Title:

Layer-Specific Molecular Dynamics of PKMζ in Sensorimotor Cortex During Acquisition and Storage of Procedural Memory

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Summary

Procedural learning and memories are thought to be supported by the reorganization and plasticity of the sensorimotor cortex. However, changes linking synaptic molecular machinery within cortical layers and procedural learning and memory have been elusive. Here we track persistent changes in a key molecule necessary and sufficient for maintaining long-term potentiation, the atypical PKC (aPKC) isoform PKMζ, in sensorimotor cortex during the acquisition and storage of procedural motor memory. We show that PKMζ increases in S1/M1 layers II/III and V as performance improves to an asymptote, and, after training ends, the increase persists for more than 1 month in M1 layer V. Perturbing PKMζ synthesis during and after learning slows the memory formation and maintenance. Blocking aPKC activity erases 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 without practice for a long time (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 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, 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 S1), 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.

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 (Figures 1E and S2) and the interhemispheric ratios (trained / untrained hemispheres) were used to compare the levels of PKMζ in each group with controls (naïve rats).

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). No significant changes were found in other regions on day 3, which indicates the initial downregulation of PKMζ is specific to S1 layer II/III.

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 (Figures 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 high level of PKMζ in S1 layer II/III, M1 layer II/III and M1 layer V (Figures 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 (Figures 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.

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

The interhemispheric ratios of PSD-95, a marker of postsynaptic structure, in sensorimotor cortex were also measured. In S1, there were no significant changes in PSD-95 puncta number associated with initial sensorimotor learning (Figures S3A-1 ~ A-5). However, its clustering size increased in layers II/III and V during the performance improvement phase (Figures S3B-2 and B-4). In M1, PSD-95 puncta number increased significantly after 9 days of training in both layers II/III and layer V (Figures S3A-7 and A-8), and the clustering size was also elevated during the performance improvement phase (Figures S3B-7). The increases of PSD-95 were not sustained in the proficiency maintenance or long-term memory storage phase.

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 the sensorimotor cortex(S1/M1) 30 minutes before daily training and examined its effect on sensorimotor learning (Figures 3 and S4). Our results showed that for the first 5 days of training, the learning with daily PKMζ-antisense injection (red line in Figure 3) was the same as the control groups injected with inactive scrambled oligodeoxynucleotides (blue line) or saline (grey line) injection. 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 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.

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 (Figures 4A and S4). 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 for days 14-16. Again, ZIP still 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 (Figures 4B and S4). 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.

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 (Figures 2, S2 and S3). 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 (von 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 (Figures 2C, D and S2). 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). The transient increase in PSD-95 is also observed in this later phase of learning (Figure S3), suggesting that structural changes also occur after an initial delay (Kleim et al., 2004). 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.

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.

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Figure 1:

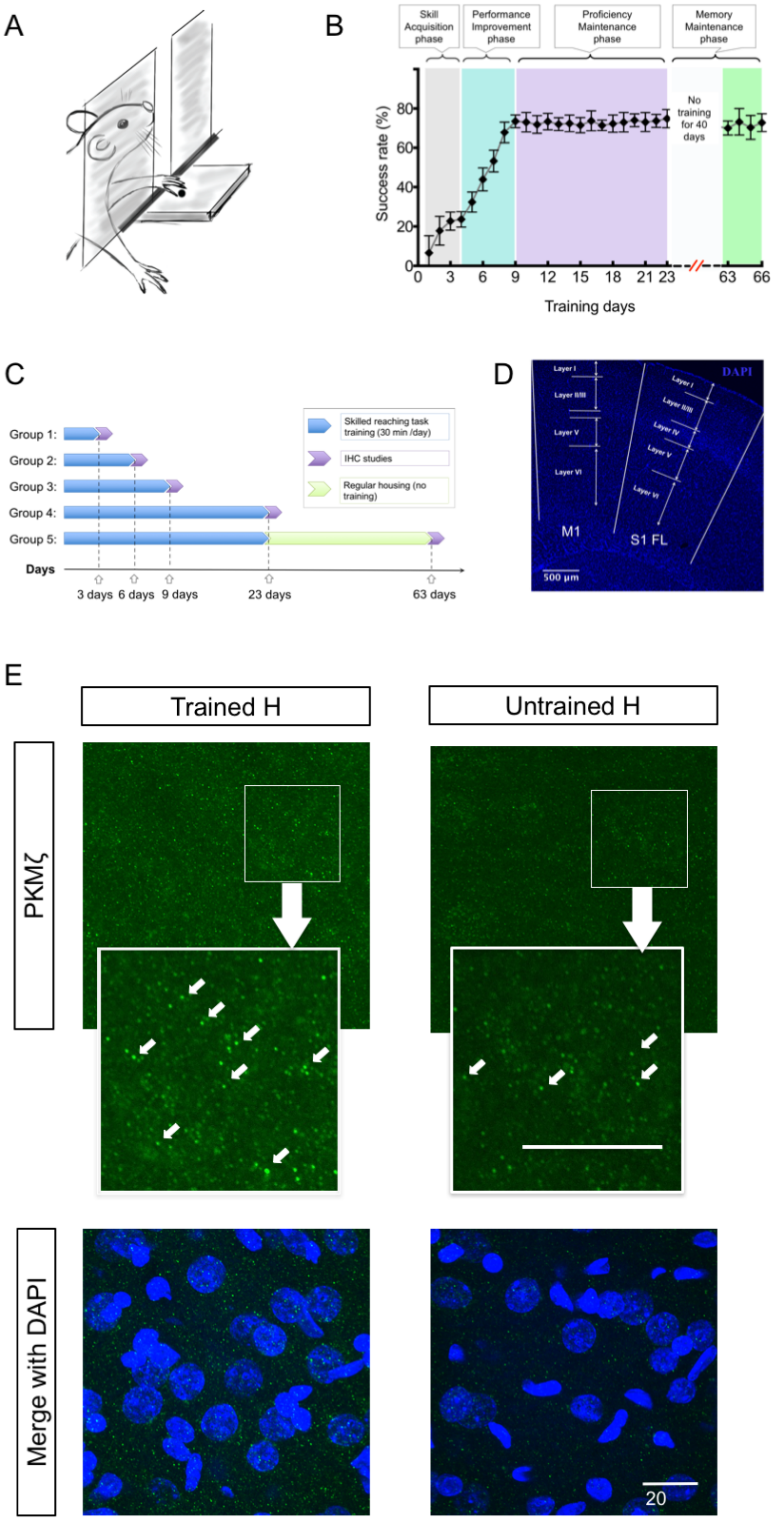

Figure 1. Skilled reaching task and PKMζ immunostaining of sensorimotor cortex.

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: 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:** 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).

Figure 2:

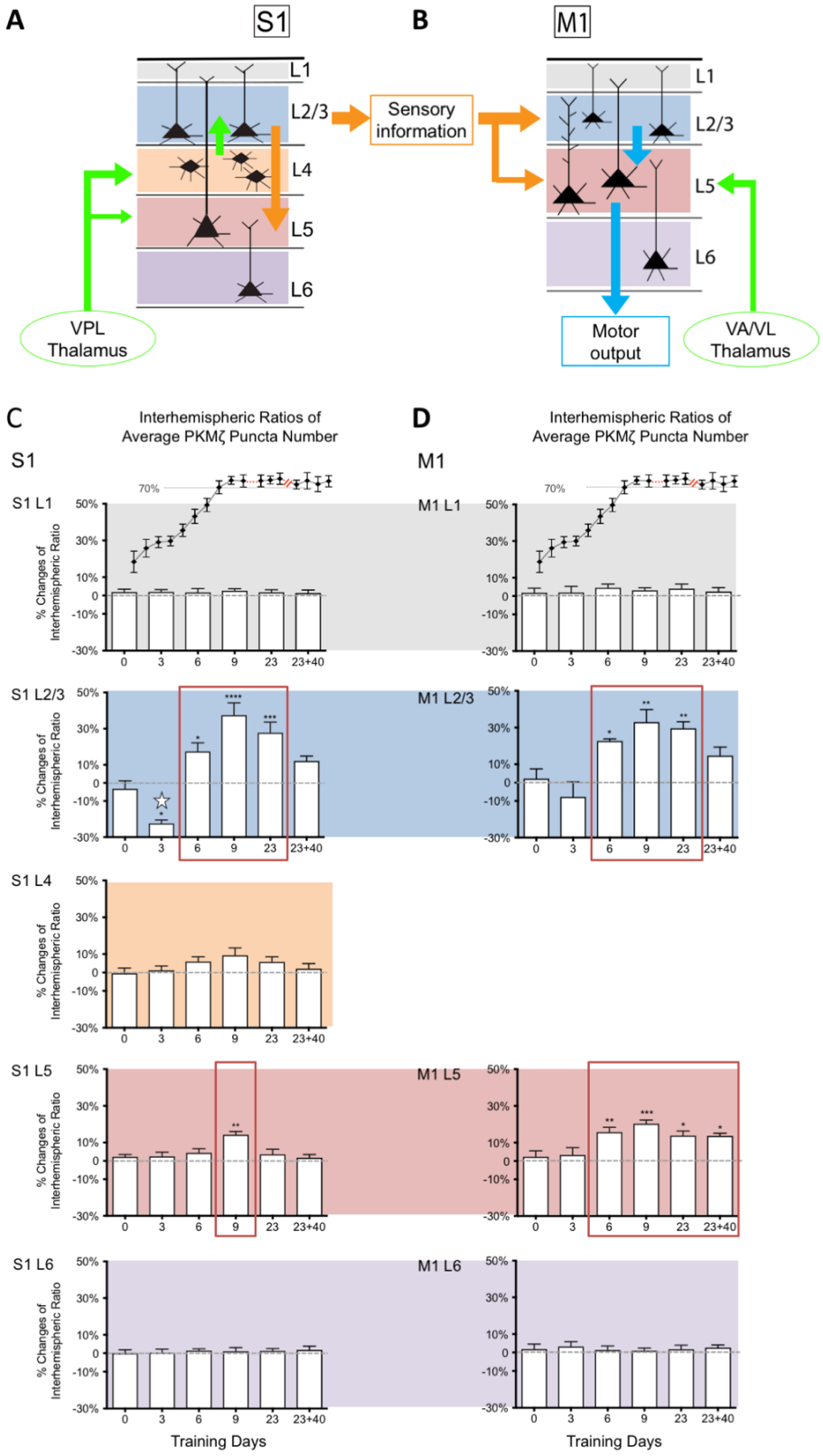

Figure 2. Spatiotemporal Changes of PKMζ Puncta Number in Sensorimotor Learning.

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 S2).

Figure 3:

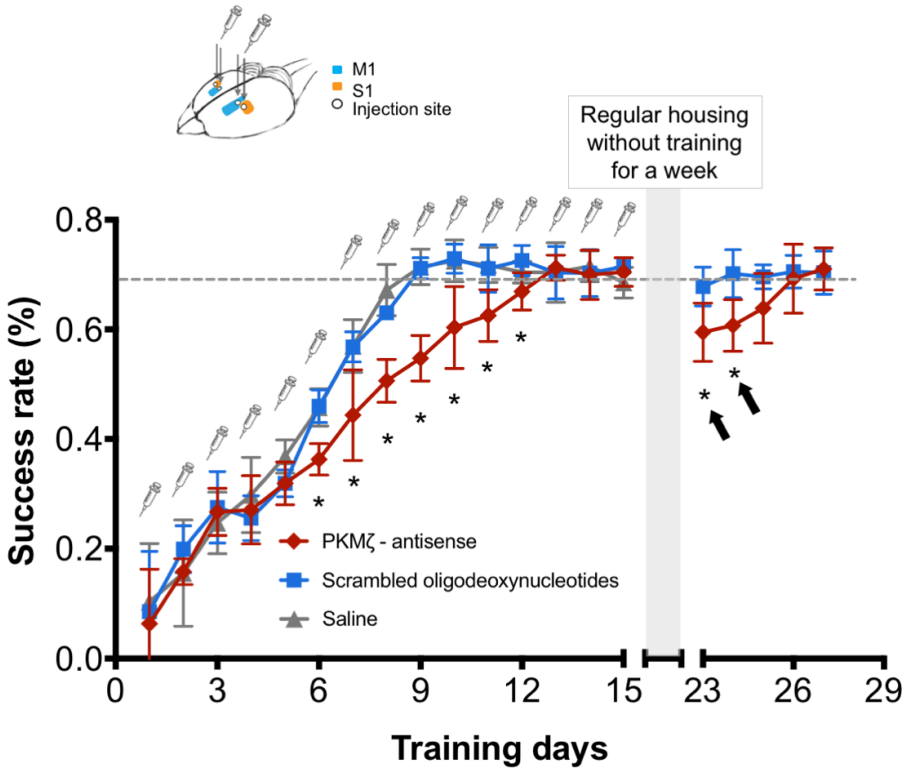

Figure 3. The effect of PKMζ-antisense on sensorimotor learning.

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* Turkey'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 ($p = 0.0183$*).

Figure 4:

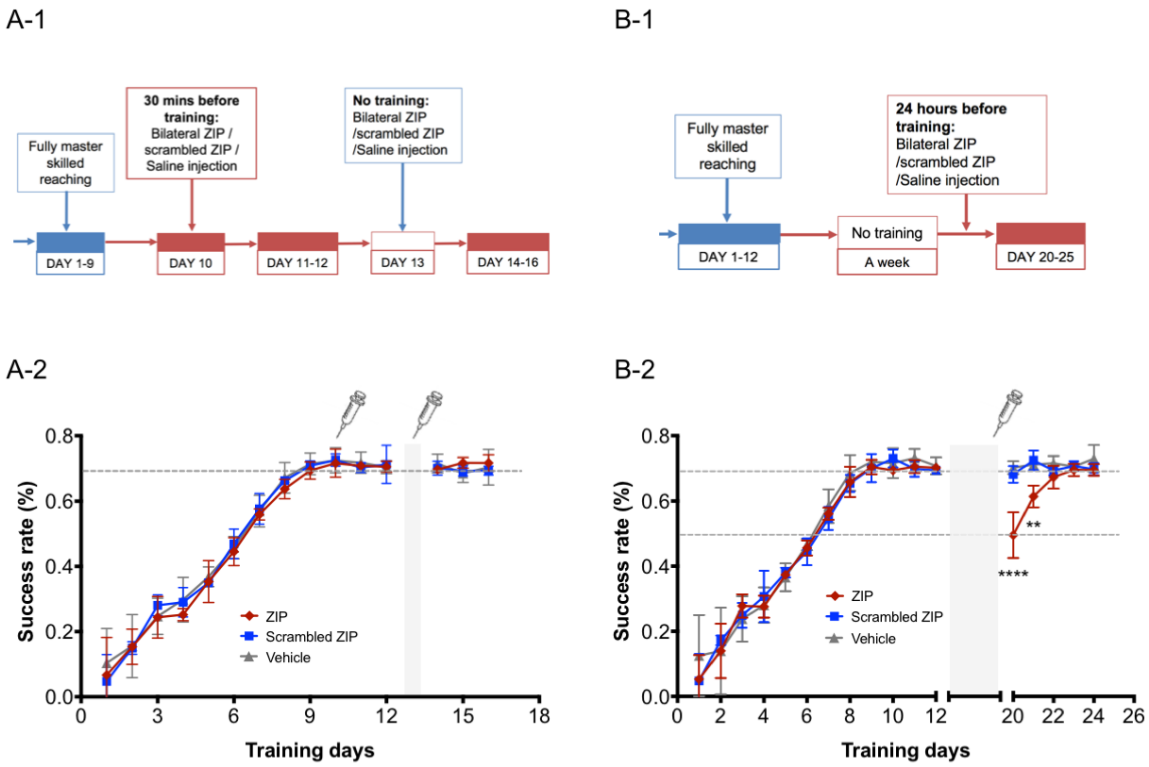

Figure 4. The effect of ZIP on sensorimotor memory maintenance.

A-1: Rats were trained on the skilled reaching task and received ZIP, scrambled ZIP, or saline injections bilaterally on day 10 and 13 (injection dosage: 2 µl / hemisphere). **A-2**: The learning curves for rats that received ZIP, scrambled ZIP or saline injections on day 10 and day 13 were marked in red (n = 3), blue (n=3) and grey (n = 5) respectively (Means ± SD). Two-way ANOVA showed no significant difference between groups ($F_{(2,8)} = 0.6555$, $p = 0.5450$). **B-1**: Rats were trained for the skilled reaching for 12 days followed by one week regular housing, and received ZIP, scrambled ZIP, or saline injections bilaterally on day 19 (2 µl / hemisphere). **B-2**: The learning curves for rats that received ZIP, scrambled ZIP and saline injections on day 19 were marked in red (n=5), blue (n=5) and grey respectively (Means ± SD). Two-way ANOVA showed significant differences between groups ($F_{(2,8)} = 6.927$, $p = 0.0180$), followed by *Post hoc* turkey's multiple comparisons. On day 20 and 21, the success rate of ZIP group was significantly lower compared scrambled ZIP ($p<0.0001$**** and $p=0.0024$**), and saline groups ($p<0.0001$**** and $p=0.0086$**).