Supplementary Materials for

THE NEURAL SUBSTRATE OF SPATIAL MEMORY STABILIZATION DEPENDS ON THE DISTRIBUTION OF THE TRAINING SESSIONS

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This PDF file includes:

Supplementary text

Figures S1 to S10

Tables S1
Supplementary Information Text

Stereotaxic surgery and focal infusion

Following surgeries, animals were allowed to recover for further 7-9 days before behavioral experiments. During the recovery, all mice were given 2 mg/kg paracetamol in drinking water as analgesic. All injection sites were verified histologically. We only included in the analysis mice with correct placements and minimum 70% of virus expression, limited to the targeted regions.

Behavioural procedure

During the whole task, the water was made black with non-toxic paint (Giotto, Italy) and water temperature was kept constant at +22°C. Familiarization phase consisted of one session of three consecutive trials (intertrial interval: 20 s). No cues were attached to the wall and a 10-cm-diameter platform protruded 1.5 cm above the water surface. During the training phase, the platform was submerged 0.5 cm beneath the surface of the water and visual cues were attached to the black curtains surrounding. Animals were randomly released from one of the three non-target quadrants. At the test phase, the platform was removed and mice were allowed a 60-s search for the platform starting from the center of the pool.

Search strategies during training were analyzed using numerical parameters from swim tracking data (adapted from4), and the respective predominant search strategy for each trial was objectively classified by a criterion-based custom-made algorithm. Trials were classified as one of the following ordered strategies (tracking criteria in parentheses): thigmotaxis: >35% of swim distance within closer wall zone (10 cm from pool wall) and total latency to the platform < 25s; random search: total latency >25 seconds; scanning: <75% surface coverage and >35% surface coverage <0.7 s.d. distance to the pool center (scanning area) and total latency >25 seconds; chaining: between 35-70% of time within chaining zone (chaining zone: corridor 20cm wide, centered in the center of platform) and total latency >25 seconds; missing- then-reaching: latency to critical area/latency to the platform = 0.2-0.5 and total latency <11s (critical area: 30cm-wide area centered in the center of the platform); directed search: >70% of time in goal corridor (rectangular goal corridor 30 cm wide, centered along direct connection between start and platform positions); focal search: <20 s.e.m. body angle, <0.25 s.d. mean distance to present goal; direct swim: >90% distance in the goal corridor. When we used these
definitions and the algorithm, only <2.5% of the trials could not be assigned univocally to one strategy. Probe trial performance was quantified as the time spent, distance travelled or annuli crossed in target compared to other three non-target quadrants.

**Video analysis and data collection**

All the trials were recorded through a camera located above the pool and videos were analyzed with the software AnyMaze-Behaviour Tracking System 5.0 (ANY-maze, Stoelting, USA). To study behavior during the training, the variables scored were escape latency (in seconds) and escape distance travelled to reach the platform (in m). For the probe trial we set with AnyMaze an arena corresponding to the pool circumference. The arena was divided in 4 quadrants (target, opposite, left and right) and 4 annuli (target, opposite, left and right), defined as an area of 18 cm diameter surrounding the center of the platform for each quadrant. The following variables were scored during the probe phase: time spent in each quadrant (in seconds), distance travelled in each quadrant (in m), annulus crossing (frequency of each annulus crossing).

**Immunohistochemistry**

Mice used for the immunohistochemical analysis were transcardially perfused with 40 ml of saline solution (NaCl 0.9%) followed by 40 ml of 4% formaldehyde. Brains were rapidly removed and post-fixed for 24 h in 4% formaldehyde in PBS and then transferred to 30% sucrose in PBS until sectioning. 30 μm-coronal sections were stored at −20 °C in cryoprotectant solution.

For the -Fos-immunoreactivity (c-Fos-IR), free-floating slices were incubated for 5 min in 3% hydrogen peroxide in PBS and rinsed three times in PBS containing 0.1% Triton X-100 (PBST). After 1 h of incubation in PBST containing 1% BSA and 1% NGS sections were incubated overnight in anti-phospho-c-Fos rabbit monoclonal antibody (5348S; Cell signaling Technology, USA) diluted 1:8000 in PBST–BSA–NGS, at 4 °C and with constant orbital rotation. Sections were washed three times in PBST and incubated in biotinylated secondary antibody diluted 1:500 in PBST-1% BSA (goat anti-rabbit IgG; Vector Laboratories, USA) for 2 h at room temperature. After three washes in PBST, sections were transferred for 1 h in avidin–biotinylated peroxidase complex diluted 1:500 in PBST (ABC Kit; Vector Laboratories, USA) and rinsed three times in PBS. Finally, the reaction was visualized using nickel intensified diaminobenzidine (DAB peroxidase substrate
kit, Vector Laboratories, USA). The reaction was stopped after exactly 4 min by washing with 0.1 M PBS (pH 7.6). Sections were mounted on slides, dehydrated through a graded series of alcohols, cleared and coverslipped. Sections from groups to be directly compared were processed at the same time and using the same conditions and reagents in order to reduce variability. In all the experiments, the number of cells displaying c-Fos-IR was measured in DMS and DLS (from bregma +0.50 mm to bregma +0.02 mm). At least three to five non-consecutive coronal sections were stained and digitized bilaterally for each brain region, for each subject. Digital images were acquired at 10× magnification (Nikon eclipse 80i microscope). After images acquisition, counting of the stained cells was carried out using Image J (NIH Image, USA). Briefly, for each region, stained nuclei were automatically detected based on their intensity of staining relative to background and their size. Counts from both hemispheres and from all rostro-caudal levels were averaged per subject, and subsequently per group (naïve, cue and spatial).

Digital reconstruction
Following c-Fos staining, one representative sections (+0.38 mm from bregma) from one representative mouse for each training protocol was digitized for the reconstruction of c-Fos expression throughout the whole striatum. Digital images were acquired at ×10 magnification, using a Nikon microscope (Nikon eclipse 80i) equipped with a digital camera (DXM1200F). Single images of 2560 × 1920 pixel were stitched together in a mosaic view with the use of Adobe Photoshop (Adobe Photoshop CC 2019). Images were then processed with a custom made script in MatLab.

Histology and viral diffusion
To verify the location of the injections in the pharmacological experiments and optic fiber location in the optogenetic experiment, at the completion of testing animals were anesthetized followed by cervical dislocation. Brains were removed and post-fixed in 4% formaldehyde (4°C). 90-μm thick coronal slices were cut using a microtome (Leica Microsystem, Germany), collected on gelatin-coated slides and stained with cresyl-violet (Sigma-Aldrich, Italy). Only mice with correct placement in the target regions were included in the analysis. Illustration of coronal sections from single animals are represented for each experiment (Figs. S7, S9, S10).
To verify the viral diffusion and optical fibers placements in the optogenetic experiments, mice were anesthetized and perfused as previously described. Brains were post-fixed overnight at 4 °C in 4% formaldehyde and then cryopreserved in 30% sucrose. Coronal slices containing DLS (40-μm thick) were cut using a freezing microtome (Leica Microsystem, Germany) and collected in PBS. For virus detection, slices were incubated 5 min with Hoechst (Termofisher, USA) diluted 1:2000, immediately mounted and covered with Fluoromount aqueous mounting medium (Sigma-Aldrich, Italy). Immunofluorescence images were acquired with a fluorescence microscope (Nikon Eclipse TE300) with 2x objective under 488nm laser/eYFP setting. Total virus diffusion was outlined and determined for each section with the use of ImageJ (NIH) and Adobe Illustrator (Adobe Systems Incorporated, USA). Only mice showing AAV diffusion in at least the 70% of total DLS extension and a correct optic fibers placement were included in statistics (Figs. S9). To verify viral diffusion in unilateral-stimulated mice, slices consecutive to the ones used for c-Fos staining were used.

Data collection and statistical analysis

For the analysis of training for all MWM experiments, escape latency and path length data were analyzed using two-way repeated measures ANOVA with procedure (MASSED or DISTRIBUTED), pre-test treatment (NBQX or PBS) or light stimulation (LIGHT ON or LIGHT OFF) as between groups factor and session (six levels: session 1 to 6) as repeated measure. If the interaction between factors was not significant a one-way repeated measure ANOVA with session (six levels: session 1 to 6) as repeated measure was performed on the two groups independently. To compare the navigational strategies in distributed vs massed, a multinomial logistic regression was conducted using 4 navigational strategies (spatial, spatial non specific, non spatial and non classified) predictors to predict the classification on the multinomial dependent variable offence type (massed vs distributed).

For probe trial time, distance and annuli were analyzed by using two-way repeated measure ANOVA with procedure (two level, DISTRIBUTED or MASSED), pre-test treatment (two level, PBS or NBQX) or light stimulation (two levels, LIGHT ON or LIGHT OFF) as between groups factor and quadrants (4 levels: target, opposite, right and left) as repeated measure. Group differences were considered statistically significant when p<0.05. If this was not the case, the repeated measures were analyzed independently in each group (PBS, NBQX or DISTRIBUTED, MASSED or LIGHT ON, LIGHT OFF) through a one-way ANOVA with
quadrants (4 levels: target, opposite, right and left) as repeated measure. If this was statistically significant Tukey HSD post-hoc analysis was used. Difference between the target distance and the mean of non-target quadrants was analyzed by using paired t-test. To compare c-Fos expression, normalized on HC controls, in mice from the massed and distributed trained groups a Mann-Whitney non-parametric test was used. To compare c-Fos expression (c-Fos/mm2) in HC, spatial and cue trained mice with the two protocols one-way ANOVA was used with training (HC, spatial, cue) as between-groups factor. If this was statistically significant Tukey HSD post-hoc analysis was used. Principal Component Analysis (PCA) was run to compare different stainings by using an open-source web tool (https://biit.cs.ut.ee/clustvis).
Figure S1. Distributed training increases memory stability. Mean annulus frequency ± SEM on test trial 24h (A) or 14 days (B) after massed or distributed training in the Morris water maze (24 h: two-way ANOVA repeated measure: quadrant F(3,69) = 22.08, p < 0.0001; protocol F(1,23) = 0.00054, p = 0.9816; quadrant x protocol F(3,69) = 0.4273, p = 0.7341; 14 days: two-way ANOVA repeated measure: quadrant F(3,84) = 17.32, p < 0.0001; protocol F(1,28) = 3.35, p = 0.077; quadrant x protocol F(3,84) = 8.335, p < 0.0001). *p < 0.05 target vs right, opposite, left (within group, Tukey HSD); # p < 0.05 target vs target (between group, Tukey HSD).
Figure S2. Training and strategy deployment of mice trained with the massed or the distributed-training protocol. (A) Path length expressed as mean distance ± SEM during spatial training in the Morris water maze of mice trained with the massed (n=28) or distributed protocol (n=27) (two-way ANOVA repeated measure: session $F_{(5, 265)} = 24.38, p < 0.0001$; protocol $F_{(1, 53)} = 0.20 p = 0.6541$; session x protocol $F_{(5, 265)} = 1.57 p = 0.1688$). (B) Mean latency ± SEM to reach the platform of mice trained in the Morris water maze with the two protocols (two-way ANOVA repeated measure: session $F_{(5, 265)} = 21.12 p < 0.0001$; protocol $F_{(1, 53)} = 0.39 p = 0.5355$; session x protocol $F_{(5, 265)} = 1.27 p = 0.2772$). (C) Schematic representation of the search strategies classified and color coded for each category. The graph shows the average prevalence of each category across sessions of mice trained with the two protocols. Comparison between the two groups did not reveal any significant difference in strategy deployment (multinomial regression).
Figure S3. Learning curves for mice trained in the spatial or the cue version of the MWM with massed or distributed training protocols. (A) Mean path length (m) ± SEM of mice massed trained in the sMWM or the cMWM (two-way ANOVA repeated measure: session $F_{(5,65)}=14.07$, $p<0.0001$; protocol $F_{(1,13)}=4.02$, $p=0.0664$; session x protocol $F_{(5,65)}=3.67$, $p=0.0055$). (B) Mean latency to reach the platform (s) ± SEM of mice massed trained in the spatial or cue version of the task (two-way ANOVA repeated measure: session $F_{(5,65)}=12.97$, $p<0.0001$; protocol $F_{(1,13)}=6.97$, $p=0.0204$; session x protocol $F_{(5,65)}=2.95$, $p=0.0184$). (C) Mean path length (m) ± SEM of mice trained with the distributed protocol in the spatial or the cue version of the task (two-way ANOVA: session $F_{(5,70)}=10.23$, $p<0.0001$; protocol $F_{(1,14)}=6.7$, $p=0.0213$; session x protocol $F_{(5,70)}=1.053$, $p=0.394$). (D) Mean latency to reach the platform (s) ± SEM of mice trained with the distributed protocol in the in the sMWM or the cMWM (two-way ANOVA repeated measure: session $F_{(5,70)}=10.77$, $p<0.0001$; protocol $F_{(1,14)}=6.15$, $p=0.0265$; session x protocol $F_{(5,70)}=0.7254$, $p=0.6067$).
Figure S4. Spatial training induced c-Fos expression in the DMS and DLS after massed or distributed training. (A) c-Fos labeling in the DMS was significantly higher in mice massed trained in the sMWM (n=6) compared to cMWM (n=7) or HC controls (n=12) (one-way ANOVA F(2,22) = 117.8, p < 0.0001). (B) Representative images of c-Fos immunoreactivity in the DMS in the three experimental groups. (C) Distributed training significantly increased c-Fos expression in the DMS in both the cue (n=6) and the spatial (n=7) trained mice compared to the HC controls (one-way ANOVA F(2,22) = 25.36, p< 0.0001). (D) Representative images of c-Fos immunoreactivity in the DMS in the three experimental groups. (E) Massed protocol induces a significantly higher c-Fos expression in the DLS following the sMWM compared to cMWM or HC controls (one-way ANOVA F(2,22) = 54.39, p < 0.0001). (F) Representative images of c-Fos immunoreactivity in the DLS in the three experimental groups. (G) Distributed training in the sMWM induces a significantly higher c-Fos expression in the DLS compared to training in the cMWM or in HC controls (one-way ANOVA F(2,22) =25.33, p < 0.0001). (H) Representative images of c-Fos immunoreactivity in the DLS in the three experimental groups. **p<0.01 (Tukey HSD). Scale bars: 200 μm.
Figure S5. Training performance for massed- and distributed-training groups in the pharmacological experiments. (A) Mean of path length (m) ± SEM after massed trained in the sMWM, before bilateral administrations in the DMS with PBS or NBQX (two-way repeated measure ANOVA of session $F_{(5,165)}=8.28$, $p<0.0001$; treatment $F_{(1,33)}=1.050$, $p=0.313$; session x treatment $F_{(5,165)}=1.659$, $p=0.1474$). (B) Mean of latency to reach the platform (s) ± SEM of mice massed trained in the sMWM before bilateral administrations in the DMS of vehicle or NBQX (two-way repeated measure ANOVA: session $F_{(5,165)}=24.56$, $p<0.0001$; treatment
F_{(1,33)}=1.406, p=0.2442; session x treatment $F_{(5,165)}=1.13, p=0.3487)$. (C) Mean of path length (m) ± SEM of massed trained mice in the sMWM before bilateral vehicle or NBQX administrations in the DLS (two-way repeated measure ANOVA: session $F_{(5,95)}=14.79, p<0.0001$; treatment $F_{(1,19)}=0.103, p=0.7516$; session x treatment $F_{(5,95)}=1.9, p=0.1009$). (D) Mean of latency to reach the platform (s) ± SEM of mice massed trained in the sMWM before bilateral DLS vehicle or NBQX administrations (two-way repeated measure ANOVA: session $F_{(5,85)}=14.26, p<0.0001$; treatment $F_{(1,19)}=0.48, p=0.4952$; session x treatment $F_{(5,95)}=1.5, p=0.1942$). (E) Mean of path length (m) ± SEM of mice trained with the distributed protocol in the sMWM before bilateral vehicle or NBQX administrations in the DMS (two-way repeated measure ANOVA: session $F_{(5,115)}=7.93, p<0.0001$; treatment $F_{(1,23)}=0.13, p=0.7234$; session x treatment $F_{(5,115)}=1.09, p=0.3697$). (F) Mean of latency to reach the platform (s) ± SEM in the sMWM of the distributed trained mice before bilateral vehicle or NBQX administrations (two-way repeated measure ANOVA: session $F_{(5,115)}=9.22, p<0.0001$; treatment $F_{(1,23)}=0.33, p=0.5706$; session x treatment $F_{(5,115)}=1.804, p=0.1174$). (G) Mean of path length (m) ± SEM of mice trained with the distributed protocol in the sMWM before bilateral vehicle or NBQX administration in the DLS (two-way repeated measure ANOVA: session $F_{(5,135)}=12.36, p<0.0001$; treatment $F_{(1,27)}=0.87, p=0.3591$; session x treatment $F_{(5,135)}=1.14, p=0.34$). (H) Mean of latency to reach the platform (s) ± SEM of mice trained on the distributed protocol in the sMWM before bilateral vehicle or NBQX administrations in the DLS (two-way repeated measure ANOVA: session $F_{(5,135)}=15.61, p<0.0001$; treatment $F_{(1,27)}=0.65, p=0.4282$; session x treatment $F_{(5,135)}=1.12, p=0.3545$).
Figure S6. Pre-test bilateral inhibition of the DMS or the DLS following massed or distributed training in the sMWM. (A) Mean of annulus frequency ± SEM on probe trial 24h after massed training of mice administered pre-test in the DMS with vehicle or NBQX (two-way ANOVA repeated measure: annuli $F_{(3,99)}=21.76, p<0.0001$; treatment $F_{(1,33)}=0.099, p=0.7553$; annuli x treatment $F_{(3,99)}=3.88, p=0.0114$). (B) Mean of annulus frequency ± SEM on probe trial test, 24h after massed training, of mice administered with either vehicle or NBQX in the DLS (two-way ANOVA repeated measure: annuli $F_{(3,57)}=16.59, p<0.0001$; treatment $F_{(1,19)}=0.13, p=0.72$; annuli x treatment $F_{(3,57)}=0.3767, p=0.77$). (C) Mean of annulus frequency ± SEM on probe trial of distributed trained mice after bilateral DMS vehicle or NBQX administrations (two-way ANOVA repeated measure: annuli $F_{(3,69)}=21.26, p<0.0001$; treatment $F_{(1,19)}=0.13, p=0.72$; annuli x treatment $F_{(3,69)}=0.3767, p=0.77$). (D) Mean of annulus frequency ± SEM on probe trial 24h after last training, on the distributed protocol, of mice administered pre-test vehicle or NBQX in the DLS (two-way ANOVA repeated measure: annuli $F_{(3,81)}=21.21, p<0.0001$; treatment $F_{(1,23)}=0.078, p=0.7815$; annuli x treatment $F_{(3,81)}=0.3585, p=0.7832$). *p<0.05 target vs right, opposite, left (within group, Tukey HSD); § p<0.05 vs target (within groups, Tukey HSD).
Figure S7. Schematic drawings of the injection sites for the pharmacological experiments. Each symbol represents the site of injection for one animal. (A) Massed training groups. Pre-test DMS vehicle (blue circles, $n = 17$) and NBQX (grey circles, $n = 18$); pre-test DLS vehicle (blue triangles, $n = 11$) and NBQX (grey triangles, $n = 10$). (B) Distributed training groups. Pre-test DMS vehicle (orange circles, $n = 11$) and NBQX (yellow circles, $n = 14$); pre-test DLS vehicle (orange triangles, $n = 14$) and NBQX (yellow triangles, $n = 15$). Coordinates are expressed as mm from bregma.
Figure S8. Light delivery in the DLS improved memory performance 14 days after massed training only in ChR2(C128S/D156A) expressing mice. (A) Mean path length (m) ± SEM during massed training of ChR-eYFP expressing mice with (Light ON) or without (Light OFF) light delivery (two-way ANOVA: session $F_{(5,75)} = 7.929$, $p < 0.0001$; treatment $F_{(1,15)} = 0.0286$, $p = 0.8679$; session x treatment $F_{(5,75)} = 0.7919$, $p = 0.5589$). (B) Mean of latency (s) ± SEM during massed training of stimulated (Light ON) and unstimulated (Light OFF) ChR-eYFP expressing mice (two-way ANOVA repeated measure: session $F_{(5,75)} = 8.054$, $p < 0.0001$; treatment $F_{(1,15)} = 0.227$, $p = 0.64$; session x treatment $F_{(5,75)} = 0.3382$, $p = 0.8883$). (C) Mean of annulus frequency ± SEM on probe trial of stimulated and unstimulated ChR-eYFP expressing mice (two-way ANOVA: annuli $F_{(3,45)} = 2.513$, $p = 0.0705$; treatment $F_{(1,15)} = 0.7932$, $p = 0.3872$; annuli x treatment $F_{(3,45)} = 4.568$, $p = 0.0071$). (D) Mean path length (m) ± SEM during massed training of saline injected groups with (Light ON) or without (Light OFF) light stimulation (two-way ANOVA repeated measure: session $F_{(5,95)} = 10.66$, $p < 0.0001$; treatment $F_{(1,19)} = 0.3007$, $p = 0.5898$; session x treatment $F_{(5,95)} = 1.874$, $p = 0.1060$).
Mean of latency (s) ± SEM during massed training of stimulated and unstimulated saline-injected mice (two-way ANOVA repeated measure: session $F_{(5,95)} = 11.78$, $p < 0.0001$; treatment $F_{(1,19)} = 0.8741$, $p = 0.361$; session x treatment $F_{(5,95)} = 2.226$, $p = 0.058$). (F) Mean of annulus frequency ± SEM on probe trial of stimulated and unstimulated saline injected groups (two-way ANOVA repeated measure: annuli $F_{(3,57)} = 0.0.126$, $p = 0.9443$; treatment $F_{(1,19)} = 0.2011$, $p = 0.6589$; annuli x treatment $F_{(3,57)} = 0.91$, $p = 0.44$). *p<0.05 target vs right, opposite, left (within group, Tukey HSD); # p<0.05 target vs target (between group, Tukey HSD).
Figure S9. AAV::ChR2(C128S/D156A) diffusion in the DLS of stimulated and unstimulated mice. Schematic representation of eYFP protein expression and fiber placements in (A) the light-OFF (n=9) and (B) the light-ON (n=8) groups. For each animal eYFP expression (lighter) and the fiber placement (darker dots) are represented in the same color. Coordinates are expressed as mm from bregma.
Figure S10. Schematic drawing of the fiber placements in stimulated and unstimulated saline controls. Schematic representation of the site of injection for saline-injected mice (A) without light delivery (LIGHT OFF) or (B) with light delivery (LIGHT ON). Coordinates are expressed as mm from bregma.
TABLE S1. Multinomial logistic regression analysis for navigational strategies across the learning sessions.

|                  | B     | Standard Error | Wald | gl | Sign. |
|------------------|-------|----------------|------|----|-------|
| Intercept        | 0.253 | 0.211          | 1.44 | 1  | 0.229 |
| S1 * strategy    | -0.154| 0.102          | 2.28 | 1  | 0.131 |
| S2 * strategy    | -0.147| 0.104          | 2.00 | 1  | 0.157 |
| S3 * strategy    | -0.148| 0.112          | 1.73 | 1  | 0.189 |
| S4 * strategy    | -0.110| 0.115          | 0.91 | 1  | 0.340 |
| S5 * strategy    | -0.067| 0.121          | 0.31 | 1  | 0.578 |
| S6 * strategy    | -0.179| 0.126          | 2.01 | 1  | 0.156 |