tate, and analyzed by gas chromatography–mass spectrometry selected ion monitoring (GC-MS-SIM). Before analysis, JA was methylated with diazomethane to give MeJA. The GC-MS instrument was a Hewlett-Packard quadrupole mass spectrometer (HP 5970B) fitted to an HP 5890 gas chromatographer equipped with an Ultra-1 fused silica capillary column (25 m by 0.2 mm, inner diameter, 0.33 μm phase thickness; Hewlett-Packard). Each sample was injected in the splitless mode at 120°C. After a 2-min isothermal pause at 120°C, the column temperature was programmed to 280°C at 16°C/min. The column pressure of carrier gas (He) was 40 kPa. GC-MS-SIM was performed by monitoring the mass-to-charge (*m/z*) ratios 226, 224, 195, 193, 153, and 151. The electron energy was 70 eV. The retention time of MeJA was approximately 8 min. The amounts of JA and MeJA in the original extracts were determined from the ratio of peak areas for *m/z* $224(^2H_0)/226(^2H_2)$. All data were corrected for losses.

40. For quantitation of SA and SAG, 2 g of leaf material was used for each assay. Quantitation of SA and SAG was performed essentially as described [J. Malamy, J. Hennig, D. F. Klessig, *Plant Cell* **4**, 359 (1992)]. HPLC was performed on a μBondasphere 100 Å, 5-μm C-18 (3.9 mm by 15 cm) column maintained at 40°C. Isocratic separation was performed at 1 ml/min with 23% (v/v) methanol in 20 mM sodium acetate, pH 5.0. Fluorescence detection was done with a Model RF-550A (Shimadzu, Tokyo, Japan). The detection limit was 10 pg of SA. All data were corrected for losses.

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Tissue Plasminogen Activator Induction in Purkinje Neurons After Cerebellar Motor Learning

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The cerebellar cortex is implicated in the learning of complex motor skills. This learning may require synaptic remodeling of Purkinje cell inputs. An extracellular serine protease, tissue plasminogen activator (tPA), is involved in remodeling various nonneural tissues and is associated with developing and regenerating neurons. In situ hybridization showed that expression of tPA messenger RNA was increased in the Purkinje neurons of rats within an hour of their being trained for a complex motor task. Antibody to tPA also showed the induction of tPA protein associated with cerebellar Purkinje cells. Thus, the induction of tPA during motor learning may play a role in activity-dependent synaptic plasticity.

Long-term memory requires the synthesis of new proteins, which are thought to bring about structural changes in synaptic connections within the brain (*1*). The cerebellar cortex has been implicated as an important area of plasticity for motor learning (*2, 3*). An increase in the number of synapses per Purkinje cell in the molecular layer of the cerebellar cortex is seen in rats trained to perform complex acrobatic tasks (*4*). In contrast, the number of parallel fiber synapses on Purkinje neurons does not change in animals subjected to forced or voluntary exercise (*4*). Thus, the synaptic changes are related to the learning of a specific motor task and not merely to increased synaptic activity. These studies (*4*) support the Marr-Albus mathematical models of cerebellar learning, in which climbing fibers are postulated to modulate mossy fiber inputs to Purkinje cells (*2, 5*). Synaptic changes have also been seen in Purkinje cells of rabbits subjected to a classical conditioning eyeblink response (*6*). Thus, structural changes in synaptic connections appear to accompany cerebellar learning.

Molecules that may participate in synaptic remodeling are the plasminogen activators (PAs), secreted serine proteases that primarily serve to cleave the zymogen plasminogen into the active protease plasmin (*7*). In the absence of plasminogen, PAs can act directly to cleave matrix molecules such as fibronectin and to activate latent growth factors, including scatter factor–hepatocyte growth factor (*8*). The PA-plasmin system plays a role in tissue remodeling, cell migration, and tumor cell invasion (*7, 9*).

Tissue plasminogen activator (tPA) is the primary PA in the brain (*10*). During embryonic and neonatal development, tPA levels are high in brain regions undergoing extensive cell migration or tissue remodeling. Similarly, PAs are secreted by neurons during axonal growth and regeneration and then bind with high affinity to the surface of the growth cone, where they are poised to facilitate nerve outgrowth through a tissue matrix (*11*). In general, tPA levels are reduced in the mature brain, where the dentate gyrus and cerebellum show the most tPA activity (*12*).

We investigated the involvement of tPA in the consolidation phase of the learning of complex motor tasks. We used a runway task in which rats had to learn to traverse a runway by placing their paws on aluminum pegs that protruded horizontally from the walls of the runway, a task that is dependent on cerebellar function (*13*) (Fig. 1, inset). Initially, the rats were shaped and trained on a regular (REG) peg pattern; then, after a 2-week break, they were tested for their performance on an irregular (IRR) peg pattern. Their speed in crossing the runway was measured for 20 trials per day. The greatest improvement in performance (that

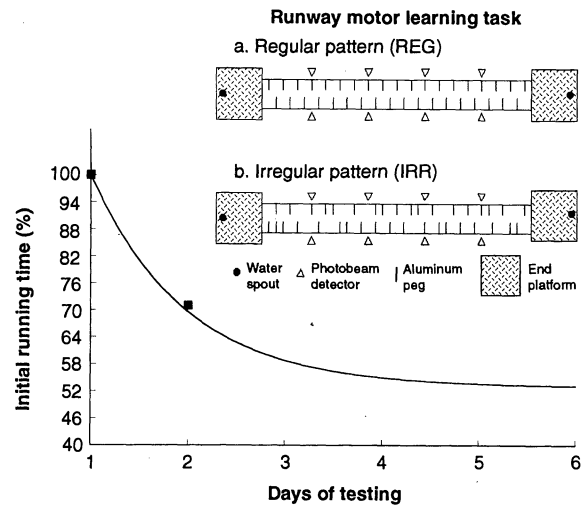

Fig. 1. Motor learning curve and apparatus. Thirty 3-month-old male Fischer rats were trained (*19*) on a complex motor task that involved learning to negotiate a runway consisting of aluminum pegs placed in the specific pattern depicted in the inset on the upper right. The solid line represents a learning curve on the IRR pattern for normal control animals from previous experiments (*13*); the squares represent the rats that were studied for tPA levels. It can be observed from the learning curve that a majority of the improvement in running times (that is, learning) takes place between days 1 and 2. Smaller improvements are observed between days 2 and 5. Rats brains were analyzed for tPA mRNA at varying intervals after training on the IRR task, starting at 1 hour after training on day 1. Two rats were examined after training on day 2, and the improvement in running times for these rats was similar to that of the 10 rats that had been previously observed and that fit the solid curve (*13*).

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is, learning) was seen between testing day 1 and day 2 (Fig. 1). Rat brains were examined during this active phase of learning for the expression of tPA mRNA.

Fresh frozen sections of rat cerebella examined by in situ hybridization 1 hour after training showed an increase in tPA mRNA expression within the cerebellum, especially in Purkinje neurons (Fig. 2B), when compared with brain sections from a naïve rat (Fig. 2A). The naïve rat showed a low but detectable level of specific tPA expression in Purkinje cells, as determined by pixel counting [ratio of the Purkinje layer to the molecular layer (P/M) = 2.4)] (*14*) or when viewed in a bright field at a higher magnification. By 4 hours after the first training session, Purkinje cells in cerebella of trained rats still expressed elevated levels of tPA mRNA; however, a greater relative percentage (P/M = 1.35) of the radiolabeled probe was bound in the cerebellar molecular layer as compared with that bound at 1 hour (P/M = 2.14) (Fig. 2C). Twenty-four hours after training, tPA mRNA levels were reduced in the cerebellum (P/M = 1.2) when compared with the levels at 1 and 4 hours after training (Fig. 2D), although they were still somewhat elevated compared with those in the naïve rat brain. One hour after the training session on day 2 (that is, at 25 hours after training session one) cerebella showed an increase in tPA mRNA compared with that seen at 24 hours after training, but the mRNA was not restricted to the Purkinje cells (P/M = 1.58) (Fig. 2E). Companion cerebellar sections taken at each time point were also incubated with a radiolabeled sense complementary RNA (cRNA) probe (Fig. 2F). Sense probes failed to show specific binding to the cerebellar tissue (P/M = 1.08). The induction of tPA mRNA in Purkinje neurons is shown in a composite view of an entire cerebellar section (Fig. 2G).

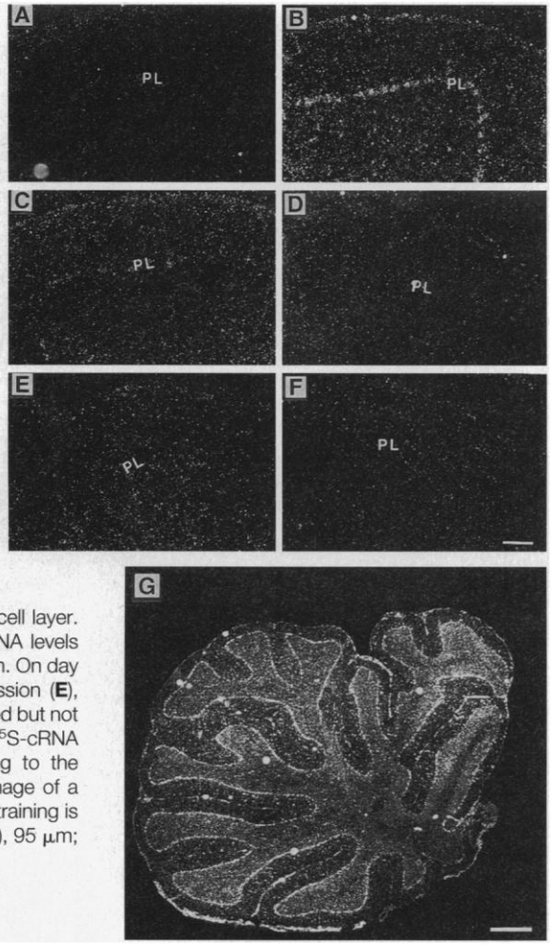

Fig. 2. In situ hybridization of tPA mRNA in rat cerebella after a complex motor learning task. A 515-base antisense ^{35}S-cRNA riboprobe complementary to part of the kringle 2 domain and to the catalytic domain of mouse tPA was used to visualize the presence of tPA mRNA in the adult rat cerebellum by in situ hybridization (*12, 20*). Dark-field illumination of the radiolabeled cerebellar sections from the paramedian lobules showed that there was a marked increase in cerebellar expression of tPA mRNA, most notably in Purkinje neurons, within 1 hour of training on the IRR pattern of pegs (**B**) as compared with expression in the cerebellum of an untrained naïve rat (**A**), who remained in his cage (PL, Purkinje layer). By 4 hours after training (**C**), the overall level of tPA mRNA had decreased somewhat and there appeared to be more tPA mRNA in the molecular layer, which lies above the Purkinje cell layer. By 24 hours after training (**D**), tPA mRNA levels were reduced throughout the cerebellum. On day 2, 1 hour after the second training session (**E**), cerebellar tPA mRNA levels were elevated but not as dramatically as on day 1. A sense ^{35}S-cRNA riboprobe indicated nonspecific binding to the cerebellar sections (**F**). A composite image of a whole cerebellar section at 1 hour after training is shown in (**G**). Scale bar in (A) through (F), 95 μm; scale bar in (G), 600 μm.

Because the naïve rats had simply spent 2 weeks in their cage after the initial training and were water-deprived but not exercised, it was important to examine whether the exercise in the runway apparatus may have led to the induction of tPA mRNA in cerebellar Purkinje cells. Another group of rats was shaped and trained as before on the REG pattern; however, in this case the control rats ran on the runway apparatus with the pegs covered by a solid plexiglass sheet (an exercise control), whereas others underwent the typical training session on the IRR pegs. Thus, all parameters of the training and testing were identical except that the rats ran on a flat surface rather than on the novel IRR peg pattern. Rats ran similar distances on the test day in both the pegs-covered group and the IRR group (20.0 m ± 0.0, n = 4 for the pegs-covered group, compared with 17.9 m ± 1.2, n = 14 for the IRR group). The rats in the pegs-covered group ran faster than did the rats in the IRR group [7.9 ± 0.5 cm/s compared with 3.5 ± 1.3 cm/s ($P < 0.001$, two-tailed t-test)]. Thus, the total movement of the pegs-covered group was equal to or greater than that of the IRR pattern group. The exercised and stressed, but untrained, control rats did not induce tPA mRNA expression in their Purkinje cells at either 1 (Fig. 3B) or 4 (Fig. 3D) hours after training. Only rats undergoing complex motor training on the IRR peg runway showed specific induction of tPA mRNA expression in Purkinje neurons (Fig. 3, A and C).

To see whether this increase in tPA mRNA led to an actual cellular increase in this serine protease, cerebellar tissue sections were fixed and incubated with an antibody to murine tPA (*15*). The cerebella of exercised but untrained rats showed tPA protein associated with the meninges, occa-

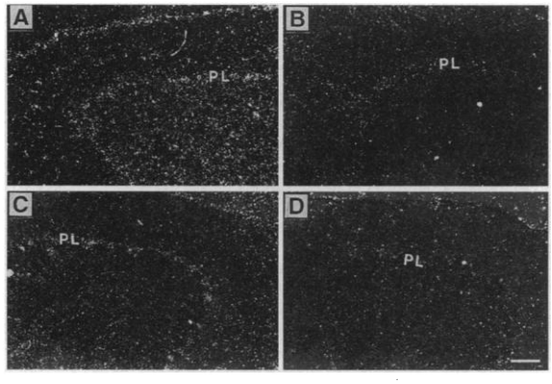

Fig. 3. Cerebella of rats trained to perform a complex motor task versus cerebella of stressed and exercised rats. In situ hybridization was performed (*15*) on paramedian lobule cerebellar sections from rats trained on the IRR peg pattern (**A** and **C**) or rats allowed to run in the apparatus with the pegs covered with plexiglass [stress and exercise control (**B** and **D**)] (PL, Purkinje layer). A 358-base antisense ^{35}S-cRNA riboprobe complementary to part of the catalytic domain of rat tPA was used in this experiment. Cerebella at 1 hour (A) and 4 hours (C) after training show tPA mRNA induction similar to that seen in Fig. 2B. The control rats show only a low level of labeling in the Purkinje layer at 1 hour (B) or 4 hours (D) after exercise, which is similar to that seen with naïve rats (Fig. 2A). Scale bar, 95 μm.

sional blood vessels, and a few presumptive astrocytes in the internal granular and molecular layers (Fig. 4A). Only rats subjected to the complex motor task of the IRR peg runway expressed tPA protein in their Purkinje cell bodies and primary dendritic shafts (Fig. 4, B and C), 4 hours after their experience in the runway apparatus. Thus, complex motor learning led to a rapid induction of tPA mRNA within minutes after the training session and to a specific increase in tPA protein within the Purkinje neurons by 4 hours after training.

The specificity of this tPA induction in Purkinje cells is supported by the identical results seen when two different tPA cRNA probes were used, one to mouse tPA and another to rat tPA, for the in situ hybridizations (Figs. 2 and 3). In addition, preliminary examination of the hippocampal region in the trained and untrained rats failed to show any difference in the expression of tPA mRNA in the dentate gyrus as a result of this motor learning experience (*16*).

Thus, the induction of tPA mRNA and protein appears to occur during an active phase of motor skill learning that has been shown to be accompanied by new synapse formation (*4*). These studies complement the observations of Qian *et al.* (*17*), demonstrating a similar induction of tPA mRNA in the dentate gyrus during hippocampal long-term potentiation, another form of synaptic plasticity implicated in the storage of long-term memory.

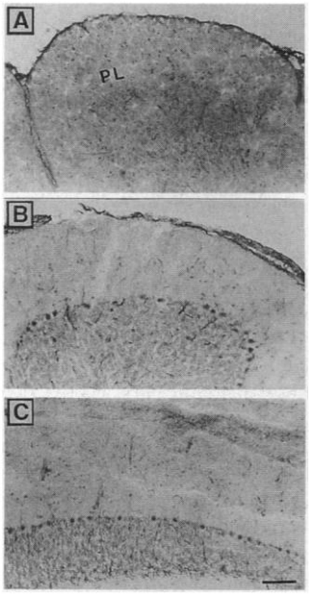

Fig. 4. Immunohistochemical localization of tPA protein after a complex motor learning task. A rabbit antibody to murine tPA (*12, 21*) was used to visualize tPA protein (dark region) in the cerebella of the rats used in Fig. 3. Untrained but exercised rats (**A**) showed tPA in the surface membranes, blood vessels, and a few presumptive astrocytes, and very little tPA in Purkinje cells (PL). However, cerebella of the trained rats showed a strong signal for tPA in Purkinje neurons and an increased signal in the granule cell layer at 4 hours after the training session (**B** and **C**). Scale bar, 95 μm.

The induction of tPA is consistent with a mechanism for synaptic plasticity in cerebellar motor learning (*2, 3, 18*), in which strong excitatory input from the climbing fibers of the inferior olive act to modulate the coordinate excitation of Purkinje neurons by parallel fiber inputs. It is possible that the secreted protease tPA may act to degrade cell surface receptors, cell adhesion molecules, or other cell surface and extracellular matrix molecules permitting structural changes and synaptic remodeling.

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14. The rats were trained as described, then killed with urethane (1.25 g per kilogram of body weight, intraperitoneal) and decapitated at specific times after the training. The cerebella were rapidly removed and frozen in 2-methylbutane at −35°C. The frozen tissue was embedded in Tissue Tek O.C.T. (Elkhart, IN) and cut in 16-μm sections. The sections were fixed in 4% paraformaldehyde phosphate-buffered saline (PBS) for 15 min and processed for in situ hybridization with the use of the procedure of Simmons *et al.* [*J. Histotechn.* **12**, 169 (1989)]. The hybridization was carried out with 10^7 cpm/ml of a 515-base ^{35}S-cRNA (antisense or sense) riboprobe to mouse tPA in 50% formamide at 58°C for 16 hours. Slides were processed through descending concentrations of 0.15 M NaCl in 30 mM sodium citrate (SSC), with a final wash in 0.1× SSC at 65°C for 30 min. The slides were dipped in Kodak NTB-2 emulsion and exposed for 2 to 3 weeks, then developed and viewed by dark-field illumination. Pixel counting of a digitized image was quantified with NIH Image software (version 1.58) for the Purkinje layer, molecular and granular layers, and the background (outside the tissue). Pixel activation in the Purkinje layer shown in Fig. 2, A through F, was 17, 45, 27, 18, 19, and 13%, respectively. The specificity of labeling is reflected in the P/M ratio.
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19. The initial phase of the motor task involved training the rats on a regularly spaced peg placement pattern (REG). Water delivery (0.3 ml) was used to reinforce running across the apparatus. Water delivery was associated with a loud tone that served as a conditioned reinforcer. Rats were deprived of water for 12 hours before training. Shaping of water drinking and running was achieved while the rods were covered by a piece of plexiglass. The rats were maintained under these conditions for 1 week or until they were able to run successfully back and forth on the runway. Rats were weighed daily, and weight loss did not exceed 15% of body weight. Animals were given 3 min of ad libitum water access after each daily session. After shaping of runway performance, training proceeded through gradual removal of sections of the plexiglass plate that covered the REG rods. Daily performance was calculated by determination of the running time for 20 successive trials per 30-min session. Sessions were conducted on five consecutive days during this phase of training. After being shaped and trained on the REG pattern, the rats were not exposed to the runway for 2 weeks. The testing phase of the experiment consisted of daily sessions of 20 trials conducted on the IRR pattern. On day 1, the running time averaged 3.26 ± 0.33 s per cross. The variability between animals' percentage of learning on any following day of training was ≤5%. Animal care was in accordance with institutional guidelines.
20. The rats were either trained on the IRR peg pattern or simply exercised in the apparatus while the pegs were covered with the plexiglass runway. The cerebella were prepared and the tissue sections were hybridized at 60°C with a 358-base antisense ^{35}S-cRNA riboprobe to rat tPA, under the conditions described above (*14*).
21. Immunostaining was done on 16-μm frozen cerebellar sections that were companion sections to those used in Fig. 3. Sections were from rats trained in the complex motor task or from exercised control rats. Cerebellar sections were warmed to room temperature, then fixed in acetone for 5 min and rinsed in Hanks' PBS. Tissue sections were treated with 0.3% H_2O_2 in PBS for 5 min, then incubated with 5% normal goat serum for 15 min. The cerebellar sections were covered with 50 μl of dilute (5.2 μg of immunoglobulin G per milliliter) rabbit antibody to murine tPA (*15*) for 90 min. The sections were rinsed and reacted with biotinylated goat antiserum to rabbit, followed by an ABC peroxidase (Vector Labs, Burlingame, CA) reaction mixture.

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