Supporting Information

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SI Text

Determination of PAI-1 Inhibition of tPA Activity. Small molecule PAI-1 inhibitors synthesized at Wyeth were formulated in DMSO at a final concentration of 10 mM, and then diluted to 100x in an HNEPA buffer [100 mM Hepes (pH 7.4), 100 mM NaCl, 1 mM EDTA, 0.1% wt/vol PEG-8000, 2 mM APO-10] (Fig. S1*A*). The assay was initiated by the addition of small molecule PAI-1 inhibitors (0.02–10 μM final concentration, maximum DMSO concentration of 1%) to recombinant human PAI-1 (24 nM in pH 7.4 buffer; Molecular Innovations). After a 5-min incubation at room temperature, 20 nM recombinant human tPA was added and the combination of compound, PAI-1 and tPA (Activase; Genentech) were incubated for an additional 10 min. After the second incubation, Spectrozyme-tPA (American Diagnostica), a chromogenic substrate for tPA, was added and absorbance was read at 405 nm over a period of 15 min to generate kinetic curves (Thermomix Emax Reader with SoftmaxPro 4.3 software; Molecular Dynamics) from which IC_{50} values were calculated. Relative PAI-1 Inhibitor activity was calculated as equal to the residual tPA activity in the compound-treated samples. Control treatments included the complete inhibition of tPA by PAI-1 at the molar ratio used (1.2:1) and the absence of any effect of the compound on tPA alone. Unless otherwise stated, all chemicals were obtained from Sigma Chemical.

PAZ-417 Specificity. To demonstrate that PAZ-417 does not directly inhibit tPA or plasminogen, two clot lysis assays were performed by using human plasma and purified human tPA and plasminogen to simulate physiologically relevant conditions (Fig. S1 *B* and *C*). PAZ-417 (up to concentrations of 20 μM) did not inhibit tPA-initiated lysis of plasma clots formed with either reptilase (Fig. S1*B*) or thrombin (Fig. S1*C*). These data indicate that tPA-initiated clot dissolution remains unperturbed by the presence of small molecule inhibitors of PAI-1.

PAZ-417 was profiled against a panel of neurotransmitter, endocrine, and enzymatic targets to identify potential selectivity and side-effect issues (Fig. 2*D*). Of the 40 assays performed, 37 revealed no significant activity (<20% inhibition) of PAZ-417 at 10 μM. The remaining 3 assays displayed low activity for histamine H2 receptors (21%), choline acetyltransferase (24%) and cholecystokinin CCK1 receptors (27%). However, no assays displayed >50% inhibition, the criteria established by NovaScreen to qualify a compound as significantly active against a target.

PAI-1 Inhibition Aβ Cleavage Assay. *In vitro* characterization of $A\beta_{42}$ peptide cleavage was assessed by incubation with various components of the tPA/plasmin proteolysis cascade and PAZ-417 (Fig. 2). $A\beta_{42}$ peptide (Anaspec) was prepared by disaggregation in hexafluoroisopropanol that was evaporated off, dissolved in F12 media, and aggregated overnight at 4°C. $A\beta_{42}$ (10 μM) was incubated in different combinations with 0.05 unit plasminogen, 1.5 μM tPA (Genentech), 5 μM PAI-1 (Molecular Innovations), and various PAZ-417 concentrations in a total reaction volume of 50 μl. Samples were incubated for 2 h at 25°C ice and then frozen on dry ice until processed for SDS/PAGE. Samples were run on 12% NuPage gels (Invitrogen), transferred onto nitrocellulose, and probed with 6E10 (Signet Laboratories) and IR800-conjugated donkey anti-mouse antibody (Rockland) for detection. Infrared densitometry analysis of Aβlevels was performed by scanning blots on an Odyssey system (LI-COR Biosciences).

Pharmacokinetic Parameters of Small Molecule Inhibitors of PAI-1. Before performing *in vivo* work with PAI-1 inhibitors the penetration of PAI-1 inhibitors across the blood–brain barrier (BBB) after a single administration (10 mg/kg, po) of PAZ-417 or PAI-749 was determined (data not shown). For PAZ-417, studies revealed drug exposures of 617 ng·h/ml in brain and 7,113 ng·hr/ml in plasma indicating approximately a 0.09 brain to plasma ratio. In contrast, the structurally diverse PAI-1 antagonist, PAI-749, was not detectable in brain despite a higher drug exposure in the plasma (15,012 ng-h/ml).

Zymography and Immunohistochemistry. For *in situ* zymography, slides were air-dried, then warmed in a humid chamber to 37°C. An overlay mixture was made that contained 0.7 ml of a 3.1% agar solution in water, 0.5 ml of a 6% nonfat dry milk solution in PBS and 0.75 ml of 0.1 M PBS. After heating the mixture to 45°C, 20 μl of a 50 mg/ml solution of purified human plasminogen was added. Prewarmed cryostat sections were covered with 200 μl of the overlay mixture spread evenly under a 24 × 60 mm glass coverslip. Slides were incubated at 37°C in a humid chamber for 6 h to allow the zymograms to develop. For immunohistochemistry, sections were postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 25 min at 4°C, washed in cold Tris-buffered saline (TBS), then preincubated for 45 min in 0.5% Triton X-100 in TBS at 4°C. Sections were then rinsed and any nonspecific staining was blocked by using TBS containing 0.1% Triton X-100 and 5% goat serum for 1 h followed by incubation with primary rabbit anti-PAI-1 antibody (1:50, Santa Cruz Biotechnology) or rabbit anti-tPA antibody (1:50, Santa Cruz Biotechnology) overnight at 4°C. The next day, sections were rinsed with TBS and incubated with FITC conjugated goat anti-rabbit secondary antibody (1:100) for 1 h then rinsed in TBS. To reduce endogenous fluorescence, sections were treated with 0.3% sudan black for 10 min and rinsed in PBS before applying coverslips.

PAZ-417 Reverses LTP Deficits in Hippocampal Brain Slices from Tg2576 Mice While Preserving Neuronal Function. To address the possibility that administration of a PAI-1 inhibitor may cause untoward effects on neuronal function, the effect of PAZ-417 administration of several measures of synaptic physiology, specifically I-O determination, paired-pulse facilitation and LTP was examined (Fig. 5 *A* and *B* and Figs. S2 and S3). Moreover, hippocampal slices from both wild-type and Tg2576 mice (20 weeks of age), which we have demonstrated to reveal an impairment in the later genotype were examined. Our data show a beneficial effect of PAZ-417 administration on measures of synaptic function (LTP) pertinent to the improvement in behavior-cognitive function previously included and the complete absence of any negative effect in these tests. Equally important is the parallel finding of no PAZ-417-mediated effect in slices prepared from WT mice. Specifically, the synaptic tests used were: (*i*) input–output (I–O) determination, a measure of basal synaptic activity widely attributed to AMPA receptor mediated synaptic transmission (Fig. S2 *A–D*); (*ii*) paired-pulse (PP) stimulation, a measure of presynaptically mediated short-term facilitation (Fig. S3 *A* and *B*); and (*iii*) high-frequency stimulation-induced LTP, a measure of synaptic plasticity known to be mediated in the dentate gyrus by NMDA receptor activation, and acknowledged to represent a

key physiological underpinning of learning and memory (Fig. 5 *A* and *B*).

The most dramatic effect of PAZ-417 administration observed was the restoration of Tg LTP to WT levels (Fig. 5*B*), an effect that may account for the cognitive-behavioral improvements in Tg2576 mice undergoing hippocampus-mediated tasks over a similar time course. More modest effects of PAZ-417 were observed in the I-O curves (Fig. S2 *A–D*), whereas no effect was seen on PP facilitation (Fig. S3 *A* and *B*).

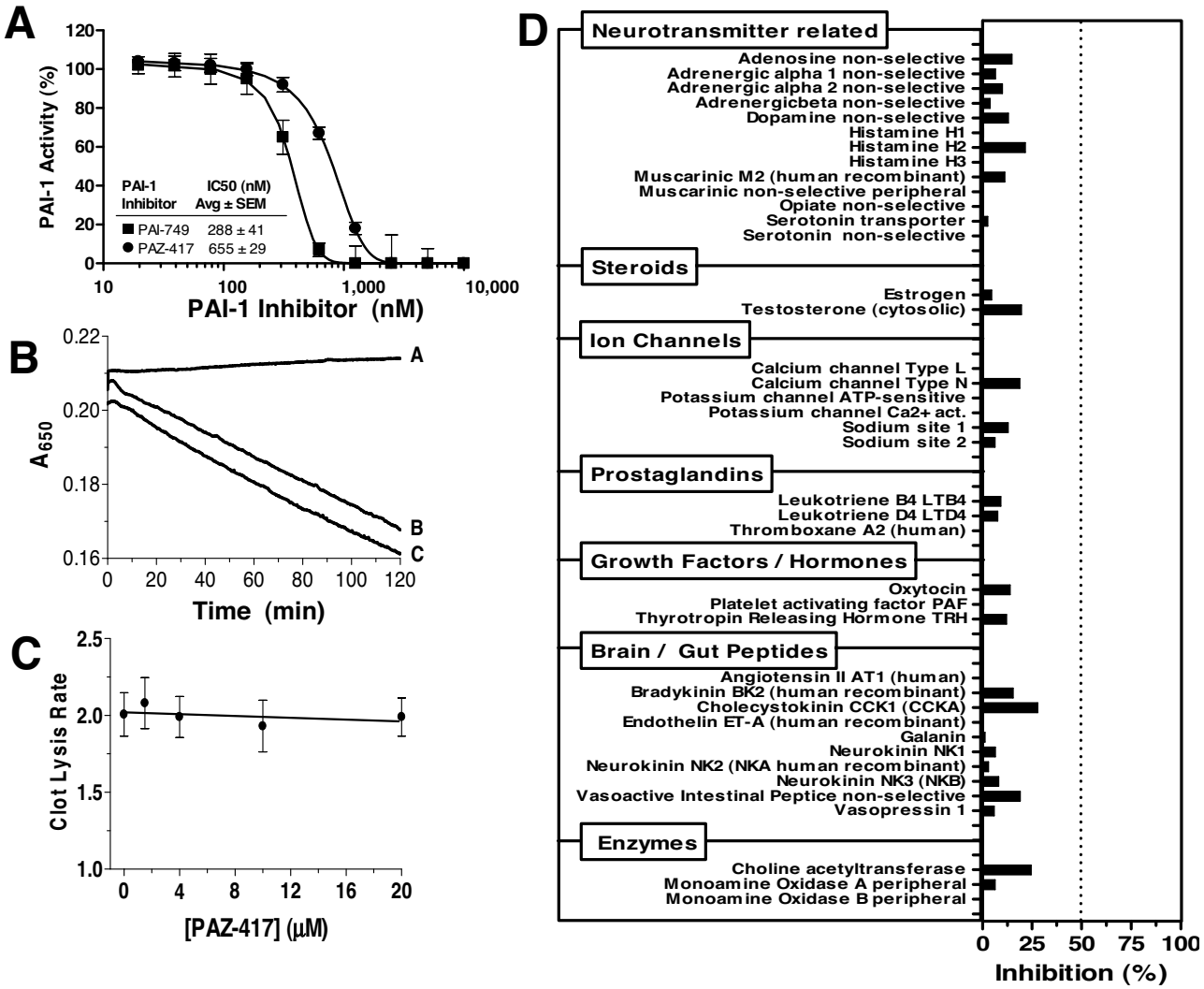

Fig. S1. Inhibition and selectivity of human recombinant PAI-1 by small molecule inhibitors *in vitro*. (*A*) Dose-response of inhibitors PAZ-417 (filled circles) and PAI-749 (filled squares) on PAI-1 activity *in vitro*. IC_{50} values are given as average nM (± SEM) for four determinations. (*B*) PAZ-417 has no impact on tPA-initiated lysis of plasma clots formed with reptilase. Reptilase was added to human plasma and the ensuing clots were aged for 60 min at 25°C. tPA and PAZ-417 (10 μM, final concentration) were added, and clot lysis was assessed by monitoring the time-dependent decrease in turbidity ($A_{650\ nm}$) at 25°C. A, No tPA; B, tPA + PAZ-417; C, tPA. Each curve shows averaged data from the 6 replicates in a single experiment and was repeated four times with comparable results. (*C*) No impact of PAZ-417 on tPA-initiated lysis of thrombin-generated plasma clots. Thrombin was added to human plasma and the ensuing clots were aged for 60 min at 25°C. tPA and varying amounts of PAZ-417 (0, 1.5, 4, 10, and 20 μM final concentrations) were added and clot lysis was assessed by monitoring the time-dependent decrease in turbidity ($A_{650\ nm}$) at 25°C. The clot lysis rate represents the slope value ($\times 10^4$) from the 30–120-min interval of the progress curve. Each data point shows the mean (± standard deviation) from three independent experiments (with 6 replicates in each of the three independent experiments). (*D*) *In vitro* selectivity of PAZ-417 (10 μM) against a panel of assays to establish a peripheral side-effects profile (NovaScreen Biosciences). Of the 40 assays performed, 37 revealed no significant activity (<20% inhibition) of PAZ-417 at 10 μM. The remaining 3 assays displayed low activity for histamine H2 receptors (21%), choline acetyltransferase (24%) and cholecystokinin CCK1 receptors (27%). However, no assays displayed >50% inhibition, the criteria established by NovaScreen Biosciences to qualify a compound as significantly active against a target. Data are expressed as percentages of inhibition of given endpoint.

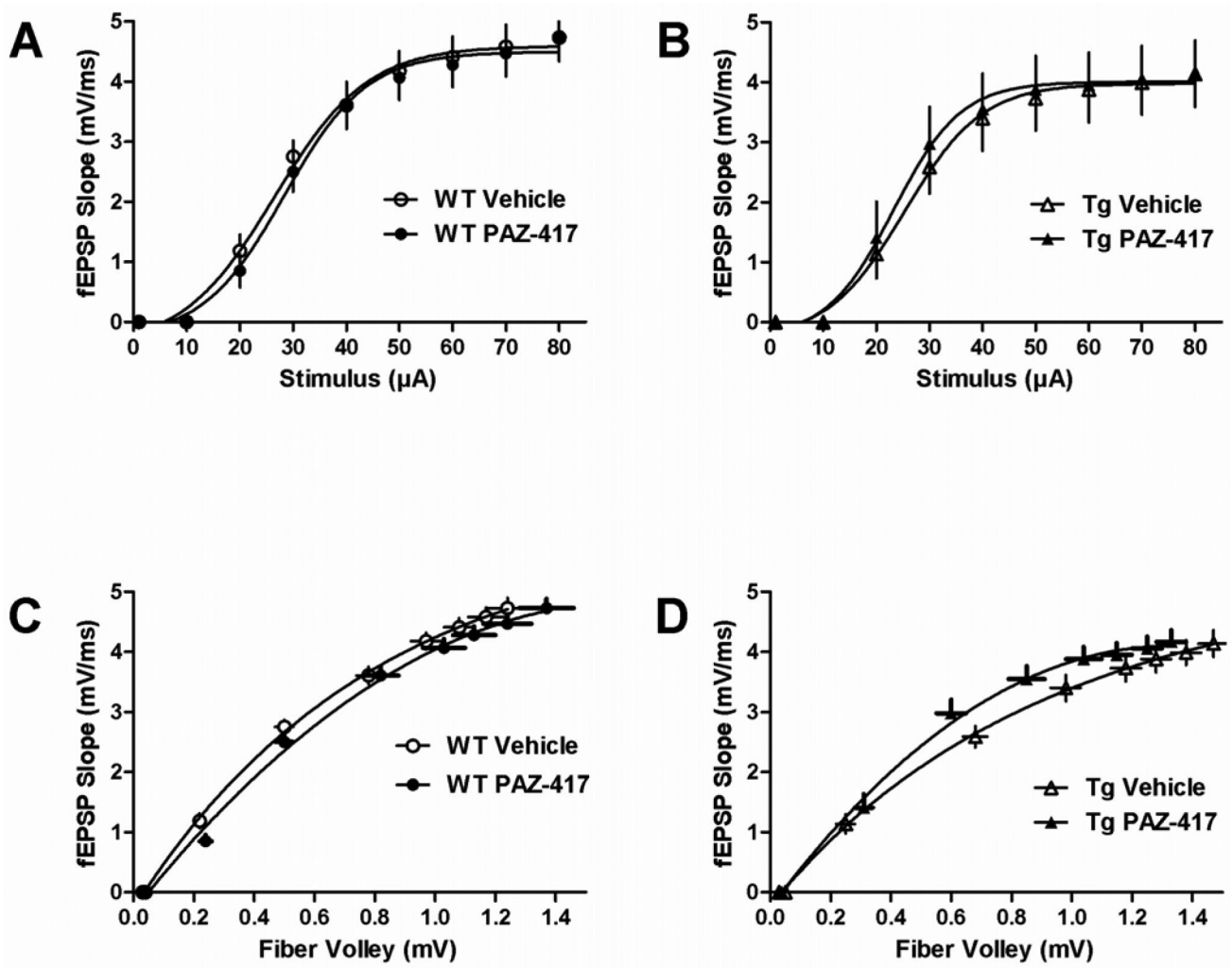

Fig. S2. Effect of PAZ-417 on basal synaptic transmission. Treatment of slices of either genotype (WT: *A* and *C*; Tg: *B* and *D*) with PAZ-417 had no effect on basal transmission, as measured by the fEPSP slope as a function of stimulus intensity (*A* and *B*). The Input-Output curves of WT (*A*) slices trended modestly, but not significantly, higher at stronger stimulus intensities compared with Tg2576 slices (*B*). At 80 μA, fEPSPs slopes $\pm$ SEM were: WT Vehicle 4.73 $\pm$ 0.38 mV/ms, n = 7 (5); WT PAZ-417 4.73 $\pm$ 0.39 mV/ms, n = 8 (5); Tg Vehicle 4.141 $\pm$ 0.55 mV/ms, n = 8 (6); and Tg PAZ-417 4.17 $\pm$ 0.54 mV/ms, n = 9 (6). Treatment with PAZ-417 also showed no significant effect of WT I-O curves when plotted as a function of presynaptic fiber volley amplitude (*C*), although there appeared a modest improvement of PAZ-treated Tg fEPSP slopes toward WT levels (*D*).

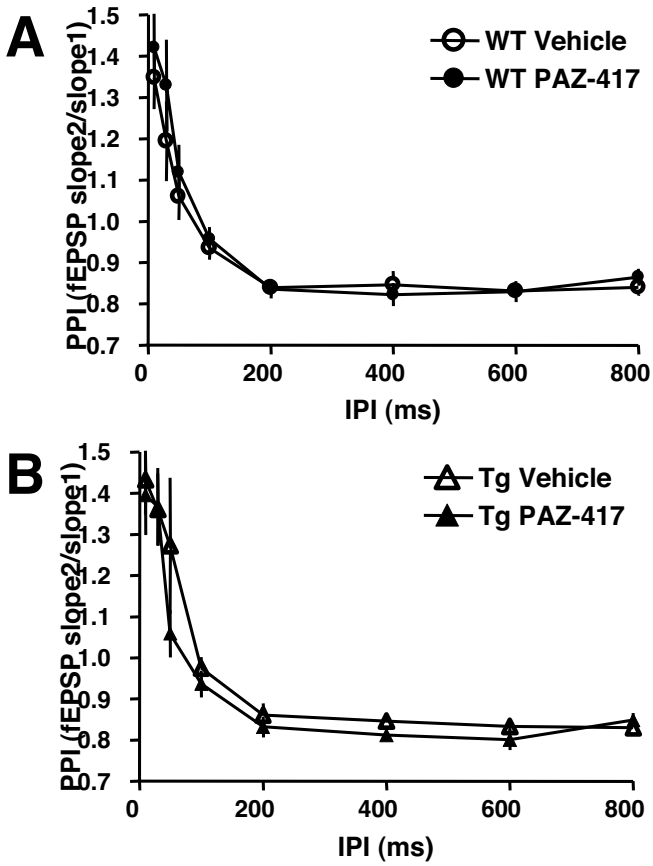

Fig. S3. Effect of PAZ-417 on paired pulse facilitation. Treatment with PAZ-417 had no effect on presynaptic short-term facilitation in WT (*A*) or Tg2576 (*B*) slices, as measured by a paired-pulse stimulation paradigm.