Title:

Tracking and Perturbing PKMζ in Sensorimotor Cortex During Acquisition and Storage of Procedural Memory

Authors

Peng P. Gao[1], Jeffrey H. Goodman[124], Todd C. Sacktor[134], Joseph T. Francis[15*]

[1] Department of Physiology and Pharmacology, The Robert F Furchgott Center for Neural and Behavioral Science, State University of New York Downstate Medical Center, Brooklyn, NY11203, United States;
[2] Department of Developmental Neurobiology, New York State Institute for Basic Research, Staten Island, New York 10314, United States;
[3] Department of Anesthesiology, University of New York Downstate Medical Center, Brooklyn, NY11203, United States;
[4] Department of Neurology, University of New York Downstate Medical Center, Brooklyn, NY11203, United States;
[5] Department of Biomedical Engineering, University of Houston, Houston, TX77204, United States;
* Correspondence: joey199us@gmail.com

Summary

Procedural learning and memories, such as those associated with learning to ride a bike, are thought to be supported by reorganization and plasticity of the sensorimotor cortex (S1/M1). Several studies have shown that procedural learning is accompanied by enhanced synaptic strength and structural modification in the primary motor cortex (M1) at distinct layers (e.g. layers II/III and V). However, changes linking synaptic molecular machinery within cortical layers and procedural learning and memory have been elusive. This study aims to fill this gap by tracking layer specific changes in a key molecule, the atypical PKC (aPKC) isoform PKMζ, that has been shown necessary and sufficient for the maintenance of long term potentiation, in S1 and M1. In addition, we correlate change in PKMζ with changes in task performance. The results show that PKMζ decreases in S1 Layers II/III during an early pause in performance gains after 3 days of training. Subsequently, PKMζ increases in S1/M1 layers II/III and V as performance improves to an asymptote on day 9, and, after training ends, the increase persists for more than 1 month in M1 layer V. Perturbing PKMζ synthesis in S1/M1 during and after learning slows the formation of the skilled motor memory, and such memories cannot be maintained without continual daily practice. Blocking aPKC activity has no effect on learned motor performance that is practiced daily, but specifically erases long-term motor memories that are maintained without reinforcement. Thus, PKMζ sustains the engram for skilled motor memories that are maintained without practice in M1 layer V.

Keywords

Procedural memory; sensorimotor cortex; PKMζ; synaptic plasticity; layer II/III; layer V; motor learning; skilled reaching task.

Introduction

Motor learning is characterized by a slow improvement of the smoothness and accuracy of skilled movements, which, once established, can then be maintained for a long time without further practice (Dayan and Cohen, 2011). A skilled reaching 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). In particular, Rioult-Pedotti and colleagues found the synaptic efficacy of horizontal connections in primary motor cortex (M1) layer II/III increased significantly, after 5 days of training, on the contralateral hemisphere to the preferred forelimb (Rioult-Pedotti et al., 1998), which lead to an LTP-like effect on synaptic transmission (Monfils and Teskey, 2004; Rioult-Pedotti et al., 2000). Although both rapid spine formation and elimination were seen earlier during learning (Xu et al., 2009), these *de novo* spines were preferentially stabilized and likely became synapses at a later phase for long-term memory storage (Kleim et al., 1996). The molecular mechanisms in sensorimotor cortex that encode and maintain motor memories remain unknown.

Persistent activity by the atypical PKC (aPKC) isoform PKMζ, has been shown to be both necessary and sufficient for the maintenance of 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, changes which have been extensively observed in sensorimotor cortex after sensorimotor learning (Chen et al., 2014; Yu and Zuo, 2011). Inhibition of persistent atypical PKC 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 long-term associative conditioned taste aversion memory (Shema et al., 2007). Studies from our own group demonstrate that intracortical injection of ZIP into sensorimotor cortex disrupts the maintenance of learned skilled reaching (von Kraus et al., 2010), without affecting subsequent relearning of the skill, suggesting that persistent increases in PKMζ may be necessary for the maintenance of sensorimotor memory.

In this study, we characterize the molecular dynamics of PKMζ in all layers of primary somatosensory (S1) and motor (M1) cortex during the acquisition and maintenance of the memory for a skilled reaching task. The results reveal that during motor skill acquisition, PKMζ levels initially decrease in S1 layer II/III during an early pause in learning on day 3 and then increase in S1/M1 layer II/III and V as performance reaches an asymptotic stable level by day 9. Without further training, the increased level of PKMζ in M1 layer V persists for at least 40 days, paralleling the memory of the motor skill. Blocking *de novo* PKMζ synthesis with antisense during learning slows the performance improvement rate and impairs the ability to maintain memory without continual practice. Likewise, the aPKC inhibitor ZIP specifically disrupts long-term memories that are maintained without practice. Thus, the PKMζ molecular engram for the storage of long-term skilled motor memories is in M1 layer V.

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 (Figure 1A). 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, in a 30-minute training session, was used to evaluate task performance on a daily basis (Figure 1B). A successful reach includes: 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).

We observed that the acquisition of skilled motor memory could be divided into four sequential phases (as shown in Figure 1B and Figure S 1), consistent with the model described by Monfils and Teskey (Monfils and Teskey, 2004): 1) a skill acquisition phase (Days 1-4), when the total reaching attempts increased rapidly but success rate was comparatively low (<30%); 2) a performance improvement phase (Days 5-9), when the success rate increased rapidly until plateauing (~70%) and the total reaching attempts remained stable; 3) the proficiency maintenance phase, when both the performance and reaching attempts remained stable; and 4) a long-term memory storage phase, consisting of a 40-day no training gap, during which performance did not diminish, as tested on days 63-66. Days 23-66 were combined as the long-term memory maintenance phase.

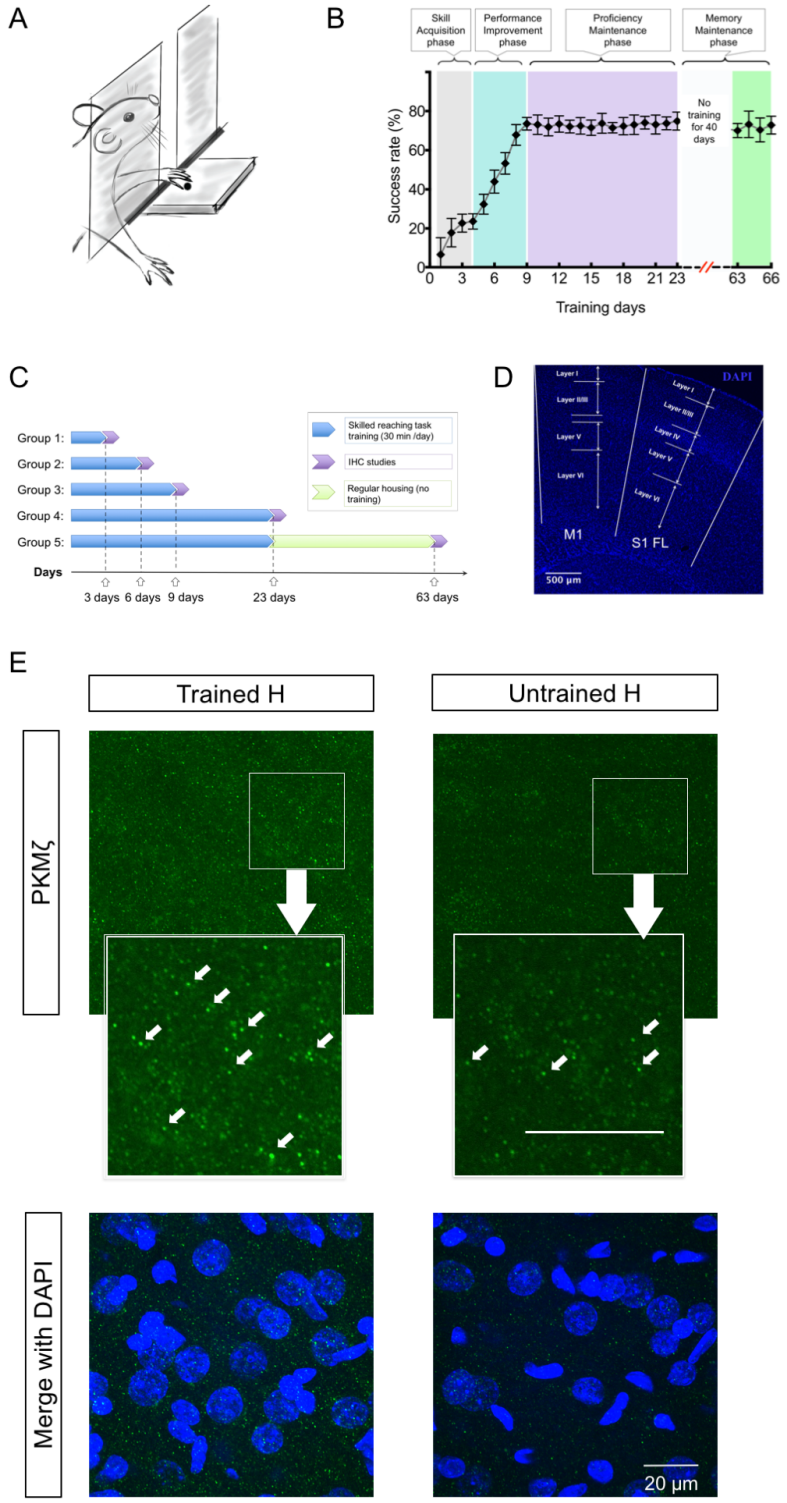

Figure 1. Skilled reaching task and PKMζ immunostaining of sensorimotor cortex.

A: Rats were trained to reach through the slot with their preferred forelimb and grasp the food pellet on the platform outside of the behavioral chamber. B: Learning Curve. X-axis – training days; Y-axis – success rate (%): successful reaches divided by the total reaching attempts (Means ± SEM); C: Experimental Paradigm. 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 methods); Green arrow: regular housing without training. The expression of proteins was tested at the following time points of motor skill learning: 1) the skill acquisition phase (group 1, n = 5 rats); 2) the performance improvement phase (group 2, n = 6 rats); 3) the transition from performance improvement to maintenance (group 3, n = 5 rats); 4) the proficiency maintenance phase (group 4, n = 5); 5) the long-term memory storage phase (group 5, n = 6 rats). D: DAPI staining was used to identify different layers of motor cortex (M1) and forelimb somatosensory cortex (S1 FL). Scale bar, 500 μm. E: Immunostaining of PKMζ could be detected as small puncta (indicated by the white arrows). Scale bar, 20 μm. Example images were from M1 layer III/III of a rat with 9 days of training (magnification 120x).

Sensorimotor training induces an initial decrease followed by a persistent increase of PKMζ in sensorimotor cortex that is maintained during long-term memory storage

PKMζ was measured in all layers of S1 and M1 forelimb regions during each phase, i.e., 1 day after the last training session with 3, 6, 9 and 23 days of training, and 40 days after the last training session for another group of rats that had received 23 days of training (Figure 1C). Confocal microscopy revealed PKMζ in sensorimotor cortex is compartmentalized in small puncta (Figure 1E), similar to its distribution in 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 levels of PKMζ in each group with controls (naïve rats) (Figure 2 and Figure S 2).

The learning-induced changes in PKMζ were selective to distinct cortical layers and specific phases of memory. One-way ANOVA showed significant changes of PKMζ puncta numbers in both S1 and M1 layers II/III and V. During the initial skill acquisition phase, the interhemispheric ratios of PKMζ puncta number decreased significantly compared with control in S1 layer II/III (Figure 2C).

As the initial skill acquisition transitioned into increased movement accuracy in the performance improvement phase, PKMζ increased in multiple 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 (Figure 2C, D). The increases reached a maximum on day 9 when memory expression reached asymptotic levels of performance. During the proficiency maintenance phase, while the animals continued daily training, there was a sustained higher level of PKMζ in S1 layer II/III, M1 layer II/III and M1 layer V (Figure 2C, D).

During the long-term memory storage phase, i.e., after a 40-day no training gap, the ratios of PKMζ puncta number remained high in M1 layer V (Figure 2C, D). These results suggest persistent increased PKMζ formed during the performance improvement phase in sensorimotor cortical networks might play an essential role in maintaining long-term sensorimotor memory.

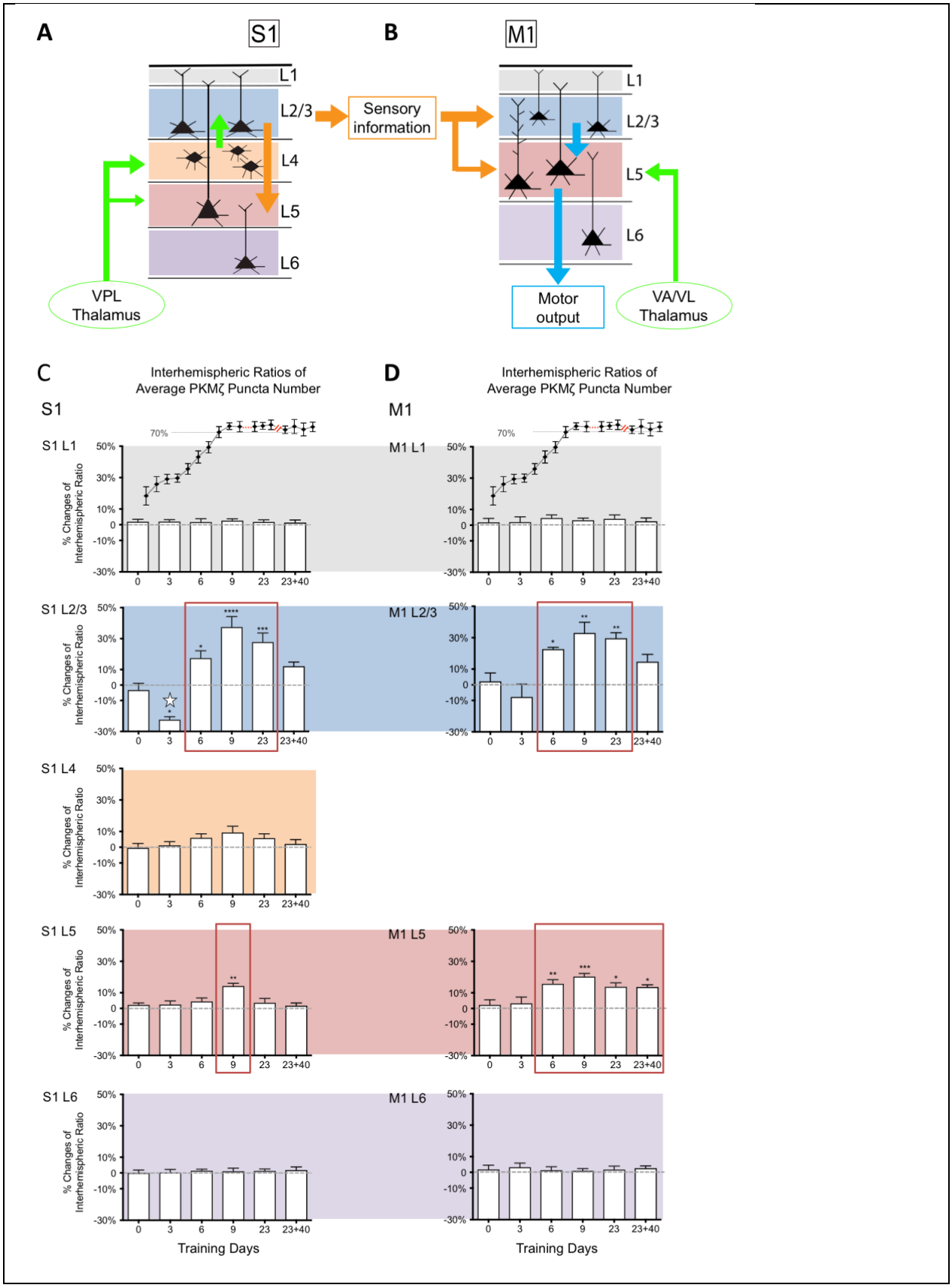
A
S1
L1
L2/3
L4
L5
L6
VPL Thalamus
Sensory information
B
M1
L1
L2/3
L5
L6
Motor output
VA/VL Thalamus
C
Interhemispheric Ratios of Average PKMζ Puncta Number
S1
S1 L1
S1 L2/3
S1 L4
S1 L5
S1 L6
% Changes of Interhemispheric Ratio
70%
50%
30%
10%
0
-10%
-30%
0
3
6
9
23
23+40
Training Days
D
Interhemispheric Ratios of Average PKMζ Puncta Number
M1
M1 L1
M1 L2/3
M1 L5
M1 L6
Training Days

Figure 2. Spatiotemporal Changes of PKMζ Puncta Number in Sensorimotor Learning.

A, B: Information propagation in S1 and M1 during sensorimotor learning. **C, D:** Layer-specific changes of PKMζ puncta number ratios in S1 and M1. X-axis - days of training; Y-axis – the % changes of interhemispheric ratio of average PKMζ puncta number (Means ± SEM). One-way ANOVA 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 test showed the difference of each training group with 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 S 2).

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 effectively blocks PKMζ synthesis and has no effect on LTP or memory formation in the absence of its specific target PKMζ mRNA (Hsieh et al., 2017; Tsokas et al., 2016). We injected PKMζ–antisense bilaterally in S1/M1 30 minutes before daily training and examined its effect on sensorimotor learning (Figure 3 and Figure S 3). Our results showed that for the first 5 days of training, learning with daily PKMζ-antisense injection (red line in Figure S 4A) was the same as the control groups with inactive scrambled oligodeoxynucleotides (blue line) or saline (grey line) injections. However, from day 6 onward, the antisense group had a significantly lower learning rate compared with other groups (Figure 3). *Post hoc* Tukey's tests after repeated two-way ANOVA showed significantly lower success rates for the antisense group compared with scrambled oligodeoxynucleotides and saline groups on days 6 – 11 (Figure 3). In the antisense group, the fast performance improvement phase was replaced with a slow learning curve extended from day 4 to day 11 (Figure 3). With continuous training, the success rate of the antisense group eventually reached the same asymptote as other groups on day 12 (Figure 3). Rats with scrambled oligodeoxynucleotides or saline injection learned the skilled reaching task as efficiently as the controls seen in Figure 1B, indicating the surgery and intracortical injections did not affect their ability in sensorimotor learning. Thus, the *de novo* synthesis of PKMζ in sensorimotor cortex is not required for the

initial skill acquisition, but necessary for the delayed rapid performance improvement during sensorimotor learning.

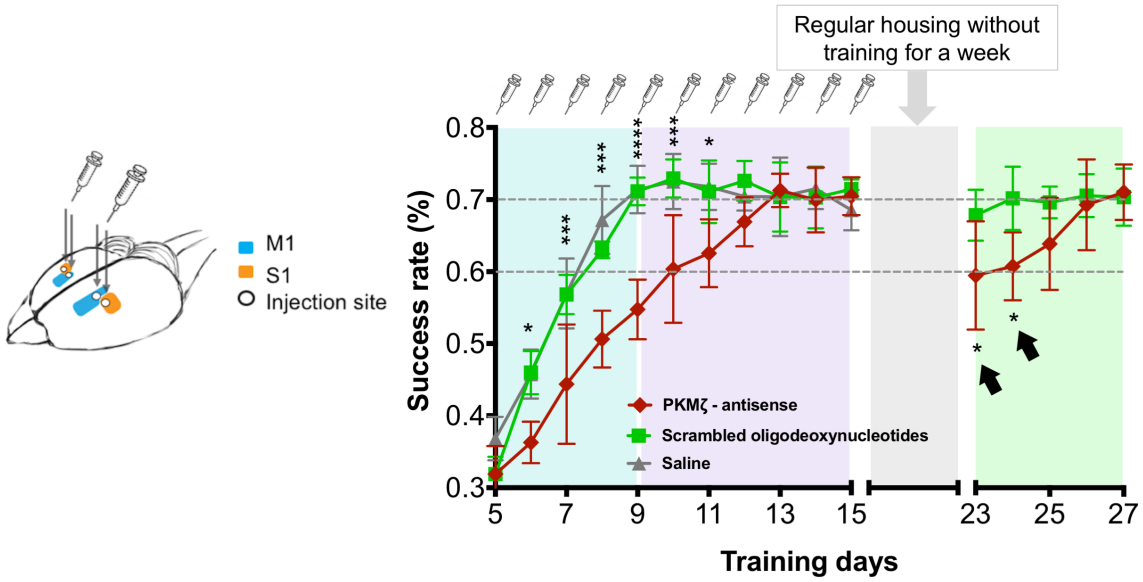

Figure 3. The effect of PKMζ-antisense on sensorimotor learning.

PKMζ-antisense, scrambled oligodeoxynucleotides, and saline was injected bilaterally 30 minutes before the skilled reaching session every day for 15 days (1 µl/ hemisphere). The learning curves for animals that received PKMζ-antisense, scrambled oligodeoxynucleotides or saline injections were marked in red (n=6), blue (n=4) and grey (n=5) (Means ± 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 oligodeoxynucleotides group and saline group on day 6 (*$p = 0.0105$, ** $p = 0.0072$), day 7 (***$p = 0.0006$, ***$p = 0.0002$), day 8 (***$p = 0.0007$, ****$p < 0.0001$), day 9 (****$p < 0.0001$, **** $p < 0.0001$), day 10 (***$p = 0.0006$, ***$p = 0.0004$) and day 11 (*$p = 0.0278$, **$p = 0.0092$). The same groups of rats that had received daily PKMζ–antisense or scrambled oligodeoxynucleotides injection in S1/M1 from day 1 to day 15, were tested again on days 23-27 after one week no training gap. Student *t-test* showed significant lower success rates of rats with PKMζ-antisense compared with scrambled oligodeoxynucleotides injections on day 23 (*$p= 0.0313$) and day 24 (*$p = 0.0183$). (See Figure S 4A for training from day 1-5).

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 the PKMζ-antisense animals eventually reached the same proficiency as controls with additional training days. We next asked how well could this memory obtained in the absence of new PKMζ synthesis be maintained without daily reinforcement. After asymptotic performance was achieved, we stopped training and 1 week later retested the rats. Whereas the control, scrambled oligodeoxynucleotides group maintained peak performance, as expected, the PKMζ–antisense group showed significantly lower performance on day 23 and 24 (Figure 3). We then resumed the daily training and found that the group previously injected with antisense relearned the task and reached the same asymptotic success rate again on day 26. These results demonstrate that PKMζ-antisense injections during learning impair the long-term maintenance of sensorimotor memory that is not reinforced by daily practice, without affecting the ability to relearn the task when training resumes.

ZIP specifically disrupts the storage of sensorimotor memory maintained without reinforcement

These results show that PKMζ-antisense prevented the formation of the long-term skilled motor memory that is maintained in the absence of reinforcement, but not the memory of the motor skill that is sustained day-to-day by practice. This suggests the hypothesis that memories maintained in the absence of daily reinforcement are sustained by persistent PKMζ activity, whereas memories maintained by daily practice are not. To test this hypothesis, we examined the effect of the aPKC inhibitory peptide ZIP on sensorimotor memory maintenance after the skill was fully mastered and maintained in two schemes: one maintained with continued daily training, the other maintained for 1 week without practice/reinforcement.

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 (Figure 4A and Figure S 3). 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 4A). The training was continued for the next two days and no further difference was found. To confirm that memories held by daily practice 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 4A).

The second set of rats were trained to asymptotic performance and after 1 week of no training were divided into 3 groups that received either ZIP, scrambled ZIP, or saline injection bilaterally in S1/M1 (Figure 4B and Figure S 3). Rats intracortically injected with scrambled ZIP or saline showed no loss of proficiency in the reaching task when tested again on day 20 (1 day after injection). In striking contrast to the animals that had been trained within 24 hours, the rats that maintained memory without practice could not perform the skilled reaching with proficiency after ZIP injection (Figure 4B). Continued daily training from day 20-25 formed a relearning curve for the ZIP group, which was similar to the skill proficiency phase of their original learning curve. Thus, in the absence of daily practice, ZIP disrupts the storage of long-term skilled motor memory.

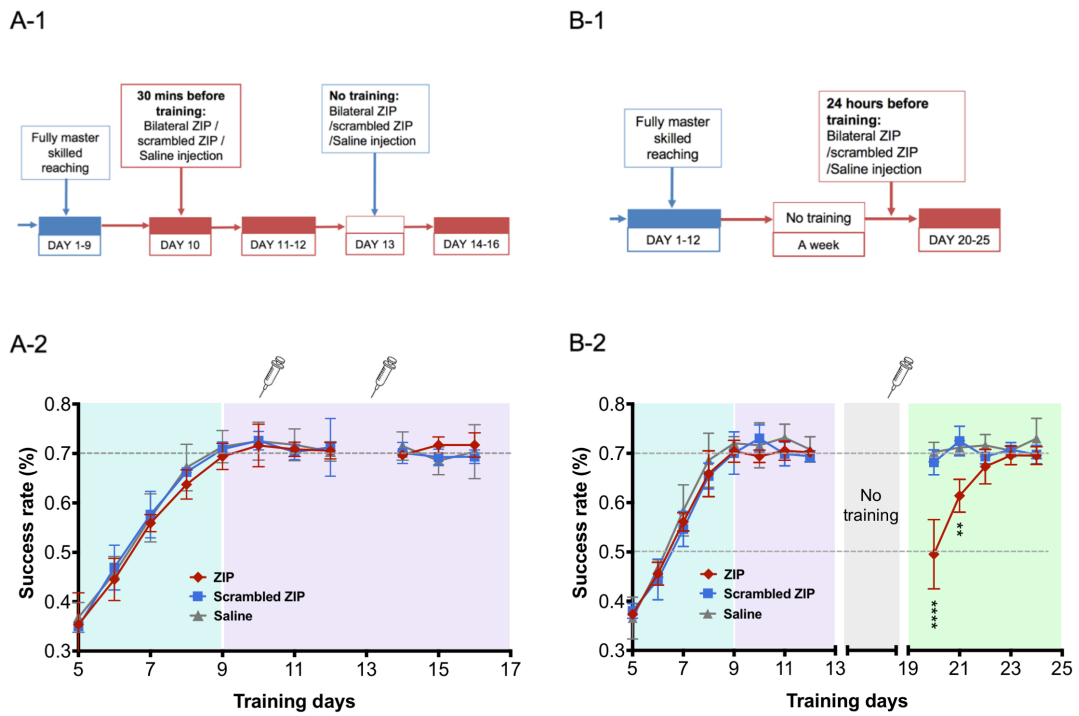

Figure 4. The effect of ZIP on motor memory maintenance.

A: Motor memory sustained by continual practice is PKMζ-independent.

A-1: Rats were trained for the skilled reaching for 9 days until the success rate reached plateau: ~70%. On day 10, the well-trained animals were randomly divided into 3 groups to receive ZIP/scrambled ZIP/ saline injection bilaterally 30 minutes before the skilled reaching task. The injection dosage of ZIP/scrambled ZIP was 1μl per site and 2 μl in total on each hemisphere. Daily training was continued for day 11 and 12. On day 13, the same injections were made to the same rats as day 10, but without training. Daily training was resumed from day 14-16 and the behaviors were recorded as the successful reaches divided by the total reaches in each session.

A-2: The learning curves for rats that received ZIP, scrambled ZIP or saline injections were marked in red (n = 3), blue (n=3) and grey (n = 5) respectively (Means ± SD). Two-way ANOVA showed no significant difference of learning rate between groups ($F_{(2,8)} = 0.6555$, $p = 0.5450$). (See Figure S 4B for training from day 1-5).

B: Motor memory sustained without practice is PKMζ-dependent.

B-1: Rats were trained daily for 12 days followed by one week regular housing, and received ZIP, scrambled ZIP, or saline injections bilaterally on day 19 (2 μl / hemisphere). Since day

20, daily training was resumed and the behaviors were recorded as the successful reaches divided by the total reaches in each session.

B-2: The learning curves for rats that received ZIP, scrambled ZIP and saline injections were marked in red (n=5), blue (n=5) and grey respectively (Means ± SD). Two-way ANOVA ($F(2,8) = 6.927$, $p = 0.0180$), followed by post hoc tukey's comparisons showed significant lower success rate of ZIP on day 20 and 21 compared with scrambled ZIP (****$p<0.0001$ and **$p=0.0024$), and saline groups (****$p<0.0001$ and **$p=0.0086$). (See Figure S 4C for training from day 1-5).

Discussion

To link dynamic changes in synaptic molecular machinery with the evolving phases of sensorimotor learning and memory, we examined changes in PKMζ in the sensorimotor cortex (Figure 2 and Figure S 2). The results reveal that the level of PKMζ initially decreases in S1 layer II/III during early skill acquisition and then increases in both S1 and M1 layer II/III and V, as skill performance improves to peak levels on Day 9. The increase in PKMζ in M1 layer V then persists through the maintenance phase of task proficiency for at least 54 additional days (40 without practice), which is the longest time point examined. The long-term increased amount of PKMζ localized to layer V motor cortex indicates that the persistent molecular changes associated with highly stable skilled motor memory are within the cellular output circuitry of primary motor cortex. This localization is in line with work showing changes to thalamocortical synaptic plasticity that target cortical layer V neurons projecting to C8 and the distal forepaw muscles used in learned grasping on this rodent reaching task (Biane et al., 2016).

The initial downregulation of PKMζ in the forelimb contralateral S1 layer II/III observed during early skill acquisition (Figure 2C) may represent a weakening of the sensory map or sensory-motor associations during the initial phase of sensorimotor learning, when there is a pause in performance improvement prior to rapid acquisition of the stable motor engram (Kraus et al., 2010; Monfils and Teskey, 2004; Wang et al., 2011). 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 and the comparatively low

success rate might act as negative feedback to disrupt previously acquired sensory-motor associations. In short, in the earliest phase of acquiring a new motor memory, the neural network could be disengaging from a local minimum that is inappropriate for the task at hand.

After the 5th day of training, the success rate of skilled reaching increases rapidly together with the interhemispheric ratio of PKMζ in S1/M1 layer II/III and V (Figure 2C, D and Figure S 2). The timing of the increase of PKMζ parallels the LTP-like potentiation of synaptic transmission observed in motor cortex after skill learning (Monfils and Teskey, 2004; Rioult-Pedotti et al., 2000). Blocking the new synthesis of PKMζ with antisense during training slows the rapid increase of skilled motor learning after day 5, but does not prevent its acquisition with extended training (Figure 3). This indicates that there is a form of motor memory that can be maintained without PKMζ that lasts at least a day, which is the interval between training episodes. But this PKMζ-independent second process cannot fully sustain the memory because when retention is examined a week after training in the absence of continual practice, motor skills acquired without new PKMζ synthesis no longer show asymptotic performance (Figure 3). Likewise, blocking aPKC activity with ZIP disrupts the long-term skilled motor memory that is maintained without practice, but has no effect on long-term memory that is sustained with continual daily practice (Figure 4). Therefore, PKMζ is crucial for sustaining skilled motor memory that is maintained without continual reinforcement, like the memory of how to ride a bicycle when one has not ridden for a while. The molecular PKMζ engram for this form of long-term motor memory is localized in layer V of primary motor cortex.

However, there are several limitations in this study, which need to be addressed in the future. First, memory consolidation, especially for motor learning, is a continuous process (Kandel et al., 2014). The time window can last for more than 24 hours and sleeping is also an important phase in memory consolidation (Luft and Buitrago, 2005). Therefore, longer temporal dynamics of molecular modifications need to be investigated to better model motor learning and memory maintenance in detail. Our antisense injection approach (Figure 3) cannot rule out the possibility that some PKMζ gets synthesized when the injected drug is cleared out from the brain. A control-released formulation of PKMζ-antisense can be tested in future experiment to ensure the total blockage of *de novo* PKMζ synthesis. In addition, our staining and ZIP study cannot separate PKMζ with PKC λ/ι. PKC λ/ι is another isoform of PKC and its persistent activation has been shown to compensate for PKMζ to maintain memory without high cognitive demand in PKMζ knock out mice (Tsokas et al., 2016). Therefore, PKC λ/ι might play a secondary role in

assisting motor learning when PKMζ antisense was injected on a daily basis in our study (Figure 3). Finally, when ZIP was injected in Figure 4, the reaching proficiency dropped significantly, but not to the pre-training level. We suspect that part of the basic reaching ability in this task becomes independent of sensorimotor cortex when the skill is fully mastered. Studies extending to multiple brain regions are necessary in the future to fully decipher the process of motor learning and memory.

Author Contributions

P.P.G. performed the experiments and analyzed the data. J.T.F., J.H.G. and T.C.S. supervised the project and provided input on experimental design. All the authors listed wrote the manuscript together.

Acknowledgments

We would like to thank Dr. Changchi Hsieh, Dr. Janina Ferbinteanu and Dr. Panayiotis Tsokas for technical advice, and thank all the members of the Francis lab for discussion and support. This study was supported by grants from DAPAR (www.darpa.mil) (Award#60806; Project#:108723) (J.T.F.) and IBR-SUNY Downstate graduate fund (J.T.F and J.H.G.).

References:

Biane, J.S., Takashima, Y., Scanziani, M., Conner, J.M., and Tuszynski, M.H. (2016). Thalamocortical Projections onto Behaviorally Relevant Neurons Exhibit Plasticity during Adult Motor Learning. Neuron *89*, 1173–1179.

Chen, S., Cai, D., Pearce, K., Sun, P.Y.-W., Roberts, A.C., and Glanzman, D.L. (2014). Author response. eLife *3*, 1–21.

Dayan, E., and Cohen, L.G. (2011). Neuroplasticity subserving motor skill learning. Neuron *72*, 443–454.

Fu, M., and Zuo, Y. (2011). Experience-dependent structural plasticity in the cortex. Trends Neurosci. *34*, 177–187.

Gámiz, F., and Gallo, M. (2011). Intra-amygdala ZIP injections impair the memory of learned active avoidance responses and attenuate conditioned taste-aversion acquisition in rats. Learn. Mem. *18*, 529–533.

Harms, K.J., Rioult-Pedotti, M.S., Carter, D.R., and Dunaevsky, A. (2008). Transient Spine Expansion and Learning-Induced Plasticity in Layer 1 Primary Motor Cortex. Journal of Neuroscience *28*, 5686–5690.

Hernández, A.I., Oxberry, W.C., Crary, J.F., Mirra, S.S., and Sacktor, T.C. (2014). Cellular and subcellular localization of PKMζ. Philos. Trans. R. Soc. Lond., B, Biol. Sci. *369*, 20130140–20130140.

Hrabetova, S., and Sacktor, T.C. (1996). Bidirectional regulation of protein kinase M zeta in the maintenance of long-term potentiation and long-term depression. Journal of Neuroscience *16*, 5324–5333.

Hrabetova, S., and Sacktor, T.C. (2001). Transient translocation of conventional protein kinase C isoforms and persistent downregulation of atypical protein kinase Mzeta in long-term depression. Brain Res. Mol. Brain Res. *95*, 146–152.

Hsieh, C., Tsokas, P., Serrano, P., Hernández, A.I., Tian, D., Cottrell, J.E., Shouval, H.Z., Fenton, A.A., and Sacktor, T.C. (2017). Persistent increased PKMζ in long-term and remote spatial memory. Neurobiology of Learning and Memory *138*, 135–144.

Kandel, E.R., Dudai, Y., and Mayford, M.R. (2014). The molecular and systems biology of memory. Cell *157*, 163–186.

Kargo, W.J. (2004). Improvements in the Signal-to-Noise Ratio of Motor Cortex Cells Distinguish Early versus Late Phases of Motor Skill Learning. Journal of Neuroscience *24*, 5560–5569.

Kleim, J. (2002). Motor Learning-Dependent Synaptogenesis Is Localized to Functionally Reorganized Motor Cortex. Neurobiology of Learning and Memory *77*, 63–77.

Kleim, J.A., Barbay, S., and Nudo, R.J. (1998). Functional reorganization of the rat motor cortex following motor skill learning. Journal of Neurophysiology *80*, 3321–3325.

Kleim, J.A., Lussnig, E., Schwarz, E.R., Comery, T.A., and Greenough, W.T. (1996). Synaptogenesis and Fos expression in the motor cortex of the adult rat after motor skill learning. J. Neurosci. *16*, 4529–4535.

Kleim, J.A., Hogg, T.M., VandenBerg, P.M., Cooper, N.R., Bruneau, R., and Remple, M. (2004). Cortical synaptogenesis and motor map reorganization occur during late, but not early, phase of motor skill learning. J. Neurosci. *24*, 628–633.

Klein, A., Sacrey, L.-A.R., Whishaw, I.Q., and Dunnett, S.B. (2012). The use of rodent skilled reaching as a translational model for investigating brain damage and disease. Neuroscience and Biobehavioral Reviews *36*, 1030–1042.

von Kraus, L.M., Sacktor, T.C., and Francis, J.T. (2010). Erasing sensorimotor memories via PKMzeta inhibition. PLoS ONE *5*, e11125.

Kwapis, J.L., Jarome, T.J., Gilmartin, M.R., and Helmstetter, F.J. (2012). Intra-amygdala infusion of the protein kinase Mzeta inhibitor ZIP disrupts foreground context fear memory. Neurobiology of Learning and Memory *98*, 148–153.

Kwapis, J.L., Jarome, T.J., Lonergan, M.E., and Helmstetter, F.J. (2009). Protein kinase Mzeta maintains fear memory in the amygdala but not in the hippocampus. Behav. Neurosci. *123*, 844–850.

Ling, D.S.F., Benardo, L.S., Serrano, P.A., Blace, N., Kelly, M.T., Crary, J.F., and Sacktor, T.C. (2002). Protein kinase Mzeta is necessary and sufficient for LTP maintenance. Nat Neurosci *5*, 295–296.

Luft, A.R., and Buitrago, M.M. (2005). Stages of motor skill learning. Mol. Neurobiol. *32*, 205–216.

Luft, A.R., Buitrago, M.M., Ringer, T., Dichgans, J., and Schulz, J.B. (2004). Motor skill learning depends on protein synthesis in motor cortex after training. J. Neurosci. *24*, 6515–6520.

Migues, P.V., Hardt, O., Wu, D.C., Gamache, K., Sacktor, T.C., Wang, Y.-T., and Nader, K. (2010). PKMzeta maintains memories by regulating GluR2-dependent AMPA receptor trafficking. Nat Neurosci *13*, 630–634.

Monfils, M.H., and Teskey, G.C. (2004). Skilled-learning-induced potentiation in rat sensorimotor cortex: a transient form of behavioural long-term potentiation. Neuroscience *125*, 329–336.

Osten, P., Valsamis, L., Harris, A., and Sacktor, T.C. (1996). Protein synthesis-dependent formation of protein kinase Mzeta in long-term potentiation. Journal of Neuroscience *16*, 2444–2451.

Pastalkova, E., Serrano, P., Pinkhasova, D., Wallace, E., Fenton, A.A., and Sacktor, T.C. (2006). Storage of spatial information by the maintenance mechanism of LTP. Science *313*, 1141–1144.

Pauli, W.M., Clark, A.D., Guenther, H.J., O'Reilly, R.C., and Rudy, J.W. (2012). Inhibiting PKMζ reveals dorsal lateral and dorsal medial striatum store the different memories needed to support

adaptive behavior. Learn. Mem. *19*, 307–314.

Rioult-Pedotti, M.S., Donoghue, J.P., and Dunaevsky, A. (2007). Plasticity of the Synaptic Modification Range. Journal of Neurophysiology *98*, 3688–3695.

Rioult-Pedotti, M.S., Friedman, D., and Donoghue, J.P. (2000). Learning-induced LTP in neocortex. Science *290*, 533–536.

Rioult-Pedotti, M.S., Friedman, D., Hess, G., and Donoghue, J.P. (1998). Strengthening of horizontal cortical connections following skill learning. Nat Neurosci *1*, 230–234.

Sacktor, T.C., Osten, P., Valsamis, H., Jiang, X., Naik, M.U., and Sublette, E. (1993). Persistent activation of the zeta isoform of protein kinase C in the maintenance of long-term potentiation. Proc. Natl. Acad. Sci. U.S.a. *90*, 8342–8346.

Sacktor, T.C. (2012). Memory maintenance by PKMζ--an evolutionary perspective. Mol Brain *5*, 31.

Sanes, J.N., and Donoghue, J.P. (2000). Plasticity and primary motor cortex. Annu. Rev. Neurosci. *23*, 393–415.

Serrano, P., Friedman, E.L., Kenney, J., Taubenfeld, S.M., Zimmerman, J.M., Hanna, J., Alberini, C., Kelley, A.E., Maren, S., Rudy, J.W., et al. (2008). PKMzeta maintains spatial, instrumental, and classically conditioned long-term memories. Plos Biol *6*, 2698–2706.

Shema, R., Sacktor, T.C., and Dudai, Y. (2007). Rapid erasure of long-term memory associations in the cortex by an inhibitor of PKM zeta. Science *317*, 951–953.

Tsokas, P., Hsieh, C., Yao, Y., Lesburguères, E., Wallace, E.J.C., Tcherepanov, A., Jothianandan, D., Hartley, B.R., Pan, L., Rivard, B., et al. (2016). Compensation for PKMζ in long-term potentiation and spatial long-term memory in mutant mice. eLife *5*, 12677.

Wang, L., Conner, J.M., Rickert, J., and Tuszynski, M.H. (2011). Structural plasticity within highly specific neuronal populations identifies a unique parcellation of motor learning in the adult brain. Proc. Natl. Acad. Sci. U.S.a. *108*, 2545–2550.

Whishaw, I.Q. (2000). Loss of the innate cortical engram for action patterns used in skilled reaching and the development of behavioral compensation following motor cortex lesions in the rat. Neuropharmacology *39*, 788–805.

Whishaw, I.Q., Alaverdashvili, M., and Kolb, B. (2008). The problem of relating plasticity and skilled reaching after motor cortex stroke in the rat. Behavioural Brain Research *192*, 124–136.

Xu, T., Yu, X., Perlik, A.J., Tobin, W.F., Zweig, J.A., Tennant, K., Jones, T., and Zuo, Y. (2009). Rapid formation and selective stabilization of synapses for enduring motor memories. Nature *462*, 915–919.

Yu, X., and Zuo, Y. (2011). Spine plasticity in the motor cortex. Current Opinion in Neurobiology *21*, 169–174.

Supplemental:

Figure S1 (refers to Results: Learning phases of a skilled reaching task)

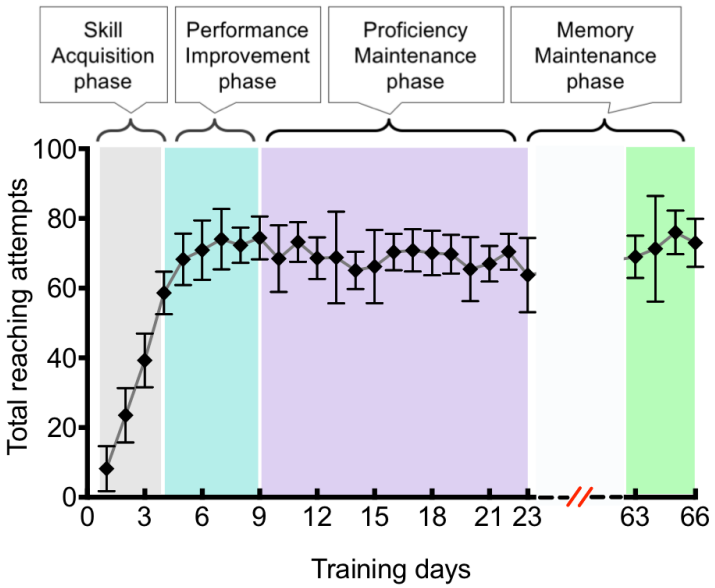

Figure S 1. Daily total reaching attempts in the skilled reaching task.

The total reaching attempts in a daily 30 minutes training session. X-axis – the number of training days; Y-axis – total reaching attempts: the average number of total reaching attempts (Means ± SEM); Grey 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 40 days no training gap, and was defined as the memory maintenance phase together with white block.

Figure S2 (refers to Results: Sensorimotor training induces an initial decrease followed by a persistent increase of PKMζ in sensorimotor cortex that is maintained during long-term memory storage)

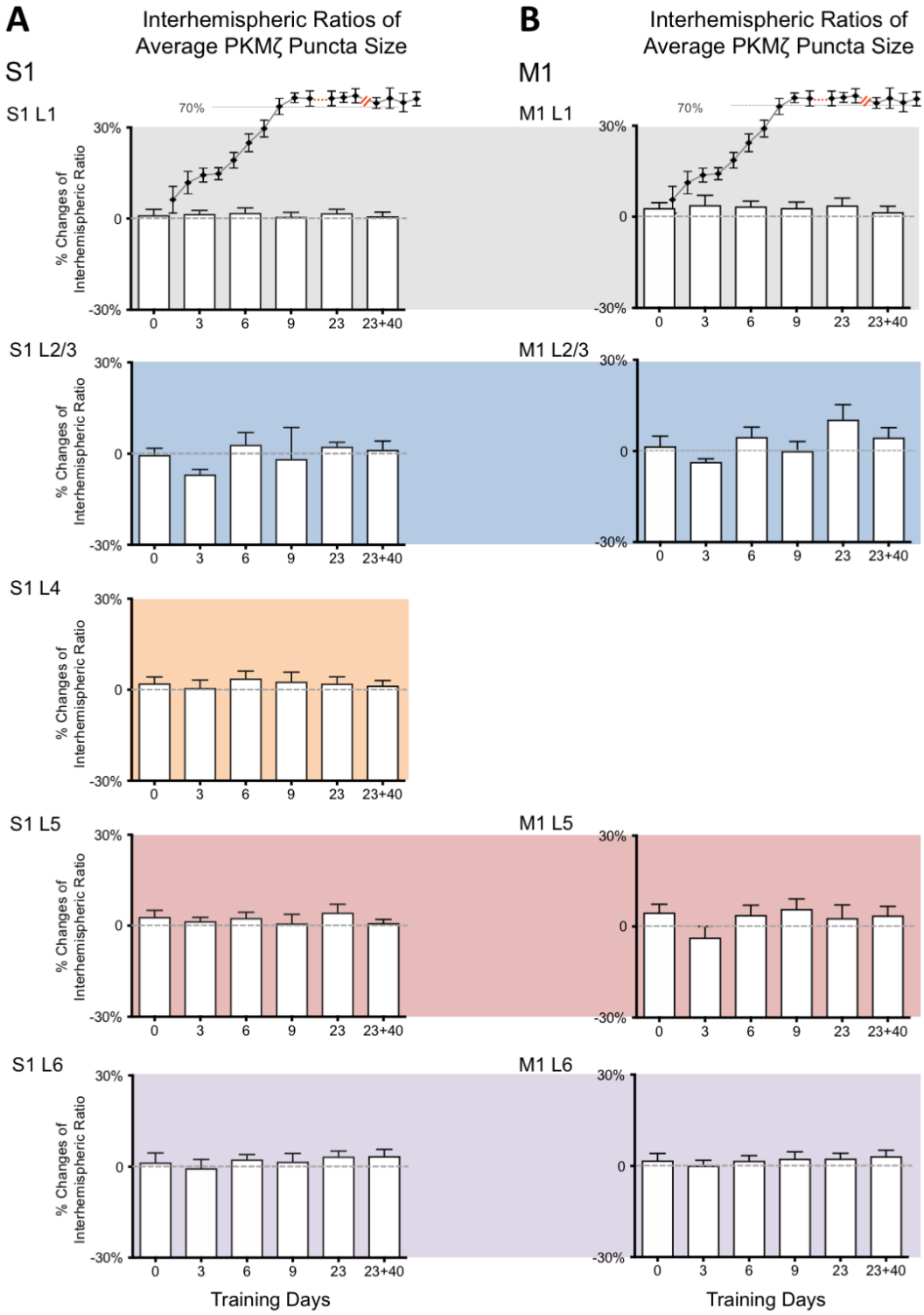

Figure S 2. Spatiotemporal Changes of PKMζ Puncta Size in Sensorimotor Learning.

A, B: The layer-specific changes of average PKMζ puncta size in S1 and M1 along with 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 another 40 days); Y-axis – the % of

interhemispheric ratio of averaged PKMζ puncta number (Means ± SEM); No significant between group effects were found with one-way ANOVA in all the regions of both S1 and M1.

Figure S3 (refers to Results PKMζ-antisense slows the performance improvement phase of sensorimotor learning and ZIP specifically disrupts the storage of sensorimotor memory maintained without reinforcement).

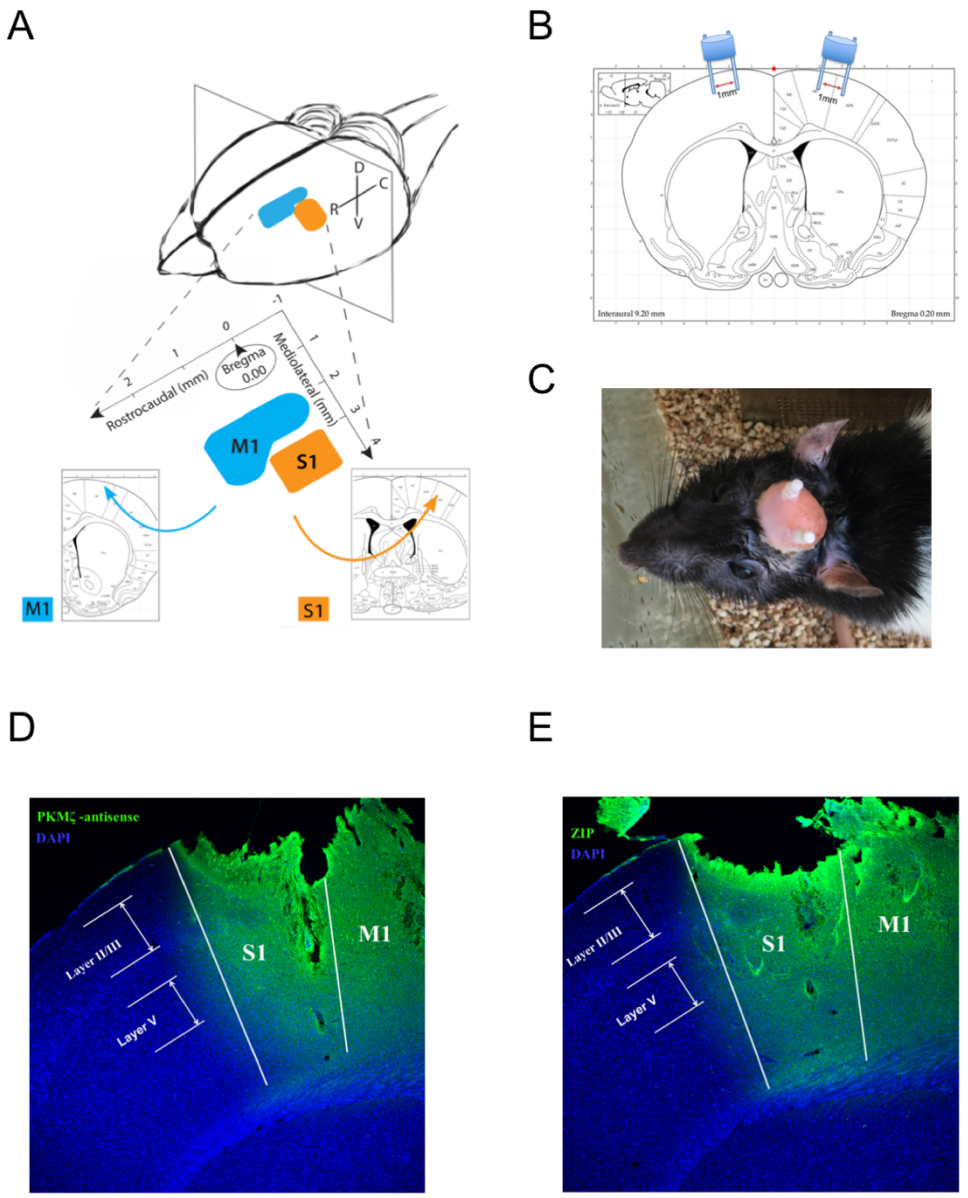

Figure S 3. Fluorescence labeling of biotinylated PKMζ-antisense injection.

A: The stereotaxic coordinates for rat forelimb M1 and S1 regions. The coronal sections for IHC study were chosen from primary motor cortex (M1, 1.6 mm ~ -0.5 mm to bregma; 1.5 mm ~ 3 mm lateral to the midline) and primary somatosensory cortex (S1, 0.5 mm ~ -0.4 mm to bregma; 3 mm ~ 4mm lateral to the midline) in rat. **B:** Demonstration of the location for implanted double guide cannula bilaterally. The center-to-center distance between two dummy wires in each double guide cannula was 1.0 mm. They were tilted 10˚ on each hemisphere from the vertical line. The coordination of guide cannula in M1: AP + 0.2 mm, ±ML 2.2 mm, DV -1.16 mm; The coordination 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 biotinylated PKMζ-antisense injection. Rat was sacrificed 1 hour

after the injections of biotinylated PKMζ-antisense in PBS. The injection dosage was 0.5μl/site and 1 μl per hemisphere in total. DAPI was counterstain. It was used to evaluate the spread of PKMζ-antisense and prove the effectivity of our injection approach. Green: biotinylated PKMζ-antisense. Blue: DAPI. **E:** Fluorescence labeling of biotinylated ZIP injection. Rat was sacrificed 1 hour after the injections biotinylated ZIP solution. The injection dosage was 1 μl/site and 2 μl per hemisphere in total. DAPI was counterstain. It was used to evaluate the spread of ZIP and prove the effectivity of our injection approach. Green: biotinylated ZIP. Blue: DAPI.

Figure S4 (refers to Results PKMζ-antisense slows the performance improvement phase of sensorimotor learning and ZIP specifically disrupts the storage of sensorimotor memory maintained without reinforcement)

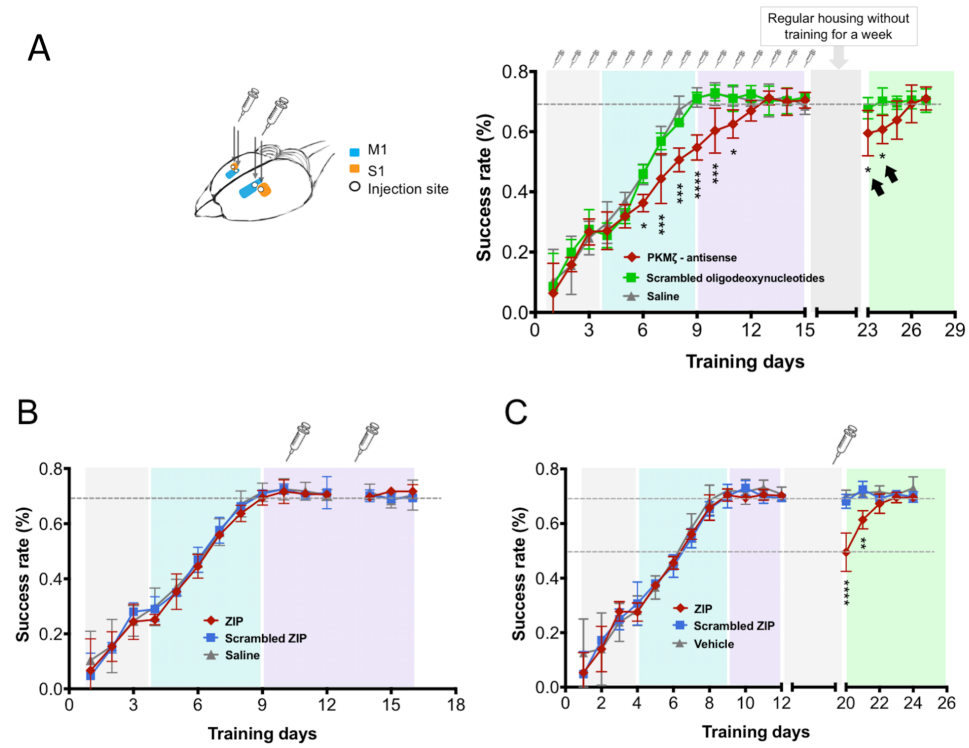

Figure S 4. Perturbing PKMζ synthesis and aPKC activity during motor learning and memory maintenance.

A: the complete learning curve for Figure 3. The effect of PKMζ-antisense on sensorimotor learning. B, C: the complete learning curve for Figure 4. The effect of ZIP on motor memory maintenance.