LETTERS

Rapid formation and selective stabilization of synapses for enduring motor memories

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Novel motor skills are learned through repetitive practice and, once acquired, persist long after training stops[1,2]. Earlier studies have shown that such learning induces an increase in the efficacy of synapses in the primary motor cortex, the persistence of which is associated with retention of the task[3–5]. However, how motor learning affects neuronal circuitry at the level of individual synapses and how long-lasting memory is structurally encoded in the intact brain remain unknown. Here we show that synaptic connections in the living mouse brain rapidly respond to motor-skill learning and permanently rewire. Training in a forelimb reaching task leads to rapid (within an hour) formation of post-synaptic dendritic spines on the output pyramidal neurons in the contralateral motor cortex. Although selective elimination of spines that existed before training gradually returns the overall spine density back to the original level, the new spines induced during learning are preferentially stabilized during subsequent training and endure long after training stops. Furthermore, we show that different motor skills are encoded by different sets of synapses. Practice of novel, but not previously learned, tasks further promotes dendritic spine formation in adulthood. Our findings reveal that rapid, but long-lasting, synaptic reorganization is closely associated with motor learning. The data also suggest that stabilized neuronal connections are the foundation of durable motor memory.

Fine motor movements require accurate muscle synergies that rely on coordinated recruitment of intracortical synapses onto corticospinal neurons[6,7]. Obtaining new motor skills has been shown to strengthen the horizontal cortical connections in the primary motor cortex[4,5]. In this study, we taught mice a single-seed reaching task (Supplementary Movie 1). The majority of 1-month-old mice that underwent training gradually increased their reaching success rates during the initial 4 days, and then levelled off ($n = 42$, Fig. 1a, b). There were a few mice ($n = 5$) that engaged in extensive reaching, but continually failed to grasp the seeds. These mice normally gave up reaching after 4–8 days (Fig. 1b). To investigate the process of learning-induced synaptic remodelling in the intact motor cortex, we repeatedly imaged the same apical dendrites of layer V pyramidal neurons marked by the transgenic expression of yellow fluorescent protein (YFP-H line) in various cortical regions during and after motor learning, using transcranial two-photon microscopy[8] (Supplementary Fig. 1). Dendritic spines are the postsynaptic sites of most excitatory synapses in the brain and changes in spine morphology and dynamism serve as good indicators of synaptic plasticity[9,10]. Spines that were formed and eliminated were identified by comparing images from two time points, and then normalized to the initial images. Imaged regions were guided by stereotaxic measurements, ensuring the imaged neurons resided in the primary motor cortex. In several experiments, intracortical microstimulation was performed at the end of repetitive imaging to confirm that images were taken from the functionally responding motor cortex (Fig. 1c, Supplementary Notes and Supplementary Fig. 2).

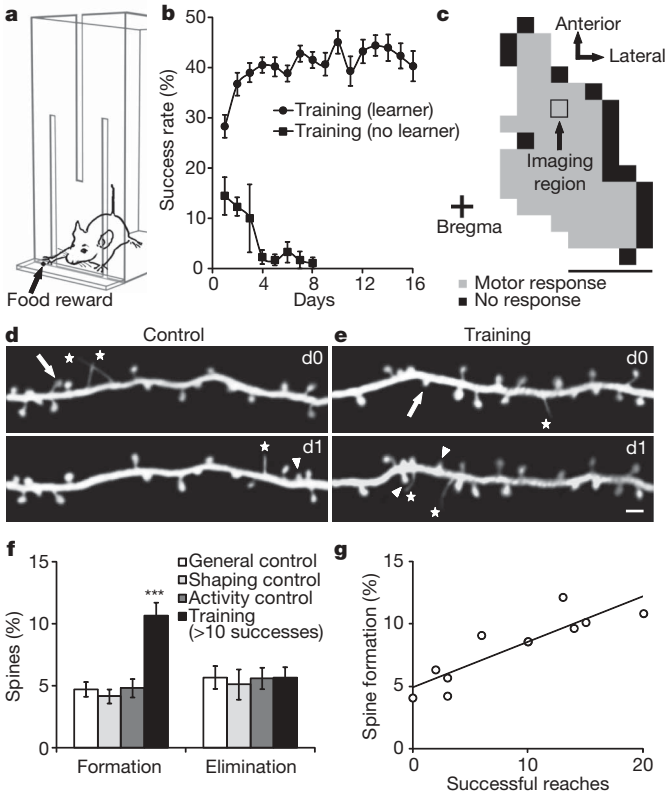

Figure 1 | Motor skill learning in adolescent mice promotes immediate spine formation in the contralateral motor cortex. a, A cartoon of motor training. **b**, Average success rates during training for learning and non-learning mice (mean ± s.e.m., 42 learners and 5 no learners). **c**, An intracortical microstimulation map indicates that the imaged region is within the motor cortex. Scale bar, 1 mm. **d**, **e**, Repeated imaging of the same dendritic branches over one-day intervals reveals spine elimination (arrows) and formation (arrowheads), and filopodia (asterisks) in a general control (**d**) and a trained (**e**) mouse. Scale bar, 2 μm. **f**, Percentage of spines formed and eliminated under various control and training conditions immediately following the first training session (mean ± s.d., $***P < 0.001$). **g**, The degree of spine formation observed following the first training session is linearly correlated with the number of successful reaches during this session ($r^2 = 0.77$).

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Unexpectedly, we found that motor learning led to rapid formation of dendritic spines (spinogenesis) in the motor cortex contralateral to the reaching forelimb. One-month-old mice that finished 30 reaches with more than 10 successes in the first day of training were imaged within 1 h of the training session and showed 10.6 ± 1.1% new spines which were not in the images acquired the day before training. This spine formation was more than double that found in age-matched controls, which were handled similarly and imaged over the same period of time, but not trained (Fig. 1d–f, 4.7 ± 0.6% in general controls, $P < 0.001$). In contrast, spine elimination measured in the same images was not significantly altered by motor learning during single training sessions (Fig. 1f, $P > 0.9$). In addition, mice that went through shaping but not training (shaping controls) or mice that were trained to reach for a seed too far away to grasp (activity controls) did not show an increase in spine formation rates (Fig. 1f, $P > 0.1$ with general control, $P < 0.001$ with trained mice; see Methods for all control conditions). This suggests that refinement of fine motor movements, rather than other training-related experiences or unskilled motor activity, drives robust spine formation. Furthermore, the percentage of spines formed immediately after the first training session is linearly correlated with the number of successful reaches during the training session, revealing a direct link between learning and spine formation (Fig. 1g, $r^2 = 0.77$).

Perfection of a motor skill often requires persistent practice over time. To examine how prolonged learning affects spine dynamics, we trained and imaged mice over different periods of time (that is, from 2 to 16 days). We found that training for 2 days and longer resulted in significant increases, not only in spine formation, but also in spine elimination (Fig. 2a, b, $P < 0.005$ at all time points). Although delayed, this increase in spine elimination ultimately resulted in the total spine density in the trained animals returning to control levels by day 16 (Fig. 2c). As a control, we measured spine formation and elimination over a 4-day training period in the ipsilateral (to the trained limb) primary motor cortex and the contralateral posterior sensory cortex, and found no significant increase in spine formation or elimination in either case (Fig. 2a, b, d, e, $P > 0.2$). In addition, mice that failed to learn also failed to show an increase in either spine formation or elimination in the contralateral motor cortex (Figs 1b and 2a, b, f, $P > 0.6$). Therefore, the observed changes in spine dynamics are region- and learning-specific, indicating that motor learning causes synaptic reorganization in the corresponding motor cortex.

The enhanced spine loss after rapid spinogenesis reflects a rewiring of the neuronal circuitry in response to learning, rather than a simple addition of new spines. To examine how learning reorganizes synaptic connections, we imaged the same mice three times, classified imaged spines into new and pre-existing spines based on their appearance in the initial two images, and then quantified their survival percentages in the third images (Fig. 3a). Our data show that new spines are less stable than pre-existing spines in general (Fig. 3b, c). Specifically, in control mice, 43.8 ± 3.1%, 25.8 ± 5.3% and 19.2 ± 4.6% of the spines that formed between days 0 and 4 remained by days 6, 8 and 16, respectively. During the same period of time, 96.7 ± 0.5%, 94.9 ± 1.1% and 92.8 ± 1.9% of the pre-existing spines remained (Fig. 3d, $P < 0.001$ compared to new spines). These results suggest that new spines are initially unstable and undergo a prolonged selection process before being converted into stable synapses. In addition, we found that new spines were significantly more stable in trained mice, with 64.1 ± 2.2%, 55.3 ± 4.1% and 51.0 ± 4.8% of the spines that formed during the initial 4-day training remaining by days 6, 8 and 16, respectively (Fig. 3d, $P < 0.001$ compared to new spines in control mice). In contrast, pre-existing spines in trained mice were significantly less stable than control mice over the same time periods (Fig. 3d, $P < 0.05$). More importantly, when the fate of the new

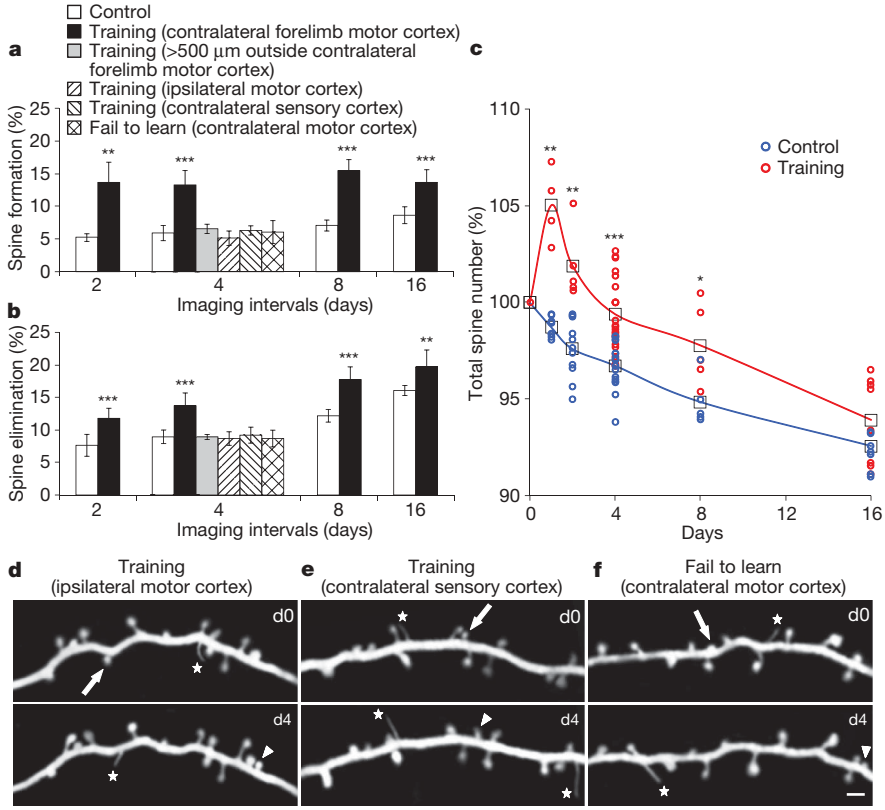

Figure 2 | Enhanced spine dynamics during adolescent motor training is region- and learning-specific. **a**, **b**, Percentage of spines formed (**a**) and eliminated (**b**) under control and training conditions. **c**, Total spine number increases during initial learning, but returns to normal levels with prolonged training. **d**, **e**, Imaging of the same dendritic branches over 4 days in the ipsilateral primary motor cortex (**d**) and the contralateral sensory cortex (**e**) of the trained mice. **f**, Imaging of the same dendritic branches over 4 days in the contralateral motor cortex of a mouse that failed to learn the task. Data are presented as mean ± s.d., $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Scale bar, 2 μm.

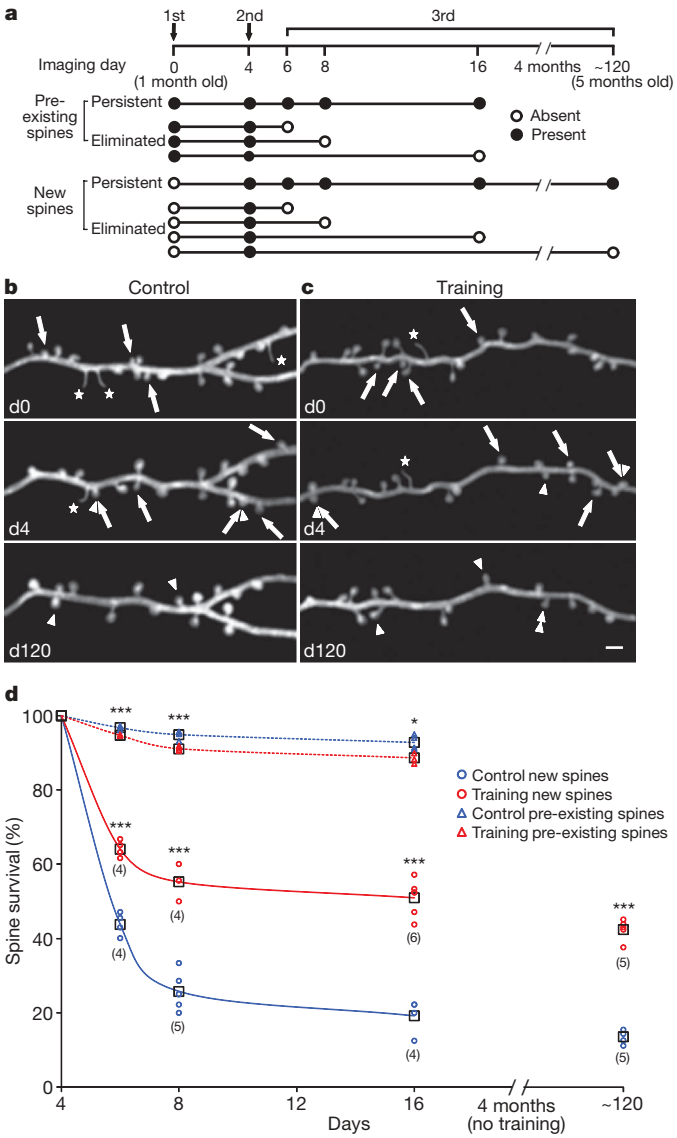

Figure 3 | Motor skill learning stabilizes newly formed spines. **a**, Timeline of experiments, showing possible outcomes. **b**, **c**, Repeated imaging of dendritic branches at 0, 4 and 120 days in a control (**b**) and a trained (**c**) mouse. Scale bar, 2 μm. **d**, Percentages of surviving new and pre-existing spines, as a function of time, for control and trained animals (mean ± s.d., $*P < 0.05$ and $***P < 0.001$). Numbers of animals examined at each time point are indicated below new spine data points.

spines formed during initial learning (day 0–4) was examined months later (day 120), we found that 42.3 ± 2.9% of new spines persisted in the mice trained for 16 days during adolescence, whereas only 13.5 ± 1.7% of new spines remained in the control mice (Fig. 3d, $P < 0.001$). In addition, we found that spine formation and stabilization were associated with behavioural improvement. More new spines were formed daily during the learning acquisition phase (days 1–4) than during the learning maintenance phase (days 5–16); the new spines that were formed during learning acquisition, but not during maintenance, were preferably stabilized with continuous training (Supplementary Notes and Supplementary Fig. 3). Taken together, these data indicate that motor learning selectively stabilizes learning-induced new spines and destabilizes pre-existing spines. The prolonged persistence of learning-induced synapses provides a potential cellular mechanism for the consolidation of lasting, presumably permanent, motor memories.

Dendrites in the mammalian brain contain not only spines but also filopodia. Filopodia are long, thin protrusions without bulbous heads, and make up ~10% of the total dendritic protrusions in the motor cortex of 1-month-old mice. Previous studies suggest that filopodia are precursors of dendritic spines[11,12]. We found that filopodia were very dynamic in the mouse motor cortex *in vivo*. Most of them turned over within 1 day in control mice (79.3 ± 12.8% formation and 87.6 ± 5.9% elimination), and motor learning had no effect on filopodial formation and elimination (91.0 ± 15.3% formation and 86.5 ± 8.8% elimination, $P > 0.2$). Among the filopodia observed in the initial images, few of them became spines over the following day in control mice (6.3%). However, this filopodium-to-spine transition was enhanced by motor skill learning (13.1%). Furthermore, 25% of new spines formed from filopodia on training day 1 persisted after another 4 days of training, indicating a contribution of filopodia to the rewired neuronal circuitry. Furthermore, when filopodia and spines were pooled together for analysis, there was a ~10% increase in the dynamics of both control and training categories. Thus, the conclusion of motor learning on total protrusions was consistent with the spine analysis alone (Supplementary Fig. 4).

One of the important characteristics of motor learning is that, once the skill is well learned, its further maintenance does not require constant practice. To test whether lasting motor memories might be contained within structurally stable neural circuits, we trained young mice for 8–16 days to acquire the reaching skill, housed them in control cage conditions for 4 months, and retrained them on the same task in adulthood. We found that these pre-trained mice maintained skilful performance with high success rates even on the first day of reintroducing the reaching task (Fig. 4a). Imaging of these pre-trained adult mice showed that spine formation and elimination during retraining were similar to those of naive adults without training (Fig. 4b, e, g, $P > 0.1$ for 4 and 8 days). In contrast, naive adults learning the reaching task for the first time had a learning curve similar to adolescent mice, and showed significantly higher spine formation and elimination than control adults (Fig. 4a–c, g, 4 and 8 days, $P < 0.01$ with control for both formation and elimination). Next, we asked if learning a novel motor skill continued to drive synaptic reorganization in the pre-trained brain. To do this, we trained mice that had been pre-trained on the reaching task with a new motor task—the capellini handling task, which also requires fine forelimb motor skills (see Methods). We found that pre-trained mice, similar to naive adults, had enhanced spine formation and elimination during the training of this novel skill task (Fig. 4d, f, g, $P < 0.001$ compared to control adults). Despite high spine dynamics induced by novel skill learning, most spines that were formed during adolescent learning of the reaching task and maintained in adults persisted after training with the capellini handling task (95.6 ± 7.7%), suggesting that already stabilized synapses are not perturbed by novel learning in adults. These results indicate that synaptic structural coding outlasts the early learning experience and persists in adulthood to support later maintenance of motor skills. The fact that novel learning experiences continue to drive synaptic reorganization without affecting the stability of synapses formed during previous learning further suggests that different motor behaviours are stored using different sets of synapses in the brain.

Our study investigated the process of synapse reorganization in the living brain during natural learning, distinguishing it from several studies where changes were triggered by non-physiological sensory manipulation[13–18]. Although rapid synapse formation has been observed during long-term potentiation *in vitro*[19,20], we show, for the first time, that synapse formation in the neocortex begins immediately as animals learn a new task in the living brain (within 1 h of training initiation). Such high spine formation does not occur with motor activity alone or later practice of the established skill. The rapidity of the response contradicts the general assumption that significant synaptic structural remodelling in motor cortex takes days to occur, following more subtle cellular activity and changes in synaptic efficacy[4,21,22]. One recent study on brain slices shows that glutamate-sensitive currents expressed in newly formed spines are indistinguishable from mature spines of comparable volumes[23], further suggesting

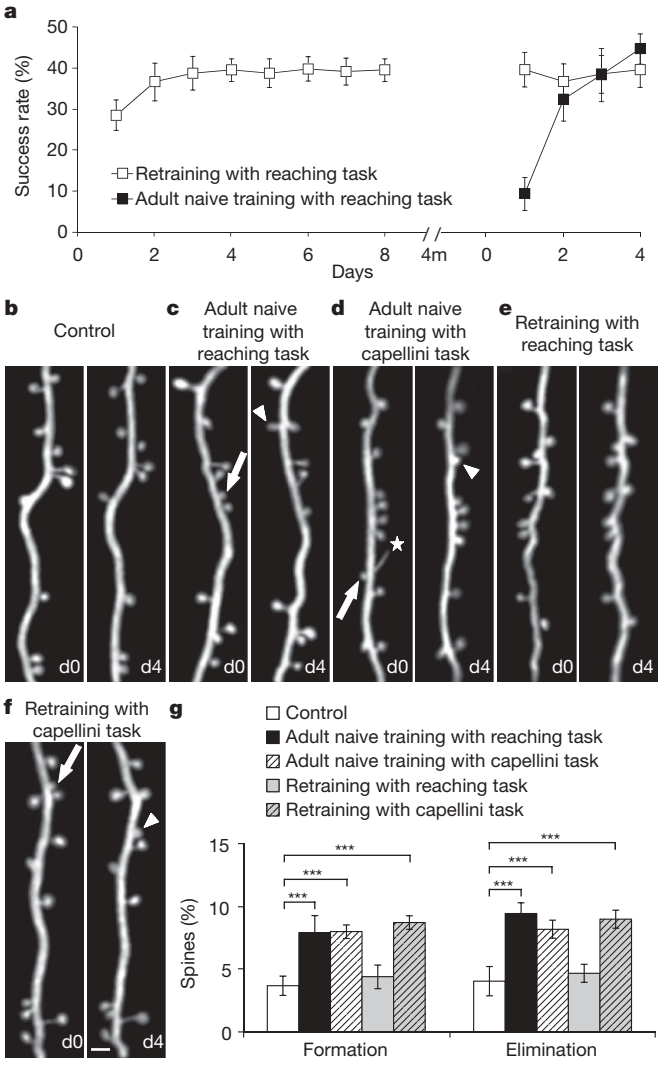

Figure 4 | Novel motor skill training promotes spine formation and elimination in adult mice. a, Pre-trained mice start with high success rates during adult retraining (mean ± s.e.m., 10 naive trained and 14 retrained adults). **b–f**, Repetitive imaging of dendritic branches over 4 days in a control adult (**b**), naive adults training with the reaching task (**c**) and capellini handling task (**d**), and pre-trained adults retraining with the same reaching task and the new capellini handling task (**f**). Scale bar, 2 μm. **g**, Percentages of spines formed and eliminated over 4 days in adult mice under different conditions (mean ± s.d., $***P < 0.001$).

that the new spines formed during learning are probably active. Furthermore, the persistence of new spines over months provides a long-lasting structural basis for the enhanced synaptic strength that is retained even when the task performance is discontinued.

Many previous studies have used fixed tissue preparation to investigate changes in synapse number and dendritic complexity after motor skill learning[24–28]. Our *in vivo* imaging of superficial dendrites from layer V pyramidal neurons revealed that postsynaptic dendritic spine addition was rapid, but eventually counteracted by the loss of pre-existing spines, resulting in a time-dependent spine density change during motor learning. Although the synaptogenesis observed in our study is compatible with earlier results, its temporal relationship with behavioural improvement and the contribution of synapse elimination in circuitry reorganization in other brain layers and regions during motor learning require further investigation. This eventual balancing of synapse number could be a homeostatic mechanism by which the output layer V neurons integrate converging inputs into superficial cortical layers to govern precise fine motor control.

METHODS SUMMARY

Young (1 month old) and adult (>4 months old) mice expressing YFP in a small subset of cortical neurons (YFP-H line[29]) were used in all the experiments. Young mice were trained on the single-seed reaching task for up to 16 days and displayed a stereotypical learning curve (Fig. 1b). Naive adult mice and mice that had been previously trained with the single-seed reaching task in adolescence were trained with either the same reaching task or a novel capellini handling task for up to 8 days (see Methods). Apical dendrites of layer V pyramidal neurons, 10–100 μm below the cortical surface, were repeatedly imaged in mice under ketamine–xylazine anaesthesia with two-photon laser scanning microscopy. Spine dynamics in the motor cortex and other regions were followed over various intervals. Imaged regions were initially guided by stereotaxic measurements. In 14 mice, intracortical microstimulation (see Methods) was performed at the end of repetitive imaging to determine the location of acquired images relative to the functional forelimb motor map (Supplementary Fig. 2). In total, 32,079 spines from 209 mice were tracked over 2–4 imaging sessions, with 121 mice imaged twice, 79 mice three times and 9 mice imaged four times. Spine formation and elimination rates in each mouse were determined by comparing images of the same dendrites acquired at two time points; all changes were expressed relative to the total number of spines seen in the initial images. The number of spines analysed and the percentage of spine elimination and formation under various experimental conditions are summarized in Supplementary Table 1. To quantify spine size, calibrated spine head diameters were measured over time[30] (Supplementary Notes). All data are presented as mean ± s.d., unless otherwise stated. *P*-values were calculated using the Student's *t*-test. A non-parametric Mann–Whitney *U*-test was used to confirm all conclusions.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions T.X. and X.Y. contributed equally to this work. Both of them performed *in vivo* imaging, analysed the data, made figures and participated in the discussion. A.J.P., W.F.T. and J.A.Z. trained all the mice used in the experiments. K.T. and T.J. developed behavioural methods, performed the intracortical microstimulation experiments, and provided comments for the manuscript. Y.Z. initiated the project, did data analysis and wrote the manuscript.

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METHODS

Single-seed reaching task. Mice were food-restricted to maintain 90% of free feeding weight before the start of training. The training chamber was constructed as a clear Plexiglas box 20 cm tall, 15 cm deep and 8.5 cm wide into which each individual mouse was placed. Three vertical slits 0.5 cm wide and 13 cm high were located on the front wall of the box: in the centre, on the left side, and on the right side (Supplementary Fig. 5). A 1.25-cm-tall exterior shelf was affixed to the wall in front of the slits to hold millet seeds for food reward. The training included two phases: 'shaping' and 'training'. The shaping phase (2–5 days in duration) was used to familiarize mice with the training chamber and task requirements and also to determine their preferred limbs. During the shaping phase millet seeds were placed in front of the centre slit and mice used both paws to reach for them. Shaping was considered finished when 20 reach attempts were achieved within 20 min, and the mouse showed >70% limb preference. Training started the day after shaping, and each training day consisted of one session of 30 trials with preferred limb or 20 min (whichever occurred first). Seeds were presented individually in front of the slit on the side of preferred limb. Occasionally a mouse used the non-preferred limb; however, because of the difficulties presented by reaching angle, such reaches usually were unsuccessful. Mice displayed three reach attempt types: fail, drop and success (Supplementary Movie 1). A 'fail' was scored as a reach in which the mouse failed to touch the seed or knocked it away. A 'drop' was a reach in which the mouse retrieved the seed, but dropped it before putting into its mouth. A 'success' was a reach in which the mouse successfully retrieved the seed and put it into its mouth. Success rates were calculated as the percentage of successful reaches over total reach attempts. About half of the mice in our experiments were right handed (55 right handed out of a total of 109 mice, 50.6%). All data collected from both left- and right-handed mice were pooled for analysis in this study. No significant difference was found in the reaching performance of left- and right-handed mice.

All our control mice were littermates that underwent the same food restriction. All mice were handled (that is, removed from their cages and placed temporarily in the training chamber into which some seeds were dropped) by the same experimenters. To ensure that the increase seen in spine dynamics was learning specific, three different controls were used in our study. The first control group was general controls comprising mice with neither training nor shaping, but with food restriction, food reward and handling. The second was shaping controls in which mice received similar shaping as trained mice. During training, they were placed into the training chamber for 20 min daily, with ~15 seeds periodically dropped into the training chamber. This control group was used to determine whether the shaping period and/or experience of the training environment had any effect on spine dynamics. The third control group was activity controls in which mice were given similar shaping as trained mice. During training, mice were placed into the training chamber and trained to reach for a seed placed outside the slit for 20 min daily. However, the seed was placed out of reach, so that they could never obtain it and, therefore, did not learn skilful reaching movements (as shown by testing their performance occasionally). Thus, both trained mice and activity control mice experienced similar amounts of forelimb activity, but only trained mice developed the motor skill. The activity control was used to determine whether enhanced spine dynamics were caused by increased motor activity or were specific to motor skill learning. Our results indicate that there is no difference in the spine dynamics between the activity controls and general controls.

Capellini handling task. This task was similar to the vermicelli handling tasks previously described for rats[31]. Mice were food-restricted to maintain 90% of free feeding weight before training began. A daily training session consisted of 10 trials with uncooked capellini pasta pieces (2.5 cm), given one piece per trial. Mice learned to use coordinated forepaw movements to eat the pasta. The average consumption time for one piece of capellini pasta decreased from 3.44 ± 0.18 min on day 1 to 1.98 ± 0.29 min on day 4 (mean ± s.e.m., $P < 0.005$, 7 mice). There was no significant behavioural difference in the capellini handling task between naive adults and adults pre-trained in the reaching task in adolescence.

***In vivo* imaging of superficial dendrites.** The procedure for transcranial two-photon imaging has been described previously[8]. Mice aged 1–6 months were anaesthetized with an intraperitoneal injection (5.0 ml per kg body weight) of 17 mg ml^{-1} ketamine and 1.7 mg ml^{-1} xylazine in 0.9% NaCl. The skull was exposed with a midline scalp incision and imaged regions were located based on stereotactic coordinates. A small region of skull (~300 μm in diameter) was manually thinned down to ~20 μm in thickness using both a high-speed drill and a microknife. To reduce respiration-induced movements, the skull was glued to a 400-μm-thick stainless steel plate with a central opening for skull access. The plate was screwed to two lateral bars located on either side of the head and fixed to a metal base. The brain of the mouse was then imaged through the thinned skull using a Prairie Ultima IV multi-photon microscope with a Ti-sapphire laser tuned to the excitation wavelength for YFP (925 nm). Stacks of image planes were acquired with a step size of 0.70 μm using a water-immersion objective (×60, NA 1.1 infrared Olympus objective) at a zoom of 3.0. For relocation of the same dendrites at subsequent imaging times, an image stack containing the dendritic structures of interest was taken without zoom with a step size of 2.0 μm and the surrounding blood vessels were imaged with a CCD camera. The patterns of blood vessels and neuronal processes in this low-resolution image stack were used for relocating the same dendrites at each subsequent imaging session (Supplementary Fig. 1). After imaging, the plate was detached from the skull, the scalp sutured, and the animal was returned to its home cage until the next imaging session.

Spine and filopodium identification. All analysis of spine dynamics was done manually using ImageJ software, blind with regard to experimental conditions. Briefly, the same dendritic segments (~5–20 μm in length) were identified from three-dimensional image stacks selected from all views having high image quality (signal-to-background-noise ratio >4-fold). Individual dendritic protrusions were tracked manually along dendrites. Three-dimensional stacks, instead of two-dimensional projections, were used for analysis to ensure that tissue movements and rotation between imaging intervals did not influence identification of dendritic protrusions. The number and location of dendritic protrusions (protrusion length >1/3 dendritic shaft diameter) were identified in each view. Filopodia were identified as long thin structures with head diameter/neck diameter <1.2 and length/neck diameter >3. The remaining protrusions were classified as spines.

Analysis of spine and filopodial dynamics. Notations of the formation and elimination of spines and filopodia were based on comparison of the images collected at two different time points. Spines or filopodia were considered the same between two views if they were within 0.7 μm of their expected positions, based on their spatial relationship to adjacent landmarks and/or their position relative to immediately adjacent spines. A stable spine is a spine that was present in both images. An eliminated spine is a spine that appeared in the initial image, but not the second image. A newly formed spine is a spine that appeared in the second image, but was absent from the initial image. Percentages of stable, eliminated and formed spines were all normalized to the initial image. Percentage changes in the total spine number over a given interval were relative to the first view and calculated as percentage of formation minus percentage of elimination measured over that interval. Data on spine dynamics are presented as mean ± s.d..

Image processing and presentation. Two-dimensional projections of three-dimensional image stacks containing in-focus dendritic segments of interest were used for all figures. We chose very sparsely labelled regions as examples and maximum projections were made from images from 2–4 focus planes. There were normally few crossing structures in the projected images from such a shallow stack, and the presented branches could be clearly isolated. Finally, images were thresholded, Gaussian filtered and contrasted for presentation.

Mapping of motor cortex by intracortical microstimulation. This method was adapted from those used in rat experiments[21]. Mice were anaesthetized with an initial cocktail of ketamine (150 mg kg^{-1}, intraperitoneal) and xylazine (10 mg kg^{-1}, intraperitoneal) and supplemented with additional ketamine and isofluorane (0.5–1% in oxygen) as necessary. The mouse was placed into a mouse stereotaxic frame (Stoelting), lidocaine (2 mg kg^{-1}, subcutaneous) was injected into the scalp, and a midline incision was made. The cisterna magna was drained to prevent cortical swelling and the skull and dura overlying the motor cortex were removed. The craniotomy was then filled with warm (37 °C) silicone oil to prevent drying. A picture of the cortical surface was taken and overlaid with a 250 μm square grid in Canvas software.

Intracortical penetrations of a glass microelectrode (diameter of 20-25 μm) with a platinum wire were made at 250 μm intervals in a systematic order throughout the cortex at a depth of 790–800 μm (corresponding to deep layer V/shallow layer VI) with a hydraulic micropositioner until the entire extent of the forelimb representation was resolved. A 40-ms train of 13 200-μs monophasic cathodal pulses was delivered at 350 Hz from an electrically isolated, constant current stimulator at a rate of 1 Hz stimulation and current was increased to a maximum of 60 μA until a visible movement was evoked. If a movement was evoked at or below 60 μA, the threshold current was determined by gradually decreasing the stimulation until the movement stopped. The lowest current that evoked a movement was taken as the threshold current. If no movement was seen at 60 μA, the site was considered non-responsive. In cases where stimulation evoked more than one movement, the site was considered responsive to the movement that was determined to have the lowest threshold. To verify that the stimulation position was located within layer V, we injected DiI in seven mice at the end of the experiments and found that all injections left deposits extending through mid layer V to mid layer VI. In addition, penetrating electrode tracts could be observed in Nissl-stained coronal sections in most mice. Most (81.3 ± 4.7%) of these tracts terminated in layer V at a measured depth of 782 ± 137 μm, with the remainder terminated in upper layer VI.

31. Allred, R. P. *et al.* The vermicelli handling test: a simple quantitative measure of dexterous forepaw function in rats. *J. Neurosci. Methods* **170**, 229–244 (2008).