The Molecular Engram of Procedural Motor Skill Memories resides in Layer 5 of the Primary Motor cortex

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Significance Statement

Procedural memories of motor skill, such as for learning to ride a bicycle, can be retained without practice for long periods of time, but the location and mechanism of storage of these long-term memories are unknown. 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. PKMζ is a protein shown necessary and sufficient for the maintenance of long term potentiation (LTP). We show that PKMζ is necessary for maintaining sensorimotor memories, only, if practice on a task is paused for more than 48 hrs, that new PKMζ is necessary for normal learning and memory, and such memories are maintained by PKMζ in layer IV of M1.

Summary

Procedural motor 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, an investigation that causally links these changes with synaptic molecular machinery and behavior has been elusive. This study aims to fill this gap in our current knowledge by tracking layer specific changes in a key molecule, PKMζ, that has been shown necessary and sufficient for the maintenance of long term potentiation (LTP), in S1 and M1. In addition, we correlate change in PKMζ with changes in task performance. We show that PKMζ levels decrease in Layers II/III of S1 during an early pause in performance gains on day 3 of training. Subsequently, PKMζ levels increase 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. Lastly, we utilized genetic and pharmacological methods to causally perturb PKMζ during and after learning, which slowed the memory formation and weakened its maintenance, but didn't change the asymptotic level of task performance. Blocking aPKC activity erased sensorimotor memories that were maintained without reinforcement after a consolidation window of greater than 48 hrs. Thus, PKMζ sustains the molecular engram for motor memories maintained without practice within 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 in the smoothness and accuracy of skilled movements, which, once established, can be maintained without practice for a long period of time (1). 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 (2-11). Performance gains and the maintenance of proficiency on this task depend on the integrity of the sensorimotor cortex (5, 12-14). Plastic changes in sensorimotor cortex, including synaptic strength modification and structural remodeling, have been correlated with different phases of the learning process (4, 8-11, 15, 16). 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 (11), which lead to an LTP-like effect on synaptic transmission (8, 9). Accompanying this post training LTP was a persistent upward shift in the synaptic modification range that was maintained for more than two months (10). Increased synaptogenesis in M1 layer V pyramidal neurons was found after 4-7 days of training (4). Although both rapid spine formation and elimination were seen earlier during learning (15), these *de novo* spines were preferentially stabilized and likely became synapses at a later phase for long-term memory storage (17). In addition, reorganized motor maps were found after 8-10 days of training (4) and the signal-to-noise ratios of M1 spiking improved after 7-12 days of training (3). More and more evidence associates these plastic changes with the performance gains during motor learning; however, the molecular and cellular basis in sensorimotor cortex to encode and maintain motor memory remains unknown.

Persistent activity by the atypical PKC (aPKC) isoform PKMζ, has been shown to be both necessary and sufficient for the maintenance of LTP (18-20). 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 (21, 22). 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 (23, 24). Overexpression of PKMζ in cortical neuronal cultures increases the size of spine heads (e.g. from thin to mushroom) (25). PKMζ also regulates the function of postsynaptic density protein 95 (PSD-95), a major postsynaptic scaffolding protein and an essential factor for spine remodeling (26-28), (29). In mouse visual cortex,

PKMζ facilitates clustering of PSD-95 at dendritic spines (30); and the increase of PSD-95 clusters can be reversed by inhibiting PKMζ activity in cultured hippocampal pyramidal cells (31). 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 (32), basolateral amygdala-dependent fear memories (21, 33-36), dorsal lateral striatum-dependent habit memory (37) and insular cortex-dependent long-term associative conditioned taste aversion memory (38). Studies from our own group demonstrate that intracortical injection of ZIP into sensorimotor cortex disrupts the maintenance of learned skilled reaching (39), 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) cortices 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 as performance reaches an asymptotic stable level of performance by day 9. Without further training, the increased level of PKMζ in M1 layer V persists for at least 40 days, the longest time point used in this study, 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 sensorimotor memories that are maintained without practice for more than 48 hours, presumably the sensorimotor memory consolidation time window. Thus, within the sensorimotor cortex, 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 1.A). 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 1.D-1). 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 (40).

We observed that the acquisition of skilled motor memory could be divided into four sequential phases (as shown in Figure 1.D), consistent with the model described by Monfils and Teskey (8): 1) a skill acquisition phase (Days 1-4), when the total reaching attempts (Figure 1.D-2) increased rapidly but success rate (Figure 1.D-1) 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 gap in training, during which performance did not diminish, as tested on days 63-66. Days 23-66 were combined as the long-term memory maintenance phase.

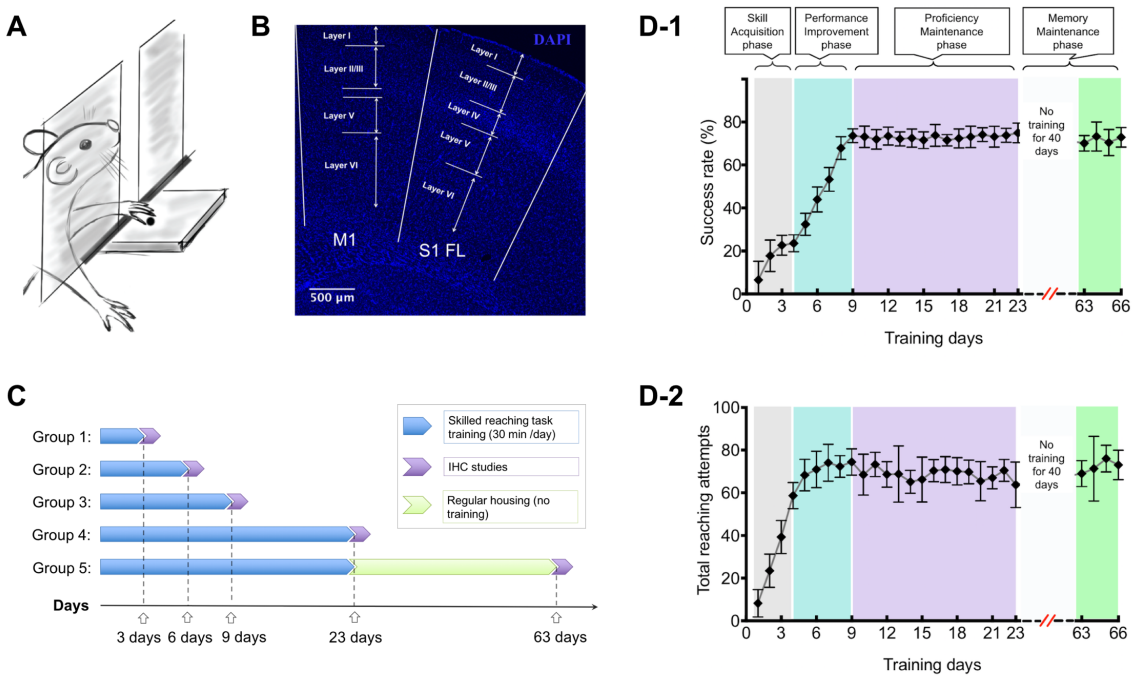

Figure 1. Rat skilled reaching task and learning curve.

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:** DAPI staining was used to identify different layers of motor cortex (M1) and forelimb somatosensory cortex (S1 FL). Scale bar, 500 µm. **C:** Experimental Paradigm. Blue arrow: daily training sessions for the skilled reaching task (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. Day 1-3, n = 29 rats (5 were sacrificed on day 4); Day 4-6, n = 24 rats (6 were sacrificed on day 7); Day 7-9, n = 18 rats (5 were sacrificed on day 10); Day 10-23, n = 13 rats (5 were sacrificed on day 24 with 23 days of training, and 6 were sacrificed on day 64 with 23 days of training plus 40 days regular housing); Day 63-66, n = 3 rats (tested for accuracy of skilled reaching, no IHC performed). **D-1:** Learning Curve. X-axis – training days; Y-axis – success rate (%): successful reaches divided by the total reaching attempts (Means ± SEM). **D-2:** Average daily total reaching attempts in a 30 minutes training session. Y-axis – total reaching attempts: the average number of total reaching attempts in each training session - 30 minutes (Means ± SEM); Grey block - the skill acquisition phase; Blue block - the performance improvement phase; Purple block - the proficiency maintenance phase; White block - regular house 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.

Sensorimotor learning induces a transient increase of PSD-95 in sensorimotor cortex during the performance improvement phase

We start by presenting results on the post synaptic density protein 95 (PSD-95), which is a marker of postsynaptic structure in sensorimotor cortex. PSD-95 is modulated during learning and memory, and this modulation is at least in part due to PKMζ (22), which we visit next.

PSD-95 immunoreactivity was measured in all layers of S1 and M1 forelimb regions (Figure 1.B and Figure 2.A) during each of the four aforementioned phases of sensorimotor learning, 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 1.C). The puncta number and size were quantified in each hemisphere and the interhemispheric ratios (trained / untrained hemispheres) were used to compare the levels of PSD-95 in each group with controls (naïve rats). In S1, there were no significant changes in PSD-95 puncta number associated with initial sensorimotor learning (Figure 2.C). However, its clustering size increased in layers II/III and V during the performance improvement phase (Figure 2.D). In M1, PSD-95 puncta number increased significantly after 9 days of training in both layers II/III and layer V (Figure 2.C), and the clustering size was also elevated during the performance improvement phase (Figure 2.D). Importantly, the increases in PSD-95 were not sustained in the proficiency maintenance or long-term memory storage phase, that is any time point after 9 days of training.

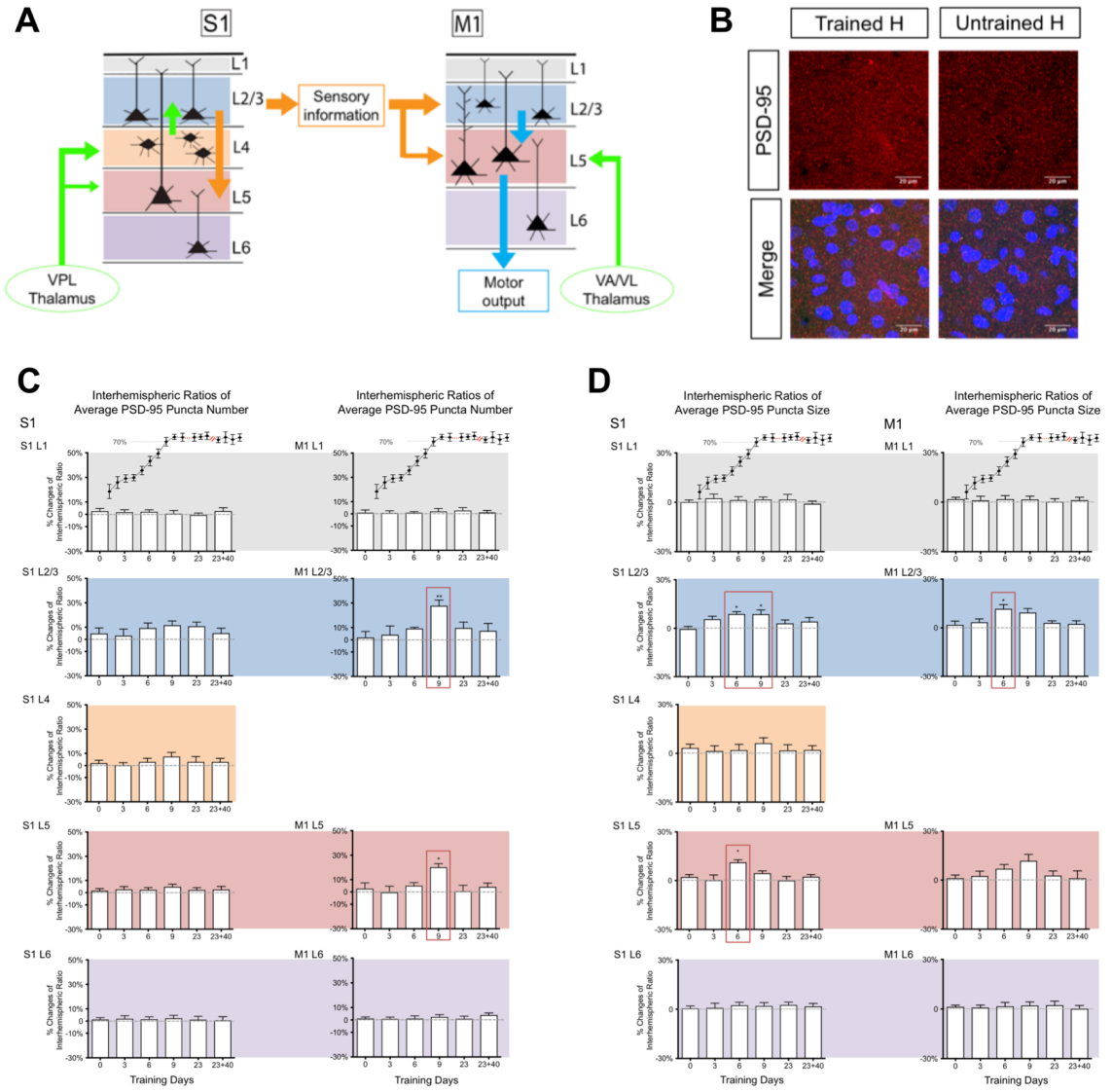

Figure 2. Spatiotemporal Changes of PSD-95 Puncta Number and Size in Motor Learning.

A: Information propagation in S1 and M1 during sensorimotor learning. **B:** Immunostaining of PSD-95. Scale bar, 20 μm. Example images were from M1 layer III/III of a rat with 9 days of training (magnification 120x). **C:** The layer-specific changes of PSD-95 puncta number in S1 and M1 along with motor learning. **D:** The layer-specific changes of PSD-95 puncta size in S1 and M1 along with 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 - the 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 average PSD-95 puncta number (A) and average PSD-95 puncta size (B) (Means ± SEM);
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 (0 - naïve rat) *(* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).*

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

Immunoreactivity to PKMζ was measured in all layers of S1 and M1 forelimb regions during each of the four aforementioned phases (Figure 1.D) as for PSD-95 (Figure 2). Confocal microscopy revealed PKMζ in the sensorimotor cortex is compartmentalized in small puncta (Figure 3), similar to its distribution in hippocampus (41). 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).

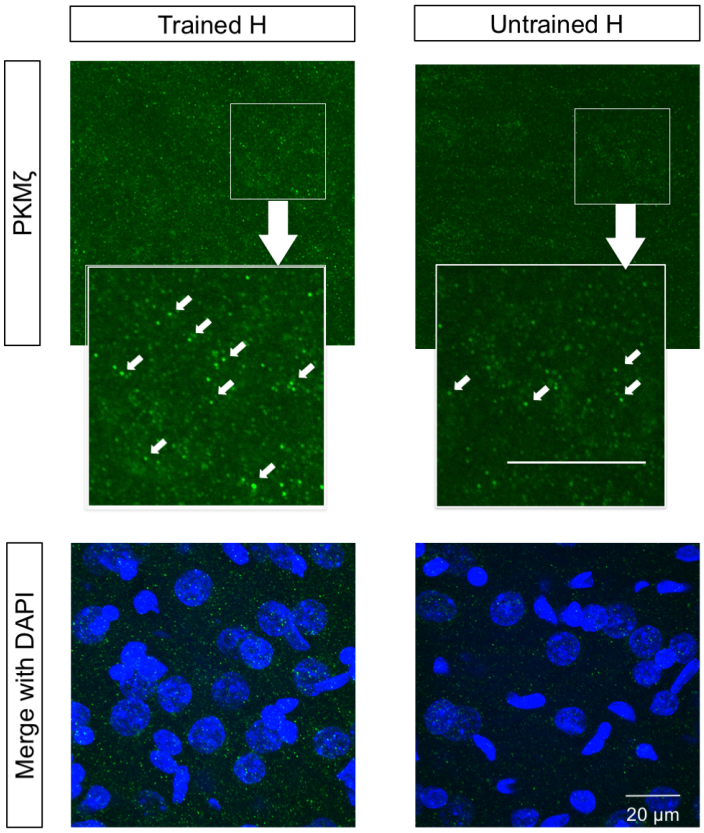

Figure 3. Immunostaining of PKMζ.

Immunostaining of PKMζ could be detected as small puncta (indicated by the white arrows) on both the trained and untrained hemisphere. DAPI was used for nucleus staining. Scale bar, 20 µm. Example images were from M1 layer II/III of a rat with 9 days of training (magnification 120x).

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 controls in S1 layer II/III (Figure 4.A). No significant changes were found in other regions on day 3, however there was a noticeable, but insignificant, decrease in M1 layer II/III (Figure 4.B), that paralleled the changes seen in S1.

As the initial skill acquisition transitioned into increased movement accuracy in the performance improvement phase, PKMζ increased in multiple cortical layers (Figure 4.A, B). 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 as well (Figure 4.A). The increases reached a maximum on day 9 when memory expression reached asymptotic levels of performance. During the proficiency maintenance phase (days 9-23), while the animals continued daily training, there was a sustained high level of PKMζ in S1 layer II/III, M1 layer II/III and M1 layer V (Figure 4).

During the long-term memory storage phase, i.e., after a 40-day gap without training, the ratio of PKMζ puncta number remained significantly high in M1 layer V (Figure 4.B), although levels remind elevated in S1/M1 layers II/III, they did not reach a significant level. These results suggest that the persistent increase in PKMζ that was formed during the performance improvement phase in sensorimotor cortical networks might play an essential role in maintaining long-term sensorimotor memories. Below represent results where we performed a set of experiments perturbing PKMζ and determine the influence on behavior in a causal manner.

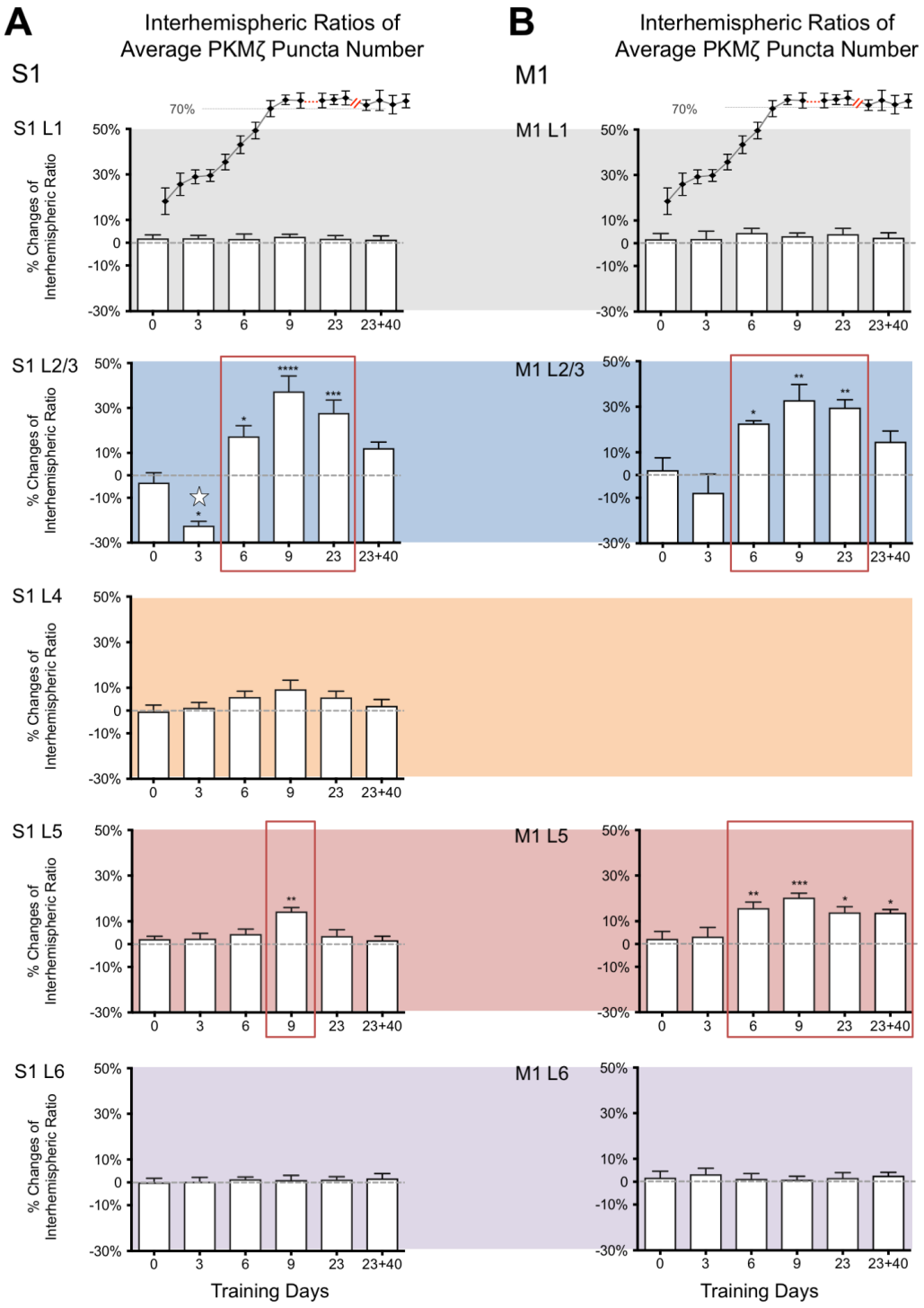

Figure 4. Spatiotemporal Changes of PKMζ Puncta Number in Sensorimotor Learning.

A, B: 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 S1).

PKMζ-antisense slows the performance improvement phase of sensorimotor learning

To determine the necessity of de novo 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 (42, 43). We injected PKMζ–antisense bilaterally in the sensorimotor cortex (S1/M1) 30 minutes before daily training and examined its effect on sensorimotor learning (Figure 5.A-D). Our results show that for the first 5 days of training, that learning with daily PKMζ-antisense injections (red line in Figure 5.E) was the same as the control groups injected with inactive scrambled oligodeoxynucleotides (green line) or saline (grey line) injections. However, from day 6 onward, the antisense group had a significantly lower learning rate compared with the other groups. In repeated two-way ANOVA, significant effects of group were found. *Post hoc* Turkey's tests for multiple comparisons showed significantly lower success rates for the antisense group compared with scrambled oligodeoxynucleotides and saline groups on days 6 – 11. In the antisense group, the fast performance improvement phase was replaced with a longer learning period that extended from day 4 to day 11. With extended training, the success rate of the antisense group eventually reached the same asymptote as the control groups on day 12. Rats with scrambled oligodeoxynucleotides or saline injection learned the skilled reaching task as efficiently as the controls seen in Figure 5.E, 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.

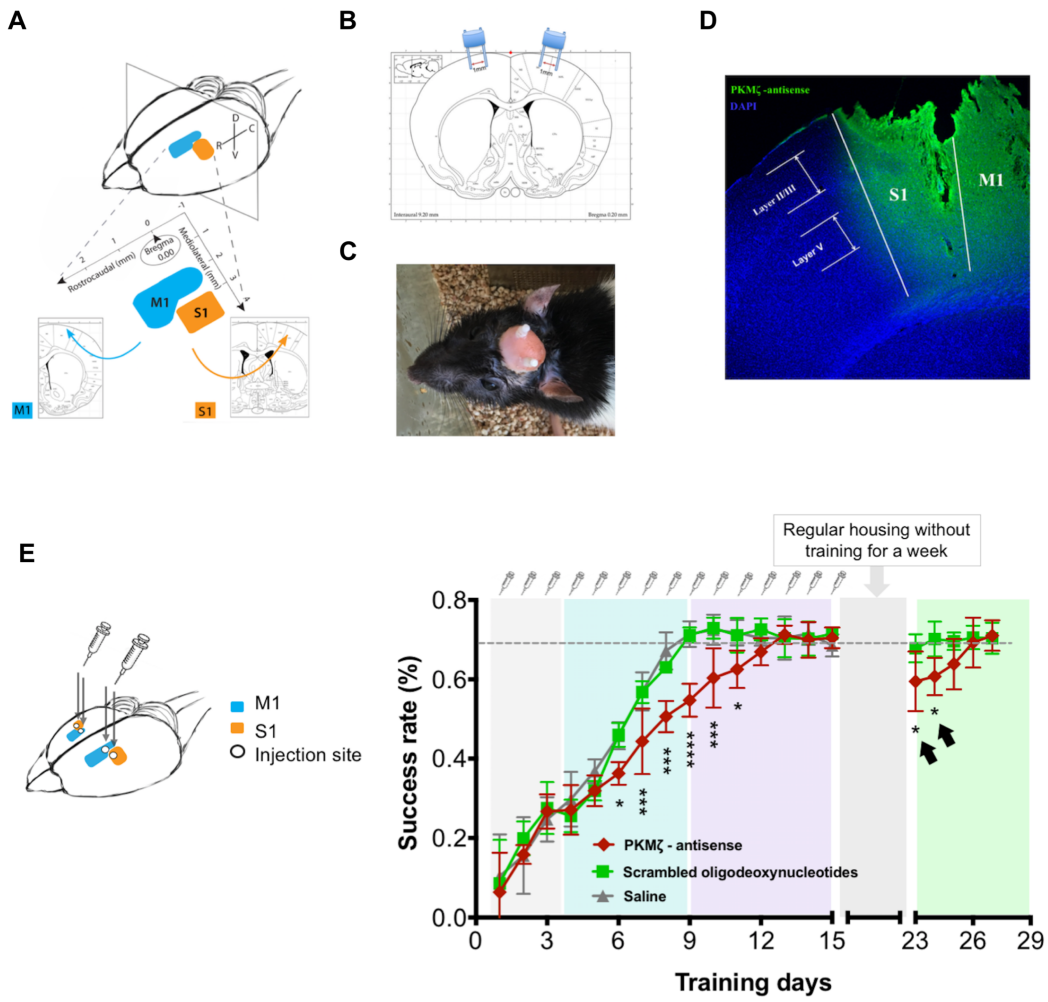

Figure 5. PKMζ stores sensorimotor memory that is maintained without practice.

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: PKMζ underlies the performance improvement phase of 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), green (n=4) and grey (n=5) (Means ± SD). Two-way ANOVA ($F_{(2, 12)} = 32.95$; $p < 0.0001$), followed by *post hoc* Turkey's comparisons showed significant lower success rate for the antisense group compared with scrambled oligodeoxynucleotides and saline groups 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 were tested again on days 23-27 after one week no training gap. Student *t-test* showed significant lower success rates of antisense group compared with scrambled oligodeoxynucleotides group on day 23 ($p= 0.0313^{*}$) and day 24 ($p = 0.0183^{*}$).

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, however, the PKMζ-antisense animals eventually reached the same proficiency as controls with additional training days (Figure 5.E). 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. The control, scrambled oligodeoxynucleotides group, maintained peak performance, as expected, however, the PKMζ–antisense group showed significantly lower performance on day 23 and 24 (Figure 5.E). We subsequently resumed 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 consolidated sensorimotor memories

The above results showed 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 under two scenarios: one maintained with continued daily training, the other maintained for 1 week without practice/reinforcement, or in other words, post consolidation.

Rats were trained for 9 days to reach maximum proficiency in the skilled reaching task. On day 10, before the training session, ZIP, scrambled ZIP, or saline was injected bilaterally in S1/M1 (Figure 6.A-1). 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 6.A-2). The training was continued for the next two days and no differences were found. To confirm that memories held by daily practice were not affected by ZIP, a second injection was given on day 13, 24 hrs after the last training session in the same rats, and 1 day later, for a total of 48 hrs from the last training session, the training resumed for days 14-16. Again, ZIP still had no effect on the task performance (Figure 6.A-2).

A second set of rats were trained to asymptotic performance and after 1 week without training were divided into 3 groups that received either ZIP, scrambled ZIP, or saline injection bilaterally in S1/M1 (Figure 6.B-1). 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, 1 week after their last training session). In striking contrast to the animals that had been trained within 24-48 hours, the rats that maintained memory without practice for one week could not perform the skilled reaching with proficiency after ZIP injection (Figure 6.B-2). 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 sensorimotor memory after more than 48 hrs. Therefore, the sensorimotor memory consolidation window is greater than 48 hrs and less than 1 week.

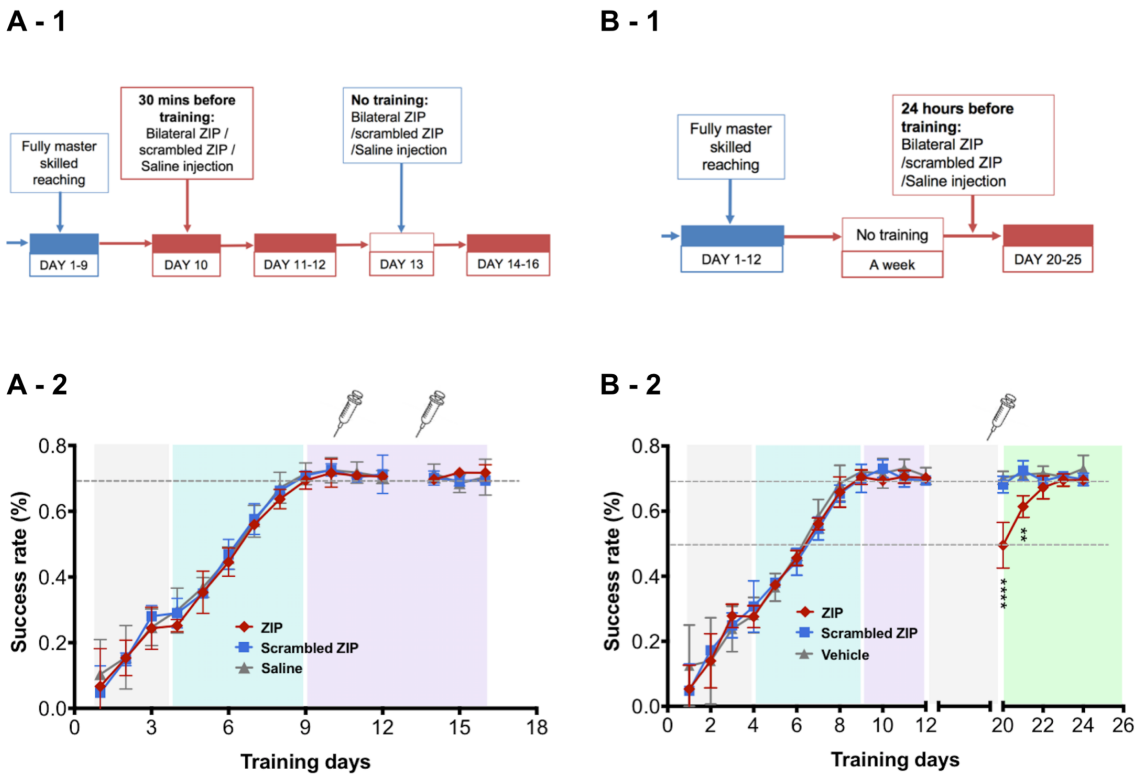

Figure 6. The effect of ZIP on motor memory maintenance.

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 ($F_{(2,8)} = 0.6555$, $p = 0.5450$).

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 turkey'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***).

Discussion

To link dynamic changes in synaptic molecular machinery with the evolving phases of sensorimotor learning and memory, we examined changes in PKMζ and PSD-95 in the sensorimotor cortex (Figure 2 and Figure 4). 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 here. 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 (44).

The initial downregulation of PKMζ in the forelimb, contralateral S1 layer II/III, observed during early skill acquisition (Figure 4.A) 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 (8, 39, 45). Downregulation of PKMζ is associated with long-term depression (46, 47), 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 a negative feedback signal to disrupt previously acquired sensorimotor associations. It should also be noted that M1 II/III PKMζ levels decreased in parallel with S1, although not significantly, on day 3. In short, in the earliest phase of acquiring a new sensorimotor 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 layers II/III and V (Figure 4). The timing of the increase of PKMζ parallels the LTP-like potentiation of synaptic transmission observed in motor cortex after skill learning (8, 9). The transient increase in PSD-95 is also observed in this later phase of learning (Figure 2), suggesting that structural changes also occur after an initial delay (4). Blocking the new synthesis of PKMζ with antisense during training slows the rapid increase of skilled sensorimotor learning after day 5, but does not prevent its acquisition with extended training (Figure 5.E). This indicates that there is a form of sensorimotor memory that can be maintained without new PKMζ that lasts at least 1 day, the time between training sessions (Figure 5.E). But this PKMζ-independent 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 5.E). Likewise, blocking aPKC activity with ZIP disrupts the long-term skilled sensorimotor memory that is maintained without practice, after a consolidation window more than 48 hrs long and less than a week, but has no effect on “long-term” memory that is sustained with continual daily practice (Figure 6). Therefore, PKMζ is crucial for sustaining skilled sensorimotor 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 sensorimotor memory is at least partially localized in layer V of primary motor cortex.

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

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Materials and Methods:

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

We used female rats only in this study for 3 reasons: i) It has been shown that sex has no effect on rat's reaching performance which is measured by daily success rate (48). ii) All the studies from Rioult-Pedotti and colleagues, showing motor learning is accompanied by enhanced synaptic plasticity and upward-shifted synaptic modification range in sensorimotor cortex, were using female rats (9-11). In their studies, the synaptic efficiency was measured on the trained hemisphere and compared with the untrained hemisphere in each animal. We utilized the same experimental paradigm to investigate the molecular changes with female rats to keep the consistency in this study. iii) Female rats were easier to handle and showed better consistency in learning this particular task.

Behavior Training: The animals with cannula implantation were housed individually, while the rest were pair-housed during the behavior training. 12/12 h light/dark cycle was maintained for all animals.

Skilled reaching task (Figure 1A): First, rats were food restricted to 85~90% of their free feeding body weight for a week. The training session lasted 30 minutes per day. On day 0, which was called the pre-training session, food pellets (45 mg 'dustless precision' food pellet, banana or chocolate flavor, Bio-Serve) were placed inside of the behavior chamber for free exploration.

The training chamber was customized with clear Plexiglas (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 1cm wide), was located at the front of the chamber and a horizontal food platform (3 cm wide and 8.5 cm long) was fixed at 3 cm height of the slot. When training started (from day 1), food pellet was placed in the metal washer (0.5 cm inside diameter, 1.5 mm away from the slot) on the platform. This design only allowed one forelimb to pass through and reach for the pellet each time. At the back of the chamber, a 1cm 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 again. In order to make them learn the pattern faster, an extra pellet was given 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, only a successful reaching attempt could get an extra pellet at the back after day 3. For each failed attempt, the unattained pellet would be removed by the experimenter and the rats had to start 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 tightly and finally retracting through the slot.

Rats without cannula implantation were divided into five groups based on the days of training (as shown in Figure 1C). 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) respectively and subjected to immunohistochemistry (IHC) 24 hours after the last training session in each group. The fifth group (n = 6 rats) were trained for 23 days followed by regular housing for another 40 days before being subjected to IHC. The control group included both naïve (n = 2 rats) and paired-control (n = 3 rats). For paired control, food pellets were placed on the other part of platform which extended inside of the behavior chamber. Skilled reaching was not necessary under this paradigm and the rats mostly ate them with their mouth directly. Paired-control animals still had to go back of the chamber to reset the trail each time. No IHC differences were found between the naïve and paired-control in our study, therefore they were combined into one group. Power analysis for one-way ANOVA was performed in G*Power, the actual power was larger than 0.9 with the total sample size (n = 32) used in this study.

Immunohistochemistry: After behavioral training, rats were fixed by cardiac perfusion and the brains were placed in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 48 hours. 40 μM coronal sections of the sensorimotor cortex were made by Leica VT 1200S vibratome. The sections of forelimb M1 and S1 regions were chosen according to the rat brain stereotaxic coordinates and previous studies (11, 17, 39) illustrated in Figure S4A. For each animal, 6~10 sections were stained accordingly.

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

Double staining of PKCζ and PSD-95: sections were incubated overnight at 4°C in primary antibodies --- anti-PKCζ rabbit polyclonal (1: 4000, purified in Dr. Sacktor's lab at SUNY Downstate) and anti-PSD95 mouse monoclonal (1:1000, abcam), followed by 6 rinses with PBST (10 minutes each). Then the sections were transferred to incubate with secondary antibodies --- Alexa Fluor® 488-AffiniPure Donkey Anti-Mouse IgG (H+L) (1:200, Jackson ImmunoResearch) and Alexa Fluor® 647-AffiniPure Donkey Anti-Rabbit IgG (H+L) (1:200, Jackson ImmunoResearch), 3 hours at room temperature.

Staining of brain with Biotinylated PKCζ-antisense or Biotinylated ZIP injection: every fifth section was selected from rats with biotinylated injection to show the spread of PKCζ-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).

Finally, DAPI (4,6'-diamidino-2-phenylindole) (1:500, Fisher Scientific) was applied for 15 minutes in the end of all the above staining. After extensive wash 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 could be performed via confocal microscopy.

Image Acquisition and Analysis: Confocal image acquisition: multi-channel images with double PKCζ and PSD-95 staining were collected using an FV1000 confocal microscope (Olympus Fluoview) at 60X magnification and zoomed in 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 18.0μm, and the step size between each 2-D image was 3.0μm.

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 identified using the green and red LUTs separately. Meanwhile, DAPI staining in blue was used as reference of nucleus.

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

Puncta counting: After thresholding, particle analyzer in Fiji was used to extract the averaged number and size of PKMζ and PSD-95 puncta on each hemisphere for all the animals. The contralateral hemisphere of the preferred forelimb was analyzed as the trained hemisphere while the ipsilateral hemisphere was analyzed as the untrained hemisphere. The interhemisphere ratios (averaged puncta number and size in the trained hemisphere divided by untrained hemisphere) were obtained in 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 $p < 0.05$.

Surgery: Animals were anesthetized with isoflurane for cannula implantation in a stereotaxic frame (Kopf instruments). At the beginning of surgery, valium (i.p. 5mg/kg body weight) was administered as sedative. Additionally, atropine (i.m. 4mg/kg body weight) was used to avoid fluid accumulation in the respiratory tract. After craniotomy, the double guide cannula (Plastics

One, 26GA, 1.0mm center-to-center distance between two dummy wires) were inserted through the burr holes and fixed onto the skull by dental cement bilaterally. The coordination for guide cannula was measured anteroposterior (AP) and dorsoventricular (DV) relative to Bregma, and mediolateral (ML) relative to Lambda according to the rat brain atlas (Figure S4 BC).

- 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.

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

Intracortical Injection: Before the skilled reaching task started, all animals with surgery were allowed to recover for 7-10 days and received bilateral injection of saline (0.5μl at each site = 1μl total on each hemisphere) once per day for 3 days to allow for habituation with the infusion procedures. During injection, the rats were restrained to remove the cannula dummy, then the injection needles (Plastics One, 33GA, 1.0mm center-to-center distance between two wires) were inserted, which protruded 0.5 mm from the guide cannulas. The infusion speed was controlled by a microinjection pump (model NE-4000, New Era Pump Systems) at 0.25μl /minute. At the end of each injection, the needles were left in place for another 3 minutes before retracting.
Antisense PKMζ: Same sequences of single-stranded oligodeoxynucleotides were used as in Sacktor's lab (42) (Gene Link, Hawthorne, NY). During training, 1nmol PKMζ-antisense / Scrambled antisense oligodeoxynucleotides in 0.5μl PBS per site (1μl total on each hemisphere) or 1 μl saline solution were injected 30 minutes before the skilled reaching task every day. The injection and training procedures lasted for 15 days and the success rate of reaching were recorded for analysis.

PKMζ-antisense: ctcTTGGGAAGGCAtgaC;
Scrambled antisense oligodeoxynucleotides: aacAATGGGTCGTCtcgG.

ZIP: During certain days of training, 1μl of ZIP / scramble ZIP (Tocris, 10nmol in 1μl PBS) was injected at each site, and the total injection amount was 2μl per hemisphere.

Biotinylated injection was given in the end 60 minutes before histology to confirm the location of cannulas and track the spread of drug diffusion. Data from rats (n = 3) with misplaced cannulas were ruled out from the analysis.

Statistical analysis: Two-way repeated measures ANOVA was used to compare the significance of time (days of training) and injection groups on performance (success reaching rate) in the skilled reaching task. *Post hoc* Tukey's honestly significant difference (HSD) test were used to compare the group effect of antisense with scrambled oligodeoxynucleotide or compare ZIP with scrambled ZIP if necessary. Significance was accepted when $p < 0.05$.

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Supplemental:

Figure S1 (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)

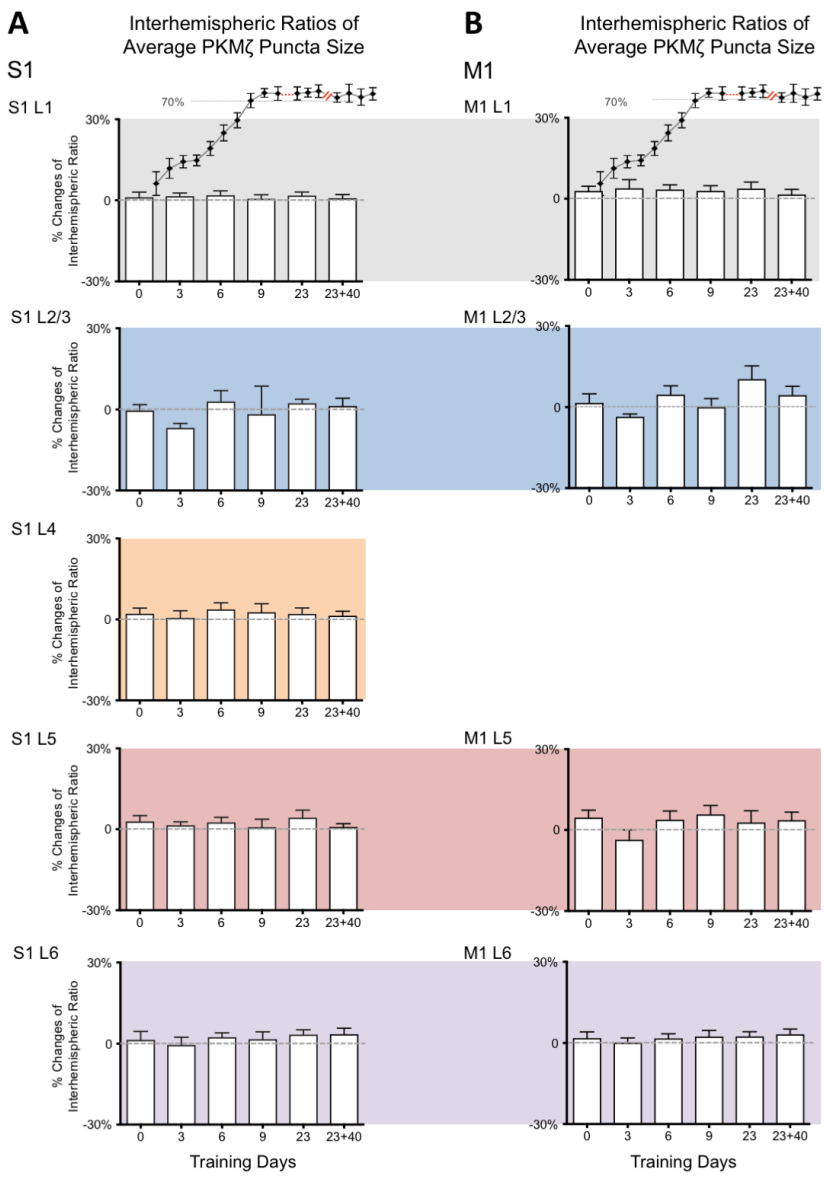

Figure S1. Spatiotemporal Changes of PKMζ Puncta Size in Sensorimotor Learning.

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.

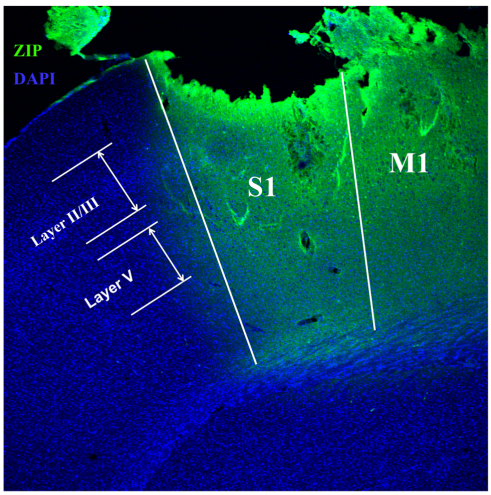

Figure S2: Fluorescence labeling of biotinylated ZIP injection.

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.