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EXTRACELLULAR PROTEOLYSIS OF REELIN BY TISSUE PLASMINOGEN ACTIVATOR FOLLOWING SYNAPTIC POTENTIATION

J. H. TROTTER[a,b,†], **A. L. LUSSIER**[a,b,†], **K. E. PSILOS**[a,b], **H. L. MAHONEY**[a,b], **A. E. SPONAUGLE**[a,b], **H.-S. HOE**[c], **G. W. REBECK**[c], and **E. J. WEEBER**[a,b,*]

[a]Department of Molecular Pharmacology and Physiology, University of South Florida, Tampa, FL 33620, United States

[b]USF Health Byrd Alzheimer's Institute, Tampa, FL 33613, United States

[c]Department of Neuroscience, Georgetown University Medical Center, Washington, DC 20057, United States

Abstract

The secreted glycoprotein reelin plays an indispensable role in neuronal migration during development and in regulating adult synaptic functions. The upstream mechanisms responsible for initiating and regulating the duration and magnitude of reelin signaling are largely unknown. Here we report that reelin is cleaved between EGF-like repeats 6–7 (R6–7) by tissue plasminogen activator (tPA) under cell-free conditions. No changes were detected in the level of reelin and its fragments in the brains of tPA knockouts, implying that other unknown proteases are responsible for generating reelin fragments found constitutively in the adult brain. Induction of NMDAR-independent long-term potentiation with the potassium channel blocker tetraethylammonium chloride (TEA-Cl) led to a specific up-regulation of reelin processing at R6–7 in wild-type mice. In contrast, no changes in reelin expression and processing were observed in tPA knockouts following TEA-Cl treatment. These results demonstrate that synaptic potentiation results in tPA-dependent reelin processing and suggest that extracellular proteolysis of reelin may regulate reelin signaling in the adult brain.

Keywords

Reelin; Tissue plasminogen activator (tPA); Hippocampus; Tetraethylammonium chloride; Long-term potentiation

*Correspondence to: E.J. Weeber, USF Health Byrd Alzheimer's Institute, University of South Florida, 4001 E. Fletcher Ave., Tampa, FL 33613, United States. Tel: +813-396-9984. eweeber@health.usf.edu (E. J. Weeber).

†Authors contributed equally to this publication.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

INTRODUCTION

The inside-out layering of the mammalian cortex requires the extracellular glycoprotein reelin, which is secreted by Cajal–Retzius cells (D'Arcangelo et al., 1995; Hirotsune et al., 1995). Loss of reelin or downstream signaling components (ApoER2/VLDLR, Disabled-1, etc.) leads to a roughly inverted cortex, perturbed hippocampal lamination, and cerebellar hypoplasia (Trommsdorff et al., 1999). In addition to playing a pivotal role in neuronal migration, reelin signaling is necessary for dendritic morphogenesis (Niu et al., 2004), synapse development (Groc et al., 2007; Qiu and Weeber, 2007; Niu et al., 2008; Rogers et al., 2011; Trotter et al., 2011), and synaptic plasticity (Weeber et al., 2002; Pujadas et al., 2010; Rogers et al., 2011; Trotter et al., 2013). Changes in reelin expression (Impagnatiello et al., 1998; Botella-Lopez et al., 2006; Chin et al., 2007; Herring et al., 2012), processing (Botella-Lopez et al., 2010; Duveau et al., 2011; Tinnes et al., 2011) and glycosylation (Botella-Lopez et al., 2006) have been associated with the pathoetiology of a range of neuropsychiatric and neurodegenerative diseases, underscoring the importance of understanding mechanisms that regulate reelin in the adult brain.

Reelin is processed at two main sites (Fig. 1A), between EGF-like repeats 2–3 (R2–3) and 6–7 (R6–7) (Jossin et al., 2004). Full-length reelin and the 5 potential fragments generated by reelin cleavage can be observed in both the developing and adult brain (Jossin et al., 2007; Krstic et al., 2012). The N-R2 region of reelin (Fig. 1A) is important for protein homopolymerization and signaling (Utsunomiya-Tate et al., 2000), but is not essential for lipoprotein receptor binding (Jossin et al., 2004). In fact, disruption of reelin aggregation with the CR-50 antibody, which binds the CR-50 region between N-R2 (Fig. 1A), perturbs neuronal migration *in vivo* (Nakajima et al., 1997). The N-R2 region has also been reported to bind $alpha_3beta_1$-integrins (Dulabon et al., 2000). Recently, it has been shown that reelin can be cleaved within the repeat 3 (R3) and that this produced N-terminal fragment is transported or diffuses to further regions than the larger fragments or full-length reelin (Koie et al., 2014). These results support a role of the N-terminal fragment in the range and duration of reelin signaling (Koie et al., 2014). The preponderance of known reelin functions require the R3–6 region (Fig. 1A), which is responsible for binding apoE receptor 2 (ApoER2), very-low-density-lipoprotein receptor (VLDLR) (D'Arcangelo et al., 1999), and amyloid precursor protein (APP) (Hoe et al., 2009). Consistent with these findings, application of reelin fragments containing R5–6 to *reeler* cortical explants is sufficient to induce Disabled-1 phosphorylation and normalize cortical lamination (Jossin et al., 2004). The highly-charged C-terminal region (R7-C) may be involved in reelin folding, secretion (D'Arcangelo et al., 1997; de Bergeyck et al., 1997), and signaling efficacy (Nakano et al., 2007), but is not known to bind to receptors.

While significant progress has been made in delineating downstream mechanisms of reelin signaling, upstream mechanisms operative in the developing and adult nervous system remain elusive. Similar to other extracellular signaling molecules, a critical locus of reelin signaling regulation is at the level of transcription (Erbel-Sieler et al., 2004; Wang et al., 2004; Chen et al., 2007; Miller and Sweatt, 2007; Cubelos et al., 2008), although receptor availability (Duit et al., 2010; Hong et al., 2010; Balmaceda et al., 2014) and secretion (Duveau et al., 2011) may also play a role. However, these mechanisms only serve to adjust

the level of reelin signaling, as they are not sufficient to initiate the reelin signal by themselves. In support of this view, preventing extracellular proteolysis of reelin by inhibiting metalloproteinases blocks signaling in the developing cortex and disrupts corticogenesis (Lambert de Rouvroit et al., 1999; Jossin et al., 2007) and also impairs reelin processing in an epilepsy model (Tinnes et al., 2013). These findings imply that reelin is tethered to the extracellular matrix following secretion, where it remains inactive until liberated by proteolysis to initiate downstream signaling.

Recent *in vitro* studies have identified a disintegrin and metalloproteinase with thrombospondin motifs 4 and 5 (ADAMTS-4/5) and tissue plasminogen activator (tPA) as candidate enzymes capable of generating major reelin fragments (Krstic et al., 2012). Although little is known about the function of ADAMTS-4 and -5 in the brain, tPA represents a promising reelin protease candidate due to its high expression in the hippocampus and important role in learning and memory (Qian et al., 1993; Barnes and Thomas, 2008). Moreover, activity-dependent secretion and activation of tPA has been found to be critical for synapse formation and hippocampal synaptic plasticity (Pang et al., 2004; Nagappan et al., 2009), processes which also require reelin signaling (Weeber et al., 2002; Pujadas et al., 2010; Rogers et al., 2011). The extent to which tPA cleaves reelin *in vivo* and its potential significance have not been previously addressed. In this study we present evidence that tPA is a critical regulator of reelin processing following synaptic potentiation.

EXPERIMENTAL PROCEDURES

Animals

Both C57BL/6 J (wild-type) and tPA knockout mice (B6.129S2_Plat^{tm1mlg}/j) were obtained from the Jackson Laboratory. For experiments, males at approximately 4 months of age were used. Animals were group housed in a standard 12-h light/dark cycle and fed ad libitum standard mouse chow. All animal care protocols were followed in accordance with the Institutional Animal Care and Use Committee of the University of South Florida.

Chemicals and reagents

Plasminogen (P7397), activated plasmin (P1867), Diisopropyl fluorophosphate (DIFP; D0879), tetraethylammonium chloride (TEA-Cl; 86616) were obtained from Sigma. Recombinant tissue plasminogen activator (tPA; #176) was obtained from American Diagnostica Inc. Plasminogen activator inhibitor-1 (PAI-1, 528205) and aprotinin (616371) were purchased from Calbiochem. HyClone Dulbecco's Phosphate-Buffered Saline (DPBS) without magnesium and calcium and Halt Protease Inhibitor Cocktail (78425) were obtained from Thermo Scientific. Mammalian protein extraction reagent (MPER) was obtained from Thermo Pierce. Reelin G10 antibody (MAB5364) was obtained from Millipore and was used to visual full-length reelin and the 370- and 180-kDa fragments. Reelin ab14 antibody was generously provided by Andre Goffinet. Beta-actin (#4967) and p-ERK1/2 antibodies (Tyr202/Tyr204; #4370S) were obtained from Cell Signaling.

Recombinant protein production

HEK293 cells were transfected with full-length reelin pCrl vector to produce recombinant reelin as described previously (Qiu and Weeber, 2007).

Cell-free reelin processing assay

Partially-purified reelin (50 nM) was incubated with recombinant tPA (0, 5, 25, 100, 200, and 400 nM) for 15 min at 37 °C in DPBS. The reactions were terminated by combining them with equal volume of Laemmli Sample Buffer (Bio-Rad) with 5% 2-mercaptoethanol. The Halt protease inhibitor cocktail (without EDTA) was used to block reelin processing by 200 and 400 nM of tPA. The cocktail was used at a 1X concentration, resulting in final concentrations of 1 mM AEBSF, 800 nM aprotinin, 50 μM Bestatin, 15 μM E64, 29 μM Leupeptin, and 10 μM Pepstatin A. A time course of reelin processing by tPA and plasminogen was established by incubating reelin (50 nM) with tPA (50 nM), plasminogen (18 μg/μl), tPA and plasminogen, or active plasmin (0.5 U/ml) in DPBS at 37 °C for 15 and 45 min. The aforementioned reactions were also mixed separately with aprotinin (40 μM), DIFP (100 μM), PAI-1 (1 ng/ul) for 45 min at 37 °C to establish the role of serine protease activity in reelin processing by the tPA/plasminogen system. Reactions were mixed with CR-50 (0.02 μg/μl) for 45 min at 37 °C to determine the role of reelin dimerization in its processing. All *in vitro* experiments were performed in triplicate.

Ex vivo slice culture assay

Mice were euthanized and their brains were dissected as described previously (Weeber et al., 2002). Brains were sectioned horizontally in ice-cold cutting solution at 400 μm. The hippocampus was dissected and acclimated in 50:50 solution (cutting:ACSF) for 10 min at room temperature. Sections were then recovered in ACSF at 32 °C for 1.5–2 h. To measure the effect of tPA on reelin processing in the intact brain, hippocampal slices (n=4 per treatment group) were treated with tPA (50 or 100 nM) diluted in ACSF for 45 min. For this and subsequent experiments, CA1 was dissected in ice-cold cutting solution and snap-frozen on dry ice. Samples were stored at −80 °C until processing for Western blot analysis. Chemical long-term potentiation was induced by exposing slices to tetraethylammonium chloride (TEA-Cl, 25 mM) diluted in ACSF (adjusted for osmolarity) for 10 min. Slices were then recovered in normal ACSF and collected 5-, 15-, and 45-min post-treatment. The tPA KO slices were treated with TEA-Cl for 10 min and recovered in ACSF for 15 min. All experiments were performed with a minimum of 6 hippocampal slices total obtained from at least 2 animals.

Western Blot analysis

For all *ex vivo* slice culture assays, total protein was extracted from dissected hippocampal area CA1 by mixing with 25 μl M-PER supplemented with Halt protease inhibitor cocktail. For evaluating reelin levels *in vivo*, supplemented M-PER was used to isolate proteins (1 ml/80 mg) from the hippocampus, cortex, and cerebellum of adult wild-type and tPA knockout mice (n=6 per genotype). Samples were incubated on ice for 30 min followed by clarification at 15,000g for 15 min. Protein concentrations were measured using a BCA Protein Assay Kit (Thermo Scientific). Samples were adjusted to equal protein

concentrations and combined with an equal volume of Laemmli Sample Buffer with 5% of 2-mercaptoethanol. Unboiled samples were electrophoretically separated on 4–15% TGX gels (Bio-Rad) and transferred to PVDF blotting membranes (Millipore). The membranes were blocked in blotting solution containing 0.1 M tris-buffered saline with 0.1% Tween-20 and 5% nonfat milk. The membranes were then incubated with primary antibody diluted at 1:1000 in blotting solution overnight. The next day, the membranes were washed and then incubated with anti-Mouse-HRP or anti-Rabbit-HRP secondary antibodies (Southern Biotech) diluted at 1:2000 in blotting solution. For Western blot analysis of tissue lysates, beta-actin was also probed to normalize for variation in protein loading. Blots were detected using Pierce ECL Western Blotting reagent (Thermo Scientific) for chemiluminescence on autoradiography X-ray film.

The films were digitized and optical densities were determined using a computerized image analysis system with a high powered scanner and the software program Image J (v1.43u, National Institutes of Health). Single autoradiographic signal bands of appropriate molecular weights were identified and quantified. An experimenter who was blind to the treatment conditions measured the optical density of the bands.

Statistical analysis

All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, v 16.0, Chicago, IL, USA). Independent samples *t*-tests (twotailed) were used to examine the statistical significance of differences between the different brain regions of tPA KO mice and wild-type mice when comparing protein levels and across the TEA-Cl and control groups in the tPA KO mice. We also examined the effects of TEA-Cl between the non-treated group and treatment groups with one-way ANOVAs and LSD post-hoc analyses where appropriate. The criterion for statistical significance was set at $p<0.05$. All data are represented as mean±the standard error of the mean.

RESULTS

Processing of reelin by tPA under cell-free conditions

To evaluate the effects of tPA on reelin processing, we combined partially-purified reelin (50 nM) with varying concentrations of tPA (0–400 nM) for 15 min at 37 °C (Fig. 1B). The reelin G10 antibody was used to look at full-length reelin and the 370-kDa (N-R6) and 180 kDa (N-R2) fragments; whereas Ab14 was used to evaluate the 80-kDa (R7–8) fragment (Fig. 1A). We found that tPA dose-dependently increased reelin processing at R6–7, revealed by increased levels of both the 370- and 80-kDa fragments. The level of the 180-kDa fragment was not significantly affected by tPA treatment, suggesting that tPA is unable to affect cleavage at the R2–R3 site. Because generation of the 370- and 80-kDa fragments both reflect cleavage of reelin at R6–7, all later experiments evaluated reelin processing using the reelin G10 antibody. To block the effect of tPA we used the Halt broad-spectrum protease inhibitor cocktail (PIC) without EDTA, at a concentration that effectively inhibits aspartic acid, cysteine and serine proteases. Inclusion of the PIC significantly inhibited tPA-mediated reelin processing (Fig. 1C).

The major *in vivo* substrate of tPA is the zymogen plasminogen, which is processed into the serine protease plasmin (Silverstein et al., 1984). As most established tPA functions in the adult brain are mediated through plasmin (Pang et al., 2004; Chen et al., 2008; Echeverry et al., 2010), we determined the effects of both tPA and plasmin on reelin processing (Fig. 2A) by incubating reelin (50 nM) with tPA (50 nM), plasminogen (18 μg/μl), plasminogen and tPA or purified plasmin (0.5 U/ml) for 15 and 45 min. Plasminogen alone had no effect on reelin levels, whereas both active plasmin and the tPA/plasminogen combination resulted in a complete degradation of full-length reelin and the 370-kDa fragment into the 180-kDa fragment and three sub-180-kDa fragments. Both tPA-activated plasminogen and purified active plasmin generated the same spectrum of reelin fragments. Reelin degradation by plasmin progressed slightly from the 15 to 45-min time-points, but revealed no additional reelin fragments. These findings suggest that tPA may influence reelin processing in both a plasminogen-independent and -dependent fashion.

We next examined the specificity of reelin processing by the tPA/plasminogen system using various serine protease inhibitors. The bovine pancreatic trypsin inhibitor, aprotinin, is capable of blocking the fibrinolytic effects of active plasmin (Kang et al., 2005) but does not directly affect tPA protease activity (Lottenberg et al., 1988). Consistent with these findings, aprotinin (40 μM) was unable to prevent tPA-dependent processing of reelin after 45 min (Fig. 2B), but potently blocked plasmin-mediated reelin degradation. The plasminogen/reelin combination was used as a negative control because plasminogen alone has no effect on reelin processing. Diisopropyl fluorophosphate (DIFP) is a general serine protease inhibitor that can be used to inhibit plasmin activity (Liu and Gurewich, 1991; Novak et al., 1997). We found that DIFP (100 μM) effectively inhibited reelin processing by both tPA and plasmin. Plasminogen activator inhibitor (PAI-1) is a member of the serpin family of serine protease inhibitors and is the principle inhibitor of both uPA and tPA (Ranby and Brandstrom, 1988). We found that PAI-1 (1 ng/μl) led to a specific inhibition of tPA- and tPA/plasminogen-mediated reelin processing, whereas it did not affect processing of reelin by active plasmin. These results confirm that both tPA and plasmin differentially affect reelin processing, and that their effects can be dissociated using aprotinin, DIFP, and PAI-1.

Secreted reelin forms homodimeric complexes comprised of more than 40 monomers (Kubo et al., 2002). Blocking reelin dimerization using the CR-50 antibody reduces its signaling efficacy (Utsunomiya-Tate et al., 2000). To determine if dimerization is required for reelin proteolysis we co-incubated reactions with the CR-50 antibody (0.02 μg/μl). Inhibition of dimerization had no effect on reelin processing by tPA or plasmin (Fig. 2B).

Processing of reelin by tPA in acute hippocampal slices

Cell-free conditions are devoid of extracellular factors which may ultimately dictate the propensity of reelin to be processed *in vivo*. For this reason, we examined the effects of exogenous tPA application on reelin processing in the hippocampus of wild-type mice (Fig. 3). Acute hippocampal slices were treated with tPA for 60 min at 50 and 100 nM (n=4 per treatment). We used 50 nM of tPA as it is sufficient to enhance long-term potentiation in hippocampal area CA1 (Zhuo et al., 2000). There were no significant differences in the levels of full-length reelin and the 370-kDa (N-R6) and 180-kDa (N-R2) fragments

following any tested concentration of tPA. Higher concentrations of tPA (up to 400 nM) were also tested (data not shown) and had no effect on reelin processing, suggesting that the R6–7 cleavage site may not be readily accessible in the native tissue environment.

Reelin levels in the brains of adult tPA KO versus wild-type mice

Since reelin fragments are observed under basal conditions in the adult brain (Krstic et al., 2012), we next determined if tPA deficiency *in vivo* altered reelin processing. The levels of full-length reelin and fragments were evaluated in 4-month-old tPA KO and wild-type mice (Fig. 4, n=6 per genotype). We found no significant differences in the levels of full-length reelin or fragments in the cortex, hippocampus, and cerebellum; brain regions demonstrated to have high levels of tPA expression in the adult (Thewke and Seeds, 1999; Teesalu et al., 2004). These findings suggest that under basal conditions, tPA has minimal effect on reelin processing in the adult brain.

Effect of chemical LTP on reelin processing

The potassium channel blocker TEA-Cl induces a robust Ca^{2+}-dependent and NMDAR-independent LTP in hippocampal area CA1 (Aniksztejn and Ben-Ari, 1991). For this reason, we evaluated changes in reelin processing in area CA1 at 5, 15, and 45 min following a 10-min exposure to TEA-Cl (Fig. 5A). There was a significant effect of TEA-Cl treatment on the level of the 370-kDa reelin fragment, [$F(3, 50)=3.689$, $p<0.05$]. Post-hoc analysis revealed a significant increase for the 15-min TEA-Cl recovery group when compared to the non-treatment and 5-min exposure groups for the 370-kDa fragment (all p-values<0.05). Because induction of LTP by TEA-Cl also requires activation of PKA and ERK1/2, we measured p-ERK1/2 levels as a positive control for TEA-Cl treatment (Kanterewicz et al., 2000). The p-ERK1/2 levels were significantly elevated by 5 min which remained elevated but decreased back toward baseline levels at the 15- and 45-min recovery time points (Fig. 5B). An overall ANOVA revealed that there were significant main effects for both p-ERK1 and p-ERK2 levels [$F(3,43)=4.455$, $p<0.05$, and $F(3, 43)=26.245$, $p<0.001$, respectively]. Post-hoc analysis revealed that the 5-min recovery group showed significant increases in p-ERK1 levels when compared to the control group and the 45-min recovery group (all p-values<0.05). For p-ERK2, each group exposed to TEA-Cl has significantly higher levels of protein than the vehicle control group (all p-values<0.05). In addition, the 5-min recovery group also showed significant increases in p-ERK2 levels when compared the 15- and 45-min recovery groups.

Given the differences in reelin processing following TEA-Cl treatment, we utilized the same paradigm in the tPA KO mice to examine the role of tPA in reelin processing following the 15-min recovery period (Fig. 5C). No differences were found in the levels of full-length reelin or the fragments. However, tPA knockout slices still responded to TEA-Cl treatment with significant increases in p-ERK1 and p-ERK2 at the 15-min recovery time compared to the non-treatment group [($t(15)= -2.246$, $p<0.05$) and ($t(15)= -2.246$, $p<0.05$), respectively; Fig. 5D].

DISCUSSION

The secreted glycoprotein reelin performs diverse roles in the developing and adult brain, including regulation of neuronal migration (D'Arcangelo et al., 1995), dendritogenesis (Niu et al., 2004), synapse development (Niu et al., 2008; Rogers et al., 2011; Ventruti et al., 2011), hippocampal synaptic plasticity (Weeber et al., 2002; Rogers et al., 2011; Trotter et al., 2011), and learning and memory (Weeber et al., 2002; Qiu et al., 2006; Rogers et al., 2011). Recent studies have suggested that changes in reelin expression and processing may contribute to cognitive deficits associated with several debilitating neuropsychiatric and neurodegenerative disorders, including schizophrenia (Impagnatiello et al., 1998; Fatemi et al., 2000), depression (Fatemi et al., 2000; Lussier et al., 2009; Lussier et al., 2011; Lussier et al., 2013), and Alzheimer's disease (Chin et al., 2007; Botella-Lopez et al., 2010; Herring et al., 2012). Although the downstream mechanisms of reelin signaling have been extensively studied, there is still little known about the upstream mechanisms that control the initiation, magnitude, and duration of the reelin signal. Here we present evidence that extracellular proteolysis by tPA could serve as a critical upstream regulator of reelin signaling following synaptic potentiation. Specifically, we demonstrate that reelin is processed by tPA at a single site between R6 and 7. Although tPA is not involved in cleaving reelin under basal conditions, induction of NMDAR-independent forms of LTP with TEA-Cl revealed dynamic changes in reelin expression and processing that required tPA.

The binding of full-length reelin and fragments containing R5–6 to the conserved ligand-binding modules of apolipoprotein E receptor 2 (ApoER2) and very-low density lipoprotein receptor (VLDLR) (Yasui et al., 2010) promotes receptor clustering and tyrosine phosphorylation of Disabled-1 by Src family kinase members (Hiesberger et al., 1999; Trommsdorff et al., 1999). Blocking cleavage of reelin through metalloproteinase inhibition prevents Disabled-1 activation and disrupts cortical development in embryonic cortical explants (Jossin et al., 2007). Although the specific metalloproteinases responsible for reelin cleavage *in vivo* have not been identified, a disintegrin and metalloproteinase with thrombospondin motifs 4 and 5 (ADAMTS-4 and -5) are both capable of cleaving reelin at N- and C-terminal cleavage sites *in vitro* (Hisanaga et al., 2012; Krstic et al., 2012). Despite the ability of ADAMTS-4 to cleave reelin, it is not the enzyme responsible for reelin processing in cultured cortical neurons (Hisanaga et al., 2012), highlighting an important limitation of cell culture models in which reelin and proteases are secreted freely. This emphasizes the need for *in vivo* models to identify and test proteases that regulate reelin processing in its native, extracellular matrix (ECM) bound state.

In the brain, reelin is enriched in the golgi apparatus, axonal secretory pathways, and throughout the extracellular matrix of dendrite-rich neuropils (Rodriguez et al., 2000; Ramos-Moreno et al., 2006). Potential mechanisms responsible for the initiation of the reelin signaling pathway may involve changes in its transcription and secretion, alterations in the availability of reelin receptors, or extracellular proteolysis. Although not studied extensively, both reelin expression and secretion do not appear to be regulated by synaptic activity (Lacor et al., 2000; Tinnes et al., 2011). Moreover, acute hippocampal slices respond to reelin stimulation with enhancement of LTP (Weeber et al., 2002), reflecting

ready availability of receptors on the cell surface. We hypothesize that extracellular proteolysis of reelin by activity-dependent proteases may initiate reelin signaling following synaptic potentiation.

A potential candidate for the activity-dependent processing of reelin is tPA, which was recently found to cleave reelin between R6 and 7 *in vitro* (Krstic et al., 2012). tPA is both secreted and activated in an activity-dependent fashion (Pang et al., 2004; Nagappan et al., 2009), allowing it to activate substrates which influence synaptic plasticity and learning and memory. In agreement with the recently published study (Krstic et al., 2012), we found that tPA cleaves reelin between R6 and 7. The ability of PAI-1 to inhibit the cleavage of reelin by tPA suggests that it could serve as an endogenous inhibitor of reelin processing and signaling in the brain. We also found that plasmin was capable of cleaving reelin at several sites, effectively degrading both full-length reelin and the 370-kDa fragment. This is unlike the brain-derived neurotrophic factor (BDNF), whose maturation is promoted by a single cleavage of pro-BDNF by plasmin (Pang et al., 2004). Although the current study did not focus on plasmin-mediated reelin processing, the additional cleavage sites that are sensitive to plasmin may represent a more complex mechanism for the control of the duration and magnitude of reelin signaling.

The inability of tPA to modulate reelin processing in *ex vivo* slice cultures, even at concentrations known to enhance LTP (Zhuo et al., 2000), may be due to the cleavage site being sterically-hindered under basal conditions in intact tissue. To determine if tPA deficiency affects reelin processing *in vivo*, we evaluated reelin fragment levels in tPA KO mice. No changes were observed in the levels of full-length reelin or its fragments in the adult cortex, hippocampus, and cerebellum. The lack of obvious changes in reelin processing under basal conditions is consistent with a requirement of high levels of synaptic activity to drive the release and activation of tPA (Lochner et al., 2006; Lochner et al., 2008). Notwithstanding, we did still observe that the reelin N-R2 (370 kDa) and N-R6 (180 kDa) fragments were expressed in all tested regions, implying that other proteases expressed in the adult brain are responsible for reelin processing under basal conditions.

Because we did not observe any effect of tPA on reelin processing under basal conditions, we hypothesized that synaptic potentiation could drive activation of tPA and processing of reelin at R6–7. To study the activity-dependent processing of reelin, we used the potassium channel blocker tetraethylammonium chloride (TEA-Cl), which induces a robust, Ca^{2+}-dependent and NMDAR-independent chemical LTP (cLTP) in hippocampal area CA1 (Aniksztejn and Ben-Ari, 1991). Unlike electrical stimulation, which allows activation of only restricted bundles of Schaffer collaterals (SC), TEA-Cl induces a more uniform activation of the SC, culminating in LTP throughout area CA1. The selective generation of the 370-kDa (NR6) fragment reveals that the proteolysis does not always occur sequentially or uniformly. In addition, the relative recovery of this processing as shown by the decrease in the 370-kDa fragment by the 45-min recovery time following TEA-Cl exposure suggests that this is a dynamic process that requires further examination to understand the importance of this transient regulation.

CONCLUSIONS

Here we present a novel mechanism whereby activity-dependent release and activation of tPA, and possibly other proteases, results in the processing of reelin into active, receptor-binding fragments. The generation of fragments that bind to and activate unique receptor pathways offers a potential mechanism to explain the robust effect of reelin on synaptic plasticity and learning and memory. Understanding these mechanisms may have particular relevance to AD, as impaired regulation of tPA has been observed in post-mortem AD tissue and in mouse models (Liu et al., 2011), a change which could contribute to deficits in reelin signaling and synaptic dysfunction. Future experiments should further explore mechanisms of activity-dependent reelin processing and establish its relevance to the regulation of synaptic function and learning and memory, particularly in the context of pathological alterations in reelin proteases such as tPA.

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Abbreviations

ADAMTS-4/5	a disintegrin and metalloproteinase with thrombospondin motifs 4 and 5
ApoER2	apoE receptor 2
APP	amyloid precursor protein
BDNF	brain-derived neurotrophic factor
DIFP	diisopropyl fluorophosphate
LTP	long-term potentiation
PAI-1	plasminogen activator inhibitor
PIC	protease inhibitor cocktail
SCs	Schaffer collaterals
tPA	tissue plasminogen activator
TEA-Cl	tetraethylammonium chloride
VLDLR	very-low-density-lipoprotein receptor

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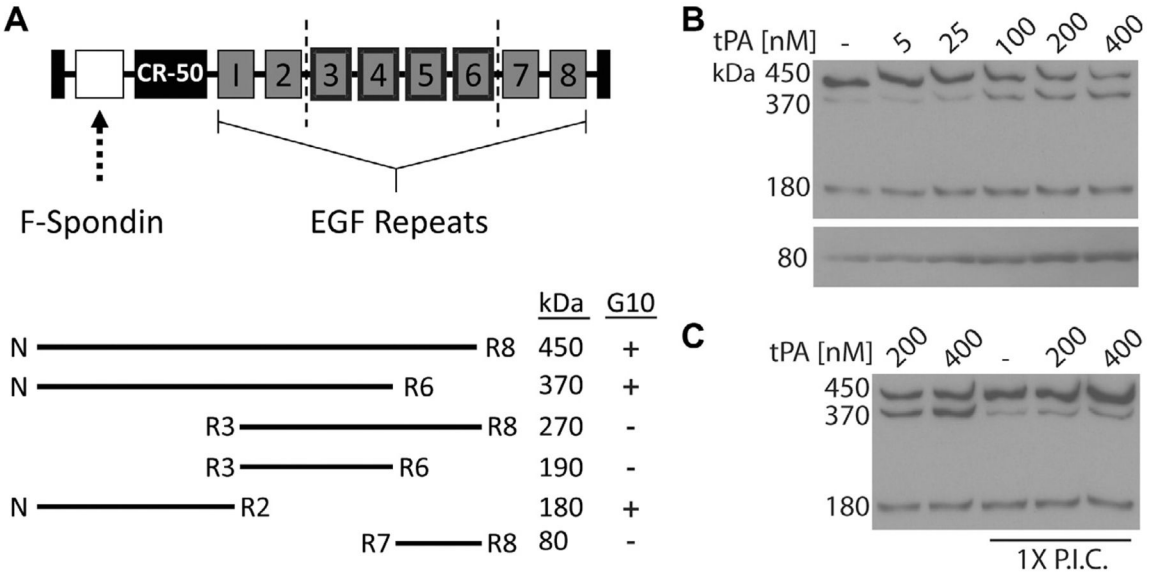

Fig. 1.

Modulation of Reelin processing by tPA. (A) Reelin is cleaved between epidermal growth factor (EGF) repeats 2–3 (R2–3) and 6–7 (R6–7), resulting in 5 potential fragments (370, 270, 190, 180, and 80 kDa). The anti-reelin G10 primary antibody detects full-length reelin (450 kDa), and the 370- and 180-kDa fragments. Ab14 was used to detect the 80-kDa fragment. (B) Recombinant reelin (50 nM) was incubated with 0–400 nM tPA at 37 °C for 15 min. tPA concentration-dependently increased processing of recombinant reelin between R6 and 7. Both the 370 and 80-kDa reelin fragments were increased, while the level of full-length reelin was decreased by tPA treatment. The 180-kDa fragment was not altered. (C) Inclusion of the broad-spectrum Halt protease inhibitor cocktail (1X P.I.C.) with tPA (200 nM and 400 nM) inhibited reelin processing. Experiments were performed in triplicate.

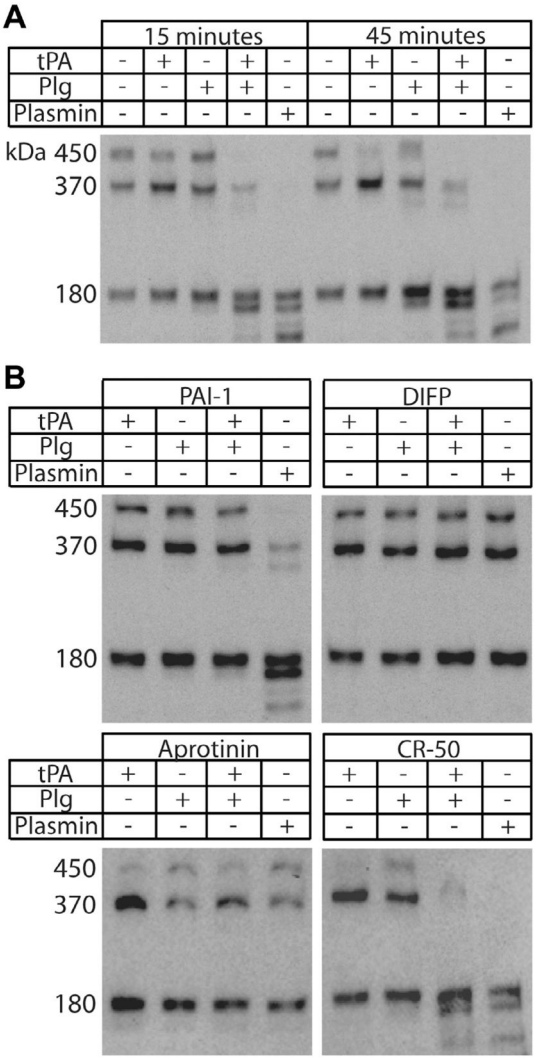

Fig. 2.

Effects of tPA and plasminogen on reelin processing under cell-free conditions. Recombinant reelin (50 nM) was incubated with tPA (50 nM), plasminogen (Plg, 18 μg/μl), plasminogen (18 μg/μl)/tpa (50 nM), and activated plasmin (0.5 U/ml) at 37 °C for 15 and 45 min under cell-free conditions. (A) At both the 15- and 45-min time points, tPA promoted the generation of the 370 fragment. Plasminogen alone had no effect. The Plg/tPA combination and active plasmin converted reelin to 180-kDa and sub-180-kDa fragments. (B) The above-mentioned reactions were co-incubated with serine protease inhibitors or CR-50 for 45 min at 37 °C. Plasminogen activator inhibitor (PAI-1, 1 ng/μl) blocked the cleavage of reelin by tPA and the plg/tPA combination, whereas it had no effect on already active plasmin. DIFP (100 μM) inhibited the processing of reelin by all protease combinations. Aprotinin (40 μM) blocked the effects of tPA-activated plasmin and purified plasmin, but did not prevent tPA-mediated reelin processing. The dimerization-inhibiting CR-50 antibody (0.02 μg/μl) had no effect on reelin processing by tPA, plg/tPA, or active plasmin. Experiments were performed in triplicate.

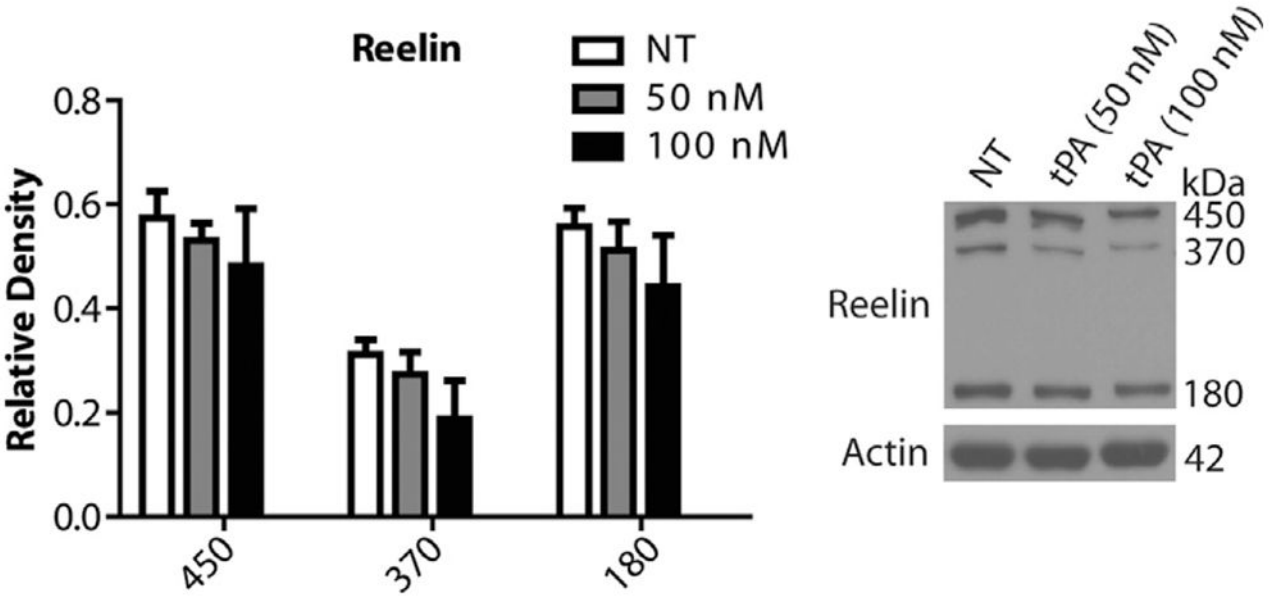

Fig. 3.

tPA does not modulate reelin processing in acute hippocampal slices. Wild-type hippocampal slices (*n*=4 per treatment) were treated with 50 or 100 nM tPA for 60 min. No significant differences were found in full-length reelin or fragment levels.

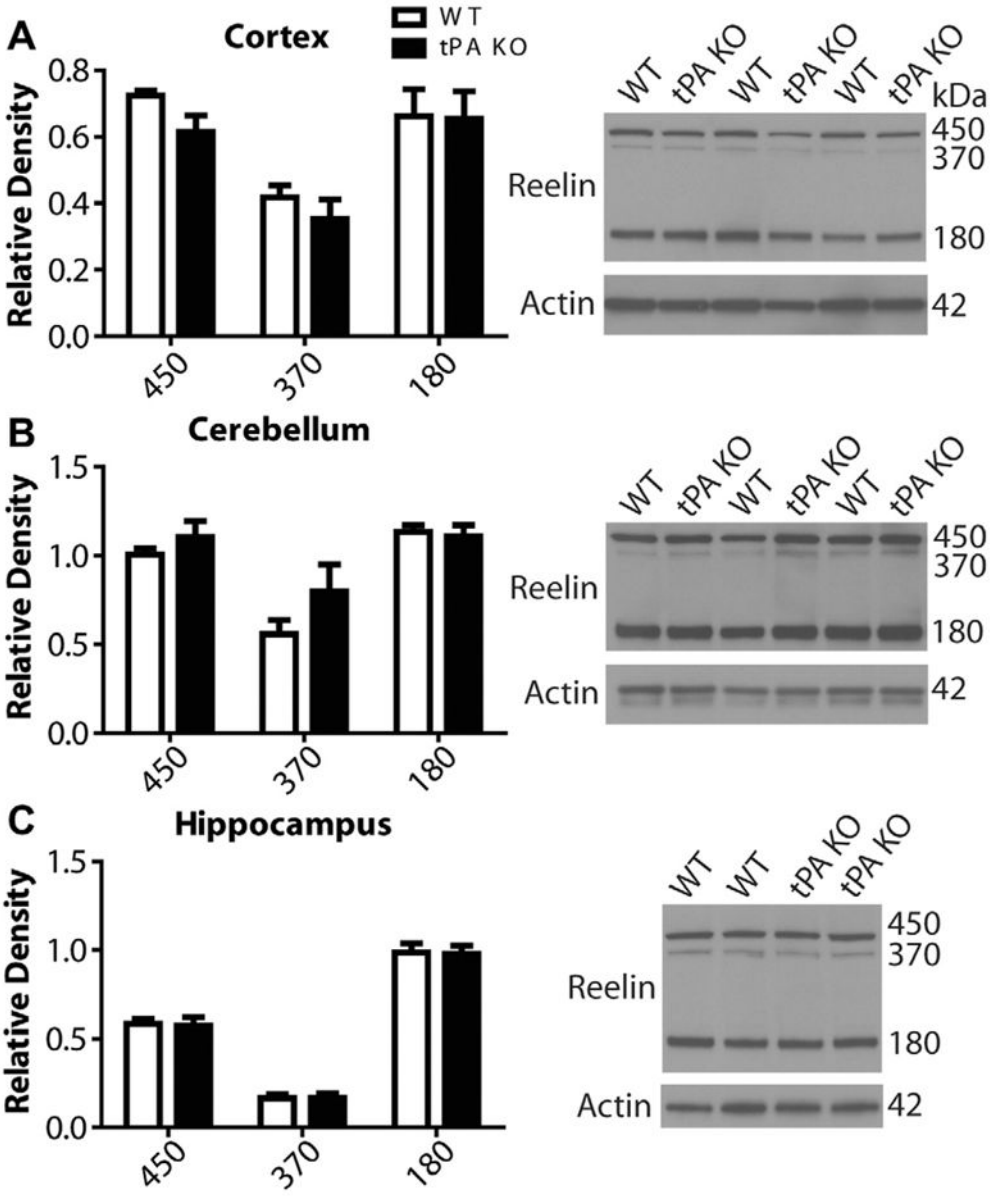

Fig. 4.

Loss of tPA *in vivo* does not affect basal reelin processing. The level of full-length reelin and the 370- and 180-kDa fragments were measured in the brains of 4-month-old wild-type (*n*=6) and tPA knockout (*n*=6) mice. No significant differences were found in the levels of full-length reelin or fragments in the cortex (A), cerebellum (B), or hippocampus (C). The values are shown as a relative density and error bars represent the mean±the standard error of the mean.

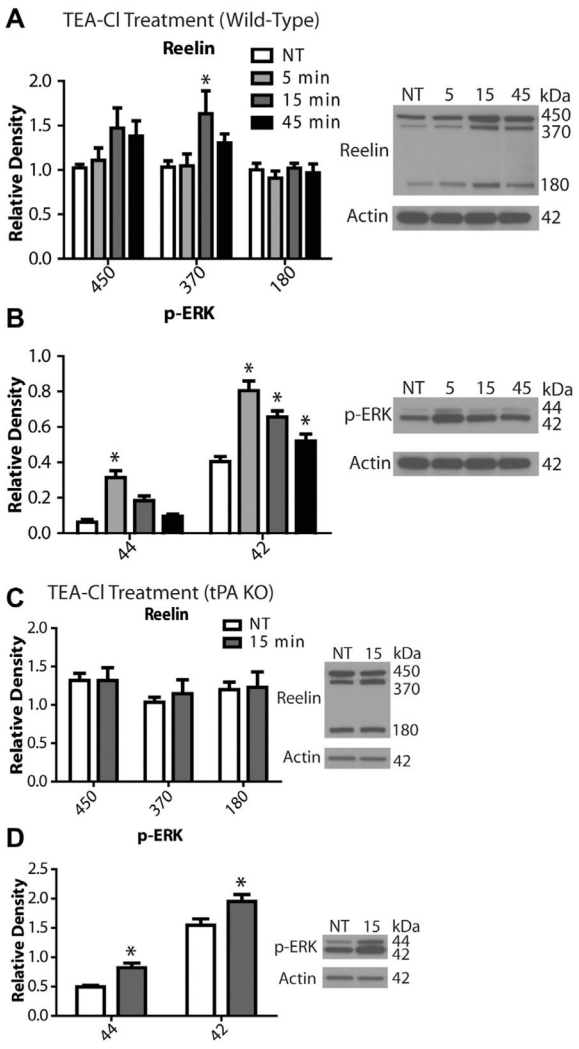

Fig. 5.

cLTP induces reelin processing in wild-type mice but tPA is required for reelin processing. Acute hippocampal slices from wild-type mice (Panels A, B) or tPA KO mice (Panels C, D) were treated with TEA-Cl (25 mM; at least 2 slices from 3 different mice) for 10 min and recovered in aCSF for 5, 15, and 45 min (wild-type mice) or 15 min (tPA KO mice). Proteins from dissected hippocampal area CA1 were evaluated for changes in reelin processing and ERK1/2 activation. (A) The level of the 370-kDa fragment was significantly increased at 15 min following TEA-Cl treatment. (B) The level of p-ERK1 (44 kDa) was increased at 5-min post-treatment compared to the non-treated group and 45-min recovery group. The levels of p-ERK2 (42 kDa) were significantly increased at 5, 15, and 45 min following TEA-Cl treatment when compared to non-treatment. The 5-min group was also significantly higher than the 15 and 45-min recovery groups. (C) There were no significant changes in full-length reelin or either of the fragments in tPA KO mice 15 min following TEACl treatment. (D) The levels of p-ERK1 (44 kDa) and p-ERK2 (42 kDa) were significantly increased 15 min following TEA-Cl treatment in the tPA KO mice. The values are shown as a relative density and error bars represent the mean±the standard error of the

mean. (*) denotes $p<0.05$ as indicated by the post-hoc analysis (wild-type mice) or as indicated by the independent samples two-tailed *t*-test (tPA KO mice).