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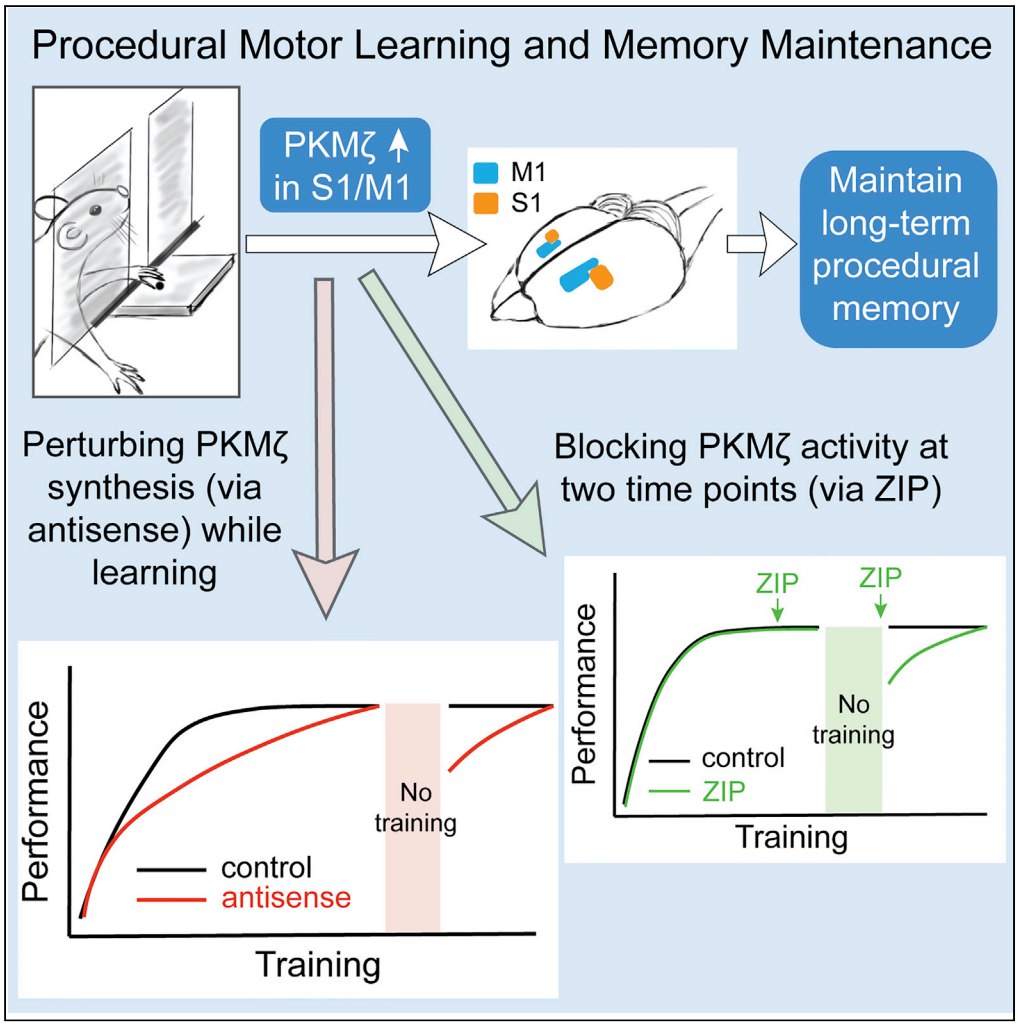

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HIGHLIGHTS

Perturbing PKMζ synthesis in S1/M1 slows the formation of skilled motor memory

Blocking PKMζ activity specifically erases memories maintained without reinforcement

Skilled motor learning induces the increase of PKMζ in S1/M1 layers II/III and V

PKMζ sustains the engram for procedural motor memory in M1 layer V

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

Peng Penny Gao,[1] Jeffrey H. Goodman,[1,2,4] Todd Charlton Sacktor,[1,3,4,6,*] and Joseph Thachil Francis[1,5,6,7,*]

SUMMARY

Procedural motor learning and memory are accompanied by changes in synaptic plasticity, neural dynamics, and synaptogenesis. Missing is information on the spatiotemporal dynamics of the molecular machinery maintaining these changes. Here we examine whether persistent increases in PKMζ, an atypical protein kinase C (PKC) isoform, store long-term memory for a reaching task in rat sensorimotor cortex that could reveal the sites of procedural memory storage. Specifically, perturbing PKMζ synthesis (via antisense oligodeoxynucleotides) and blocking atypical PKC activity (via zeta inhibitory peptide [ZIP]) in S1/M1 disrupts and erases long-term motor memory maintenance, indicating atypical PKCs and specifically PKMζ store consolidated long-term procedural memories. Immunostaining reveals that PKMζ increases in S1/M1 layers II/III and V as performance improved to an asymptote. After storage for 1 month without reinforcement, the increase in M1 layer V persists without decrement. Thus, the persistent increases in PKMζ that store long-term procedural memory are localized to the descending output layer of the primary motor cortex.

INTRODUCTION

Motor learning is characterized by a slow improvement of the smoothness and accuracy of skilled movements, which, once established, are maintained for long periods of time without further practice (Dayan and Cohen, 2011). A skilled motor task in which rodents are trained to reach with their preferred forelimb through a small slot to grasp food pellets has been widely used to study the neural substrate underlying motor learning (Fu and Zuo, 2011; Kargo, 2004; Kleim, 2002; Kleim et al., 1998, 2004; Luft et al., 2004; Monfils and Teskey, 2004; Rioult-Pedotti et al., 2007, 2000, 1998). Performance gains and the maintenance of proficiency on this task depend on the integrity of the sensorimotor cortex (Luft et al., 2004; Sanes and Donoghue, 2000; Whishaw, 2000; Whishaw et al., 2008). Plastic changes in sensorimotor cortex, including synaptic strength modification and structural remodeling, have been correlated with different phases of the learning process (Harms et al., 2008; Kleim et al., 2004; Monfils and Teskey, 2004; Rioult-Pedotti et al., 1998, 2000, 2007; Xu et al., 2009). Rioult-Pedotti and colleagues, for example, found that after 5 days of training, the synaptic efficacy of horizontal connections in primary motor cortex (M1) layer II/III increased significantly on the contralateral hemisphere to the preferred forelimb (Rioult-Pedotti et al., 1998), indicating a long-term potentiation (LTP)-like modification of synaptic transmission (Monfils and Teskey, 2004; Rioult-Pedotti et al., 2000). Both spine formation and elimination were seen immediately after the first motor learning session in mice (Xu et al., 2009) and were sustained for up to 20 days with continued daily training in rodents (Kleim et al., 1996; Xu et al., 2009). However, the molecular mechanisms that store motor memories in the sensorimotor cortex remain unknown.

The persistent increase in the autonomously active, atypical protein kinase C (aPKC) isoform PKMζ is both necessary and sufficient for maintaining LTP (Ling et al., 2002; Osten et al., 1996; Sacktor et al., 1993). PKMζ activity retains increased amounts of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors (AMPARs) in postsynaptic sites to maintain synaptic potentiation (Migues et al., 2010; Sacktor, 2012). PKMζ also contributes to maintaining the structural modifications of dendritic spines and synapses (Chen et al., 2014; Shao et al., 2012), changes that have been extensively observed in the sensorimotor cortex after sensorimotor learning (Yu and Zuo, 2011). Inhibition of persistent aPKC activity in specific brain structures by zeta inhibitory peptide (ZIP) disrupts the maintenance of various types of memory, including hippocampus-dependent spatial memory (Pastalkova et al., 2006), basolateral amygdala-dependent fear memories (Gámiz and Gallo, 2011; Kwapis et al., 2012, 2009; Migues et al., 2010; Serrano et al., 2008), dorsal lateral striatum-dependent habit memory (Pauli et al., 2012), and insular cortex-dependent

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long-term associative conditioned taste aversion memory (Shema et al., 2007), as well as sensorimotor cortex-dependent motor learning in the reaching task (von Kraus et al., 2010). However, ZIP affects PKC isoforms in addition to PKMζ, particularly the other aPKC, PKCι/λ, which can compensate for PKMζ in PKMζ-knockout mice (Tsokas et al., 2016). Therefore, we started by investigating the role of PKMζ in motor learning with PKMζ-antisense oligodeoxynucleotides directed against the translational start site of the PKMζ mRNA, which specifically disrupts PKMζ synthesis and not PKCι/λ synthesis when applied during LTP in brain slices (Hsieh et al., 2017; Tsokas et al., 2016) and effectively blocks learning-induced increases in PKMζ when injected intracranially (Hsieh et al., 2017).

RESULTS

Learning Phases of a Skilled Reaching Task

We used a skilled reaching task to study the role of PKMζ in sensorimotor learning and long-term memory maintenance. Rats were trained to reach with their preferred forelimbs through a small slot to grasp food pellets. Repeated training sessions were required to obtain good performance, which provided an extended time window to examine in detail any changes in sensorimotor cortex. The success rate, defined as % successful reaches/total reaching attempts, was used to evaluate task performance in a daily 30-min training session. A successful reach includes the following motor sequence: (1) lift and advance the preferred forelimb through the slot; (2) pronate and grasp food pellet; (3) retrieve without dropping the pellet (Klein et al., 2012).

Consistent with the model described by Monfils and Teskey (Monfils and Teskey, 2004), we observed that the acquisition and maintenance of skilled motor memory can be divided into four phases: (1) a skill acquisition phase (days 1–4), when success rate is comparatively low (<30%); (2) a performance improvement phase (days 5–9), when the success rate increases rapidly until plateauing (at ∼70%); (3) the proficiency maintenance phase, when performance is stable with continual training; and (4) a long-term memory storage phase, when performance is stable without training (Figure S1 and Transparent Methods). Because ZIP appears to specifically disrupt the long-term sensorimotor memory storage phase (von Kraus et al., 2010), we first focused on the effects of PKMζ-antisense on the acquisition and subsequent maintenance of sensorimotor learning and memories.

PKMζ-Antisense Slows the Performance Improvement Phase of Sensorimotor Learning

To determine the necessity of PKMζ in sensorimotor cortical networks during sensorimotor learning and memory maintenance, we utilized antisense oligodeoxynucleotides against the translation start site of PKMζ mRNA, which specifically and effectively block PKMζ synthesis during LTP in brain slices and learning *in vivo* (Hsieh et al., 2017; Tsokas et al., 2016). We injected PKMζ-antisense bilaterally in S1/M1 30 min before daily training and examined its effect on sensorimotor learning (Figures 1 and S2 and Transparent Methods). The antisense group (red) had a significantly lower learning rate compared with the control groups that received inactive scrambled oligodeoxynucleotide (green) or saline (gray) injections (Figure 1). *Post hoc* Tukey's tests after repeated two-way ANOVA showed significantly lower success rates for the antisense group compared with scrambled oligodeoxynucleotide and saline groups on days 6–11 (Figure 1). In the antisense group, the fast performance improvement phase was replaced with a slow learning curve that extended from days 5 to 11 (Figure 1). With continued training the success rate of the antisense group eventually reached the same asymptote as the other groups on day 12 (Figure 1). Rats with scrambled oligodeoxynucleotide or saline injections learned the skilled reaching task as efficiently as uninjected animals (Figures 1 and S1), indicating the surgery and intracortical injections did not affect their ability to acquire the skilled sensorimotor memory.

PKMζ-Antisense Disrupts the Stability of Long-Term Sensorimotor Memory

The injection of PKMζ-antisense in S1/M1 slowed the rate of learning in the proficiency acquisition phase, but with additional training days the antisense-injected animals eventually reached the same proficiency as the control animals. This result suggests there are two distinct mechanisms of motor learning—one that requires *de novo* PKMζ synthesis and a second that does not. Because our experiments involve continual training and reinforcement, we asked how well the PKMζ-independent mechanism maintains memory without this reinforcement. After asymptotic performance was reached, we ceased training and retested the rats 1 week later. The control, scrambled oligodeoxynucleotide group maintained peak performance even without reinforcement, whereas the PKMζ-antisense group showed significantly lower performance

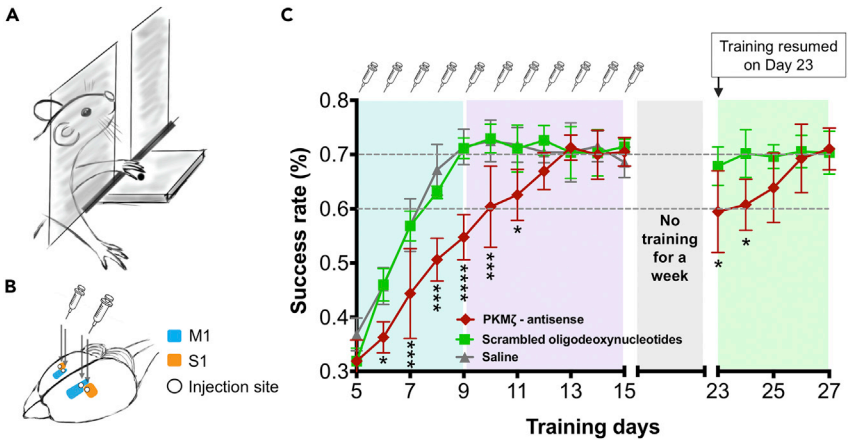

Figure 1. PKMζ-Antisense Slows Sensorimotor Learning

(A) Illustration showing rats trained to reach through a slot with their preferred forelimb and grasp a food pellet on a platform outside of the behavioral chamber.

(B) Bilateral injection sites of M1 (blue) and S1 (orange).

(C) PKMζ-antisense, scrambled oligodeoxynucleotides, and saline were injected bilaterally 30 min before the skilled reaching session every day for 15 days (1 μL/hemisphere) in each group. The learning curves for animals that received PKMζ-antisense, scrambled oligodeoxynucleotide, or saline injections were marked in red (n = 6), green (n = 4), and gray (n = 5) (mean ± SD). Two-way ANOVA showed significant effect between injection groups on learning ($F_{(2, 12)} = 32.95$; $p < 0.0001$). *Post hoc* Tukey's tests for multiple comparisons showed significant lower success rate for the antisense group compared with scrambled oligodeoxynucleotide group (antisense vs. scrambled) or saline group (antisense vs. saline). The p value for anti vs. scrambled: day 6 (*p = 0.0105), day 7 (***p = 0.0006), day 8 (***p = 0.0007), day 9 (****p < 0.0001), day 10 (***p = 0.0006), and day 11 (*p = 0.0278); the p value for antisense vs. saline: day 6 (p = 0.0072), day 7 (p = 0.0002), day 8 (p < 0.0001), day 9 (p < 0.0001), day 10 (p = 0.0004), and day 11 (p = 0.0092). The asterisks in the figure represent the p value of antisense vs. scrambled. The same groups of rats that had received daily PKMζ-antisense or scrambled oligodeoxynucleotide injection in S1/M1 from day 1 to 15 were tested again on days 23–27 after a 1-week period of no training. Student's t-test showed significant lower success rates of rats with PKMζ-antisense compared with those with scrambled oligodeoxynucleotide injections on day 23 (*p= 0.0313) and day 24 (*p = 0.0183). (See Figure S3A for training from days 1 to 5.)

on days 23 and 24 (Figure 1). We then resumed daily training and found that the group previously injected with antisense relearned the task and reached the asymptotic success rate again on day 26. These results indicate that new PKMζ synthesis during learning is required specifically for the long-term storage of sensorimotor memory that is maintained without daily reinforcement.

ZIP Specifically Disrupts the Storage of Sensorimotor Memory Maintained without Reinforcement

To test this hypothesis further, we examined the effect of the aPKC inhibitory peptide ZIP on sensorimotor memory maintenance after the skill was fully mastered and maintained in two protocols: one maintained with daily training and the other maintained without reinforcement for 1 week.

We first examined if long-term memory sustained by daily practice is disrupted by injections of ZIP. Rats were trained for 9 days to reach maximum proficiency in the skilled reaching task. One day after the last training session, ZIP, scrambled ZIP, or saline was injected bilaterally in S1/M1 (Figures 2A and S2 and Transparent Methods). Thirty minutes after injection, reaching proficiency was tested. The success rate of rats with ZIP injection was not significantly different from those with scrambled ZIP or saline injections (Figure 2A). The training was continued for the next 2 days and no difference was found. To further assess whether memories held by daily practice and reinforcement were not affected by ZIP, a second injection was given on day 13 in the same rats, and 1 day later the training resumed from days 14–16. Again, ZIP had no effect on the task performance (Figure 2A).

A second set of rats were trained to asymptotic performance and after 1 week of no training were divided into three groups that received ZIP, scrambled ZIP, or saline injection bilaterally in S1/M1 (Figures 2B and S2 and Transparent Methods). Rats intracortically injected with scrambled ZIP or saline controls showed no

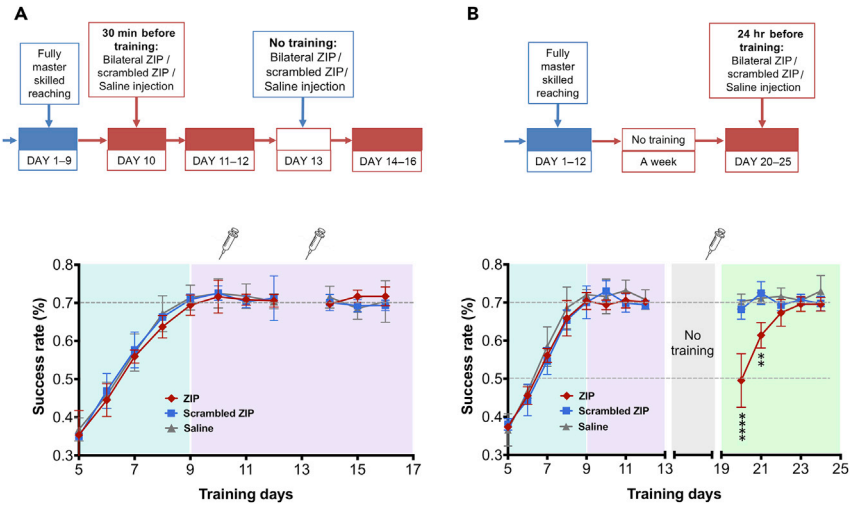

Figure 2. ZIP Specifically Disrupts the Storage of Unreinforced Skilled Motor Memory

(A) Motor memory sustained by practice is PKMζ-independent. Rats were trained for 9 days until skilled reaching performance plateaued at ~70% success rate. On day 10, the animals then were randomly divided into three groups that received ZIP, scrambled ZIP, or saline injections bilaterally 30 min before the skilled reaching task. The injection dosage of ZIP/scrambled ZIP was 1 μL per site (2 μL total in each hemisphere). Daily training was continued for days 11 and 12. On day 13, the same injections were made in the same rats as day 10 but without training after injection. Daily training was resumed from days 14 to 16. The learning curves for rats that received ZIP, scrambled ZIP, or saline injections are marked in red (n = 3), blue (n = 3), and gray (n = 5), respectively (mean ± SD). Two-way ANOVA showed no significant difference in the learning rate among the groups ($F_{(2,8)}$ = 0.6555, p = 0.5450). (See Figure S3B for training from days 1 to 5.)

(B) Motor memory sustained without practice is PKMζ-dependent. Rats were trained daily for 12 days followed by 1 week of regular housing. The animals then received ZIP, scrambled ZIP, or saline injections bilaterally on day 19 (2 μL/hemisphere). On day 20, daily training resumed. The learning curves for rats that received ZIP, scrambled ZIP, or saline injections are marked in red (n = 5), blue (n = 5), and gray (n = 5), respectively (mean ± SD). Two-way ANOVA ($F_{(2,8)}$ = 6.927, p = 0.0180), followed by *post hoc* Tukey's comparisons, showed a significantly lower success rate of ZIP on days 20 and 21 compared with scrambled ZIP (****p < 0.0001 and **p = 0.0024) or saline groups (p < 0.0001 and p = 0.0086). The asterisks in the figure represented the p value of ZIP vs. scrambled ZIP. (See Figure S3C for training from days 1 to 5.)

loss of proficiency in the reaching task when tested again on day 20 (1 day after injection). But in striking contrast to its lack of effect on memory sustained by reinforcement, the ZIP injection disrupted long-term motor memory that was maintained without reinforcement (Figure 2B). On resuming daily training from days 20 to 25, a relearning curve revealed that the group previously injected with ZIP acquired motor memory with a proficiency indistinguishable from the original learning curve, indicating no savings of motor memory after memory erasure by ZIP.

Sensorimotor Training Induces a Persistent Increase of PKMζ in Sensorimotor Cortex

To localize the persistently increased PKMζ that stores unreinforced long-term motor memory, we next measured the changes of PKMζ expression throughout S1 and M1 forelimb regions during each learning phase. Confocal microscopy revealed PKMζ in the sensorimotor cortex is compartmentalized in small puncta (Figure 3), similar to its distribution in the hippocampus (Hernández et al., 2014). The puncta number and size were quantified in each hemisphere, and the interhemispheric ratios (trained/untrained hemispheres) were used to compare the amounts of PKMζ in each group with those in controls (naive rats) (Figures 4 and S5 and Transparent Methods).

The results revealed that learning-induced changes in PKMζ, as well as the persistence of these changes during memory storage, are selective to distinct cortical layers. One-way ANOVAs showed significant changes of PKMζ puncta numbers in both S1 and M1 layers II/III and V. In the initial skill acquisition phase, which is not affected by PKMζ-antisense or ZIP, the interhemispheric ratios of PKMζ puncta number decreased significantly compared with control in S1 layer II/III (Figure 4C). In contrast, during the performance improvement phase, which is disrupted by these PKMζ antagonists, PKMζ increased in multiple

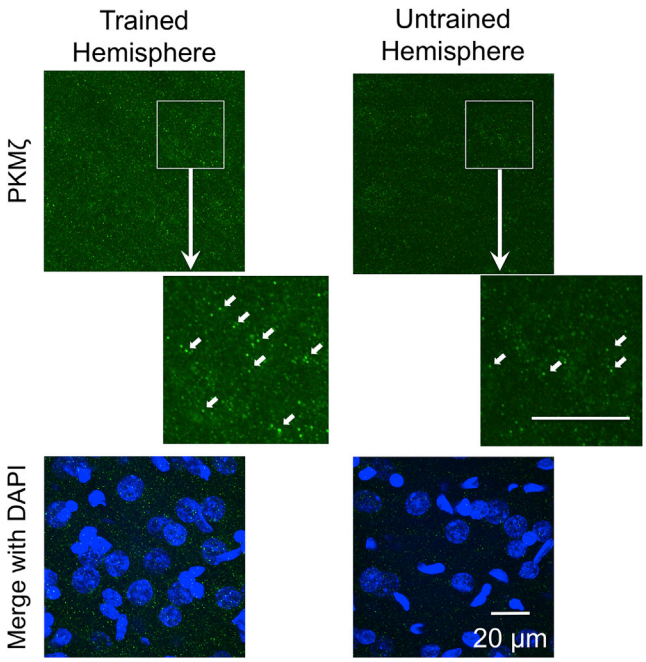

Figure 3. PKMζ Immunostaining of Sensorimotor Cortex

Immunostaining of PKMζ detected as small puncta (white arrows). Green, PKMζ staining; blue, DAPI staining; scale bar, 20 μm. Sample images were from M1 layer II/III of a rat after 9 days of training (magnification 120x).

cortical layers. After 6 and 9 days of training, PKMζ puncta numbers increased in S1 layer II/III, M1 layer II/III, and M1 layer V, and, after 9 days of training, in S1 layer V (Figures 4C and 4D). The increases reached a maximum on day 9 when memory expression reached asymptotic levels of performance. During the proficiency maintenance phase, when the animals continued daily training, the increased amounts of PKMζ in S1 layer II/III, M1 layer II/III, and M1 layer V were maintained, whereas those in S1 layer V returned to basal levels (Figures 4C and 4D).

During the long-term memory storage phase, i.e., after 40 days without training, the increases in PKMζ persisted specifically in M1 layer V (Figure 4D). As a control, we examined in all S1/M1 cortical layers a second protein, postsynaptic density protein-95 (PSD-95), which is associated with learning-induced structural changes in the sensorimotor cortex. In contrast to PKMζ, PSD-95 showed a transient increase at the end of the performance improvement training phase, which did not persist into the maintenance or storage phases (Figure S6). These results demonstrate that skilled motor training induces selective increases in PKMζ that can be either transient or highly stable at long timescales within specific layers of the sensorimotor cortex.

DISCUSSION

Here we show that persistent increases in PKMζ maintain the long-term storage of skilled motor memory that is sustained without continual daily reinforcement. The locus of this persistent molecular storage mechanism during unrehearsed, long-term motor memory is the output cortical layer of M1.

Both PKMζ-specific antisense oligodeoxynucleotides and the aPKC-selective kinase inhibitor ZIP disrupted the maintenance of long-term skilled motor memory that is maintained without practice but not skilled motor memory that is reinforced daily. These results are in line with earlier findings with ZIP on conditioned taste aversion (CTA), in which the inhibitor erased the stored CTA memory but had no effect on CTA memories that were recently reconsolidated by re-exposure to the conditioning stimulus (Levitan et al., 2016). ZIP, however, cannot exclude the possibility of an additional role for PKCι/λ (Tsokas et al., 2016). Because both PKMζ-antisense and ZIP specifically disrupt memory maintained without reinforcement (Figures 1C and 2B), our results indicate that another mechanism of memory can maintain the expression of enhanced motor performance for ~2 days during continual daily practice/reinforcement

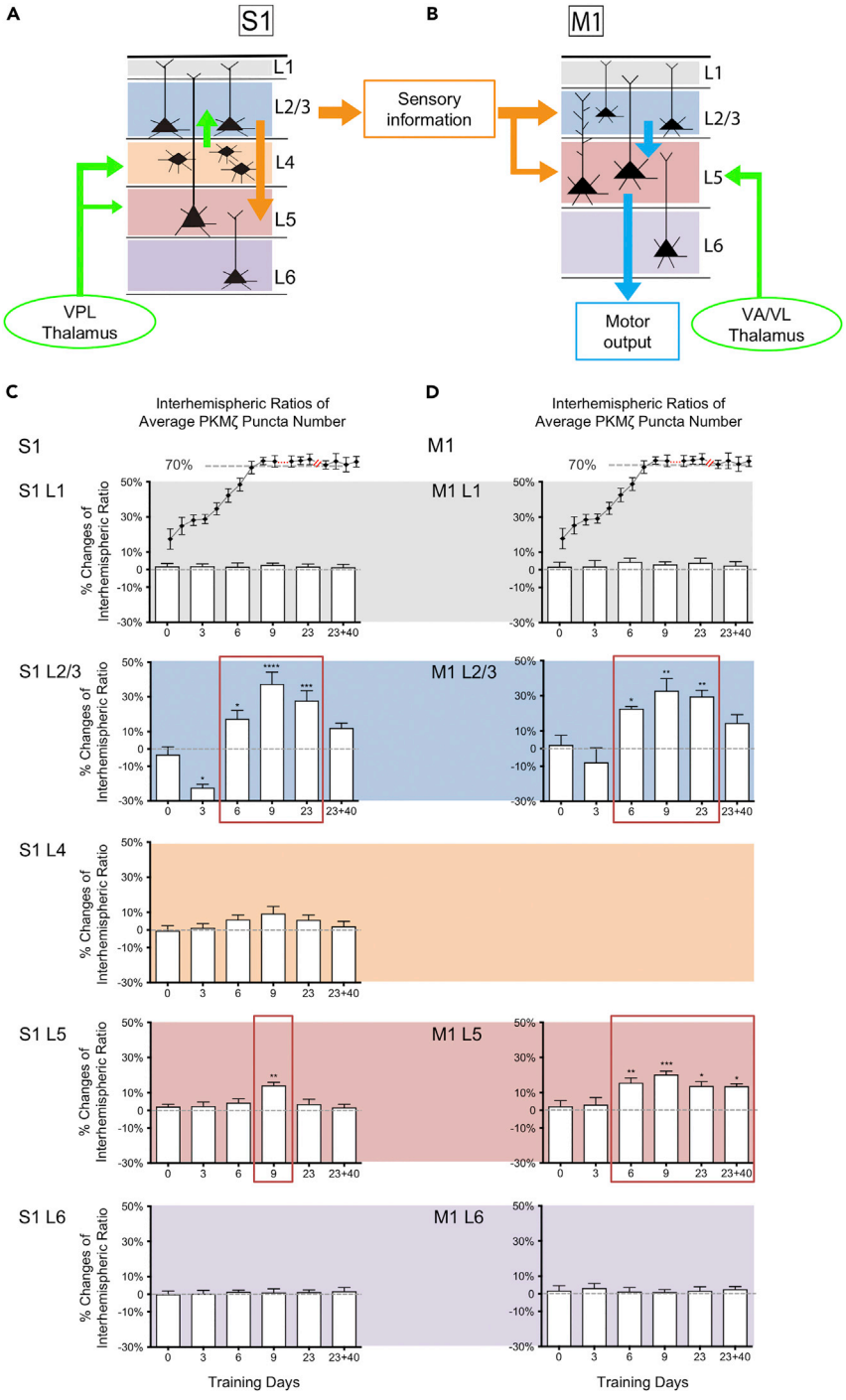

Figure 4. Spatiotemporal Changes of PKMζ Puncta Number during Sensorimotor Learning and Memory

(A and B) Illustration of information propagation in S1 and M1 during sensorimotor learning.

(C and D) Layer-specific changes of PKMζ puncta number ratios in S1 and M1. For clarity, the learning curves of the skilled reaching task for the animals (same as Figure S1A) are shown as inserts. X axis, days of training; Y axis, % changes of interhemispheric ratio of average PKMζ puncta number (mean ±SEM). One-way ANOVAs showed significant changes in S1 layer II/III ($F_{(5,26)} = 20.55$; $p < 0.0001$), S1 layer V ($F_{(5,26)} = 5.079$; $p = 0.0022$), M1 layer II/III ($F_{(5,26)} = 8.764$; $p < 0.0001$), and M1 layer V ($F_{(5,26)} = 6.857$; $p = 0.0003$). Dunnett's multiple comparison tests showed differences between each training

Figure 4. *Continued*
group and control (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). In contrast to puncta number, the interhemispheric ratios of the average PKMζ puncta size did not change significantly (see Figure S5). Different from PKMζ, PSD-95 only showed a transient increase at the end of the performance improvement phase (See Figure S6). The training paradigm and sample size are shown in Figure S4.

(Figure 2A). Such mechanisms might include the less persistent action of other PKC isoforms or other kinases such as CaMKII.

The changes of PKMζ in the forelimb contralateral S1 layer II/III are biphasic. During early skill acquisition, PKMζ is initially downregulated in S1 layer II/III (Figure 4C). This downregulation may represent a weakening of the sensory map or sensorimotor associations during the initial sensorimotor training. Downregulation of PKMζ is associated with long-term depression (Hrabetova and Sacktor, 1996, 2001), and, therefore, the initial decrease of PKMζ in S1 layer II/III might represent an LTD-like process involving a weakening of pre-existing neuronal networks. Because rats were still actively exploring the training chamber and food pellet, each reaching attempt at this phase could induce new sensory stimuli patterns, and the comparatively low success rate might act as negative feedback to disrupt previously acquired sensorimotor associations. In contrast to the rapid acquisition of the stable motor engram in the performance improvement phase, this initial phase of motor learning is unaffected by PKMζ antagonists. Notably, there is a pause in performance improvement at day 4 that appears to separate this initial skill acquisition phase and the performance improvement phase (Figure S1) (Monfils and Teskey, 2004; von Kraus et al., 2010; Wang et al., 2011).

After initially downregulating in the skill acquisition phase, the amount of PKMζ rebounds to increase above baseline during the performance improvement phase that begins on day 5. These increases are selective to S1/M1 layers II/III and V (Figures 4C, 4D, and S5). The timing of the increase of PKMζ parallels that of the LTP-like potentiation of synaptic transmission observed in motor cortex after skill learning (Monfils and Teskey, 2004; Rioult-Pedotti et al., 2000). PKMζ-antisense specifically delays the acquisition of this performance improvement phase, in line with the critical role of PKMζ in maintaining LTP.

The persistence of these learning-induced increases in PKMζ is layer-specific, revealing the location of the molecular mechanism storing very long-term skilled motor memory. Even after 40 days without reinforcement, the increases of PKMζ in layer V of motor cortex are stable. In contrast, the initial PKMζ increases observed at the end of the performance improvement phase in the sensory cortex and layers II/III of the motor cortex return toward baseline. These results indicate that the persistent molecular mechanism specifically associated with stable skilled motor memory is within the cellular output circuitry of the primary motor cortex. The location of the stable PKMζ increases in layer V of M1 is in line with work showing that this rodent reaching task induces synaptic plasticity of thalamocortical pathways that target cortical layer V neurons, which then project to C8 and distal forepaw muscles used in learned grasping (Biane et al., 2016). Further research will be required to localize within M1 layer V neurons the PKMζ that persistently potentiates synaptic transmission and thus stores long-term procedural memory.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods and six figures and can be found with this article online at https://doi.org/10.1016/j.isci.2018.07.002.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.T.F. and T.C.S.; Methodology, P.P.G. and J.T.F.; Investigation, P.P.G.; Writing, P.P.G., J.T.F., J.H.G., and T.C.S.; Funding Acquisition, J.T.F., J.H.G., and T.C.S.; Supervision, J.T.F., J.H.G., and T.C.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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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.

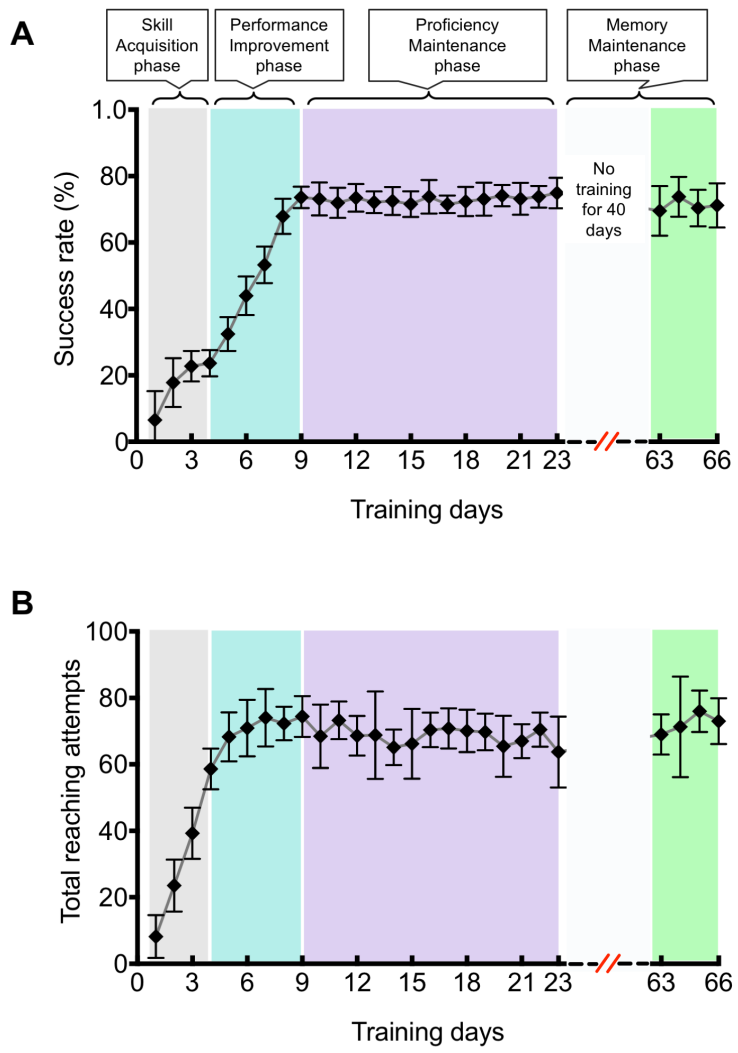

Figure S1. Daily success rate and total reaching attempts in the skilled reaching task.

(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.

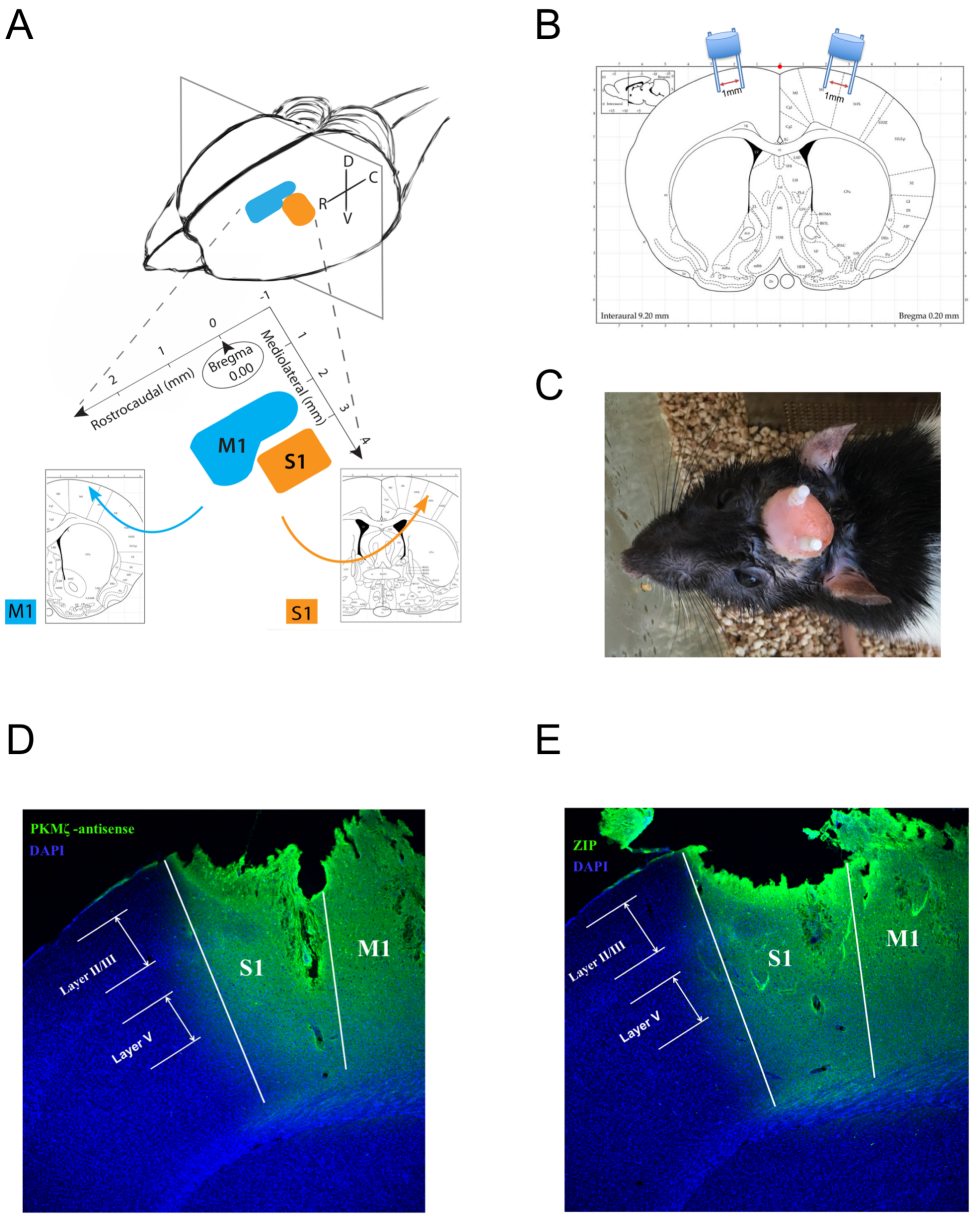

Figure S2. Fluorescence labeling of biotinylated PKMζ-antisense and biotinylated ZIP injections.

(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.

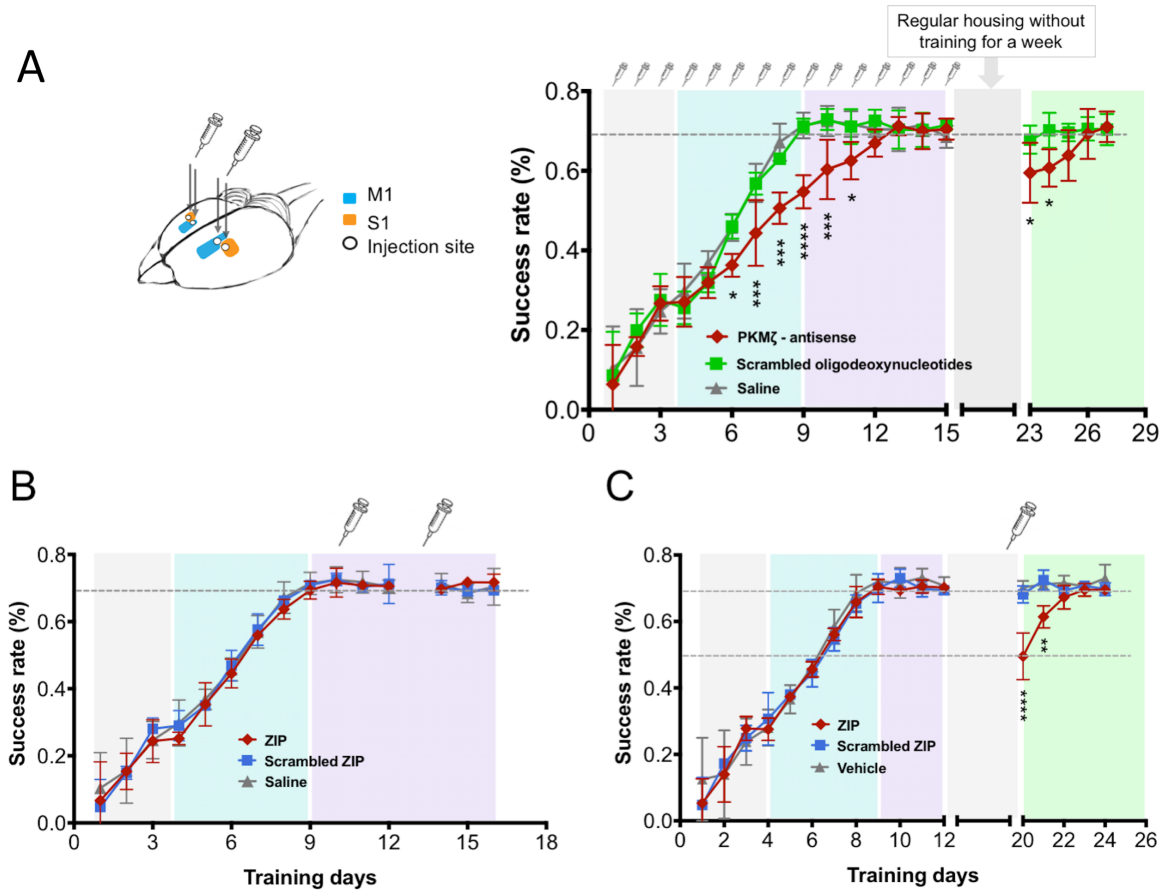

Figure S3. Perturbing PKMζ synthesis and aPKC activity during motor learning and memory maintenance.

(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.

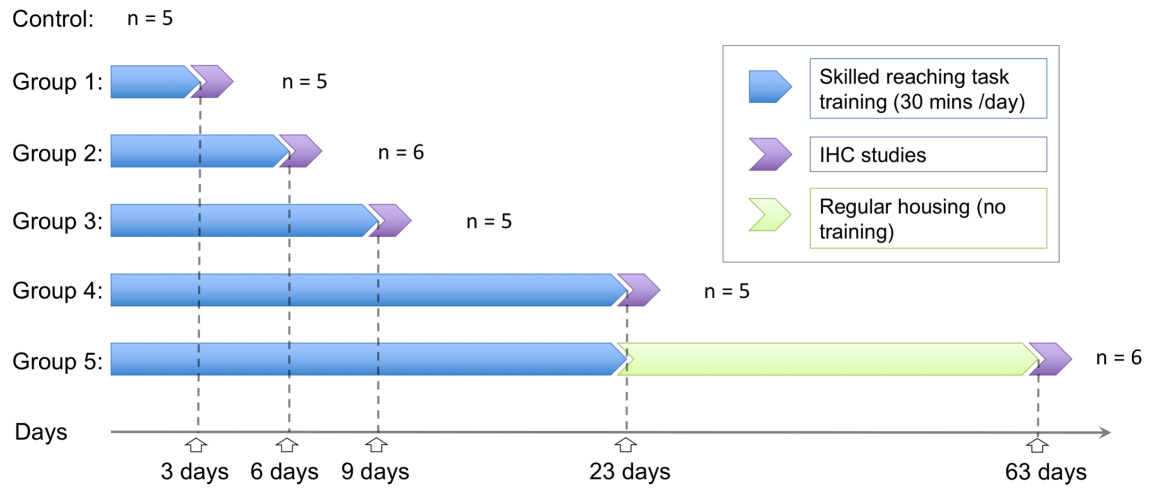

Figure S4. Experimental paradigm of PKMζ immunostaining.

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.

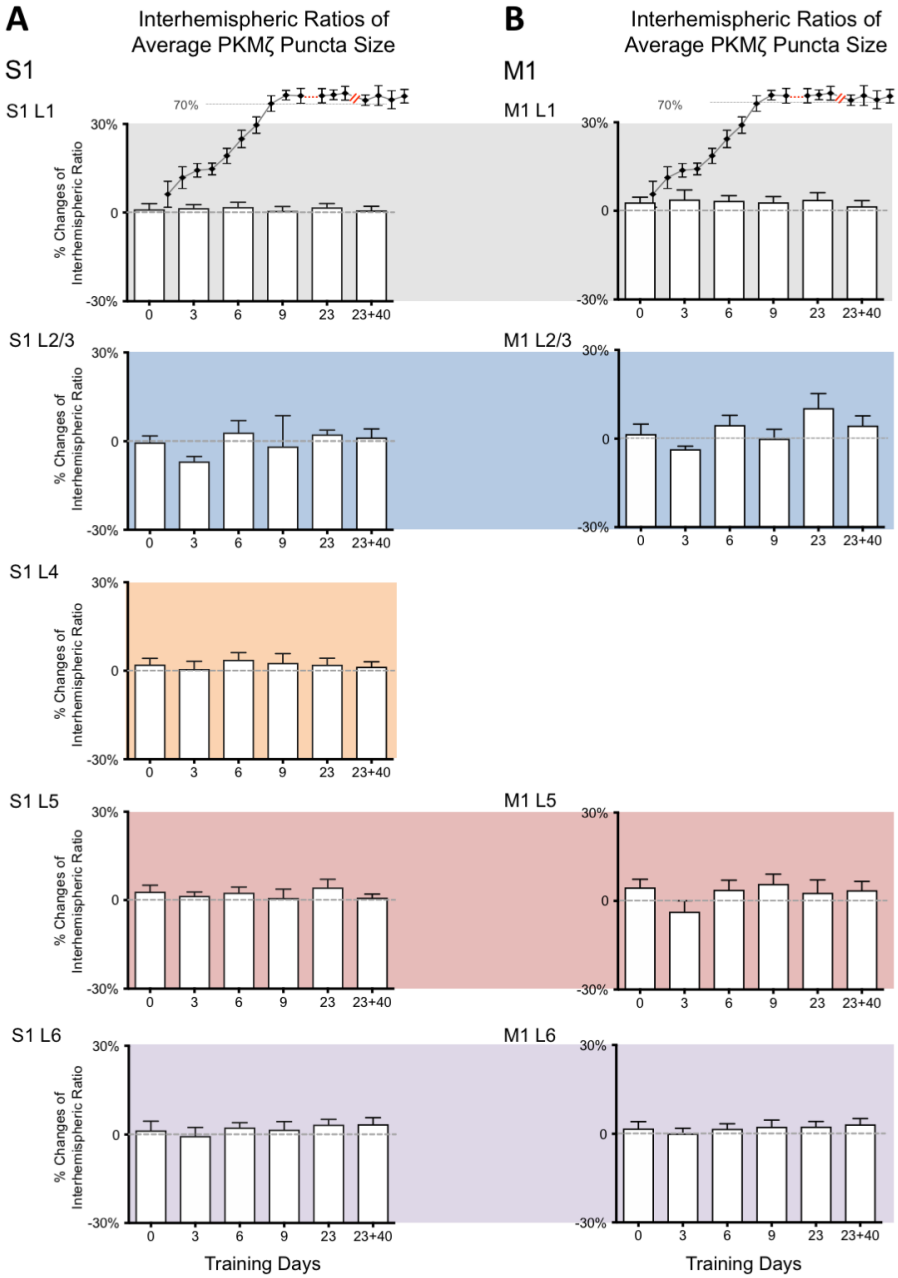

Figure S5. PKMζ puncta size does not change during sensorimotor learning and memory.

(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.

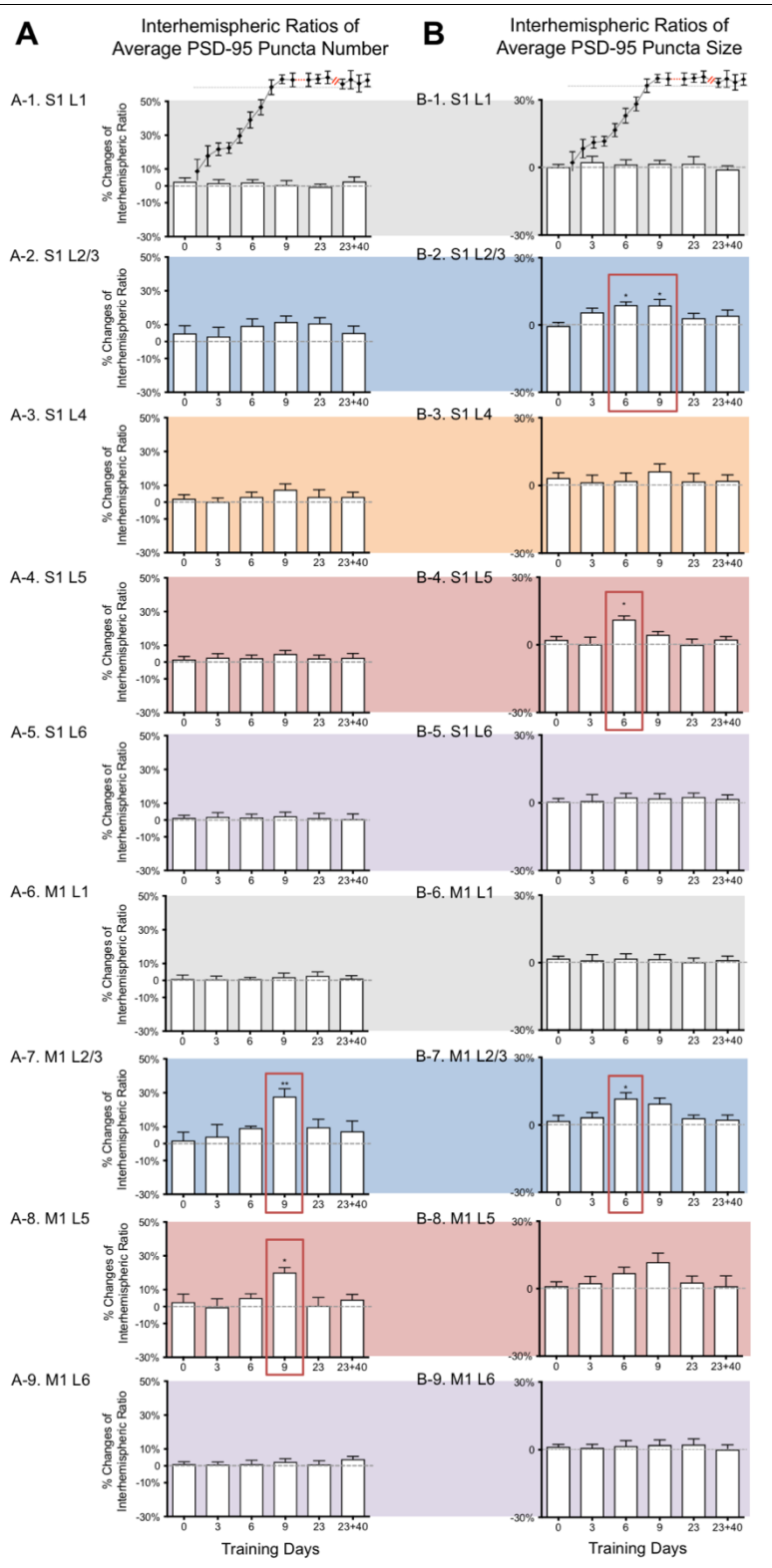

Figure S6. Spatiotemporal changes of PSD-95 puncta number and size during motor learning and memory.

(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

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