Plasmin-Mediated Degradation of Laminin γ-1 Is Critical for Ethanol-Induced Neurodegeneration

Anna E. Skrzypiec, Rajani Maiya, Zulin Chen, Robert Pawlak, and Sidney Strickland

Background: Alcoholism may result in severe neurological deficits and cognitive impairments. Many of the central effects of ethanol (EtOH) can be explained by upregulation of *N*-methyl-D-aspartate (NMDA) and downregulation of gamma-aminobutyric acid (GABA) A receptors (GABAA) in response to long-term EtOH consumption. Abrupt ethanol withdrawal (EW) may result in neuronal hyperexcitability leading to hallucinations, seizures, neurodegeneration, and sometimes death.

Methods: Using a multidisciplinary approach in wild-type and genetically modified mice, we examined the contribution of the tissue plasminogen activator (tPA), plasminogen, and laminin to EW-induced cell death.

Results: Here we show that EW-induced neurodegeneration is mediated by the tPA/plasmin system. During EW, tPA is upregulated in the hippocampus and converts plasminogen to plasmin, which in turn degrades an extracellular matrix component laminin, leading to caspase-3-dependent cell death. Consequently, mice in which the tPA or plasminogen genes have been deleted do not show EW-induced laminin degradation, mitochondrial dysfunction, and neurodegeneration. Finally, we demonstrated that disruption of the hippocampal laminin γ-1 renders the mice resistant to neurotoxic effects of EW.

Conclusions: Our data identify laminin γ-1 as a novel target to combat neurodegeneration.

Key Words: Ethanol, hippocampus, laminin, neurodegeneration, plasmin

Alcoholism is a devastating condition that, beyond the personal and family impact, generates a cost of $185 billion annually in the United States alone (1). Ethanol (EtOH) produces a wide variety of physiological and behavioral effects, such as initial hyperactivity and euphoria followed by sedation, hypothermia, motor and sensory incoordination, and loss of righting reflex (2,3). Beyond its toxic effects on various tissues and organs, chronic ethanol abuse often leads to a number of neurological deficits, such as cortico-hippocampal atrophy with the resulting cognitive impairments (4–6).

Most of ethanol's effect can be explained by its neuromodulatory actions at several neurotransmitter-gated ion channels (2,7,8). Ethanol blocks the activity of *N*-methyl-D-aspartate (NMDA) receptors and enhances gamma-aminobutyric acid (GABA) A receptor (GABAA)-mediated signaling, therefore elevating neuronal excitability threshold (2,7,8). In contrast, chronic ethanol administration causes adaptive upregulation of NMDA receptors and downregulation of GABAA receptors in neurons aimed to counteract ethanol-induced sedation. Thus, abrupt ethanol withdrawal (EW) results in a dramatic increase in neuronal excitability, which may lead to hallucinations, seizures, neurodegeneration, and eventually death (8–10).

Beyond its effect on ion channels, ethanol modulates a variety of neurotransmitter systems. We have recently shown that a serine protease tissue plasminogen activator (tPA) is induced by EW and facilitates EW-induced seizures (11). This effect of tPA is mediated through its interaction with ethanol-sensitive, NMDA receptor 2B (NR2B)-containing NMDA receptors (11).

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Whether tPA contributes to ethanol-induced neurodegeneration has not been investigated. In this study, we show that tPA dose-dependently facilitates EW-induced neuronal death both in vitro and in vivo. This effect is plasminogen-dependent and can be prevented by deletion of either the tPA or plasminogen gene. Ethanol withdrawal-induced neurodegeneration correlates with the tPA/plasmin-mediated degradation of laminin in the hippocampus. Finally, we show that conditional disruption of the laminin γ-1 in the CA1 region of the hippocampus renders the mice resistant to neurotoxic effects of EW.

Methods and Materials

Animals

Experiments were performed on male, 3-month-old wild-type (WT) C57/Bl6 mice, tPA-/- (12) or plasminogen-/- (13,14), and mice in which laminin γ-1 was deleted from the forebrain by crossing mice in which laminin γ-1 gene was flanked by loxP sites (fLAMγ1) with mice expressing Cre recombinase under the control of the calcium/calmodulin-dependent protein kinase II promoter (CaMKII-Cre) mice (15). Tissue plasminogen activator-/- and plasminogen-/- mice have been backcrossed to Jackson Laboratory C57/BL6J for at least nine generations. Mice homozygous for the knockout allele were used to set up breeding colonies. We used Cre(-) littermates as control animals for fLAMγ1 × CaMKIIα-Cre animals.

Induction of Physical Dependence

The mice were housed individually and given a measured amount of liquid diet (Bio-Serv, Frenchtown, New Jersey) containing 2.3% vol/vol to 10% vol/vol ethanol and vitamin supplement as their sole nutrient source. The mice were gradually introduced to the ethanol diet as follows: days 1 to 3, 2.3%; days 4 to 6, 4.7%; days 7 to 10, 7%; days 11 to 14, 10% ethanol. The pair-fed control mice were given the same volume of isocaloric ethanol-free liquid diet as the ethanol-exposed mice had consumed the previous day (11,16). Every 24 hours, the mice were rated for behavioral signs of ethanol intoxication by an observer who was unaware of the kind or amount of diet consumed as well as genotype of the animals, as previously described (11,16).

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Ethanol withdrawal was initiated on day 15 at 8:00 AM by removing the ethanol-containing diet and replacing it with ethanol-free diet.

In Situ Zymography

In situ zymography was performed as previously described (17). The slides were incubated at 37°C in a humid chamber for 2 to 4 hours, and the developed zymograms were photographed under dark-field illumination.

Quantification of Ethanol and EW-Induced Cell Death in the Hippocampus

To assess the extent of cell death, animals were sacrificed after 14 days of ethanol administration (without withdrawal) or up to 2 days following EW. Their brains were embedded in paraffin and 4 μm-thick sections were cut at 1.7 to 2.2 mm posterior to bregma. To estimate the extent of cell loss, sections were deparaffinized, rehydrated, and stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. At least four sections per animal were analyzed. The cells were counted in three consecutive fields of 300 μm of the CA1 or the CA3 region and averaged.

To confirm cell death (either necrotic or apoptotic), the above sections were processed for detection of broken DNA strands with terminal deoxynucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick-end labeling (TUNEL) method (fluorescein-based kit, Boehringer Mannheim, Mannheim, Germany), performed according to manufacturers instructions.

Fluoro-ade B staining (Millipore, Temecula, California) was performed as previously described (18).

Determination of the Lineage of Dying Cells in the Hippocampus Following EW

Animals were sacrificed 2 days following EW and 4-μm paraffin sections were prepared (see above). The TUNEL staining was performed in conjunction with neuron-specific (neuron-specific nuclear protein [NeuN]) or astrocyte-specific (glial fibrillary acidic protein [GFAP]) markers (Supplement 1).

To distinguish the type of neurons undergoing degeneration, we performed immunohistochemistry for a glutamate transporter, vesicular glutamate transporter 1 (VGLUT1) (a marker for excitatory neurons), glutamic acid decarboxylase-67 (GAD67) (a marker for inhibitory interneurons), or active caspase-3 in conjunction with TUNEL staining (Supplement 1).

Laminin Immunohistochemistry

Laminin staining was performed on 15-μm thick paraformaldehyde fixed sections as previously described (19).

Brain Ethanol Levels

Hippocampal samples were deproteinized and ethanol concentration was measured using Ethanol UV-method Kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions (Supplement 1).

Cell Culture Experiments

Hippocampal primary neuronal cultures were prepared from embryonic day 18.5 WT and tPA-/- mice (Supplement 1). On day 10 after plating, the cultures were exposed to culture media containing 50 mmol/L or 100 mmol/L ethanol for 3 days. Ethanol withdrawal was induced on day 4 by replacing ethanol-containing media with regular growth media. Neuronal death was determined on day 6.

Lactate Dehydrogenase Measurement

Lactate dehydrogenase (LDH) released into the culture media was determined using the LDH assay kit (Sigma, St. Louis, Missouri) according to manufacturer's instructions.

Determination of Mitochondrial Permeability

Tissue plasminogen activator+/+ or tPA-/- neurons (with or without prior administration/withdrawal of ethanol) were stained for the presence of mitochondrial J-aggregates using MitoPT kit (Immunochemistry Technologies, Bloomington, Indiana) according to the manufacturers instructions and observed under Zeiss LSM5 Exciter (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) confocal microscope.

Statistical Analysis

Mann-Whitney U test was used for statistical evaluation and $p < .05$ was considered statistically significant. Diet consumption and intoxication levels were compared between genotypes using Kruskall-Wallis nonparametric analysis of variance (ANOVA).

Results

Ethanol Withdrawal Causes Hippocampal Cell Death in a Chronic Consumption Model

It has been widely accepted that ethanol abuse causes cell death in the brain (4–6), which depends on the genetic background of the animals used, as well as the schedule and route of EtOH administration (20–22). The above parameters not only determine the extent but also the time when cell death is observed (23–25). To investigate the occurrence and severity of degeneration in our model, we determined the cell number in the CA1 and CA3 regions of the hippocampus after 14 days of EtOH administration with or without withdrawal. We found that chronic administration of ethanol did not change the number of cells in the CA1 or CA3 region as compared with EtOH-naive animals (Figure 1; $p > .05$). Similarly, the cell number was not affected 6 hours following ethanol withdrawal (Figure 1; $p > .05$). However, a 14% to 17% drop was observed in the CA1 2 days after EtOH diet had been replaced by the control one (Figure 1; $p < .05$ and Figure 2; $p < .01$). This result indicates that in our model EW is necessary for cell death to occur in the CA1 region and implicates delayed cell death mechanisms in its pathogenesis.

To confirm the presence of degenerating neurons in the CA1 after EW, we performed Fluoro-ade B staining. Ethanol withdrawal resulted in an appearance of bright green, Fluoro-ade B positive cells in the CA1, but not CA3, region of the hippocampus (Figure 2), which was consistent with neuronal damage we had observed in that area.

To further analyze cell death, 2 days after EW in the hippocampus we performed TUNEL staining. The number of TUNEL-positive cells increased 12-fold in the CA1 region as compared with mice fed ethanol-free diet (Figure 2; $p < .05$). These cells were characterized by condensed chromatin in DAPI staining (Figure 2), which is consistent with apoptotic nucleus morphology (26). Again, cell death was specific to CA1, as we did not observe TUNEL-positive cells in other regions of the hippocampus (not shown).

To investigate which cell type is predominantly affected by EW, we analyzed the phenotype of TUNEL-positive cells in wild-type animals by multiple immunohistochemistry. The TUNEL-positive cells were co-labeled using antibodies against neuron-specific antigen NeuN and astrocyte-specific antigen (GFAP). We found that 74.4% ± 9% of dying cells were NeuN-positive and therefore of a neuronal lineage (Figure 3). Among the TUNEL-positive

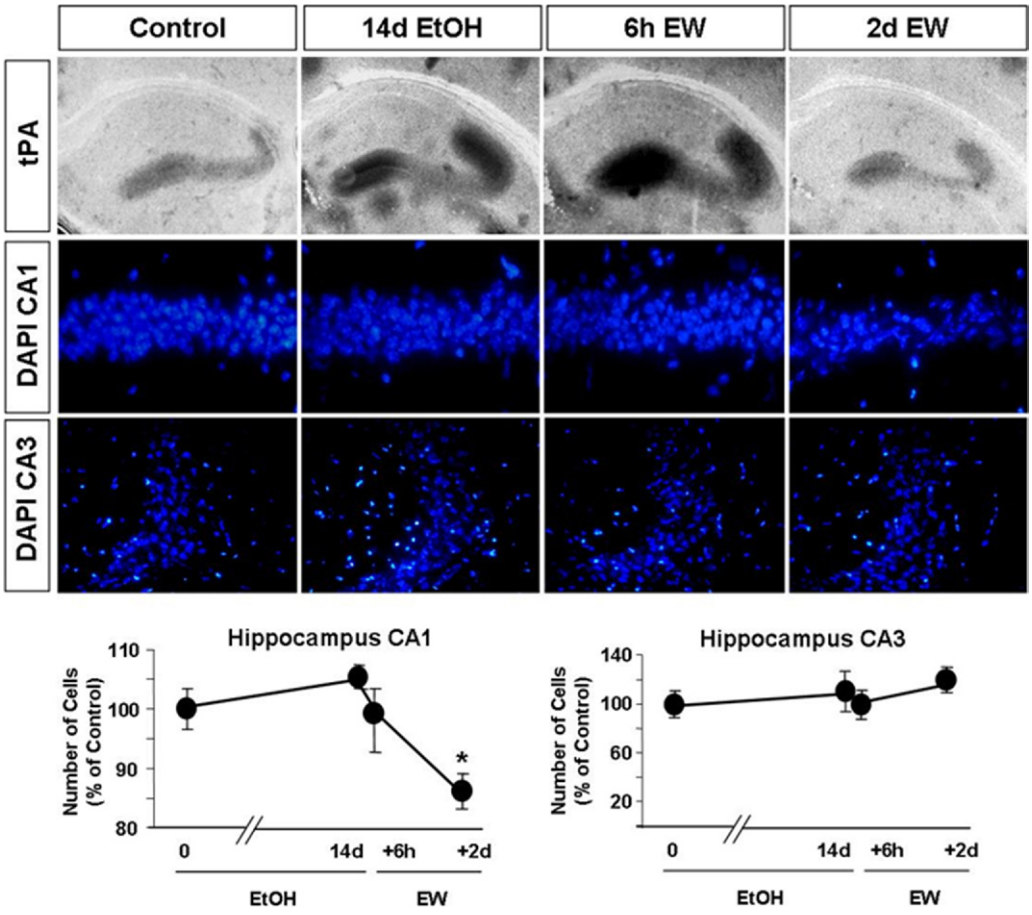

Figure 1. Ethanol withdrawal causes upregulation of tPA activity and triggers cell death in the hippocampus. Wild-type mice were sacrificed after 14 days of EtOH administration or 6 hours to 2 days after EW. EtOH-naïve mice served as control animals. The brains were collected and in situ zymography was performed. Ethanol administration caused an upregulation of tPA activity in the hippocampus (dark lytic zones in the upper panels), which was even more dramatic after EW. The correlation between tPA levels and cell loss was determined by counting cell numbers in the CA1 and CA3 region of the hippocampus using DAPI-stained sections (middle panels). Fourteen days of EtOH administration (14 days) or 6 hours of EW (6 hours) did not affect the number of cells in the CA1 or CA3 as compared with EtOH-naive animals (shown as 0 days). However, a 14% decrease was observed 2 days after EW in the CA1 region (lower panels). *$p < .05$; $n = 3$ to 4 per time point. The results are presented as mean ± SEM. DAPI, 4′,6-diamidino-2-phenylindole; EtOH, ethanol; EW, ethanol withdrawal; tPA, tissue plasminogen activator.

cells, 14.6% ± 4% co-localized with GFAP and were identified as astrocytes (Figure 3). The remaining 11% ± 6% did not express either of the cell-type specific markers and remained unidentified. These results suggest that neurons are predominantly affected by EW in the hippocampus ($p < .001$).

To determine the lineage of degenerating neurons, we performed immunohistochemistry for VGLUT1 (a marker of excitatory neurons) and GAD67 (a marker for inhibitory interneurons) in conjunction with TUNEL (Figure 3). The majority of GAD67-positive cells (distinguished as bright red in contrast to punctuate red GAD67-positive synaptic buttons found on most CA1 pyramidal neurons; Figure 3) were localized in stratum radiatum and were always TUNEL-negative. We found that only 5% ± 6% of TUNEL-positive cells were GAD67-positive in stratum pyramidale of the CA1. In contrast, 70% ± 5% of degenerating cells were VGLUT1-positive ($p < .001$), which points to excitatory neurons as most vulnerable to EW.

To investigate the relationship between EW-induced neuronal death and activation of caspase-3, a critical effector protease-promoting neurodegeneration, we performed immunohistochemistry (Figure 4). We found that TUNEL staining correlated well with high levels of active caspase-3 in the tissue after EW (96% ± 6% of TUNEL-positive cells showed high caspase-3 levels; $p < .001$), indicating an important role for caspases in promoting cell death in our model.

Neuronal death is often preceded by mitochondrial dysfunction leading to depolarization and protein leakage from these structures. To examine mitochondrial integrity after EW, we subjected hippocampal neurons in culture to EW and visualized J-aggregates (Figure 4). Ethanol withdrawal resulted in a dramatic increase in the percentage of cells displaying signs of mitochondrial disruption (2% ± 1% vs. 61% ± 7% in control and EW groups, respectively; $p < .001$) as evidenced by disappearance of J-aggregates.

EW-Mediated Increase in tPA Activity in the Hippocampus Precedes Neurodegeneration

High tPA activity has previously been associated with neuronal death (27,28). To investigate whether hippocampal tPA activity correlated with EW-induced neurodegeneration, we performed in situ zymography using brain sections of wild-type

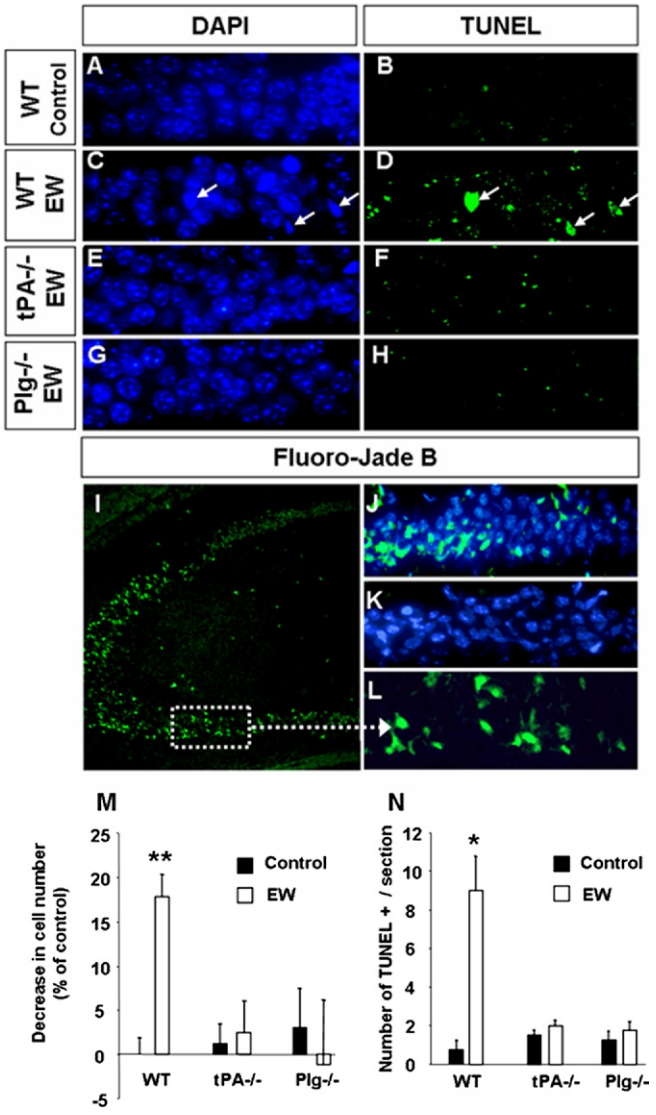

Figure 2. Ethanol withdrawal-induced neurodegeneration is tPA- and plasminogen-dependent. Wild-type, tPA-/- or plasminogen-/- mice were sacrificed 2 days following EW. EtOH-naive mice of the same genotype served as control animals. Cell loss was determined by counting cell number in the CA1 region of the hippocampus using DAPI-stained sections. To confirm cell death, the above sections were processed for detection of broken DNA strands with TdT-mediated dUTP nick-end labeling (TUNEL) method. Analysis of the CA1 region revealed that the number of cells decreased in the wild-type mice **(A,C,M)** but not in tPA-/- **(E,M)** or plasminogen-/-animals **(G,M)** 2 days after EW. Similarly, we did not observe TUNEL-positive cells in CA1 region of tPA- or plasminogen-deficient mice **(F,H,N)** typically seen in wild-type mice after EW **(B,D,N**; arrows in **D)**. Staining using another marker of neurodegeneration, Fluoro-ade B, confirmed the presence of bright green damaged cells (as seen in the kainic acid-injected positive control; **[I,L]**) in the CA1 region in wild-type **(J)** but not tPA-/- mice **(K)** 2 days after EW. Altogether, these results demonstrate that EW-mediated neurodegeneration is tPA- and plasminogen-dependent. Sections derived from EtOH-naive tPA-/- or plasminogen-/- mice did not show any signs of neurodegeneration and are not shown but are included in quantification in **M** and **N**. *$p < .05$; **$p < .01$; $n = 4$ to 5 per group. The results are presented as mean ± SEM. DAPI, 4′,6-diamidino-2-phenylindole; EtOH, ethanol; EW, ethanol withdrawal; tPA, tissue plasminogen activator; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick-end labeling.

mice collected at various time ,points during ethanol intoxication and/or EW. In line with our previous observations (11) extracellular tPA activity increased after 14 days of ethanol treatment and even more dramatically during EW (Figure 1). This increased caseinolytic activity was due to tPA and not urokinase-type plasminogen activator (uPA), since it was not seen in tPA-/- mice (not shown).

EW-Induced Neurodegeneration Is tPA- and Plasmin-Dependent

Since high tPA activity and seizures often correlate with cell death (10,19,27,29,30), we investigated if EW-induced neurodegeneration is tPA-dependent. To this end, we induced EW in tPA-/- mice and analyzed their hippocampi 2 days later. Histological analysis of the CA1 region revealed that, unlike wild-type mice (Figure 2; $p < .01$), the number of cells in tPA-/- mice was not affected by EW ($p > .05$). Similarly, we did not observe TUNEL-positive cells in CA1 region (Figure 2; $p > .05$) typically seen in wild-type mice after EW ($p < .05$) or disruption of mitochondrial J-aggregates in tPA-deficient hippocampal neurons subjected to EW (Figure 4).

To investigate if plasminogen is necessary for EW-mediated neuronal death, we subjected plasminogen-/- mice to EW and determined the number of cells in CA1 region. Plasminogen-/- mice that were fed ethanol-free diet served as control animals. Similar to tPA-/- animals, we did not observe any decrease in the number of cells in the CA1 region as the result of EW in plasminogen-/- mice (Figure 2; $p > .05$). Moreover, TUNEL-positive cells were not seen in these animals (Figure 2; $p > .05$), which is consistent with the role of plasmin in EW-mediated neuronal death.

tPA and Plasminogen Do Not Affect Ethanol Levels, Consumption, or Behavioral Signs of Intoxication

To investigate whether attenuation of EW-induced neuronal death in tPA-/- and plasminogen-/- mice can be attributed to reduced ethanol drinking, we measured the volume of the EtOH diet consumed during the course of the 14-day intoxication. We did not find any differences in this parameter between wild-type animals and mice in which the tPA or plasminogen genes have been disrupted (Figure 1 in Supplement 1; $p > .05$). The amount of ethanol consumed on day 14 was 26.7 ± 2 g/kg/day, 25.5 ± 7 g/kg/day, and 27.7 ± 3 g/kg/day for wild-type mice, tPA-/-, and plasminogen-/- mice, respectively, and did not differ between the genotypes. Weight loss was similar during the course of ethanol intoxication between groups (Figure 1 in Supplement 1) and reached the maximum of ~20% at day 14.

To check if tPA deletion affects EtOH metabolism, we measured hippocampal ethanol levels in wild-type and tPA-/- mice before EtOH diet was introduced, after 14 days of its consumption, and 6 hours following its withdrawal. Hippocampal ethanol levels on day 14 amounted to 43 ± 11 mmol/L and 42 ± 9 mmol/L in wild-type and tPA-/- mice, respectively (Figure 1 in Supplement 1; $p > .05$ between the genotypes), and were consistent with those known to produce sedation (2,8). These levels dropped sharply 6 hours after the ethanol had been withdrawn and were similar in the wild-type and tPA-deficient animals (Figure 1 in Supplement 1; $p > .05$).

In spite of lack of differences in brain ethanol metabolism, tPA-/- mice could still have subtle receptor/circuit anomalies that would render them resistant to behavioral effects of ethanol. To determine the effect of tPA and plasmin on EtOH-induced sedation, we measured behavioral signs of ethanol intoxication

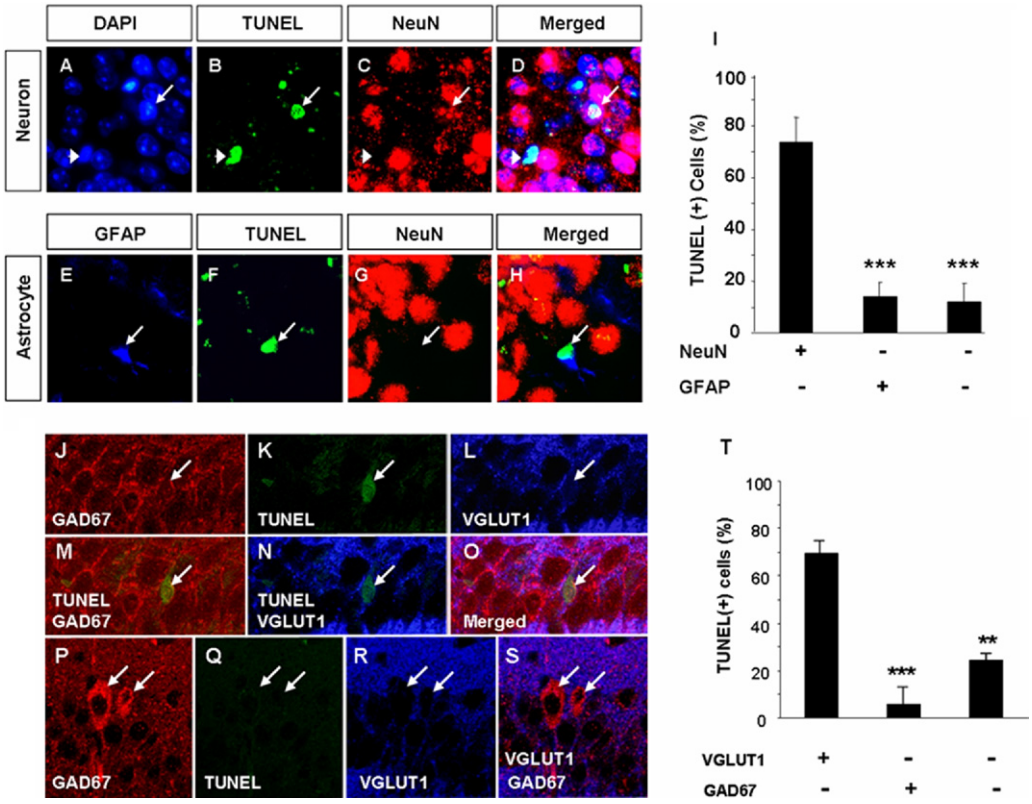

Figure 3. Glutamatergic neurons are predominant cell type affected by ethanol withdrawal in the hippocampus. To investigate which cell type is primarily affected by EW, we analyzed the phenotype of TUNEL-positive cells in the CA1 region of wild-type mice 2 days after withdrawal. To this end, we performed co-labeling using antibodies against neuron-specific antigen NeuN and astrocyte-specific antigen glial fibrillary acidic protein (GFAP). Panels **A**–**D** show two TUNEL-positive cells with chromatin condensation visualized by the DAPI staining. One of these cells (arrowhead) co-localized with NeuN and therefore was identified as a neuron. The second cell (arrow) was NeuN-negative. Panels **E** to **H** show a GFAP-positive, damaged astrocyte (arrow). We found that 74.4% of dying cells were neurons (NeuN-positive) and 14.6% were astrocytes (GFAP-positive; quantified in **I**). Immunohistochemistry for VGLUT1 (a marker of excitatory neurons) and glutamic acid decarboxylase-67 (GAD67) (a marker for inhibitory interneurons) in conjunction with TUNEL staining revealed that the majority of dying cells were VGLUT1-positive and GAD67-negative excitatory neurons (**J**–**O**). GAD67-positive cells (bright red in **P** as opposed to punctuate red GAD67-positive synaptic buttons found on most CA1 pyramidal neurons; see **J**) were TUNEL-negative (**P**–**S**). Quantification of the above results shown in **T**. ***$p < .001$. The above estimation was performed using paraffin sections containing CA1 regions of four mice. The results are presented as mean ± SEM. DAPI, 4′,6-diamidino-2-phenylindole; EW, ethanol withdrawal; GAD67, glutamic acid decarboxylase-67; GFAP, glial fibrillary acidic protein; NeuN, neuron-specific nuclear protein; tPA, tissue plasminogen activator; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick-end labeling; VGLUT1, vesicular glutamate transporter 1.

in mice deficient in the above proteases and compared them with wild-type control mice. We did not observe any differences in this parameter between wild-type tPA-/- or plasminogen-/- mice during the course of 14 days of ethanol administration (Figure 1 in Supplement 1; $p > .05$).

EW-Induced Neurodegeneration and Its tPA-Dependence Can Be Mimicked in Vitro

Many of the effects of EtOH in the central nervous system are mediated at the systems level and ethanol dependence is often considered an aberrant form of neuronal plasticity. This form of plasticity requires the presence of well-defined synaptic circuits that are modified by EtOH (31,32). To investigate if the facilitation of EW-induced neurodegeneration by tPA was due to its effects at the circuit level or rather at the cellular level, we subjected dissociated forebrain neurons to EtOH and induced EW. In wild-type neurons, EW resulted in a 10-fold increase in the release of LDH, an indicator of degeneration, to the medium (Figure 5, lower panel) followed by a dramatic decrease in the cell number (Figure 5, upper panels). In contrast, neurons from mice deficient in tPA were resistant to EW-induced degeneration, as measured by either mitochondrial disruption (Figure 4), LDH release, or cell loss (Figure 5).

EW-Induced Neurodegeneration Correlates with tPA-Dependent Degradation of Laminin

Chen and Strickland (19) demonstrated that neurodegeneration after injection of an excitotoxin kainic acid is promoted by plasmin-catalyzed degradation of laminin (19). To investigate if EW-induced neurodegeneration correlates with laminin degradation, we assessed laminin levels by immunohistochemistry in the hippocampal CA1 region of wild-type mice 6 hours after EW, when seizures were most pronounced. We observed a marked decrease in laminin levels surrounding neuronal bodies 6 hours after EW in the CA1 (Figure 6), the region that typically undergoes degeneration 2 days later (Figures 1 and 3). This result suggests that degradation of laminin could be responsible for EW-induced neuronal death.

To investigate if EW-induced degradation of laminin was tPA-dependent, we performed analysis of laminin levels in the hippocampus of tPA-/- mice with or without EW. We did not

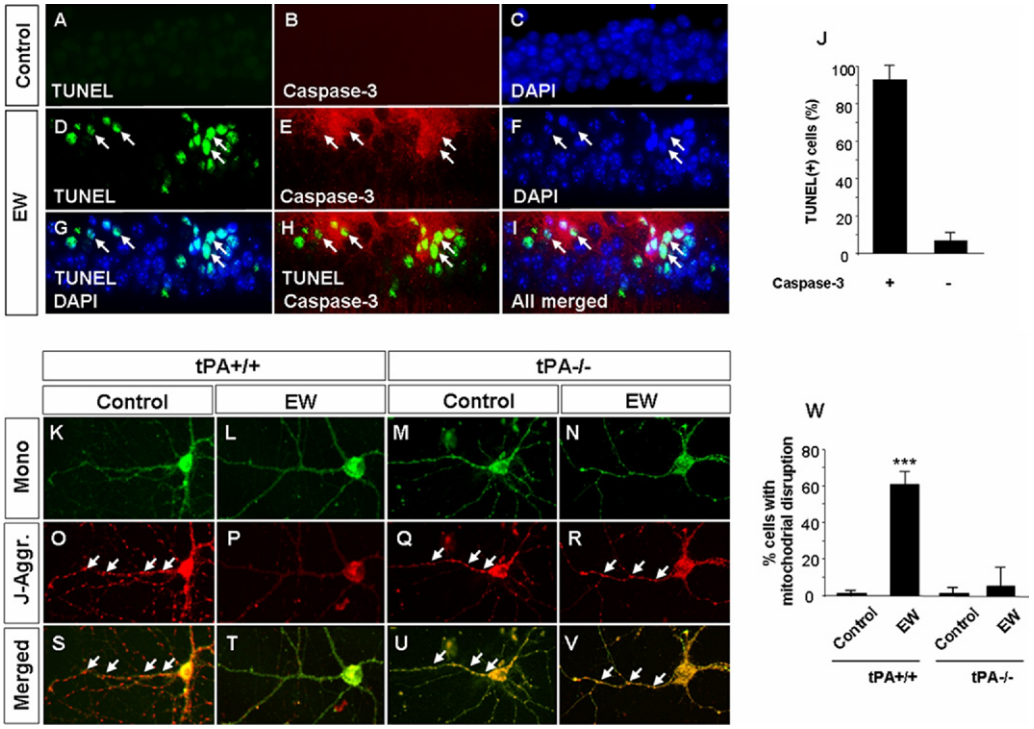

Figure 4. Ethanol withdrawal-induced neuronal death is caspase-dependent and accompanied by mitochondrial dysfunction. Upper panels: Wild-type mice received either ethanol-free diet **(A–C)** or ethanol-containing diet followed by EW **(D–I)**. Ethanol withdrawal resulted in neurodegeneration in the CA1 region of the hippocampus as indicated by the presence of TUNEL-positive cells **(D,G,H,I)**. Double immunohistochemistry revealed that the majority of dying cells spatially and temporarily coincided with activation of caspase-3 (arrows in **E,H,I** and quantified in **J**). Lower panels: Wild-type **(K,L,O,P,S,T)** and tPA-/- **(M,N,Q,R,U,V)** hippocampal neurons were subjected to ethanol withdrawal and mitochondrial integrity was investigated by J-aggregate staining. EW resulted in mitochondrial disruption in wild-type neurons as evidenced by disappearance of red J-aggregates **(P,T)**. In contrast, tPA-/- neurons were protected against EW-induced injury (arrows in **R,V**; quantified in **W**). ***$p < .001$. The above experiments were repeated at least four times with similar results. The results are presented as mean ± SEM. EW, ethanol withdrawal; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick-end labeling.

observe laminin degradation typically seen in the wild-type mice at the same time point after EW (Figure 6).

CA1-Specific Deletion of Laminin γ-1 Protects Neurons from EW-Induced Degeneration

Degradation of laminin γ-1 chain has previously been implicated in tPA/plasmin system-mediated neuronal death after kainic acid injection (33,34). To investigate if laminin γ-1 was involved in EW-mediated cell death, we generated mice in which the gene encoding laminin γ-1 chain has been floxed (F/F) and conditionally deleted in the CA1 region of the hippocampus (15). We treated these F/F Cre+ and their F/F Cre- control littermates with ethanol 2 days after EW cell number was assessed. We found 12% ± 2% decrease in the cell number in the CA1 region of F/F Cre- control mice 2 days after EW (Figure 7). On the other hand, the animals in which laminin γ-1 had been deleted were protected from EW-induced cell loss ($p < .05$).

To confirm that deletion of laminin γ-1 chain rendered neuroprotection, we performed TUNEL staining using coronal brain sections collected 2 days after EW. We observed a dramatic increase in the number of TUNEL-positive cells in the CA1 region in F/F Cre- animals (to 6 ± 2 cells per section; $p < .05$; Figure 7), while hippocampi of the F/F Cre+ mice subjected to EW were TUNEL-negative. Similar to C57/Bl6 mice (Figure 3), the majority of these TUNEL-positive cells were NeuN-positive and were therefore identified as neurons (Figure 7).

These results indicate that the presence of laminin γ-1 during EW is necessary for cell death in the CA1 region of the hippocampus and that its degradation by plasmin may constitute an important mechanism mediating neurodegeneration in this region.

Discussion

One of the most common complications of alcoholism is degenerative changes observed in the course of ethanol dependence (4–6). Ethanol-related degenerative changes are characterized by cell death and damage of neurites and synapses and the myelin sheath in the cortex, hippocampus, thalamus, and pons (4–6). However, depending on the dose of ethanol, route of its administration, and the strain and genetic background of the animals used, the above changes can be observed early after EtOH treatment or only after EW (20–24,35–40). In our study, EW was necessary to trigger cell death, which may reflect lower susceptibility of C57/BL6 mice to EtOH toxicity as compared with other strains (41), and it was limited to the CA1 region of the hippocampus. We found that EW predominantly affects excitatory neurons, as 74.4% of TUNEL-positive cells co-express neuronal marker NeuN and 70% ± 5% of them were also VGLUT1-positive. These findings are in agreement with previous studies demonstrating neuronal death following EW both in animal models and in human alcoholics (4–6).

What is the mechanism of EW-induced neurodegeneration? Ethanol acts as an antagonist at excitatory NMDA receptors and at the same time stimulates inhibitory GABAA binding sites (8). Because of its pharmacological profile, chronic administration of

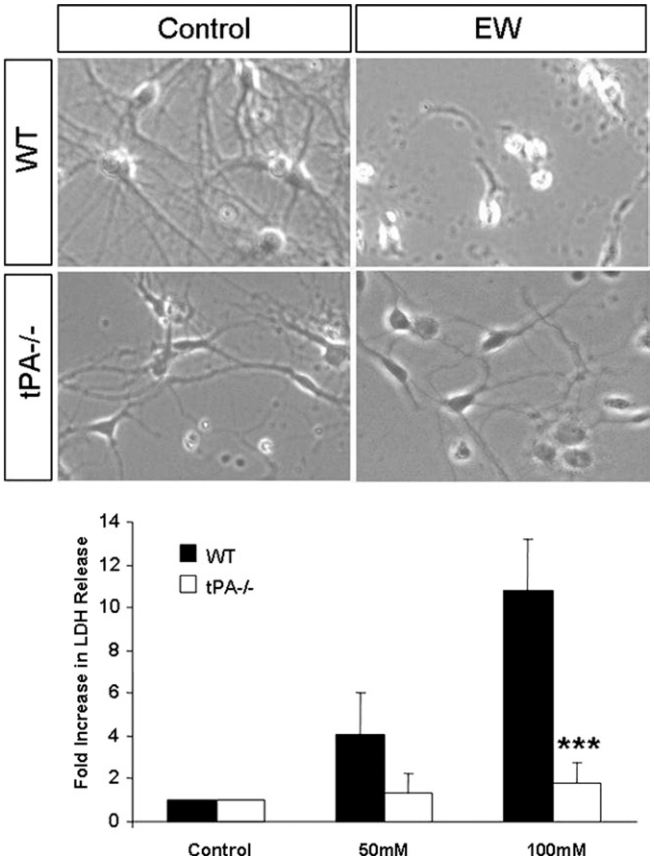

Figure 5. tPA-/- neurons in culture are resistant to EW-induced neurodegeneration. To determine whether ethanol mediates its effects at the cellular or rather circuit level, we investigated the effects of EW on the survival of dissociated wild-type (WT) and tPA-/- hippocampal neurons in culture. Neurons were exposed to 50 mmol/L or 100 mmol/L ethanol for 3 days and subject to withdrawal. Bright field images of neurons exposed to 100 mmol/L ethanol revealed signs of cell death (rounded cell bodies, short neurites) in wild-type neurons undergoing EW, whereas tPA-/- neurons were resistant to EW-induced neurodegeneration (upper panels). EW-induced neurodegeneration was quantified using the LDH assay (lower panel). Withdrawal from 50 mmol/L and 100 mmol/L ethanol resulted in a dose-dependent increase in LDH release from wild-type neurons ($n = 4$), whereas tPA$^{-/-}$ neurons did not show a significant increase in LDH release ($n = 5$). ***$p < .001$. The results are presented as mean ± SEM. EW, ethanol withdrawal; LDH, lactate dehydrogenase; tPA, tissue plasminogen activator.

EtOH causes an adaptive upregulation of NMDA receptors and downregulation of GABAA receptors. This creates a potential state of hyperexcitability temporarily inhibited by high doses of EtOH. However, upon cessation of drinking, this predominance of excitatory transmission is unmasked, which results in EW seizures and cell death. There are numerous factors that can facilitate excitotoxic neuronal death further. One critical molecule is a serine protease tissue plasminogen activator. Tissue plasminogen activator is induced and released from neurons by various forms of neuronal activity (30,42–44) including EW (11). We have previously demonstrated that tPA-/- mice are resistant to seizures and cell death after injection of kainic acid (19,27,45). We have also found that tPA interacts with NR2B, the ethanol-sensitive subunit of NMDA receptor, and promotes EW seizures (11). To investigate if tPA similarly promotes EW-induced neurodegeneration, we administered EtOH to tPA-/- mice and determined cell death in the hippocampus upon its withdrawal. Mice or neurons deficient in tPA were resistant to EW-induced mitochondrial disruption and neurodegeneration, as indicated by the presence of J-aggregates, cell number, and lack of TUNEL-positive neurons. This finding is in line with the role of tPA in facilitating physical dependence to EtOH and promoting neuronal death in different animal models.

High seizure activity during EW can deteriorate brain damage and facilitate cell death (46–48). Considerable evidence suggests that EW-induced epileptic activity emanates from either brain stem or forebrain and is propagated and augmented within specific neuronal circuits (31). To investigate if the tPA-dependent element of EW-induced neurodegeneration is secondary to convulsions or whether the two phenomena can be separated, we eliminated the neuronal network component. Dissociated mixed forebrain neuronal/astrocytic cultures were treated with EtOH and EW was initiated. Faster development of ethanol dependence in vitro compared with the in vivo model may be due to continuous EtOH exposure (no circadian variations in ethanol intake that is typical for mouse models) and limited potential for compensatory negative feedback from inhibitory interneurons. We have found that in the wild-type cultures neuronal death was still observed, suggesting that EW-induced

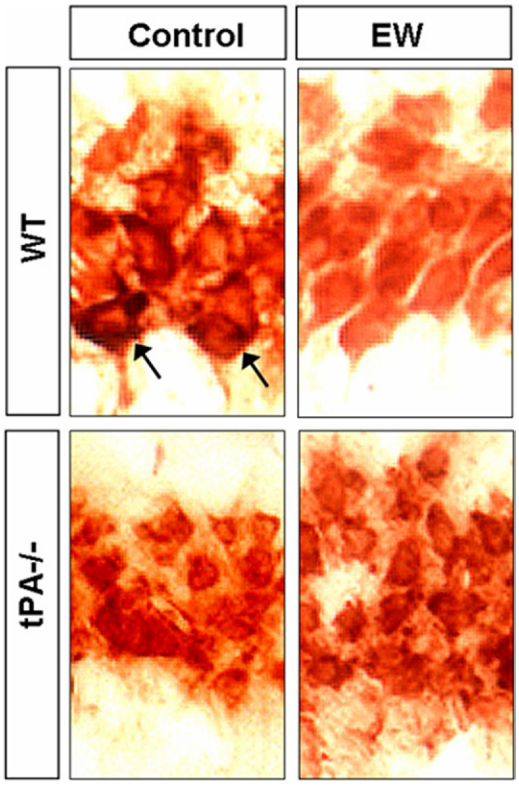

Figure 6. EW-induced neurodegeneration correlates with tPA-dependent degradation of laminin. To investigate if EW-induced neurodegeneration correlates with laminin degradation we assessed laminin levels by immunohistochemistry in the hippocampal CA1 region of wild-type (WT) and tPA-/- mice during EW. EtOH-naive mice of the same genotype served as controls. Laminin was localized mainly around cell bodies. In wild-type mice a marked decrease in laminin levels was observed 6 hours after EW in the CA1 (strong pericellular staining indicated by arrows), the region that degenerates 2 days later. Laminin degradation was not observed in tPA-/- mice, which were also resistant to neurodegeneration. These results suggest that degradation of laminin is tPA-dependent and could be responsible for EW-induced neuronal death. This experiment was repeated four times with similar results. EtOH, ethanol; EW, ethanol withdrawal; tPA, tissue plasminogen activator.

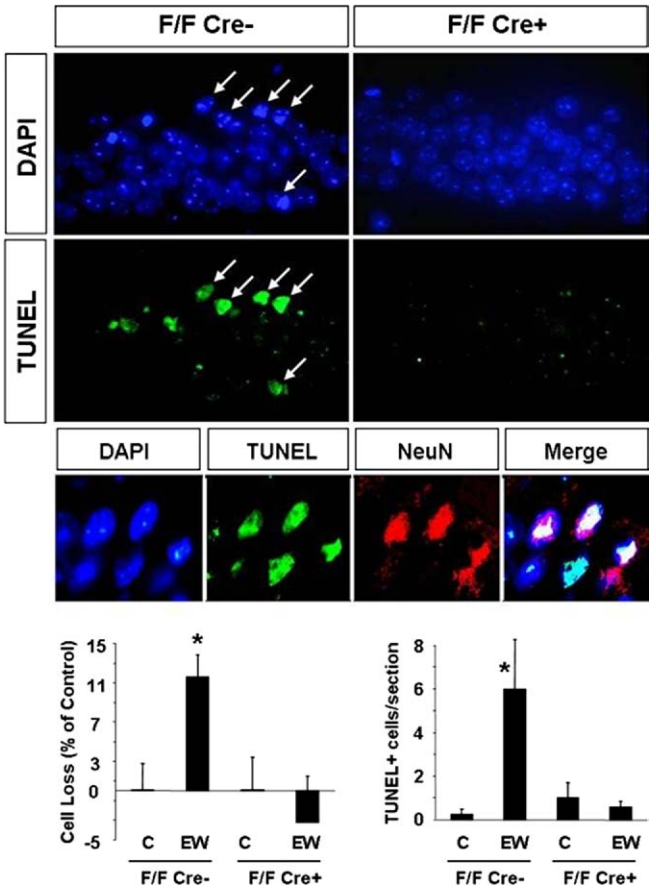

Figure 7. Conditional deletion of the laminin γ-1 gene from the CA1 region of the hippocampus protects neurons from EW-induced degeneration. To delete laminin γ-1 in the CA1 region, mice in which both copies of the laminin γ-1 gene has been floxed (F/F) were crossed with CaMKIIα-Cre transgenic mice (offspring referred to as F/F Cre+). Immunohistochemistry confirmed a complete absence of laminin γ-1 protein in the CA1 region of F/F Cre+ mice (54). The F/F Cre- littermates served as control animals. The mice were administered EtOH for 14 days and were sacrificed 2 days following EW. EtOH-naive mice of the same genotype served as control animals. Cell loss was determined by counting cell number in the CA1 region of the hippocampus using DAPI-stained sections. Cell death was further confirmed by visualizing broken DNA strands with the TUNEL method. Analysis of the CA1 region 2 days after EW revealed that the number of cells decreased in F/F Cre- mice but not in F/F Cre+ animals, in which laminin γ-1 has been deleted (upper panels and quantification in the lower panels). Similarly, we did not observe TUNEL-positive cells in CA1 region of F/F Cre+ mice, which are typically seen in F/F Cre- (middle panels and quantification in the lower panels) as well as C57/Bl6 animals (see Figures 2 and 3) after EW. Most TUNEL-positive cells co-localized with the neuronal marker NeuN (small middle panels). Sections derived from EtOH-naive F/F Cre+ and F/F Cre- mice did not show any signs of neurodegeneration and therefore are not shown but are included in the quantification. *$p < .05$; $n = 4$ to 10 per group for each experiment. The results are presented as mean ± SEM. CaMKIIα-Cre, ; Cre-, ; Cre+, ; DAPI, 4′,6-diamidino-2-phenylindole; EtOH, ethanol; EW, ethanl withdrawal; F/F, floxed; NeuN, neuron-specific nuclear protein; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick-end labeling.

neurodegeneration has a cellular component independent of the epileptic circuitry. Deletion of the tPA gene completely prevented cell death in vitro. This confirmed our in vivo results pointing to the role of tPA as a critical factor facilitating EW-induced neuronal death.

Tissue plasminogen activator can use several mechanisms to exert its effects in the nervous system. These involve direct or indirect interactions with NMDA receptors (11,29,49), low density lipoprotein receptor-related protein (LRP) receptors (50), activation of brain-derived neurotrophic factor (BDNF) (51), and matrix metalloproteinase-9 (MMP-9) (52). One well-established substrate for tPA is plasminogen, which it activates to a broad-spectrum protease, plasmin. To investigate whether tPA-mediated neurodegeneration following EW is plasmin-dependent, we assessed cell death in plasminogen-/- mice. We found that these mice were resistant to neuronal death after EW, suggesting that the effect of tPA is mediated by activation of plasminogen. This result is in line with our previous findings pointing to the role of plasmin in the central nervous system injury after kainic acid injection (19,27,45). However, in our previous article (11), we

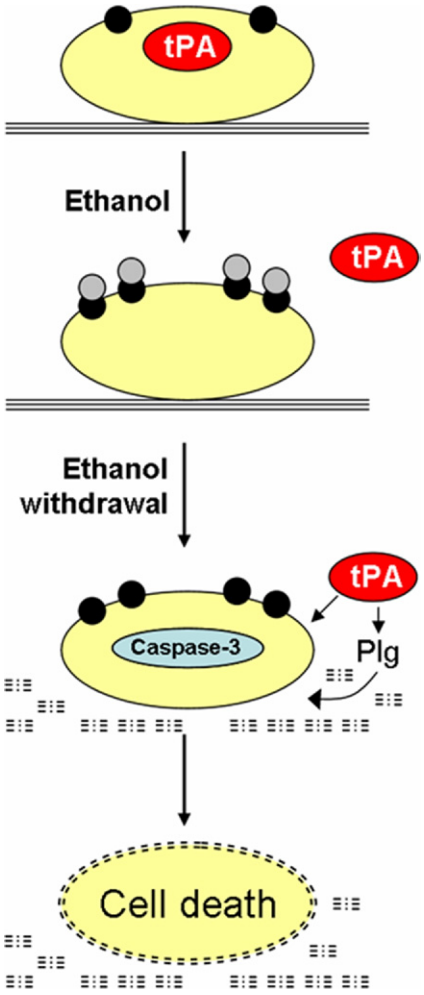

Figure 8. A proposed model of EW-induced neurodegeneration. In basal conditions, tPA is stored intracellularly and is released into the extracellular space following EtOH administration (Figure 1). During intoxication, EtOH (gray circles) blocks NMDA receptors (black circles) and together with extracellular tPA (see [11]) is responsible for their upregulation. Abrupt withdrawal of EtOH leaves NMDA receptors unblocked and the resulting hyperexcitability causes further release of tPA from neurons (Figure 1). High extracellular levels of tPA activate plasminogen to plasmin, which in turn degrades a component of extracellular matrix, laminin (Figure 6). Laminin γ-1 degradation renders neurons more sensitive to damaging effects of EW, which results in caspase-3-dependent cell death, while the absence of laminin γ-1 is protective (Figure 7). EtOH, ethanol; EW, ethanol withdrawal; NMDA, *N*-methyl-D-aspartate; tPA, tissue plasminogen activator.

found that the facilitating effect of tPA on EW seizures was plasminogen-independent. Our results collectively suggest that the mechanism of EW seizures and EW-induced neurodegeneration can be genetically dissected by the deletion of the plasminogen gene and that both plasminogen-dependent (cleavage of laminin) and plasminogen-independent (interaction with NR2B subunit of NMDA receptor) play different, albeit complementary, roles in promoting EW-induced seizures and neuronal death.

What could be the mechanism that the tPA/plasmin system uses to promote EW-induced cell death? One well-characterized substrate for plasmin is an extracellular matrix protein, laminin (19,33). Through its interactions with membrane-bound integrins, laminin provides means of communication and attachment between the cell and its outside surroundings. We have previously shown that degradation of laminin by plasmin during excitotoxic insult triggers cell death (19). We found here that, similar to kainic acid injection, laminin is degraded in hippocampal regions that eventually undergo degeneration. Laminin degradation occurred 6 hours after EW was initiated, when tPA activity was the highest and seizures were most pronounced. Both laminin degradation and neurodegeneration were prevented by the deletion of the tPA gene. This result strongly suggests a correlation between the tPA/plasmin-mediated loss of laminin and EW-induced neuronal death.

If EW-induced neurodegeneration was caused by a loss of laminin support, then deletion of laminin should sensitize mice toward EW-mediated insult. That was not the case. Deletion of laminin γ-1, a primary substrate for plasmin, rendered CA1 neurons resistant to degeneration. What could be the reason for such an effect? One possibility is that the presence of laminin γ-1 could ensure proper functioning of some cellular mechanisms involved in neurodegeneration, such as correct membrane placement and trafficking or anchoring of excitatory ion channels. Another possibility is that laminin degradation products, released after EW, could facilitate neurodegeneration. We have recently reported that laminin degradation products are essential for upregulation of kainate receptor subunit KA1 in the hippocampus in response to excitotoxic injury (53). Whether similar mechanisms operate after EW requires further investigation.

In summary, we have found that plasmin-mediated degradation of laminin is critical for neurodegeneration after EW, a common complication seen in alcoholics (see Figure 8 for summary). Consequently, deletion of the tPA, plasminogen, or laminin γ-1 genes prevents EW-induced neuronal loss in the hippocampus. These findings add to our knowledge of the mechanism of EW and may contribute toward developing better therapies against central nervous system injury.

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Supplementary material cited in this article is available online.

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