Enhanced clearance of Aβ in brain by sustaining the plasmin proteolysis cascade

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The amyloid hypothesis states that a variety of neurotoxic β-amyloid (Aβ) species contribute to the pathogenesis of Alzheimer's disease. Accordingly, a key determinant of disease onset and progression is the appropriate balance between Aβ production and clearance. Enzymes responsible for the degradation of Aβ are not well understood, and, thus far, it has not been possible to enhance Aβ catabolism by pharmacological manipulation. We provide evidence that Aβ catabolism is increased after inhibition of plasminogen activator inhibitor-1 (PAI-1) and may constitute a viable therapeutic approach for lowering brain Aβ levels. PAI-1 inhibits the activity of tissue plasminogen activator (tPA), an enzyme that cleaves plasminogen to generate plasmin, a protease that degrades Aβ oligomers and monomers. Because tPA, plasminogen and PAI-1 are expressed in the brain, we tested the hypothesis that inhibitors of PAI-1 will enhance the proteolytic clearance of brain Aβ. Our data demonstrate that PAI-1 inhibitors augment the activity of tPA and plasmin in hippocampus, significantly lower plasma and brain Aβ levels, restore long-term potentiation deficits in hippocampal slices from transgenic Aβ-producing mice, and reverse cognitive deficits in these mice.

Alzheimer | plasminogen activator inhibitor | tissue plasminogen activator

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the presence of intracellular neuronal tangles and extracellular parenchymal and vascular amyloid deposits containing β-amyloid peptide (Aβ). Aβ is a 39- to 42-aa peptide derived from the proteolytic processing of the amyloid precursor protein (APP) (1). The "amyloid hypothesis" of AD postulates a central causal role for Aβ in AD pathogenesis and is supported by genetic and physiological evidence. All known early onset familial AD mutations result in enhanced levels of cytotoxic Aβ species, amyloid plaque deposition, and dementia. Furthermore, Aβ peptide is reported to be neurotoxic and synaptotoxic *in vitro* and *in vivo*, inhibiting long-term potentiation (LTP), a physiological correlate of memory (2). Based on these observations, a number of strategies to reduce brain Aβ levels are being pursued as therapeutic approaches to treat AD (3, 4).

If the amyloid hypothesis of AD is correct and Aβ levels are pivotal to disease etiology, then the balance between Aβ production and catabolism is likely to be a key determinant of disease progression. It has been suggested that insufficient clearance of Aβ may account for elevated Aβ levels in the brain and the accumulation of pathogenic amyloid deposits in sporadic AD (5). A number of proteases have been implicated in the proteolytic clearance of Aβ from the CNS, including neprilysin, insulin-degrading enzyme, endothelin converting enzyme, and plasmin (3, 6–8). The relative contribution of these enzymes to Aβ catabolism remains unclear, but each protease may play a significant role in the degradation and clearance of Aβ, resulting in a slowing of Aβ accumulation and aggregation and ultimately Aβ's deposition into amyloid plaques.

Plasmin has received little attention as an Aβ catabolizing protease. The plasmin cascade initiates with tissue plasminogen activator (tPA) cleaving plasminogen to generate plasmin, an active serine protease (9, 10). All components of the tPA/plasmin cascade are present in the CNS, with tPA expressed in neurons and microglia and plasminogen predominantly expressed in neurons (11). Reports assessing the structure, turnover, and neurotoxicity of soluble and aggregated Aβ species indicate that both $Aβ_{40}$ and $Aβ_{42}$ are substrates for plasmin resulting in their catabolism (10, 12–14). Aggregated Aβ induces expression of tPA and urokinase plasminogen activator (uPA), in cultured neurons and in the brains of plaque-bearing transgenic Tg2576 mice (13, 15, 16). Although tPA is up-regulated, plasmin activity remains low in the brains of these mice, a finding consistent with the low plasmin activity reported in the brains and sera of AD patients. This suggests that the tPA/plasmin cascade may be inhibited in AD (17, 18). A known inhibitor of this cascade is plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor (serpin) gene family and the primary inhibitor of tPA and uPA (19, 20). Binding of PAI-1 to tPA irreversibly inhibits the serine protease activity of tPA and thus inhibits the conversion of plasminogen to plasmin (19). Of particular relevance, PAI-1 expression is increased in the vicinity of amyloid deposits in brain (11) and is elevated at sites of inflammatory response in AD patients (21). PAI-1 expression is also increased in the brains of aged mice and in transgenic APP mice with increased Aβ levels (22). Therefore, it is possible that increased levels of PAI-1 in the brains of AD patients reduce Aβ catabolism by inhibiting the production of plasmin (Fig. 1 *A–B*).

This mechanism predicts that blocking PAI-1 will remove inhibition of the tPA/plasmin cascade, reestablishing normal levels of plasmin activity and thereby increasing clearance of Aβ (Fig. 1*C*). To test this hypothesis, we developed an orally active, CNS penetrant, small-molecule inhibitor of PAI-1, which we named PAZ-417. We demonstrate that PAZ-417 is a potent inhibitor of PAI-1 that promotes plasmin formation and proteolysis of Aβ. In transgenic mouse models of AD, PAZ-417

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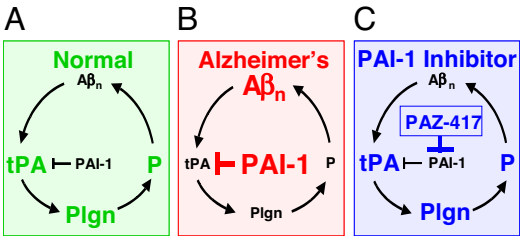

Fig. 1. Aβ activates and is cleaved by the tPA/plasmin cascade. Schematic representation to demonstrate the proteolytic clearance of β-amyloid (Aβ) in a cascade including tissue plasminogen activator (tPA), plasmin (P), and the tPA inhibitor plasminogen activator inhibitor-1 (PAI-1). (*A*) Normal. Aβ monomers aggregate into oligomers and/or fibrils; Aβ aggregates ($A\beta_n$) induce the expression of tPA and enhance the activation of tPA; and tPA cleaves plasminogen (Plgn) to liberate active plasmin, which cleaves Aβ. (*B*) Alzheimer's disease. PAI-1 binds to tPA inhibiting its activity and preventing the activation of plasmin and the proteolytic clearance of Aβ. (*C*) Treatment with PAI-1 inhibitor. The small-molecule inhibitor of PAI-1, PAZ-417, prevents formation of the PAI-1/tPA complex, resulting in sustained proteolytic tPA activity, activation of plasmin, and the proteolytic clearance of Aβ.

reduces Aβ levels in both plasma and brain and reverses both LTP and cognitive deficits. Here, we report a pharmacological enhancement of Aβ degradation by increased proteolytic catabolism. This approach provides a disease-modifying strategy for the treatment of AD.

Results

Identification of Potent and Selective PAI-1 Inhibitors. To identify inhibitors of PAI-1, an *in vitro* assay was developed, which spectrophotometrically measures tPA activity (23). The assay uses recombinant human PAI-1 and tPA, which, when associated, abolishes tPA activity and the cleavage of a chromogenic tPA substrate. Preincubation of potent small-molecule inhibitors with PAI-1 preserves the proteolytic activity of tPA. Screening the Wyeth compound library in this assay resulted in identification of numerous inhibitors including PAI-749 (24) and PAZ-417 exhibiting PAI-1 inhibitory activities (IC_{50} values) of 288 and 655 nM, respectively, for PAI-1 inhibition [supporting information (SI) Fig. S1*A*]. To examine specificity, *in vitro* studies were performed and demonstrate that PAZ-417 does not directly inhibit tPA or plasminogen (Fig. S1 and *C*) and that PAZ-417 is selective against a panel of 40 neurotransmitter, endocrine, and enzymatic targets (Fig. S1*D*).

PAZ-417 enhances the cleavage of Aβ in vitro. To investigate whether PAI-1 is able to modulate the tPA/plasmin cascade and cleavage of Aβ, monomeric and oligomerized preparations of synthetic $A\beta_{42}$ were used as the substrate to be cleaved by plasmin (Fig. 2*A*). Conditions were established to show that tPA together with plasminogen (lane 3), but not plasminogen (lane 2) or tPA (lane 4) alone, induces the cleavage of monomeric and oligomeric Aβ. Preincubation of PAI-1 with tPA, followed by the addition of plasminogen and Aβ, resulted in little proteolytic degradation of Aβ (lane 5). The pretreatment of PAI-1 with PAZ-417 (5 μM) restored the cleavage of Aβ (lane 6). Cleavage of monomeric and oligomeric $A\beta_{42}$ in response to PAZ-417 displayed dose-dependent and submicromolar potency for blocking PAI-1 activity (Fig. 2*B*). These data demonstrate that the inhibition of PAI-1 augments tPA activity, generates plasmin, and enhances the degradation of monomeric and oligomeric Aβ.

PAI-1 inhibitors sustain tPA activity in the brain. Immunohistochemical and zymograph analyses were performed on brain sections from WT and PSAPP transgenic mice. High levels of tPA protein expression were observed in the CA2/CA3 region of the hippocampus (Fig. 3*A*) and in the dentate gyrus (data not shown) of both WT and PSAPP mice. However, PAI-1 levels were

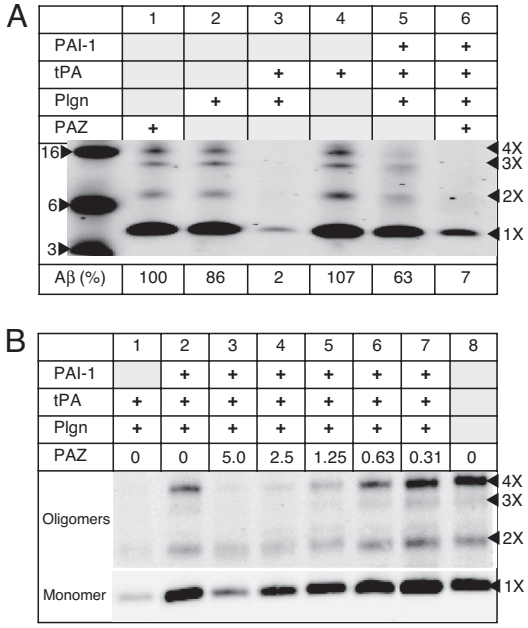

Fig. 2. A small-molecule inhibitor of PAI-1 results in cleavage of monomeric and oligomeric $A\beta_{42}$ in an *in vitro* assay. $A\beta_{42}$ peptide cleavage was assessed by Western blot after *in vitro* incubation with recombinant human PAI-1 and purified tPA and plasminogen proteins as described (see *SI Text*). (*A*) Cleavage of monomeric and oligomeric $A\beta_{42}$ in response to PAZ-417. Control Aβ was incubated in assay buffers (lane 1), with plasminogen alone (lane 2), with tPA alone (lane 4), or with tPA and plasminogen (lane 3). Only the combination of both tPA and plasminogen resulted in cleavage (98%) of monomeric and oligomeric Aβ. Cleavage of Aβ was inhibited with the preincubation of PAI-1 and subsequent addition of plasminogen (lane 5) but is restored by 93% when the mixture is preincubated with 5 μM PAZ-417 (lane 6). Indicated are the molecular weight markers (on the left); positions of Aβ monomer, dimer, trimer, and tetramer (on the right); and the percentage of remaining monomeric Aβ (*Lower*). (*B*) Dose-dependent cleavage of monomeric and oligomeric $A\beta_{42}$ in response to PAZ-417. Control for maximal cleavage Aβ (tPA and plasminogen, no PAI-1, lane 1); control for minimal cleavage of Aβ (PAI-1, tPA, plasminogen and vehicle, lane 2); and dose-response with decreasing treatment with PAZ-417 at 5, 2.5, 1.25, 0.63, and 0.31 μM (lanes 3–7); untreated Aβ control displaying starting levels of monomer and oligomers (lane 8).

markedly elevated in PSAPP compared with WT mice (Fig. 3*B*) similar to those described in Tg2576 mice (22). To further examine the endogenous activity of tPA in the brains of these animals, enzyme activities were examined in brain slices of mice treated with a single dose of PAZ-417 [30 mg/kg *per os* (po)]. Robust tPA activity was detected in the dentate gyrus and CA2 and CA3 regions of WT mice (Fig. 3*B Upper Left*). However, tPA activity was significantly reduced (64%, $P < 0.004$; Fig. 3*C*) in the hippocampus of PSAPP mice (Fig. 3*B Lower Left*) as might be predicted given the significant increase in PAI-1 expression levels observed in these mice (Fig. 3*A Lower Right*) compared with WT mice (Fig. 3*A Upper Right*). Furthermore, treatment of PSAPP animals with PAZ-417 (30 mg/kg, po; Fig. 3*B*, *Lower Right*) increased tPA activity 2-fold ($P < 0.04$, Fig. 3*C*) over vehicle-treated animals (Fig. 3*B Lower Left*) without any significant effect on WT mice (Fig. 3*C*, $P = 0.34$; and Fig. 3*B*, comparing *Upper Left* and *Upper Right*). These data demonstrate that a PAI-1 inhibitor is able to augment hippocampal tPA activity *in vivo*.

PAZ-417 reduces plasma and brain Aβ levels in transgenic APP mice. To further explore the consequences of PAI-1 inhibition on Aβ levels in the periphery and brain, PAI-1 inhibitors were administered to Tg2576 mice. Single high-dose administration of PAZ-417 (100 mg/kg, po) was initially used to determine a time

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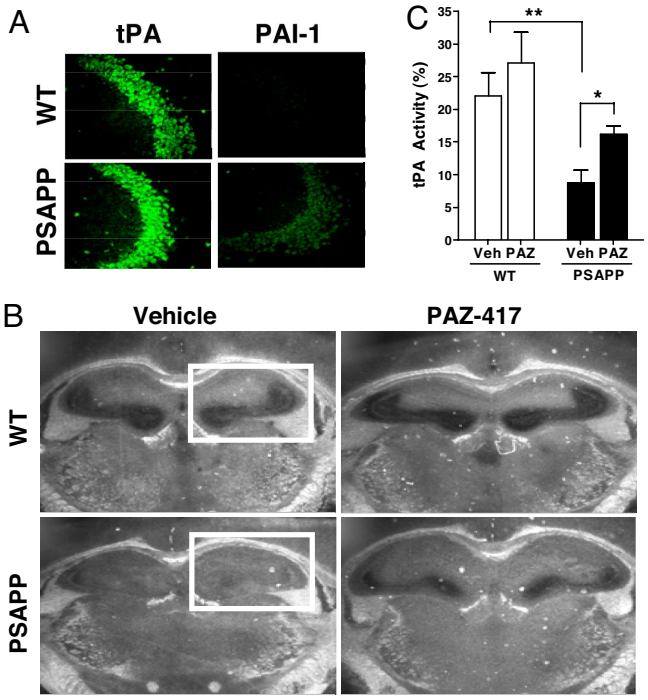

Fig. 3. A small-molecule inhibitor of PAI-1 restores tPA activity in the hippocampus of PSAPP mice. (*A*) Immunohistochemical analysis demonstrating protein levels of tPA (*Left*) and PAI-1 (*Right*) in the hippocampal CA2/CA3 brain region of WT and PSAPP mice. (*B*) Representative zymograms highlighting regions of the brain containing tPA protease activity visualized by dark-field micrographs shown for each genotype and treatment. Areas of dark exposure (example indicated by white boxes) reflect zones of substrate hydrolysis localizing tPA protease activity. (*C*) Quantitative analysis of tPA activity measured from zymograms of hippocampal brain regions ($n = 5$). The areas of tPA-associated lysis visualized by dark-field illumination are expressed as percentages of the area of hippocampus in the same plane (*, $P < 0.004$; **, $P < 0.04$).

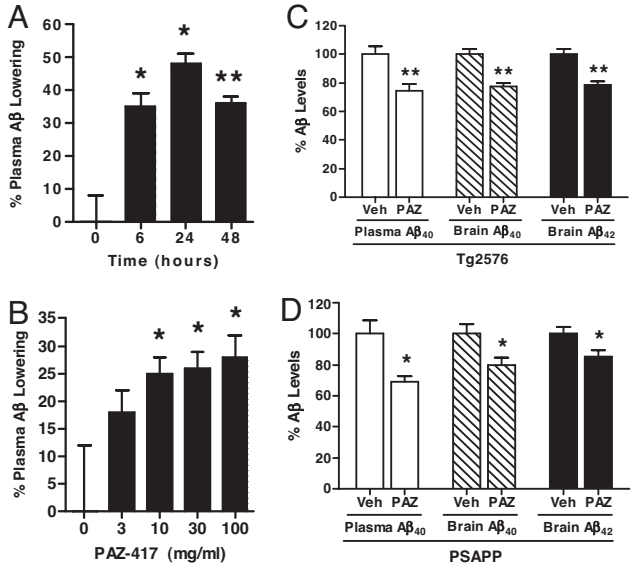

Fig. 4. A small-molecule inhibitor of PAI-1 reduces plasma and brain Aβ levels in transgenic APP mice. (*A*) Time-course of plasma $A\beta_{40}$ lowering measured in response to a single administration of PAZ-417 (100 mg/kg, po) or vehicle at the indicated posttreatment times (*, $P < 0.005$; **, $P < 0.001$) to Tg2576 mice. (*B*) Dose-response of PAZ-417 on $A\beta_{40}$ lowering at 6 h after treatment (*, $P < 0.02$). (*C* and *D*) Plasma $A\beta_{40}$ or brain $A\beta_{40}$ and $A\beta_{42}$ levels after a single administration of PAZ-417 (20 mg/kg, po) 6 h after treatment to (*C*) Tg2576 or (*D*) PSAPP mice (*, $P < 0.01$; **, $P < 0.001$). Aβ levels are presented as percentages (%) of vehicle treatment.

course of Aβ lowering and resulted in a significant reduction of plasma $A\beta_{40}$ levels by 35% at 6 h ($P < 0.005$) and 36% at 48 h ($P < 0.001$) and a peak reduction of 48% at 24 h ($P < 0.005$; Fig. 4*A*). The combination of low abundance and high animal to animal variability makes a determination of statistically significant differences extremely difficult. Nonetheless, we performed these experiments and observed trends for the 15% lowering of plasma $A\beta_{42}$ with PAZ-417 treatment. A dose-response analysis with PAZ-417 demonstrated that 10 mg/kg (po) was the minimally effective dose ($P < 0.02$), reducing plasma $A\beta_{40}$ levels by 25%. Doses of 30 and 100 mg/kg resulted in Aβ reductions comparable to those achieved at 10 mg/kg. The 3 mg/kg dose reduced plasma Aβ levels by ≈18%, but this was not significantly different from vehicle-treated animals (Fig. 4*B*). Clearance of plasma and brain Aβ was examined in both Tg2576 (nonplaque bearing age) and PSAPP (plaque-bearing age) transgenic mouse models. In Tg2576 mice, PAZ-417 (20 mg/kg, po) lowered plasma $A\beta_{40}$ by 26% ($P < 0.001$) and brain $A\beta_{40}$ and $A\beta_{42}$ by 22% ($P < 0.001$) and 21% ($P < 0.001$), respectively (Fig. 4*C*). In PSAPP mice, $A\beta_{40}$ levels were reduced by 31% ($P < 0.01$) in plasma, and in brain $A\beta_{40}$ and $A\beta_{42}$ by 20% ($P < 0.01$) and 15% ($P < 0.01$), respectively (Fig. 4*D*). These data demonstrate that PAZ-417 not only reduced plasma $A\beta_{40}$ levels but also reduced $A\beta_{40}$ and $A\beta_{42}$ levels in the brains of transgenic AD mice. In contrast, PAI-749, a non-brain penetrant PAI-1 inhibitor, had no effect on plasma and brain Aβ levels in transgenic AD mice (data not shown). Furthermore, the data demonstrate that CNS penetration of PAI-1 inhibitors is essential for lowering of plasma and brain Aβ. These data suggest that inhibition of PAI-1 in the brain and not the periphery is necessary for the observed Aβ lowering activity

PAZ-417 reverses hippocampal LTP and memory deficits in Tg2576 mice. As described in ref. 25, Tg2576 mice show a significant hippocampal LTP deficit. Administration of PAZ-417 (100 mg/kg, po) 24 h before slice preparation significantly reversed the LTP deficits in Tg2576 mice ($P < 0.05$, Fig. 5*B*) while having no effect on WT LTP (Fig. 5*A*). In Tg2576 slices 90 min after induction, field excitatory postsynaptic potential (fEPSP) slopes (as a percentage of baseline ± SEM) were 121 ± 16.5% ($n = 8$ slices, 6 animals), and 167 ± 16.0% ($n = 9$ slices, 6 animals), for vehicle- and PAZ-417-treated animals, respectively. fEPSP slopes in WT slices were 151 ± 12.5% ($n = 7$ slices, 5 animals), and 159 ± 19% ($n = 8$ slices, 9 animals), for vehicle- and PAZ-417-treated animals, respectively. To address concerns that administration of a PAI-1 inhibitor may adversely alter neuronal function, we tested the effect of PAZ-417 administration on several measures of synaptic physiology. The lack of altered basal synaptic transmission (see Fig. S2) or paired pulse facilitation (see Fig. S3), together with the observed reversal of LTP deficits in the transgenic AD mice (Fig. 5 *A* and *B*), suggests the preservation of neuronal function, and it is unlikely that PAZ-417 alters synaptic transmission or presynaptic short-term facilitation.

To examine the ability of PAZ-417 to reverse memory deficits in Tg2576 mice, we used contextual fear conditioning (CFC), a test of hippocampal-dependent learning and memory as described in refs. 25 and 26. Contextual learning involves the association of an aversive stimulus (footshock) with a novel testing environment (context). In this model, memory is expressed as a context-dependent freezing behavior in the absence of the shock 24 h after learning. PAZ-417 administered to Tg2576 mice completely reversed cognitive deficits when dosed orally 4 h before training at 30 and 100 mg/kg, but not at 10 mg/kg ($P < 0.02$, Fig. 5*C*). PAZ-417 had no effects on CFC perfor-

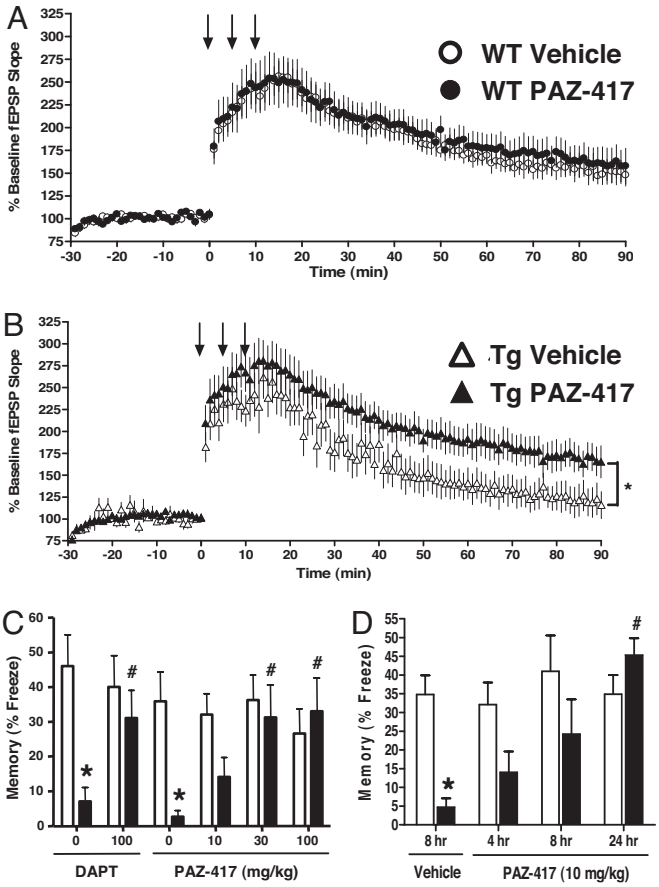

Fig. 5. PAZ-417 reduces both hippocampal LTP and contextual memory deficits in Tg2576 mice. (*A*) Administration of PAZ-417 (100 mg/kg, po) to WT mice (21–25 weeks of age) 24 h before hippocampal slice preparation has no effect on LTP induced in the dentate gyrus (DG) by high-frequency stimulation of the perforant path. (*B*) In contrast, when similarly administered to age-matched Tg2576 (Tg) mice, PAZ-417 restored DG LTP to WT levels ($P < 0.05$). (*C*) Dose-dependent effects of drug on reversing contextual memory deficits in Tg2576 and WT mice after administration of a single dose of PAZ-417 (10, 30 or 100 mg/kg, po) 4 h before training or of DAPT (100 mg/kg, po) 3 h before training. Transgenic animals exhibited significantly reduced contextual memory compared with WT mice (*, $P < 0.002$). Drug-treated transgenic animals exhibited significantly improved contextual memory compared with vehicle-treated transgenic animals (#, $P < 0.02$). (*D*) Time-dependent effects of PAZ-417 on reversing contextual memory deficits in Tg2576 and WT mice over a 24 h period after administration of a single dose (10 mg/kg, po). Transgenic animals exhibited significantly reduced contextual memory compared with WT mice (*, $P < 0.002$). Drug-treated transgenic animals exhibited significantly improved contextual memory compared with vehicle-treated transgenic animals (#, $P < 0.0001$). Twenty week-old Tg2576 (filled bars) and WT (open bars) mice.

mance in WT animals. These data are consistent with observations in Tg2576 mice (26) showing that administration of an Aβ lowering gamma-secretase inhibitor (DAPT) also completely reversed CFC deficits (Fig. 5*C*). To understand the relationship between Aβ lowering and reversal of cognitive deficits, PAZ-417 (10 mg/kg, po) was administered at 4, 8, and 24 h before training, and memory performance was examined 20 h later. Complete reversal of the memory deficits was observed with pretreatment 24 h before training ($P < 0.0001$), and a partial but nonsignificant reversal was observed with a pretreatment of 8 h ($P < 0.06$, Fig. 5*D*). These results demonstrate that the time of drug administration is important for reversal of the memory deficit and likely depends on the time at which peak Aβ lowering is occurring in the brain (i.e., 24 h, Fig. 4*A*). Treatment with the non-CNS penetrant compound PAI-749 (100 mg/kg, po) was ineffective in reversing CFC deficits in these transgenic AD mice (data not shown).

Discussion

The amyloid hypothesis postulates that brain Aβ is neurotoxic and synaptotoxic and a key driver of disease progression and cognitive decline (5). Recent studies have shown that proteases, such as neprilysin, insulin-degrading enzyme, and plasmin, may contribute to the catabolism and clearance of Aβ and that an imbalance between production and catabolism of Aβ may be a critical driver of AD pathogenesis (3). Although genetic manipulation of Aβ catabolizing enzymes has added credence to the critical role these proteases play in maintaining low levels of brain Aβ, pharmacological augmentation of these proteolytic pathways has not been possible (8). In this study, we demonstrate that sustaining the plasminogen-plasmin pathway, using a small-molecule inhibitor of PAI-1, can enhance Aβ catabolism *in vitro*, lower plasma and brain Aβ levels *in vivo*, reverse LTP deficits in transgenic Aβ-producing hippocampal slices, and improve cognition in transgenic Aβ-producing mice.

The molecular components of the tPA/plasmin cascade are all expressed in brain (11) and regulated in a manner similar to that seen in the periphery for cleavage of the prototypical substrate fibrin (12, 13). Several lines of evidence support a role for the tPA/plasmin cascade in the clearance of Aβ (9). Plasmin, the active zymogen generated by tPA from plasminogen, cleaves both monomeric and aggregated forms of $Aβ_{40}$ and $Aβ_{42}$ at multiple sites (10, 13), and the cleavage of Aβ by plasmin is protective against Aβ mediated cell death (12). Importantly, injection of Aβ into the brains of mice lacking either tPA or plasminogen confirmed that both these components are required for the effective clearance of Aβ from brain (22), although it should be noted that another study showed no effect of plasminogen deficiency on endogenous nonaggregated murine Aβ levels (27). Finally, aggregated forms of Aβ induce the expression of tPA *in vitro* and *in vivo* (13, 15, 16) and serve as cofactor for tPA-mediated plasminogen activation (10). Hence it is surprising that amyloid accumulation in AD patients is not better controlled by the tPA/plasmin cascade. One explanation for this may be that in AD the aggregation and deposition of Aβ is associated with a number of inflammatory responses, leading to an induction of PAI-1 in the brain (21). This increased expression of PAI-1 is predicted to inhibit the tPA/plasmin cascade, resulting in a reduced ability to degrade Aβ, and a cycle of increased Aβ accumulation and further PAI-1 induction. Taken together these observations suggest that the tPA/plasmin cascade contributes to the proteolytic clearance of Aβ and that Aβ can also participate in autoregulation of its steady-state levels (see Fig. 1). To test this hypothesis, we developed a potent PAI-1 inhibitor, PAZ-417, and tested its ability to degrade Aβ *in vitro* and *in vivo*.

Initial evidence demonstrating that PAI-1 inhibition with PAZ-417 could lead to a dose-dependent degradation of soluble monomeric and oligomeric Aβ species was obtained by using an *in vitro* tPA/plasmin assay (Fig. 2). Detection and colocalization of tPA and PAI-1 protein levels in the hippocampus of transgenic AD mice was observed as reported in ref. 28. Inhibition of PAI-1, using PAZ-417, resulted in the preservation of tPA activity in the hippocampus, as demonstrated by *ex vivo* zymography studies in brain slices (Fig. 3). Furthermore, PAZ-417 reduced both plasma and brain Aβ levels (Fig. 4), reversed LTP deficits (Fig. 5 *A–D*) and improved hippocampal-dependent cognitive performance in transgenic AD mice (Fig. 5 *C* and *D*). Although we have demonstrated a reversal of CFC deficits by reducing Aβ levels with other approaches (e.g., gamma-secretase inhibitors; Fig. 5*C*) (26), we cannot exclude that PAZ-417 also improves cognition by mechanisms independent of Aβ-lowering. For example, PAI-1 levels have been reported to

modulate NMDA receptor signaling, providing an alternative mechanism for modulating learning and memory via a tPA-dependent mechanism (29). It is also possible that activation of uPA, a functional analog of tPA (19, 30), contributes to efficacy in our experiments, because PAI-1 is also the principal inhibitor of uPA. This may be of relevance given that single nucleotide polymorphisms in the uPA gene have been associated with elevated $A\beta_{42}$ levels in the plasma of patients with late onset AD (31).

To examine the importance of central versus peripheral PAI-1 inhibition, we compared the activity of the brain penetrant PAZ-417 with the non-brain penetrant PAI-749. PAI-749 was inactive in all $A\beta$ lowering and behavioral assays, demonstrating that inhibition of PAI-1 in the brain is critical for activity. Consistent with studies showing that PAZ-417 directly inhibits PAI-1 in the brain, immunohistochemistry studies showed that PAI-1 protein levels are reduced in the hippocampus of transgenic AD mice after drug treatment (data not shown). This observation is consistent with studies demonstrating that the half-life of PAI-1 is significantly reduced when bound by small-molecule inhibitors (32).

Given the pivotal role of tPA and plasmin in fibrinolysis and blood clot dissolution, one concern with the use of PAI-1 inhibitors might be a potential for increased risk of bleeding. Complete PAI-1 deficiency in a human kindred with a null mutation in the PAI-1 gene results in severe bleeding after trauma or surgery (33). However, heterozygous individuals with a partial PAI-1 deficiency demonstrate normal clotting and bleeding times. Hemostasis and the effects of PAI-1 gene inactivation have also been studied in PAI-1-deficient mice, with neither spontaneous bleeding nor delayed rebleeding evident (34). In addition, PAI-1 inhibitors have been evaluated in preclinical models of coronary occlusion and shown to exert an antithrombotic effect without altering homeostasis (35). Furthermore, *in vitro* studies demonstrated that PAZ-417 does not inhibit tPA-initiated lysis of clots formed with either reptilase or thrombin, further showing that clot dissolution is unperturbed. Finally, safety pharmacology studies in mouse, rat, and dog evaluating PAZ-417 at high exposure multiples over the efficacious exposure have demonstrated no significant effects on the CNS or respiratory function, arterial blood pressure, heart rate, or bleeding times (data not shown). It is therefore expected that pharmacological manipulation of PAI-1, using PAZ-417, should not affect bleeding in humans.

Although the primary role of PAI-1 is to regulate tPA and uPA activities, elevated PAI-1 levels have also been suggested to contribute to vascular disease, fibrosis, obesity, metabolic syndromes, and cancer (19, 20, 28, 36, 37). Neurodegeneration and other pathophysiological conditions have also been associated with overexpression (38, 39) or with exogenously added components of the tPA/plasmin cascade (28, 40, 41). For example, increased synthesis of tPA leading to neuronal death after seizure or stroke (42, 43) and the subsequent generation of plasmin resulting in degradation of the extracellular matrix (42, 44) are reported. The formation of cerebral amyloid angiopathies (CAA) and the generation of plasmin may contribute to the loss of vessel integrity and cerebral microhemorrhage (45). Nevertheless, beneficial roles of plasmin and tPA in the CNS have also been described. For example, plasmin enhances the cleavage of APP by α-secretase (46) and tPA contributes to a number of functions including synaptic plasticity, learning, and memory processes in the hippocampus and motor learning processes in the cerebellum (28).

In summary, we demonstrate that activation of an endogenous $A\beta$ catabolic pathway, using a small-molecule inhibitor of PAI-1, significantly lowers plasma and brain $A\beta$ *in vivo* and reverses LTP and cognitive deficits in transgenic AD mice. These data provide evidence that pharmacological enhancement of $A\beta$ catabolism may be a useful strategy for the treatment of AD. Future clinical investigations will evaluate PAI-1 inhibitors as potential novel therapeutics for the disease-modifying treatment of AD.

Materials and Methods

Transgenic APP Mice. We used heterozygous male transgenic mouse models Tg2576 [expressing human amyloid precursor protein with the Swedish mutation (47)] and PSAPP (expressing human presenilin-1 with the Met-146-Leu mutation crossed to Tg2576) or their respective WT littermates. Tg2576 mice at 20 weeks of age were used for measuring $A\beta$ levels in plasma and brain (n = 8–12), and for behavioral studies (n = 8–18 per genotype per age), whereas PSAPP mice at 24 weeks of age were used for measuring plasma and brain levels (n = 10–12), or at 28 weeks of age for zymography (n = 5). Animals received oral doses of PAZ-417 in vehicle (2% Tween-80 and 0.5% methylcellulose) as indicated and were killed after dosing at the time points indicated, and brains and/or plasma collected.

Zymography and Immunohistochemistry. PAZ-417 (30 mg/kg, po) or vehicle (0.5% methylcellulose/0.5% Tween 80) was administered to PSAPP or the WT mice. Twenty-eight hours later, mice were anesthetized and perfused transcardially with 5 ml of ice-cold PBS. Brains were extracted and frozen, and 18-μm cryostat sections were cut onto slides. Slides were stored at −20°C until processed for *in situ* zymography or immunohistochemistry. *In situ* zymography was performed essentially as described in ref. 48 with modification (see *SI Text*). Areas of tPA activity were visualized by dark-field illumination, and photographs taken. Areas of lysis were measured by using Image Pro Software and expressed as percentages of the area of hippocampus in the same plane. All area measurements were taken from coronal sections 1.94 mm caudal to bregma. For immunohistochemistry, sections were postfixed in paraformaldehyde as described (see *SI Text*). Fluorescent images were obtained with a Zeiss Axioplan 2 microscope with appropriate filter. All images were photographed under identical exposure and magnification conditions with a Diagnostic Instruments RT Slider Spot camera, using ImagePro Plus software.

Electrophysiological Evaluation. The procedure for hippocampal slice preparation, recording of fEPSPs and assessment of LTP deficits in Tg2576 mice are described elsewhere in ref. 25. Briefly, hippocampal slices (n = 7–9) were prepared from WT or Tg2576 (Tg) 21- to 25-week-old mice, an age at which the latter show pronounced synaptic deficits in the dentate gyrus after lateral perforant path stimulation. Twenty-four hours before slice preparation, animals were treated with PAZ-417 (100 mg/kg, po) or vehicle (10 ml/kg, 2% tween and 0.5% methylcellulose in water).

Plasma and Brain $A\beta$ measurements. Blood was collected and plasma prepared from male Tg2576 or PSAPP mice, or their age-matched WT controls, and brains were saline perfused and snap frozen on dry ice. Extraction of brain in guanidine and plasma $A\beta_{40}$ and brain $A\beta_{40}$ and $A\beta_{42}$ measurement were conducted by sandwich ELISA as described in ref. 25. All samples were analyzed in duplicate, and the average of two to three independent assays is reported in Fig. 4.

Behavioral CFC Testing. Tg2576 or WT mice were trained and tested in operant chambers controlled by Med-PC software (Med-Associates) on two consecutive days in the contextual fear conditioning (CFC) paradigm as described in refs. 25 and 26.

Statistical Analysis. For $A\beta$ measurements, data are expressed either as mean percentage (%) of control samples from vehicle-treated animals or mean absolute $A\beta$ levels with standard errors. Statistical analyses were performed by the student t test. Behavior results were analyzed by using a two-way ANOVA followed by posthoc pairwise comparison made by using SAS statistical software (SAS Institute).

For analysis of zymographs, the tPA activity associated with hippocampal area was measured and calculated as a percentage of total hippocampal area demonstrating proteolysis. Data were transformed by square root based on the test by Box and Cox. The transformed data were analyzed by using ANOVA. The P value of an F test was found to be 0.0023. The assumption of normal distribution of errors was checked to be reasonable (P = 0.1368) by the Shapiro–Wilk test applied on residuals. Two-tailed least significant difference tests were used to compare treatments. SAS 9.1 (SAS Institute) was the software used for all statistical computation.

For LTP, statistical differences were assessed by t test on the terminal end

of the timecourse. Input-Output curves were analyzed by ANOVA. Values are means ± SEM. Sample sizes are number of slices with the number of originating animals in parentheses.

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