT left, RSPd right, RSPv right |
| 43 | Alp left, AON left, PIR left, Alv left, GU left, AAA right, CP right |
| 44 | AAA left, CP left, VISp left, VISA left, VISam left, VISP left, VISI left, POST left, PRE left, AAA right, CP right, AUDd right, PTLP right, ENT right, PAR right |
| 45 | SSS left, CP left, ECT left, TEa left, ENTI right |
| 46 | SI right, FS right, SUBv-sp right |
| 47 | ACB left, OT left, PIR left, CP left, SI left |
| 48 | SSP left, GU left, OT right, SI right, ACB right, CP right, POST right, ec right, VISpm right, VISp right, RSPv right, RSPd right |
| 49 | MOs right |
| 50 | ECT right, PERI right, ENTI right |
| 51 | VISP left, VISp left, ec left, dhc left, POST left, MOs right, MOp right, PRE right, HPF right, SUBv-sp right, alv right, ec right |
| 52 | OT left |
| 53 | SUBv-sp right |
| 54 | PIR right, PAR right, ENTI right, ENTI right |
| 55 | SSS left, FS left, act left, CP left |
| 56 | SSP right |
| 57 | CP right |
| 58 | TEa right, SUBv-sp right, PAR right |
Table S3. List of analytical approaches considered for ordering brain areas based on c-fos activation.

| Approach   | Brief explanation                                      | Not pursued because:                                                                 |
|------------|--------------------------------------------------------|--------------------------------------------------------------------------------------|
| Clustering | Clustering to reduce dimensions, then order the cluster groups. The pseudo-time would then have resolution equal to the number of clusters. Ordering could be achieved by comparing to a simulated model of possible clusters out of theory (example: cluster with only initial activation at t_{30}; cluster with activation at t_{90}; clustering with activation at t_{90} as well as t_{180}). | All brain areas were activated; therefore, very minimal difference would appear between clusters. Furthermore, creating “expected” cluster models is not trivial. |
| Derivatives| Identify the steepest derivative between each two consecutive time points. This can be performed per sample (probabilistic approach) or on the median across samples. It might be able to identify multiple activations (e.g. if first and third derivatives are steeper than the second). | Too many rules (e.g. only one derivative is the steepest, two derivatives are the steepest...), therefore it has the same problem as creating the “expected cluster” model as described above. Furthermore, very pseudo-time resolution (n = 3). |
| Peaks      | Fit a loess curve for each sample and identify the maxima. Each maxima is considered a peak. Advantage that it can identify multiple activations for a single brain area | Since all brain areas were so activated, many peaks would appear within the same range (poor pseudo-time resolution). As a consequence, too much importance would be given to the type of curve used to fit the data. |
Table S4. Functional categorization of brain areas. Brain areas were classified in functional groups relevant to the stress response, by adapting (15). cx = cortex.

| Functional categorization | Brain area                                      | Acronym |
|---------------------------|-------------------------------------------------|---------|
| Amygdala                  | Anterior amygdalar area                         | AAA     |
| Amygdala                  | Basolateral amygdalar nucleus                   | BLA     |
| Amygdala                  | Basomedial amygdalar nucleus                    | BMA     |
| Amygdala                  | Central amygdalar nucleus                       | CEA     |
| Amygdala                  | Cortical amygdalar area                         | COA     |
| Amygdala                  | Intercalated amygdalar nucleus                  | IA      |
| Amygdala                  | Lateral amygdalar nucleus                       | LA      |
| Amygdala                  | Medial amygdalar nucleus                        | MEA     |
| Amygdala                  | Posterior amygdalar nucleus                     | PA      |
| Amygdala                  | Piriform-amygdalar area                         | PAA     |
| Hippocampus               | Field CA1                                       | CA1     |
| Hippocampus               | Field CA2                                       | CA2     |
| Hippocampus               | Field CA3                                       | CA3     |
| Hippocampus               | Dentate gyrus                                   | DG      |
| Hippocampus               | Entorhinal area                                 | ENT     |
| Hippocampus               | Parasubiculum                                   | PAR     |
| Hippocampus               | Postsubiculum                                   | POST    |
| Hippocampus               | Presubiculum                                    | PRE     |
| Hippocampus               | Subiculum                                       | SUB     |
| Hypothalamus              | Anterior hypothalamic nucleus                   | AHN     |
| Hypothalamus              | Arcuate hypothalamic nucleus                    | ARH     |
| Hypothalamus              | Dorsomedial nucleus of the hypothalamus         | DMH     |
| Hypothalamus              | Lateral hypothalamic area                       | LHA     |
| Hypothalamus              | Lateral preoptic area                           | LPO     |
| Hypothalamus              | Hypothalamic lateral zone                       | LZ      |
| Hypothalamus              | Hypothalamic lateral zone                       | LZ      |
| Hypothalamus              | Mammillary body                                 | MBO     |
| Hypothalamus              | Medial preoptic nucleus                         | MPN     |
| Hypothalamus              | Posterior hypothalamic nucleus                  | PH      |
| Hypothalamus              | Parastrial nucleus                              | PS      |
| Hypothalamus              | Paraventricular hypothalamic nucleus descending division | PVHd |
| Hypothalamus              | Periventricular region                          | PVR     |
| Hypothalamus              | Periventricular zone                            | PYZ     |
| Hypothalamus              | Tuberal nucleus                                 | TU      |
| Hypothalamus              | Ventromedial hypothalamic nucleus               | VMH     |
| Motor cx                  | Somatomotor areas                               | MO      |
| Prefrontal cx             | Anterior cingulate area                         | ACA     |
| Prefrontal cx             | Orbital area                                    | ORB     |
| Prefrontal cx | Prelimbic area | PL |
|---------------|----------------|----|
| Prefrontal cx | Taenia tecta | TT |
| Primary somatosensory cx | Somatosensory areas | SS |
| Thalamus | Anterior group of the dorsal thalamus | ATN |
| Thalamus | Thalamus polymodal association cortex related | DORpm |
| Thalamus | Thalamus sensory-motor cortex related | DORsm |
| Thalamus | Epithalamus | EPI |
| Thalamus | Intralaminar nuclei of the dorsal thalamus | ILM |
| Thalamus | Intermediodorsal nucleus of the thalamus | IMD |
| Thalamus | Lateral group of the dorsal thalamus | LAT |
| Thalamus | Mediodorsal nucleus of thalamus | MD |
| Thalamus | Paraventricular nucleus of the thalamus | PVT |
| Thalamus | Reticular nucleus of the thalamus | RT |
| Thalamus | Ventral anterior-lateral complex of the thalamus | VAL |
| Thalamus | Ventral medial nucleus of the thalamus | VM |
| Thalamus | Ventral posterior complex of the thalamus | VP |
| Visual cx | Visual areas | VIS |
**Movie S1 (separate file).** Visualization of parts of the brain removed due to unspecific binding (in color).

**Movie S2 (separate file).** Cartoon visualization of all brain areas that become activated after foot-shock over time. For visualization purposes, the colors correspond to categorization into different functional networks.

**Movie S3 (separate file).** 3D visualization of single cells over time within the basolateral amygdala. Each dot corresponds to a cell within a high-density area. Color of the dots corresponds to the different time points. In legend, \( t = \) represent the time expressed in minutes.
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