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Learning causes synaptogenesis, whereas motor activity causes angiogenesis, in cerebellar cortex of adult rats

(paramedian lobule/neural plasticity/exercise)

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ABSTRACT **The role of the cerebellar cortex in motor learning was investigated by comparing the paramedian lobule of adult rats given difficult acrobatic training to that of rats that had been given extensive physical exercise or had been inactive. The paramedian lobule is activated during limb movements used in both acrobatic training and physical exercise. Acrobatic animals had greater numbers of synapses per Purkinje cell than animals from the exercise or inactive groups. No significant difference in synapse number or size between the exercised and inactive groups was found. This indicates that motor learning required of the acrobatic animals, and not repetitive use of synapses during physical exercise, generates new synapses in cerebellar cortex. In contrast, exercise animals had a greater density of blood vessels in the molecular layer than did either the acrobatic or inactive animals, suggesting that increased synaptic activity elicited compensatory angiogenesis.**

Although many aspects of experience can alter synaptic connectivity (1–5), it has been difficult to relate unequivocally these changes to learning and memory because the morphological effects of learning could not be isolated from those of behaviors required to perform the task. For example, maze training (3) and forelimb reach training (5) can alter neuronal morphology, but substantial repetition of movements is required for learning these tasks. Thus it is not possible to ascribe the morphological effects to learning *per se*.

The cerebellar cortex may be particularly appropriate for testing hypotheses about synaptic plasticity because empirical evidence has implicated cerebellar cortex in motor skill learning (6, 7, 36), and there is some indication that synapse formation underlies cerebellar cortical learning, as suggested by dendritic-field changes in Purkinje cells of rodents and monkeys exposed to challenging sensory-motor environments (8–10). Synaptogenesis in adult rat cerebellar cortex also occurs when afferents are cut (11, 12). Furthermore, theorists have noted the suitability of cerebellar cortex for motor learning, with its convergence of two afferent systems conveying extensive somatic and cerebral state information upon the Purkinje cell, a single-output neuron that modulates motor activity (13–17).

The results of the present study show that learning, as opposed to the motor activity necessary for learning a complex motor task, is responsible for synapse formation in the cerebellar cortex. We report a dissociation of learning and motor activity, in which animals provided with complex visuomotor learning and minimal motor activity (acrobatic training) form substantial numbers of new synapses in cerebellar cortex, whereas animals given extensive locomotor exercise with minimal opportunities for learning (repetitive exercise) formed new blood vessels but formed no more new synapses than animals in an inactive control group.

MATERIAL AND METHODS

Animals and Training. Thirty-eight adult Long–Evans hooded female rats, kept in small groups until 10 months old, were housed individually for 30 days in one of four experimental groups. To the extent possible, each litter contributed equally to each group. Rats in the acrobatic condition (AC) were given progressively longer and more difficult trials on an elevated path consisting of balance beams, see-saws, rope bridges, and other obstacles, until they reached five trials of seven obstacles each day after the first week. Gentle physical encouragement, as well as small portions of sweetened and/or chocolate-flavored rat chow, provided the rats with motivation to traverse the path. (Equivalent portions were given to the other rats in their home cages.) Initially the AC rats had difficulty traversing wide stable platforms, but at the end of training they were easily able to pass over the most difficult obstacles, such as pencil-wide dowels and loosely suspended ropes and chains (Fig. 1). These trials required from 5.6 to 8.6 hr total training time for members of this group. Fig. 2 shows one aspect of the substantial improvement in visuomotor skills acquired by these rats. During the first week of training, AC animals needed considerable time to traverse one simple segment of the pathway (e.g., a 10-cm wide and 1-m long board), but during the last week these same animals were able to rapidly traverse a much more difficult segment (e.g., a rope bridge or thin dowel). This dramatic improvement in their ability to perform such tasks thus reflects a substantial amount of motor learning.

Rats in the forced exercise (FX) condition walked quickly at 10 m/min on a treadmill for progressively longer periods each day until they were walking for 1 hr daily. While their limited physical endurance demanded a gradually progressive running schedule, all of the FX rats were able to master locomotion on the treadmill by the first day, evidence that the amount of learning required by this task was relatively small. Rats in the voluntary exercise (VX) group had free access to a running wheel attached to their cage, with the number of wheel rotations recorded daily. Within the first 3 days all of the VX animals had completed over 200 wheel rotations, evidence that they had mastered the simple balancing and coordination required to run in a wheel. Animals in the inactive condition (IC) were kept in standard laboratory cages with minimal opportunities for learning or exercise. The IC and VX groups were handled for 1 min daily, serving

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Abbreviations: AC, acrobatic condition; IC, inactive condition; FX, forced exercise; VX, voluntary exercise; PML, paramedian lobule; PST, postsynaptic thickening; NS, not significant.

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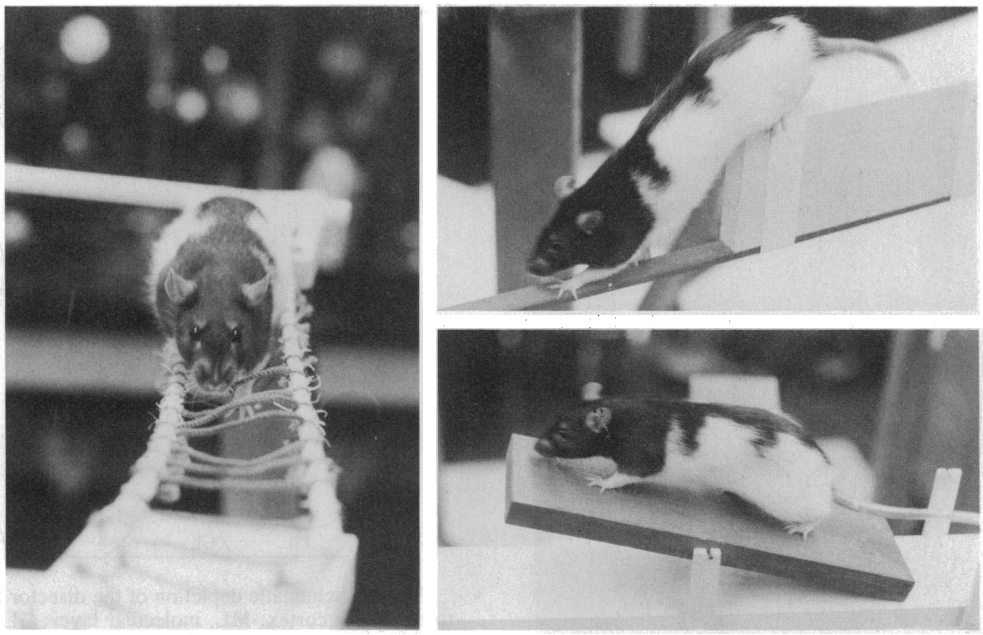

FIG. 1. AC rat traversing a rope ladder, high step, and seesaw during acrobatics training. Relatively simple tasks were noticeably difficult for the rats at the beginning of the training, whereas more complicated tasks were quite easily negotiated at the end, indicating that a substantial amount of visuomotor learning had occurred.

to control for the handling of the AC and FX groups required during their training.

It is important to emphasize that a fundamental component of this experimental design is a contrast between learning and exercise. The AC rats had to keep learning new obstacle paths for 30 days, whereas rats in the FX and VX groups had each completely mastered their simple task within a few days and were consequently involved in much less visuomotor learning. In the exercise dimension, however, the FX and VX were involved in much more repetitive exercise than the AC rats. The IC rats served as a control group with very little opportunity for learning or exercise.

Tissue Preparation. The rats were anesthetized with ketamine (120 mg/kg) and xylazine (13 mg/kg) and then perfused with physiological saline solution followed by a paraformaldehyde/glutaraldehyde fixative. Tissue from each animal was assigned a code number that did not reveal individual treatment conditions. Tissue blocks were removed from the left and right paramedian lobules (PMLs) of the cerebellum and prepared for conventional transmission electron microscopy. Forty serial 1-μm-thick sections cut at random intervals along the transverse length of the folia and aligned to the Purkinje dendritic tree were then stained with toluidine blue (Fig. 3). Silver thin sections (approximately 70 nm thick) from these blocks were then stained with uranyl acetate and lead citrate.

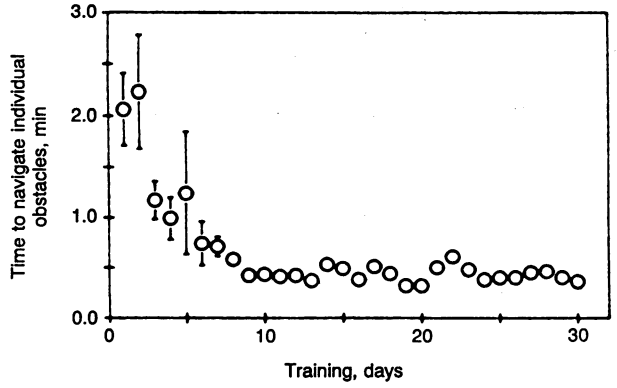

FIG. 2. Learning curve for the AC rats reveals considerable improvement in their ability to perform the acrobatic tasks. "Time to negotiate individual obstacles" is the time (mean ± SEM) required to traverse one segment of the path, approximately 1 m long. Note that the tasks were performed more quickly even as they became more difficult each day. SEM bars were not plotted if they were smaller than the group symbol.

The PML does not have any known vestibular function or connections (16), and its function is strongly associated with movements of the limbs (18). Tactile stimulation of rat forelimbs elicits electrophysiological activity from the PML cortex in a "fractured somatotopy" type of pattern (19). Direct stimulation of forelimb muscle (20), as well as central stimulation of forelimb movements (21, 22), causes increased uptake of 2-deoxyglucose in the PML region. Both the acrobatic and the exercise rats produced complex locomotor movements that involved limbs, trunk, and head and thus would have activated a wide region of the PML cortex. So the inherent complexity of the required movements, as well as the fractured somatotopy of the PML, makes it important to sample randomly along the length of the folia to assure that multiple functional regions are included. Lastly, the stereological methods used here describe the entire PML as the study volume, hence the tissue samples must be random within its extent so as to be representative of the entire folia and not just one specific segment of it.

Quantitative Methods. In conditions of stable neuron number, stereological estimates of the number of synapses per neuron accurately reflect the magnitude of synaptogenesis or synapse loss (23). To obtain an estimate of the volume of molecular layer per Purkinje cell in the PML, the molecular layer (a strip running along the top and sides of the folia between the pial surface and a line through the Purkinje cell nuclei) was drawn with the aid of a camera lucida from four 1-μm thick sections from each block of tissue, spaced about 10 μm apart (Fig. 4). The disector method (25) was then applied to determine the density of Purkinje cells within the strip of molecular layer. This stereological method for determining particle density is superior to others because it is unbiased with respect to particle size or shape. All sections were pooled and yielded a mean volume of 4×10^7 μm^3 of

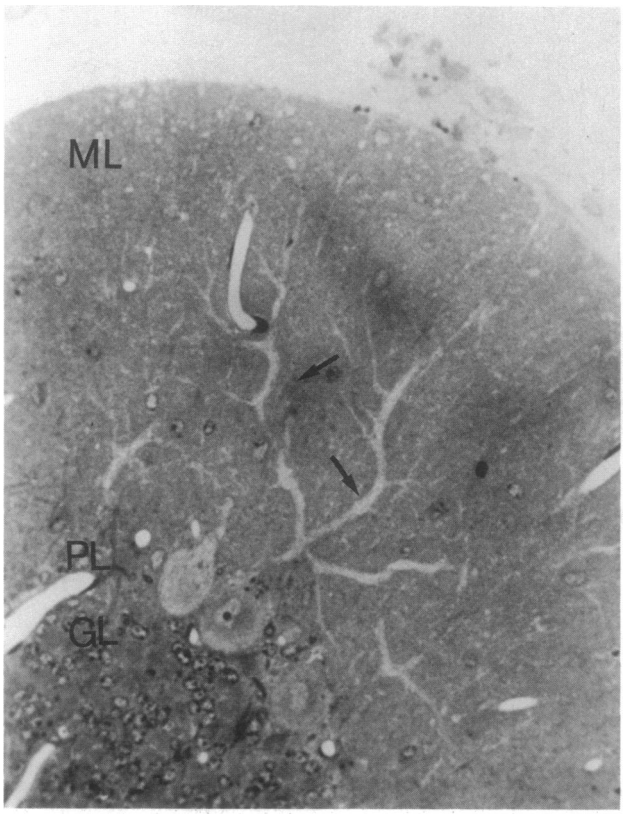

FIG. 3. Light micrograph of PML molecular layer in a 1-μm-thick section stained with 0.5% toluidine blue and 1% borax and differentiated with ethanol. Purkinje cell somata and nuclei are large and easily distinguished from other neuron types and glia. Large Purkinje cell dendrites extend and branch into the molecular layer (arrows). ML, molecular layer; PL, Purkinje cell layer; GL, granule cell layer. (Bar = 10 μm.)

cerebellar cortex sampled from each animal. The mean of the individual coefficients of error in this measure was 9.1%.

Two strips of electron micrographs from the pial surface to the Purkinje cell layer (average total area of about 3000 μm^2) were taken for each animal and printed at a final magnification of ×30,000. By using synapse criteria of a postsynaptic thickening (PST), apposed membranes, and at least two presynaptic vesicles, all of the synapses in the micrographs were counted. The area density of synapses (N_a) was calculated as an intermediate variable, with a coefficient of error of approximately 8.0%. From this set of 9243 synapses, approximately 25% were randomly selected and their PST lengths were measured using a data tablet. The coefficient of error for PST length was approximately 5.3% for each rat. The volume density of synapses for each rat was estimated as $N_v = N_a/(D + t)$, where N_a is the number of synapses per unit area, D is the mean PST length, and t is section thickness. This formula corrects synapse density for possible differences in particle size, and it assumes that synapses are flat, circular, and randomly oriented (24). An estimate of the number of synapses per Purkinje cell was then computed for each rat as the product of synapse density N_v and the volume of molecular layer per Purkinje cell. Of course, the ratio can also be affected by the loss of Purkinje cells themselves, but no evidence of pyknotic neurons was observed in the 1-μm sections. This ratio compensates for changes in reference volume, but it does not literally reflect just the synapses making contact with Purkinje cell dendrites because it also includes synapses in the molecular layer between non-Purkinje neurons.

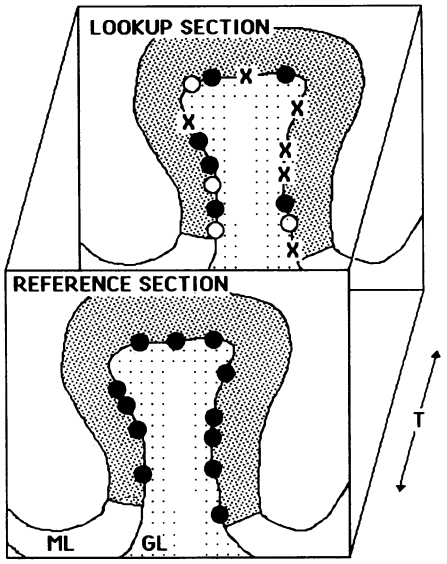

FIG. 4. Schematic depiction of the disector method (24) applied to cerebellar cortex. ML, molecular layer; GL, granule cell layer. Drawn in the "reference section" is a strip of molecular layer (dark stipple) with area A, running along the top and sides of the folia and excluding the interfolia troughs. A nearby "lookup section" a distance of T μm away defines a slab of molecular layer with a volume equal to the product of thickness T and area A. Purkinje cell nuclei apparent in the reference section are indicated by dark circles lined up at the bottom of the molecular layer (see asterisks in Fig. 2). The nuclei that are also present in the lookup section are marked with solid circles, whereas the nuclei newly appearing in the lookup section are marked with open circles, and they are both ignored in the calculation. Most importantly, nuclei that dropped out between the reference and lookup sections are marked with an X, and they are by definition uniquely present in this volume. The number of nuclei present in the slab of molecular layer is estimated by the number that dropped out and were marked X, six of them in this example. The average volume of molecular layer per Purkinje cell is simply the volume TA divided by the number of dropout nuclei (i.e., $TA/6$).

To obtain a measure of vascularity, camera lucida drawings were made of the molecular layer from two sections per block, about 40 μm apart. With our perfusion methods all blood vessels are easily identified by their clear lumens contrasting with stained neuropil. However, due to inadequate pressure during perfusion, five rats had to be dropped from the vasculature part of the study because their blood vessels had collapsed and could not be reliably identified. A total of 16,353 vessels, each of which had more than half its lumen in the molecular layer, were then noted on the drawings. The area density of blood vessels can be affected directly by angiogenesis or secondarily by changes in tissue volume (e.g., a falling vessel density with an expanding tissue volume).

The estimates for each animal of the volume of molecular layer per Purkinje cell, PST length, synaptic density, the number of synapses per Purkinje cells, and capillary density were analyzed separately with the general linear model (26) using group as a main factor. Planned comparisons of the groups included: AC vs. the other groups to test for effects of learning and VX vs. IC to test for physical exercise effects. An additional test for physical exercise effects was derived from the VX group by computing within-group correlations of the brain measures and the distance each VX rat travelled during the study.

RESULTS

At the end of 30 days the animals in the AC group had traveled a total of 0.9 km at a slow walk, those in FX had gone 10.8

km at a trot, the VX rats had run 19 ± 4 km (mean ± SEM), and the IC group had received nominally zero exercise. Fig. 5 presents the results for capillary density, with the AC group not differing significantly from the others [$F = 3.93$, not significant (NS)] and instead the two exercise groups have a higher density of capillaries than the AC and IC groups. In contrast to this pattern, the volume of overlying molecular layer per Purkinje cell in AC rats was greater than that in the other groups (Fig. 5; $F = 48.26$, $P < 0.0001$). Mean PST lengths for AC, FX, VX, and IC groups were 0.33 ± 0.02, 0.32 ± 0.01, 0.33 ± 0.01, and 0.32 ± 0.01 μm, respectively, all with very similar size distributions, and there were no statistical or apparent indications of any effect of learning ($F = 0.18$, NS). Our values are compatible with published estimates of synapse size in the molecular layer, which range from 0.29 to 0.44 μm (27, 28). The density of synapses in the molecular layer was unaffected by motor learning or exercise (Fig. 5; $F = 0.77$, NS) and is compatible with published estimates of synapse density ranging from 2.2×10^8 to 8.2×10^8 synapses per mm^3 (29, 30). Because the volume of molecular layer per Purkinje cell increased in the AC group while synapse density remained constant across groups, in the planned comparison for the effects of learning, AC rats had approximately 25% more synapses per Purkinje cell than the other groups (Fig. 5; $F = 10.28$, $P < 0.003$). Thus the effect of acrobatic training was to increase synapse number while blood vessel density remained unaffected and while the effect of the two exercise conditions was to increase vascular density without detectable change in the number of synapses. It should be noted that, since vessel density was unchanged and molecular layer volume increased in the AC group, there was also an increase in capillaries in this group that kept pace with the increase in neuropil volume. We have described (31, 32) this type of synaptogenesis-associated angiogenesis in visual cortex of young and middle-aged rats.

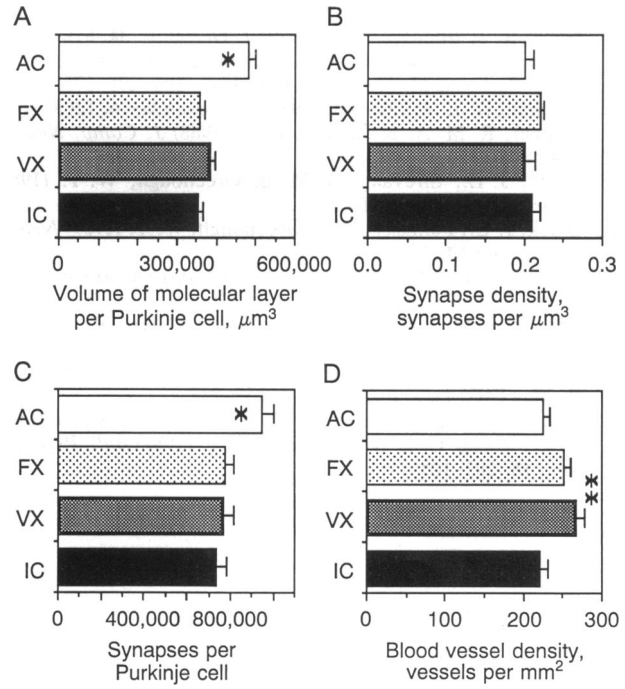

FIG. 5. Summary of neuroanatomical measures. (*A*) Volume of molecular layer per Purkinje cell. (*B*) Synapse density. (*C*) Synapses per cell. (*D*) Blood vessel density. AC rats had more volume in the molecular layer per Purkinje cell, with no difference in synaptic density, so AC rats also had more synapses per Purkinje cell. Single asterisks denote that the AC group was significantly different from other groups, $P < 0.05$. Double asterisks denote that the VX group was significantly different from the IC group, $P < 0.05$.

In clear contrast to the learning effects, the planned comparison of VX vs. IC revealed no effects of exercise for the volume of molecular layer per Purkinje cell, PST length, synaptic density, or synapses per Purkinje cell ($F = 1.80$, NS; $F = 0.20$, NS; $F = 0.04$, NS; $F = 0.24$, NS, respectively). However, the VX group did differ significantly from the IC group in the density of capillaries ($F = 10.80$, $P < 0.003$). Within the VX group there were no significant correlations of wheel-running distance with synapse density, volume of molecular layer per Purkinje cell, synapses per neuron, or vessel density (Spearman rank, $r = 0.25$, $r = -0.49$, $r = -0.07$, and $r = -0.43$, respectively, all NS).

The stereological formula used for N_v synapse can be biased by group differences in particle shape and size (33). However, PST length was unaffected by treatment and therefore synapse size effects would not have obscured group differences in synaptic density (34). Similarly, any group differences in synapse shape would probably be small and thus unlikely to significantly affect synapse density (35). Thus the larger estimate of the number of synapses per neuron in the AC group primarily reflects the larger amount of molecular layer over each Purkinje cell. The latter estimate was made using the disector method and is free of bias due to particle shape and size.

DISCUSSION

Based on distance traveled, animals in the two exercise conditions made more than 10 times the number of repetitive locomotor movements that were made by the AC or IC rats. Because the PML region is involved in controlling these movements (18, 19) and is metabolically activated as a consequence of such behaviors (20–22), the FX and VX rats presumably used the existing PML synapses and neurons considerably more than the AC or IC rats did. As a consequence of the extensive physical activity, there was an increase in the density of capillaries in the PML that was not evident in the less active AC and IC rats. On the other hand, the AC rats were involved in learning new motor skills as their performance on the changing obstacle course improved. In these animals there was an increase of approximately 25% in the number of synapses per Purkinje cell but no increase in vascular density. What we see here are apparently two very different patterns of adaptation to environmental demands in the cerebellar cortex. When the environment presents a need for skilled movements, alterations in the pattern of synaptic connections occur. When the environment demands extensive repetition of a small set of simple, well-practiced movements, the vasculature support is altered to handle the increased metabolic load associated with higher levels of neural activity. An interesting perspective is that both of these changes are in some sense "memory" (i.e., an adaptive change in brain organization brought about by behavioral experience). The memory for complex behavioral skills that appears to be mediated by synaptogenesis may be of more interest to some than the adaptation to metabolic demands, but from the viewpoint of the animals' success in the environment, their relative importance varies with the situation.

In spite of the increased synaptic activity in the exercised animals, the size and the number of synapses per Purkinje cell are similar in the VX, FX, and IC groups. This indicates that mere repetitive neuronal activation or use of synapses does not generate new synapses in this region, nor does either activity or learning substantially affect synapse size. Similarly, the lack of a significant correlation in the VX group between amount of running and synapses per Purkinje cell or PST length also suggests that repetitive activity does not generate new synapses or modify the size of existing ones in PML cortex. It is possible, of course, that changes occurred

in the sizes of particular types of synapses that were not evident in the aggregate values. It is also possible, even probable, that the differences in synapse number reflect selective changes in particular classes of synapses, rather than equivalent changes in all synapse types.

Theorists have proposed that cerebellar plasticity may depend upon the coordinated activation of parallel fiber and climbing fiber inputs to Purkinje cells. Although they have differed on the specific aspects of cerebellar connectivity that might be involved in motor learning, as well as in the involvement of new synapse formation, there has been wide agreement that the organization of cerebellar cortex, with two excitatory informational inputs converging on a single output neuron, is an easy configuration with which to work in designing functional models (13–17). The present results cannot directly test these theories until the various synapse types in the molecular layer are described. Indirectly, however, our findings indicate the importance of coordinated input that involves a "teaching signal," as is often proposed. In other words, intense synaptic activity sufficient to elicit angiogenesis does not produce new synapses, whereas neural activity that was less metabolically intense, judging by vascular measures, drives an impressive amount of synaptogenesis.

These results show changes in synapse numbers specific to learning in adult cerebellar cortex and show experience effects on cerebellar vasculature, both of which are consonant with cerebellar cortical involvement in learning. Other types of synaptic plasticity, such as changes in synaptic structure or molecular configuration, were not examined in this study and may also have been initiated in PML by either learning or repetitive exercise. These results do indicate, however, that motor learning of this type, and not mere motor activity unaccompanied by learning, is associated with a net increase in the number of synapses in the PML of the cerebellar cortex. This study thus strongly supports prior research with paradigms of reach training and maze learning (1–5) that had indicated that learning can alter patterns of synaptic connectivity.

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1. Volkmar, F. R. & Greenough, W. T. (1972) *Science* **176,** 1445–1447.
2. Greenough, W. T., Juraska, J. M. & Volkmar, F. R. (1979) *Behav. Neural Biol.* **26,** 287–297.
3. Chang, F.-L. F. & Greenough, W. T. (1982) *Brain Res.* **232,** 283–292.
4. Bailey, C. H. & Chen, M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9356–9359.
5. Greenough, W. T., Larson, J. R. & Withers, G. S. (1985) *Behav. Neural. Biol.* **44,** 301–314.
6. Yeo, C. H., Hardiman, M. & Glickstein, M. (1984) *Behav. Brain Res.* **13,** 261–266.
7. Gilbert, P. F. C. & Thatch, W. T. (1977) *Brain Res.* **128,** 309–328.
8. Floeter, M. K. & Greenough, W. T. (1979) *Science* **206,** 227–229.
9. Pysh, J. J. & Weiss, G. M. (1979) *Science* **206,** 230–232.
10. Greenough, W. T., McDonald, J. W., Parnisari, R. M. & Camel, J. E. (1986) *Brain Res.* **380,** 136–143.
11. Chen, S. & Hillman, D. E. (1982) *Brain Res.* **240,** 205–220.
12. Rossi, F., Wiklund, L., van der Want, J. J. J. & Strata, P. (1989) *Soc. Neurosci. Abstr.* **15,** 89.
13. Marr, D. (1969) *J. Physiol. (London)* **202,** 437–470.
14. Albus, J. S. (1971) *Math. Biosci.* **10,** 25–61.
15. Eccles, J. C. (1977) *Brain Res.* **127,** 327–352.
16. Ito, M. (1984) *The Cerebellum and Neural Control* (Raven, New York).
17. Fujita, M. (1982) *Biol. Cybern.* **45,** 207–221.
18. Brodal, A. (1981) *Functional Neuroanatomy* (Oxford Univ. Press, New York).
19. Shambes, G. M., Gibson, J. M. & Welker, W. (1978) *Brain Behav. Evol.* **15,** 94–140.
20. Santori, E. M., Der, T. & Collins, R. C. (1986) *J. Neurosci.* **6,** 463–474.
21. Collins, R. C., Santori, E. M., Der, T., Toga, A. W. & Lothman, E. W. (1986) *J. Neurosci.* **6,** 448–462.
22. Sharp, F. R. & Ryan, A. F. (1984) *J. Comp. Neurol.* **224,** 286–306.
23. Turner, A. M. & Greenough, W. T. (1985) *Brain Res.* **329,** 195–203.
24. Anker, R. L. & Cragg, B. G. (1974) *J. Neurocytol.* **3,** 725–735.
25. Brændgaard, H. & Gunderson, H. J. G. (1986) *J. Neurosci. Methods* **18,** 39–78.
26. SAS Institute (1985) *SAS User's Guide: Statistics* (SAS Institute, Cary, NC), Version 5.
27. Glick, R. & Bondareff, W. (1979) *J. Gerontol.* **34,** 818–822.
28. Ruela, C. L., Matos-Lima, C., Sobrinho-Simões, M. A. & Paula-Barbosa, M. M. (1980) *Acta Anat.* **106,** 270–275.
29. Hillman, D. E. & Chen, S. (1981) *Neuroscience* **6,** 1263–1275.
30. Napper, R. M. A. & Harvey, R. J. (1988) *J. Comp. Neurol.* **274,** 168–177.
31. Black, J. E., Sirevaag, A. M. & Greenough, W. T. (1987) *Neurosci. Lett.* **83,** 351–355.
32. Black, J. E., Polinsky, M. & Greenough, W. T. (1989) *Neurobiol. Aging* **10,** 353–358.
33. Weibel, E. R. (1979) *Stereological Methods* (Academic, New York), Vol. 1.
34. Colonnier, M. & Beaulieu, C. (1985) *J. Comp. Neurol.* **231,** 175–179.
35. Wesa, J. M., Chang, F.-L. F., Greenough, W. T. & West, R. W. (1982) *Dev. Brain Res.* **4,** 253–257.
36. McCormick, D. A., Lavond, D. G., Clark, G. A., Kettner, R. E., Rising, C. E. & Thompson, R. F. (1981) *Bull. Psychon. Soc.* **18,** 103–105.