Motor Skill Training Induces Coordinated Strengthening and Weakening between Neighboring Synapses

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Motor skill training promotes the formation of parallel fiber multiple-synapse boutons (MSBs) contacting dendritic spine pairs of Purkinje cells in the rat cerebellum. However, the dendritic origin of such spine pairs is unknown. Here, we used three-dimensional electron microscopy reconstruction of synaptic connectivity to demonstrate that motor skill training selectively induced MSBs contacting two spines arising from the same dendrite, consistent with strengthening of local synaptic efficacy. However, excitatory synapses near MSBs were smaller in motor-trained animals, suggesting compensatory depression of MSB-neighbor synapses. Concerted strengthening and weakening of adjacent synapses may enhance synaptic weight differences for information encoding while maintaining stable overall activity levels within local dendritic segments.

Introduction

Motor skills are acquired through repetitive practice and persist without additional reinforcement. Cerebellar cortex is critical for controlling motor movements (Ito, 2000). Purkinje cells (PCs), the sole efferent neurons of the cerebellar cortex, possess elaborate dendritic arbors bearing numerous dendritic spines, small protrusions that represent the postsynaptic sites of most excitatory synapses, and exhibit experience-dependent remodeling (Hering and Sheng, 2001). In the adult brain, PC primary dendrites receive excitatory input from a single climbing fiber, whereas higher-order branches are innervated by multiple parallel fibers (PFs) (Fig. 1A). Coactivation of these two inputs induces long-term depression (LTD) of PF–PC synapses (Linden and Connor, 1995). Although LTD is widely considered to underlie motor learning (Aiba et al., 1994; Feil et al., 2003; Hansel et al., 2006), several studies argue against this model (Shibuki et al., 1996; Welsh et al., 2005; Schonewille et al., 2011). Thus, it is unclear to what extent LTD contributes to cerebellar motor learning.

Ultrastructural experiments have consistently demonstrated formation of new PF–PC synapses after complex motor skill training but not with simple motor activity such as locomotion (Black et al., 1990; Kleim et al., 1998). Motor training also markedly increases the incidence of PF multiple-synapse boutons (MSBs) contacting spine pairs on PCs (Fig. 1B) (Federmeier et al., 2002; Kim et al., 2002). MSBs have been observed in several brain regions, including hippocampus, visual cortex, motor cortex, and cerebellum, after various behavioral paradigms (Jones et al., 1997, 1999; Geinisman et al., 2001; Federmeier et al., 2002; Kim et al., 2002) and long-term potentiation (LTP) (Toni et al., 1999; Fiala et al., 2002), suggesting that MSBs may contribute to widespread forms of learning. A key unresolved question regards the dendritic origin of spine pairs on MSBs induced by learning paradigms, which has important functional implications. MSB spine pairs arising from the same dendrite would enhance local synaptic efficacy via simultaneous activation of two adjacent synapses (Fig. 1C), whereas MSB spines originating from different dendritic segments suggests network reorganization by recruitment of postsynaptic partners from neighboring dendrites (Fig. 1D).

Here, we investigated this issue using serial section transmission electron microscopy (TEM) in the cerebellum of rats trained with a motor skill task. Training selectively induced MSBs contacting spine pairs originating from the same dendrite, suggesting local synaptic strengthening. Remarkably, synapses adjacent to MSBs were significantly smaller in trained animals. These findings suggest that coordinated strengthening and weakening between neighboring synapses may enhance synaptic weight differences for improved motor performance while maintaining stable overall activity levels in local dendritic segments.

Materials and Methods

Behavioral training. Three-month-old Sprague Dawley male rats were randomly allocated to “acrobatic” motor skill (AC), simple motor activ-

Received Feb. 21, 2012; revised April 1, 2013; accepted May 3, 2013.

Author contributions: W.T.G. and I.J.R. designed research; K.J.L. and I.S.P. performed research; H.K. contributed unpublished reagents/analytic tools; K.J.L., D.T.S.P., and I.J.R. analyzed data; K.J.L. and D.T.S.P. wrote the paper.

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (NRF-2010-0012753), Brain Korea 21 Program for Biomedical Science (K.J.L. and I.J.R.), Korea Brain Research Institute (KBRI) Basic Research Program of the Ministry of Science, ICT, and Future Planning (2031-415; K.J.L.) and National Institutes of Health Grant AG010154 (W.T.G.). We thank Dr. I. J. Weiler and Pak laboratory members for their critical reading and comments on this manuscript and Jee-Woong Kim and Sang-Hoon Lee for technical assistance.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.0848-12.2013

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Figure 1. Dendritic origin of spine pairs on MSBs. **A**, Types of excitatory synapses onto cerebellar PCs. **B**, Electron microscopic image of an MSB. Scale bar, 0.5 μm . **C**, **D**, Spine pairs on MSBs may originate from the same (**C**) or different (**D**) dendritic segments. CF, Climbing fiber; S, spine.

ity control (MC), or inactive (IC) conditions ($n = 6$ per group). AC animals were trained to traverse an elevated obstacle course with rods, ladders, chains, barriers, parallel bars, and grid platform for 25 d (four trials per day) as described previously (Kleim et al., 1998; Jones et al., 1999; Lee et al., 2007). MC animals traversed a flat, obstacle-free runway equal in length and height to the AC task. IC rats remained sedentary and received equal amounts of daily handling as other groups. All animal experiments were performed within guidelines of the Korean Academy of Medical Sciences.

Stereological analyses. Tissues were prepared using standard procedures for TEM as described previously (Kim et al., 2002). Briefly, animals were anesthetized with pentobarbital (100 mg/kg) and perfused with 2% paraformaldehyde/2.5% glutaraldehyde/0.1 M phosphate buffer, pH 7.4. Sagittal 300- μm -thick slices were postfixed in 2% OsO_4 , dehydrated, embedded in Eponate-12 resin (Ted Pella), and trimmed into the paramedian lobule (PML). Two sets of 60 serial 1- μm -thick sections were obtained per animal, stained with toluidine blue, and imaged using a Carl Zeiss Axio microscope. We used the physical disector (Sterio, 1984) to measure molecular layer volume per PC as described previously (Kleim et al., 1998). A 400- μm -width counting frame was placed on the molecular layer and frame height defined by molecular layer thickness. Five thickness measures per section were obtained by measuring PC layer-to-pial surface distance. Pairs of adjacent sections within each series were then successively compared. The first section in the series became “Reference” and the second “Lookup” section; then the second section became Reference for the third, etc. Because each PC has only one nucleolus, the number of nucleoli in the Reference but not Lookup section was counted. Disector volume was calculated as (counting frame area) $\times$ (section thickness) $\times$ (number of sections).

Blocks were further trimmed into the outer one-third of the molecular layer. Twelve to nineteen serial 70-nm-thick sections per animal were collected on Formvar-coated slot grids and recorded on an H-7500 TEM (Hitachi) (8000 $\times$ magnification, 75 kV). Section thickness was calibrated against minimal folds and cylindrical objects (e.g., mitochondria) (Fiala and Harris, 2001). Using the disector, we obtained PF–PC synaptic densities from three different PML areas per animal (33–54 disector pairs per animal; frame area, 25 μm^2), which were averaged to produce animal means. For each condition, six animal means were compared for statistics. Total sample numbers per group were 244–260 PCs, 132–168 synapses. Synapses were counted when the postsynaptic density (PSD) in a Reference section had completely disappeared in the Lookup. PF boutons with small clusters of synaptic vesicles concentrated near active

zones were readily distinguished from large climbing fiber boutons containing uniformly and densely packed synaptic vesicles and some dense-core vesicles (Palay and Chan-Palay, 1974; Cesa et al., 2003). PC dendrites were identified by characteristic hypolemmal cisterns of smooth endoplasmic reticulum (Martone et al., 1993; Kleim et al., 1998; Cesa et al., 2003). Synapse number per PC was calculated as (cubic micrometers molecular layer volume/PC) $\times$ (synapses/cubic micrometers). All analyses were performed blind to experimental conditions.

Three-dimensional reconstruction of MSBs. For individual MSBs in the outer one-third of the molecular layer, 30–50 serial 70-nm-thick sections were obtained by ultramicrotome. MSBs were randomly selected and recorded at 20,000–25,000 $\times$ magnification using TEM. Reconstruct software (<http://synapses.clm.utexas.edu/tools/index.stm>) was used for aligning and tracing serial MSB profiles. Ninety-five MSBs ($n = 31$ –32, 5–6 per animal) were classified as same- or different-dendrite MSBs. In each animal, the number of same- and different-dendrite MSBs per PC was calculated by multiplying percentage of same- and different-dendrite MSBs by the number of MSBs per PC obtained from disector analysis.

PSD areas. We analyzed serial PSD profiles of MSBs ($n = 24$ –28 per condition) and MSB-neighbor synapses (same-dendrite, $n = 84$ –91 from 12–17 segments per condition; different-dendrite, $n = 87$ –88 from 12–14 segments per condition). Based on magnification and series length, nearby synapses could be visualized $\leq 5 \mu\text{m}$ from MSBs. PSD areas were calculated as (sum of PSD lengths) $\times$ (section thickness) $\times$ (section numbers) as described previously (Harris and Stevens, 1988). Synapses on the same dendrite of MSBs in AC animals ($n = 87$) were analyzed for correlation of PSD size with distance to MSBs. PSDs were selected for optimal angle of sectioning (i.e., perpendicular to synaptic cleft). Synapses with incomplete PSD profiles or negative stain precipitates were excluded.

Statistical analyses. One-way ANOVA with Tukey’s HSD *post hoc* test was used for multiple group comparisons, ANOVA with repeated measures for behavioral data analysis, and Kolmogorov–Smirnov (KS) test to compare distributions between groups for PSD analysis. $p < 0.05$ was considered a statistically significant difference. Data are expressed as means $\pm$ SEM.

Results

Motor training induces same-dendrite MSBs

To induce formation of PF MSBs contacting spine pairs of PCs, rats were trained in the AC task, a well-established “acrobatic” motor skill task (Black et al., 1990; Kleim et al., 1998; Jones et al., 1999; Federmeier et al., 2002; Kim et al., 2002; Lee et al., 2007) involving an elevated obstacle course requiring considerable motor coordination and balance to complete (Fig. 2A). Latency to complete the task and number of errors (slips or foot faults) in each trial significantly decreased with training (Fig. 2B,C), indicating substantially improved motor performance in AC animals (latency, $F_{(24, 120)} = 39.14$, $p < 0.001$; errors, $F_{(24, 120)} = 30.61$, $p < 0.001$).

Previous studies reported an increase in cerebellar molecular layer thickness and astrocytic volume after motor skill learning (Black et al., 1990; Kleim et al., 1998, 2007). Indeed, our motor skill training caused a significant $\sim 10\%$ increase in molecular layer thickness in the cerebellar PML, a region strongly associated with limb movements (Collins et al., 1986), in AC animals compared with MC or IC animals (in μm : IC, 255 ± 5 ; MC, 261 ± 8 ; AC, 280 ± 5 , $p = 0.03$; $n = 6$; one-way ANOVA with Tukey’s HSD test) (Fig. 2D,E). Despite the larger molecular layer, there was no reduction in the mean density of PF–PC synapses in AC animals (synapses/ μm^3 : IC, 0.50 ± 0.02 ; MC, 0.53 ± 0.03 ; AC, 0.57 ± 0.03 , $p = 0.11$; $n = 6$; one-way ANOVA), suggesting new synapse formation during motor skill training.

PF–PC synapses were classified as single-synapse boutons (SSBs) contacting one spine, MSBs contacting spine pairs, and multiple-synapse spines (MSSs) in which one spine contacts two

Figure 2. Acrobatic motor skill training. **A**, The motor skill task. **B**, Latency to complete a trial. **C**, Number of errors per trial. **D**, Sagittal section from the cerebellar PML. Scale bar, 0.5 mm. Dotted lines indicate regions of stereological analysis. **E**, **F**, Molecular layer (ML) thickness (**E**) and molecular layer volume per PC (**F**) in the PML. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are means $\pm$ SEM.

Figure 3. Motor learning promotes synapse formation. **A**, Example of SSB. A PF bouton synapses with one PC dendritic spine (S). Scale bar, 0.5 μm. **B**, Quantification of SSBs per PC; * $p = 0.02$. **C**, Example of MSB. **D**, Quantification of MSBs per PC; ** $p = 0.007$. **E**, Example of MSS contacting two PF boutons. **F**, Quantification of MSSs per PC; $p = 0.96$. Data are means $\pm$ SEM.

PF boutons (Fig. 3*A,C,E*). Again, we observed no significant decrease in the mean density of each type of synapse with AC training (number/μm³ SSBs: IC, 0.43 ± 0.02 ; MC, 0.45 ± 0.02 ; AC, 0.48 ± 0.03 , $p = 0.28$; number/μm³ MSBs: IC, 0.023 ± 0.001 ; MC, 0.022 ± 0.003 ; AC, 0.032 ± 0.004 , $p = 0.06$; number/μm³ MSSs: IC, 0.015 ± 0.005 ; MC, 0.017 ± 0.004 ; AC, 0.014 ± 0.005 , $p = 0.92$; $n = 6$; one-way ANOVA).

Because of the enlarged molecular layer, measures of synapse density alone may not reveal changes in synapse number. Because PC number in mature cerebellum is stable under physiological conditions (Grimaldi and Rossi, 2006), the synapses/neuron ratio accurately reflects changes in synapse number (Anker and Cragg, 1974). Using unbiased stereological analysis, we found significantly expanded molecular layer volume per PC in AC animals (Fig. 2*F*) ($\times 10^5$ μm³/PC: IC, 2.51 ± 0.12 ; MC, 2.50 ± 0.09 ; AC, 3.07 ± 0.15 , $p = 0.007$; $n = 6$; one-way ANOVA). Together with synapse density measurements, we then calculated synapses/PC (see Materials and Methods). Acrobatic training markedly increased the mean number of MSBs as well as SSBs per PC (Fig. 3*A–D*) (MSBs/PC: IC, 5600 ± 300 ; MC, 5500 ± 900 ; AC, 9800 ± 1300 , $p = 0.007$; SSBs/PC: IC, $105,400 \pm 7300$; MC, $110,900 \pm 6900$; AC, $148,300 \pm 15,200$, $p = 0.02$; $n = 6$; one-way ANOVA). These data support previous studies demonstrating increased dendritic spine density on PC dendrites after AC training (Lee et al., 2007). However, there were no group differences in MSSs (Fig. 3*E,F*) (MSSs/PC: IC, 3900 ± 1300 ; MC, 4200 ± 900 ; AC, 4400 ± 1500 , $p = 0.96$). MC animals exhibited no difference in any type of PF–PC synapse compared with IC animals (Fig. 3*B,D,F*), excluding the possibility that simple motor activity increased synapse formation.

Next, we examined the dendritic origin of spine pairs on PF MSBs using serial section TEM. Reconstructed MSBs were classified as same- or different-dendrite MSBs (Fig. 4*A,B*). There were no significant group differences in the percentage of same- or different-dendrite MSBs (% same-dendrite MSBs: IC, 78.6 ± 7.4 ; MC, 76.7 ± 6.2 ; AC, 85.0 ± 5.3 ; % different-dendrite MSBs: IC, 21.4 ± 7.4 ; MC, 23.3 ± 6.2 ; AC, 15.0 ± 5.3 , $p = 0.63$; $n = 6$; one-way ANOVA). Based on disector analysis data (Fig. 3*C,D*), we estimated the mean number of same- and different-dendrite MSBs per PC. AC animals had 95% more same-dendrite MSBs per PC compared with controls (Fig. 4*C*) (IC, 4300 ± 300 ; MC, 4300 ± 800 ; AC, 8400 ± 1300 , $p = 0.008$; $n = 6$; one-way ANOVA), but there were no differences in the number of different-dendrite MSBs among groups (IC, 1300 ± 500 ; MC, 1200 ± 300 ; AC, 1400 ± 500 , $p = 0.98$; $n = 6$; one-way ANOVA). Thus, motor skill training promotes formation primarily of same-dendrite MSBs.

Weakening of synapses adjacent to MSBs

Dendritic spines on MSBs displayed typical mature morphologies with PSDs apposed to presynaptic vesicles, suggestive of functional synapses. Most PF–PC synapses had macular PSDs, whereas perforated or segmented PSDs were rare. Because PSD size is proportional to postsynaptic receptor density and presynaptic docked vesicle number (Nusser et al., 1998; Schikorski and Stevens, 1999), we calculated PSD areas on individual MSBs by measuring serial PSD profiles (Fig. 4*D*). Interestingly, the two PSDs on an MSB were different in size (“large” PSD, 0.16 ± 0.01 μm²; “small” PSD, 0.10 ± 0.004 μm², $p < 0.001$; $n = 78$; paired Student’s *t* test), which suggests that the PSDs of an MSB may be formed at different times. There were no group differences in large, small, or mean PSD areas of MSBs (Fig. 4*E*; data not shown) (mean MSB PSD area: IC, 0.13 ± 0.01 μm²; MC, 0.13 ± 0.01

Figure 4. Motor learning induces same-dendrite MSBs. **A, B**, MSBs were classified as same-dendrite MSBs (sdMSBs) (**A**) or different-dendrite MSBs (ddMSBs) (**B**) depending on dendritic origin of spine pairs. S, Spine; D, dendrite. Scale bar, 0.5 μm. **C**, Quantification of sdMSBs and ddMSBs per PC; ** $p = 0.008$. **D**, Image showing PSDs of an MSB (arrows) and a neighboring synapse (arrowhead). Scale bar, 0.5 μm. **E, F**, Cumulative distributions of PSD sizes of MSBs (**E**) ($p > 0.92$) and MSB-neighboring synapses on the same dendrite (**F**) ($p < 0.01$). **G**, PSD size of synapses with distance to MSB (** $p = 0.002$). Data are means $\pm$ SEM. **H**, Hypothetical model of motor training-induced plasticity. Training promotes formation of sdMSBs and increases total synapse number but weakens MSB-neighbor synapses. Numbers inside lightning symbols denote relative synapse strength (arbitrary values).

μm², $p = 0.95$ vs IC; AC, 0.13 ± 0.01 μm², $p = 0.92$ vs IC; $n = 24$ –28; KS test), suggesting that MSBs newly generated during motor learning were comparable with those of preexisting MSBs in control animals. Because the sum of two PSDs on MSB synapses was twice the PSD size of SSBs [sum of PSD areas on MSBs ($n = 78$), 0.26 ± 0.01 μm²; PSD area on single synapse in IC ($n = 179$), 0.13 ± 0.004 μm²; $p < 2.5 \times 10^{-40}$; Student's t test], same-dendrite MSBs may enhance local synaptic efficacy.

We next measured PSDs of synapses close to MSBs (<5 μm distance), originating from either the same or different local dendritic segments. Interestingly, PSDs of nearby same-dendrite synapses were significantly smaller in the AC group compared with both MC and IC groups (Fig. 4F) (IC, 0.13 ± 0.01 μm²; MC, 0.13 ± 0.01 μm², $p = 0.87$ vs IC; AC, 0.11 ± 0.01 μm², $p < 0.01$ vs IC; $n = 84$ –91; KS test). However, PSD areas of nearby different-dendrite synapses were unchanged [IC, 0.13 ± 0.01 μm²; MC, 0.13 ± 0.01 μm², $p = 0.93$ vs IC; AC, 0.14 ± 0.01 μm², $p = 0.15$ vs IC; $n = 87$ –88; KS test]. Finally, we examined the relationship between PSD size and distance to the MSB along the same dendrite in AC animals (Fig. 4G). Synapses in the close vicinity (<2 μm linear distance) of MSBs had smaller PSDs than those of MSBs, whereas PSDs located 2–5 μm from MSBs were not significantly different [MSB ($n = 26$), 0.13 ± 0.01 μm²; PSDs 0–2 μm from MSBs ($n = 45$), 0.11 ± 0.01 μm², $p = 0.002$; PSDs 2–5 μm from MSBs ($n = 42$), 0.12 ± 0.01 μm², $p = 0.06$; KS test]. These findings indicate a strong correlation between PSD size and MSB proximity, suggesting potential compensatory weakening of synapses immediately adjacent to MSBs.

Discussion

We have demonstrated that motor skill training promoted the selective formation of same-dendrite MSBs *in vivo*. Most MSBs contacted separate adjacent spines rather than branched spines, suggesting formation by spine outgrowth rather than splitting of existing spines (Fiala et al., 2002). Similar same-dendrite MSBs are produced in hippocampal CA1 neurons after LTP stimulation *in vitro* (Toni et al., 1999). Because combined activation of two adjacent synapses would produce a greater net response than single synapses, we propose that motor training strengthens a subset of PF–PC synapses by LTP-induced formation of same-dendrite MSBs. Indeed, LTP may be associated with cerebellar motor learning (Lev-Ram et al., 2002; Schonewille et al., 2010). Although we focused on the PML, we observed similar MSBs in other regions important for limb movement, lobules IV and V of the cerebellar vermis (Kim et al., 2002), suggesting a more widespread mechanism. However, our data do not reveal what percentage of PCs or dendritic branches are affected in these regions. Another caveat is that our complex behavioral paradigm engages motor learning as well as familiarity, motivation, and non-motor learning that could affect other brain areas. It would be informative to examine whether same-dendrite MSBs are pro-

duced in more cerebellum-specific behavioral tasks, such as vestibulo-ocular reflex (VOR) adaptation or eyeblink conditioning (Shibuki et al., 1996; Schonewille et al., 2011).

In addition to increased formation of same-dendrite MSBs, motor skill training also increased the number of single PF–PC synapses (but not PF-to-interneuron synapses) (Kleim et al., 1998). Because the intervaricosity distance along each PF was not reduced in AC animals (Federmeier et al., 2002), motor training may induce elongation of preexisting PFs to add more synapses and recruit additional PCs to existing neural circuits for improved coordination.

Importantly, we found that PSDs in the immediate vicinity of MSBs on the same dendrite were smaller in AC animals, suggesting compensatory depression of neighboring synapses. Our measurements of PSD areas and the percentage of MSBs were comparable with values reported previously (Harris and Stevens, 1988; Xu-Friedman et al., 2001). Smaller PSDs adjacent to MSBs is consistent with our previous report that AC animals had more thin spines on PC distal dendrites (Lee et al., 2007). Indeed, a previous study has reported heterosynaptic depression after LTP induction in amygdala (Royer and Paré, 2003), although the precise spatial relationship between neighboring synapses was not addressed. We speculate that reallocation of synaptic components from neighboring synapses to MSBs could be one molecular mechanism to account for such a phenomenon. LTD of synapses adjacent to MSBs may be valuable computationally to enhance synaptic weight differences between neighboring PF synapses, thereby increasing signal-to-noise and facilitating optimal encoding of motor skills.

The role of LTD in motor learning is controversial. Various strains of LTD-lacking mutant mice exhibit impairment of motor learning (Aiba et al., 1994; Feil et al., 2003; Hansel et al., 2006). However, pharmacological blockade of LTD does not affect motor learning (Welsh et al., 2005), and some mutant mice strains lacking LTD show normal motor coordination measured by eyeblink conditioning and VOR adaptation (Shibuki et al., 1996; Schonewille et al., 2011), suggesting that LTD is not essential for cerebellar motor learning. However, if LTD acts in synergy with LTP (on different groups of synapses on the same PC dendritic segments) (Gao et al., 2012), both processes may contribute under physiological circumstances and possibly compensate for each other when one is selectively impaired. Indeed, several lines of evidence implicate both strengthening and weakening of PF–PC synapses after motor learning (McCormick and Thompson, 1984; Ojakangas and Ebner, 1992). In line with previous work on balanced structural plasticity (Bourne and Harris, 2011), experience-dependent coordinated recruitment of opposing plasticity mechanisms in local dendritic segments may help unify disparate findings in the field variously implicating either LTP or LTD in motor learning.

The current work cannot distinguish which synaptic modification comes first during motor learning: formation of new MSBs or generation of smaller PSDs. Initial formation of MSBs may induce weakening of adjacent synapses in a homeostatic manner to maintain overall activity in a dendritic segment relatively stable, thereby preserving network stability (Rabinowitch and Segev, 2006; Pozo and Goda, 2010) (Fig. 4H). Alternatively, selective weakening of certain synapses may be compensated by subsequent strengthening of others through MSB formation. Future studies at multiple time points could address this issue. Smaller synapses in the close vicinity of MSBs could also represent a volume adjustment to help accommodate more synapses.

Thus, synaptic remodeling events may not necessarily represent specific acts of information storage but structural consequences of such encoding.

In summary, complex motor skill training induces orchestrated strengthening and weakening of neighboring PF synapses onto PCs, providing insight into how structural changes may underlie behavior in the cerebellar molecular layer and perhaps more generally in the brain.

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