Supplementary Notes:

1. Cluster analysis based on distance

Previous brain slice experiments have suggested mechanisms by which spines within 5 μm from each other on the same dendrite can interact for plasticity[33,34]. Measuring the distance of neighboring new spines (***neighborhood analysis***), we found that all but one cluster occurred within 2.5 μm on the dendrite, with an average cluster spanning over 1.0 μm. To extend the analysis to cover longer distances, we considered all new spines that formed within 5 μm of each other as a 'cluster', even if there were interspersed stable spine(s) (***distance analysis***). Using distance analysis, 64 clusters were identified in 18 mice during early training (days 0-4). Among them, 62 clusters contained at least two contiguous new spines, suggesting that clusters composed of neighboring new spines (*i.e.*, new spines immediately adjacent to one another) were the predominant form of new spine clusters. In comparison with neighborhood analysis, distance analysis revealed more large clusters (*i.e.*, cluster with three or more new spines). We found that 7 out of 9 of large clusters contained stable existing spines. In one case, the large cluster identified by distance analysis comprised 2 small clusters (*i.e.*, cluster of two neighboring new spines) and a stable spine in between. Thus, this configuration "new spine – new spine – stable spine – new spine – new spine" (n-n-s-n-n) would count as one cluster in distance analysis, but as two clusters in neighborhood analysis (Supplementary Fig. 2a). Similar to the results revealed by neighborhood analysis, 35.0±2.9% new spines that formed during early training appeared in clusters by distance analysis, significantly higher than that observed over the same period of time in control mice (10.3±5.1%, $P<0.01$) and in mice imaged during late training (7.4±4.3%, , $P<0.01$, Supplementary Fig. 2b).

2. Cluster analysis including dendritic filopodia

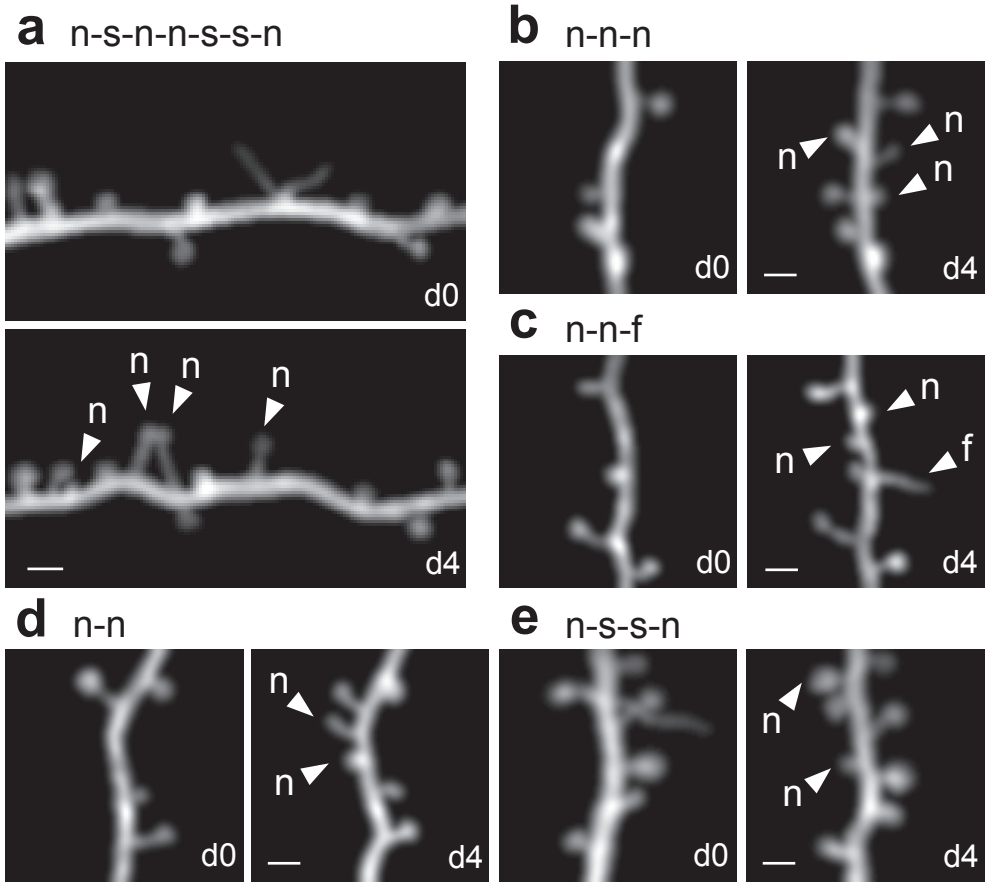
Dendrites in the mammalian brain contain not only spines, but also filopodia. Filopodia are long, thin protrusions without bulbous heads (see Methods), and make up 5-10% of the total dendritic protrusions in the motor cortex of one-month-old mice. While the analyses described in the main text focus on the formation of spine clusters, we also included filopodia in another set of analyses. Consistent with previous studies[35], we found that filopodia were very dynamic in the mouse motor cortex *in vivo*. Almost all filopodia observed before training disappeared after 4 days of training (192 out of 207 filopodia were lost in 18 mice); meanwhile, a similar number of filopodia were added. Thus, including filopodia in our analyses increases the overall turnover rate of dendritic protrusions (Supplementary Fig. 3b).

We also found that clustering of filopodia *per se* was rare during motor skill learning. In total, we identified two clusters that consisted of two neighboring new filopodia, and 1 cluster that comprised two new filopodia with one existing stable spine in between (Supplementary Fig. 3a). When we combined filopodia with spines for cluster analyses, 11 clusters comprising new spine(s) and a new filopodium were identified during early training by distance analysis. Except one case that was composed of one new spine and one neighboring new filopodium (n-f), all the other 10 clusters were composed of a contiguous spine cluster with an additional filopodium (Supplementary Fig. 3a). Overall, filopodia only made a minor contribution to clustered new protrusions and, thus, including filopodia in cluster analysis did not alter our conclusion that new spine clusters (or dendritic protrusion clusters) emerge during learning acquisition (Supplementary Fig. 3c,d).

References for Supplementary Information

33. Harvey, C.D. & Svoboda, K. Locally dynamic synaptic learning rules in pyramidal neuron dendrites. *Nature* **450,** 1195-1200 (2007).
34. Murakoshi, H., Wang, H. & Yasuda, R. Local, persistent activation of Rho GTPases during plasticity of single dendritic spines. *Nature* **472,** 100-104 (2011).

35. Zuo, Y., Lin, A., Chang, P. & Gan, W.B. Development of long-term dendritic spine stability in diverse regions of cerebral cortex. *Neuron* **46,** 181-189 (2005).



Supplementary Figure 1: Examples of clustered spine formation during early training. Images were taken before the training (day 0) and on training day 4. Arrowheads point to all new protrusions observed. Scale bar, 1 μm. n: new spine, f: new filopodium, s: stable spine.

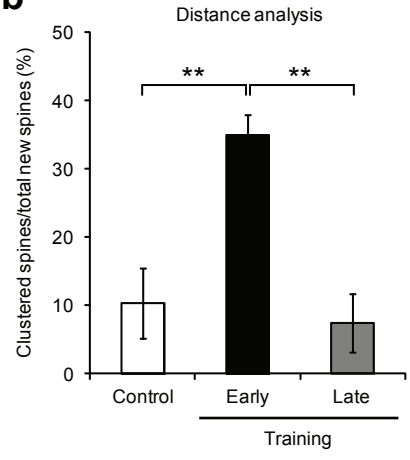
a

Illustrations	Distance analysis				Neighborhood analysis			
	Configurations	Number of cases			Configurations	Number of cases		
		Control	Training (early)	Training (late)		Control	Training (early)	Training (late)
	n-xs-n	1	2	0	NA	-	-	-
	n-n	3	53	2	n-n	3	61	2
	n-n-xs-n	0	5	0				
	n-n-s-n-n	0	1	0				
	n-xs-n-n-s-n	0	1	0				
	n-n-n	0	2	0	n-n-n	0	2	0

 Existing stable spine  New spine

n: 1 new spine; **s**: 1 stable spine; **xs**: 1-3 stable spine(s)

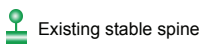
b



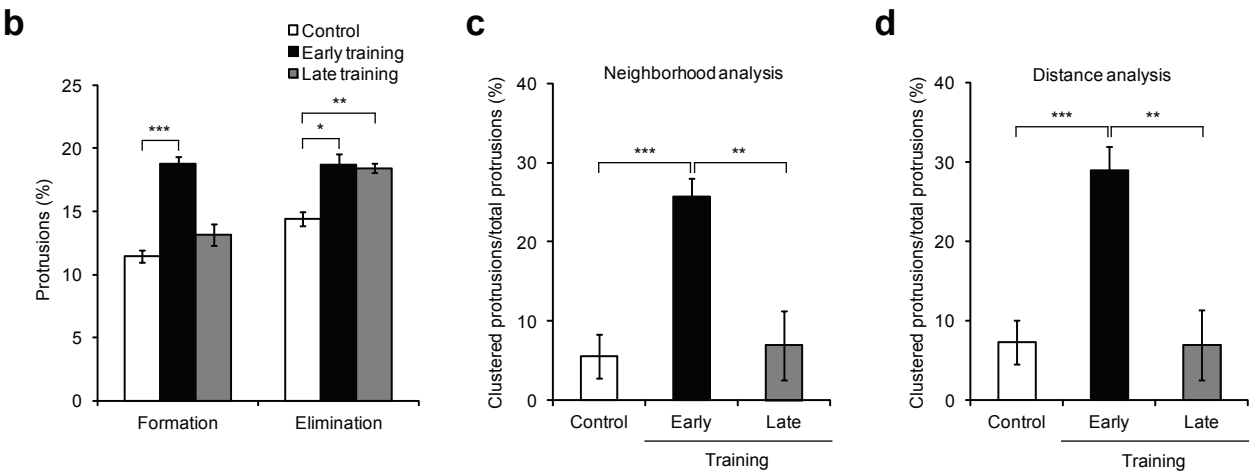
Supplementary Figure 2: Distance analysis reveals that more new spine clusters form during early training compared to control and late training. **a**, Illustration of cluster configurations based on distance and neighborhood analyses, and the number of cases observed in each configuration. **b**, Distance analysis indicates that a significantly higher percentage of new spines formed in clusters over 4 days during early training (n=18) than under the control condition (n=7) and during late training (n=4). $^{**}P<0.01$. Error bars, s.e.m.

a

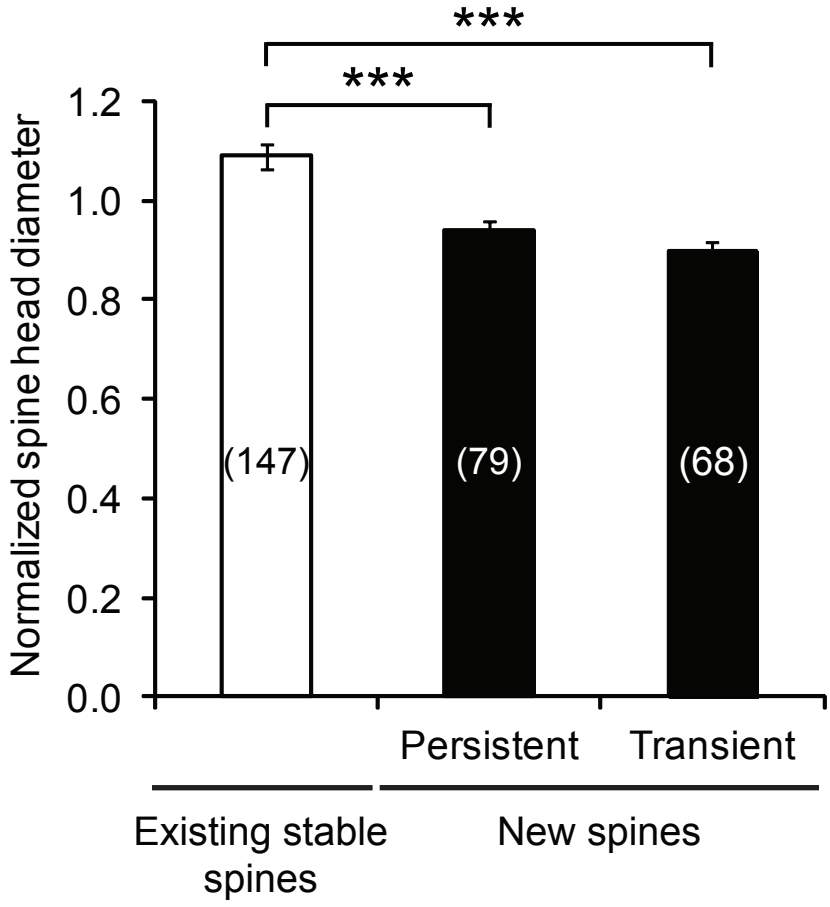
Illustrations	Distance analysis				Neighborhood analysis			
	Configurations	Number of cases			Configurations	Number of cases		
		Control	Training (early)	Training (late)		Control	Training (early)	Training (late)
	n-xs-n	1	2	0	NA	-	-	-
	f-s-f	0	1	0				
	n-n	3	44	2	p-p	4	58	3
	f-f	0	2	0				
	n-f	1	1	1				
	n-n-xs-n	0	5	0				
	n-n-xs-f	0	3	0				
	n-n-s-n-n	0	1	0				
	n-xs-n-n-s-n	0	1	0				
	n-n-n	0	1	0	p-p-p	0	8	0
	n-n-f	0	6	0				
	n-n-n-s-f	0	1	0				



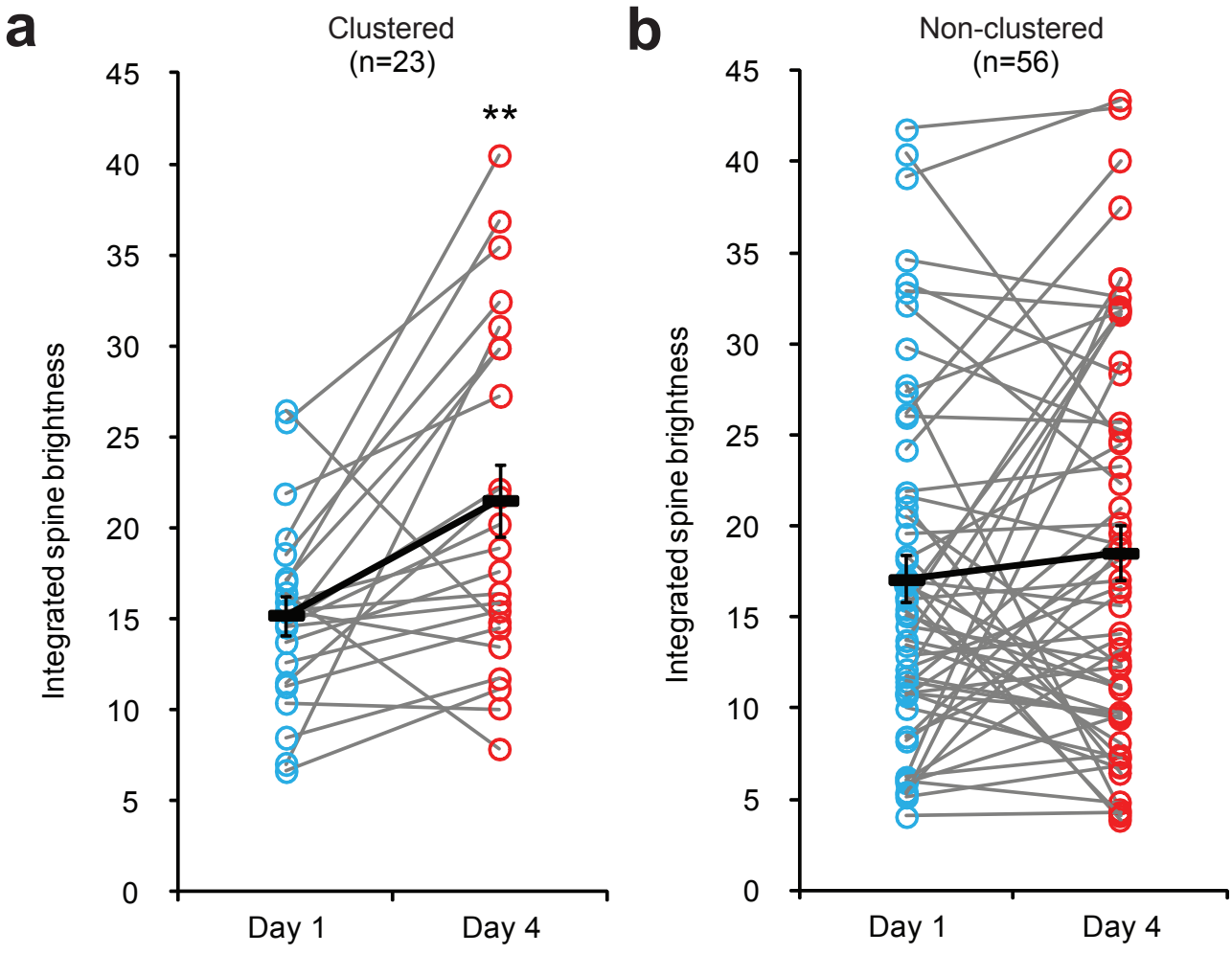
n: 1 new spine; **s**: 1 stable spine; **f**: 1 filopodium; **xs**: 1-3 stable spine(s); **p**: 1 protrusion



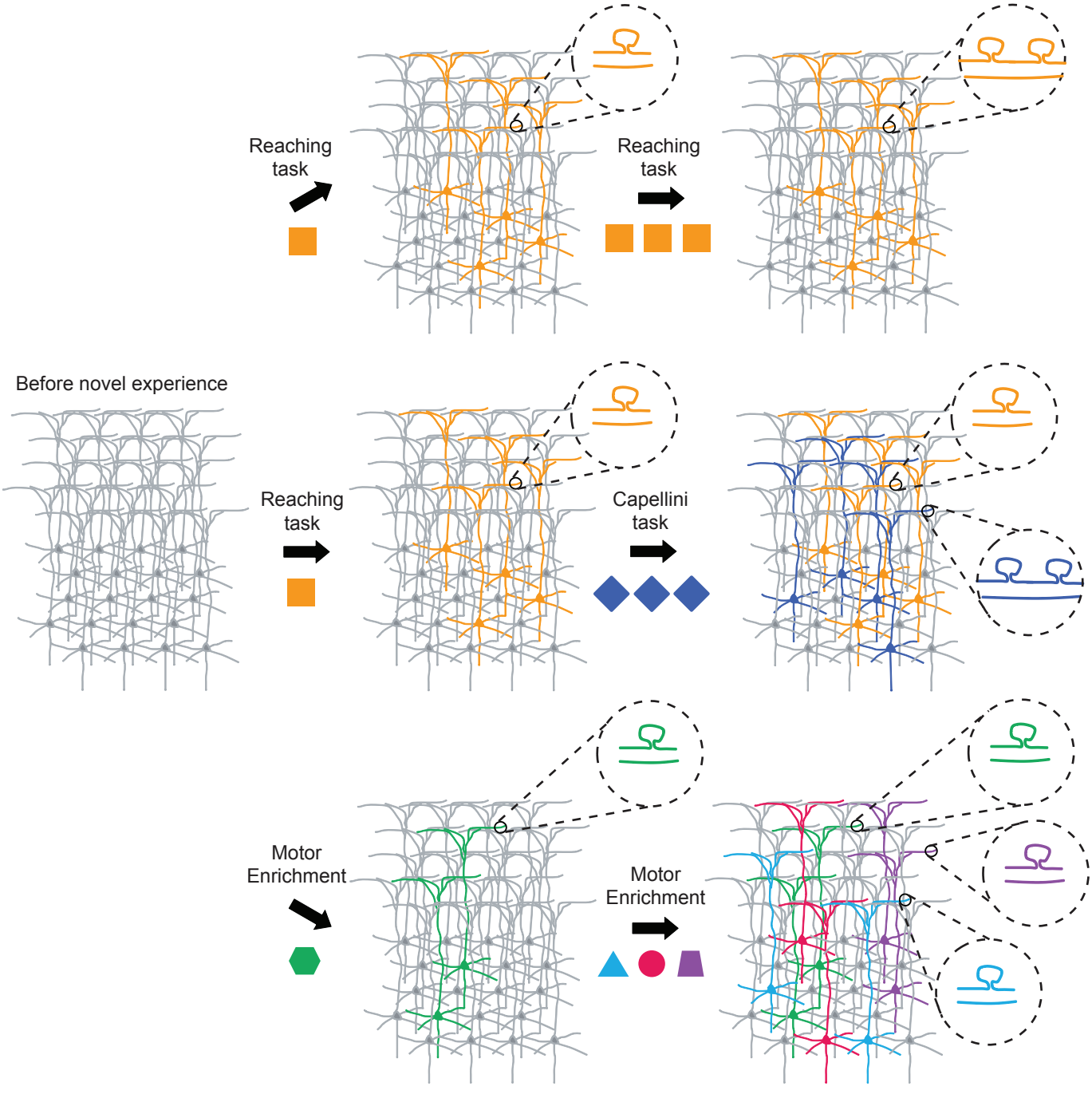
Supplementary Figure 3: A significantly higher percentage of new dendritic protrusions (spines and filopodia) formed in clusters over 4 days during early training, compared to that in control and late training. **a**, Illustration of cluster configurations including filopodia, based on distance and neighborhood analyses, and the number of cases observed in each configuration. **b**, Percentages of dendritic protrusions formed and eliminated over 4 days in control and during different phases of learning. **c, d,** Both neighborhood analysis (c) and distance analysis (d) reveal a significantly higher percentage of new dendritic protrusions formed in clusters over 4 days during early training (n=18), compared to that in control (n=7) and late training (n=4). **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars, s.e.m.



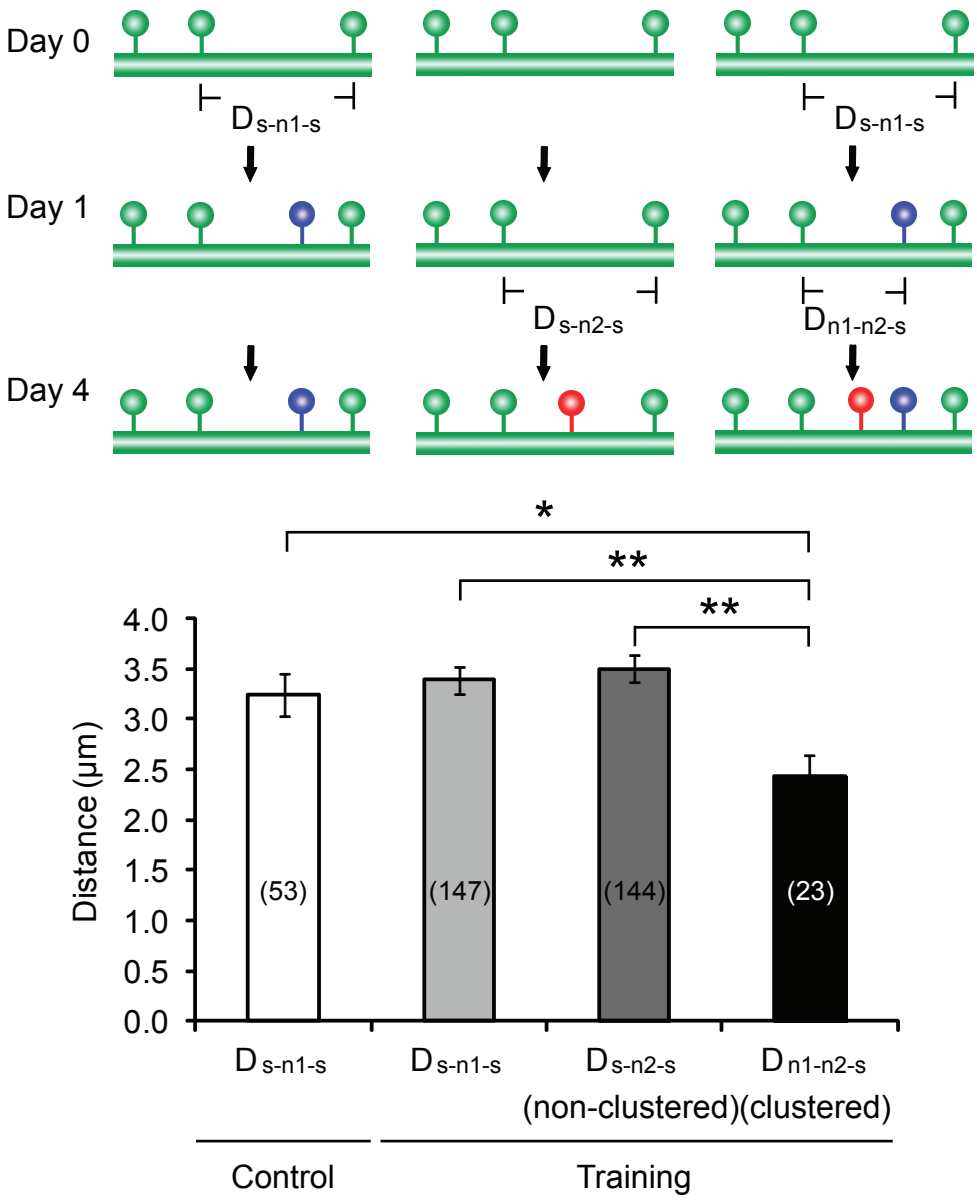
Supplementary Figure 4: Normalized spine head diameters of existing stable spines are significantly larger than those of transient and persistent new spines measured on training day 1. The number of spines analyzed in each condition is indicated on each column. ***P<0.001. Error bars, s.e.m.



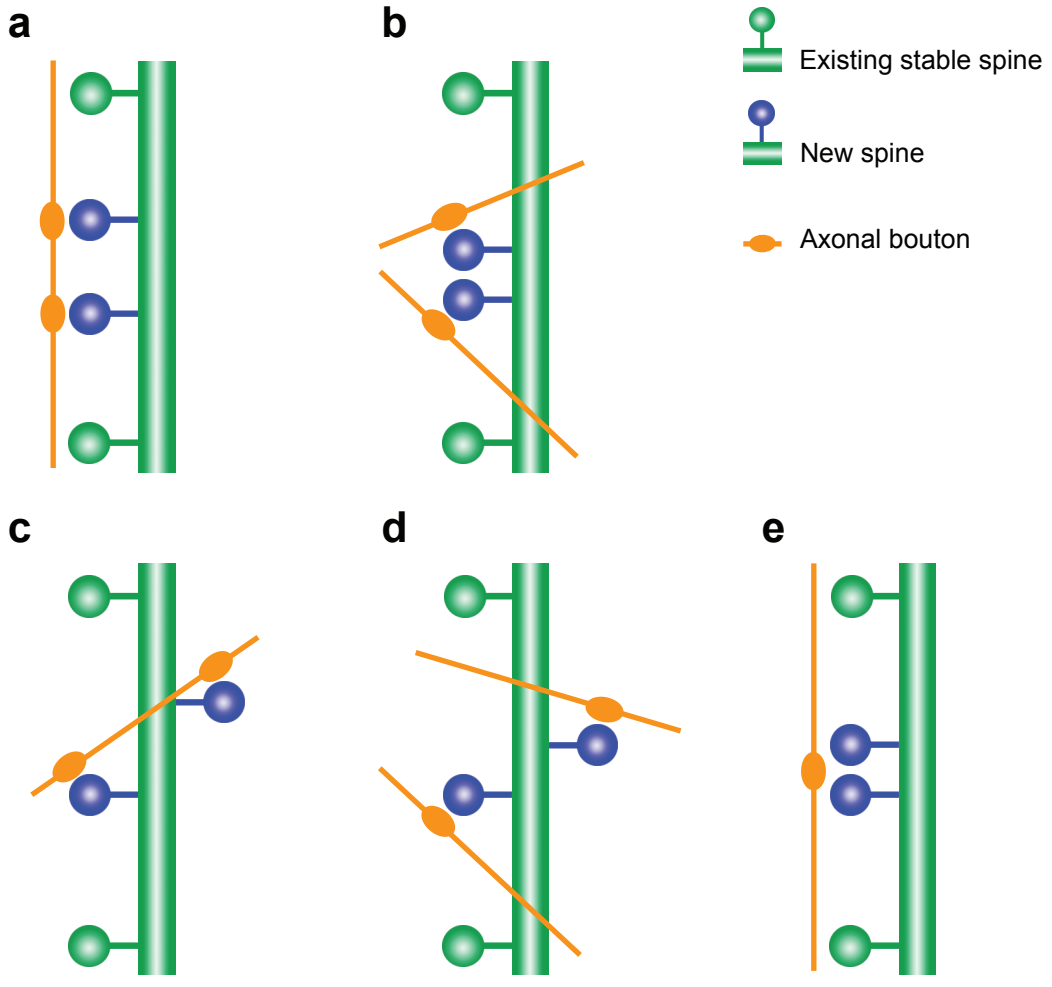
Supplementary Figure 5: The integrated spine brightness increases between training days 1 and 4 in the persistent clustered new spines (**a**), but remains unchanged in persistent non-clustered new spines (**b**). $^{**}P<0.01$. Error bars, s.e.m.



Supplementary Figure 6: A schematic summary of neuronal activation and spine formation in motor-skill training and motor enrichment. Upper panels: the same population of neurons is repetitively activated when animals are trained with the reaching task (orange squares) for four days. Sequentially formed new spines emerge as neighboring pairs on these activated neurons. Middle panels: two different populations of neurons are activated when animals are trained with the reaching task on day 1 (the orange square) and the capellini-handling task (blue diamonds) on days 2 to 4. While new spines formed during the capellini-handling task form in pairs, new spines formed in different tasks do not pair with each other. Lower panels: different populations of neurons are activated during motor enrichment, where stimuli (the green hexagon, the cyan triangle, the red circle and the purple trapezoid) change on a daily basis. New spines formed during motor enrichment participate in different neuronal circuits, and are unlikely to occur next to each other.



Supplementary Figure 7: The distances between two adjacent stable spines, where n1 (Ds-n1-s, left column in illustration), a non-clustered n2 (Ds-n2-s, middle column) or a clustered n2 (Dn1-n2-s, right column) formed under control and training conditions. Ds-n1-s is comparable in control and trained mice. In trained mice, Dn1-n2-s is significantly smaller than Ds-n1-s and Ds-n2-s, while Ds-n2-s is comparable to Ds-n1-s. The number of spines analyzed in each condition is indicated on each column. **P*<0.05, ***P*<0.01. Error bars, s.e.m.



Supplementary Figure 8: Clustered new spines may contact the pre-synaptic partner(s) in different configurations. **a**, Neighboring new spines with the same orientation synapse with two boutons of the same axon. **b**, Neighboring new spines with the same orientation synapse with two different axons. **c**, Neighboring new spines with different orientations synapse with two boutons of the same axon. **d**, Neighboring new spines with different orientations synapse with different axons. **e**, Neighboring new spines with the same orientation synapse with the same bouton of the same axon.

Supplementary Table 1

Dendritic length and spines analyzed under different experimental conditions. The motor cortex contralateral to the trained limb was imaged in all training categories, unless otherwise stated. Data are presented as mean ± s.e.m., **P*<0.05, ***P*<0.01, ****P*<0.001 compared to controls.

Experimental conditions	Total length (μm)	Total spine number analyzed on day 0	New Spine formation		Neighboring new spines in clusters	
			New spine number	Mean±SEM (%)	Clustered spine number	Mean±SEM (%)
Control	1586.72	963	69	7.1±0.5	6	6.8±4.6
Early training	4752.36	2681	386	14.4±0.6***	128	32.5±2.2**
Late training	915.77	573	49	8.6±0.7	4	7.4±4.3
Cross-training	1280.27	718	106	14.8±0.6**	24	22.7±2.3*
Motor enrichment	1822.30	1088	144	13.1±0.7***	12	7.6±2.6