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Impact of tissue storage time on immunodetection of c-Fos and GAD67 in the rat brain

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ABSTRACT

Background: Activity-dependent markers such as c-Fos, a rapid indicator of neuronal activation, and GAD67, an enzyme essential for GABA synthesis in inhibitory neurons, are extensively employed to elucidate neural circuit dynamics. Given that many studies span extended periods with multiple experimental groups, it is crucial to ensure long-term storage of non-frozen brain tissue does not compromise immunodetection.
New method: Here, we evaluated the impact of storage duration on the immunodetection of c-Fos and GAD67 in rat brains. Intact brains, fixed in paraformaldehyde, were stored at 4 °C in phosphate-buffered saline with sodium azide to prevent bacterial growth. Brains were assessed at two storage durations - short (1.5 months) and prolonged (10 months). Brain sections were immunostained for c-Fos and GAD67 and imaged by confocal microscopy.
Results: We observed robust c-Fos immunoreactivity across multiple regions of the medial prefrontal cortex, hippocampus, and neocortex, with no significant differences attributable to storage duration. Additionally, quantifications of GAD67-positive cells and cells co-labeled for c-Fos/ GAD67 confirmed that immunodetection of inhibitory neurons remains intact when whole brains are stored for up to 10 months. In contrast, prolonged storage of brain slices strongly reduced c-Fos, but increased GAD67 staining.
Comparison with existing method(s): The stability of c-Fos and GAD67 in tissue stored long-term at 4 °C remains untested.
Conclusions: These findings underscore that whereas intact brains can be safely stored for prolonged periods at 4°C without compromising antigenicity, brain slices are highly susceptible to storage-induced deterioration - insights important for planning and interpreting immunohistochemical studies in neuroscience.

1. Introduction

Immunohistochemistry is an indispensable technique in neuroscience enabling the visualization of cellular and molecular substrates underlying brain function (Burry, 2011; Werner et al., 2021). One key class of markers for activity mapping is the immediate-early genes (IEGs) which are rapidly and transiently expressed in neurons in response to synaptic stimulation, independent of de novo protein synthesis (Kovács, 1998; Lara Aparicio et al., 2022). For decades, IEGs such as c-Fos have provided breakthroughs in our ability to capture snapshots of neuronal activation in vivo. As a prototypical IEG, c-Fos is widely used to mark neuronal activation, whereas glutamate decarboxylase (GAD)67, as the key enzyme for gamma-aminobutyric acid (GABA) synthesis, marks inhibitory neurons (Herrera and Robertson, 1996; Kobelt et al., 2004). These markers have been crucial for mapping neural circuits and probing functional states in vastly different contexts - from developmental neurobiology and injury models to studies of learning and memory. Numerous studies, including our own work (Contreras et al., 2024; Shan et al., 2022, 2023) demonstrate how early-life experiences drive lasting changes in c-Fos and GAD67 expression at adulthood, underscoring the need for reliable long-term immunodetection.

Because studies often involve diverse experimental conditions including many animals that are, sometimes tracked over extended (e.g., developmental) timelines, a dependable method for long-term storage of

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brain tissue is essential. Protocols often involve equilibrating paraformaldehyde (PFA)-fixed brains in cryoprotectant media (e.g., 30 % sucrose or 15 % glycerol) and storing them at −20°C or −80°C to preserve antigenicity and prevent ice-crystal damage (Ameen-Ali et al., 2021; Estrada et al., 2017; Numa et al., 2019; Sadler et al., 2009; Vetere et al., 2021). However, freezing in cryoprotectant media can partially damage tissue integrity, cryostat slicing - typically involving slide-mounted sections - is technically more demanding, especially when cutting multiple sections across different bregma levels. To avoid the disadvantages of freezing and cryostat sectioning, other protocols have been developed allowing to store intact brains at 4 °C in phosphate-buffered saline (PBS), which is supplemented with sodium azide to inhibit bacterial growth, after fixing them in PFA for 24 h and thoroughly washing in PBS (Egorova et al., 2025; Khoury et al., 2025; McKenzie et al., 2024; Muza et al., 2023; Needham et al., 2022; Shan et al., 2022). This non-cryogenic approach preserves whole brains, which can later be sectioned more efficiently with a vibratome as free-floating sections, offering greater flexibility when cutting multiple regions across different rostrocaudal levels. However, the feasibility of long-term storage of fixed brains at 4°C over more than a couple of days is unknown.

Here we aimed at refining non-cryogenic storage protocols by evaluating whether PFA-fixed, PBS-washed rat brains can be stored at 4 °C for extended intervals without compromising immunofluorescence detection of c-Fos and GAD67. Specifically, we compared intact brains stored in PBS with sodium azide at 4 °C for short (1.5 months) and prolonged (10 months) intervals, followed by the markers evaluation in key regions of rat medial prefrontal cortex, hippocampus and posterior cortical regions. In addition, we prepared vibratome-cut slices from defined bregma levels (in medial prefrontal cortex) and stored these slices under identical conditions and similar durations, enabling a direct comparison of long-term storage effects on intact versus sliced tissues.

2. Materials and methods

2.1. Animals and behavioral procedures

A total of 27 male Long-Evans rats were used in the experiments, all obtained from Janvier Labs (Le Genest-Saint-Isle, France). For Experiment 1 (investigating short versus prolonged storage of intact brains), two groups (each, n = 6) were ordered to arrive at postnatal day (PD) 67 with orders placed 8.5 months apart. For Experiment 2 (investigating prolonged storage of intact brains versus prolonged storage of brain slices), the animals arrived already at PD8 or PD9. (These animals belonged to a larger study on memory formation in infant and adult rats, Shan et al., unpublished results).

Following arrival, all animals had at least 7 days of acclimatization before any manipulation. The pups of Experiment 2 were maintained with their dam until weaning (PD21) and were kept pairwise after the weaning day. All animals were kept at room temperature (22 ± 1°C) on a controlled 12/12 h light/dark cycle (lights on at 7:00 h), and had free access to food and water.

In both Experiment 1 and Experiment 2, the animals' brains were collected after a retrieval test on a spatial Object-place recognition (OPR) task, performed at the same age (PD80) in all animals. Testing on the OPR task took always place during the light phase (between 7:00–14:00 h) and comprised a 3-day habituation phase, followed by a sampling phase (encoding) and the retrieval test (for details see, (Shan et al., 2025)). After retrieval testing, the rats returned to the home cage. All procedures were performed in accordance with the European animal protection laws and were approved by the Baden-Württemberg state authority.

2.2. Study design and tissue storage

Brains for both Experiment 1 and 2 were collected on PD80 in defined behavioral conditions, i.e., 90 min after OPR retrieval testing (to allow maximal c-Fos protein expression, (Holstein et al., 2012; Kovács, 2008). For brain collection, the animals were transcardially perfused with PBS, followed by 4 % PFA in 0.1 M phosphate buffer. The brains were post-fixed at 4 °C for 24 h and stored in PBS with 0.1 % sodium azide at 4 °C until further processing.

In Experiment 1, brains in the "prolonged-storage" (PS) condition were stored for 10 months, whereas those in the "short-storage" (SS) condition were stored for 1.5 months. In Experiment 2, brains in the "prolonged-storage of intact brains" (PSB) condition were stored for 8.5 months, whereas those in the "prolonged-storage of slices" (PSS) condition were maintained intact for approximately 0.5 months post-perfusion; subsequently, the regions of interest (ROIs) were sectioned at the respective bregma levels, and the resulting slices were stored for an additional 8.5 months in PBS with 0.1 % sodium azide at 4 °C.

2.3. c-Fos and GAD67 immunocytochemistry in regions of interest

After storage, brain tissues in all conditions underwent basically identical further processing until final mounting on slides: Coronal slices (70 μm) at the respective bregma levels were cut with a vibratome (Microm HM 650 V, Thermo Fisher Scientific MICROM GmbH, Germany) in all conditions except in the PSS condition, where slices were cut in the same way before storage. Free-floating sections were blocked in PBS containing 10 % horse serum and 0.3 % Triton-X for 90 min, then incubated in 0.2 % Triton-X in PBS with mouse monoclonal anti-GAD67 (1:1000; Sigma-Aldrich, MI, #MAB5406) and rabbit polyclonal (1:1000; Synaptic Systems, Germany, #226 003) or monoclonal (1:2000; Synaptic Systems, Germany, #226 008) anti-c-Fos primary antibodies for 48 h at 4 °C. After four washes with PBS, the sections were incubated with donkey anti-mouse Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 555 (1:1000; Thermo Fisher Scientific) in 0.2 % Triton-X in PBS for 24 h at 4 °C, followed by incubation with NeuroTrace (1:200; Thermo Fisher Scientific). Finally, the sections were washed with PBS, mounted on gel-coated slides, coverslipped with Vectashield antifade mounting medium (Vector Laboratories), and rendered ready for confocal imaging.

The ROIs were selected based on previous literature implicating them in spatial memory formation and were anatomically defined according to the atlas by Paxinos and Watson (2006). ROIs and their distance (in mm) from Bregma were as follow: + 3.24 mm for prelimbic (PL), infralimbic (IL), and cingulate cortex (CG) and −3.96 mm for cornu ammonis (CA)1, CA3, dentate gyrus (DG), retrosplenial cortex granular (RSG), retrosplenial cortex dysgranular (RSD), parietal (PAR), perirhinal cortex (PRH) and lateral entorhinal cortex (ENT).

Images were obtained using a confocal laser scanning microscope (LSM 710; Carl Zeiss, Germany) with a low magnification (plan-apochromat oil-immersion objective, 25 ×/0.8NA; z- stack of 11 slices (50 μm thickness); voxel size 0.33 μm × 0.33 μm × 3.5 μm. Settings for laser intensity, gain, offset and pinhole were optimized initially and held constant throughout the experiments. Automated (for c-Fos$^+$ cells) and manual (for GAD67$^+$ cells) counts were performed using CellProfiler software. For automated counting, a fixed manual threshold for c-Fos was applied uniformly to all specimens in each region. Counting was done on the maximum intensity projection of the z-stacks of the entire slice volume. The sizes of the ROIs were 693,600 μm2 for PL, 462,400 μm2 for IL, 578,000 μm2 for CG, 601,800 μm2 for CA1, 612,000 μm2 for CA3, 346,800 μm2 for DG, RSD, and RTN, 683,400 μm2 for RSG, 275,400 μm2 for PAR, 404,600 μm2 for PRH, 173,400 μm2 for ENT (Fig. 1). Fig. 2 shows representative confocal images of the PL, CA3, and PRH regions (a small part of the regions defined for quantification in Fig. 1 is displayed).

2.4. Statistical analyses

Statistical comparisons were performed using SPSS software (IBM,

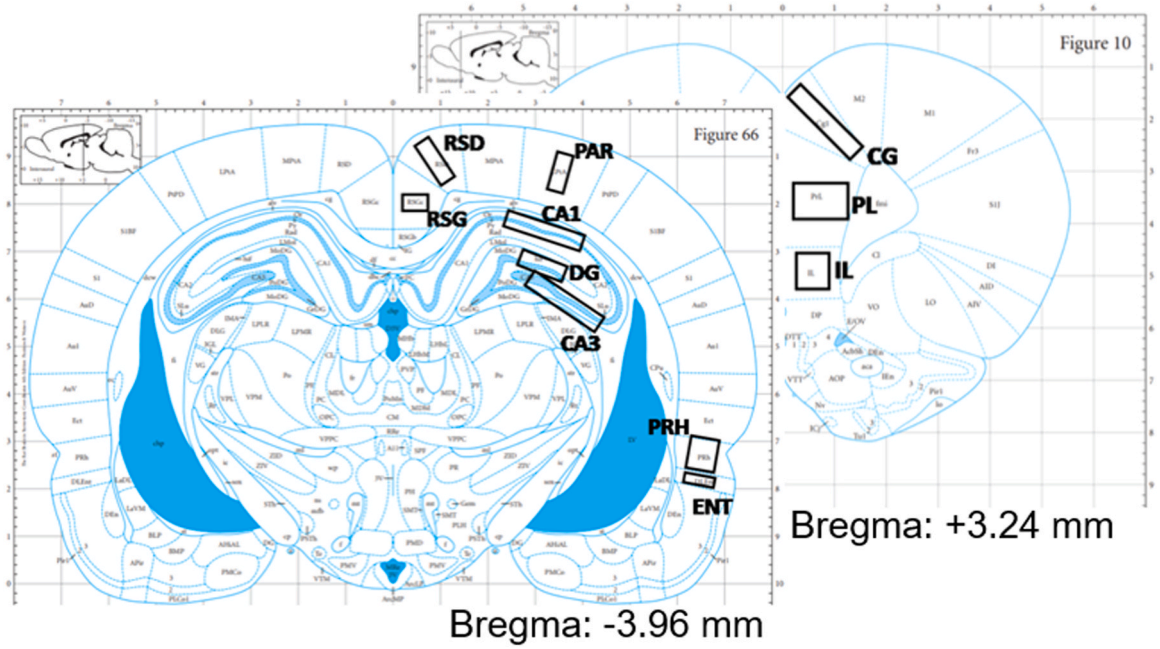

Fig. 1. Regions of interest (ROIs) for c-Fos and GAD67 analyses. ROIs indicated by black frames. Corresponding bregma levels were used in all rats (adapted from Paxinos and Watson, 2006). Medial prefrontal cortex: PL - prelimbic, IL- infralimbic, CG - cingulate cortices; hippocampus: CA1 - cornu ammonis 1, CA3 - cornu ammonis 3, DG - dentate gyrus, posterior cortex: RSG and RSD - granular and dysgranular retrosplenial cortices, PAR - parietal, PRH - perirhinal, ENT - entorhinal cortices.

Armonk, NY, USA). For Experiment 1, a global analysis of variance (ANOVA) was conducted with two factors: a between-subjects factor (Storage: SS vs. PS) and a repeated measures factor (Region). In the c-Fos analyses, the Region factor comprised 11 regions (PL, IL, CG, CA1, CA3, DG, PRH, ENT, PAR, RSD, and RSG). For the GAD67 analyses, RSD and RSG were excluded due to quantification difficulties caused by high background signal, resulting in a total of 9 regions. For Experiment 2, a global ANOVA was performed comparing two Storage conditions (PSB and PSS) with a repeated measures Region factor that included three prefrontal regions (PL, IL, and CG). When significant Storage main effects or Storage x Region interactions were found, post hoc pairwise *t*-tests were used to determine the specific differences. A significance level of $p < 0.05$ was adopted for all statistical tests, and data are presented as mean ± SEM. Additionally, to confirm stability of measures after storage, i.e., to test the null hypothesis of no difference between SS and PS conditions, we used Bayesian statistics, with a BF_{10} between 0 and 1 indicating evidence in favor of "no difference" between conditions (Schönbrodt and Wagenmakers, 2018)

3. Results

3.1. Experiment 1: storage duration of whole brains does not affect regional detection of c-Fos, GAD67 or c-Fos/ GAD67 co-labeling

In these experiments, intact brains were stored in a prolonged-storage (PS) condition for 10 months versus a short-storage (SS) condition for 1.5 months. Using a polyclonal anti-c-Fos antibody, we did not reveal any difference in c-Fos$^+$ cell counts between the storage conditions for any of the 11 ROIs (Fig. 3A; $F(2.1, 20.5) = 0.591$, $p = 0.567$, and $F(1, 10) = 0.818$, $p = 0.387$, for Storage x Region interaction and Storage main effect, $p > 0.156$, for all pairwise conditions). Bayes factors (BF_{10}) of 0.049 and 0.496 for respective Storage x Region and Storage main effects and of $BF_{10} < 0.940$ for all pairwise comparisons confirmed that in favor of the null-hypothesis distributions between the two storage conditions were highly comparable. Basically the same results were obtained using the monoclonal anti-c-Fos antibody (Fig. 3B; $F(1.9, 18.8) = 0.218$, $p = 0.793$, and $F(1, 10) = 0.049$, $p = 0.829$, $BF_{10} = 0.018$ and $BF_{10} = 0.364$ for Storage x Region interaction and Storage main effect, respectively, all $p > 0.290$, for pairwise test, all $BF_{10} < 0.687$. Finally, the overall neuronal density, as determined by NeuroTrace labeling, also did not differ between the Storage conditions ($F(3.5, 35.4) = 0.076$, $p = 0.984$, and $F(1, 10) = 0.007$, $p = 0.935$, $BF_{10} = 0.030$ and $BF_{10} = 0.443$, for Storage x Region and Storage main effect, respectively, all $p > 0.491$ for all pairwise tests, all $BF_{10} < 0.550$).

Like c-Fos$^+$ cell counts, also total GAD67$^+$ counts (Fig. 4A, $F(3.1, 30.6) = 0.521$, $p = 0.674$, $F(1, 10) = 0.497$, $p = 0.497$, $BF_{10} = 0.046$ and $BF_{10} = 0.410$, for Storage x Region interaction and Storage main effect, respectively) and double-positive c-Fos/ GAD67 cells counts (Fig. 4B, $F(1.9, 19) = 0.181$, $p = 0.825$, and $F(1, 10) = 0.059$, $p = 0.813$, $BF_{10} = 0.025$ and $BF_{10} = 0.392$) did not differ between storage conditions. Pairwise comparisons and Bayes factors further confirmed comparability of the distributions between storage conditions for each ROI for GAD67$^+$ counts (all $p > 0.105$, all $BF_{10} < 1.169$) as well as double-positive c-Fos$^+$/ GAD67$^+$ cells counts (all $p > 0.620$, all $BF_{10} < 0.508$)

3.2. Experiment 2: slices stored over long time show reduced c-Fos, but enhanced GAD67 expression

In Experiment 2, we compared c-Fos expression between two storage conditions, prolonged-storage of intact brains (PSB condition) versus prolonged-storage of brain slices (PSS condition), in three medial prefrontal cortex regions (PL, IL, CG). Representative confocal microscopy images from the PL cortex (Fig. 5A) illustrate the observed difference: storage of the intact brain (left panel) leads to robust c-Fos immunofluorescence, whereas the corresponding brain slice that was stored as slice (right panel) shows a marked reduction in staining. The reduction in c-Fos$^+$ cell counts in the stored slices (PSS condition) was stronger in the PL and IL regions showing higher c-Fos expression in the PSB condition than in the CG region relatively with low expression in the PSB condition ($F(1.2, 16.2) = 4.725$, $p = 0.038$, and $F(1, 13) = 7.465$, $p = 0.017$, for Storage x Region interaction and Storage main effect, respectively). Accordingly, post hoc pairwise comparisons indicated significant differences between the storage conditions in the PL

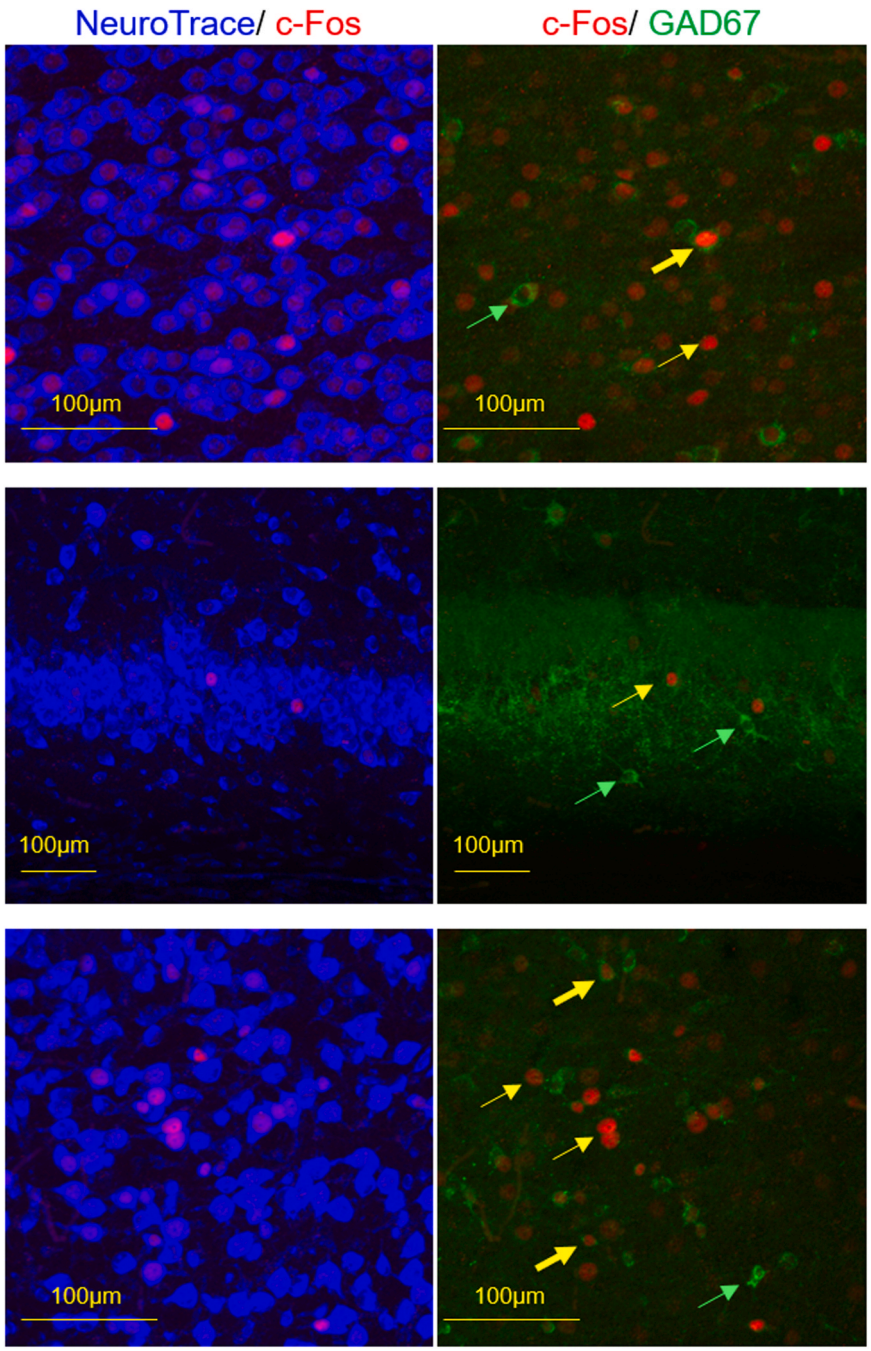

Fig. 2. NeuroTrace, c-Fos, and GAD67 expression in PL, CA3, and PRH regions. Representative confocal images**:** Maximum intensity projections of an 11 z-stack confocal scan (from a brain slice of the short-storage, SS condition). From top to bottom; Prelimbic (PL) cortex, CA3 region of the hippocampus, Perirhinal (PRH) cortex. Left panels show combined staining for NeuroTrace and c-Fos, while right panels display combined staining for c-Fos and GAD67. Thin yellow arrows indicate c-Fos–positive cells, thin green arrows GAD67-positive cells, and thick yellow arrows cells double-labeled for both c-Fos and GAD67.

($p = 0.037$) and IL ($p = 0.005$) regions, but not in the CG region ($p = 0.111$, Fig. 5B).

In addition to c-Fos expression, we examined GAD67 and c-Fos/ GAD67 co-labeling in the same three regions. Representative confocal images from the PL cortex are shown in Fig. 6A. Unlike c-Fos, expression of GAD67 was in general slightly increased in the stored-slice (PSS) compared to the intact-brain (PSB) condition ($F(1, 13) = 9.275$, $p = 0.009$, for main effect of Storage; $F(1.4, 18.7) = 0.357$, $p = 0.636$, for Storage x Region interaction; see Fig. 6B, for pairwise comparisons). c-Fos/ GAD67 double-positive cells – like c-Fos$^+$ counts – were reduced in the PSS compared to the PSB condition ($F(1, 13) = 7.304$, $p = 0.018$, main effect of Storage) with this effect failing to reach significance in the CG region where c-Fos/GAD67 double-positive cells counts were lowest ($F(1.8, 23.7) = 5.167$, $p = 0.016$; Storage × Region interaction; Fig. 6C).

4. Discussion

Our study examined to what extent long-term storage at 4 °C of PFA-fixed, washed rat brains affects antigenicity for immunofluorescence detection of c-Fos and GAD67. We found that in intact brains stored in PBS with sodium azide robust c-Fos and GAD67 immunoreactivity was maintained over prolonged storage interval of 10 months and was closely comparable to that observed in brains stored for only 1.5 months.

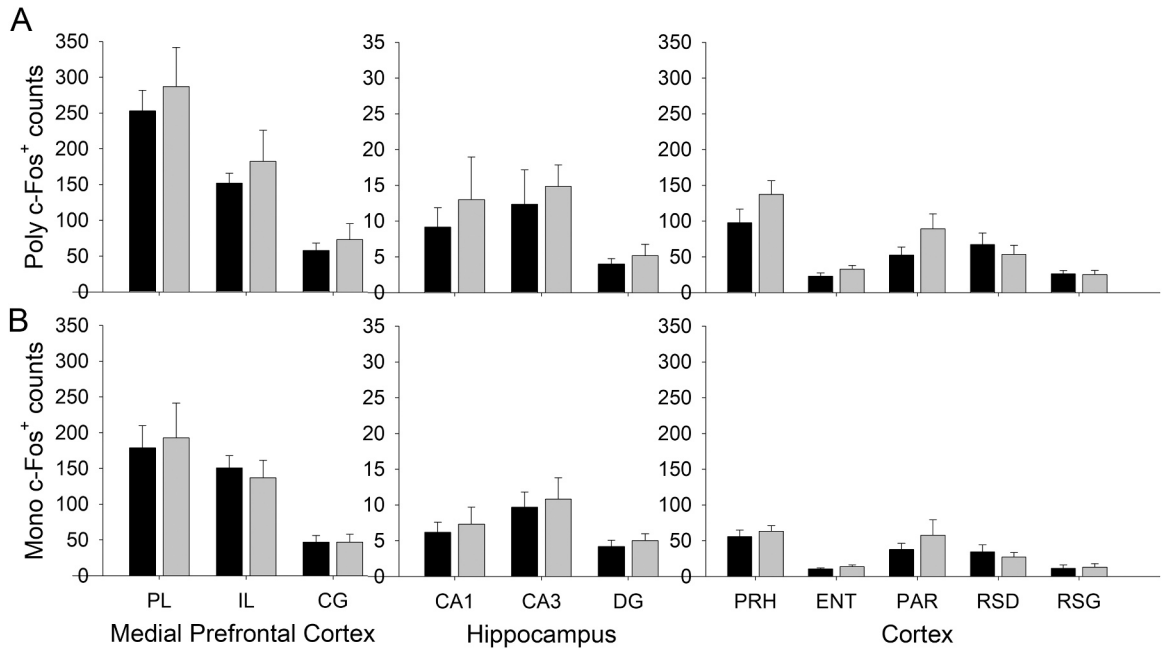

Fig. 3. Storage duration of intact brains does not alter regional c-Fos detection. (A) c-Fos⁺ cell counts (mean ± SEM) in 11 ROIs (see legend to Fig. 1 for abbreviations) for the short-storage (SS, n = 6, black bars) and prolonged-storage condition (PS, n = 6, grey bars) using a polyclonal antibody against c-Fos (Poly) or (B) a monoclonal antibody (Mono). Note. Despite large regional differences in c Fos⁺ cell counts, within each region counts were closely comparable between storage conditions.

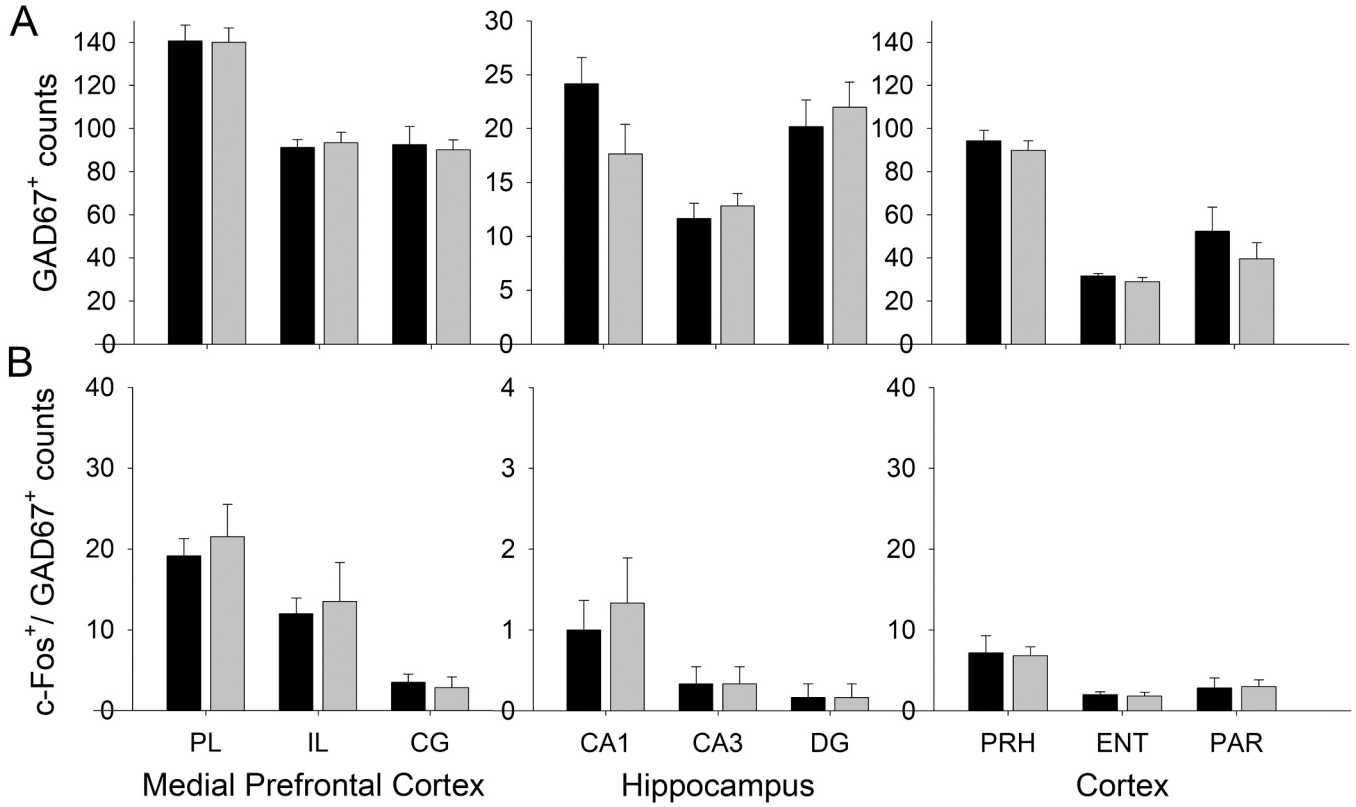

Fig. 4. Storage duration of intact brains does not affect GAD67 expression or c-Fos/ GAD67 co-labeling. Counts (mean ± SEM) of (A) GAD67^{+} cells and (B) cells co-labeled for c-Fos and GAD67 in 9 ROIs (see legend to Fig. 1 for abbreviations) for the short-storage (SS, n = 6, black bars) and prolonged-storage condition (PS, n = 6, grey bars).

On the other side, compared with the storage of intact brains, the storage of vibratome-cut slices for 8.5 months in the same conditions led to a substantial decrease in c-Fos immunofluorescence, but an increase in GAD67 staining.

c-Fos expression in intact brains was not affected by storage time, regardless of whether a polyclonal or monoclonal antibody was used.

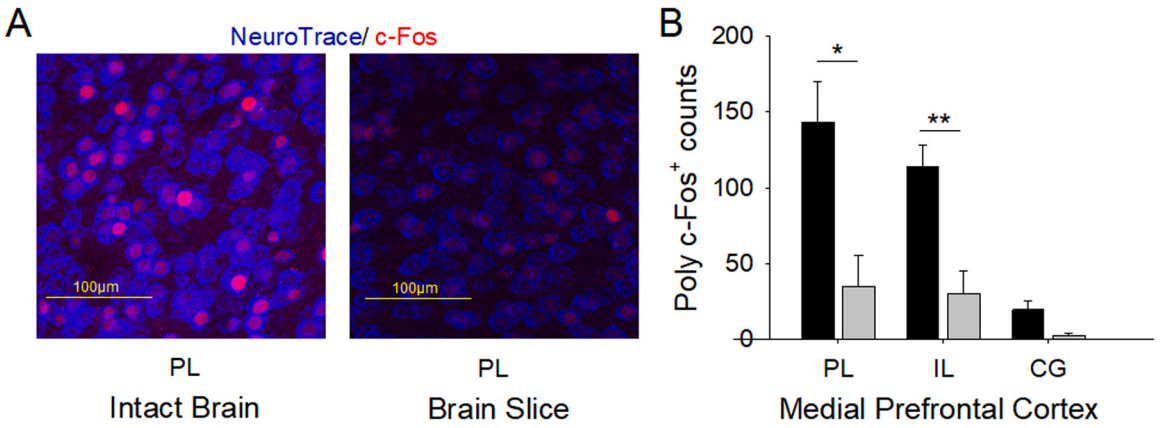

Fig. 5. Long-term brain slice storage impairs medial prefrontal cortex c-Fos detection. (A) Representative confocal microscopy images of NeuroTrace/c-Fos immunostaining in the prelimbic (PL) medial prefrontal cortex. Corresponding sections obtained from stored intact brain (left) versus slice (right). (B) c-Fos$^+$ cell counts (mean ± SEM) in the PL, infralimbic (IL), and cingulate cortex (CG) regions from samples of stored intact brains (n = 11; black bars) and stored brain slices (n = 4; gray bars). c-Fos immunostaining used the same rabbit polyclonal antibody as in Experiment 1 ** $p < 0.01$, * $p < 0.05$, for pairwise comparison.

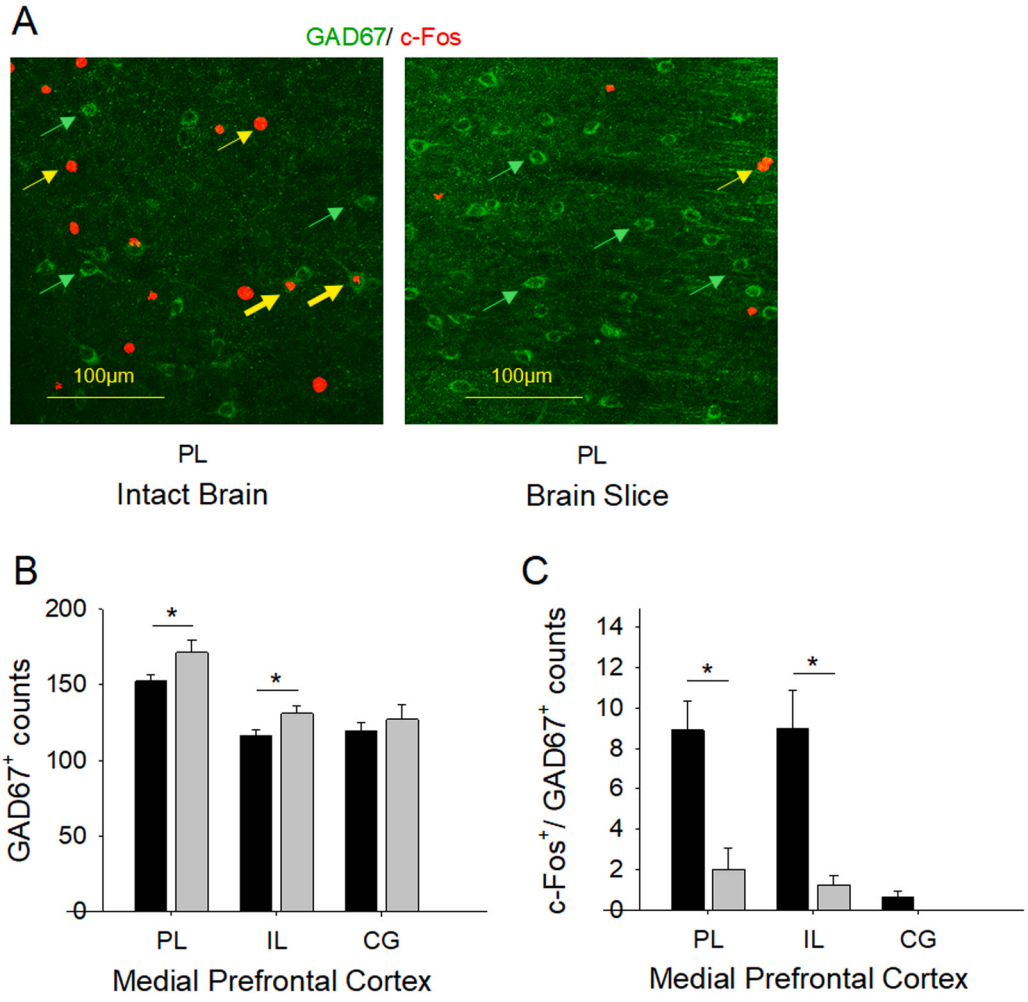

Fig. 6. Long-term brain slice storage enhances medial prefrontal cortex GAD67 expression, but impairs c-Fos/ GAD67 detection. (A) Representative confocal microscopy images of c-Fos/ GAD67 immunostaining in the prelimbic (PL) medial prefrontal cortex. Corresponding sections obtained from stored intact brain (left) versus slice (right). Thin yellow arrows indicate c-Fos–positive cells, thin green arrows GAD67-positive cells, and thick yellow arrows cells double-labeled for both c-Fos and GAD67. (B) GAD67$^+$ cell and (C) c-Fos$^+$/ GAD67$^+$ cell counts (mean ± SEM) in PL, infralimbic (IL), and cingulate cortex (CG) regions from samples of stored intact brains (n = 11; black bars) and stored brain slices (n = 4; gray bars). * $p < 0.05$, for pairwise comparison.

Both of our antibodies were from Synaptic Systems (polyclonal #226 003, monoclonal #226 008) a widely used in many studied (Attarpour et al., 2025; Aubry et al., 2025; Li and Yang, 2024; Zorab et al., 2025). In 2023, the polyclonal antibody was discontinued to be replaced by the monoclonal antibody, notably, with the manufacturer's recommendation to store tissue sections at –20 °C in cryoprotectant solution for optimal immunohistochemistry, warning that prolonged storage at 4 °C would lead to substantial signal loss. Although we did not evaluate the monoclonal antibody in stored slices, our findings of strongly compromised immunofluorescence detection in stored slices stained with the polyclonal antibody confirm that slices cannot be stored over longer times at 4° C for later immunofluorescent detection of c-Fos and GAD67. Yet, this does not account for intact brains, which show robust c-Fos detection after storage for up to 10 months at 4 °C. Thus, both the polyclonal and the newer monoclonal antibody can be safely used for prolonged storage of intact brains and thus offer a reliable alternative for future studies when one wants to avoid freezing the tissue.

In contrast, when brains were sectioned into slices prior to storage, there was a significant reduction in regional c-Fos–positive cell counts, indicating that once sliced, the tissues are less stable for prolonged storage. We did not assess shorter storage durations, but staining of vibratome-cut slices is commonly assumed to remain stable for a few weeks (Giacomoni et al., 2023). Nevertheless, in light of the strong decline of c-Fos^{+} cells we observed in slices stored at 4 °C for 8.5 months, such declines although less pronounced may also occur after shorter storage periods, which needs further clarification. The present results advise to reduce storage time of slices to a minimum, to ensure optimal efficacy of antigen staining.

In addition to the marked reduction of c-Fos expression in stored slices, our analysis of GAD67 and c-Fos/ GAD67 co-labeling revealed distinct changes in inhibitory marker detection. Specifically, prolonged storage of vibratome-cut slices was associated with a slight but significant increase in GAD67-positive cell counts, while the number of c-Fos single-positive and c-Fos/ GAD67 double-positive cells was markedly reduced in the same regions. These opposing effects suggest that prolonged storage of slices at 4 °C differentially affects antigen preservation: whereas activity-dependent markers such as c-Fos appear more susceptible to epitope degradation, GAD67 labeling may increase due to altered tissue properties favoring nonspecific antibody binding.

Considering that studies employing immunofluorescence detection of markers like c-Fos and GAD67 very often extend over periods of several months, the storage of intact brains at 4 °C in PBS with sodium azide offers a simpler alternative to freezing the tissue in cryoprotectant for preserving antigenicity. In particular, it avoids the complexities of cryostat sectioning across when slicing at multiple bregma levels is required. Nevertheless, our assessment of storage time was limited to an interval of 10 months. For storage periods beyond one year traditional freezing methods may still be the safer and more reliable option. On the other hand, our findings clearly caution against the use of vibratome-cut brain slices for long-term storage.

CRediT authorship contribution statement

Jan Born: Writing – review & editing, Validation, Supervision, Funding acquisition, Conceptualization. **Marion Inostroza:** Writing – review & editing, Validation, Supervision, Funding acquisition, Formal analysis. **Stoyan Dimitrov:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Xia Shan:** Writing – review & editing, Validation, Project administration, Methodology.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Data availability

Data will be made available on request.

References

Ameen-Ali, K.E., Sivakumaran, M.H., Eacott, M.J., O'Connor, A.R., Ainge, J.A., Easton, A., 2021. Perirhinal cortex and the recognition of relative familiarity. Neurobiol. Learn. Mem. 182, 107439. https://doi.org/10.1016/j.nlm.2021.107439.

Attarpour, A., Osmann, J., Rinaldi, A., Qi, T., Lal, N., Patel, S., Rozak, M., Yu, F., Cho, N., Squair, J., et al., 2025. A deep learning pipeline for three-dimensional brain-wide mapping of local neuronal ensembles in teravoxel light-sheet microscopy. Nat. Methods 22, 600–611. https://doi.org/10.1038/s41592-024-02583-1.

Aubry, A.V., Durand-de, C.R., Karpman, E., Fisher-Foye, R.L., Parise, L.F., Cathomas, F., Burnett, C.J., Yang, Y., Yuan, C., LaBanca, A.R., et al., 2025. A crucial role for the cortical amygdala in shaping social encounters. Nature 639, 1006–1015. https://doi.org/10.1038/s41586-024-08540-4.

Burry, R.W., 2011. Controls for immunocytochemistry: an update. J. Histochem. Cytochem 59, 6–12. https://doi.org/10.1369/jhc.2010.956920.

Contreras, M.P., Mendez, M., Shan, X., Fechner, J., Sawangjit, A., Born, J., Inostroza, M., 2024. Context memory formed in medial prefrontal cortex during infancy enhances learning in adulthood. Nat. Commun. 15, 2475. https://doi.org/10.1038/s41467-024-46734-6.

Egorova, D., Kerever, A., Inada, M., Itoh, Y., Arikawa-Hirasawa, E., Miyata, S., 2025. Microglial depletion increases aggrecan and hyaluronan levels in the diffuse and aggregated extracellular matrix of the mouse brain. Sci. Rep. 15, 9376. https://doi.org/10.1038/s41598-025-94224-6.

Estrada, L.I., Robinson, A.A., Amaral, A.C., Giannaris, E.L., Heyworth, N.C., Mortazavi, F., Ngwenya, L.B., Roberts, D.E., Cabral, H.J., Killiany, R.J., et al., 2017. Evaluation of long-term cryostorage of brain tissue sections for quantitative histochemistry. J. Histochem. Cytochem 65, 153–171. https://doi.org/10.1369/0022155416686934.

Giacomoni, J., Habekost, M., Cepeda-Prado, E., Mattsson, B., Ottosson, D.R., Parmar, M., Kajtez, J., 2023. Protocol for optical clearing and imaging of fluorescently labeled exÂ vivo rat brain slices. Star. Protoc. 4, 102041. https://doi.org/10.1016/j.xpro.2022.102041.

Herrera, D.G., Robertson, H.A., 1996. Activation of c-fos in the brain. Prog. Neurobiol. 50, 83–107. https://doi.org/10.1016/s0301-0082(96)00021-4.

Holstein, G.R., Friedrich, Jr, V.L., Martinelli, G.P., Ogorodnikov, D., Yakushin, S.B., Cohen, B., 2012. Fos expression in neurons of the rat vestibulo-autonomic pathway activated by sinusoidal galvanic vestibular stimulation. Front. Neurol. 3, 4. https://doi.org/10.3389/fneur.2012.00004.

Khoury, N., Pizzo, M.E., Discenza, C.B., Joy, D., Tatarakis, D., Todorov, M.I., Negwer, M., Ha, C., De Melo, G.L., Sarrafha, L., et al., 2025. Fc-engineered large molecules targeting blood-brain barrier transferrin receptor and CD98hc have distinct central nervous system and peripheral biodistribution. Nat. Commun. 16, 1822. https://doi.org/10.1038/s41467-025-57108-x.

Kobelt, P., Tebbe, J.J., Tjandra, I., Bae, H.G., RÃ¼ter, J., Klapp, B.F., Wiedenmann, B., MÃ¶nnikes, H., 2004. Two immunocytochemical protocols for immunofluorescent detection of c-Fos positive neurons in the rat brain. Brain Res. Brain Res. Protoc. 13, 45–52. https://doi.org/10.1016/j.brainresprot.2004.01.003.

Kovács, K.J., 1998. c-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochem. Int. 33, 287–297. https://doi.org/10.1016/s0197-0186(98)00023-0.

Kovács, K.J., 2008. Measurement of immediate-early gene activation- c-fos and beyond. J. Neuroendocr. 20, 665–672. https://doi.org/10.1111/j.1365-2826.2008.01734.x.

Lara Aparicio, S.Y., Laureani, F.Á.J., Aranda Abreu, G.E., Toledo, Cr.R., García Hernández, L.I., Coria Ávila, G.A., Rojas, D.F., Aguilar, M.E.H., Manzo, D.J., Chi-Castañeda, L.D., et al., 2022. Current opinion on the use of c-Fos in neuroscience. NeuroSci 3, 687–702. https://doi.org/10.3390/neurosci3040050.

Li, M., Yang, G., 2024. A mesocortical glutamatergic pathway modulates neuropathic pain independent of dopamine co-release. Nat. Commun. 15, 643. https://doi.org/10.1038/s41467-024-45035-2.

McKenzie, A.T., Nnadi, O., Slagell, K.D., Thorn, E.L., Farrell, K., Crary, J.F., 2024. Fluid preservation in brain banking: a review. Free Neuropathol. 5, 10. https://doi.org/10.17879/freeneuropathology-2024-5373.

Muza, P.M., Pérez, M., Noy, S., Kurosawa, M., Katsouri, L., Tybulewicz, V.L.J., Fisher, E.M.C., West, S.J., 2023. Affordable optical clearing and immunolabelling in mouse brain slices. BMC Res. Notes 16, 246. https://doi.org/10.1186/s13104-023-06511-y.

Needham, B.D., Funabashi, M., Adame, M.D., Wang, Z., Boktor, J.C., Haney, J., Wu, W.L., Rabut, C., Ladinsky, M.S., Hwang, S.J., et al., 2022. A gut-derived metabolite alters brain activity and anxiety behaviour in mice. Nature 602, 647–653. https://doi.org/10.1038/s41586-022-04396-8.

Numa, C., Nagai, H., Taniguchi, M., Nagai, M., Shinohara, R., Furuyashiki, T., 2019. Social defeat stress-specific increase in c-Fos expression in the extended amygdala in mice: involvement of dopamine D1 receptor in the medial prefrontal cortex. Sci. Rep. 9, 16670. https://doi.org/10.1038/s41598-019-52997-7.

Paxinos, G., Watson, C., 2006, 6th ed The Rat Brain in Stereotaxic Coordinates. Elsevier Academic Press. eBook, London. ISBN: 9780080475158.

Sadler, T.R., Khodavirdi, A.C., Hinton, D.R., Holschneider, D.P., 2009. Snap-frozen brain tissue sections stored with desiccant at ambient laboratory conditions without chemical fixation are resistant to degradation for a minimum of 6 months. Appl. Immunohistochem. Mol. Morphol. 17, 165–171. https://doi.org/10.1097/PAI.0b013e3181853001.

Schönbrodt, F.D., Wagenmakers, E.J., 2018. Bayes factor design analysis: planning for compelling evidence. Psychon. Bull. Rev. 25, 128–142. https://doi.org/10.3758/s13423-017-1230-y.

Shan, X., Contreras, M.P., Mendez, M., Born, J., Inostroza, M., 2022. Unfolding of spatial representation at systems level in infant rats. Hippocampus 32, 121–133. https://doi.org/10.1002/hipo.23392.

Shan, X., Contreras, M.P., Sawangjit, A., Dimitrov, S., Born, J., Inostroza, M., 2023. Rearing is critical for forming spatial representations in pre-weanling rats. Behav. Brain Res. 452, 114545. https://doi.org/10.1016/j.bbr.2023.114545.

Shan, X., Sawangjit, A., Born, J., Inostroza, M., 2025. Rearing behavior as indicator of spatial novelty and memory in developing rats. Eur. J. Neurosci. 61, e70162. https://doi.org/10.1111/ejn.70162.

Vetere, G., Xia, F., Ramsaran, A.I., Tran, L.M., Josselyn, S.A., Frankland, P.W., 2021. An inhibitory hippocampal-thalamic pathway modulates remote memory retrieval. Nat. Neurosci. 24, 685–693. https://doi.org/10.1038/s41593-021-00819-3.

Werner, C., Sauer, M., Geis, C., 2021. Super-resolving microscopy in neuroscience. Chem. Rev. 121, 11971–12015. https://doi.org/10.1021/acs.chemrev.0c01174.

Zorab, J.M., Li, H., Awasthi, R., Schinasi, A., Cho, Y., O'Loughlin, T., Wu, X., 2025. Serotonin and neurotensin inputs in the vCA1 dictate opposing social valence. Nature 642, 154–164. https://doi.org/10.1038/s41586-025-08809-2.