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The neural substrate of spatial memory stabilization depends on the distribution of the training sessions

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Distributed training is known to lead to more robust memory formation as compared to training experiences with short intervals. Although this phenomenon, termed distributed practice effect, ubiquitous over a wide variety of tasks and organisms, has long been known by psychologists, its neurobiological underpinning is still poorly understood. Using the striatum as a model system here we tested the hypothesis that the ability of distributed training to optimize memory might depend upon the recruitment of different neural substrates compared to those engaged by massed training. First, by contrasting the medial and the lateral domains of the dorsal striatum after massed and distributed training we demonstrated that neuronal activity, as assessed using c-Fos expression, is differentially affected by the training protocol in the two striatal subregions. Next, by blocking the AMPA receptors before recall we provide evidence to support a selective role of the medial and the lateral striatum in the storage of information acquired by massed and distributed training, respectively. Finally, we found that optogenetic stimulation of the dorsolateral striatum during massed training enables the formation of an enduring memory similar to what is observed with distributed learning. Overall, these findings identify a possible mechanism for the distributed practice effect, a still poorly understood aspect of learning.

striatum | spatial memory | memory systems

Significance

Distributed training has long been known to lead to more robust memory formation as compared to massed training. Using the water maze, a well-established task for assessing memory in laboratory rodents, we found that distributed and massed training differentially engage the dorsolateral and dorsomedial striatum, and optogenetic priming of dorsolateral striatum can artificially increase the robustness of massed training to the level of distributed training. Overall, our findings demonstrate that spatial memory consolidation engages different neural substrates depending on the training regimen, identifying a therapeutic avenue for memory enhancement.

Practice is fundamental to learning, and increasing practice is essential to convert learning into more stable memories. Interestingly, memory improves not only when the number of repetitions increases but also when repetitions are spaced in time. This phenomenon, termed distributed practice effect, has long been known by psychologists and has been widely studied in both basic and applied research due to its relevance for education, therapy, and advertising (1, 2). Although the distributed practice effect seems to be a fundamental principle of learning, spanning a great variety of learning tasks, study materials, and organisms (3–6), its neurobiological underpinnings are still poorly understood. Recent studies demonstrated that increasing the time intervals between training episodes improves synaptic plasticity (7–11), supporting the hypothesis that the superiority of distributed training over massed training might depend upon enhanced memory consolidation (9). However, changes in synaptic efficacy at the level of individual brain sites cannot fully explain how learning is translated into effective memory. Another fundamental process for the efficient stabilization of memories is the reorganization of neural circuits recruited by learning as experience accumulates. This is well established for skill and goal-directed behavior for which gains accrued with increasing training are implemented through the engagement of new neural networks that take control over the memory representation. For example, the dorso-medial striatum (DMS) is engaged during the initial phases of skill learning and in the rapid acquisition of action–outcome contingencies (12, 13). In contrast, the dorso-lateral striatum (DLS) is critical at later stages of acquisition, when performance reaches its asymptotic level (12, 13). A similar difference between DMS and DLS activity was also observed when comparing short or prolonged spatial training in the water maze task: the DMS showed greater activation in the initial phase of learning than in the late phase, while the DLS showed sustained activity from early to late stages, in both mice and humans (14).

Since distributed training improves memory in a similar way to prolonged training, we asked whether these two processes share similar neurobiological mechanisms. By contrasting the DMS and the DLS, we tested the hypothesis that optimization of spatial memory when learning episodes are presented in a distributed fashion, might depend upon the involvement of the DLS, in analogy to what has been observed after extended training.

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Results

Distributed Training Increases Long-Term Retention of Spatial Memory without Affecting Acquisition. Considering the suggestion that irregular intervals might be more effective in optimizing memory (9), we selected a 3-d training procedure consisting of two sessions per day, over three consecutive days, with a 4-h within-day interval in the spatial version of the Morris water maze (sMWM). To test the better efficacy of the distributed training protocol on the acquisition and storage of spatial information, we compared distributed training groups with groups trained with a massed protocol, consisting of the same number of sessions administered consecutively with an intersession interval of 10 to 15 min (15, 16). Separate groups of mice, trained with the two sMWM protocols, were tested at two different time intervals (24 h and 14 d) after the last training session (Fig. 1*A*). The probe trial 24 h after training did not reveal any difference between the two groups, both being able to correctly reach the platform location (Fig. 1*B* and *SI Appendix*, Fig. S1*A*). On the contrary, 14 d after training, mice of the massed trained group spent a similar amount of time exploring the four quadrants, demonstrating that spatial memory declined over time. Mice trained with the distributed protocol, instead, maintained intact memory for the correct quadrant location (Fig. 1*C* and *SI Appendix*, Fig. S1*B*). This difference cannot be attributed to an effect of the training protocol on the learning capabilities, since no differences were found in the latencies to locate the platform during training (*SI Appendix*, Fig. S2 *A* and *B*) or in the progression of the navigational strategies deployed (17) (*SI Appendix*, Fig. S2*C* and Table S1) by the two groups across sessions. These findings demonstrate the efficacy of distributed training in creating durable spatial memories, replicating the increased memory performance observed in humans when interrepetition lag increases (2).

Massed and Distributed Training Differentially Affect Neural Activity in DMS and DLS. To provide direct evidence supporting the hypothesis that the different dorsal striatal domains might be differentially engaged by massed or distributed spatial training, we explored learning-induced changes in neuronal activity, by c-Fos labeling, in the DMS and DLS after training with the two protocols in the sMWM (*SI Appendix*, Fig. S3). To verify the specificity of the effects, further groups of mice were trained in the cue version of the MWM (cMWM) (*SI Appendix*, Fig. S3). A group of holding caged (HC) controls was used to normalize data obtained from trained mice. Mirroring what has been reported on the contribution of the DMS and DLS after short or prolonged training (14), we found that compared to HC controls the DMS was specifically activated by massed but not distributed training (Fig. 1 *D*, *E*, and *G* and *SI Appendix*, Fig. S4). On the contrary, increased c-Fos expression in the DLS was independent of the training protocol (Fig. 1 *D*, *F*, and *H* and *SI Appendix*, Fig. S4). Importantly, with the notable exception of the DMS after distributed training, which showed similar c-Fos expression in the cue and the spatial groups, cell activity-dependent labeling was higher in all the spatial trained groups compared to those trained in the cue version of the task (*SI Appendix*, Fig. S4).

DMS and DLS Dissociation in the Retrieval of Spatial Information Acquired through Massed or Distributed Practice. To assess whether increased neuronal activity in DMS after massed training and in DLS after massed and distributed training had a causal role in memory storage, we performed a loss of function manipulation of the two striatal domains in mice trained with the two protocols. To this aim the AMPA-R antagonist, NBQX, was administered immediately before testing 24 h after the last training sessions, a time point at which both massed and distributed training induced effective memory. We first examined the effects of DMS and DLS manipulations on mice trained with the massed protocol (Fig. 2*A*). As expected, both groups of vehicle-injected mice were perfectly able to locate the platform. However, different from what could be expected from c-Fos labeling data, pretest administration of NBQX impaired the ability of mice to locate the platform only when injected in the DMS but not in the DLS (Fig. 2 *B* and *C* and *SI Appendix*, Figs. S5 *A–D*, S6 *A* and *B*, and S7*A*) compared to vehicle controls. Notably, the opposite effect was found when testing the effects of DMS and DLS manipulations on spatial memory acquired with the distributed protocol (Fig. 2*A*). Mice receiving pretest NBQX in the DMS showed intact ability to locate the platform compared to vehicle-injected controls (Fig. 2*D* and *SI Appendix*, Figs. S5 *E* and *F* and S6*C*). On the contrary, pretest NBQX administrations in the DLS impaired mice performance on the probe trial, 24 h after the last training session (Fig. 2*E* and *SI Appendix*, Figs. S5 *G* and *H*, S6*D*, and S7*B*). Overall, these data demonstrate that the two striatal domains are both involved in the processing of spatial information, providing a causal role to neural activation reported in the previous experiment as well as in imaging studies in humans and rodents (14), but more importantly, they suggest a dissociation in their involvement depending on the timing rule of the learning protocol.

Long-Term Stability of Spatial Information Can Be Driven by Optogenetic Priming of the DLS. Having shown that interference with DLS neuronal activity impairs retrieval of spatial information acquired through distributed training, we wondered whether the engagement of this region could be a mechanism responsible for the establishment of more enduring memories. Cell activity-dependent labeling of the DLS after massed training suggests that short intertrial intervals provide sufficient stimulation for activation but not for the stabilization of the memory trace. Based on recent findings demonstrating that increasing the excitability of a subset of neurons increases the probability that those neurons will participate in a memory trace (18), we reasoned that the artificial priming of cell activity in the DLS could bias the information acquired through massed training within this striatal domain and in this way create a more stable trace. To verify this possibility, we injected in the DLS an AAV (Adeno-associated virus) carrying a light-activated excitatory channelrhodopsin, ChR2(C128S/D156A), that responds to light delivery (473 nm) with a long-lasting effect (29 min) (19) (Fig. 3*A*). As expected, control mice not receiving light stimulation were not able to locate the correct quadrant on the probe trial 14 d after massed training in the sMWM (Fig. 3*B*). On the contrary, light (473 nm) stimulated mice showed intact ability to locate the platform on the 14-d probe trial despite the massed training (Fig. 3*B* and *SI Appendix*, Figs. S8 *A–C* and S9). Further control experiments demonstrated the efficacy of the light stimulation in promoting increased neuronal activity in the DLS (Fig. 3*C*) and ruled out possible effects of light delivery alone on learning (Fig. 3*D* and *SI Appendix*, Figs. S8 *D–F* and S10). These findings demonstrate that artificial stimulation of the DLS in massed training conditions enables the constitution of a more stable spatial memory representation, artificially mimicking the increased memory efficacy induced by distributed training.

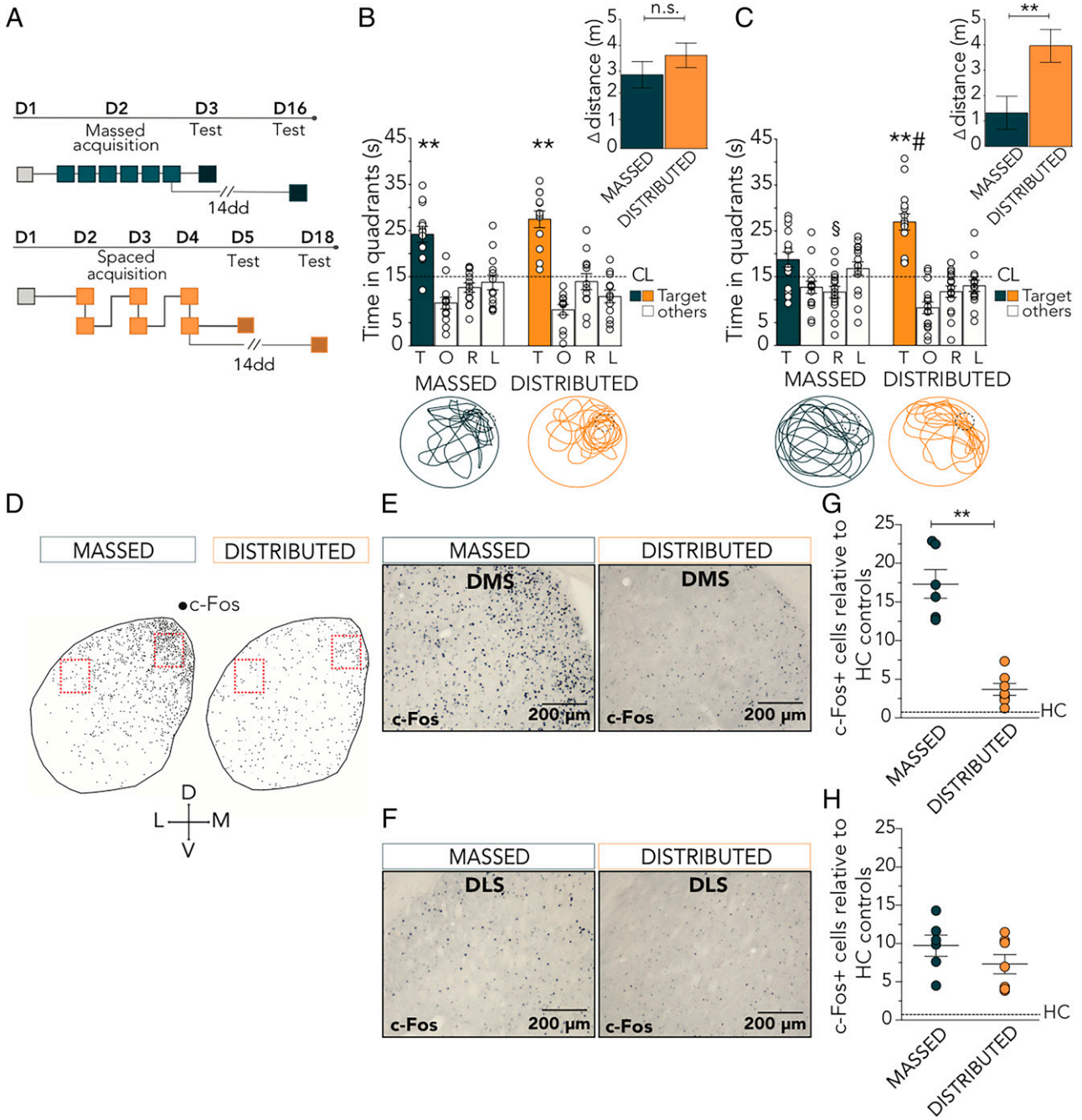

Fig. 1. Distributed training increased memory stability and differentially affected c-Fos expression in the DMS and in the DLS. (*A*) Schematic of the experimental design. (*B*) Mice trained with the massed (n = 13) or the distributed (n = 12) protocol were equally able to locate the target quadrant 24 h after the last training session (two-way ANOVA repeated measure: quadrant preference $F_{(3,\ 69)}$ = 38.41, $P < 0.0001$; protocol $F_{(1,\ 23)}$ = 1.53, P = 0.228; quadrant preference × protocol $F_{(3,\ 69)}$ = 1.35, P = 0.265). (*C*) Fourteen d after the last training session, mice trained with the distributed (n = 15) but not the massed (n = 15) protocol maintained intact ability to locate the target quadrant (two-way ANOVA repeated measure: quadrant preference $F_{(3,\ 84)}$ = 23.27, $P < 0.0001$; protocol $F_{(1,\ 28)}$ = 0.086, P = 0.770; quadrant preference × protocol $F_{(3,\ 84)}$ = 6.374, P = 0.0006). Dotted lines represent chance level (CL). (*Insets*) The distance traveled on test trial expressed as difference (Δ) between target and mean of nontarget quadrants, for each group (t_{23} = 1.063, P = 0.298, 24 h and t_{28} = 2.883, P = 0.0075, 14 d after last training session; unpaired t test). (*Bottom*) Representative path from massed and distributed trained animals. $^{**}P < 0.05$ target vs. right, opposite, left; $^{\S}P < 0.05$ vs. target (within groups, Tukey honestly significant difference [HSD]), $^{\#}P < 0.05$ target vs. target (between groups, Tukey HSD). (*D*) Mapping of c-Fos immunoreactivity in the whole dorsal striatum of two representative mice trained with the two protocols. Representative images showing training-induced c-Fos immunoreactivity (*E*) in the DMS and (*F*) in the DLS 1 h after massed or distributed training in the sMWM. (Scale bar: 200 µm.) (*G* and *H*) Quantification of c-Fos expression in DMS and DLS relative to holding cage (HC) mice (n = 12). (*G*) Cell activity in the DMS was significantly increased in the massed (n = 6) compared to distributed (n = 7) trained group (U = 0.0, P = 0.0012). (*H*) In the DLS, trained mice showed higher levels of c-Fos expression compared to HC controls; no significant differences were found between mice trained with the two protocols (U = 11, P = 0.18). Scatterplots represent mean ± SEM, $^{**}P < 0.01$ (Mann–Whitney U test).

Discussion

Using the striatum as a model system, we found that the timing rules of the training experience determine the neural substrate underpinning the storage of spatial information. We established that the DMS is required for the recall of information acquired through massed training, while the DLS is required when learning experiences are distributed over time. More importantly, we demonstrated that stimulation of the DLS is sufficient to enhance memory retention after massed training. These results suggest that the efficiency of distributed training, compared to

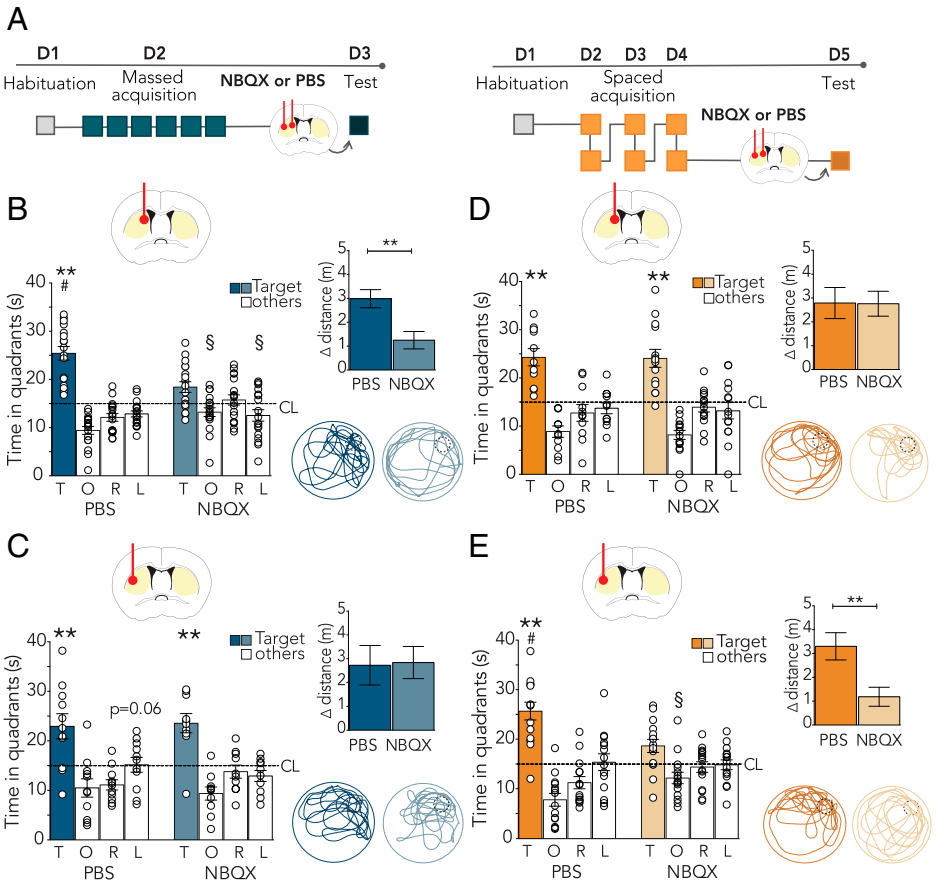

Fig. 2. Differential involvement of the DMS and DLS in the retrieval of spatial memory acquired through massed or distributed training. (*A*) Schematic of experimental design. (*B*) Pretest administrations of NBQX in the DMS ($n = 18$) impaired mice ability to correctly locate the platform on probe trial 24 h after massed training, compared to vehicle injected controls ($n = 17$) (two-way ANOVA repeated measure: quadrant preference $F_{(3,99)} = 32.63$, $P < 0.0001$; treatment $F_{(1,33)} = 0.78$, $P = 0.38$; quadrant preference × treatment $F_{(3,99)} = 9.16$, $P < 0.0001$). (*Inset*) The distance traveled on probe trial expressed as difference (Δ) between target and nontarget quadrants, in the two groups ($t_{33} = 3.29$, $P = 0.0024$; unpaired *t* test). (*C*) Pretest administrations of NBQX in the DLS ($n = 10$) did not affect the ability of mice to correctly locate the target quadrant on probe trial 24 h after massed training compared to vehicle controls ($n = 11$) (two-way ANOVA repeated measure: quadrant preference $F_{(3,57)} = 18.15$, $P < 0.0001$; treatment $F_{(1,19)} = 0.41$, $P = 0.53$; quadrant preference × treatment $F_{(3,57)} = 0.62$, $P = 0.61$). (*Inset*) The distance traveled on probe trial expressed as difference (Δ) between target and nontarget quadrants, in the two groups ($t_{19} = 0.10$, $P = 0.918$; unpaired *t* test). (*D*) Pretest administrations of NBQX in the DMS ($n = 14$) did not affect the ability of mice to correctly locate the target quadrant on probe trial 24 h after distributed training, compared to their vehicle controls (vehicle, $n = 11$) (two-way ANOVA repeated measure: quadrant preference $F_{(3,69)} = 30.02$, $P < 0.0001$; treatment $F_{(1,23)} = 0.59$, $P = 0.45$; quadrant preference × treatment $F_{(3,69)} = 0.13$, $P = 0.94$). (*Inset*) The distance traveled on probe trial expressed as difference (Δ) between target and nontarget quadrants, in the two groups ($t_{23} = 0.035$, $P = 0.972$; unpaired *t* test). (*E*) Pretest administrations of NBQX in the DLS ($n = 15$) impaired mice ability to correctly locate the platform on probe trial performed 24 h after distributed training, compared to controls ($n = 14$) (two-way ANOVA repeated measure: quadrant preference $F_{(3,81)} = 22.82$, $P < 0.0001$; treatment $F_{(1,27)} = 0.0057$, $P = 0.94$; quadrant preference × treatment $F_{(3,81)} = 5.49$, $P = 0.0017$). (*Inset*) The distance traveled on probe trial expressed as difference (Δ) between target and nontarget quadrants, in the two groups ($t_{27} = 3$, $P = 0.0057$; unpaired *t* test). Dotted line represents CL. Representative paths from control and treated mice in the different groups are also shown. Histograms represent mean ± SEM. $^{**}P < 0.05$ target vs. right, opposite, left; $^{\S}P < 0.05$ vs. target (within groups, Tukey honestly significant difference [HSD]); $^{\#}P < 0.05$ (between groups, Tukey HSD).

massed training, in optimizing memory depends on its ability to recruit a neural substrate capable of conferring increased stability to the memory trace.

In line with previous evidence (4, 20–23), our behavioral findings support the view that distributed practice does not affect the acquisition but rather yields better retention of information at remote time intervals. Indeed, in both training conditions, mice acquired equally well the ability to reach the escape platform during the training sessions. The main effect of the longer intersession intervals was to promote remote retention of the platform's location memory 2 wk after training, while retention 24 h after training was not affected by the training procedure (Fig. 1 *A*–*C*). These results are well aligned with previous findings in humans demonstrating a correlation between interrepetition intervals and retention intervals (1, 2, 24).

The difference in the ability to recall information at late time points was paralleled by differential activation of DLS and DMS immediately after training with the two protocols, as assessed by changes in c-Fos expression. We found enhanced c-Fos labeling in the DMS after massed spatial training and in the DLS after both massed and distributed spatial training, compared to expression in HC control mice (Fig. 1 *D*–*G*) and in mice trained with the same protocol in the cue version of the task (*SI Appendix*, Fig. S4). The most parsimonious explanation of this finding is that both striatal domains are actively encoding spatial information, but the DLS is more sensitive to distributed training than the DMS. Differential activation of the anterior and the posterior hippocampus has been recently correlated to retrieval of face-scene associations presented in a massed or a distributed fashion (25), suggesting that differential sensitivity to the interval between training episodes might be a common feature of distinct brain regions. However, such changes in neuronal activity do not necessarily imply a causal role of the activated brain region in the ability to increase retention, as demonstrated by the relatively preserved effect of distributed practice in hippocampal lesioned patients (26, 27).

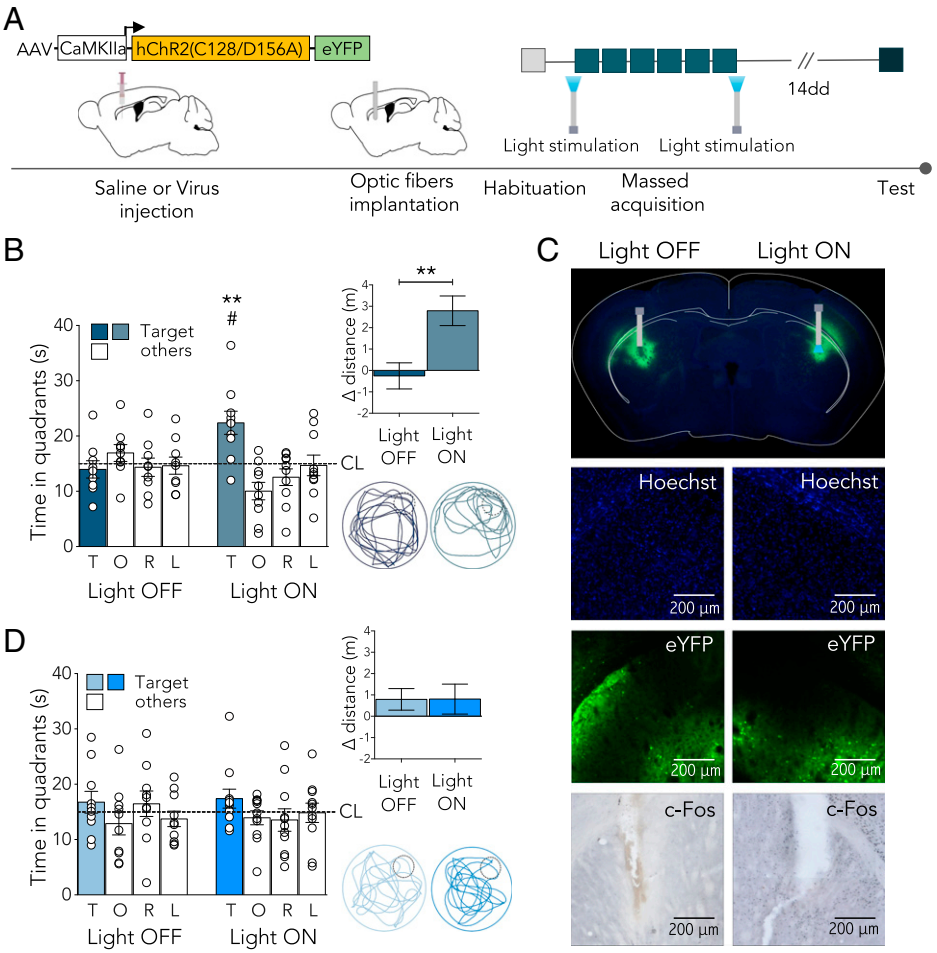

Fig. 3. Optogenetic stimulation of DLS enhances memory stability in massed-trained mice. (*A*) Schematic illustration of the experimental design. (*B*) Light delivery in DLS improved the ability to correctly locate the target quadrant on probe trial 14 d after the massed training, in stimulated ($n = 8$) but not unstimulated infected mice ($n = 9$) (two-way ANOVA repeated measure: quadrant preference $F_{(3,45)} = 4.23$, $P = 0.010$; treatment $F_{(1,15)} = 1.359$, $P = 0.26$; quadrant preference × treatment $F_{(3,45)} = 7.88$, $P = 0.0002$). Dotted line represents CL. (*Inset*) The mean difference (Δ) between the distance traveled in the target compared to nontarget quadrants ($t_{15} = 3.312$, $P = 0.0047$; unpaired *t* test). (*C*) Microphotographs showing ChR2-eYFP expression counterstained with Hoechst and representative fiber location in a mouse receiving light delivery unilaterally in the DLS. Representative images of Hoechst, eYFP, and c-Fos immunoreactivity in the two hemispheres showing increased c-Fos labeling only in the stimulated side. (Scale bar: 200 µm.) (*D*) Light delivery in mice bilaterally administered with saline did not affect performance in the probe trial 14 d after massed training, both groups (light on $n = 10$; light off $n = 11$) being equally unable to locate the target platform 14 d after the last training session (two-way ANOVA repeated measure: quadrant preference $F_{(3,57)} = 1.094$, $P = 0.359$; protocol $F_{(1,19)} = 0.564$, $P = 0.461$; quadrant preference × protocol $F_{(3,57)} = 0.421$, $P = 0.738$). Dotted line represents CL. (*Inset*) The mean difference (Δ) between the distance traveled in the target compared to nontarget quadrants ($t_{19} = 0.0133$, $P = 0.989$; unpaired *t* test). Representative paths from control and treated mice in the different groups are also shown. Histograms represent mean ± SEM. $^{**}P < 0.05$ target vs. right, opposite, left (within group, Tukey honestly significant difference [HSD]); $^{\#}P < 0.05$ (between-group interaction, Tukey HSD).

Loss-of-function manipulation of the two striatal domains before the probe test at 24 h after training provides evidence for a causal role of the two striatal subregions in the storage of information, dependent on the timing rule of the training protocol. Inhibition of DLS or DMS selectively impaired the recall of information acquired with the distributed or the massed protocol, respectively (Fig. 2), confirming that the two striatal components are engaged differentially in the early storage of spatial information, as a function of the time interval between learning episodes. These findings provide evidence of an impairing effect of a loss-of-function manipulation of the DLS on the ability to retrieve information regarding the platform's location in the spatial version of the MWM. Previous studies reported a lack of effect of DLS manipulation performed immediately after massed training (28, 29) or before distributed training in the sMWM (30, 31). Differences in the duration of the training or in the time window of DLS manipulation could explain these discrepancies.

Our results provide also compelling evidence that the time interval between training sessions determines the neural substrates sustaining the memory representation, regardless of the amount of training or the type of learning task, which were the same in the two conditions.

The rationale for choosing the striatum as a model system was based on behavioral evidence showing that the DLS is recruited in later stages of extended practice when memory reaches the plateau, both in skill (12, 32) and spatial learning tasks (14). We hypothesized that the DLS could have a more general role in memory optimization, whether it is achieved through prolonged or distributed training. Functional differences between the DMS and the DLS are generally framed within the dichotomy between goal-directed and habitual learning (33). However, in our study, both massed-trained and distributed-trained mice improved over the sessions by refining spatial search strategies similarly (*SI Appendix*, Fig. S2*C*), making it difficult to attribute the engagement of the DLS in distributed training to a change in the ability of goals to control action, as proposed in the framework of goal-directed behavior. Thus, an

interesting convergence of behavioral data suggests that regardless of the kind of information to be acquired, striatal compartment-specific activity is able to promote the formation of more enduring memories. The demonstration that optogenetic stimulation of the DLS during massed training allows memory optimization (Fig. 3) establishes that activation of this striatal component is sufficient to confer increased stability to the memory trace.

By contrasting the two striatal domains, we showed that DMS and DLS are differentially sensitive to the rate of interrepetition intervals. The ability of longer intervals to increase memory stability has been attributed to the greater efficacy of spaced stimuli in activating molecular pathways important for memory formation (6–8, 10, 11, 34–36). Therefore, it seems conceivable that the intrinsic dynamics of the molecular machinery of neural plasticity in the two striatal components might be a key element in understanding their differential sensitivity to stimuli presented with a different frequency. Further studies will be needed to shed light on the precise cellular and molecular mechanisms underlying these differences at the striatal level. Nevertheless, it is interesting to note that previous electrophysiological studies demonstrated an increase in DMS neuronal activity during the early phases of training, while DLS activity changed more gradually (12). Differences in the temporal dynamics of learning-induced responses were also observed when comparing DLS and hippocampus. For example, it has been shown that in the hippocampus, acetylcholine release increases at the onset of training in the cross-maze, while in the DLS it reaches its plateau level at later phases (37). Similarly, learning-induced rRNA transcription in the hippocampus increases immediately after training in the sMWM, while in the DLS it increases progressively over time after the end of the learning experience (38). These findings suggest that the DLS could be characterized by slower dynamics in learning-induced cellular and molecular adaptations, compared to other striatal or extrastriatal regions.

Using the stability of the performance in the sMWM as behavioral readout and the distinct striatal domains as anatomical counterparts, in this study we demonstrated that spatial memory engages different neural substrates depending on the training regimen. We also showed that gain of function manipulation of the DLS strengthens the memory representation. By demonstrating that both striatal domains are involved in spatial information processing but their participation depends on the temporal spacing of the training sessions, our data support the view that differences among memory systems could be addressed based on neurobiological determinants of the processing operations involved, rather than the kind of information processed (39). At a translational level, stimulation of brain regions such as the DLS, not traditionally included in memory circuits, could be exploited to ameliorate memory deficits in Alzheimer's disease and other forms of degenerative dementia.

Materials and Methods

Subjects. The experiments were conducted on naïve CD1 male mice (Charles River). Mice were at least 7 wk old, weighing about 35 g at the onset of the experiments. Animals were always housed in groups of three to five mice in standard cages (26.8 × 21.5 × 14.1 cm), with water and food ad libitum, under a 12-h light/dark cycle and constant temperature (22 ± 1 °C). Behavioral training and testing were conducted during the light period (from 9:00 AM to 5:00 PM). All animals were treated according to current Italian and European laws for animal care, and the maximum effort was made to minimize animal suffering. Procedures were conducted under the authorization n° 658/2019 from the Italian Ministry of Health, according to Italian (DL. 26/2014) and European laws and regulations on the use of animals in research, and NIH guidelines on animal care.

Stereotaxic Surgery and Viral Injections. Mice were deeply anesthetized with 3% isoflurane (Isovet; Piramal Healthcare) and secured on the stereotaxic apparatus (David Kopf Instruments). For the pharmacological experiment, two stainless-steel guide cannulae (0.50/0.25 × 7 mm; Unimed) were implanted at the following coordinates relative to bregma, according to the mouse brain atlas (40): AP = +0.30 mm, ML = ±1.6 mm, DV = −1.3 mm for the DMS and AP = +0.30 mm, ML = ±2.8 mm, DV = −1.3 mm for the DLS. Guide cannulae were fixed with acrylic cement (Riccardo Ilic) to be stably held on the calvarium.

For the optogenetic experiment, adeno-associated virus was used to express the eYFP and hChR2(C128S/D156A) under the control of CaMKIIa promoter (AAV-DJ-CaMKIIa-hChR2(C128S/D156A)-eYFP; Stanford University, Gene Vector and Virus Core). A glass pipette was bilaterally lowered in the DLS at the coordinates AP = +0.30 mm, ML = ±2.8 mm, DV = −3.0 mm, and a volume of 0.2 μL of the AAV (titer, 1.87e13) was inoculated bilaterally at the flow rate of 0.1 μL/min. The pipette was left in place for 5 additional minutes to allow viral diffusion. Control animals were injected with the same volume of saline solution (NaCl 0.9%) following an identical procedure. After 2 wk, mice underwent further surgery for fiber implantation. Custom-made optic fiber cannulae (200-nm core diameter; 0.39NA, Thorlabs) were lowered above the viral injection site (AP = +0.30 mm, ML = ±2.8 mm, DV = −2.6 mm) and secured to the skull with dental cement as described for the cannula fixation.

Drugs and In Vivo Focal Injection Procedure. General infusion procedure was performed for all experiments 20 min before the probe test. A total of 0.25 μL of 0.95 μg/μL of the AMPA receptor antagonist 1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium salt hydrate (NBQX; Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS 0.1 M) and bilaterally infused in the brain. Before drug injection the needle (length, 9 mm; diameter, 0.25 mm; Unimed) was connected by a plastic tube to a 2-μL Hamilton syringe and lowered into the guide cannula. The drug was delivered at the flow rate of 0.125 μL/min for 2 min and left in place for additional 1 min to allow the drug diffusion. Control mice were injected with the same volume of PBS. During the procedure, mice were awake and free to move in the HC.

Behavioral Procedures. The sMWM consisted of three different phases: familiarization, training, and test. Familiarization was the same for both massed and distributed protocols. The massed training consisted of six consecutive sessions (intersession interval: 10 to 15 min) of three trials (intertrial interval: 30 s) (15, 16), while the distributed version of MWM consisted of six sessions distributed over 3 d, with two sessions per day (intersession interval: 4 h) of three trials (intertrial interval: 30 s). Depending on the experiment, a single probe trial was performed 24 h or 14 d after the last training session. Search strategies during training were analyzed using numerical parameters from swim tracking data (adapted from ref. 41).

In the cMWM task the training was similar to the spatial version, with the exception that all the distal cues were completely removed and the proximal cue was constituted by a green ball hanging 5 cm above the hidden platform. The position of the platform and the ball changed across sessions to prevent animals from using spatial bias. Behavioral data from training trials were acquired and analyzed using an automated tracking system (ANY-maze, Stoelting).

Immunohistochemistry: c-Fos Staining. One hour after completing the last training session in the Morris water maze, each animal was deeply anesthetized with a mixture of Zoletil (500 mg/kg, Virbac Italia) and Xylazine (100 mg/kg, Bayer) and transcardially perfused. Brains were postfixed and 30-μm coronal sections were obtained using a freezing microtome (Leica CM 1950, Leica Microsystems). For c-Fos detection in the optogenetic experiment, the day after the test, animals underwent unilateral optical stimulation, were left undisturbed in their HCs for 1 h, then anesthetized and perfused as described to collect 40-μm coronal sections. For both experiments, free-floating sections were processed for c-Fos immunoreactivity (c-Fos-IR)

Optical Stimulation. The hChR2 (C128S/D156A) we used is a step-function opsin with a spontaneous deactivation time constant of 29 min (19). Before training, mice were acclimatized to their HCs for at least 30 min. After acclimatization, implanted optic fiber cannulae were connected to a blue-light laser

(473-nm wavelength, Wuhan Besram Technology Inc.) through a 1-m optic fiber (Thorlabs) and a single 1-s pulse was delivered (0.8 to 1 mW at the fiber tip) (PM100D power sensor, Thorlabs). Light-off control mice underwent to the same procedure omitting the light delivery. At the end of the stimulation, mice were detached from the fiber and left in their HC for 10 additional minutes, before starting the training. At the end of the training, mice underwent again the same procedure of light delivery and waited 30 min before being placed back in their HCs.

Data Collection and Statistical Analysis. All data were represented as mean ± SEM. One-way or two-way ANOVA as well as post hoc analysis, Student's paired or unpaired *t* test, and nonparametric analysis (Mann–Whitney *U* test) were performed with Statistica Software (Dell Software). The linear regression was performed by using Statistical Package for the Social Sciences (SPSS, IBM). Group differences were considered statistically significant when $P \leq 0.05$.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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