

# Enhanced hippocampal long-term potentiation and learning by increased neuronal expression of tissue-type plasminogen activator in transgenic mice

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**Adult cortical neurons can produce tissue-type plasminogen activator (tPA), an extracellular protease that plays a critical role in fibrinolysis and tissue remodelling processes. There is growing evidence that extracellular proteolysis may be involved in synaptic plasticity, axonal remodelling and neurotoxicity in the adult central nervous system. Here we show that transgenic mice overexpressing tPA in post-natal neurons have increased and prolonged hippocampal long-term potentiation (LTP), and improved performance in spatial orientation learning tasks. Extracellular proteolysis catalysed by tPA may facilitate synaptic micro-remodelling, and thereby play a role in activity-dependent neuronal plasticity and learning.**

**Keywords:** LTP/memory/synaptic plasticity/tPA/transgenic mice

## Introduction

Extracellular proteolysis is one mechanism whereby cells can remodel their interactions with their environment, by degradation of extracellular matrix proteins or matrix-binding cell surface proteins. Proteolysis can also modify membrane receptors or activate growth factor precursors (Saksela and Rifkin, 1988). Amongst extracellular proteases, the serine proteases appear to play an important role in tissue remodelling processes (Vassalli *et al.*, 1991). A number of different serine proteases have been identified in the rodent central nervous system (CNS); a systematic study of serine protease mRNAs in mouse and rat hippocampus has identified that encoding tissue-type plasminogen activator (tPA) as the most abundant (Davies *et al.*, 1998). Adult cortical neurons, including the pyramidal and the granular neurons of the hippocampus, can produce tPA (Sappino *et al.*, 1993), and tPA is an immediate-early gene induced in hippocampal neurons during seizure, kindling and long-term potentiation (LTP) (Qian *et al.*, 1993).

Activity-dependent synaptic plasticity plays an important role during learning, and extracellular proteolysis could be implicated in this process by catalysing synaptic micro-remodelling. In this context, the observation that mice

made genetically deficient (Carmeliet *et al.*, 1994) in tPA show a decrease in the late phase of hippocampal LTP, points towards this enzyme as a mediator of such plasticity; although these mice do not exhibit detectable deficits in certain memory tests, they do have a deficit in two-way active avoidance, a form of learning (Frey *et al.*, 1996; Huang *et al.*, 1996). This concept is reinforced by the demonstration that reverse occlusion-induced plasticity in the cat visual cortex is prevented by inhibitors of tPA or plasmin (Müller and Griesinger, 1998). A contribution of tPA to neuronal excitotoxic damage in mice has also been documented (Tsirka *et al.*, 1995).

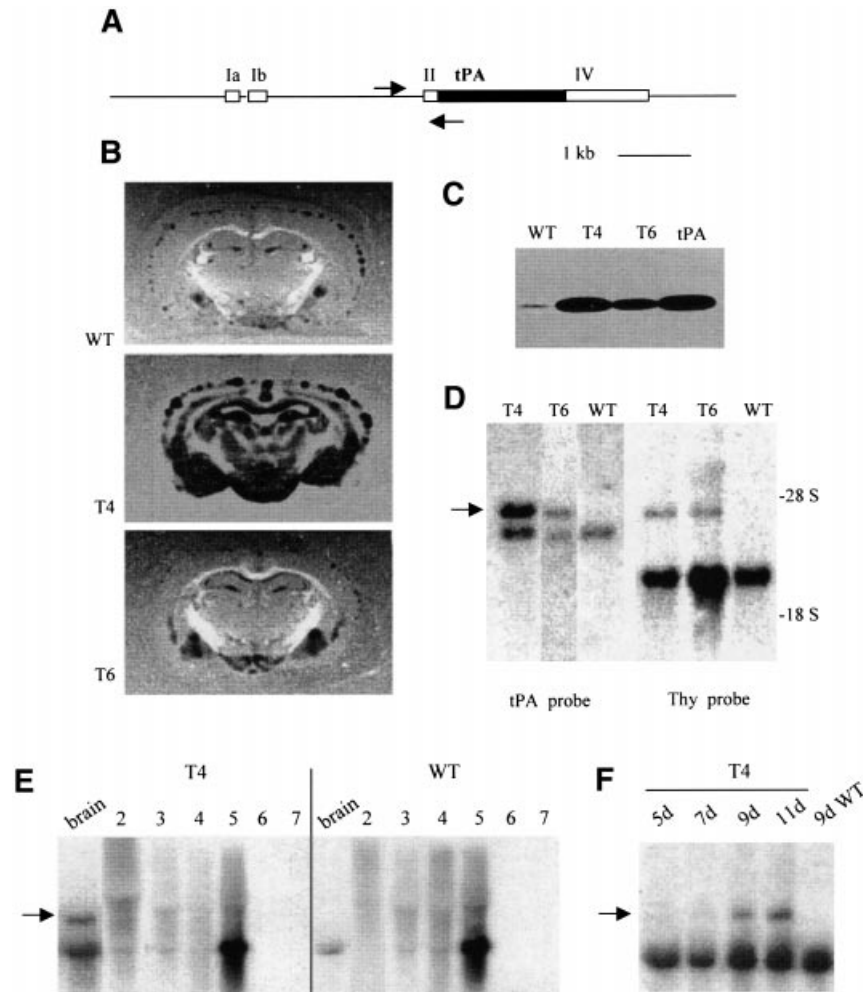
To explore further the role of tPA-catalysed extracellular proteolysis in CNS function, we generated transgenic mice that constitutively produce increased levels of the protease in post-natal neurons. Analysis of hippocampal LTP and of learning in spatial orientation tasks reveals a marked effect of transgene expression, and thus provides novel evidence in favour of an important role for tPA in synaptic plasticity and learning.

## Results

### **Generation of transgenic mice with neuronal overexpression of tPA**

The transgene used to achieve increased neuronal expression of tPA (Figure 1A) comprised the entire coding sequence of murine tPA under the control of the Thy1.2 promoter, which drives post-natal neuron-specific transcription (Kelley *et al.*, 1994). Two independent transgenic families were established (T4 and T6). By histo-enzymatic analysis of brain sections (Figure 1B), increased tPA-catalysed proteolytic activity was observed in transgenics as compared with controls; this was particularly evident in regions such as the hippocampus, amygdala and hypothalamus, which express the highest levels of tPA-dependent proteolysis in control mice (Sappino *et al.*, 1993). Zymographic analysis of brain tissue (Figure 1C) confirmed the presence of high levels of tPA in the transgenic families; by quantitative zymography (not shown) we estimate tPA levels to be increased by 3- (T6) to 10-fold (T4) as compared with non-transgenic littermates. The tissue specificity and developmental timing of transgene expression was examined by Northern blot hybridization: transgene-encoded mRNA could be detected with probes hybridizing to tPA and to Thy1.2 sequences in adult brain tissue (Figure 1D), but not in the other adult tissues analysed (Figure 1E); it was first detectable in brain tissue starting 7 days after birth (Figure 1F).

Transgenic mice appeared healthy, their life-span was comparable with that of their non-transgenic littermates, and no obvious neurological or behavioural differences were observed. Histological analysis did not reveal signs

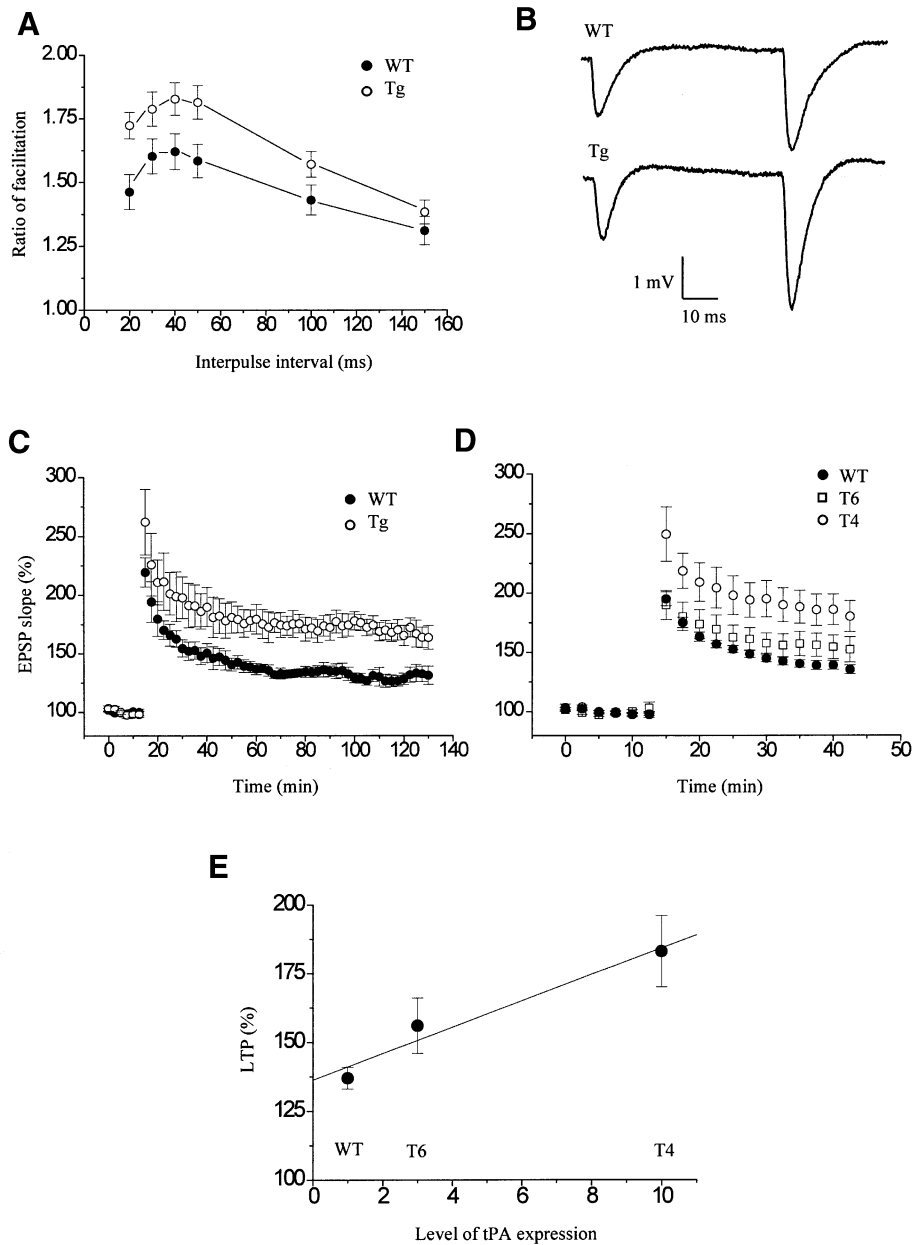


**Fig. 1.** Generation of Thy-tPA transgenic mice and analysis of transgene expression. (A) Thy-tPA construct: the mouse tPA cDNA coding sequence (black box) was inserted in a modified Thy1.2 promoter cassette (exons are indicated as white boxes), which drives neuronal expression of transgenes starting 7 days after birth and is devoid of activity in thymocytes. Arrows indicate primer positions used to determine transgenesis by PCR. (B) Histo-enzymatic analysis of sections of adult brains and (C) zymographic analysis of total brain protein from T4 and T6 Thy-tPA transgenics and wild-type (WT) mice; (tPA), murine tPA standard. No proteolysis was observed when the assays were performed in the absence of plasminogen (not shown). (D) Northern blot analysis of RNA extracted from whole brain of WT, T4 and T6 transgenic mice, using tPA and Thy1.2 probes. Transgene-encoded mRNA (arrow) was detected at the expected size with both probes. The ratio of transgene-encoded to endogenous tPA mRNAs was estimated to be 3 in T4 and 2 in T6 transgenics. (E) No transgene expression was detected in RNAs prepared from testis (lane 2), heart (lane 3), lung (lane 4), thymus (lane 5), pancreas (lane 6) and liver (lane 7) of transgenic mice and probed with the Thy1.2 probe; similar results were obtained with animals from the T6 family (not shown). (F) Expression of the transgene starts 7 days after birth, as revealed by Northern blot analysis of brain tissue using a Thy1.2 probe.

of spontaneous neurodegeneration, as might have been expected based on the contribution of tPA to the effects of excitotoxic agents (Tsirka *et al.*, 1995). This indicates that increased expression of tPA is not, by itself, neurotoxic, in accordance with the result of hippocampal tPA infusion in the absence of excitotoxic challenge (Tsirka *et al.*, 1996). No differences in brain or hippocampal anatomy were observed and no alteration in the pattern of hippocampal mossy fibres could be detected by Timm's silver-sulfide stain. Ultrastructural analysis of hippocampal CA1 synapses (five mice from each genotype) did not suggest differences in their morphology or in the shape or the width of the synaptic cleft ( $24.5 \pm 1.3$  and  $23.5 \pm 1.4$  nm, as determined by morphometry of 277 and 233 synapses from wild-type (WT) and transgenic mice, respectively).

### **LTP is increased in hippocampal slices of transgenic mice**

Mice genetically deficient in tPA have been shown to have alterations in hippocampal synaptic transmission, such as a significant decrease in the late phase of LTP in their CA1 area (Frey *et al.*, 1996; Huang *et al.*, 1996). To investigate the possibility that tPA overexpression was associated with modifications of synaptic functions in the hippocampus, we analysed different aspects of synaptic properties, including paired-pulse facilitation, LTP and long-term depression (LTD). As illustrated in Figure 2A, we found that paired-pulse facilitation, a transient enhancement of synaptic responses elicited at short interval, was larger in transgenics as compared with controls. The change was statistically significant only in the T4, and not the T6 family. The increase in facilitation was observed at all time intervals tested and



**Fig. 2.** Increased facilitation and LTP in transgenic mice. **(A)** Degree of facilitation (mean  $\pm$  SEM) obtained in slices prepared from 13 WT and 13 Thy-tPA T4 transgenic mice and calculated for various interpulse intervals as the ratio of the amplitude of the second over the first response elicited by a paired stimulation. Differences are statistically significant for interpulse intervals of 20–100 ms ( $p < 0.05$ , *t*-test). **(B)** Illustration of the responses elicited at 50 ms interpulse interval in slices from a WT and a T4 transgenic animal. **(C)** Degree of LTP induced by theta burst-patterned stimulation in slices prepared from 11 WT and 10 T4 transgenic mice. The LTP obtained 2 h after stimulation in transgenic mice ( $166 \pm 9\%$ ;  $n = 10$ ) is statistically significantly larger than that found in control animals ( $133 \pm 7\%$ ;  $n = 10$ ;  $p < 0.01$ , *t*-test). **(D)** Degree of LTP induced by theta burst-patterned stimulation in slices prepared from 11 WT, nine T6 and 10 T4 transgenic mice. **(E)** Summary of the LTP measured 30 min after stimulation in WT and in the two families of transgenic mice (T6 and T4). The increase in LTP found in transgenic mice correlates with the increased level of tPA expression (WT:  $137 \pm 4\%$ ,  $n = 19$ ; T6:  $156 \pm 10\%$ ,  $n = 9$ ; T4:  $183 \pm 13\%$ ,  $n = 10$ ).

was not associated with a change in the time course of facilitation (Figure 2B). In addition, we also found that application of theta burst-patterned stimulation to Schaffer collaterals induced a larger potentiation in slices prepared from transgenic (T4) mice than in control animals (Figure 2C–E). This statistically significant difference was present from the onset of LTP and lasted up to 2 h after stimulation, thus affecting both the early and later phases of LTP (Figure 2C). Interestingly, this greater LTP was directly

correlated with the level of tPA overexpression obtained in the two families of transgenic mice (Figure 2D and E). In contrast, LTD was similar in control and transgenic animals, and there was no difference in input–output curves relating stimulation intensity and size of excitatory potentials (not shown). Taken together, these experiments indicate that tPA overexpression is associated with a selective enhancement of paired-pulse facilitation and LTP in the hippocampus.

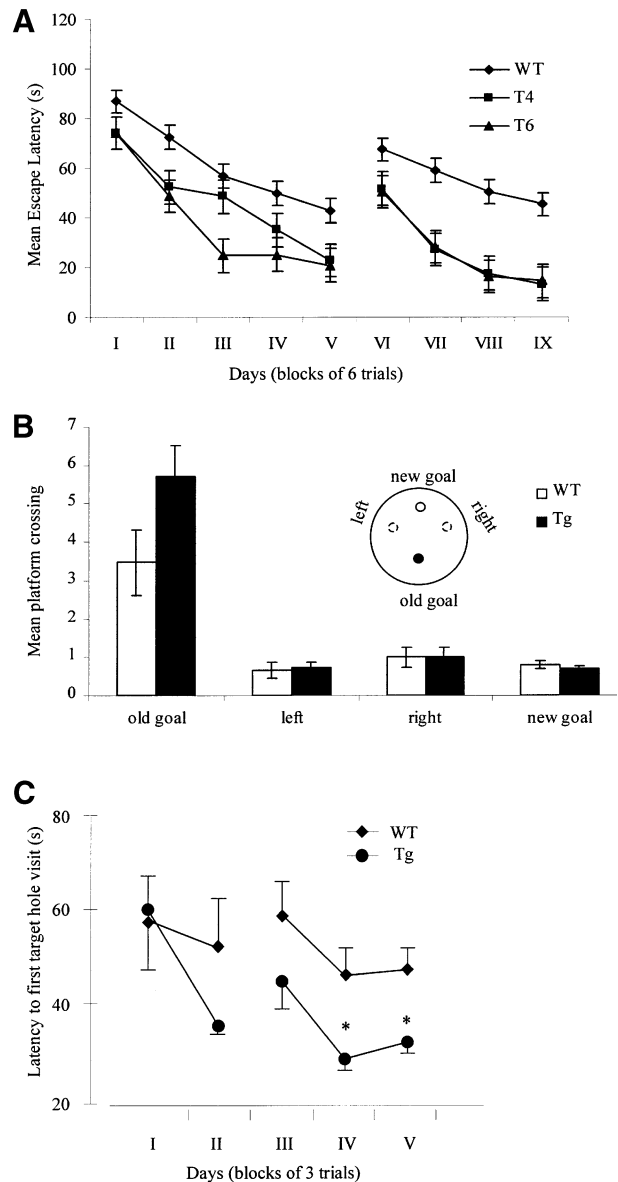
### Enhanced performance of transgenic mice in hippocampus-dependent learning tasks

LTP is considered to be a model of processes relevant to learning and memory, and we therefore tested whether the performance in hippocampus-dependent learning tasks might be modified in Thy-tPA transgenic mice. Together with their non-transgenic littermates, they were subjected to two spatial memory tasks. In the hidden platform water maze protocol of Morris, transgenic mice from the two families tested learned significantly more rapidly to find the platform, both in a first series of tests (days I–V) and after the platform was moved to a different quadrant (days VI–XI) (Figure 3A). During the first trial on day VI, the number of times the mice crossed the previous platform position and two other positions in the pool was also determined (Figure 3B); transgenic mice returned to the previous goal more often than did the controls. In a second test of spatial memory, the homing holeboard, the learning ability of transgenics was also significantly enhanced as compared with their non-transgenic littermates (Figure 3C). This increased ability of transgenics did not appear to result from differences in swimming or motor capacity: neither the speed of swimming nor the mean distance reached in a rotating wheel assay differed significantly according to the genotype of the mice (see Materials and methods). We conclude that neuronal overexpression of tPA is accompanied by an improvement in learning ability and memory storage that may be related to an effect of extracellular proteolysis on hippocampal plasticity.

### Discussion

The results reported here, both with respect to hippocampal LTP and learning performance, are unlikely to be due to genetic differences other than transgene expression, since we compared transgenic and non-transgenic siblings from the same litters. Furthermore, since similar phenotypes were observed with animals from two independently-derived transgenic families, they are most probably the consequence of increased tPA expression and not an effect of transgene integration. Interestingly, differences in LTP were observed between the two transgenic families, suggesting a dosage-related effect of tPA on this parameter. In contrast, learning performance was affected to a similar degree in both families; this may be due to a lower resolution of the behavioural tests, or to a threshold effect whereby even moderate increases in tPA expression are sufficient to achieve maximal improvement in learning capacity. The fact that LTP is affected in opposing ways by genetically engineered decreases (Huang *et al.*, 1996) and increases in tPA levels provides strong support for a role of the enzyme in this paradigm of synaptic plasticity and learning.

How might tPA affect hippocampal function? The enzyme's major substrate is plasminogen, which is present in the mouse hippocampus. Plasminogen is synthesized in neurons and, together with tPA, is implicated in neuronal degeneration following exposure to excitotoxins (Tsirka *et al.*, 1997). Activation by tPA converts plasminogen to the broad spectrum protease plasmin, which in turn can cleave a variety of extracellular substrates that may be relevant to the structure and function of the hippocampus, including laminin and NCAM (Chen and Strickland, 1997;



**Fig. 3.** Performance of transgenic mice in spatial memory tasks. Performance in the hidden-platform version of the Morris water maze tasks. (A) Escape latency in the training session (I–V) and the reversal session (VI–IX). Mean search time for each block is plotted (s). One-way ANOVA analysis reveals that performance of the wild-type and transgenic mice is improved during training days [F(4, 90) = 16.94,  $p = 0.0001$ ] for WT, [F(4, 40) = 8.92,  $p = 0.0001$ ] for T4 and [F(4, 50) = 7.75,  $p = 0.0001$ ] for T6. ANOVA with one repeated measure revealed a significant effect of genotypes during the reversal session [F(1, 27) = 10.39,  $p = 0.003$ ] for T4 and [F(1, 26) = 4.13,  $p = 0.052$ ] for T6. (B) Crossing of the trained platform position. Mean number of crossings over the old goal versus two other positions and the new goal were determined during the first trial on day VI. Data from the two transgenic families were pooled and analysed with the Tukey HSD test. Transgenic mice crossed the trained platform position (old goal) significantly more often than their wild-type littermates [F(1, 41) = 5.74,  $p = 0.02$ ]. Performance of transgenic mice in the homing holeboard task. (C) Latency for finding the connected hole in the external place condition of the homing holeboard task. ANOVA with one repeated measure revealed significant effect of genotypes at the last 2 days (\*) [F(1, 13) = 6.19,  $p = 0.02$ ].

Endo *et al.*, 1998). Alternatively, or in addition, tPA may exert plasminogen-independent effects (Tsirka *et al.*, 1997); although these are not as yet completely character-

ized they include the conversion of pro-hepatocyte growth factor (HGF) to the active neurotrophic cytokine (Mars *et al.*, 1993; Thewke and Seeds, 1996). With the promoter used, transgene expression starts only 7 days after birth, but we cannot exclude post-natal developmental effects. However, it is noteworthy that in adult rodents, transcription of the tPA gene is increased in the hippocampus by seizure, kindling and LTP (Qian *et al.*, 1993), and in cerebellar Purkinje cells by the learning of complex motor tasks (Seeds *et al.*, 1995). Furthermore, transcription of this gene is under the control of cAMP in certain cell types (Ohlsson *et al.*, 1988), and cAMP-responsive transcription is involved in synaptic plasticity and long-term memory (Guzowski and McGaugh, 1997). Taken together, these results suggest that tPA-mediated extracellular proteolysis may be activated during the learning process itself.

Our results add to the growing body of evidence in favour of a contribution of extracellular events to synaptic plasticity and learning. The remodelling of adhesive interactions between cells, and with components of the extracellular matrix, has been proposed to play a part in the modification of synaptic connections: experimental modulation of such adhesive interactions can affect plasticity and learning (Muller *et al.*, 1996). Proteolytic processing of membrane receptors could also be involved; in this respect, the demonstration that mice lacking the nociceptin receptor have a LTP and learning phenotype similar to that of Thy-tPA transgenic mice may be relevant (Manabe *et al.*, 1998). The activation of growth factor precursors, and in particular of pro-HGF, may also play a regulatory role. Extracellular proteases are obvious candidates as effectors in such events and, together, the phenotypes of tPA-deficient (Frey *et al.*, 1996; Huang *et al.*, 1996) and tPA-overexpressing mice suggest that one of the physiological functions of this enzyme may be to control synaptic plasticity. Other serine proteases [neurotrypsin (Gschwend *et al.*, 1997), neuropsin (Scarlsbrick *et al.*, 1997), neurosin (Yamashiro *et al.*, 1997) and BSP2 (Davies *et al.*, 1998)] have recently been characterized in the CNS, and it will be important to elucidate their respective roles.

One constant feature of events involving extracellular proteolysis is the combined contributions of both proteases and inhibitors. Different inhibitors of serine proteases belonging to the serpin family have been identified in the brain: protease nexin-1 is a preferential inhibitor of thrombin (Stone *et al.*, 1987), while neuroserpin is a potent inhibitor of tPA (Osterwalder *et al.*, 1998). The remarkable increase in spatial memory that we have observed in tPA-overexpressing mice may be due to the displacement of a putative protease-antiprotease balance in favour of tPA-catalysed processes. Inversely, a decrease in the proteolytic balance, either because of decreased enzyme or increased inhibitor production, may result in impaired synaptic plasticity and thereby play a part in pathological processes associated with defects in learning and memory.

## Materials and methods

### Generation of transgenic mice

A blunted *SacI*-*BglI* fragment of the mouse tPA cDNA coding sequence (Ricklees *et al.*, 1988) (positions 17–1848 bp) was inserted in the blunted

*XhoI* site of the linker sequence of a modified Thy1.2 promoter cassette (Evans *et al.*, 1984; Kelley *et al.*, 1994) (a gift from Dr F. Botterio, Friedrich-Miescher-Institute, Basel, Switzerland). This cassette drives neuronal expression of transgenes starting 7 days after birth, and it is devoid of activity in thymocytes (Vidal *et al.*, 1990). The construct was purified and injected into pronuclei of CBA/B6 F<sub>1</sub> zygotes according to established procedures (Hogan *et al.*, 1994). Transgenesis was determined on tail DNA by PCR using primers specific for the transgene (see Figure 1A) and by Southern blot hybridization with a random probe generated from an *EcoRI*-*BamHI* fragment of tPA. Two transgenic founders were used to establish independent lines, named T4 and T6, by crossing the transgenic offspring with their negative littermates.

### Analysis of transgene expression

For histo-enzymatic analysis, 5- $\mu$ m cryosections of adult brain tissue were analysed as described previously (Sappino *et al.*, 1991). The substrate solution was prepared with or without plasminogen (40  $\mu$ g/ml) and slides were incubated for 1 h at 37°C. For the enzymatic assay, 27  $\mu$ g of total brain protein and a murine tPA standard (tPA), were subjected to SDS-PAGE under non-reducing conditions. The gel was analysed by zymography (Sappino *et al.*, 1991); the zymogram was allowed to develop overnight at 37°C. Photographs for Figure 1B and C were taken under dark-ground illumination. Northern blot hybridization was done as described previously (Sappino *et al.*, 1993). Five micrograms of total RNA were loaded in each lane, and the riboprobes used were complementary to tPA mRNA (positions 1397–1848 bp), or to mRNA encoded by the *XhoI*-*BamHI* fragment of the exon IV of Thy1.2.

### Electrophysiological studies

Electrophysiological recordings were carried out as described previously (Muller *et al.*, 1996). In brief, excitatory postsynaptic potentials (EPSPs) were elicited with a stimulation electrode placed in the Schaffer collateral pathway of the hippocampus slice. Extracellular recordings were obtained from the dendritic area (stratum radiatum) of the CA1 region. In all slices, LTP was induced by theta burst-patterned stimulation (five bursts at 5 Hz, each composed of four pulses at 100 Hz) repeated twice consecutively at 10 s intervals. LTD was induced by low frequency stimulation (1 Hz applied for 7 min). To quantify the effect of LTP and LTD induction, the EPSP slope was monitored continuously and the results expressed as the ratio of the changes observed 30 min and 2 h after stimulation versus baseline values.

### Learning tasks

Water maze learning was tested as described previously (Morris, 1984), with modifications. The training apparatus consisted of a circular pool (diameter 160 cm); water was made opaque by adding skimmed milk and a 15 cm diameter circular platform was submerged 1.5 cm below water level. Mice (*n*: WT = 18, T4 = 13, T6 = 12) were given one block of trials per day for 5 consecutive days for training session (I–V). On day VI, the platform position was changed to the opposite quadrant and the same protocol was applied for reversal session for 4 days (VI–IX). A block of trials comprised six swimming turns each starting from one of three different positions in random order. Mice swam until they found the hidden platform or for 120 s. The homing holeboard task was as described previously (Schenk, 1989). In this protocol, the escape hole remained at a fixed position and olfactory traces were made irrelevant by intertrial rotations of the board. Mice (*n*: WT = 7, Tg = 11) were given one block of three trials over 5 consecutive days. The target hole was completely covered by a disk only on the third day.

The swim paths and the hole visit were videotaped and experimenters were blinded as to the genotype of the animals tested. The mice that performed the tests were F<sub>2</sub> to F<sub>4</sub> males, 10–13 weeks old. Transgenics and WT had similar body weights (26.45  $\pm$  0.48 and 26.98  $\pm$  0.48 g, respectively) and similar vigour; determined by measuring the swimming speed during the first trial of the first day (WT, 18.7  $\pm$  3.7 cm/s; Tg, 21.6  $\pm$  4.5 cm/s; *p* = 0.1) and of the fifth day (WT, 16.8  $\pm$  4.4 cm/s; Tg, 16.4  $\pm$  4.2 cm/s; *p* = 0.4) and by measuring the mean distance reached in a rotating wheel assay (WT, 197.5  $\pm$  69 m/h; Tg, 103.6  $\pm$  30 m/h; *p* = 0.1).

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