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Supplemental Information

Persistent Increases of PKMζ in Sensorimotor Cortex Maintain Procedural Long-Term Memory Storage

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Supplemental Items

Supplemental figures and legends

Figure S1. Related to Figures 4, S5, S6, and Results: Learning Phases of a Skilled Reaching Task.

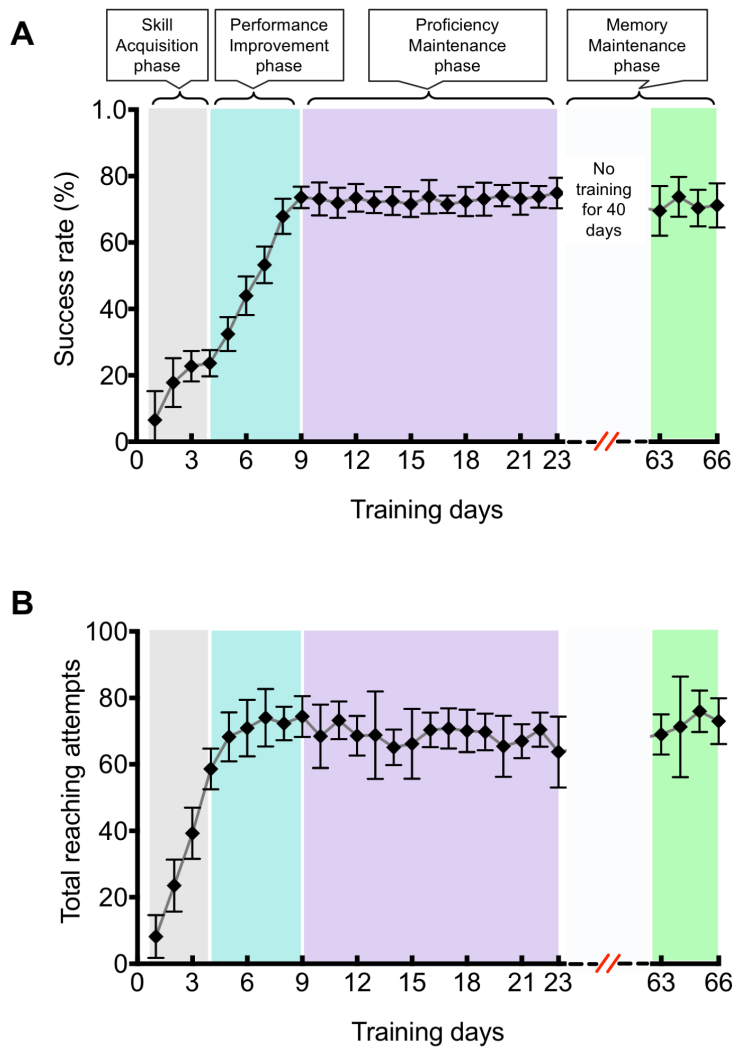

Figure S1. Daily success rate and total reaching attempts in the skilled reaching task.

(A) The learning curve for skilled reaching task. X-axis – the number of training days; Y-axis – success rate (% successful reaches divided by the total reaching attempts; means ± SD). (B) The total reaching attempts in a daily 30-minute training session. X-axis – the number of training days; Y-axis – total reaching attempts (means ± SD). Gray block - the skill acquisition phase; blue block - the performance improvement phase; purple block - the proficiency maintenance phase; white block - regular housing without training for 40 days; green block - testing after the 40-day no training gap.

Figure S2. Related to Figures 1 and 2.

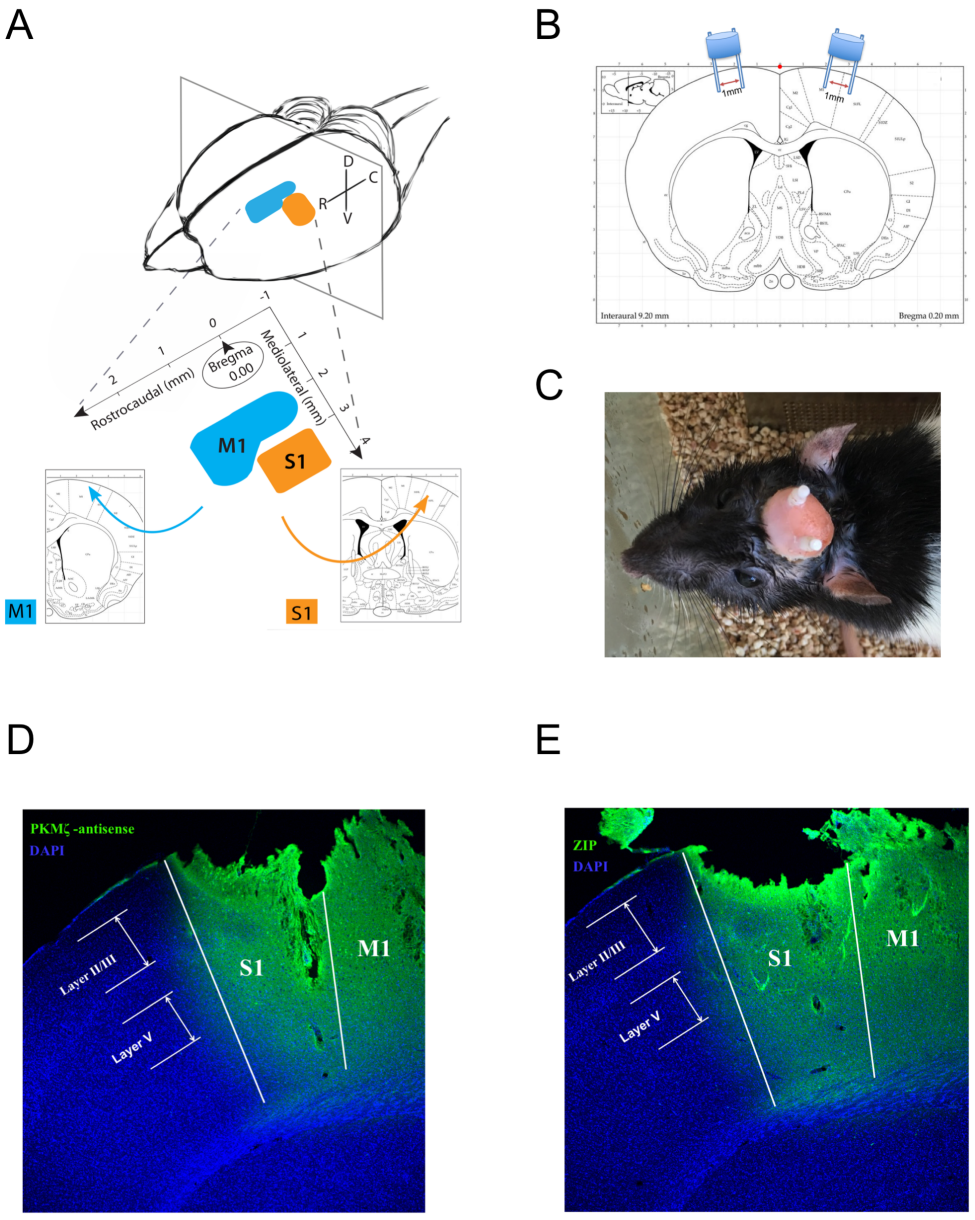

Figure S2. Fluorescence labeling of biotinylated PKMζ-antisense and biotinylated ZIP injections.

(A) The stereotaxic coordinates for rat forelimb M1 and S1 regions. The coronal sections for IHC study were chosen from primary motor cortex (M1, from 1.6 to -0.5 mm to the bregma and from 1.5 to 3 mm lateral to the midline) and primary somatosensory cortex (S1, from 0.5 to -0.4 mm to the bregma and from 3 to 4 mm lateral to the midline). (B) Illustration of the location for the bilaterally implanted double-guide cannula. The center-to-center distance between two dummy wires in each double-guide cannula was 1.0 mm, and the cannulae were tilted 10˚ from the vertical line in each hemisphere. The coordinates of guide cannula in M1: AP + 0.2 mm, ± ML 2.2 mm, DV -1.16 mm; the coordinates of guide cannula in S1: AP + 0.2

mm, ± ML 3.2 mm, DV - 0.99 mm. (C) Top view of a rat 12 hours after surgery with cannula implantation. (D) Fluorescence labeling of injected biotinylated PKMζ-antisense used to evaluate the spread of PKMζ-antisense and demonstrate the efficacy of the injection approach. Rat was sacrificed 1 hour after the injections of biotinylated PKMζ-antisense in PBS. The injection dosage was 0.5 µl/site (1 µl/hemisphere in total). DAPI was counterstain. Green: biotinylated PKMζ-antisense. Blue: DAPI. (E) Fluorescence labeling of injected biotinylated ZIP used to evaluate the spread of ZIP and to demonstrate the efficacy of the injection approach. Rat was sacrificed 1 hour after the injections of biotinylated ZIP. The injection dosage was 1 µl/site (2 µl/hemisphere in total). DAPI was counterstain. Green: biotinylated ZIP. Blue: DAPI.

Figure S3. Related to Figures 1 and 2.

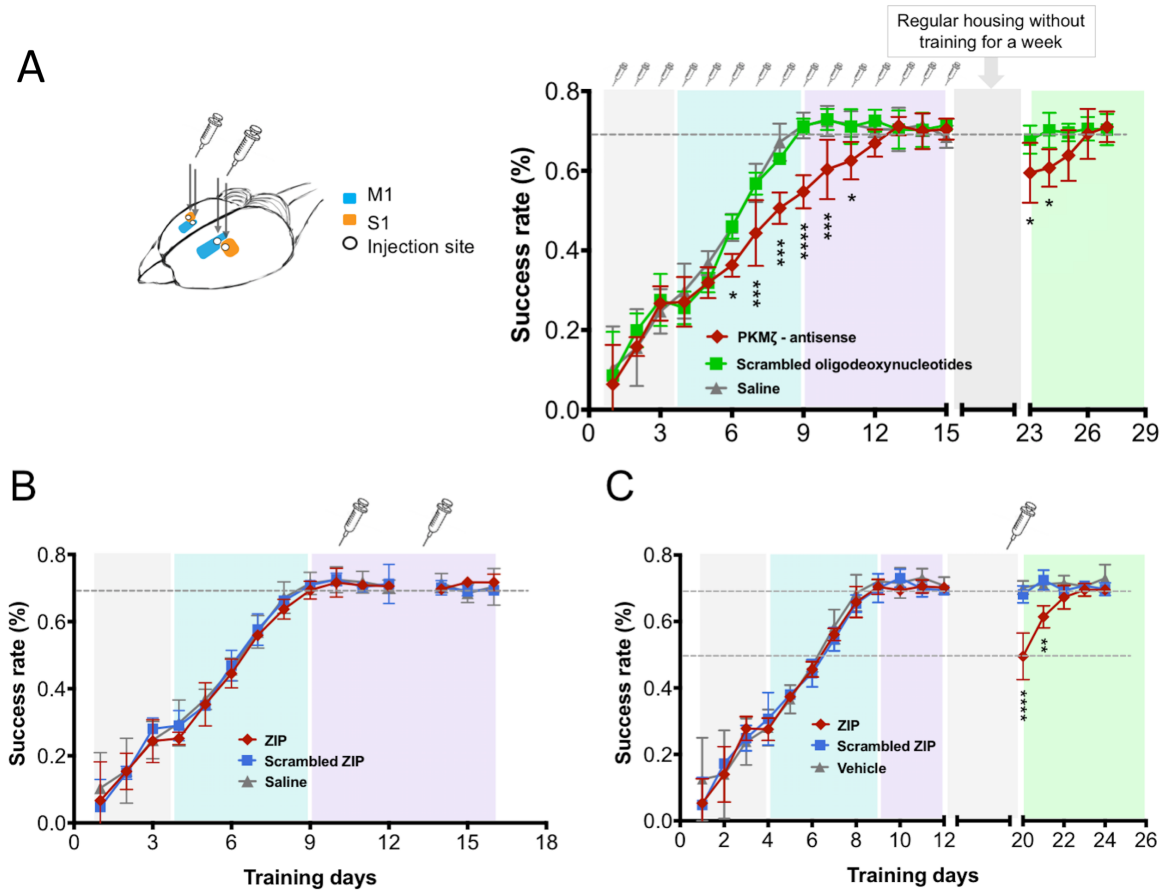

Figure S3. Perturbing PKMζ synthesis and aPKC activity during motor learning and memory maintenance.

(A) Left, schematic representation of injection sites. Right, the complete learning curve for Figure 1 shows PKMζ-antisense specifically affects the performance improvement phase and not the initial skill acquisition phase of motor learning. (B and C) The complete learning curve for Figure 2 and the effect of ZIP on motor memory maintenance.

Figure S4. Related to Figures 4, S5, and S6.

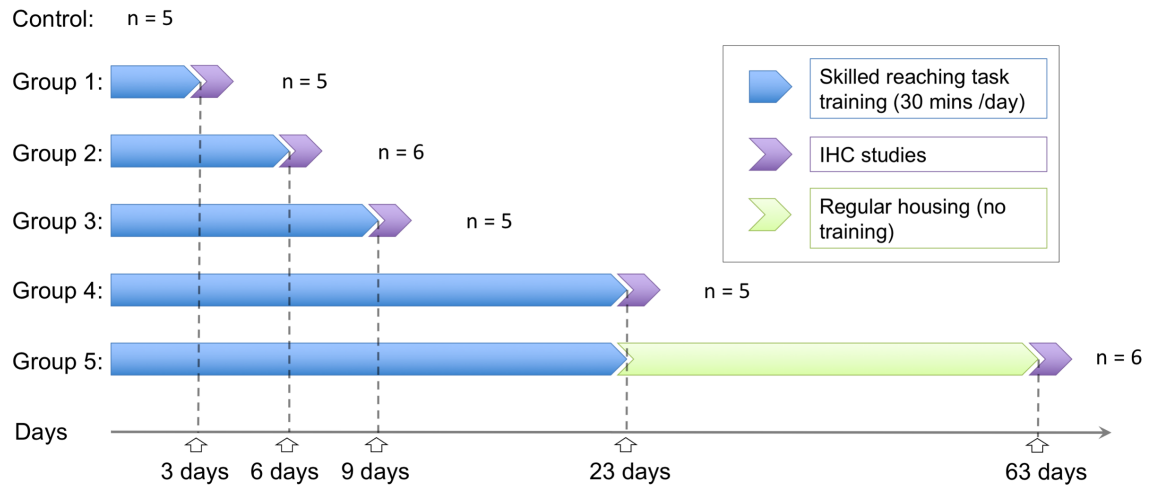

Figure S4. Experimental paradigm of PKMζ immunostaining.

Blue arrow: number of training days (30 minutes/day); purple arrow: immunohistochemistry (IHC), performed 24 hours after the last training session for each group except for the 40-day training gap group (see Transparent Methods); green arrow: regular housing without training. The expression of PKMζ (Figures 4 and S5) and PSD-95 (Figure S6) were tested at the following time points of motor skill learning: 0 days of training (control group, n = 5 rats); 3 days of training - the skill acquisition phase (group 1, n = 5 rats); 6 days of training - the performance improvement phase (group 2, n = 6 rats); 9 days of training - the performance plateau phase (group 3, n = 5 rats); 23 days of training - the proficiency maintenance phase (group 4, n = 5 rats); 23 days of training + 40 days no training gap - the long-term memory storage phase (group 5, n = 6 rats).

Figure S5. Related to Figure 4.

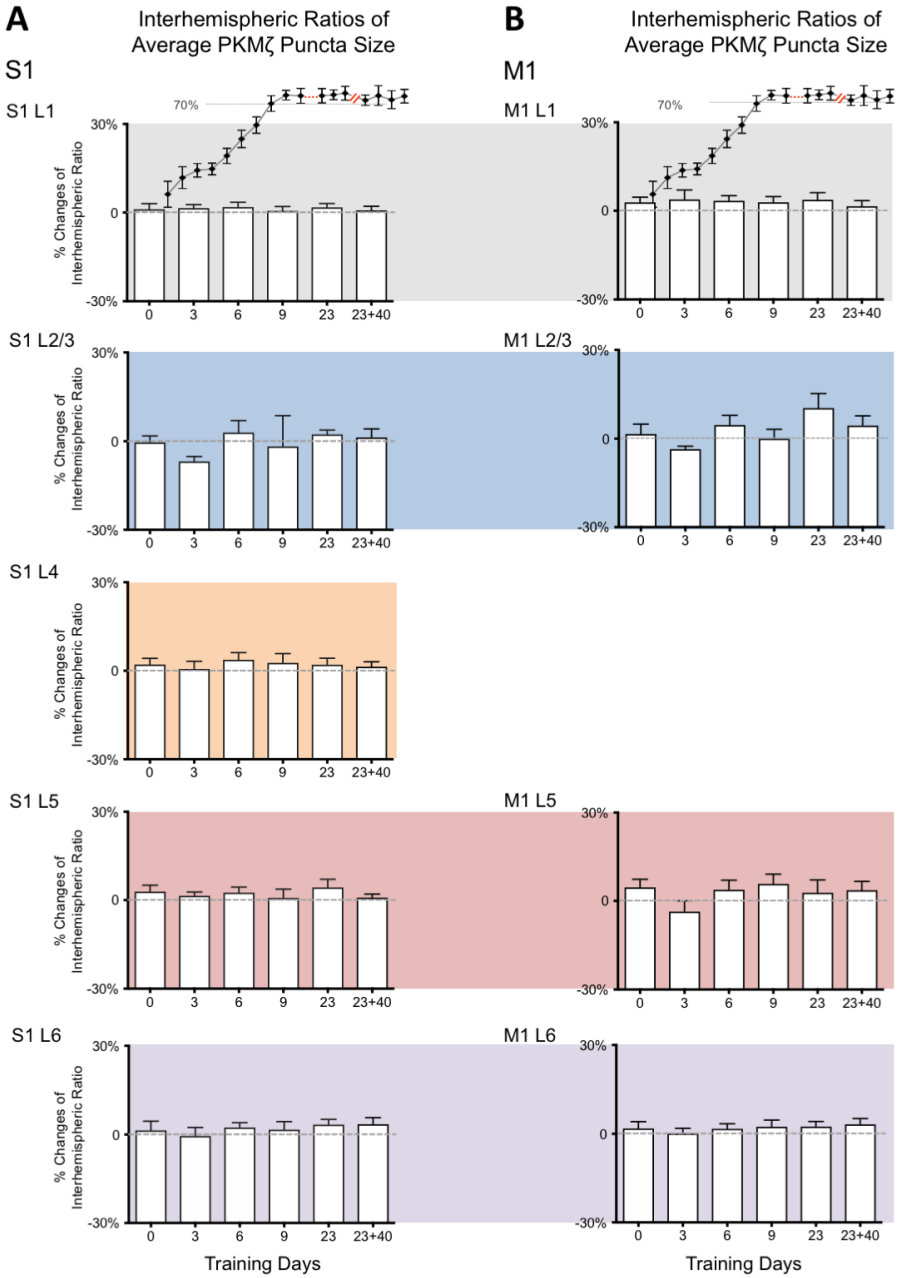

Figure S5. PKMζ puncta size does not change during sensorimotor learning and memory.

(A and B) Layer-specific, average PKMζ puncta size in S1 and M1 during motor learning. X-axis – days of training (0 - naïve rat; 3, 6, 9, 23 - rats trained for 3, 6, 9 or 23 days; 23+40 – rats trained for 23 days and regular housed for an additional 40 days); Y-axis – % interhemispheric ratio of average PKMζ puncta size (means ± SEM). No significant group effects were found with one-way ANOVAs in all regions of S1 and M1.

Figure S6. Related to Figure 4 and Results: Sensorimotor Training Induces a Transient Increase of PSD-95 in Sensorimotor Cortex.

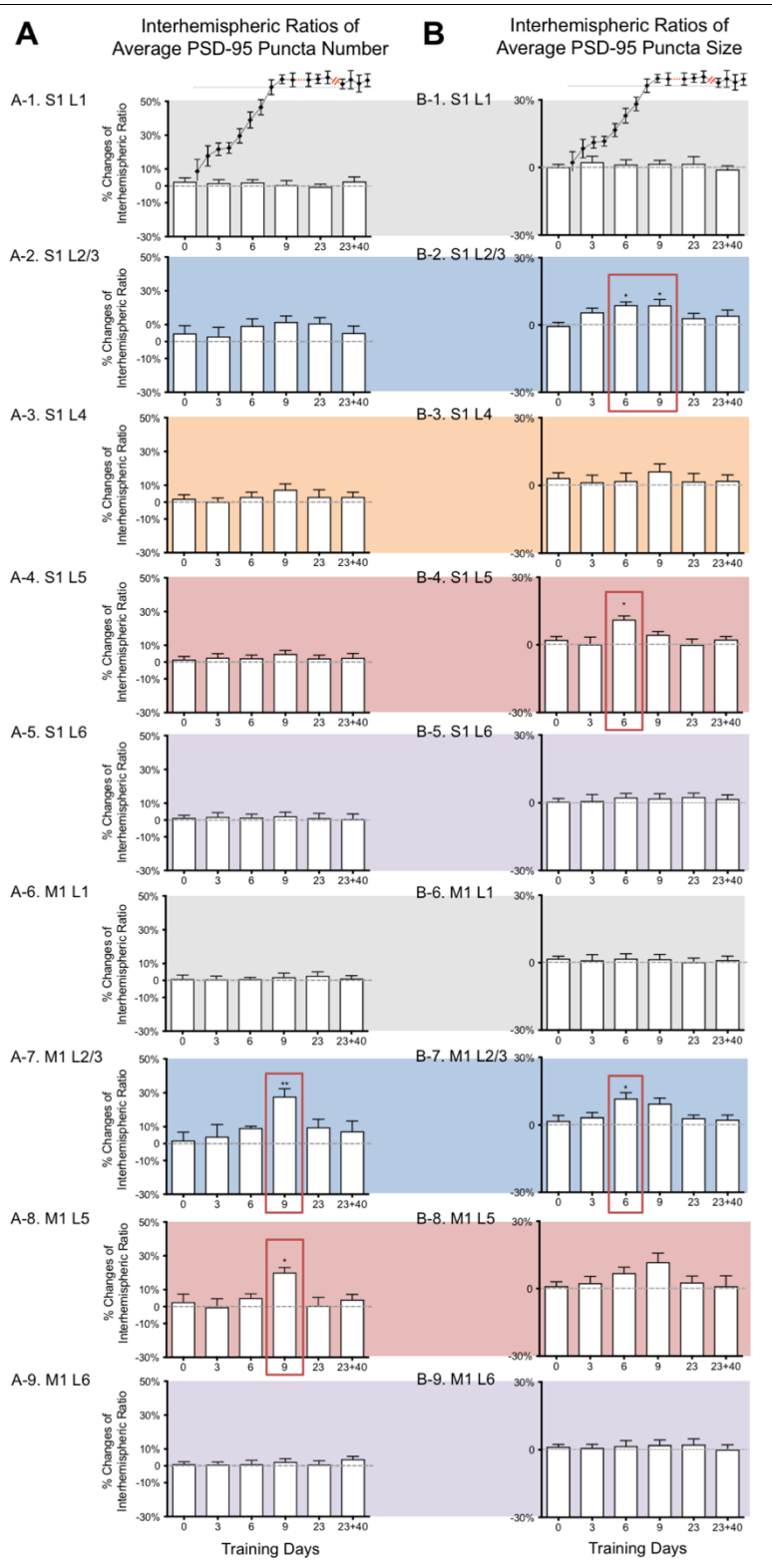

Figure S6. Spatiotemporal changes of PSD-95 puncta number and size during motor learning and memory.

(A) Layer-specific changes of PSD-95 puncta number in S1 and M1 during motor learning. (B) Layer-specific changes of PSD-95 puncta size in S1 and M1 during motor learning. A-1 and B-1: S1 layer I; A-2 and B-2: S1 layer II/III; A-3 and B-3: S1 layer IV; A-4 and B-4: S1 layer V; A-5 and B-5: S1 layer VI; A-6 and B-6: M1 layer I; A-7 and B-7: M1 layer II/III; A-8 and B-8: M1 layer V; A-9 and B-9: M1 layer VI. X-axis - days of training (0 - naïve rat; 3, 6, 9, 23 - rats trained for 3, 6, 9 or 23 days; 23+40 - rats trained for 23 days and regular housed for an additional 40 days). Y-axis - % of interhemispheric ratio of average PSD-95 puncta number (A) and average PSD-95 puncta size (B) (means ± SEM). Statistical analysis was conducted using one-way ANOVAs for between group effects, followed by Dunnett's multiple comparison tests to show the difference of each training group with control (0 - naïve rat) (*$p < 0.05$, **$p < 0.01$).

The level of PSD-95 was not maintained as long as PKMζ or for as long as the memory persisted. The transitory increases of PSD-95 in S1 and M1 layer II/III and layer V are likely due to the synaptic reorganization during motor learning (Kleim et al., 2004; Xu et al., 2009). A similar skilled reaching task in mice has been shown to increase both spine formation and elimination in the contralateral motor cortex of the preferred forelimb (Xu et al., 2009). Therefore, more synapses might be formed than eliminated at the early learning phases, which could be detected as the increased total level of PSD-95 in our study. When the skill is fully acquired, the newly formed, memory-related synapses are likely to be stabilized, while non-memory-related synapses will gradually be eliminated (Xu et al., 2009). Therefore, the overall spine density might return to the same level as before training, which could be indicated by the gradual decrease of PSD-95 to the untrained level after 9 days.

Transparent Methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Joseph Francis (joey199us@gmail.com).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals: Sixty-eight adult female rats (Long-Evans from Hilltop Lab Animals, Inc., 2-6 months old, weighing between 200-250g) were used in the study. All experiments were performed under protocol #13-10365, approved by the Institutional Animal Care and Use Committee of SUNY Downstate Medical Center.

Only female rats were used in this study for 3 reasons: 1) All the studies of Rioult-Pedotti and colleagues that showed that motor learning is accompanied by enhanced synaptic plasticity and an upward-shifted synaptic modification range in sensorimotor cortex used female rats (Rioult-Pedotti et al., 2007; 2000; 1998). In their studies, synaptic efficiency was measured on the trained hemisphere and compared with the untrained hemisphere in each animal. Therefore, we utilized the same experimental paradigm to investigate the molecular changes in female rats to maintain consistency with these prior studies. 2) It has been shown that sex has no effect on rat's reaching performance measured by daily success rate (Field and Whishaw, 2005). 3) The weights of female rats are comparatively stable.

METHOD DETAILS

Behavioral Training: For behavioral training, animals with cannula implantation were housed individually, and animals without cannulae were pair-housed. A 12/12 h light/dark cycle was maintained for all animals.

Skilled reaching task: rats were food restricted to 85-90% of their free-feeding body weight for 1 week. The training session lasted 30 minutes per day. During the pre-training session (day 0), food pellets (45 mg Dustless Precision Pellets, banana or chocolate flavor, Bio-Serve) were placed inside of the behavioral chamber during free exploration.

The training chamber was customized with clear Plexiglass (30 cm tall, 20 cm wide and 43 mm long with the thickness of 0.5 cm). A narrow vertical slot (10 cm tall and 1 cm wide) was located at the front of the chamber, and a horizontal food platform (3 cm wide and 8.5 cm long) was fixed in the slot at a height of 3 cm from the floor. Training begins on day 1 when a food pellet was placed in a metal washer (0.5 cm inside diameter) on the platform 1.5 mm away from the slot. This design allowed only one forelimb to pass through and reach for the pellet during each attempt. At the back of the chamber, a 1 cm diameter hole was opened to allow for delivery of extra food pellets. After each reaching attempt, the rats had to go back and reset the trial before a new pellet was placed on the platform. In order that the animals learn the sequence faster, an extra pellet was provided for every attempt during the first two days of training, whether successful or not. Then starting from day 3, a 0.2 cm diameter plastic dowel was glued between the metal washer and the slot to increase the difficulty of task. In addition, after day 3, an extra pellet at the back of the chamber would be provided only after a successful reaching attempt. After each failed attempt, the unattained pellet would be removed by the experimenter, and the

rats would begin a new trial. The required movements for successful reaching included extending the preferred forelimb through the slot, grasping the food pellet accurately, supinating the paw while holding the pellet, and then retracting the forelimb with the pellet through the slot.

Rats without cannula implantations were divided into five groups based on the days of training (as shown in Figure S4). The first four groups were trained for 3 days (n = 5 rats), 6 days (n = 6 rats), 9 days (n = 5 rats), and 23 days (n = 5 rats), and each group subjected to immunohistochemistry (IHC) 24 hours after the last training session. The fifth group (n = 6 rats) was trained for 23 days followed by regular housing for an additional 40 days before IHC. The control group included both naïve (n = 2) and paired-control rats (n = 3). For the paired controls, food pellets were placed on the side of the platform that extended inside of the behavioral chamber. Skilled reaching was not necessary in this paradigm, and the rats ate most of the pellets with their mouths without reaching. The paired-control animals still had to go to the back of the chamber to reset the trail each time. No IHC differences were found between the naïve and paired-controls in our study, and therefore they were combined into one group.

Immunohistochemistry: After behavioral training, rats underwent cardiac perfusion, and their brains were placed in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB) [pH 7.4] for 48 hours. Forty µm coronal sections of the sensorimotor cortex were made by a Leica VT 1200S vibratome. The sections of forelimb M1 and S1 regions were chosen according to rat brain stereotaxic coordinates and previous studies (Kleim et al., 1996; von Kraus et al., 2010; Rioult-Pedotti et al., 1998), as illustrated in Figure S2 A. For each animal, 6-10 sections were stained.

Free-floating sections were rinsed with PBST solution (0.1 M phosphate-buffered saline [pH 7.4] + 0.3% Triton X-100) 6 times (10 minutes each rinse) at room temperature. The sections were then transferred to blocking buffer (10% normal donkey serum and 1% bovine serum albumin in PBST) for 5 hours at room temperature. After blocking, primary antibodies were used as described below.

Double-staining of PKMζ and PSD-95: sections were incubated overnight at 4°C in primary antibodies: anti-PKMζ rabbit polyclonal (1: 4000, generated in Dr. Sacktor's lab at SUNY Downstate (Hernández et al., 2014)) and anti-PSD-95 mouse monoclonal (1:1000, Abcam), followed by 6 rinses with PBST (10 minutes each). The sections were then incubated with secondary antibodies: Alexa Fluor® 647-AffiniPure Donkey Anti-Rabbit IgG (H+L) (1:200, Jackson ImmunoResearch) and Alexa Fluor® 488-AffiniPure Donkey Anti-Mouse IgG (H+L) (1:200, Jackson ImmunoResearch), for 3 hours at room temperature.

Staining of brain with biotinylated PKMζ-antisense or biotinylated ZIP injection: every fifth section was selected from rats injected with biotinylated-modified agents to indicate the spread of PKMζ-antisense or ZIP. These sections were incubated with mouse anti-biotin antiserum (1:400, Jackson ImmunoResearch) overnight at 4°C, followed by 6 rinses of PBST (10 minutes each) and Alexa Fluor® 488-AffiniPure Donkey Anti-Mouse IgG (H+L) (3 hours at room temperature).

DAPI (4,6'-diamidino-2-phenylindole): the nuclear stain DAPI (1:500, Fisher Scientific) was applied for 15 minutes at the end of all immunostaining. After washing with PBST (10 minutes), the sections were mounted onto microscope slides with antifade mounting medium VECTASHIELD (Vector Laboratories) and stored at -20°C until image acquisition was performed by confocal microscopy.

Surgery: Animals were anesthetized with isoflurane for cannula implantation in a stereotaxic frame (Kopf instruments). At the beginning of surgery, valium (i.p. 5 mg/kg body weight) was administered as a sedative. Additionally, atropine (i.m. 4 mg/kg body weight) was used to avoid fluid accumulation in the respiratory tract. After craniotomy, the double-guide cannulae (Plastics One, 26GA, 1.0 mm center-to-center distance between two dummy wires) were inserted bilaterally through the burr holes and fixed onto the skull by dental cement (Figure S2 B and C). The coordinates for guide cannula were measured anteroposterior (AP) and dorsoventral axis (DV) relative to bregma, and mediolateral (ML) relative to lambda, according to a rat brain atlas (Figure S2 A) (Paxinos and Watson, 2006). The coordinates were:

M1: AP +0.2 mm, ±ML 2.2 mm, DV -1.16 mm
S1: AP +0.2 mm, ±ML 3.2 mm, DV - 0.99 mm

At the end of surgery, buprenorphine (s.c. 0.01-0.05 mg/kg body weight) was administered as analgesic, and antibiotic ointment was applied to prevent infection.

Intracortical Injection: Prior to the skilled reaching task, all animals that had undergone surgery were allowed to recover for 7-10 days and received bilateral injections of saline (0.5 µl in each site, 1 µl total in each hemisphere) once per day for 3 days to allow habituation to the infusion procedure. Prior to injection, the rats were restrained to remove the cannula dummy and then the injection needles (Plastics One, 33GA, 1.0 mm center-to-center distance between two wires) were inserted. The needles protruded 0.5 mm from the guide cannulae. The infusion speed was controlled by a microinjection pump (model NE-4000, New Era Pump Systems) at 0.25 µl/minute. At the end of each injection, the needles were left in place for 3 minutes before retracting.

Antisense PKMζ: the sequences of single-stranded oligodeoxynucleotides used, shown below, in which the lower case signifies phosphorothioate linkage 5'-3', were as previously described (Tsokas et al., 2016) (Gene Link, Hawthorne, NY).

PKMζ-antisense: ctcTTGGGAAGGCAtgaC
Scrambled antisense oligodeoxynucleotides: aacAATGGGTCGTCtcgG

During training, 1 nmol PKMζ-antisense/scrambled antisense oligodeoxynucleotide in 0.5 µl PBS per site (1 µl total in each hemisphere) or an equivalent volume of saline solution were injected daily 30 minutes before the skilled reaching task (Figures 1 and S3). The injection and training procedures lasted for 15 days, and the success rate of reaching was recorded for analysis.

ZIP: during specified days of training, 1 µl of ZIP/scrambled ZIP (Tocris, 10 nmol in 1 µl PBS) was injected at each site (2 µl total in each hemisphere) (Figures 2 and S3).

Biotinylated drugs were injected 1 hour before sacrifice to confirm the location of the cannulae and to track drug diffusion (Figure S2 D and E). Data from 3 rats with misplaced cannulae were eliminated from the analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Confocal image acquisition: Multi-channel images with double PKMζ and PSD-95 staining were collected using an FV1000 confocal microscope (Olympus Fluoview) at 60X magnification and zoom at 2X. All parameters (pinhole, contrast, and brightness) were held constant for all sections from the same batch of staining. The microscope was equipped with a 60X, 1.42 numerical aperture oil immersion lens. Multi-wavelength images were acquired in all layers on forelimb primary motor cortex and primary somatosensory cortex from both hemispheres for each animal in Z-stacks. Each stack consisted of six 2-D images with a total thickness of 18.0 μm, and the step size between each 2-D image was 3.0 μm.

Z-projection: the projection of six 2-D images along the z-axis was analyzed off-line using custom-written macros in Fiji (Image J). PKMζ and PSD-95 puncta were separately identified using the green and red LUTs (Figure 3). Nuclei were identified by DAPI staining in blue.

Threshold calculation: Z-projections were converted to 8-bit first, and the thresholds were then set in each channel according to the non-primary staining from the same batch. The threshold values were the mean light intensity plus one standard deviation calculated from 6 images of the non-primary staining.

Puncta counting: After thresholding, the particle analyzer in Fiji was used to extract the average number and size of PKMζ and PSD-95 puncta in each hemisphere for all the animals (Figure 3). The contralateral hemisphere of the preferred forelimb was analyzed as the trained hemisphere and the ipsilateral hemisphere as the untrained hemisphere. The interhemisphere ratios (averaged puncta number and size in the trained hemisphere divided by the untrained hemisphere) were obtained for each animal. Statistical analysis was conducted using one-way ANOVA for between group effects, followed by Dunnett's multiple comparison test to show the difference of each training group with control if necessary. Significance was accepted when $p < 0.05$.

Statistical analysis for the effect of drug injection on training: Two-way repeated measures ANOVA was used to compare the significance of time (day of training) and injection groups on performance (reaching success rate). *Post hoc* Tukey's test for multiple comparisons was used to compare the group effect of antisense with scrambled oligodeoxynucleotide or saline and to compare ZIP with scrambled ZIP or saline, as necessary. Significance was accepted when $p < 0.05$.

Supplemental References

Field, E.F., and Whishaw, I.Q. (2005). Sexually dimorphic postural adjustments are used in a skilled reaching task in the rat. Behav Brain Res. *163*, 237–245.

Paxinos, G., and Watson, C. (2006). The Rat Brain in Stereotaxic Coordinates (Academic Press).