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# Novel Roles for the Tissue Plasminogen Activator System in the Development of Fetal Alcohol Syndrome and the Regulation of Contextual Learning After Stress

Melissa Noel-Castro

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NOVEL ROLES FOR THE TISSUE PLASMINOGEN ACTIVATOR  
SYSTEM IN THE DEVELOPMENT OF FETAL ALCOHOL  
SYNDROME AND THE REGULATION OF CONTEXTUAL LEARNING  
AFTER STRESS

A Thesis Presented to the Faculty of  
The Rockefeller University  
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the degree of Doctor of Philosophy

by

Melissa Noel-Castro

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# NOVEL ROLES FOR THE TISSUE PLASMINOGEN ACTIVATOR SYSTEM IN THE DEVELOPMENT OF FETAL ALCOHOL SYNDROME AND THE REGULATION OF CONTEXTUAL LEARNING AFTER STRESS

Melissa Noel-Castro, Ph.D.

The Rockefeller University 2009

Ethanol exposure during synaptogenesis can result in brain and behavior neurotoxic defects referred to as fetal alcohol syndrome (FAS). Since tissue plasminogen activator (tPA) has been implicated in mediating excitotoxic neurodegeneration we subjected neonatal WT and tPA<sup>-/-</sup> to an acute ethanol paradigm that serves as a model of FAS. We observed persistent upregulation of tPA and extensive neurodegeneration after ethanol in the forebrain of WT. However, tPA<sup>-/-</sup> mice were protected from neuronal death, suggesting tPA mediates ethanol-induced neurodegeneration and FAS in mice. Commensurate with neuronal death, we observed ethanol-induced cognitive impairments in adult WT, but not tPA<sup>-/-</sup> mice. To understand how ethanol affects tPA we subjected mice to drugs that mimic the actions of ethanol in the brain. tPA<sup>-/-</sup> mice were protected from neurodegeneration after treatment with NMDA-R antagonist, but not after GABA<sub>A</sub>-agonist, suggesting tPA acts via an NMDA-R mediated mechanism. We also investigated the role of the tPA inhibitor plasminogen activator inhibitor-1

(PAI-1) in FAS. We observed PAI-1<sup>-/-</sup> mice were more vulnerable to ethanol-induced neurodegeneration than WT mice. In addition, we found that tPA<sup>-/-</sup>:PAI-1<sup>+/-</sup> mice treated with ethanol showed increased neurodegeneration than tPA<sup>-/-</sup> mice. These results suggest PAI-1 is involved in neuronal survival after ethanol. Finally, we explored the role of tPA in hippocampal synaptic plasticity after chronic stress. We restrained WT, tPA<sup>-/-</sup>, and PAI-1<sup>-/-</sup> mice for 21 days. We found chronic stress increased tPA activity in WT and PAI-1<sup>-/-</sup> mice. Stress greatly reduced PAI-1 below baseline levels in WT mice. These stress-induced changes in tPA activity and PAI-1 resulted in decreased contextual fear conditioning in WT and PAI-1<sup>-/-</sup> mice. Chronic stress did not affect contextual learning in the tPA<sup>-/-</sup> mice. These results suggest tPA is necessary for the synaptic plasticity cascade that causes cognitive deficits after chronic stress exposure.

*I wish to dedicate my thesis to my wonderful family for their constant love  
and support throughout my graduate education and the preparation of my  
thesis*

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## **CHAPTER 1: INTRODUCTION**

### **1.1 Fetal alcohol syndrome**

Ethanol exposure during synaptogenesis can result in neurotoxic brain defects referred to as fetal alcohol syndrome (FAS). The syndrome can lead to myriad of mental health problems including cognitive deficits, mental retardation, psychosis, depression, hyperactivity, and schizophrenia (Jones and Smith, 1973; Swayze et al, 1977; Kerns et al, 1977; Clarren and Smith, 1978; Sulik et al, 1981; Famy et al, 1998). According to the Center for Disease Control and Prevention, FAS is the leading preventable cause of mental retardation in the U.S. with a rate of 1 case developing the disorder for every 500 live births. There is no cure for FAS, yet the syndrome afflicts more babies born every year in the United States than the combined total of infants born with Down's syndrome, muscular dystrophy, and HIV combined.

Following the ethanol exposure that leads to FAS, there is significant brain mass loss and widespread neuronal death. The developing brain is more vulnerable to ethanol-induced neurodegeneration during synaptogenesis (West, 1987; Dobbing and Sands, 1979; Goodlet and West, 1992), an activity-dependent process. Rats exposed to ethanol during the synaptogenesis period at postnatal day 7 are vulnerable to ethanol-induced



neuronal death, while animals exposed to the same dose at postnatal day 21, when synaptogenesis has ended, are resistant to neurodegeneration (Heaton et al, 2003).

Ethanol reduces neuronal activity through a dual mechanism: antagonism of the N-Methyl-D-Aspartate receptor (NMDA-R) and potentiation of the gamma amino butyric acid type A receptor (GABA<sub>A</sub>-R) (Hoffman et al, 1989; Lovinger et al, 1989; Harris et al, 1995; Ikonomidou et al, 1999 and 2000). Drugs that mimic ethanol via inhibition of NMDA-R or hyperactivation of GABA<sub>A</sub>-R lead to a pattern of neurodegeneration that when composited together resemble what is observed after exposure to ethanol in neonatal rats (Ikonomidou et al, 1999 and 2000).

During synaptogenesis multiple subsets of CNS neurons depend on depolarization-induced elevation of intracellular calcium and the subsequent activation of multiple signaling cascades to survive. NMDA-R blockade by ethanol can inhibit these survival signals (Mennerick and Zorumski, 2000). A single large dose of ethanol administered during synaptogenesis leads to widespread neurodegeneration, and subsequent behavioral impairments in cognitive tasks in rodents that mimic those seen in humans with FAS (Ikonomidou et al, 2000; Dikranian et al, 2001, Maas et al, 2005; Olney et al, 2002; Ieraci and Herrera, 2006).

Furthermore, mice deficient in adenylyl cyclases 1 and 8, which are crucial components of the cAMP signal transduction pathway and are directly stimulated by calcium, are more vulnerable to ethanol-induced neurodegeneration (Maas et al, 2005).

Neuronal death is dependent on the activation of the key apoptotic protease caspase-3 (Olney et al, 2002), and entails the release of cytochrome c from the mitochondrial membrane (Young et al, 2003). Treatment with nicotinamide following ethanol exposure can diminish activation of the apoptotic cascade, reduce neurodegeneration, and consequently prevent ethanol-induced behavioral impairments such as hyper-activity and decreased contextual fear conditioning in mice (Ieraci and Herrera, 2006). Neurodegeneration is observed most prominently in the cerebral cortex, thalamus, hippocampus, and cerebellum in rodents (Ikonomidou et al, 2000; Dikranian et al, 2005). The improper development of neural circuits due to ethanol-induced mass deletion of neuronal populations in these brain regions is thought to underlie the deficits in learning, memory, and locomotion observed in adult animals in a model of FAS. Furthermore, identification of the mechanisms by which ethanol induces neurodegeneration in the developing brain is relevant to other clinical scenarios, including pediatric

anesthesia, antiepileptic therapy, and exposure of the immature brain to other NMDA-R antagonist drugs or GABA<sub>A</sub>-R agonists.

## **1.2 Tissue-type plasminogen activator in CNS physiology**

Tissue plasminogen activator (tPA) is a highly specific serine protease expressed in the vasculature where it initiates fibrinolysis via activation of the inactive zymogen plasminogen into plasmin (Carmeliet et al, 1994; Collen D, 1999). tPA is also expressed in the central nervous system (CNS), in neurons and microglia, where it plays multiple extravascular roles, both plasminogen dependent and independent (for a reviews see Melchor and Strickland, 2005; Benchenane et al, 2004). In neurons, tPA is stored in dense-core vesicles, transported to axon terminals, and released into the extracellular space in a regulated manner (Gualandris et al, 1996). tPA activity in the CNS is specifically regulated by the serine protease inhibitors (serpins) plasminogen activator inhibitor-1 (PAI-1) and neuroserpin.

The tPA/plasmin system is involved in neuronal growth cone penetration (Krystosek and Seeds, 1981), and migration of cerebellar granule neurons during development (Friedman and Seeds, 1995). tPA is also critical in synaptic plasticity processes that are necessary for learning and memory and long-term potentiation (LTP) (Qian et al, 1993; Seeds et al 1995; Baranes et

al, 1998; Pawlak et al, 2003; Norris and Strickland, 2007), and there is evidence that strongly suggests tPA regulates these functions via direct modulation of the NMDA-R (Norris and Strickland, 2007). In addition to normal CNS physiology, inappropriate tPA activity also plays a role in CNS pathology. tPA mediates excitotoxic neuronal death in the hippocampus after kainic acid (KA) treatment, and tPA-deficient mice are protected from KA-induced neurodegeneration (Tsirka et al, 1995; Chen et al, 1997; Indyk et al, 2003). Furthermore, chronic ethanol increases tPA activity in the hippocampus of adult mice. tPA interacts with the NR2B subunit of the NMDA-R, and ethanol-induced increase of tPA is required for increase in NR2B in the hippocampus, consequently resulting in ethanol withdrawal seizures (EW) in adult animals and neurodegeneration (Pawlak et al, 2005; Skrzypiec et al, 2008-submitted). Since tPA mediates ethanol-induced modulation of the NMDA-R, which is a main target for ethanol in the CNS, it became clear tPA was an appropriate candidate to investigate in the development of FAS.

### **1.3 NMDA-R**

Excitatory glutamatergic signaling mediated by the NMDA-R, a subtype of glutamate ionotropic receptor, is critical during developmental synaptogenesis. The heightened level of NMDA receptor activity during development allows for increased influx of calcium, which acts as a secondary signal, eventually leading to the translation of proteins required for neuronal differentiation through activation of immediate early genes (IEG) (Ghiani et al, 2007).

The NMDA-R complex consists of obligatory NR1 subunits that bind the co-agonist glycine, assembled with NR2 subunits (NR2A, NR2B, NR2C) that bind glutamate. A functional NMDA-R consists of two NR1, and at least one type of NR2 (NR2A-D) (Cull-Candy et al, 2001). The composition of NR2 is usually homomeric (i.e. NR2A/NR2A or NR2B/NR2B) and very rarely heteromeric (e.g. NR2A/NR2B). Each subunit consists of 4 transmembrane domains and an intracellular carboxyl terminal-domain (CTD) and an extracellular amino-terminal domain (ATD).

The NMDA-R is a central target in the CNS for the neurobehavioral effects of ethanol (Hoffman et al, 1989; Lovinger et al, 1989). Ion flux through the NMDA-R is inhibited by ethanol, with the NR2A- and NR2B-containing receptors showing higher sensitivity to ethanol inhibition than

NR2C- and NR2D-containing receptors (Masood et al, 1994; Mirshani and Woodward, 1995). Furthermore, chronic ethanol exposure of adult animals results in increased NMDA-R number, as determined by ligand binding studies, and increased expression of NR1, NR2B, and NR2A (Grant et al, 1990; Follesa and Ticku, 1995; Snell et al, 1996).

The NR2 subunit composition confers different biophysical and pharmacological properties on the receptor. NR2B-containing receptors have greater and longer acting excitatory post-synaptic currents (EPSCs) than NR2A-containing NMDA-R (Cull-Candy et al, 2001; Sheng and Kim, 2002). In the mature brain, NR2B-containing receptors are mostly found at extrasynaptic sites requiring higher concentrations of glutamate (spill-over) than synaptic receptors to be stimulated, which has been speculated to underlie the developmental changes observed in excitotoxicity (Zhou and Baudry, 2006). Indeed, the expression and localization of the NR2 subunits within the synapse are developmentally regulated (Sheng et al, 1994). The NR2B subunit is predominant during synaptogenesis, while NR2A expression does not rise until later postnatal stages near the end of the synaptogenesis period in rodents. The localization of the subunits is also developmentally regulated since increased NR2A replaces NR2B-containing receptors as the main NMDA-R in the synapse, and NR2B becomes

localized to extra-synaptic sites with its robust expression decreasing slightly after the end of synaptogenesis (Liu et al, 2004).

Whether the NMDA-R contains NR2A or NR2B subunits can influence neuronal survival or death. NR2B-specific blockade has been shown to preclude apoptosis and necrosis after NMDA treatment, suggesting NR2B is involved in promoting neuronal death. Conversely, antagonism of NR2A-containing NMDA-Rs enhances apoptosis after NMDA, suggesting NR2A is involved in neuronal survival (Liu et al, 2007). The differences in neuronal survival due to subunit composition could stem from the coupling of different signaling pathways downstream of the receptor complex and/or differences in subcellular localization. In addition, the roles for NR2B and NR2A might possibly vary depending on the developmental stage and brain region (Lee et al, 2002, Hardingham et al, 2002). The role of NR3 subunits is unclear, although reports suggest they have an inhibitory role on the receptor and might be important for NMDA-R signaling at early developmental stages (Perez-Otano and Ehlers, 2005; Yang et al, 2006; Corlew, 2007).

The NMDA-R can be regulated in several ways that affect the function and cell surface expression of the complex. The receptor can be internalized via clathrin-dependent endocytosis (Lavezzari et al, 2004). This route of

internalization is mediated by the phosphorylation state of tyrosine 1472 of the NR2B subunit by Src kinases (Yu et al, 1997; Prybylowski, et al, 2005; Zhang et al, 2008). NR2B contains a binding sequence to the adaptor protein complex 2 (AP-2), which is involved in the formation of clathrin-coated pits, and when NR2B is phosphorylated at Y1472, AP-2 cannot bind the subunit, and consequently the receptor is not internalized. The retention of NR2B at the membrane also depends on the interactions of its CTD with scaffolding proteins of the post-synaptic density such as post-synaptic density protein 95 (PSD-95), which couples the NMDA-R to intracellular signaling molecules that cluster and promote stabilization of the receptor (Brenman et al, 1996; Sheng, 2001). Finally, the motility of the receptor at the cell surface can also be determined by NR2 subunit composition, with NR2A-containing receptors being more stable at the synapse than NR2B-containing ones (Groc et al, 2006). It is possible the stability of NR2A is due to reduced interaction with PSD scaffolding proteins, since perturbations of the NR2A CTD interactions with PSD scaffold proteins does not affect its retention at the synapse (Groc et al, 2006).



## **1.4 NMDA-R-dependent signaling in FAS: nNOS and neurotrophic factors**

### **nNOS**

Neuronal nitric oxide synthase (nNOS), the enzyme that produces nitric oxide (NO) in the brain, is closely localized to the NMDA-R in the post-synaptic density. nNOS produces NO in response to NMDA-R-mediated increases in  $\text{Ca}^{2+}$  influx (Bredt and Snyder, 1989; Garthwaite et al., 1989; Kornau et al., 1997; Christopherson et al., 1999). nNOS has been implicated as a source of protection from ethanol-induced neurotoxicity during brain development. Mice that are deficient in nNOS are more vulnerable to neurodegeneration after exposure to ethanol in a mouse model of FAS (Bonthius et al, 2006), indicating nNOS protects neurons from ethanol-induced toxicity (Bonthius et al, 2002). In vitro studies suggest nNOS-mediated neuroprotection of developing neurons after ethanol is part of a neurotrophic survival pathway stimulated by nerve growth factor (NGF) that also requires CREB activation (Bonthius et al, 2003; Karacay et al, 2007).

nNOS possesses other neuroprotective effects in the CNS. These include post-translational S-nitrosylation of the NMDA-R (Lipton and Stamler, 1994), caspases (Kim et al, 2002; Fiorucci, 2001) and p21ras (Yun et al, 1998; Chiueh, 1999; Ahern et al, 2002). NO can also lead to increased expression of cytoprotective genes such as HSP70 (Sharp et al, 1999) and

Bcl-2 (Shimazaki et al, 1994; Kang et al, 2004). Furthermore ischemic preconditioning-mediated neuroprotection is mediated by the NO and cyclic guanosine monophosphate (cGMP) pathway and new protein expression (Andoh, 2000; Atochin et al, 2003). Under pathological circumstances, however, overproduction of NO and increased levels of reactive oxygen species that can lead to the formation of peroxynitrite, a reactive agent implicated in mechanisms of excitotoxicity after stroke (Keynes and Garthwaite, 2004). S-nitrosylation and nitration of proteins by peroxynitrite also exacerbate neurological disorders such as dementia (Giasson et al., 2000), Parkinson's disease (Chung et al., 2004), and Alzheimer's disease (Uehara et al., 2006).

The principal mechanism for NO action in the brain is through activation of cGMP, which is stimulated by calcium via calmodulin and PKG activation resulting in the modulation of ion channel function (Garthwaite et al, 1988). Interestingly, the cGMP pathway also activates the anti-apoptotic serine/threonine kinase Akt by PKG-dependent activation of phosphatidylinositol 3-kinase (PI3 kinase) (Waxman and Lynch, 2005). S-nitrosylation has recently emerged as a form of post-translational modification of ion channels, thus providing a route for NO to regulate electrical activity without stimulating production of cGMP. In the brain,

PKG and S-nitrosylation enhance the activity of large conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  (BK) channels.

### **Neurotrophic factors: BDNF and NGF**

As discussed previously, a great portion of the neuronal population is eliminated through programmed cell death in early postnatal stages during developmental synaptogenesis. Neurons enter apoptosis depending on their proliferative or metabolic state, or the presence of extracellular signals, and the subset that survives, do so through their ability to effectively connect to appropriate targets that deliver neurotrophic factors essential for survival (Hidalgo, 2003; Benn et al, 2004; Buss et al, 2006), forming synapses as a result, and eventually, neural circuits.

The formation of new synapses relies on electrical activity between afferent and target neurons (Katz and Shatz, 1996; Komuro and Rakic, 1998; Penn et al., 1998; Spitzer et al., 2000; Stellwagen and Shatz, 2002), which allow delivery of neurotrophic support and maturation of the synapse. This model of activity-dependent survival comes from in vivo and in vitro studies where cortical neurons have shown dependence on the activation of the NMDA-R and the consequent synthesis/release of BDNF for survival (Lessman et al, 2003; Suzuki et al, 2007). In the human brain this process

starts in gestation and lasts for a few years after birth. In rodents, synaptogenesis is solely postnatal, occurring during the first 2 weeks after birth.

Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are indispensable during developmental, and adult synaptogenesis (Garofalo et al, 1992; Aguado et al, 2003). Ethanol exposure can decrease the expression of BDNF and NGF, and the results vary depending on the manner of administration, length of exposure, brain region, and developmental period. In vitro data support a neuroprotective role for BDNF and NGF after ethanol exposure (Heaton et al, 1994; Climent et al, 2002).

tPA can aid in the maturation of pro-BDNF and pro-NGF through the activation of the extracellular protease plasmin (Pang et al, 2004; Bruno and Cuello, 2006). This process is essential for the formation of LTP in the adult hippocampus (Pang et al, 2004). The survival-related properties of these neurotrophic factors are mediated by the neurotrophic tyrosine kinase receptors (Trk): BDNF binds TrkB and NGF binds and activates TrkA. However, activation of the low affinity nerve growth factor receptor (also known as the p75 neurotrophin receptor) by BDNF or NGF has been associated with apoptotic neuronal death (Huang and Reichardt, 2001).

### **1.5 Plasminogen activator inhibitor type 1 (PAI-1) in CNS physiology**

As discussed earlier, inappropriate, elevated levels of tPA activity can have detrimental effects in the brain. Plasminogen activator inhibitor type 1 (PAI-1), a member of the serine protease inhibitor (serpin) gene family, is the primary inhibitor of tPA and urokinase (uPA) activity in the brain, and a key regulator of fibrinolysis in the vasculature. PAI-1 irreversibly inhibits tPA activity and abrogates the production plasmin (Lawrence et al, 1990). Eventually, the tPA:PAI-1 complex can be cleared from the extracellular space through LRP (Cao et al, 2006).

Most of PAI-1 is produced by astrocytes (Buisson et al, 1998; Hino et al, 2001), and previous work implicated astrocyte-derived PAI-1 in neuronal anti-apoptotic signaling (Kimura et al, 2000; Soeda et al 2001). These studies established that PAI-1 deficiency, in pheochromocytoma (PC12) cells differentiated with NGF, resulted in a significant reduction of Bcl-2 and Bcl-XL mRNAs, which typically promote cellular survival, and an increase in Bcl-XS and Bax mRNAs, which typically promote apoptosis (Soeda et al., 2001). As a result, pro-apoptotic signaling was favored leading to cellular death.

Although basal PAI-1 levels are relatively low in the brain, they can rapidly increase upon stimulation. An example of this is seen after intra-

hippocampal delivery of the glutamate analog KA. The treatment increases tPA activity and induces excitotoxicity in WT mice, whereas tPA<sup>-/-</sup> mice are protected from neuronal injury (Tsirka et al, 1995). PAI-1 mRNA is upregulated following KA-induced increase in tPA activity (Masos and Miskin, 1997), suggesting an elevation in tPA can trigger a subsequent increase in PAI-1 leading to a decrease of the protease activity. Furthermore, when exogenous PAI-1 was administered during KA injection, neurodegeneration was abrogated (Tsirka et al, 1996), suggesting PAI-1 promotes neuronal survival via inhibition of tPA. However, since WT mice not infused with PAI-1 do undergo neurodegeneration after KA, it is unclear whether the reported increase in PAI-1 mRNA in WT reflects an increase in protein levels that is sufficient to abolish tPA activity in a timely manner.

PAI-1 has also been implicated in cellular migration and neuritogenesis during development. Recent research showed stimulation of muscarinic receptors in astrocytes triggered the release of PAI-1, laminin, and fibronectin, facilitating the extension of neurites during development (Guizzetti et al, 2008). In addition, PAI-1 upregulation was required for laminin- and fibronectin-mediated neuritogenesis. It remained unclear whether the involvement of PAI-1 was dependent or independent of tPA. It is possible the presence of PAI-1 in the extracellular space precludes tPA-

mediated degradation of laminin and possibly other components of the matrix. In light of tPA's involvement in ethanol-induced neurodegeneration in the developing brain, we became interested in determining if PAI-1 is also associated to ethanol-induced neurotoxicity. Since neuronal death plays a pivotal role in the development and outcome of FAS, the identification of the molecules that influence neurons' vulnerability to ethanol's effects is of critical importance.

### **1.6 Chronic stress effects on hippocampal-dependent learning**

Exposure to short-lasting stressful situations can induce plasticity changes in the hippocampus that are necessary for learning, and ultimately survival. However, the prolonged exposure to stressful stimuli can result in maladaptive pathological effects on the hippocampus that might lead to the development of anxiety disorders, posttraumatic stress disorders, and depression (Miller and McEwen, 2006). Male rodents that are chronically stressed by immobilization (6 hours/day for 21 days) have retracted dendritic spines of hippocampal CA3 neurons, decreased body weight, and impairments in spatial memory tasks (Tsien et al, 1996; McEwen BS, 1999; Pawlak et al, 2005; McLaughlin et al, 2007).

Chronic stress in the rat also suppresses dentate gyrus neurogenesis and causes dendrites of hippocampal and medial prefrontal cortical neurons to shrink (Gould, et al, 1998; Bennur et al, 2007; Cameron and Gould, 1994; Cerqueira et al, 2005; Cook and Wellman, 2004). Interestingly, the same treatment increases dendritic complexity and sprouting of new synapses in neurons of the basolateral amygdala (Duvarci and Pare, 2007). Repeated stress also increases fear and aggression and reduces spatial memory (Wood et al, 2003; Conrad et al, 1996).

tPA is highly expressed in the adult hippocampus, and heavily involved in synaptic plasticity events that underlie learning and memory. Basal extracellular tPA activity can be observed in the mouse hippocampus within the mossy fiber pathway, with little or no activity or expression within the perforant path, the Schaffer collaterals, or neuronal cell bodies (Salles and Strickland, 2002). Hippocampal tPA has been implicated in LTP (Frey et al., 1996; Huang et al., 1996; Baranes et al., 1998; Madani et al., 1999; Pang et al, 2004), long-term depression (Calabresi et al., 2000), NMDA-R-mediated signaling (Nicole et al., 2001; Norris and Strickland, 2007), and synaptic remodeling (Baranes et al., 1998; Neuhoff et al., 1999). Additionally, overexpression of tPA enhances long-term potentiation (LTP) and improves performance in the Morris water maze (Madani et al., 1999).



tPA is a key mediator of the structural and molecular changes that ensue in the hippocampus as a result of repeated exposure to stressful stimuli. Mice deficient in tPA and plasminogen are protected from stress-induced decrease in NMDA-Rs and reduction in dendritic spines, and show impaired acquisition, but not retrieval, of hippocampal-dependent spatial learning in the Morris water maze (Pawlak et al, 2005). Since tPA is a key target of the stress response in the hippocampus, we sought to determine if chronic stress regulates the tPA/PAI-1 system in this brain region. We observed chronic stress modulated the tPA system in the hippocampus, and the changes observed were associated to impairments in learning and memory.

## **CHAPTER 2: MATERIALS AND METHODS**

### **2.1 Animals**

C57/BL6/J WT, tPA<sup>-/-</sup>, PAI-1<sup>-/-</sup>, and Plg<sup>-/-</sup> mice backcrossed for at least 10 generations were used in all the experiments. Mice were maintained in the Comparative Bioscience Center (CBC) with a controlled humidity, temperature, and light/dark cycle (7:00 am to 7:00 pm light). All animal procedures were according to protocols approved by the Institutional Animal Care and Use Committee (IACUC).

### **2.2 Polymerase Chain Reaction (PCR)**

DNA from tail tissue was obtained at the moment of sacrifice. Tails were digested overnight at 55 degrees in a mix of lysis buffer & Proteinase K.

DNA was extracted and PCR product samples run on 2% agarose gel. The tPA primers were: T881 TTGTGTCGTGCCAGGCCGAGTGCC, T731 GCTATGTTGGGAAAGGTGTGACTTACCG, PGKnSATTAAGGGCCAG CTCATTCCTCCC. PAI-1 primers: 2726 GAGTGGCCTGCTAGGAAATTCATTC, 2312 GACCTTGCCAAGGTGA TGCTTGGCAAC, PGKpAAATGTGTCAGTTTCATAGCC.

### **2.3 Materials**

Human recombinant tPA was provided by Genentech (South San Francisco, CA) and dissolved in phosphate buffered saline (PBS). Plasminogen for gel zymographies was isolated from human plasma provided by the New York Blood Center (Deutsch and Mertz, Science, 1970). The tPA inhibitor, tPA-STOP, was purchased from American Diagnostica (Stamford, CT) and the mutant S481A inactive tPA was obtained from Molecular Innovations (Novi, MI). MK801 was purchased from Calbiochem (San Diego, CA), Ro25-6981 was purchased from Tocris (Ellisville, MO). Diazepam was purchased from Sigma (St. Louis, MO).

### **2.4 Acute ethanol treatment**

Although synaptogenesis begins during pre-natal stages in humans, this process occurs post-natally in mice during the first two weeks after birth. Therefore pups were used at post-natal day 7 (P7) in these studies. Pups were injected subcutaneously (s.c.) with two injections of 2.5g/kg ethanol (prepared as a 20% solution in sterile saline) at 0 hour and 2 hours. Control pups were injected with an equal volume of sterile saline.

## **2.5 tPA in-gel zymography**

Forebrains from saline and ethanol-treated WT mice were removed and homogenized in 100 mM Tris containing 0.2% Triton X-100 and phosphatase inhibitors. After protein determination by the BCA Assay (Pierce, Rockford, IL), the homogenates were prepared to equal concentrations in non-reducing loading buffer and kept on ice. Samples were loaded on 10% separating gels containing casein and plasminogen. Upon completion, gels were rinsed in 2.5% Triton X-100 twice for 45 min at 37°C and then incubated overnight at 37°C in 0.25% Triton X-100. The following day, gels were stained with Coomassie Blue R-250 for 30 minutes and then destained with 10% acetic acid to visualize tPA activity. The bands representing tPA activity were quantified with Scion software (NIH).

## **2.6 *in situ* zymography**

To determine hippocampal extracellular tPA activity after 21 days of chronic restraint stress, 20 µm thick sections were incubated with an overlay mixture containing casein and plasminogen. The sections were then coverslipped and incubated at 37° C until dark zones of lysis became visible in the hippocampus. The sections were then observed in the microscope using a dark field setting, and pictures were taken and stored for analysis at a later

time. tPA activity (lytic zones) was analyzed using Image J (NIH). Briefly, the area of lysis was determined, and divided by the total hippocampal area, and multiplied by 100 to determine the percentage area of lysis.

## **2.7 Fluorescent *in situ* zymography**

To determine extracellular tPA activity in neonatal mice after ethanol (or saline) exposure, 20  $\mu$ m thick sections were incubated with an overlay mixture containing plasminogen and fluorescently quenched casein (Molecular Probes, Oregon), that fluoresces when cleaved by plasmin. The sections were then coverslipped and incubated at 37° C for 2. The sections were then observed in a fluorescent microscope (Zeiss Axioscope) using the fluorescein filter to visualize the areas of lysis, and pictures were taken and stored for analysis at a later time.

## **2.8 Immunoprecipitation and western blotting**

### **Immunoprecipitation**

Samples of forebrain homogenates from ethanol- or saline-treated mice were adjusted to equal protein concentration and precleared with preimmune IgG and Gamma Bind Plus Sepharose beads (Amersham Biosciences, Piscataway, NJ). The monoclonal anti-NR2B antibody (NeuroMab facility,

UC Davis, CA) was added for 1 hr, followed by incubation with Sepharose beads overnight at 4°C. Beads were washed with PBS and heated to 100°C for 5 min in loading buffer containing DTT. Following immunoprecipitation, we probed for  $\beta$ -adaptin and NR2B by western blot.

### **Western blotting**

Forebrain homogenates of saline and ethanol-treated WT and tPA<sup>-/-</sup> mice were prepared as described above. All samples were prepared to equal amounts of protein concentration, as previously determined by BCA assay. After protein separation on 4-15% pre-cast gradient Tris-Glycine polyacrylamide gels and transfer onto nitrocellulose membranes, blots were blocked in 5% milk and incubated in primary antibody overnight at 4°C. Blots were rinsed in Tris buffered saline containing 0.1% Tween-20 (TBS-T), and then incubated in secondary antibody for 1 hr at room temperature followed by rinsing in TBS-T before exposure to film. Bands were scanned and quantified with the Scion software (NIH), and normalized to beta-tubulin.

## **2.9 Antibodies**

Polyclonal anti-caspase-3 (1:500) antibody was obtained from Cell Signaling; monoclonal anti-cytochrome c (1:500) from BD Pharmingen; polyclonal anti-NGF (1:500), anti-BDNF (1:500), anti-NR1 (1:1000) and anti-cFOS (1:500), and  $\beta$ -adaptin (1:500) were purchased from Santa Cruz Biotechnology; polyclonal anti-tPA from Molecular Innovations and American Diagnostica; polyclonal anti-phospho GAP-43 (1:1000) and anti-GAP-43 (1:1000) from Chemicon; polyclonal anti-nNOS (1:1000) from Biomol; anti-cleaved caspase-3 from Cell Signaling; anti-NR1 from Santa Cruz Biotechnology; monoclonal anti-PSD-95 from Sigma; polyclonal anti-NR2B from PhoshoSolutions; monoclonal anti-NR2B from the NeuroMab facility; polyclonal anti-phospho NR2B (Y1472) from Imgenex; and anti-NR2A from Upstate Biotechnologies.

## **2.10 Cleaved Caspase-3 Immunostaining**

Saline- and ethanol-treated WT and tPA<sup>-/-</sup> mice were deeply anesthetized and transcardially perfused with normal saline and 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 48 hrs, followed by immersion in 30% sucrose until sinking. Brains were then frozen, sectioned at 20  $\mu$ m, and mounted on slides. The slides were allowed

to air-dry overnight. The following morning they were incubated in blocking buffer (1% BSA, 5% goat serum, 0.5% Triton X-100, in PBS) for 2 hours at room temperature, followed by primary antibody (anti-cleaved caspase-3 alexa fluor 488-conjugated, Cell Signaling, 1:500) incubation in blocking buffer at 4°C overnight. The following morning sections were washed in PBS with Triton X-100, coverslipped, and observed in the fluorescent microscope.

### **2.11 tPA Immunostaining**

tPA<sup>-/-</sup> P7 mice that had been treated with S481A tPA and ethanol (or saline), were deeply anesthetized and transcardially perfused with normal saline and 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 48 hrs, followed by immersion in 30% sucrose until sinking. Brains were then frozen, sectioned at 20  $\mu$ m, and mounted on slides. The slides were allowed to air-dry overnight. The following morning they were incubated in blocking buffer (1% BSA, 5% goat serum, 0.5% Triton X-100, in PBS) for 4 hours at room temperature followed by primary antibody incubation (anti-tPA, Molecular Innovations 1:500; anti-tPA, American Diagnostica 1:500) or preabsorbed primary antibody incubation in blocking buffer at 4°C overnight. Preabsorption was done by incubation of



the primary antibodies with tPA<sup>-/-</sup> tissue for 4 hrs at room temperature (Salles and Strickland, 2002). Slides were then washed with Triton X-100, and incubated with fluorescently tagged secondary antibody. After incubation, the sections were washed coverslipped, and observed in the fluorescent microscope.

### **2.12 PAI-1 Enzyme-Linked Immuno Sorbent Assay (ELISA)**

Forebrain homogenates from saline- and ethanol-treated WT mice were prepared as described above. Samples were prepared in duplicates (200 ug of protein each) and the ELISA was performed using a kit (Molecular Innovations, Novi, MI) according to the manufacturer's instructions.

### **2.13 Fluoro-Jade B staining**

Mice were deeply anesthetized and transcardially perfused with normal saline and 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 48 hrs, followed by immersion in 30% sucrose until sinking. Brains were then frozen and sectioned at 40 um on a cryostat. Sections were collected in PBS, mounted on slides, and allowed to air dry for 24 hrs. After drying, the slides were stained with Fluoro-Jade B (FJB) as has been previously described (Schmued and Hopkins 2000; Toscano et al.

2008). FJB detects neuronal damage by staining neurons undergoing necrosis or apoptosis a fluorescent green color.

#### **2.14 Fluoro-Jade B quantitation and analysis**

Quantitation was done as described previously (Paul et al, 2007). Upon completion of FJB staining the slides were observed on the fluorescent microscope with the appropriate filter. All pictures from animals from the same experiment were taken the same day with the same exposure settings. A minimum of 3 slices per animal and brain region was used for quantification purposes. Images taken with the 20X objective were obtained using a camera attached to the microscope and stored for later analysis. Image J (NIH software) was used to quantify FJB-positive staining. Images of the area of interest (e.g cingulate cortex, hippocampus, thalamus) were converted to 1-bit, the background subtracted from the image, and the positive-labeled cells counted by the program.

### **2.15 Blood Ethanol level determination**

Plasma EtOH levels were analyzed with the EnzyChrome kit by BioAssay Systems according to the manufacturer's directions.

### **2.16 Evans Blue extravasation**

Neonatal P7 and adult P90 WT and tPA<sup>-/-</sup> mice were injected transcardially with a solution of 2% Evans blue/PBS (4 ml/kg) dye, or control buffer. Fifteen minutes after Evans blue injection, animals were perfused with PBS, and their brains collected for analysis. The brains were sectioned in a freezing cryostat and observed in the fluorescent microscope, the presence of Evans Blue was observed in the brain parenchyma of young, but not the adult animals.

### **2.17 Restraint stress**

Adult (10-12 weeks of age) WT, tPA<sup>-/-</sup>, and PAI-1<sup>-/-</sup> mice were immobilized in wire mesh restrainers and placed in their homecage. Chronic stress lasted 6 hours a day, for 21 consecutive days. The restrainers were secured with clips to avoid having the mice escape from them. On an occasion, a mouse bit through the restrainer and escaped immobilization, this mouse was sacrificed and eliminated from the study. Control animals were not

restrained, and were kept in a different room than stress mice. We fed, changed water bottles, and cleaned their cages in order to minimize unnecessary stress by the CBC staff.

## **2.18 Fear Conditioning**

Fear conditioning was done on adult mice (8-10 weeks of age) that had been exposed to ethanol or saline at P7. In a separate study, chronically stressed (or non-stressed control) mice, were also tested in this paradigm. The conditioning chambers were equipped with a speaker, house light, and a video camera. The chamber floors consisted of rods that were connected to a shock generator. Mice were habituated to the behavioral room for 1 hour on the day of training and placed individually into a conditioning chamber cleaned with an ammonia-based solution for 2 minutes, this time was considered the “baseline period”. Mice were then exposed to three tone-footshock pairings (tone, 20 sec, 85 dB, 3.5 kHz; footshock, 1 sec, 0.6 mA) with an inter-trial interval of 60 sec. Upon completion of training, mice were returned to their home cages and room and tested 24 hrs later. The training sessions were video recorded in order to analyze the animals’ baseline freezing behavior (every 5 sec) during training. On testing day, mice were habituated to the behavioral room for 1 hr. Mice were then

placed individually into the same chamber (same scent, lighting, etc.) as the previous day for a 2-minute period, during which time their behavior was recorded. Freezing behavior, or the complete lack of movement, in response to the context, was scored every 5 sec.

### **2.19 Statistical analyses**

Data are presented as mean  $\pm$  SEM. Between-group comparisons were performed with factorial ANOVA and Tukey post-test. *P* values  $<0.05$  were considered significant. Numbers of animals in each experiment and the statistical significance are presented in figure legends.

## CHAPTER 3: RESULTS

### 3.1 Ethanol treatment increased neuronal-associated tPA activity in perinatal mouse pups

tPA and PAI-1 activities were determined following the administration of sedating doses of ethanol (2.5 g/kg at 0hr and 2 hr) to wild-type (WT) mouse pups postnatal day 7 (P7). Gel zymography of forebrain homogenates showed tPA activity slightly, but not significantly, increased 30 minutes after ethanol intake (Figure 1A and 1C). Three hours post-ethanol administration tPA activity was significantly elevated over 1.5 fold above control levels. tPA activity peaked with a 2-fold increase at 6 hours, and continued to be significantly increased at 24 hrs (Figure 1B and 1C) following ethanol administration. Increased tPA activity, represented as green fluorescence, was visibly localized to the cortex, hippocampus, and thalamus of WT mice treated with ethanol using *in situ* zymography, which detects extracellular activity of the protease, 3 hours post-ethanol. (Figure 1D, 1E, and 1F respectively).

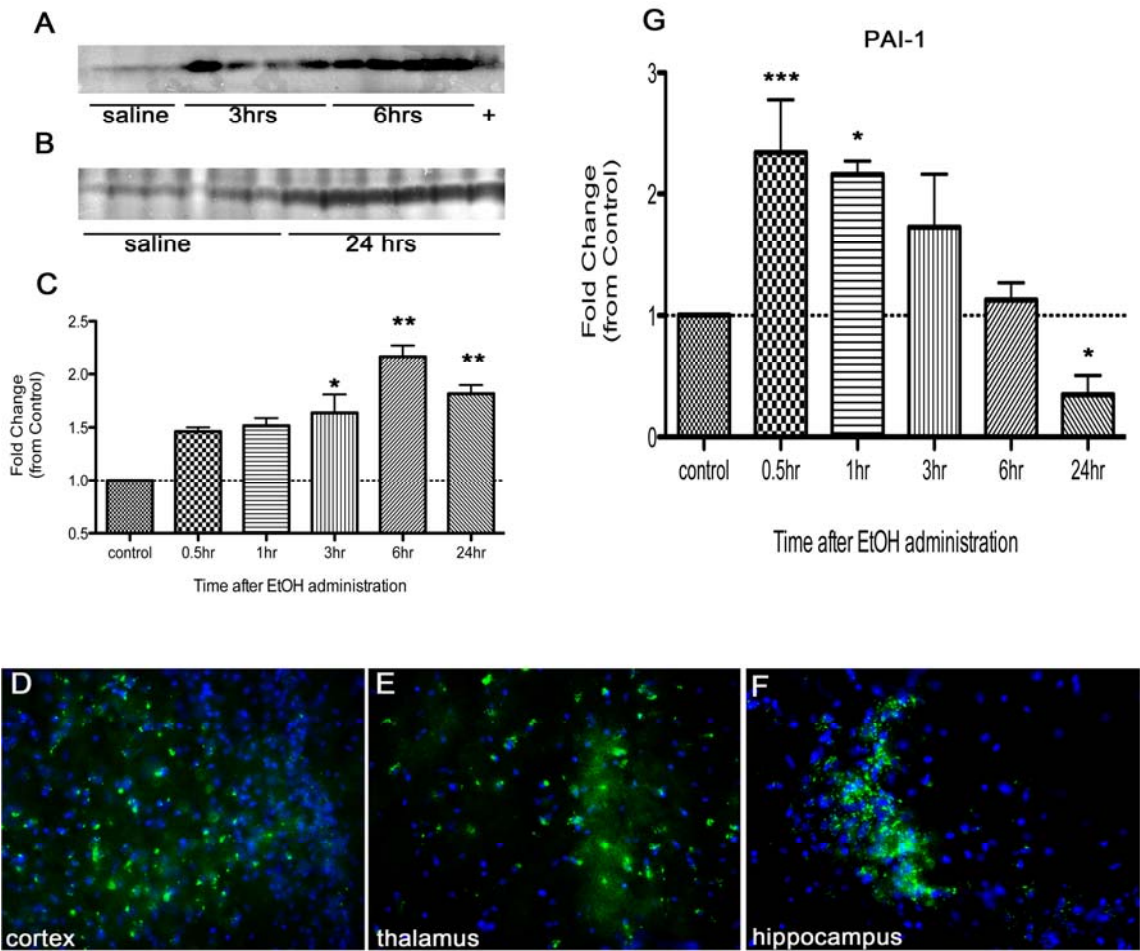
Localized increase of tPA activity could be the result of upregulation of tPA expression or a decrease in PAI-1 expression. Therefore, tissue homogenates from WT P7 mice treated with ethanol were also examined for the expression of free and complexed (i.e. total) PAI-1 using an enzyme-linked immunosorbent assay (ELISA). PAI-1 antigen was increased approximately 3-

fold above baseline levels in the WT brain 30 minutes after ethanol treatment, but was reduced to baseline at 3 hours, and downregulated below the baseline by 24 hours following the administration of ethanol (Figure 1G). The pattern of neurotoxic insult after ethanol leading to elevated tPA activity and increased PAI-1 is not consistent with the pattern previously observed by Masos and Miskin in adult mice following kainic acid (KA) treatment (Masos and Miskin, 1997). In the KA scenario, the hippocampal increase in tPA was followed by a subsequent increase in PAI-1. Here we observe an opposite pattern where ethanol increased PAI-1 levels prior to the increase of tPA activity. Also, ethanol induced downregulation of PAI-1 below the baseline 24 hours after treatment when tPA levels remain significantly elevated.

**Figure 1. Ethanol treatment increased neuronal-associated tPA activity in perinatal mouse pups.** WT mice (P7) were injected subcutaneously (s.c) with ethanol (referred to as EtOH in the figures) in 2 doses of 2.5g/kg at times 0 hr and 2hr (N=5-6). Control mice were injected with equal volumes of saline. Mice were sacrificed at different time-points, the forebrain was homogenized, and tPA activity assayed by in-gel zymography (A and B) and *in situ* zymography (D, E, F). Quantification of gel zymographs (C) showed tPA activity was significantly increased in the forebrain of WT mice 3 hr after EtOH (\*,  $P < 0.05$ ). tPA activity remained increased at later time-points up to 24 hrs (\*\*,  $P < 0.001$ ). *In situ* zymography showed extracellular tPA activity is localized to the cortex (D), thalamus (E), and hippocampus (F) of WT mice 3 hrs after ethanol, as observed by green fluorescence (blue is DAPI staining). PAI-1 was assayed with an ELISA. PAI-1 levels were increased significantly (\*\*\*,  $P < 0.0001$ ) 0.5 hr after treatment, returned to baseline at 3 hr post-ethanol, and were downregulated (\*,  $P < 0.05$ ) 24 hrs post-ethanol.



Figure 1

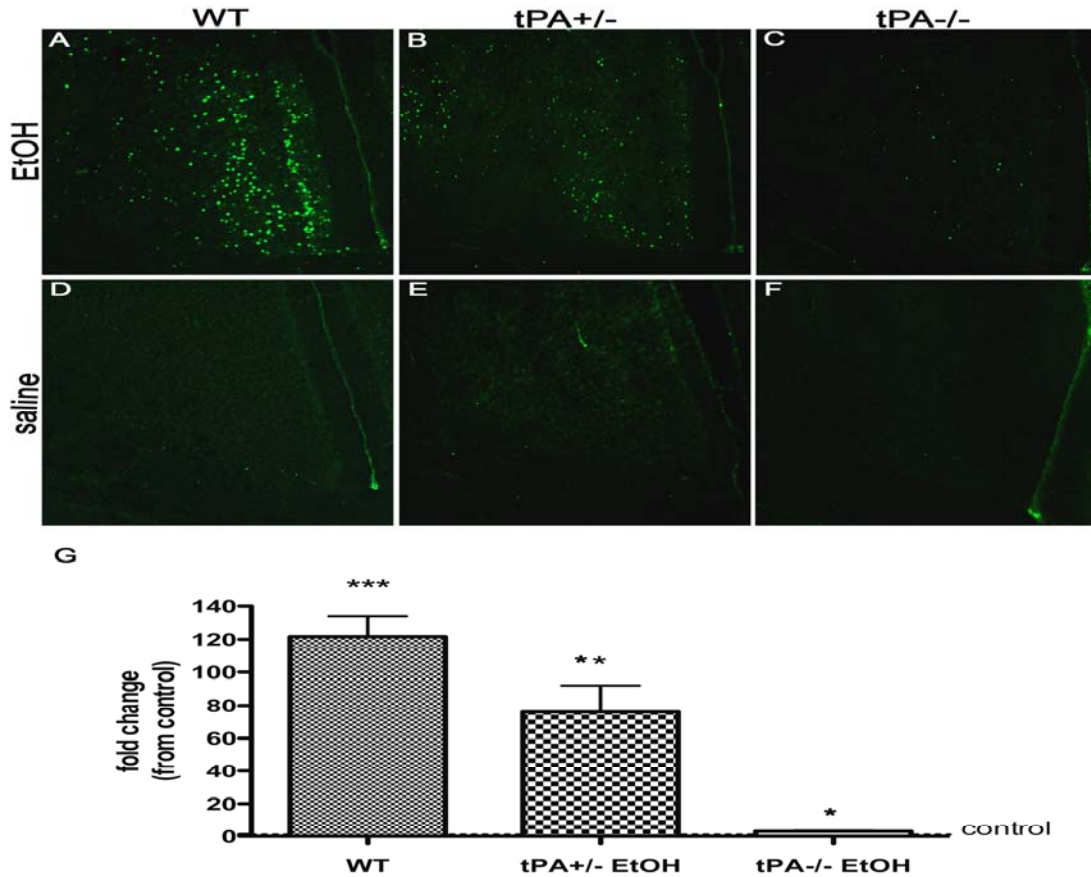


### **3.2 tPA upregulation corresponds to increased apoptotic neurodegeneration after ethanol**

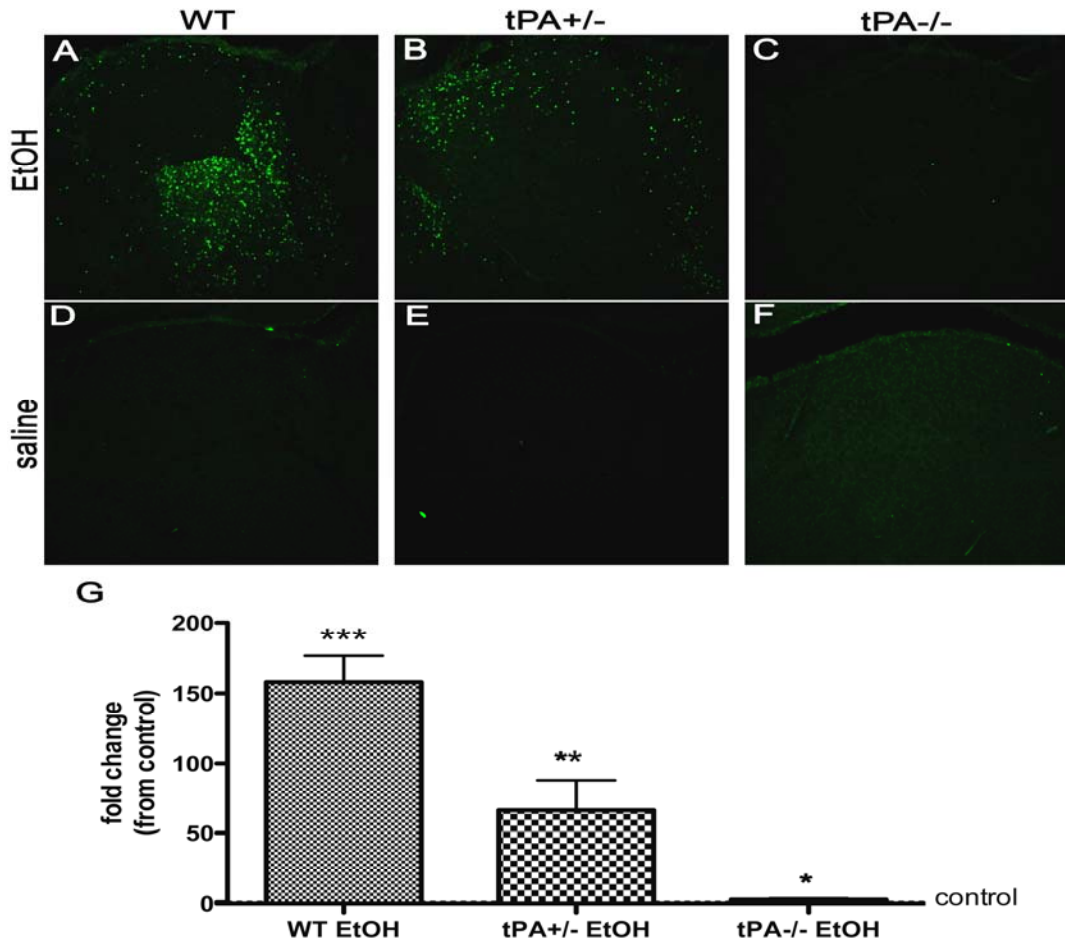
Increased tPA activity promotes excitotoxin-induced neurodegeneration as seen following kainic acid treatment and ethanol withdrawal seizures in the hippocampus of adult mice. To determine whether the increased tPA activity seen in P7 mouse brain following ethanol treatment was similarly associated with neuronal loss, we used Fluoro-Jade B staining to identify areas of neurodegeneration in WT and tPA<sup>-/-</sup> mice 24 hours after ethanol treatment. Neuronal loss was widespread throughout the WT forebrain (Figure 2 and Figure 3). The most vulnerable areas included the cingulate cortex which exhibited ~120-fold increase in neuronal loss above saline-treated controls (Figure 2A and 2D), and the thalamus with ~150-fold increase in neurodegeneration above control (Figure 3A and 3D). In a stark contrast, tPA<sup>-/-</sup> mice showed significant attenuation in neuronal loss in both the cortex (Figure 2C and 2F) and thalamus (Figure 3C and 3F) after ethanol treatment.

The effect of tPA on neurodegeneration as detected by Fluoro-Jade B staining was gene dosage-dependent, as tPA mice heterozygous for tPA deficiency showed an intermediate phenotype in both the cortex (Figure 2B and 2F) and thalamus (Figure 3B and 3F) when compared to WT and tPA<sup>-/-</sup> animals. Ethanol induced a ~3.6-fold increase in neurodegeneration in the WT

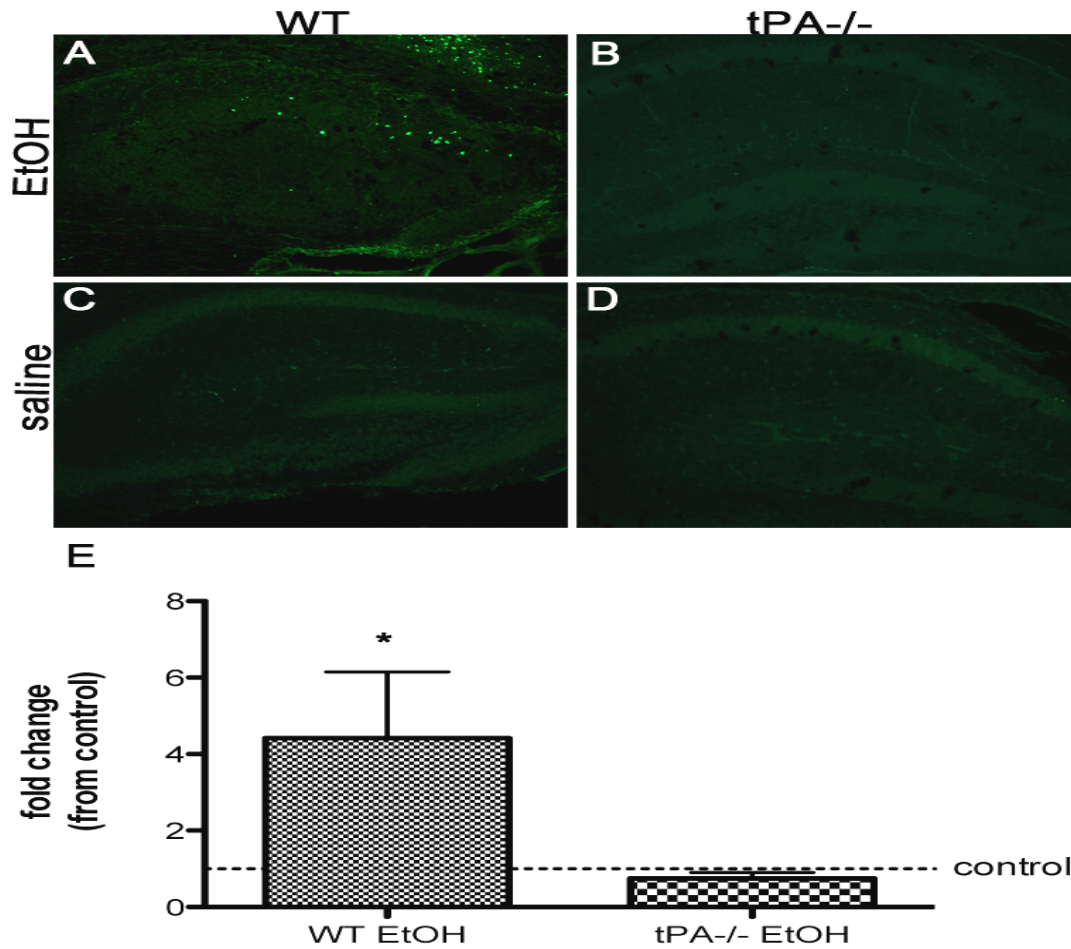
hippocampus (Figure 4A and 4E) compared to a ~0.8-fold increase seen in the tPA<sup>-/-</sup> (Figure 4B and 4E). The absence of significant neuronal death in the hippocampus observed in this study is consistent with previous studies of FAS in neonatal mice (Ikonomidou et al, 2000) in which the same protocol did not affect the hippocampus to the same magnitude it did the cortex and thalamus.



**Figure 2. Ethanol induces neurodegeneration in the cortex of WT mice, but not tPA<sup>-/-</sup>.** WT, tPA<sup>-/-</sup>, and tPA<sup>+/-</sup> P7 mice were injected with ethanol or saline as control (N = 6-7/group). 24 hrs after treatment, animals were sacrificed and brain sections analyzed histologically with Fluoro-Jade (FJB) to visualize neurodegeneration. Ethanol induced extensive neuronal death in the cortex of WT (A) when compared to saline-treated mice (D) (\*\*\*,  $P < 0.0001$ ). tPA<sup>-/-</sup> mice, however, are significantly protected from neurodegeneration after ethanol (C) in comparison to the saline group (F) (\*,  $P < 0.05$ ). tPA<sup>+/-</sup> showed intermediate neurodegeneration (B) when compared to the WT (A) and tPA<sup>-/-</sup> (C) brains after ethanol (\*\*,  $P < 0.001$ ). There was no difference in basal (saline-treated) neurodegeneration between any of the groups.

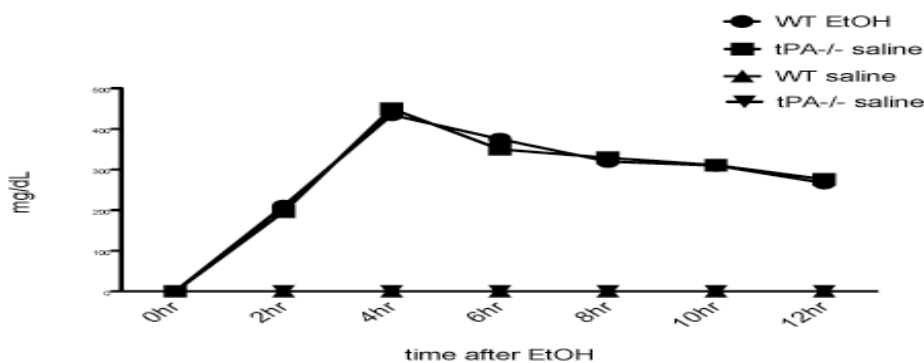


**Figure 3. Ethanol induces neurodegeneration in the thalamus of WT mice, but not tPA<sup>-/-</sup>.** WT, tPA<sup>-/-</sup>, and tPA<sup>+/-</sup> P7 mice were injected with ethanol or saline as control (N = 6-7/group). 24 hrs after treatment, animals were sacrificed and brain sections analyzed histologically with Fluoro-Jade (FJB) to visualize neurodegeneration. Ethanol induced extensive neuronal death in the thalamus of WT (A) when compared to saline-treated mice (D) (\*\*\*,  $P < 0.0001$ ). tPA<sup>-/-</sup> mice, however, are significantly protected from neurodegeneration after ethanol (C) in comparison to the saline group (F) (\*,  $P < 0.05$ ). tPA<sup>+/-</sup> showed intermediate neurodegeneration (B) when compared to the WT (A) and tPA<sup>-/-</sup> (C) brains after ethanol (\*\*,  $P < 0.001$ ). There was no difference in basal (saline-treated) neurodegeneration between any of the groups.



**Figure 4. Ethanol induces neurodegeneration in the hippocampus of WT mice, but not tPA<sup>-/-</sup>.** WT and tPA<sup>-/-</sup> P7 mice were injected with ethanol or saline as control (N = 6-7). 24 hrs after treatment, animals were sacrificed and brain sections analyzed histologically with Fluoro-Jade (FJB) to visualize neurodegeneration. Ethanol induced modest, but significant neuronal death in the hippocampus of WT (A) when compared to saline-treated mice (C) (\*;  $P > 0.05$ ). tPA<sup>-/-</sup> mice did not display significant neurodegeneration in the hippocampus after ethanol (B) either ( $P > 0.05$ ). There was no difference in basal (saline-treated) neurodegeneration between any of the groups.

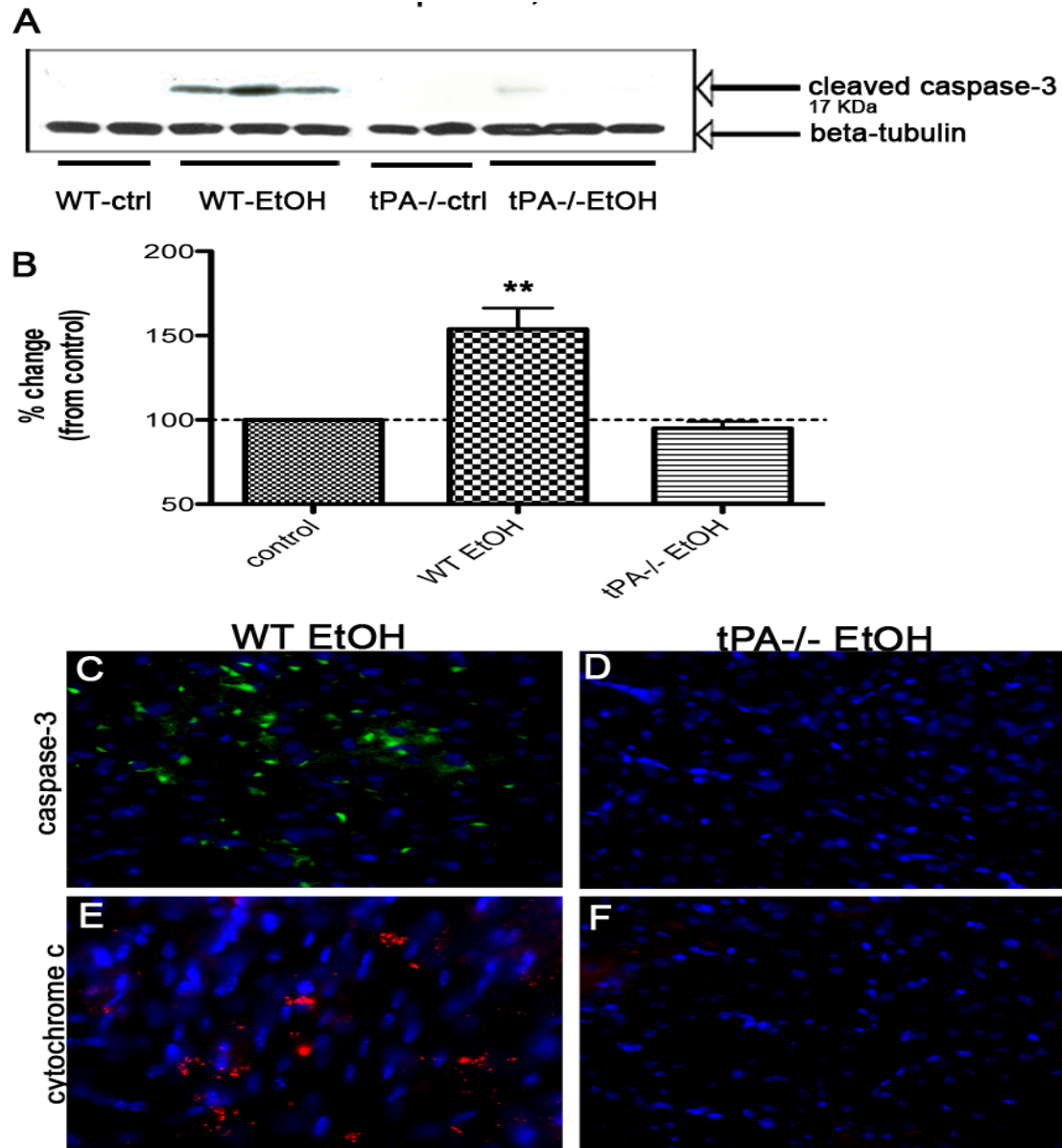
It was possible that the difference in neurodegeneration during synaptogenesis in  $tPA^{-/-}$  and wild type could have been an artifact of differing blood ethanol concentrations, perhaps reflective of underlying metabolic differences between the two genotypes, and not to a primary difference in neuronal physiology. To address this possibility, we determined the blood ethanol concentrations following treatment with the acute binge paradigm described earlier (2.5g/kg ethanol at 0 and 2 hours). We found no difference in blood ethanol concentrations between  $tPA^{-/-}$  and WT mice (Figure 5) at any timepoint studied. This result verifies that the observed neuroprotection in the  $tPA^{-/-}$  mice was not due to differences in the rate of ethanol metabolism between mouse genotypes.



**Figure 5. Blood ethanol concentrations are similar in WT and  $tPA^{-/-}$  pups.** WT and  $tPA^{-/-}$  P7 mice were injected with ethanol as previously described. Mice were decapitated at different time-points after treatment, trunk blood was collected, and plasma was assayed to determine ethanol concentration. WT and  $tPA^{-/-}$  mice show similar concentration of ethanol in plasma at all the time-points evaluated. ( $P > 0.05$ )

Ethanol-induced apoptotic neurodegeneration during synaptogenesis is mediated by BAX, requires the release of cytochrome c from the mitochondrial membrane, and culminates with the activation of caspase-3 (Olney et al, 2000 and 2002; Young et al, 2003 and 2005). To determine if activation of ethanol-induced apoptosis during synaptogenesis is tPA dependent, we examined tissue homogenates and sections for cleaved caspase-3 and cytochrome c immunoreactivity. WT mice showed an increase in activation of caspase-3 as observed by western blot (Figure 6A and 6B) and immunohistochemistry (Figure 6C), and cytochrome c immunoreactivity (Figure 6E) eight hours after ethanol treatment as previously noted (Olney et al, 2000 and 2002; Young et al, 2003 and 2005). tPA<sup>-/-</sup> mice did not exhibit changes in active caspase-3 (Figure 6A, 6B, and 6D) or cytochrome c (Figure 6F) immunoreactivity concomitant with the lack of neuronal loss observed in these mice.



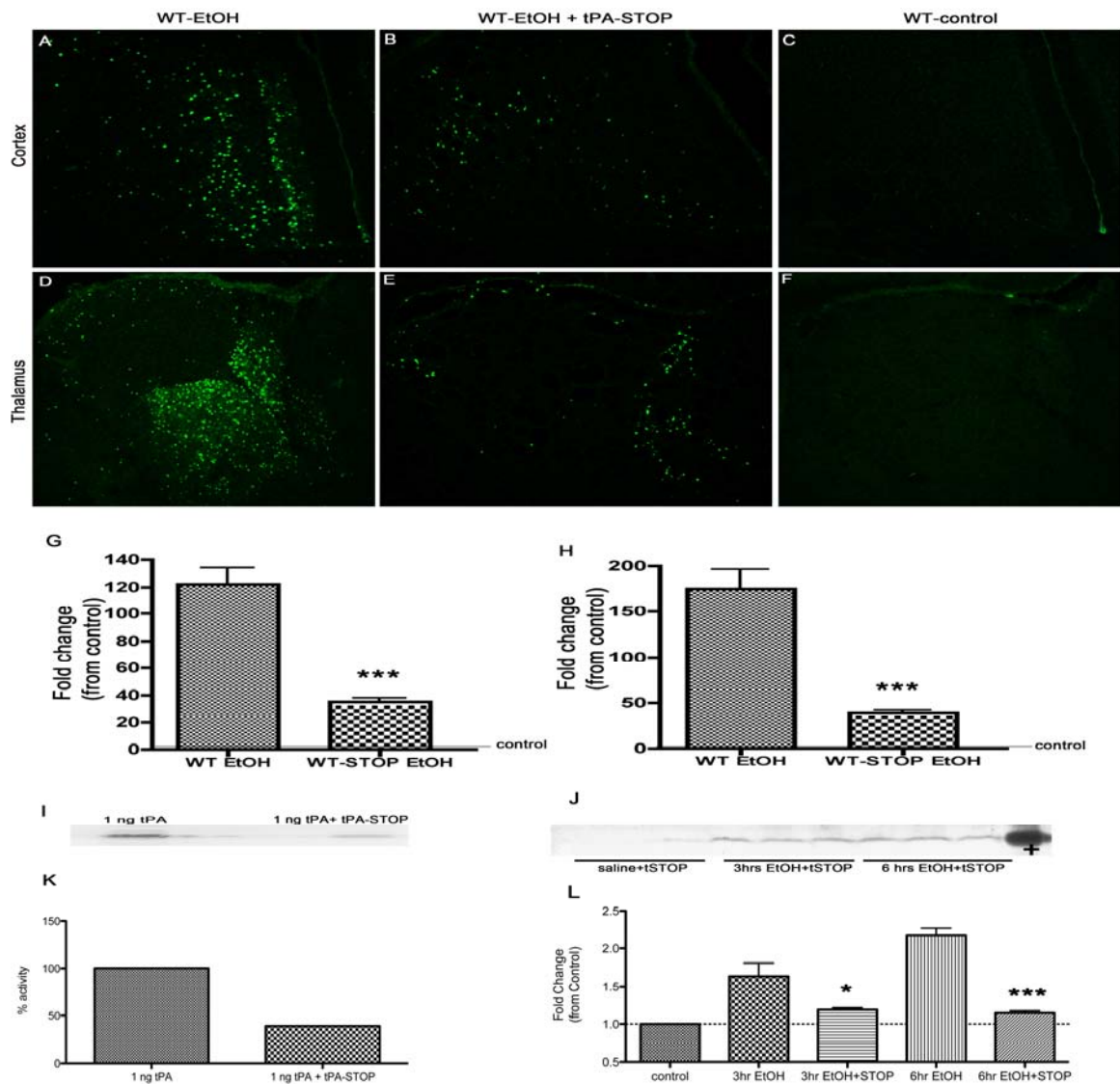


**Figure 6. Ethanol induces caspase-3 activation and cytochrome c immunoreactivity in WT, but not tPA<sup>-/-</sup> mice.** WT and tPA<sup>-/-</sup> P7 mice were injected with ethanol or saline and sacrificed 8 hrs post-treatment. Ethanol induced the activation of caspase-3 in the forebrain of WT mice (\*\*, P < 0.001) but this activation was not seen in the tPA<sup>-/-</sup> (A and B). Immunohistochemistry showed ethanol dramatically increased caspase-3 and cytochrome-c reactivity (C and E) in WT mice, but not tPA<sup>-/-</sup> (D and F).

In case tPA<sup>-/-</sup> mice were resistant to ethanol-induced neurodegeneration as a result of underlying developmental abnormalities distinct from the acute ethanol insult and not as a direct effect of tPA activity upregulation following ethanol ingestion, we used the small molecular weight tPA-specific inhibitor tPA-STOP to deplete tPA activity (Figure 7I and 7K) instead of making use of genetically deficient tPA mice. WT mice were injected intra-peritoneally (i.p.) with tPA-STOP (10ug/g) 30 minutes before the first ethanol treatment. Fluoro-Jade B staining showed that the mice pre-treated with tPA-STOP exhibited reduced neurodegeneration after ethanol when compared to the group treated only with ethanol (Figure 7). The cingulate cortex showed ~80 fold reduction in neurodegeneration when compared to the ethanol-treated group (Figure 7B and 7G). The thalamus showed greater reduction in neuronal loss than the cortex ~100 fold (Figure 7E and 7H). Forebrain samples collected from WT mice treated with both tPA-STOP and ethanol did not have as extensive an upregulation of tPA activity compared to WT mice that did not receive tPA-STOP at 3 and 6 hrs post-Ethanol (Figure 7J and 7L). This result is consistent with that seen using tPA<sup>-/-</sup> mice and suggests that the effect of tPA in ethanol-induced neurodegeneration is an acute effect of tPA upregulation.

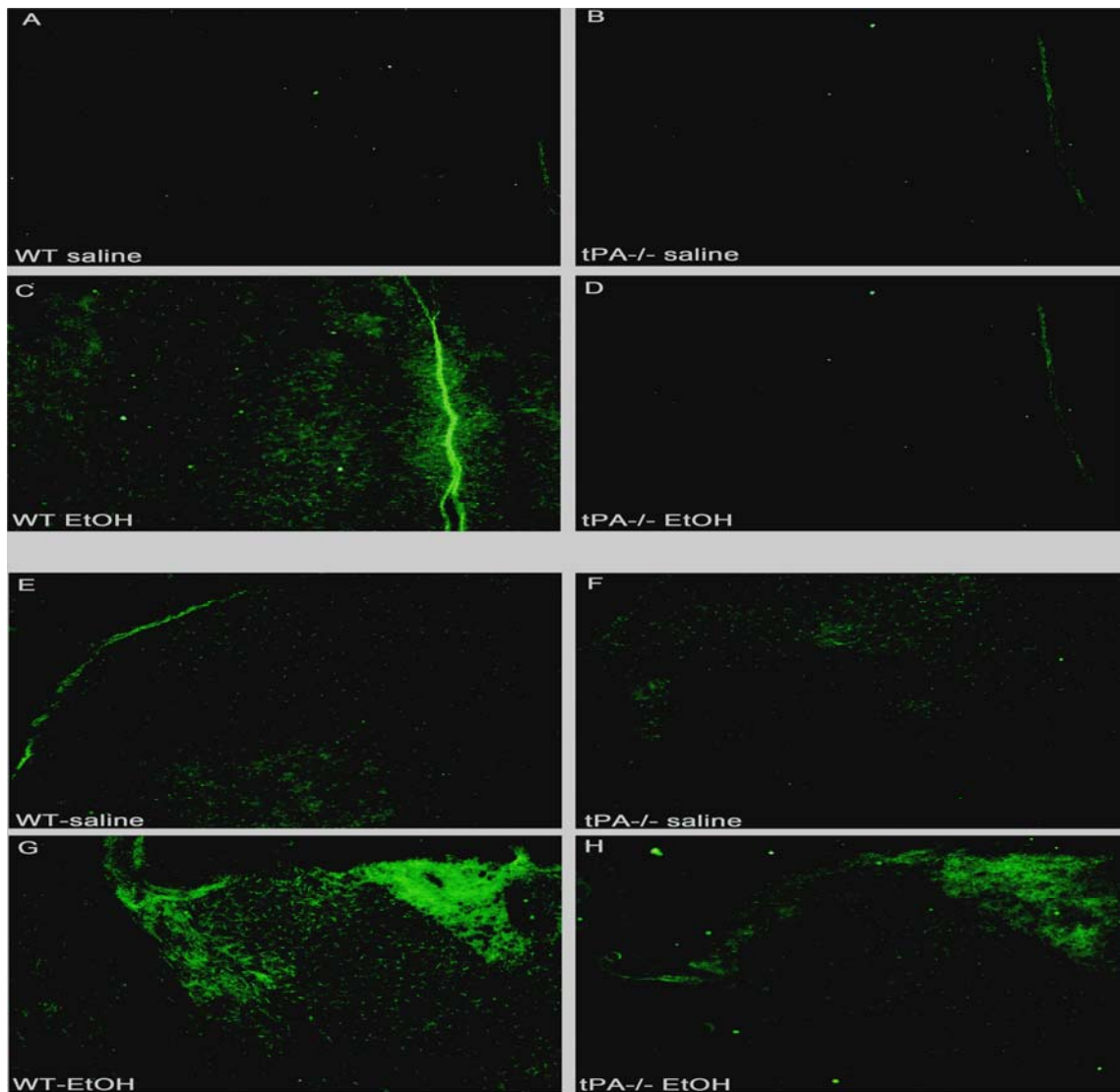
**Figure 7. tPA-STOP attenuates ethanol-induced neurodegeneration in WT mice.** WT P7 mice were injected with tPA-STOP (10ug/g body weight) 30 mins prior to the first ethanol injection. tPA-STOP pre-treated mice exhibited reduced neurodegeneration when compared to mice treated with EtOH alone. tPA-STOP attenuated neurodegeneration by ~80 fold in the cortex (A, B, and G; \*\*\*,  $P < 0.0001$ ) and over 100 fold in the thalamus (D, E, and H; \*\*\*,  $P < 0.0001$ ). tPA-STOP administered along with saline did not result in any changes in neurodegeneration (C and F). 1 ng of active tPA was incubated with 14 ug of tPA-STOP (1ug/ul) for 3 hrs at room temperature. tPA activity was reduced by ~60% (I and K). Mice pre-treated with tPA-STOP were sacrificed 3 and 6 hrs post-EtOH, their forebrains removed, and the homogenates assayed for tPA activity by in-gel zymography (J). Mice pre-treated with tPA-STOP before EtOH showed an attenuated increase in tPA activity compared to what was previously observed when treated with EtOH alone (L; \*  $P < 0.05$ , \*\*\*  $P < 0.0001$ ).

Figure 7

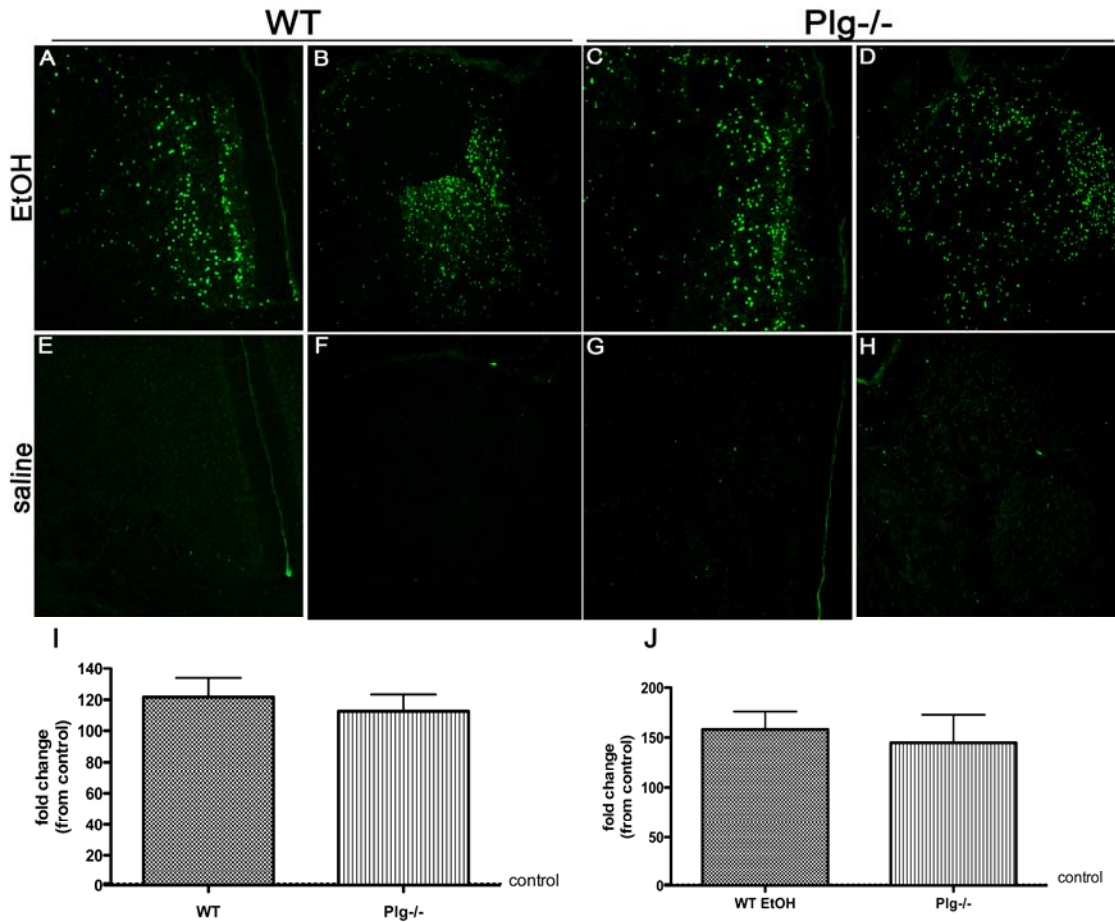


### **3.3 tPA induction of neurodegeneration is plasminogen-independent**

The mechanisms by which tPA has previously been shown to induce neurodegeneration include 1) activation of plasminogen to plasmin with subsequent proteolysis of the supporting extracellular matrix surrounding neurons; 2) microglial activation; and 3) via direct modification of the NMDA-R. We observed increased CD11b immunoreactivity, characteristic of microglia reactivity, in the cortical (Figure 8C) and thalamic (Figure 8G) regions of WT, but not tPA<sup>-/-</sup> mice (Figure 8D and 8H) 24 hrs after ethanol exposure. To determine whether tPA activation of plasminogen to plasmin was necessary for tPA-induced neurodegeneration in our system, plasminogen-deficient mice (Plg<sup>-/-</sup>) mouse pups were treated with ethanol at postnatal day-7, and examined for neurodegeneration as previously done with tPA<sup>-/-</sup> and wild type animals. There was no difference between the extent of neurodegeneration in Plg<sup>-/-</sup> and wild type mice (Figure 9), suggesting that tPA acts independently of plasmin in this system.



**Figure 8. WT mice, but not tPA<sup>-/-</sup>, show increased CD11b immunoreactivity after ethanol.** WT and tPA<sup>-/-</sup> P7 mice treated with ethanol or saline (N = 6-7) were sacrificed 24 hrs after treatment, and their brains examined histologically for a marker of microglial activation. WT mice showed enhanced immunoreactivity to an anti-CD11b FITC-conjugated antibody 24 hrs after ethanol treatment in the cortex (C) and thalamus (G). tPA<sup>-/-</sup> mice did not show increased immunoreactivity after ethanol in either brain region (D and H). There were no baseline differences in CD11b immunoreactivity in any group (A, B, E, F).



**Figure 9. tPA-mediated neurodegeneration after ethanol is plasmin-independent.** To investigate if tPA-mediated neuronal death after ethanol is plasmin dependent, we injected WT and plasminogen-deficient (Plg<sup>-/-</sup>) P7 mice with ethanol or saline as control (N = 6/group). 24 hrs after treatment, animals were sacrificed and brain sections analyzed histologically with Fluoro-Jade (FJB) to visualize neurodegeneration. Ethanol induced extensive neuronal death in the cortex (A) and thalamus (B) of WT mice. Plg<sup>-/-</sup> deficient mice showed ethanol-induced neurodegeneration in the cortex (C) and thalamus (D) to a similar degree and pattern previously observed in the WT mice. There was no difference in basal (saline-treated) neurodegeneration between any of the groups.

### **3.4 tPA induction of neurodegeneration is independent of its proteolytic activity**

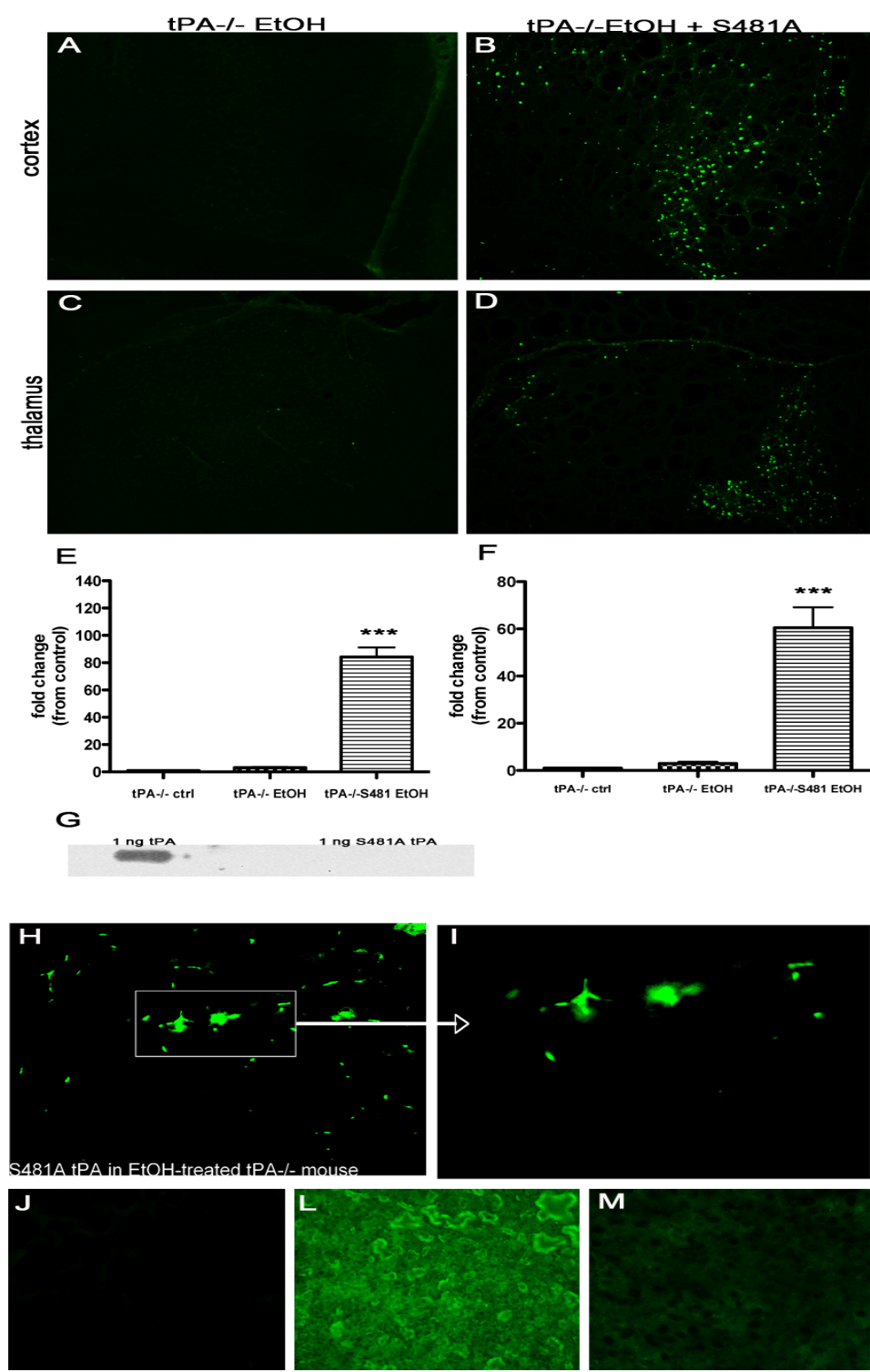
To determine if restoration of tPA antigen to tPA<sup>-/-</sup> mice was sufficient to restore neurodegeneration as observed in the WT mouse after ethanol exposure, we treated P7 tPA<sup>-/-</sup> mice with exogenous tPA. Since P7 mice cannot undergo stereotaxic surgery, and peripherally circulating active tPA can compromise the integrity of the blood brain barrier (BBB) (Yepes et al, 2003), we opted for peripheral administration of tPA which has a mutation from serine to alanine at residue 481 (S481A). This mutation renders the proteinase catalytically inactive (see Figure 10G) and (Olson et al, 2001). tPA<sup>-/-</sup> mice were injected with S481A tPA (1µg/g) 30 minutes before the first ethanol treatment. Fluoro-Jade B analysis revealed a partial, but significant, restoration of the ethanol-induced neurodegeneration in the tPA<sup>-/-</sup> mice that were pre-treated with S481A tPA (Figure 10). The effect was surprisingly greater in the cortex with a ~80-fold increase (Figure 10B and 10E) than in the thalamus where the increase was in neuronal death was ~50-fold (Figure 10D and 10F). To verify that S481A reached the brain parenchyma in our P7 mice, we examined S481A-treated P7 tPA<sup>-/-</sup> mouse brain sections by immunohistochemistry (IHC). The commercially available antibodies against tPA were not specific when observed by western blot (data not shown) and IHC, yielding high cross-reactivity with a milieu of



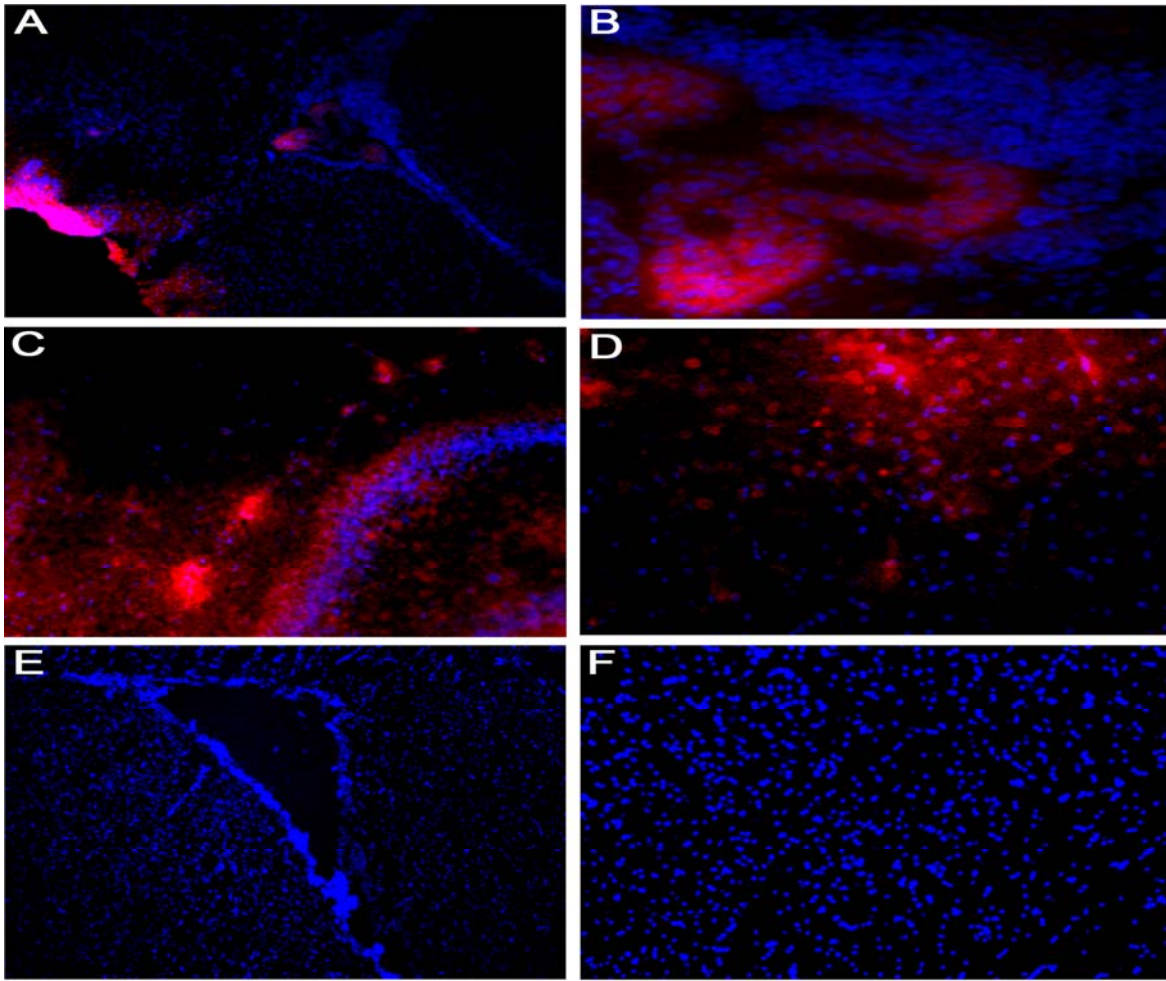
other molecules (Figure 10L). When the antibody was incubated with a tPA<sup>-/-</sup> forebrain slice at room temperature for 4 hrs the specificity of the antibody greatly increased (Figure 10M). Our results show there was positive staining for tPA in the brain of tPA<sup>-/-</sup> mice treated with S481A (Figure 10H and 10I) but not in the tPA<sup>-/-</sup> brain of mice treated with saline (Figure 10M).

**Figure 10. S481A tPA partially restores ethanol-induced neurodegeneration in the tPA<sup>-/-</sup> mouse.** tPA<sup>-/-</sup> P7 mice were injected with S481A tPA (1ug/g) 30 mins prior to ethanol exposure. Mice were also injected with saline as a control, or with ethanol alone. Mice pre-treated with S481A tPA before ethanol showed a partial restoration of neurodegeneration (B and D) when compared to mice treated with ethanol alone (A and C). Significant FJB-positive staining was seen in the cortex (B and E; \*\*\*,  $P < 0.0001$ ) and thalamus (D and F; \*\*\*,  $P < 0.0001$ ) of tPA<sup>-/-</sup> mice pre-treated with S481A tPA prior to ethanol. S481A tPA lacked enzymatic activity when compared to the same amount of active tPA by in-gel zymography (G). S481A tPA could be observed in the brain of tPA<sup>-/-</sup> mice with an anti-tPA that had been pre-adsorbed in tPA<sup>-/-</sup> tissue (H and I). We did not observe immunoreactivity when the 1° antibody was not present (J). When the anti-tPA antibody was not pre-adsorbed, immunostaining of saline treated tPA<sup>-/-</sup> samples was non-specific (L). Specificity greatly increased when the antibody was pre-adsorbed as seen by the reduction in background intensity (M).

Figure 10



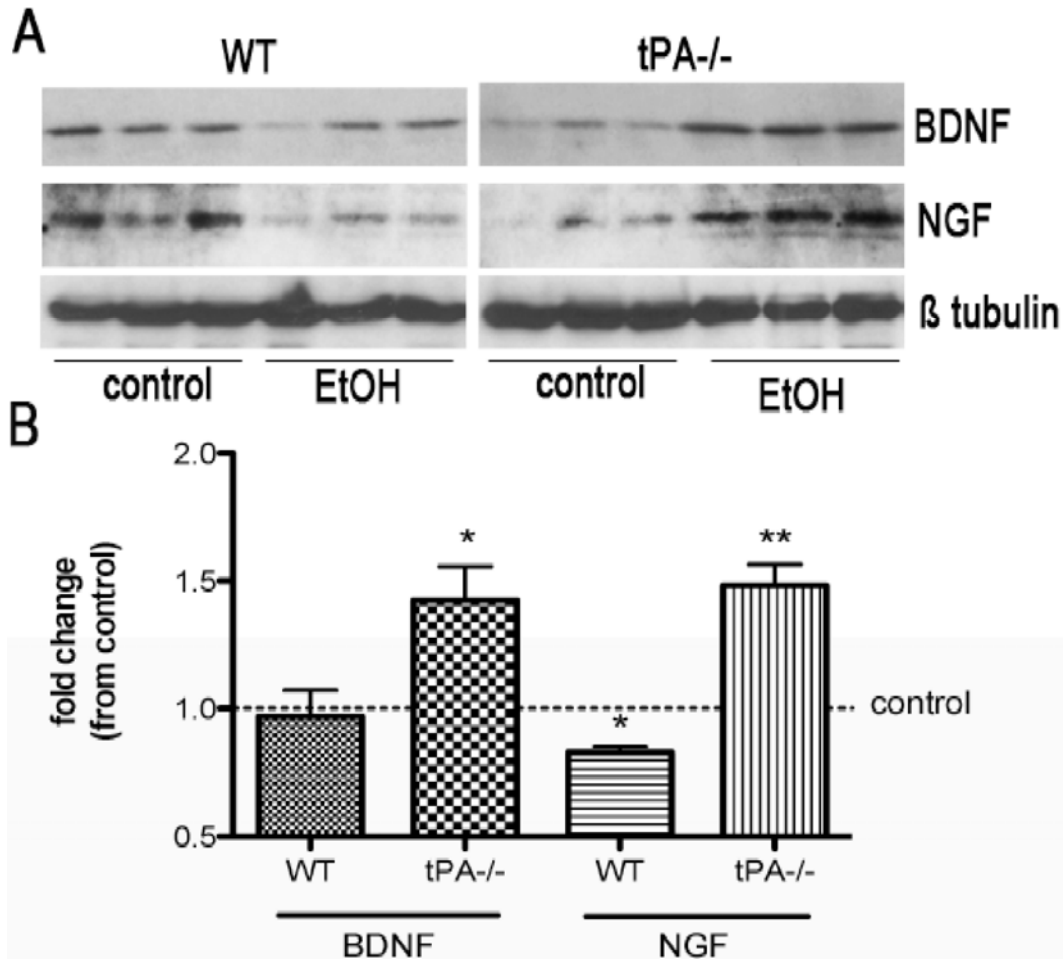
This result is particularly interesting because the blood brain barrier (BBB) does not permit the entrance of macromolecules into the brain and raises the question of whether there might be an activity-independent transport mechanism for tPA. An alternative possibility was that the BBB is incompletely developed in P7 mice facilitating passive transport of S481A tPA (Risau et al, 1994; Xu et al, 1993; Butt et al, 1990). To determine which of these possibilities was the case, BBB permeability was examined in P7 and P90 mice that were injected transcardially with Evans Blue, a dye with high affinity for albumin, which does not cross the mature BBB under physiologic conditions. Fifteen minutes after Evans Blue injection, mice were perfused with PBS, their brains collected and sectioned in a freezing cryostat, and the sections examined by fluorescent microscopy. Evans Blue dye was observed in the brain parenchyma of the P7 WT and tPA<sup>-/-</sup> mice (Figure 11A and 11B), but not of the adult P90 mice (Figure 11C and 11D). As expected, the mice injected with saline were not positive for Evans Blue (Figure 11E and 11F). These data suggest the BBB was incomplete at the younger age, and tPA transport into the CNS parenchyma was not the result of a tPA-specific transporter.



**Figure 11. The P7 mouse blood brain barrier is permeable to Evans Blue extravasation.** Since peripherally administered S481A tPA reached the brain parenchyma, we sought to determine the permeability of the blood brain barrier (BBB) in P7 mice using Evans Blue (EB). We observed the presence of EB (red fluorescence) in the ventricle of tPA<sup>-/-</sup> mice (A and B), around the hippocampal formation (C), and around the cortex (D). There was no EB fluorescence in the untreated P7 tPA<sup>-/-</sup> ventricle (E). Adult tPA<sup>-/-</sup> treated with EB did not show its presence in the cortical area (F). (Blue is DAPI staining)

### **3.5 tPA promotes neurodegeneration by downregulating neurotrophic factor expression following ethanol ingestion**

Brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) promote neuronal growth and survival through activation of the TrkB receptor, and are of particular importance during early developmental stages of synapse formation (for reviews see Luikart and Parada, 2006; Nagappan and Lu, 2005). tPA can convert pro-BDNF and pro-NGF to their mature forms through the activation of the extracellular protease plasmin (Pang et al, 2004; Bruno and Cuello, 2006). We observed ethanol treatment significantly increased the protein levels of both NGF 1.5-fold and BDNF 1.5-fold in the tPA-deficient pups 24 hours after treatment (Figure 12). The effect of was opposite in WT mice, ethanol decreased NGF and had no effect on BDNF (Figure 12). Interestingly, WT pups showed higher baseline protein levels of NGF than tPA<sup>-/-</sup> (Figure 12). These data suggest that prolonged and elevated levels of tPA activity in the WT brain after ethanol might lead to inappropriate processing of pro-BDNF and pro-NGF, perhaps leading to their degradation. However, since tPA-mediated neurotrophic maturation requires plasmin, and Plg<sup>-/-</sup> mice show a phenotype after ethanol that resembles WT, it is more likely the changes in BDNF and NGF observed are due to proteolysis-independent effects of tPA.



**Figure 12. Ethanol increases forebrain neurotrophic factor expression in the absence of tPA.** WT and tPA<sup>-/-</sup> P7 mice were treated with ethanol or saline and sacrificed 24 hrs later. The brains were removed and the forebrains homogenized and analyzed by western blot for BDNF and NGF levels (A). Baseline levels of BDNF were similar in both genotypes, while NGF was initially higher in the WT than in the tPA<sup>-/-</sup> (A). Ethanol significantly increased both BDNF and NGF in the tPA<sup>-/-</sup>, but not the WT (B). The effect was stronger for NGF (\*\*,  $P < 0.001$ ) than BDNF (\*,  $P < 0.05$ ) in the tPA<sup>-/-</sup> brain. BDNF remained the same after ethanol in the WT, and NGF was downregulated below the baseline (\*,  $P < 0.05$ ).

### **3.6 Ethanol does not suppress synaptic activity markers in the tPA<sup>-/-</sup> mice**

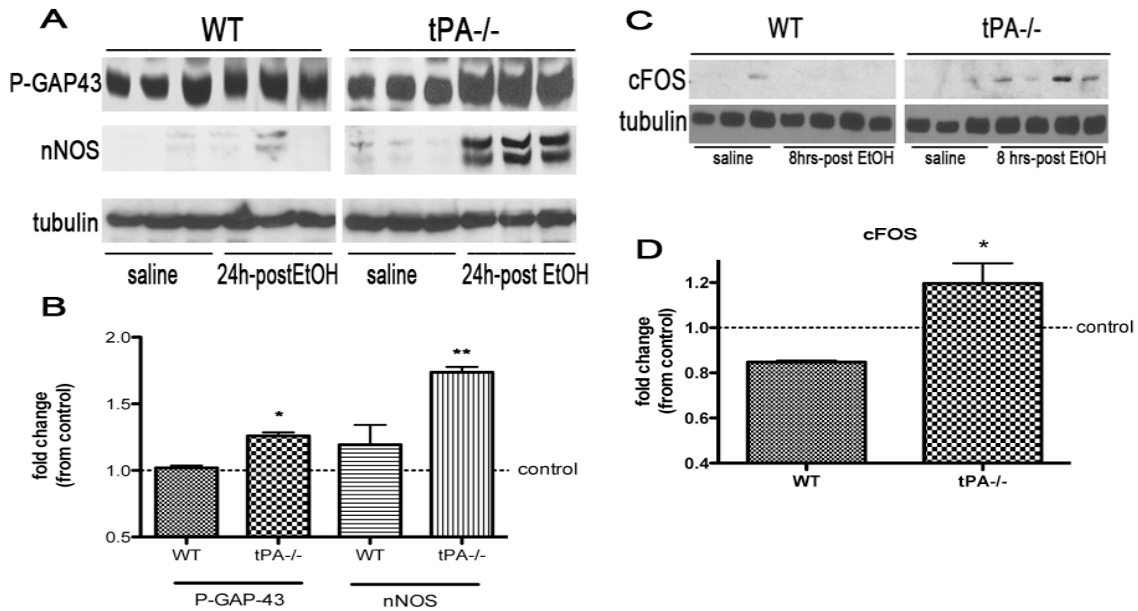
One mechanism by which NGF may protect against neurodegeneration is via induction of nitric oxide (Bonthius et al, 2003). Nitric oxide (NO) has recently been shown to protect against ethanol-induced damage in the developing brain (Bonthius et al, 2002 and 2006) via a cGMP/PKG pathway. NO is produced by neuronal nitric oxide synthase (nNOS) after calcium induced activation of calmodulin. Mice deficient in nNOS exhibited increased vulnerability to ethanol-induced neurodegeneration, particularly in the cingulate cortex and nuclei of the thalamus (Bonthius et al, 2006), which were the brain regions of most interest to us. In our experimental paradigm, nNOS levels were significantly increased in tPA<sup>-/-</sup> mice, but not WT, 24 hrs after ethanol treatment (Figure 13A and 13B). This suggests that there might have been a neuroprotective pathway in the tPA<sup>-/-</sup> mice that included both NGF and nNOS.

The immediate early gene c-fos encodes a transcription factor induced by neuronal activity that is critical in regulating neuronal cell survival. Mice in which c-fos expression is largely eliminated in the hippocampus show decreased BDNF transcription and protein expression, more severe KA-induced seizures, increased neuronal excitability and neuronal death than control mice (Zhang et al, 2002). We found that 8 hours after treatment, when ethanol levels in the circulation are still significantly elevated, the expression of cFOS was



suppressed in the forebrain of WT mice (Figure 13C and 13D). The tPA<sup>-/-</sup> mice showed elevated expression of cFOS after ethanol treatment (Figure 13C and 13D).

Another molecule of interest was the growth and plasticity associated protein 43 (GAP-43), a neuronal protein with a critical role in axon guidance during synaptogenesis, as phosphorylation of GAP-43 on Ser41 by PKC promotes cytoskeletal remodeling (Widmer and Caroni, 1993; Aigner et al., 1995). Phosphorylated GAP-43 (Ser 41) was significantly increased above control levels in the tPA<sup>-/-</sup>, but not in WT, 24 hrs after ethanol treatment (Figure 13A and 13B). However, total levels of GAP-43 remained unchanged in both genotypes and treatment groups (data not shown). These results suggest ethanol is not properly inhibiting synaptic activity in the tPA<sup>-/-</sup> mice, as the neuroprotective properties of these molecules depend on excitatory, calcium-dependent signaling pathways. Consistent with our results, recent research shows NMDA-induced currents were larger in tPA<sup>-/-</sup> cortical neurons than in WT mice (Park et al, 2008), giving support to the idea that tPA regulates the NMDA-R.



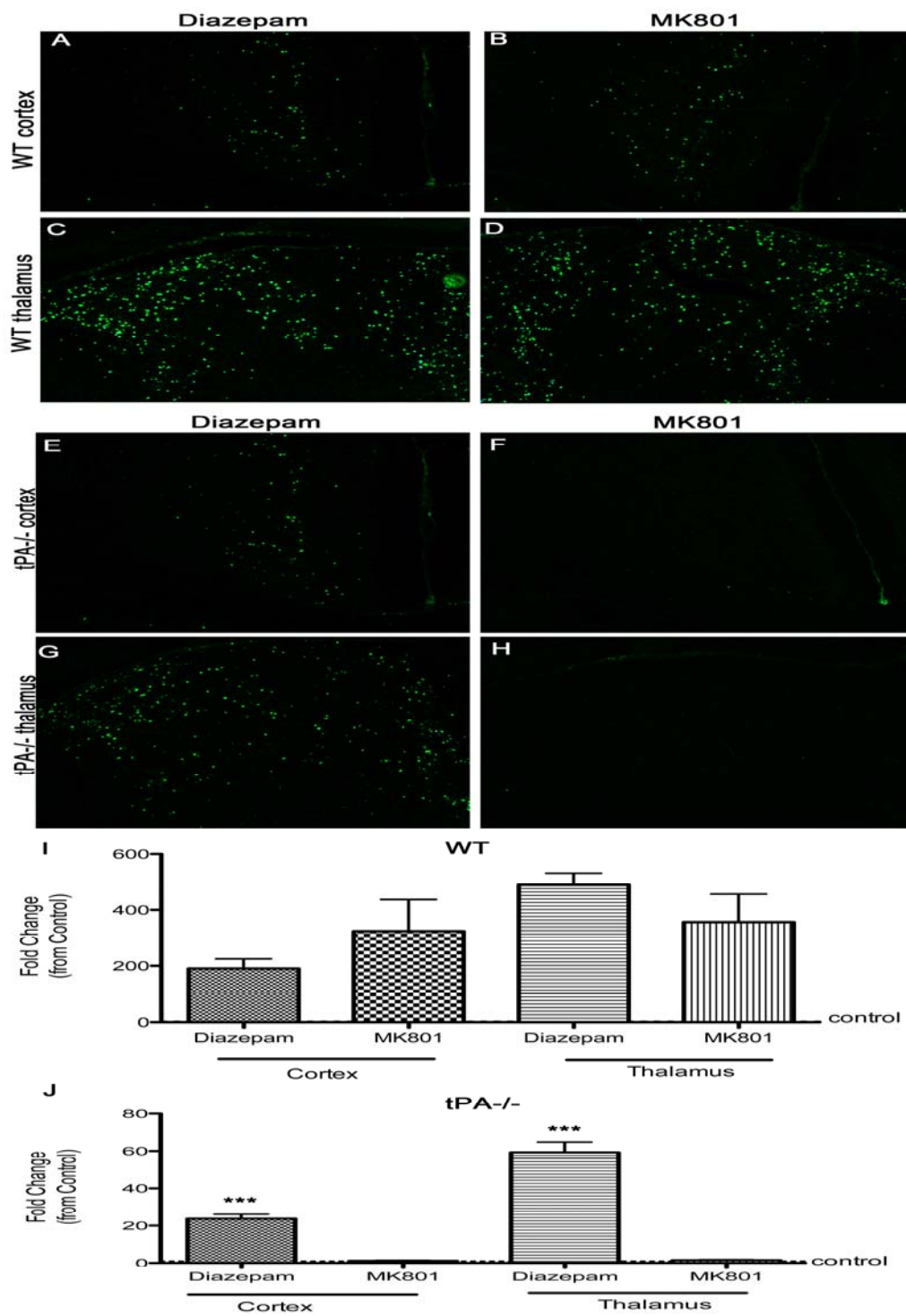
**Figure 13. Ethanol does not suppress synaptic activity markers in the tPA<sup>-/-</sup> mice.** WT and tPA<sup>-/-</sup> P7 mice were treated with ethanol or saline and sacrificed 8 hrs and 24 hrs later. The brains were removed and the forebrains homogenized and analyzed by western blot for cFOS expression 8 hrs after ethanol treatment, and for phospho-GAP-43 and nNOS 24 hrs post-ethanol. Baseline levels of all molecules were similar in both genotypes. Ethanol significantly increased both phospho-GAP-43 and nNOS in the tPA<sup>-/-</sup>, but not the in WT (A and B). The effect was stronger for nNOS (\*\*,  $P < 0.001$ ) than phospho-GAP-43 (\*,  $P < 0.05$ ) in the tPA<sup>-/-</sup> brain. nNOS and phospho-GAP-43 remained the same after ethanol in the WT mouse. cFOS expression was evaluated 8 hrs after ethanol exposure (C). Expression of cFOS was higher in the tPA<sup>-/-</sup> mouse than the WT (D) (\*,  $P < 0.05$ ).

### **3.7 tPA promotes neurodegeneration via the NMDA-R**

Ethanol decreases synaptic activity by a dual mechanism that involves inhibition of the NMDA-R, and potentiation of the GABA<sub>A</sub>-R. To determine which neurotransmitter system mediates tPA's effects after ethanol, we injected WT and tPA<sup>-/-</sup> mice with high doses of either Diazepam (30 mg/kg; GABA<sub>A</sub>-R agonist) or MK801 (1.5mg/kg; non-selective antagonist of NMDA-R) and analyzed neuronal loss 24 hours after injection. As previously reported (Ikonomidou et al, 2000), both Diazepam and MK801 treatment induced extensive neuronal death in the developing WT cortex (Figure 14A, 14B, and 14I), and thalamus (Figure 14C, 14D, and 14I). tPA<sup>-/-</sup> mice showed neuronal death after Diazepam in the cortex (Figure 14E and 14J) and thalamus (Figure 14G and 14J), though to a significantly lesser degree than the WT. tPA<sup>-/-</sup> mice demonstrated neuroprotection after MK801 (Figure 14F, 14H, and 14J), suggesting tPA effects on neurodegeneration are mediated by the NMDA-R.

**Figure 14. tPA promotes neurodegeneration via the NMDA-R.** WT and tPA<sup>-/-</sup> P7 mice were injected with the non-selective NMDA-R antagonist MK801 (1.5mg/kg) or the GABA<sub>A</sub>-R agonist Diazepam (30mg/kg). Control mice were injected with saline as (N = 6/group). 24 hrs after treatment, animals were sacrificed and brain sections analyzed histologically with FJB to visualize neurodegeneration. Diazepam induced extensive neuronal death in the cortex (A) and thalamus (C) of WT when compared to saline-treated mice (\*\*\*, P < 0.0001). Diazepam also induced neurodegeneration in the cortex (E) and thalamus (G) of tPA<sup>-/-</sup> mice, albeit to a significantly lesser degree than WT mice. WT mice were also vulnerable to NMDA-R-induced neurodegeneration since MK801 treatment resulted in increased FJB-positive staining in the cortex (B) and thalamus (D) (\*\*\*, P < 0.0001). tPA<sup>-/-</sup> mice, however, were significantly protected from NMDA-R-mediated neuronal death since MK801 did not increase FJB staining in the cortex (F) or thalamus (H) of these mice (P > 0.05).

Figure 14

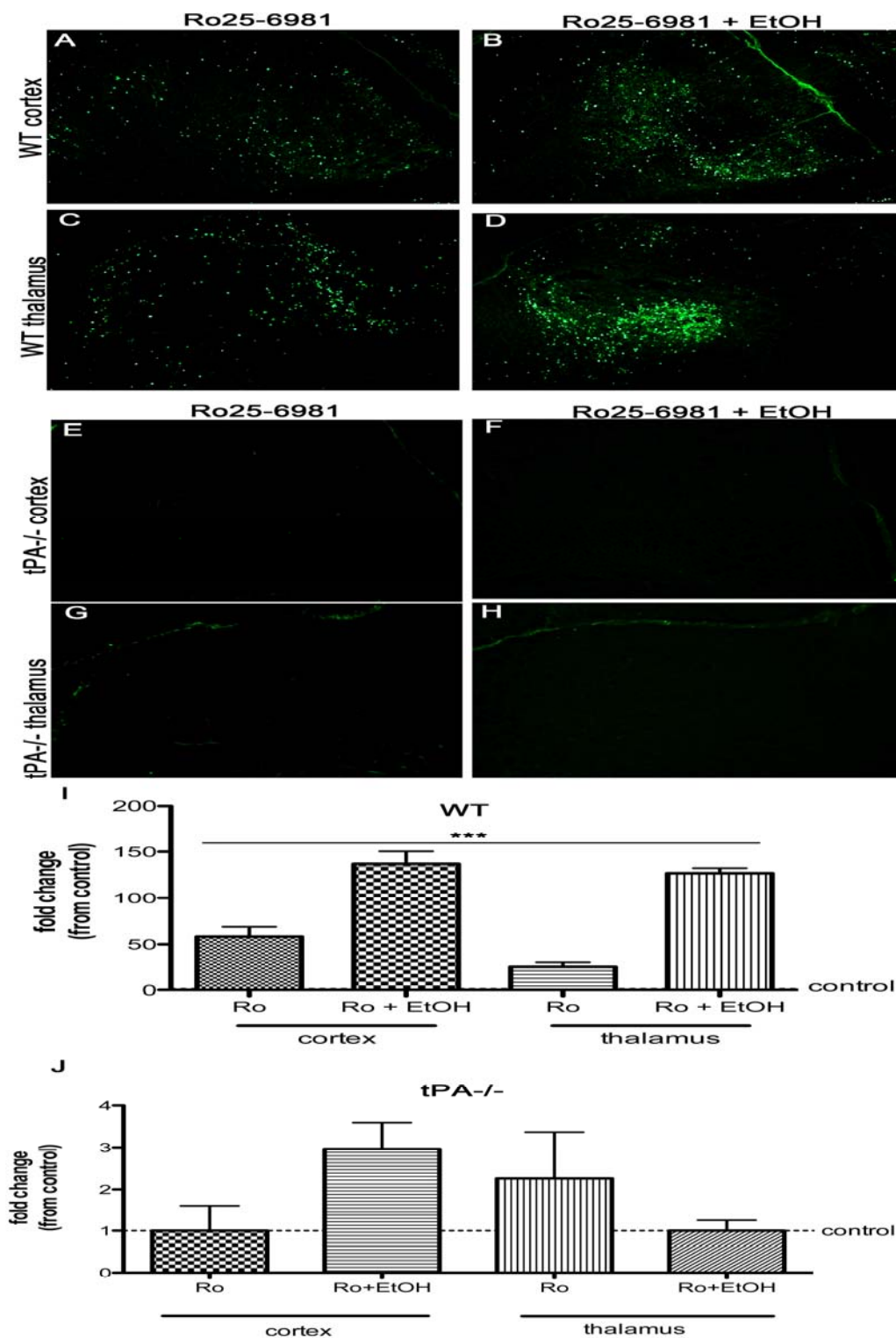


Since NR2B is the prominent NR2 subunit during synaptogenesis, and results from prior studies suggest that tPA can interact with NR2B in the adult brain (Pawlak et al, 2005; Norris and Strickland, 2007), we examined neurodegeneration after treatment with the NR2B-specific antagonist Ro25-6981 (6mg/kg) in WT and tPA<sup>-/-</sup> P7 pups. Neurodegeneration was observed in the cortex (Figure 15A and 15I) and thalamus (Figure 15C and 15I) of WT mice after drug treatment. tPA<sup>-/-</sup> mice were protected from Ro25-6981 neurodegeneration (Figure 15E, 15G, 15J). We wondered if a combination of Ro25-6981 and ethanol would augment neurodegeneration in the WT mice. Pups were treated with Ro25-6981 (6mg/kg) 30 minutes prior to treatment with ethanol. Ethanol potentiated Ro25-6981-induced neurodegeneration in the cortex (Figure 15B and 15I) and thalamus (Figure 15D and 15I). Next, we administered the same combination of treatments to the tPA<sup>-/-</sup> pups as an attempt to potentiate inhibition of the NMDA-R neurotransmitter system in these mice. tPA<sup>-/-</sup> mice were still protected from neurodegeneration even in these conditions (Figure 15F, 15H, and 15J), suggesting tPA promotes neurodegeneration via an NR2B-dependent mechanism.

**Figure 15. tPA<sup>-/-</sup> mice are resistant to NR2B-mediated neuronal death.**

WT and tPA<sup>-/-</sup> P7 mice were injected with either a NR2B selective NMDA-R antagonist Ro25-6981 (6mg/kg), a combination of Ro25-6981 (6mg/kg) administered 30 mins prior to acute ethanol binge treatment (2.5g/kg at 0hr and 2hr). Control mice were injected with saline as (N = 5/group). 24 hrs after treatment, animals were sacrificed and brain sections analyzed histologically with FJB to visualize neurodegeneration. Ro25-6981 induced extensive neuronal death in the cortex (A) and thalamus (C) of WT when compared to saline-treated mice (\*\*\*,  $P < 0.0001$ ). When Ro25-6981 was administered with ethanol neurodegeneration was increased in the cortex (B) and thalamus (D) (\*\*\*,  $P < 0.0001$ ). tPA<sup>-/-</sup> mice, however, were protected from NMDA-R-mediated neuronal death after administration of Ro25-6981 on its own (E and G) or in combination with ethanol (F and H).

Figure 15



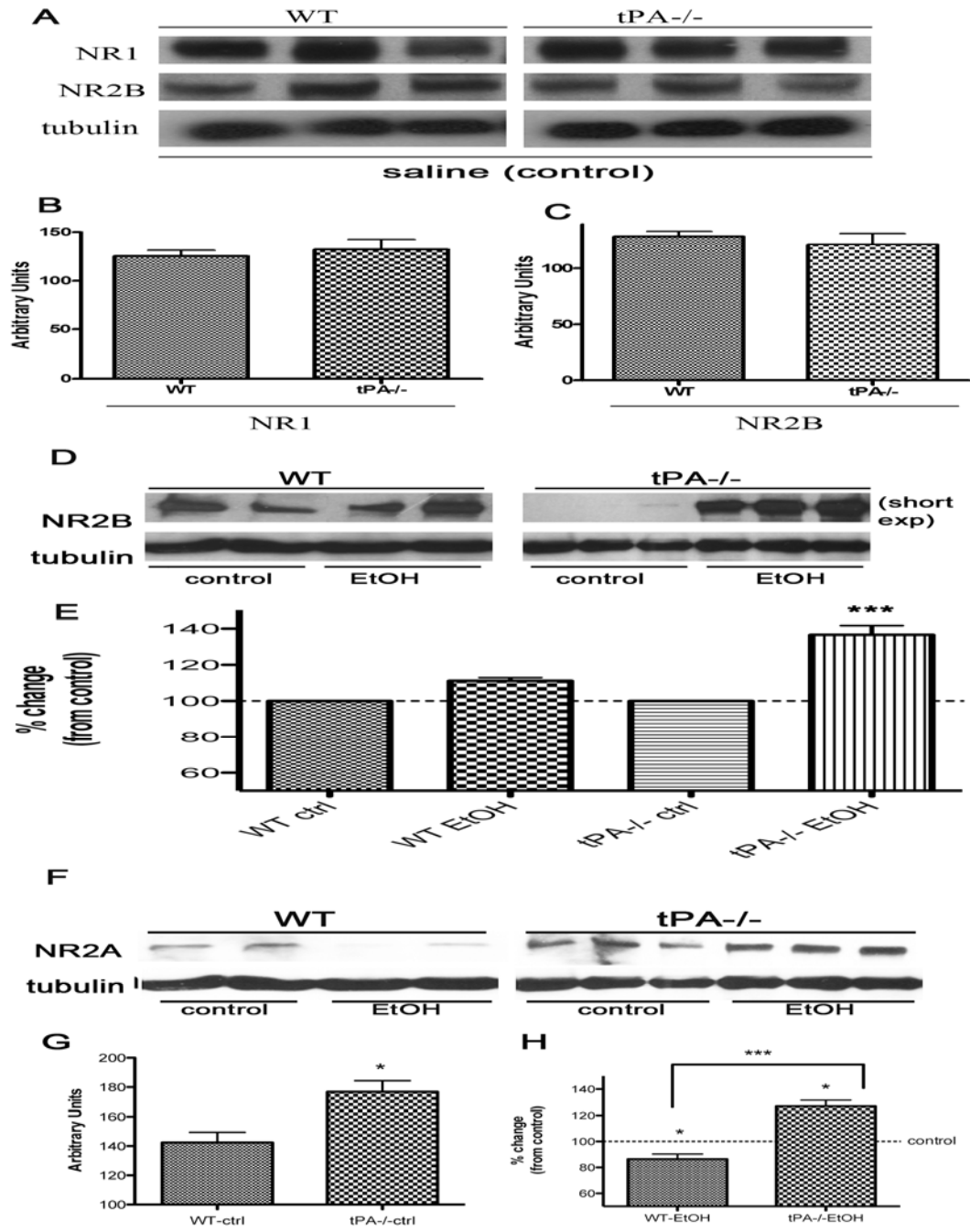


### **3.8 Ethanol differentially modulates the NMDA-R in WT and tPA<sup>-/-</sup> mice**

Since tPA<sup>-/-</sup> mice were protected from NMDA-R-mediated neurodegeneration, we determined the expression levels of individual NMDA-R subunits in tPA<sup>-/-</sup> and WT P7 pups before and after ethanol treatment. Western blot analysis of forebrain homogenates showed no differences in NR1 expression between genotypes before and after ethanol (Figure 16A and 16B). NR2B baseline levels were also similar in tPA<sup>-/-</sup> and WT early postnatal aged 7 mice. (Figure 16A and 16C). However, NR2B was upregulated 24 hours after ethanol-treatment in tPA<sup>-/-</sup> mice, but not wild type mice (Figure 16D and 16E). Baseline NR2A levels were higher in the tPA<sup>-/-</sup> brain than in the WT (Figure 16F and 16G), and ethanol treatment further augmented NR2A expression in the tPA<sup>-/-</sup> 24 hrs after treatment (Figure 16F and 16H). It has been postulated that NR2A and NR2B can have different and opposing roles in the adult brain. NR2A has been shown to activate survival-signaling pathways, whereas NR2B activates cell death pathways (Liu et al, 2007). It might be possible then that at P7, enhanced baseline NR2A expression, and subsequent ethanol-induced increase in NR2B in the neonatal tPA<sup>-/-</sup> brain, may be part of the observed neuroprotection from NMDA-R mediated antagonism.

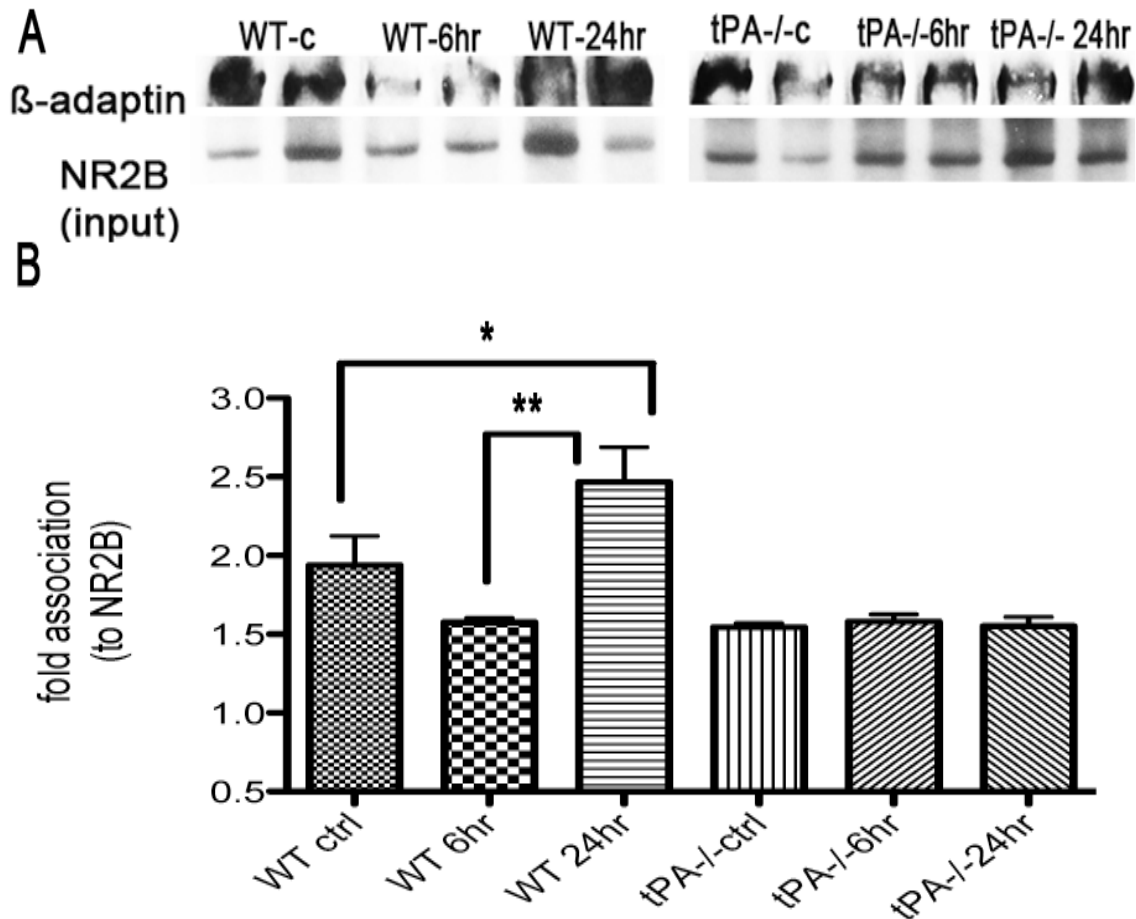
**Figure 16. Ethanol differentially modulates the NMDA-R in WT and tPA<sup>-/-</sup> mice.** WT and tPA<sup>-/-</sup> P7 mice were injected with either ethanol or saline, and the forebrain NMDA-R subunit composition analyzed by western blot. WT and tPA<sup>-/-</sup> have no baseline differences in total NR1 or NR2B (A, B, and C;  $P > 0.05$ ). However, 24 hours after ethanol administration, NR2B was significantly increased above the baseline in the tPA<sup>-/-</sup> but not WT forebrain (D and E; \*\*\*,  $P < 0.0001$ ). We found NR2A baseline levels were higher in the tPA<sup>-/-</sup> than in the WT (G; \*,  $P < 0.05$ ), and ethanol further increased NR2A in tPA<sup>-/-</sup> 24 hours after ethanol (F and H; \*,  $P < 0.05$ ). While ethanol increased NR2A in the tPA<sup>-/-</sup>, it decreased the subunit expression in the WT mice (H; \*,  $P < 0.05$ ). The effect of ethanol on NR2A was contingent on the genotype of the animals (H; \*\*\*,  $P < 0.0001$ ).

**Figure 16**



There are multiple mechanisms of NMDA-R regulation. The NR2B subunit contains an adaptor protein complex (AP-2) binding site in its C-terminal domain. AP-2 is a component of endocytic clathrin coated pits which regulate cell surface expression of NR2B (Prybylowski et al, 2005). Binding of AP-2 to NR2B is inversely related to the phosphorylation state of Y1472 residue by Src kinases (Roche et al, 2001; Yaka et al, 2002). Therefore phosphorylation at this residue leads to decreased AP-2 binding and enhanced NR2B surface expression. We explored the association of AP-2 to NR2B by immunoprecipitating  $\beta$ -adaptin, a component of the AP-2 complex, with an NR2B antibody. We studied WT and tPA<sup>-/-</sup> mice before and after 6hrs and 24 hrs of ethanol exposure. We found that the association of  $\beta$ -adaptin with NR2B was unchanged from control levels in the tPA<sup>-/-</sup> brain at any of the studied timepoints after ethanol (Figure 17). WT mice however showed reduced association of  $\beta$ -adaptin 6 hrs after ethanol (Figure 17). However, a significant enhancement of  $\beta$ -adaptin association to NR2B was observed 24 hrs after ethanol treatment (Figure 17). These findings suggest that ethanol's blockade of the NMDA-R led to increased receptor internalization, possibly leading to impaired synaptogenesis and neurodegeneration in the WT brain. The decreased association of  $\beta$ -adaptin 6 hrs after ethanol in the WT mice could be due to a homeostatic mechanism induced to counterbalance inhibition of NR2B

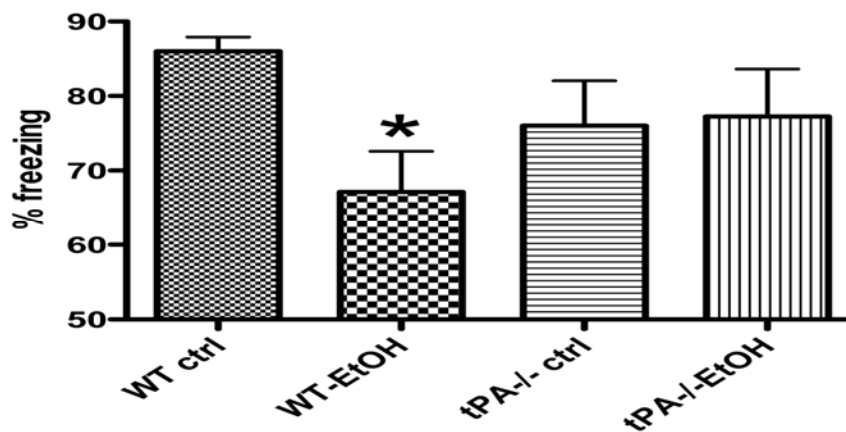
in the presence of ethanol. However, the reduced association observed at 24 hrs is consistent with the decrease in circulating levels of ethanol and concomitant with neurodegeneration.  $tPA^{-/-}$  mice, however, did not show any changes in  $\beta$ -adaptin association to NR2B after ethanol (Figure 17), suggesting the stability of the NMDA receptor complex was not affected by ethanol, and as a result the establishment of new synaptic connections and cell survival processes was not hindered.



**Figure 17. Ethanol modulates  $\beta$ -adaptin association to NR2B in WT, but not  $tPA^{-/-}$  mice.** WT and  $tPA^{-/-}$  P7 mice were injected with ethanol or saline and sacrificed 24 hours after treatment. Forebrain homogenates were immunoprecipitated with an anti-NR2B antibody, and subsequently probed for  $\beta$ -adaptin to investigate whether ethanol affects NR2B subunit internalization. We found ethanol significantly decreases the association of  $\beta$ -adaptin to NR2B 6 hours post administration (B; \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ ) but this association was increased above the baseline 24 hours later.  $\beta$ -adaptin association to NR2B in the  $tPA^{-/-}$  was unchanged at any time-point after ethanol treatment.

### **3.9 tPA-deficient mice were protected from cognitive defects induced by early postnatal ethanol treatment**

We evaluated the cognitive ability of adult mice (8-10 weeks of age) that were exposed to ethanol or saline at P7. We found, consistent with previous reports (Ieraci and Herrera, 2005), that WT mice that had undergone ethanol treatment at P7 had approximately a 20% reduction in contextual fear conditioning compared to the saline treatment group (Figure 18). In contrast, tPA<sup>-/-</sup> mice that received ethanol at the same age did not show impairments when compared to their saline-treated counterparts (Figure 18). Interestingly, there is a baseline difference between WT and tPA<sup>-/-</sup> mice, since saline-treated tPA<sup>-/-</sup> animals exhibited slightly decreased freezing (i.e increased locomotor activity) during the contextual test when compared to WT saline-treated mice. But this reduction in freezing between WT and tPA<sup>-/-</sup> saline treated animals did not reflect a lack of learning of the paradigm in the tPA<sup>-/-</sup> mice since contextual freezing for these mice was significantly greater than that observed during training. This suggests that exposure of the immature brain to a binge-like dose of ethanol has long-term effects on the brain and as a result, on behavior. These effects are commensurate to the vulnerability of the immature brain to ethanol-induced neurodegeneration, and support a role for tPA as a regulator of ethanol-induced neurodegeneration and FAS development.



**Figure 18. Neonatal exposure to ethanol induces learning and memory deficits in WT, but not tPA<sup>-/-</sup> mice.** WT and tPA<sup>-/-</sup> P60-90 that had been treated with ethanol or saline at P7 were tested on the fear conditioning paradigm. WT mice that had been treated with ethanol at P7 showed a decrease in fear conditioning as observed by a 20% reduction in freezing behavior when compared to WT mice that had been exposed to saline (\*,  $P < 0.05$ ). Ethanol did not induce differences in the adult tPA<sup>-/-</sup> mice, since ethanol- and saline-treated mice showed similar freezing behavior.



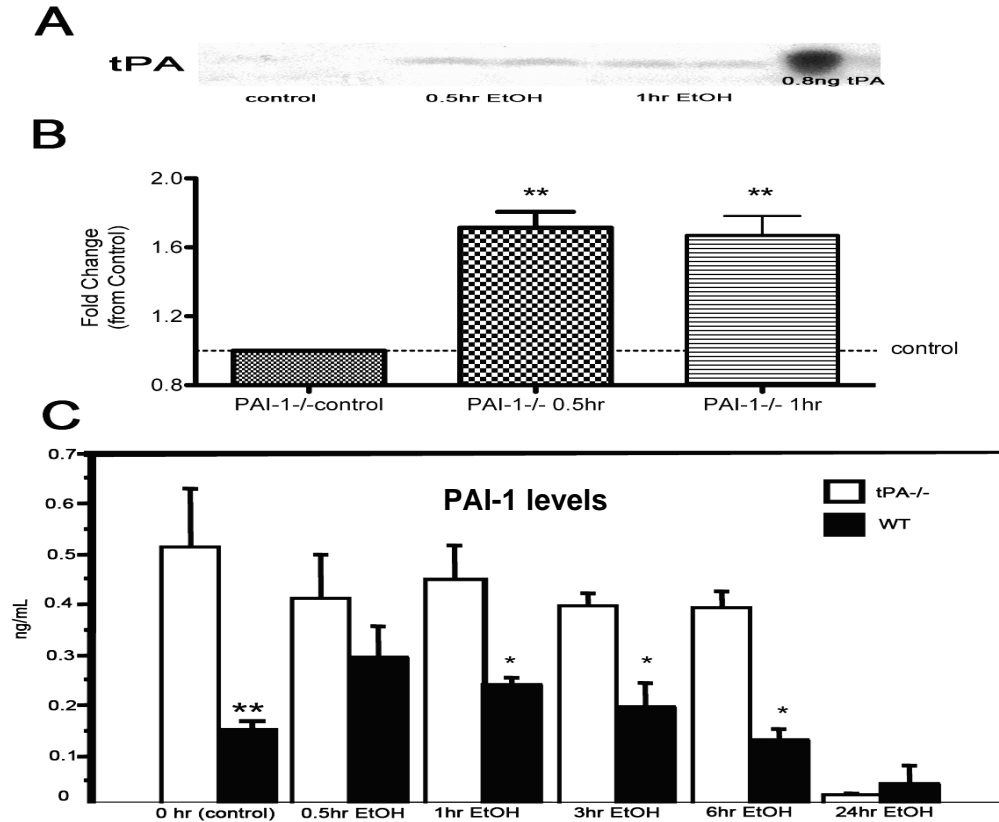
### **3.10 Ethanol treatment increased neuronal-associated tPA activity in perinatal PAI-1<sup>-/-</sup> mouse pups**

Since PAI-1, the main inhibitor for tPA in the brain, was regulated by ethanol in WT mice we decided to investigate what role, if any, PAI-1 plays in ethanol-induced neuronal death. We subjected WT, tPA<sup>-/-</sup>, and PAI-1<sup>-/-</sup> postnatal age 7 to the acute ethanol binge paradigm that serves as a model for FAS in mice. Mice were injected as described earlier with ethanol at a dose of 2.5g/kg at 0 and 2 hrs. Control mice were injected with equal volumes of saline. We sacrificed mice at different time-points after ethanol treatment, and visualized tPA activity in PAI-1<sup>-/-</sup> forebrain homogenates using in-gel zymography. We found tPA activity was significantly increased over 1.5-fold 30 mins and 1 hr (Figure 19A and 19B) after ethanol. However, contrary to the long-lasting tPA increase observed in WT mice, the increase in activity was reduced back to the baseline in the PAI-1<sup>-/-</sup> mice 8 hrs after ethanol, and continued to be decreased at 24 hours (data not shown).

### **3.11 Ethanol modulates PAI-1 in a tPA-independent manner**

Since PAI-1 levels were regulated by ethanol in the WT mice, we were then interested in examining PAI-1 in the tPA<sup>-/-</sup> mice using an enzyme-linked immunosorbent assay (ELISA). We were surprised to find out that

tPA<sup>-/-</sup> mice have significantly higher baseline levels of PAI-1 than WT (Figure 19C). Contrary to what was observed in the WT brain, ethanol treatment did not enhance PAI-1 levels further in the tPA<sup>-/-</sup>. However, 24 hours after ethanol, PAI-1 was downregulated below the baseline for both WT and tPA<sup>-/-</sup> mice, suggesting ethanol-induced modulation of PAI-1 occurs independently of tPA. The results also raise the possibility that PAI-1 might be involved in the ethanol response in the tPA<sup>-/-</sup> mice, possibly as a source of neuroprotection.

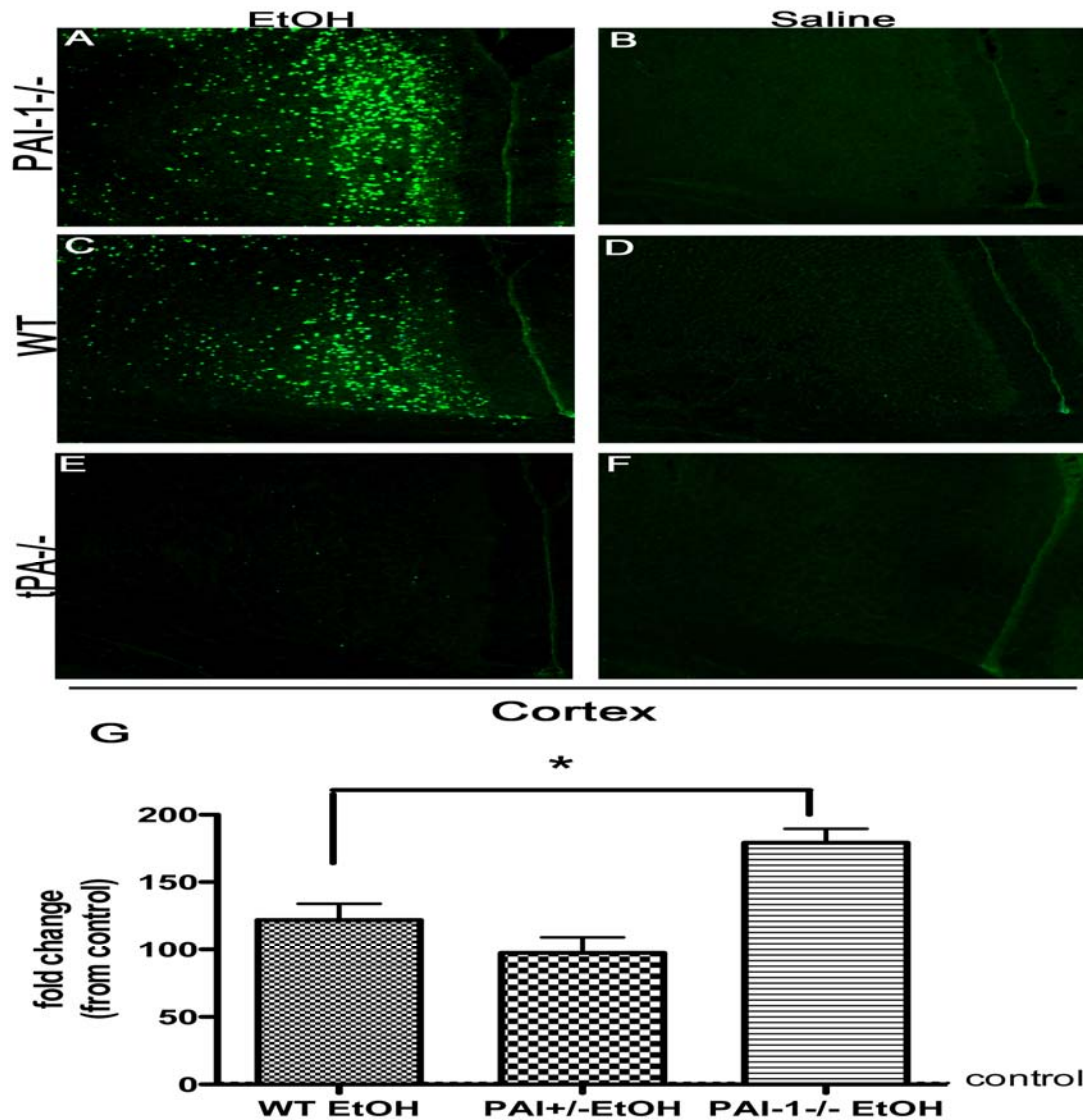


**Figure 19. Ethanol treatment increased tPA activity in perinatal PAI-1<sup>-/-</sup> mouse pups, and modulated PAI-1 in a tPA-independent manner.** WT, tPA<sup>-/-</sup>, and PAI-1<sup>-/-</sup> P7 pups were injected with ethanol or saline as a control. In-gel zymography revealed tPA activity is significantly increased in the PAI-1<sup>-/-</sup> mice 0.5hr and 1 hr after ethanol exposure (A and B; \*\*, P < 0.001). ELISA of WT and tPA<sup>-/-</sup> homogenates showed baseline PAI-1 levels are significantly higher in the tPA<sup>-/-</sup> when compared to WT mice (C; \*\*, P < 0.001). PAI-1 levels were decreased below the baseline for both WT and tPA<sup>-/-</sup> 24 hrs after ethanol, indicating ethanol-mediated modulation of PAI-1 occurs independently of tPA.

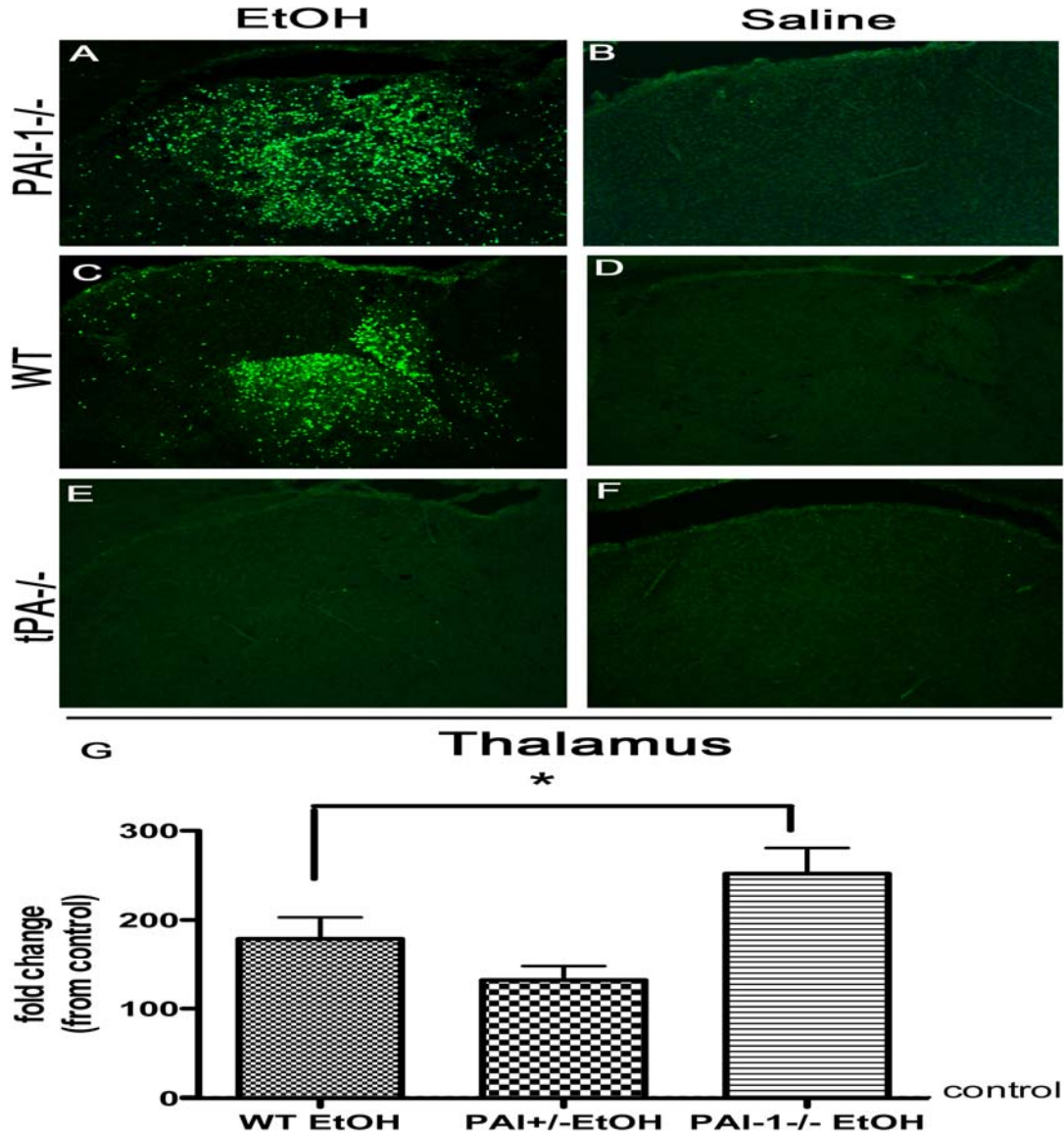
### **3.12 PAI-1-deficient pups are more vulnerable to ethanol-induced neurodegeneration**

Since baseline levels of PAI-1 were higher in the tPA<sup>-/-</sup> animals, we hypothesized PAI-1-deficient mice might be more vulnerable to ethanol-induced neurodegeneration. We found that 24 hours after ethanol administration PAI-1<sup>-/-</sup> mice showed enhanced neuronal death than WT mice, as observed by increased FJB staining. PAI-1<sup>-/-</sup> showed neurodegeneration most prominently in the cerebral cortex (Figure 20A), and in the thalamus (Figure 21A). Neurodegeneration in the cortex of PAI-1<sup>-/-</sup> mice was close to 100-fold greater in the cortex (Figure 20G), and over a 100-fold in the thalamus (Figure 21G) of PAI-1<sup>-/-</sup> mice when compared to WT. PAI<sup>+/-</sup> mice showed neurodegeneration similar to WT mice (Figure 20G and 21G). It was possible that the difference in neurodegeneration in PAI-1<sup>-/-</sup> and WT could have been an artifact of differing blood ethanol concentrations, perhaps reflective of underlying metabolic differences between the two genotypes, and not to a primary difference in neuronal physiology. To address this possibility, we determined the blood ethanol concentrations following treatment. We found no difference in blood ethanol concentrations between PAI-1<sup>-/-</sup> and WT mice (Figure 22) at any timepoint studied. This result verifies that the observed difference in the

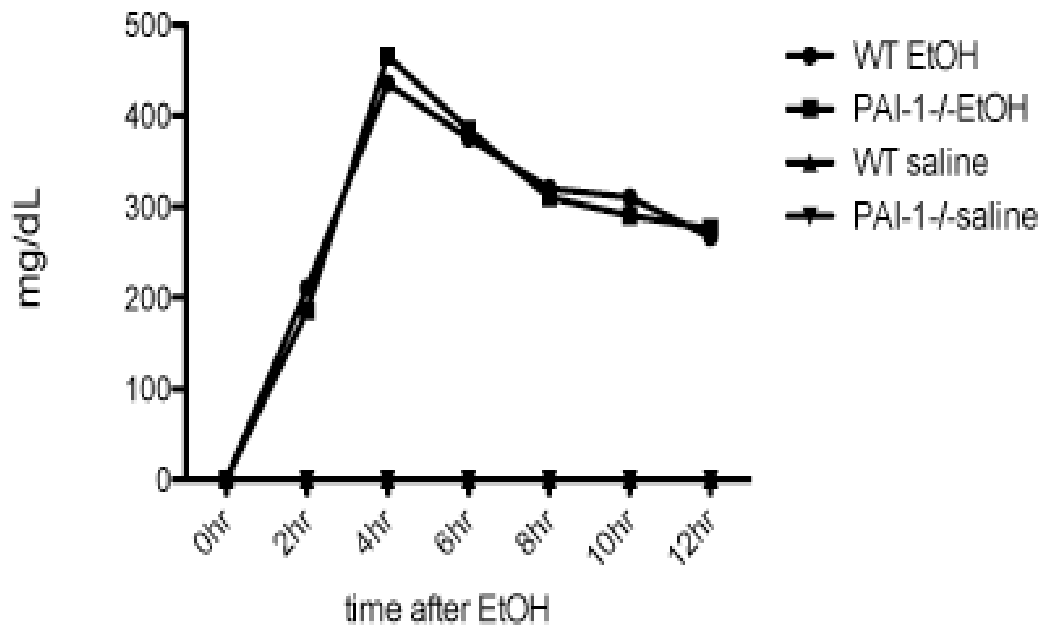
severity of neurodegeneration in the PAI-1<sup>-/-</sup> mice was not due to differences in the rate of ethanol metabolism between mouse genotypes. These data show that lack of PAI-1 exacerbates the neurotoxic effects of ethanol, and provides support to the hypothesis that PAI-1 promotes neuronal survival after ethanol exposure.



**Figure 20. PAI-1-deficient pups were more vulnerable to ethanol-induced neurodegeneration in the cortex.** WT, tPA<sup>-/-</sup>, and PAI-1<sup>-/-</sup> P7 pups were injected with ethanol or saline, and sacrificed 24 hrs later to determine neuronal death using FJB staining. PAI-1<sup>-/-</sup> mice showed increased neurodegeneration in the cortex when compared to WT treated mice (A and C; \*,  $P < 0.05$ ).



**Figure 21. PAI-1-deficient pups were more vulnerable to ethanol-induced neurodegeneration in the thalamus.** WT, tPA<sup>-/-</sup>, and PAI-1<sup>-/-</sup> P7 pups were injected with ethanol or saline, and sacrificed 24 hrs later to determine neuronal death using FJB staining. PAI-1<sup>-/-</sup> mice showed increased neurodegeneration in the cortex when compared to WT treated mice (A and C; \*, P < 0.05).



**Figure 22. Blood ethanol concentrations are similar in WT and PAI-1<sup>-/-</sup>.**

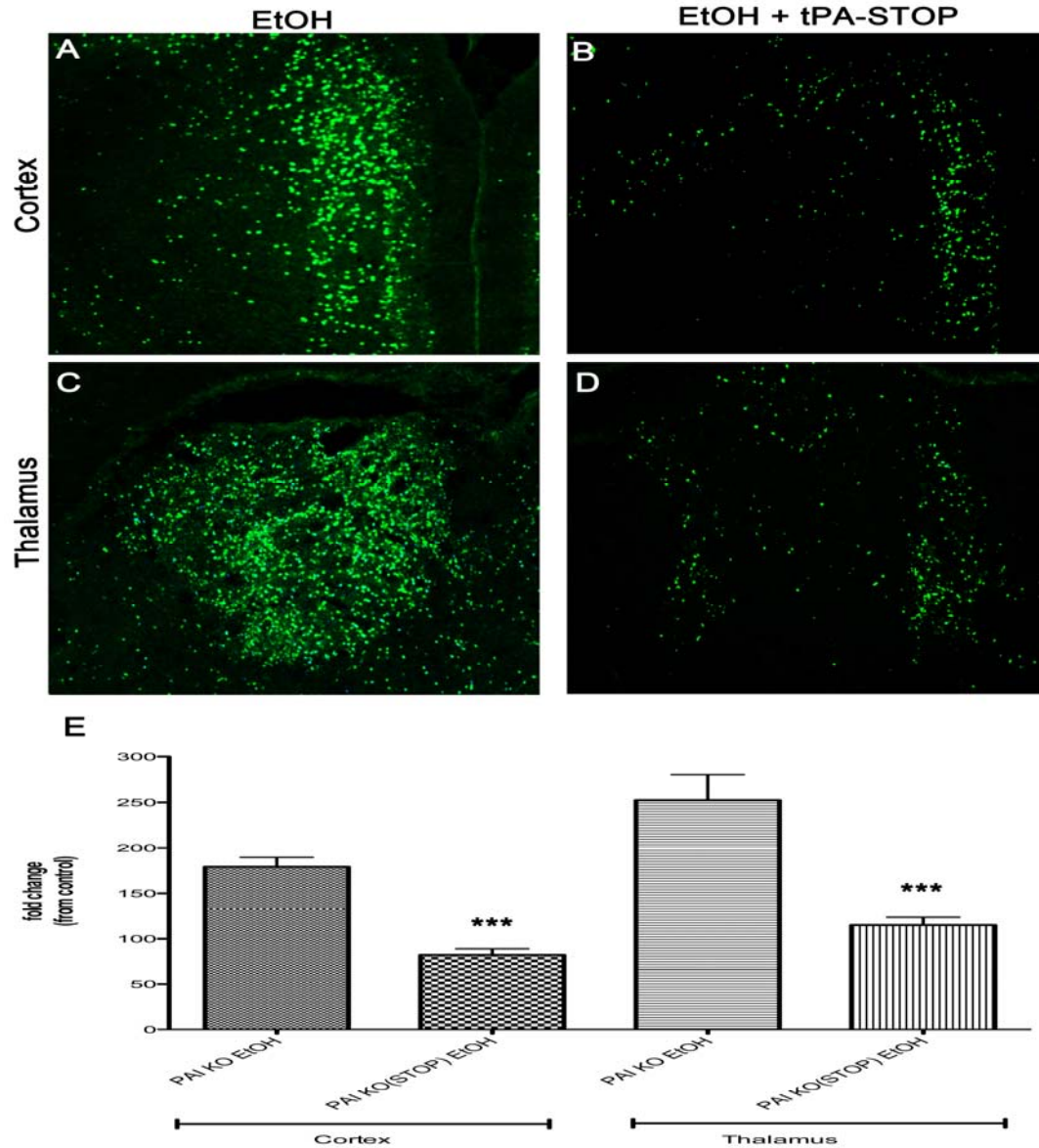
WT and PAI-1<sup>-/-</sup> P7 pups were injected with ethanol or saline as a control (N=5/group). Mice were sacrificed at 0hr after treatment, and every 2 hrs for the remaining 12 hrs after injection, trunk blood was collected, and ethanol concentrations were obtained from plasma. There were no differences in ethanol levels between WT and PAI-1<sup>-/-</sup>, suggesting the rate of metabolism was similar in both genotypes.

### 3.13 tPA promotes ethanol-induced neurodegeneration in PAI-1<sup>-/-</sup> mice

To determine whether the increased tPA activity seen in the PAI-1<sup>-/-</sup> brain following ethanol treatment was associated with neuronal loss, we injected i.p. PAI-1<sup>-/-</sup> pups with the small molecular weight inhibitor tPA-STOP (10ug/g) 30 minutes before ethanol treatment. We have previously shown



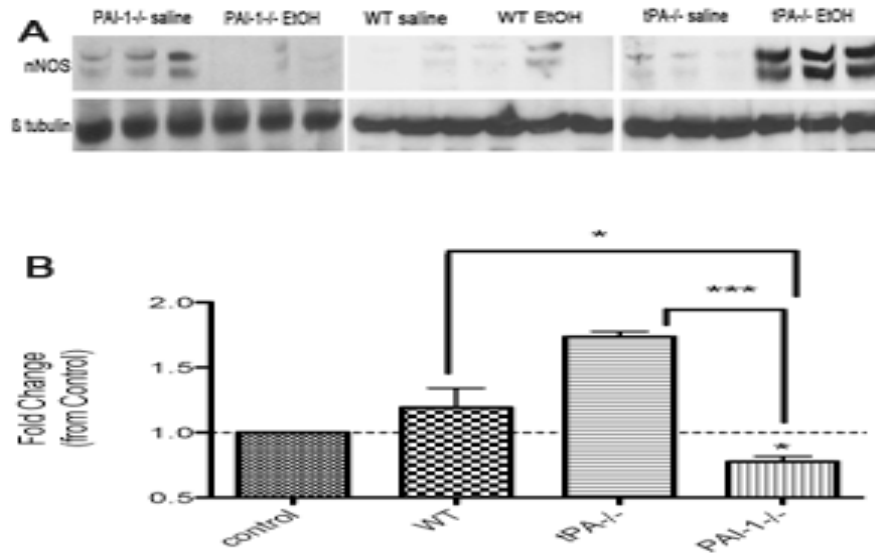
this drug can significantly decrease tPA activity both in vitro and in vivo (Figure 7I and 7K), and consequently attenuated ethanol-induced neurodegeneration in WT mice (Figure 7). PAI-1<sup>-/-</sup> mice pretreated with tPA-STOP showed a significant attenuation of neurodegeneration after ethanol as observed by decreased FJB staining (Figure 23). The number of FJB-positive cells after ethanol was reduced ~100-fold, in both the cortex (Figure 23B and 23E) and the thalamus (Figure 23D and E), in comparison to PAI-1<sup>-/-</sup> animals treated only with ethanol (Figure 23A and 23C). These findings indicate that tPA promotes ethanol-induced neurodegeneration in the PAI-1<sup>-/-</sup> brain.



**Figure 23. tPA promotes ethanol-induced neurodegeneration in PAI-1<sup>-/-</sup> mice.** PAI-1-deficient P7 pups were administered different treatments: tPA-STOP (10ug/g) 30 mins prior to acute binge ethanol, tPA-STOP alone, acute binge ethanol alone, or saline control. Pre-treatment with tPA-STOP significantly attenuated ethanol-induced neurodegeneration as observed by decrease FJB staining in both the cortex (B) and thalamus (D), when compared to non-tPA STOP-treated ethanol exposed PAI-1<sup>-/-</sup> mice (A and C; \*\*\*,  $P < 0.0001$ ).

### **3.14 Ethanol decreased nNOS expression in PAI-1<sup>-/-</sup> mice**

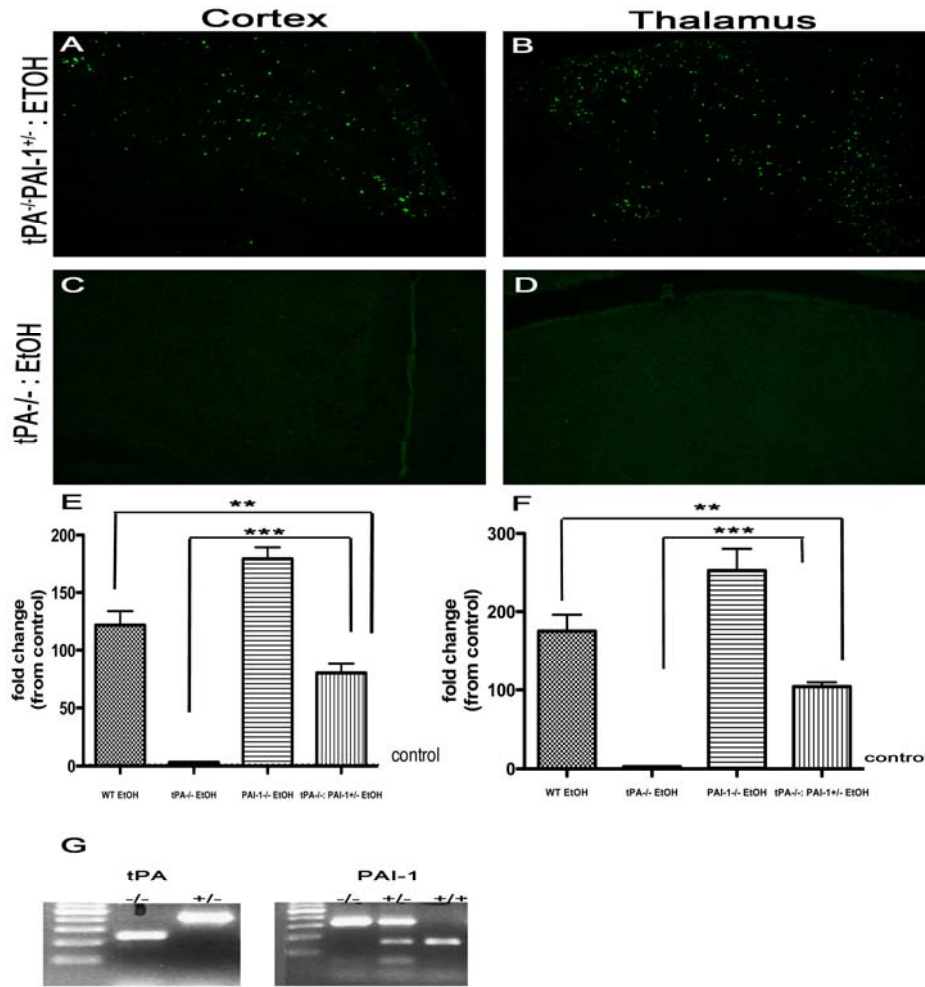
NO is produced by neuronal nitric oxide synthase (nNOS) after calcium induced activation of calmodulin. Mice deficient in nNOS exhibited increased vulnerability to ethanol-induced neurodegeneration, particularly in the cingulate cortex and nuclei of the thalamus (Bonthius et al, 2006). Since ethanol increased nNOS expression in the tPA<sup>-/-</sup> mice suggesting it might be part of a survival pathway that protects these mice from ethanol-induced neurotoxicity, we evaluated nNOS levels in the PAI-1<sup>-/-</sup> brain after ethanol treatment. Western blot analysis showed ethanol decreased below baseline levels nNOS expression 24 hours after treatment in PAI-1<sup>-/-</sup> mice (Figure 24). The ethanol-induced reduction in nNOS in the PAI-1<sup>-/-</sup> mice was significant when compared to WT and tPA<sup>-/-</sup> treated with ethanol (Figure 24). These results are consistent with the pro-survival role that has been described for nNOS activity after ethanol exposure of the immature brain, and concomitant with our neurodegeneration findings.



**Figure 24. Ethanol decreased nNOS expression in PAI-1<sup>-/-</sup> mice.** Since ethanol increased nNOS expression in the tPA<sup>-/-</sup> mice suggesting it might be part of a survival pathway that protects these mice from ethanol-induced neurotoxicity. We evaluated nNOS levels in the PAI-1<sup>-/-</sup> P7 mice after ethanol treatment (N=4). Western blot analysis showed ethanol decreased below baseline levels nNOS expression 24 hours after treatment in PAI-1<sup>-/-</sup> mice (A). The ethanol-induced reduction in nNOS in the PAI-1<sup>-/-</sup> mice was significant when compared to WT and tPA<sup>-/-</sup> treated with ethanol (B; \*,  $P < 0.05$ ; \*\*\*,  $P < 0.0001$ ).

### **3.15 PAI-1 promotes neuronal survival and tPA promotes neuronal death after ethanol treatment**

To investigate the potential role of PAI-1 in the neuronal protection from ethanol-induced toxicity observed in tPA<sup>-/-</sup> mice, we developed mice that are deficient in tPA and heterozygous for PAI-1 (i.e. tPA<sup>-/-</sup>:PAI-1<sup>+/-</sup>) by crossing double knock-out (tPA<sup>-/-</sup>:PAI-1<sup>+/-</sup>) mice with tPA<sup>-/-</sup>. Analysis of FJB-positive staining revealed tPA<sup>-/-</sup>:PAI<sup>+/-</sup> mice are more vulnerable to neurodegeneration than tPA-deficient mice, but less susceptible than WT mice (Figure 25). Ethanol-induced neurodegeneration, as reflected by FJB staining, was observed in the cortical (Figure 25A) and thalamic (Figure 25B) regions of tPA<sup>-/-</sup>:PAI<sup>+/-</sup>. These results suggest PAI-1 promotes neuronal survival in tPA<sup>-/-</sup> mice after ethanol treatment, since lack of a PAI-1 allele, which presumably results in reduced protein levels, is sufficient to enhance neuronal death in the absence of tPA.

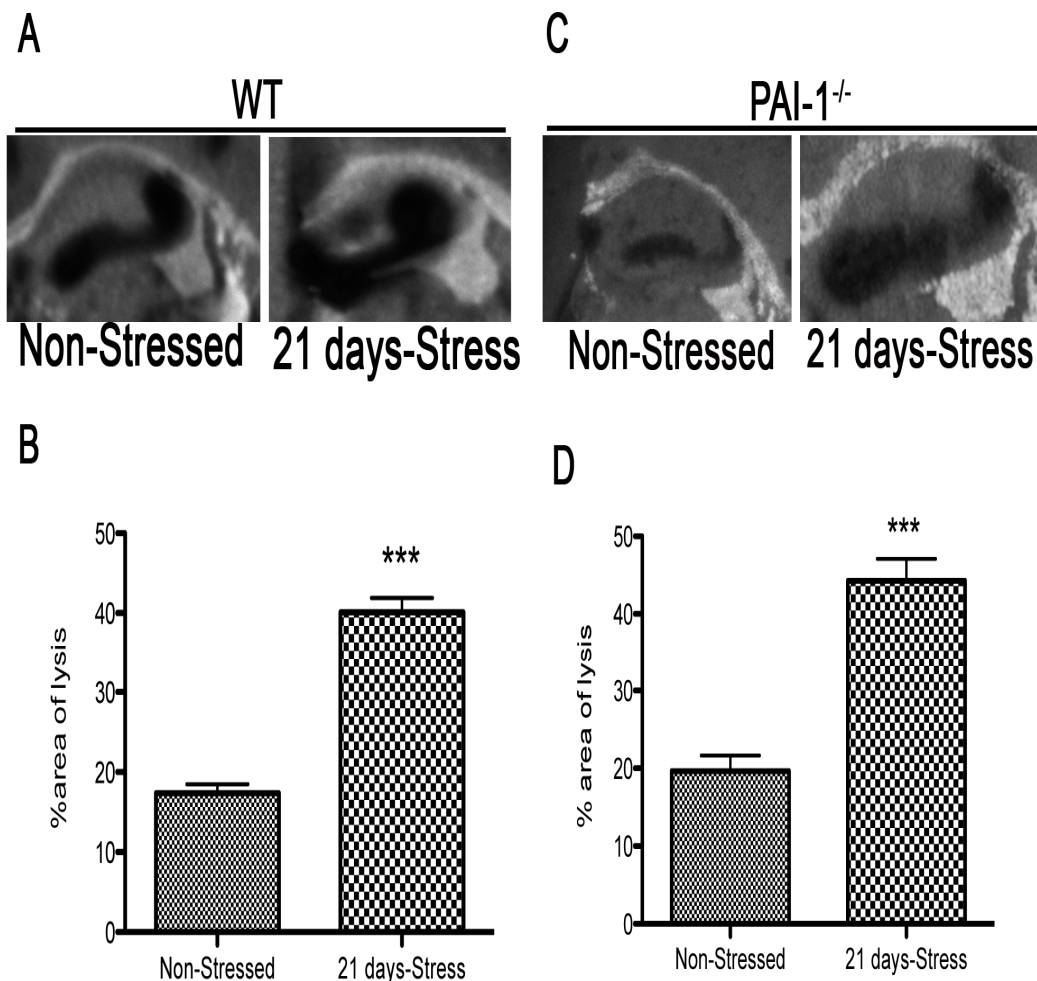


**Figure 25. PAI-1 promotes neuronal survival and tPA promotes neuronal death after ethanol treatment.**

To investigate the potential role of PAI-1 in the neuronal protection from ethanol-induced toxicity observed in tPA<sup>-/-</sup> mice, we developed mice that are deficient in tPA and hemizygous in PAI-1 (i.e. tPA<sup>-/-</sup>:PAI-1<sup>+/-</sup>). Analysis of FJB-positive staining revealed tPA<sup>-/-</sup>:PAI-1<sup>+/-</sup> mice are more vulnerable to neurodegeneration than tPA-deficient mice, but less susceptible than WT mice. Ethanol-induced neurodegeneration, as reflected by FJB staining, was observed in the cortical (A) and thalamic (B) regions of tPA<sup>-/-</sup>:PAI-1<sup>+/-</sup> (\*\*,  $P < 0.001$ ; \*\*\*,  $P < 0.0001$ ). Figure 25 G shows PCR analysis used to determine the genotype of the animals used in the experiment.

### **3.16 Chronic stress increases hippocampal tPA activity in WT and PAI-1-deficient adult mice**

Chronic restraint stress causes hippocampal-dependent cognitive deficits that are consistent with hippocampal structural changes. Chronic stress results in the atrophy of dendritic spines, and decreased expression of NR1 and NR2B subunits in the CA1 region that lead to impaired learning of the Morris water maze task in WT mice. tPA<sup>-/-</sup> mice are protected from these stress-induced molecular, structural and behavioral effects (Pawlak et al, 2005). In addition, PAI-1, has also been shown to be modulated by stress (Norris and Strickland, 2007). To investigate whether chronic stress leads to increased tPA activity in the hippocampus, adult WT and PAI-1<sup>-/-</sup> mice were restrained 6 hours a day for 21 days, and sacrificed the following morning after the end of stress. Increased extracellular tPA activity was observed via *in situ* zymography in the hippocampus of WT and PAI-1<sup>-/-</sup> mice that had been chronically stressed (Figure 26). The area of lysis (indicative of tPA activity) was increased 200% in the WT mice that had been chronically stressed over the non-stressed levels (Figure 26A and B). The increase in area of lysis observed in the PAI-1<sup>-/-</sup> mice exceeded 200% (Figure 26C and D), when compared to non-stressed animals of the same genotype.

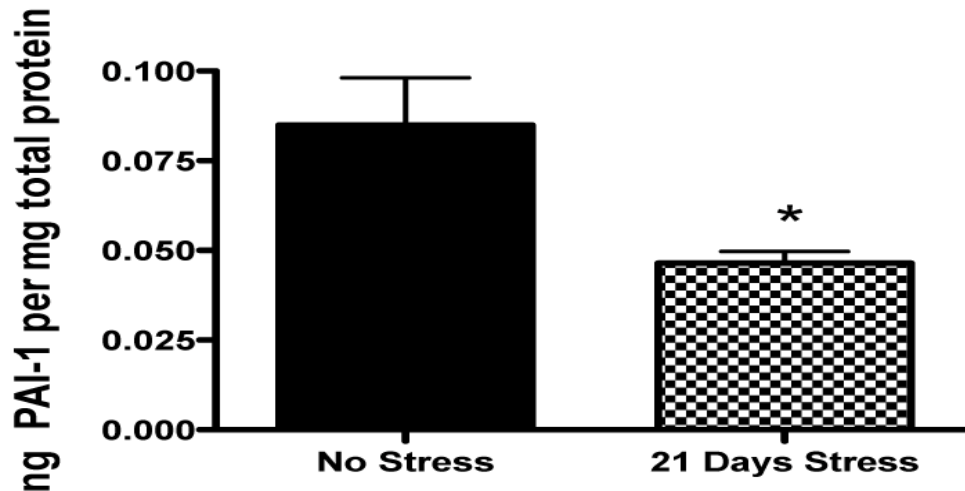


**Figure 26. Chronic stress increases hippocampal tPA activity in WT and PAI-1<sup>-/-</sup> mice.** To investigate if chronic stress regulates hippocampal tPA, adult WT and PAI-1<sup>-/-</sup> mice were restrained for 21 days and sacrificed on day 22 following the end of stress. The brains were sectioned and analyzed for extracellular tPA activity. Increased tPA activity was observed in the mossy fiber pathway of the hippocampal formation of chronically restrained WT and PAI-1<sup>-/-</sup> mice (A and C). Stress increased the area of lysis in the hippocampus from ~20% to ~40% in both genotypes (B and D; \*\*\*,  $P < 0.0001$ ).



### 3.17 Chronic stress decreases hippocampal PAI-1 levels in WT mice

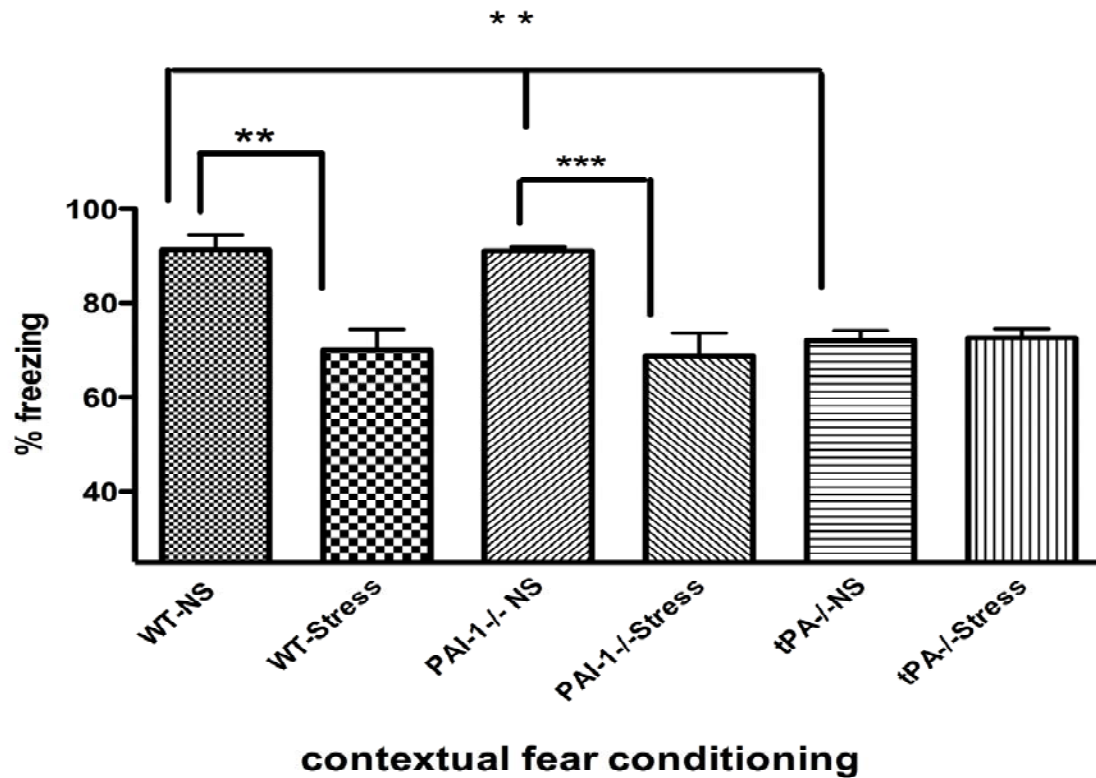
Since chronic stress increased extracellular tPA activity in the hippocampus of WT and PAI-1<sup>-/-</sup> mice, we investigated if PAI-1 is also regulated by chronic stress. Upon completion of 21 days of restraint, stressed and non-stressed WT mice were sacrificed and PAI-1 levels in hippocampal homogenates were determined using an ELISA. Chronic stress significantly decreased by 200% total PAI-1 antigen when compared to non-stressed WT mice (Figure 27).



**Figure 27. Chronic stress decreased hippocampal PAI-1 levels in WT mice.** To further determine if stress modulates the tPA/PAI-1 system, we analyzed PAI-1 levels in the hippocampus of chronically restrained WT mice with ELISA. Results indicate PAI-1 levels were significantly decreased after chronic stress (\*,  $P < 0.05$ ).

### **3.18 Chronic stress decreases fear conditioning in WT and PAI-1<sup>-/-</sup> mice, but not in tPA<sup>-/-</sup>**

To investigate whether the stress-induced changes in tPA activity and PAI-1 result in changes in learning and memory, we tested WT, tPA<sup>-/-</sup>, and PAI-1<sup>-/-</sup> adult animals in fear conditioning. Animals were restraint stressed for 21 days, or non-stressed as controls, and upon completion of restraint the mice were tested in fear conditioning. Baseline freezing behavior to the conditioning chambers was measured on the first day of the protocol, before starting the conditioning to electrical shocks. There were no differences in baseline freezing between genotypes (Figure 28). When animals were tested for contextual conditioning on the second day, chronically stressed WT and PAI-1<sup>-/-</sup> mice showed ~ 20% decrease in freezing when compared to their non-stress counterparts, reflective of impaired learning and/or memory probably due to chronic stress. Restraint stressed tPA<sup>-/-</sup> mice did not show differences in freezing behavior when compared to non-stressed tPA<sup>-/-</sup> controls. Also, tPA<sup>-/-</sup> mice appear to be naturally more hyperactive than WT and PAI-1<sup>-/-</sup>. Non-stressed tPA<sup>-/-</sup> mice show reduced contextual freezing when compared to non-stressed WT and PAI-1<sup>-/-</sup>.



**Figure 28. Chronic restraint impairs contextual fear conditioning in WT and PAI-1<sup>-/-</sup> mice but not tPA<sup>-/-</sup>.** Adult WT, PAI-1<sup>-/-</sup>, and tPA<sup>-/-</sup> mice that had been chronically restrained or non-restrained (control) were tested on fear conditioning. Stressed WT and PAI-1<sup>-/-</sup> show a significant reduction in freezing behavior when compared to the non-stressed animals of the same genotype (\*\*,  $P < 0.001$ ). Stress did not have an effect on the tPA<sup>-/-</sup> mice. Furthermore, there were basal differences in freezing between the non-stressed WT and PAI-1 when compared to non-stressed tPA<sup>-/-</sup> (\*\*\*,  $P < 0.0001$ ).

## **CHAPTER 4: DISCUSSION**

### **4.1 Ethanol and neurotoxicity in a murine model of FAS**

FAS is a prevalent disease in our society and there is currently no cure, other than prevention, for this life-long affliction. The effects of ethanol on the developing brain are particularly important because these often result in behavioral and cognitive complications that not only have a serious impact on the individual, but also place a burden on society. It is speculated that the behavioral sequelae, and specifically the cognitive deficits that are associated with FAS originate from brain mass loss and massive forebrain neurodegeneration resulting from high doses of ethanol. Therefore we sought to determine whether tPA is a component of the molecular cascade leading to neuronal death.

The data presented in this study identify tPA as a key molecular regulator of the neurotoxic effects of alcohol in a well-accepted murine model of FAS (Ikonomidou et al, 2000; Ieraci and Herrera, 2006; Maas et al, 2005; Heck et al, 2008). We found tPA signaling is upstream of the apoptotic cascade and of the NMDA-R. Our results also suggest tPA does not require proteolysis or plasmin activation to induce neurodegeneration after ethanol. Except for mice that are deficient in core apoptotic molecules (e.g BAX), no other transgenic mouse line has shown resistance to the ethanol-induced

neurotoxicity and the subsequent cognitive deficits that are characteristic of FAS.

Furthermore, our data implicates the major inhibitor for tPA in the brain, PAI-1, as an important regulatory component of neuronal survival after ethanol. In our model of FAS, PAI-1<sup>-/-</sup> mice were more vulnerable to neurodegeneration than WT after ethanol treatment. Our data indicate PAI-1 can provide trophic support to neurons in the presence of ethanol, tPA<sup>-/-</sup>: PAI-1<sup>+/-</sup> mice are more susceptible to neurodegeneration after ethanol than tPA-deficient animals.

#### **4.2 Ethanol modulates tPA activity and PAI-1 expression in WT pups**

We found that postnatal day 7 WT mice exposed to acute-binge ethanol showed changes in tPA activity and PAI-1 expression. Ethanol treatment elevated tPA activity for a period close to 24 hrs. tPA up-regulation was accompanied by an increase in PAI-1 levels. PAI-1 expression, however, was increased for less than 6 hrs after ethanol. Interestingly, PAI-1 was downregulated below baseline levels at 24 hours, suggesting ethanol modulation of PAI-1 is independent of tPA activity. It is probable the increase in PAI-1 observed after ethanol was not sufficient to counterbalance the increase in tPA, and consequently interfere with its activity and/or

interactions. Moreover, PAI-1 modulation after ethanol exposure occurred independently of changes in tPA activity, which favored the hypothesis of a tPA-independent modulation of PAI-1 after ethanol exposure.

#### **4.3 tPA<sup>-/-</sup> mice are resistant to ethanol-induced apoptotic signaling and neurodegeneration**

Imaging studies of humans with FAS show a high incidence of microencephaly (Swayze et al, 1997), reductions in cortical gray matter and total brain volumes (Rivkin et al, 2008), decreased size of the basal ganglia (Mattson et al, 2006), and abnormal development or in some cases absence of the corpus callosum (Bookstein et al, 2002). In our experiments, WT mice showed over a 100-fold increase in FJB staining in the thalamus and cortex when compared to the saline-treated control mice. In comparison, tPA-deficient mice showed less than a 4-fold increase in FJB staining after ethanol. Concomitantly, activation of caspase-3, a key effector molecule in the apoptotic cascade, was observed in WT, but not tPA<sup>-/-</sup>, confirming the results observed with FJB. The effects of tPA appeared to be dependent on gene-dosage since hemizygous tPA mice (tPA<sup>+/-</sup>), which probably exhibit reduced production of tPA in the brain, showed intermediate susceptibility to neuronal death between what was observed in the WT and tPA<sup>-/-</sup> animals.

Furthermore, since blood ethanol levels were similar in WT and tPA<sup>-/-</sup> animals after treatment, we concluded the effects of tPA on ethanol-induced neurodegeneration were not due to underlying differences in the rate of metabolism, but on the acute effects of tPA on neuronal physiology. In addition, ethanol treatment resulted in plasma concentrations that were consistent with the criteria required to induce neurodegeneration (Ikonomidou et al, 2000). In addition, there were no obvious differences in the time of loss of righting reflex after ethanol between WT and tPA<sup>-/-</sup> mice (personal observation), supporting that rate of ethanol metabolism is not the underlying mechanism for tPA-mediated ethanol-induced neurodegeneration.

#### **4.4 tPA-induced neurodegeneration after ethanol is independent of proteolysis and plasmin production**

Since tPA involvement in excitotoxic neuronal death is contingent on plasminogen activation (Tsirka et al, 1995), we investigated ethanol-induced neurodegeneration in plasminogen-deficient animals. We found Plg<sup>-/-</sup> mice were just as vulnerable to ethanol-induced neurotoxicity as WT mice, suggesting a plasmin-independent pathway was activated in tPA-mediated neuronal death. Indeed, in the brain tPA can function independently of the

typical plasminogen/plasmin proteolysis cascade through the modulation of the NMDA-R or stimulation of the LRP receptor (Pawlak et al, 2005; Norris and Strickland, 2007; Wang et al, 2003). It was reported tPA can directly cleave the NR1 subunit of the NMDA-R leading to increased calcium influx and excitotoxicity (Nicole et al, 2001). However, our laboratory has not been able yet to replicate this finding. Our data suggest tPA proteolytic activity is not a requirement to promote ethanol-induced neurodegeneration. The catalytically inactive S481A tPA, when administered to tPA-deficient mice, partially restored ethanol-induced neurodegeneration. The attenuation in neurodegeneration observed after tPA-STOP treatment might arise from preclusion of tPA-PAI-1 acyl-enzyme complex formation and downstream LRP interactions. It is also possible that association of tPA-STOP to tPA might have altered protease interactions with the NMDA-R or LRP, which has been reported to mediate a tPA-induced increase in matrix metalloproteinase-9 (MMP-9) resulting in neuronal death (Wang et al, 2003; Hu et al, 2006), or with a yet to be described binding partner. Future research will address the nature of tPA's interactions and signaling partners in ethanol-induced neurodegeneration.



#### **4.5 tPA-mediated ethanol-induced neurodegeneration occurs via the NMDA-R**

The NMDA-R is critical during development particularly since excitatory signaling is crucial for the formation of new synaptic contacts. During brain development, up to 25% of the neuronal population is eliminated in early postnatal stages during synaptogenesis (for reviews see Hidalgo et al, 2003; Benn and Woolf, 2004). Blockade of the NMDA-R exacerbates the basal rate of apoptosis that occurs developmentally, consequently impairing the appropriate formation of synapses, and eventually of neuronal circuitry (Ikonomidou et al, 1999 and 2000; Ghiani et al, 2007). Our data suggest ethanol and, NMDA-R antagonists MK801 and Ro25-691, do not obliterate NMDA-R-dependent function in tPA<sup>-/-</sup> mice. Interestingly, basal NMDA-R function might have been more robust in the tPA<sup>-/-</sup> mice since increased NR2A expression was observed in these mice when compared to WT. Consistent with our results, enhanced basal NMDA-R currents in tPA<sup>-/-</sup> slices were observed in adult animals (Park et al, 2008). It remains unclear what the underlying cause is for increased excitatory signaling in these mice.

Additionally, NMDA-R function in the tPA<sup>-/-</sup> mice might have been further enhanced as a result of ethanol exposure. tPA-deficient animals showed elevated NR2B, NR2A, BDNF, NGF, and nNOS, 24 hours post

ethanol administration. These are proteins that function downstream of the NMDA-R and are dependent on calcium signaling. Ethanol also up-regulated the expression of the activity-dependent immediate early gene cFOS 8 hours post-treatment in the tPA<sup>-/-</sup>, but not WT mice which suggested excitatory signaling was surprisingly improved or enhanced after ethanol. Increased NR2A and NR2B might be responsible for downstream increases in cFOS, nNOS, and neurotrophic factors.

In addition, western blot analysis of crude synaptosomal homogenates showed reduced basal PSD-95 protein in the tPA<sup>-/-</sup> forebrain, consistent with a previous study that found reduced association of PSD-95 with the NR2B subunit of the NMDA-R (Norris and Strickland, 2007). These results are incredibly fascinating, and support the hypothesis of tPA as a key modulator of the receptor, perhaps enabling the appropriate interactions between NR2B and PSD-95. Indeed NR2B<sup>-/-</sup> mice die within the first week of birth (Kutsuwada et al, 1996; Tovar et al, 2000), while PSD-95<sup>-/-</sup> mice survive into adulthood (Kim et al, 2003) suggesting there might be other molecules that are essential during early development. A potential candidate is synaptic ras GTPase-activating protein (SynGAP), and just like NR2B<sup>-/-</sup> mice, SynGAP null mutants die early after birth (Komiyama et al, 2002). Although SynGAP binds PSD-95 in the adult PSD, both genes have distinct,

as well as overlapping expression. The expression of SynGAP in the murine brain peaks during synaptogenesis and developmental plasticity in contrast to PSD-95, which is expressed throughout the brain from early embryonic stages. Furthermore, SynGAP shows a spatial pattern restricted mostly to the forebrain in contrast to PSD-95, which is also found in mid- and hindbrain (Porter et al, 2005). These reports indicate that synaptic signaling complexes are heterogeneous, and individual components show temporal and spatial specificity during development, and might account for the discrepancies observed between the neonatal WT and tPA<sup>-/-</sup> forebrain.

As discussed in the introductory section, the NR2 subunits undergo a developmental shift after birth. NR2B is the predominant NR2 subunit at birth and early post-natal stages. NR2A expression begins close to post-natal day 6, and slowly becomes the predominant subunit at the synapse, displacing most of NR2B to extrasynaptic sites (Sheng et al, 1994; Liu et al, 2004). NR2A-containing receptors promote neuronal survival while NR2B-containing receptors promote apoptosis in adult neurons. The mechanism for NR2A-mediated survival depends on the activation of the Akt pathway, and this molecular cascade involves NO (Liu et al, 2007). Synaptic NMDA-R activation leads to the stimulation of the cAMP response element binding (CREB) and extracellular signal-regulated kinase (ERK), whereas

extrasynaptic NMDA-R stimulation leads to shutoff of the CREB pathway (Hardingham et al, 2002). In cerebellar granule neurons, NO-mediated survival after ethanol-induced neurodegeneration required CREB activation and NGF, which we found was increased in the “ethanol-resistant” tPA<sup>-/-</sup> brain. Consistent with our data, neonatal nNOS-deficient mice showed increased neurodegeneration after ethanol in the same FAS paradigm we used in our studies (Bonthius et al, 2006). And ethanol-induced increase in nNOS and NGF, which are in the same neuroprotective pathway (Bonthius et al, 2003 and 2006), was observed in the tPA<sup>-/-</sup> mice. Enhanced NR2A in the tPA<sup>-/-</sup> brain, as opposed to the decreased observed in the WT, might have engaged downstream signaling molecules like NO and NGF thus serving as a starting advantage against neurotoxicity from ethanol.

We also observed increased association of the clathrin-associated  $\beta$ -adaptin to the NR2B subunit of ethanol-treated WT 24 hours after treatment. We did not observe changes in the tPA<sup>-/-</sup> mice after ethanol. Clathrin-mediated endocytosis serves as a regulatory mechanism for NMDA-R surface expression and function (Lavezzari et al, 2004). Our immunoprecipitation results suggest increased clathrin-mediated endocytosis of the NMDA-R complex after ethanol exposure. The observed reduction

in NR2A in conjunction to endocytosis of NR2B-containing receptors in the WT mice after ethanol might underlie neurodegeneration after treatment.

#### **4.6 tPA<sup>-/-</sup> mice have increased neurotrophic factor expression**

A “neurotrophic theory” has been proposed based on data from the sympathetic and motor nervous system (Linden 1994; Sendtner, 2000) where the select subset of neurons that survive during synaptogenesis do so via connection to appropriate targets that supply them with neurotrophic factors. This model of activity-dependent survival comes from in vivo and in vitro studies where cortical neurons have shown dependence on the activation of the NMDA-R and the consequent synthesis/release of BDNF for survival (Lessman et al, 2003; Suzuki et al, 2007). tPA<sup>-/-</sup> mice showed enhanced expression of BDNF and NGF 24 hrs after ethanol. It is likely the increase in these proteins began at an earlier time-point and provided a source of trophic support from ethanol toxicity. Furthermore, it is likely the changes in these proteins in the tPA<sup>-/-</sup> arise from initial changes observed in the NMDA-R subunit composition.

#### **4.7 Neonatal ethanol-induced neurodegeneration leads to adult cognitive impairments**

Early exposure to ethanol interferes with the formation of synaptic contacts possibly affecting the later development of neural circuits. The cortex, thalamus, and hippocampus, which comprise part of the limbic system, are some of the most vulnerable brain regions to ethanol-induced neurodegeneration during synaptogenesis. Consistent with previous reports, we show that neonatal exposure of the WT brain, but not tPA<sup>-/-</sup>, to ethanol results in decreased contextual fear conditioning, a learning task that depends on intact hippocampal function (Chen et al, 1996). Indeed, the hippocampus is essential for memory storage, and the thalamus, typically referred to as the relay station of the brain, allows communication between the cortex and other brain regions, including the hippocampus. Therefore, it was not unexpected that ethanol-induced neurodegeneration was commensurate with the behavioral deficits observed in the WT mice.

#### **4.8 tPA is a key regulator of ethanol-induced neurodegeneration and FAS development**

Overall, our data suggests a novel role for the NMDA-R in neuroprotection from ethanol's effects that are opposite from what is

observed during excitotoxicity. Increased basal levels of NR2A in the tPA<sup>-/-</sup> brain might be an advantageous starting point through which the NMDA-R compensates the blockade of ethanol. The advantage that increased NR2A might provide to the tPA<sup>-/-</sup> brain is probably enhanced by increased NR2B and NR2A at a later timepoint in the presence of ethanol. These NR2 subunit differences, and, subsequent changes after ethanol probably boost NMDA-R function, nNOS-dependent signaling, and neurotrophic factor support. In fact, nNOS has been shown to protect cultured cerebellar neurons from ethanol-induced death via the PKG pathway and NGF production. So it is possible that increased NR2A and NR2B can lead to downstream changes such as enhanced nNOS activity, and increased neurotrophic signaling that protects cells from the toxic effects of ethanol.

It still remains unclear what the trigger for the increased tPA in the WT brain might be. Ethanol depresses synaptic activity through inhibition of the NMDA-R and the GABA<sub>A</sub>-R. However, tPA is not a constitutively secreted protease, it is synthesized and stored in synaptic vesicles in axon terminals, and released into the extracellular space upon depolarization (Gualandris et al, 1996). In the adult brain, the answer to ethanol-induced increase in tPA might lie in the NMDA-R since chronic exposure stimulates the production of more receptors, probably as a compensatory mechanism from ethanol-

induced inhibition. As a result, tPA was also increased, and its interaction with the NR2B subunit was found necessary for ethanol-induced NMDA-R upregulation (Pawlak et al, 2005), thus leading to a positive feedback mechanism between the NMDA-R and tPA that was initially stimulated by ethanol. However, this mechanism does not account for what we observed in our model with neonatal animals, since we did not observe any evidence of ethanol-induced NMDA-R stimulation in the WT brain. An explanation could lie in the cellular source of increased tPA in the brain after acute ethanol exposure. Besides neurons, tPA is also synthesized by microglia cells in the brain (Rogove et al, 1999), and these cells can also be activated by tPA independently of proteolysis. As a result, the ethanol-induced increase in tPA we observe could be partly due to microglia activation. This is consistent with our findings that suggest tPA induces neurodegeneration after ethanol independently of plasminogen and proteolytic activity. An experiment could evaluate neuronal death after the administration of ethanol to neonatal mice that are deficient in neuronal- or microglial-specific tPA. Furthermore, microglia involvement is likely to only be part of the explanation since we observed many basal and ethanol-induced differences in NMDA-R subunit composition, NMDA-R-dependent signaling, and PAI-1 involvement between WT and tPA<sup>-/-</sup> mice.



Our study shows tPA is a novel molecule crucial in the development of FAS. Exposure of neonatal mice to ethanol leads to widespread neurodegeneration, prolonged increase in tPA activity in WT mice, and cognitive impairments in adult animals. tPA<sup>-/-</sup> mice however do not undergo neurodegeneration or develop subsequent cognitive impairment characteristic of FAS. The pharmacological manipulation of tPA levels in the brain might be a therapeutic avenue for the treatment of FAS and NMDA-R blockade related pathologies.

#### **4.9 Ethanol treatment increased neuronal-associated tPA activity in perinatal PAI-1<sup>-/-</sup> mouse pups**

PAI-1 serves as the major inhibitor of tPA in the brain and as such is an important regulatory component of the tPA/plasmin pathway that is engaged in excitotoxicity (Tsirka et al, 1996). We have shown here that ethanol exposure during synaptogenesis differentially modulates tPA activity and PAI-1 levels in the forebrain of WT neonatal P7 mice. Ethanol induced a prolonged increase in tPA activity and temporarily upregulated PAI-1 soon after treatment. Levels of PAI-1, however, were downregulated 24 hrs after ethanol exposure. Since PAI-1 was regulated by ethanol in WT mice, we were interested in evaluating tPA activity in PAI-1-deficient animals.

Ethanol treatment increased tPA activity in the forebrain of PAI-1<sup>-/-</sup> mice, but contrary to what is observed in WT, the observed increase in tPA was rapid and short-lasting. Though the timeline for increased tPA activity is slightly different in WT and PAI-1<sup>-/-</sup> mice, the increase in tPA in both genotypes precede the activation of caspase-3 and consequently neurodegeneration, suggesting tPA is an important component of the neurodegenerative cascade after ethanol in PAI-1<sup>-/-</sup> mice.

#### **4.10 Ethanol modulates PAI-1 in a tPA-independent manner**

PAI-1 modulation by ethanol had been previously observed in cultured human endothelial cells where ethanol treatment induced a transcriptional decrease of PAI-1 mRNA (Grenett et al, 2000). In our acute binge paradigm that serves as a model for FAS, ethanol initially increased, and progressively decreased PAI-1. The effects observed in PAI-1 after ethanol occur independently of tPA, since tPA<sup>-/-</sup> mice showed a similar pattern of modulation after ethanol exposure. Interestingly, we observed PAI-1 baseline levels were significantly greater in the tPA<sup>-/-</sup> mice than in WT, and since tPA<sup>-/-</sup> mice are resistant to neurodegeneration, we hypothesized elevated levels of PAI-1 might confer neuroprotection from ethanol toxicity to these mice. The feasibility for this proposition is supported by studies

that have implicated PAI-1 in neuritogenesis and neuronal survival (Kimura et al, 2000; Soeda et al 2001; Guizzetti et al, 2008).

#### **4.11 tPA promotes ethanol-induced neurodegeneration in PAI-1<sup>-/-</sup> mice**

Indeed, PAI-1-deficient mice showed enhanced vulnerability to neurodegeneration after ethanol exposure seen by increased FJB staining in the cortical and thalamic regions of the forebrain in comparison to the WT mice. It was possible that the difference in neurodegeneration in PAI-1<sup>-/-</sup> and WT could have been an artifact of differing blood ethanol concentrations, perhaps due to underlying metabolic differences between the WT and PAI-1<sup>-/-</sup> mice, and not to a primary difference in neuronal physiology. To address this possibility, we determined the blood ethanol concentrations following treatment. We found no differences in blood ethanol concentrations at any time-point studied, suggesting the differences observed are not due to the rate of ethanol metabolism.

Enhanced neuronal death vulnerability after ethanol exposure was partially dependent on increased tPA activity, since treatment with the synthetic inhibitor tPA-STOP before EtOH exposure led to attenuation of the severity of neurodegeneration in these animals. This evidence is not surprising, since in addition to tPA, PAI-1 interacts with other molecules in

the brain (e.g. uPA, vitronectin, and LRP). Our data suggests that PAI-1 is acting independently of tPA, and an interaction of PAI-1 with any of these molecules could potentially influence the effects of ethanol, additionally to the effects stemming from increased tPA activity. Furthermore, these results suggest that while tPA mediates ethanol-induced neurotoxicity, PAI-1 mediates a survival-signaling cascade that protects neurons from neurodegeneration. As a result, absence of PAI-1 exacerbates the neurotoxic insult by ethanol, supporting the hypothesis that PAI-1 promotes neuronal survival after ethanol exposure.

What could be the mechanism for PAI-1 in neuronal survival? PAI-1 is secreted by astrocytes and a potential mechanism could entail modifications of the extracellular matrix that favor the extension of neurites, thus abrogating the effects of ethanol on neuronal activity and consequently synaptogenesis. A recent report showed that PAI-1 upregulation was deemed necessary for laminin- and fibronectin-mediated neuritogenesis and the extension of neurites during development (Guizzetti et al, 2008). It is unclear though, whether PAI-1 actions are dependent or independent of tPA in this study.

Another possibility entails PAI-1 in neuronal anti-apoptotic signaling (Kimura et al 2000 and Soeda et al 2001). These studies showed that PAI-1

deficiency in NGF-differentiated PC12 cells resulted in a significant reduction of Bcl-2 and Bcl-XL mRNAs, which typically promote cellular survival, and an increase in Bcl-XS and Bax mRNAs, which typically promote apoptosis (Soeda et al., 2001). As a result, changes in the balance between mRNAs expressed by the anti- and pro-apoptotic Bcl-2 family favored pro-apoptotic signaling, and resulted in cell death. Ethanol-induced neurodegeneration in our model is apoptotic, and requires caspase-3 activation and cytochrome c release. It might have been possible that elevated levels of PAI-1 in the tPA<sup>-/-</sup> favored expression of anti-apoptotic signaling molecules, thus protecting against ethanol-induced neuronal death in these mice. Thus there are several scenarios through which PAI-1 might be exerting neuroprotection, and future studies will address the mechanism for PAI-1-mediated neuronal survival after ethanol in neonatal mice.

#### **4.12 Ethanol decreased nNOS expression in PAI-1<sup>-/-</sup> mice**

Previous studies have reported that PAI-1 can prevent serum deprivation-induced loss of neurites in differentiated PC12 cells (Soeda et al., 2004). In the same model system, it was later shown that adding PAI-1, in the absence of NGF, can lead to the phosphorylation of Trk A receptors, and promote neurite outgrowth and the survival of PC12 cells (Soeda et al.,

2006). In the presence of NGF, however, PAI-1 was found to act as a synergist since the treatment of NGF-differentiated PC12 cells with anti-Trk A receptor antibodies caused neurite disappearance and cell death. We've shown tPA<sup>-/-</sup> mice are resistant to neurodegeneration, and concomitantly, these mice show enhanced PAI-1, NGF, BDNF, and nNOS. WT mice, however, have decreased NGF and PAI-1, and lack increased BDNF and nNOS. A feasible mechanism for PAI-1-mediated neuroprotection could then be through the synergistic activation of TrkA receptors with NGF.

Concomitantly, PAI-1 and NGF could promote neuroprotection from ethanol toxicity through nNOS activation. Particularly since the pro-survival properties of nNOS after ethanol seem to require NGF (Bonthius et al, 2003). nNOS was significantly up-regulated in the tPA<sup>-/-</sup> mice. However, nNOS expression was unchanged in the WT, and significantly decreased in the PAI-1<sup>-/-</sup> brain. These results are consistent with the pro-survival role that has been described for nNOS activity after ethanol exposure of the immature brain, and concomitant with our neurodegeneration findings. Decreased nNOS expression in the PAI-1<sup>-/-</sup> brain after ethanol exposure, suggest nNOS could be part of the neuroprotective pathway engaged by PAI-1 after ethanol.

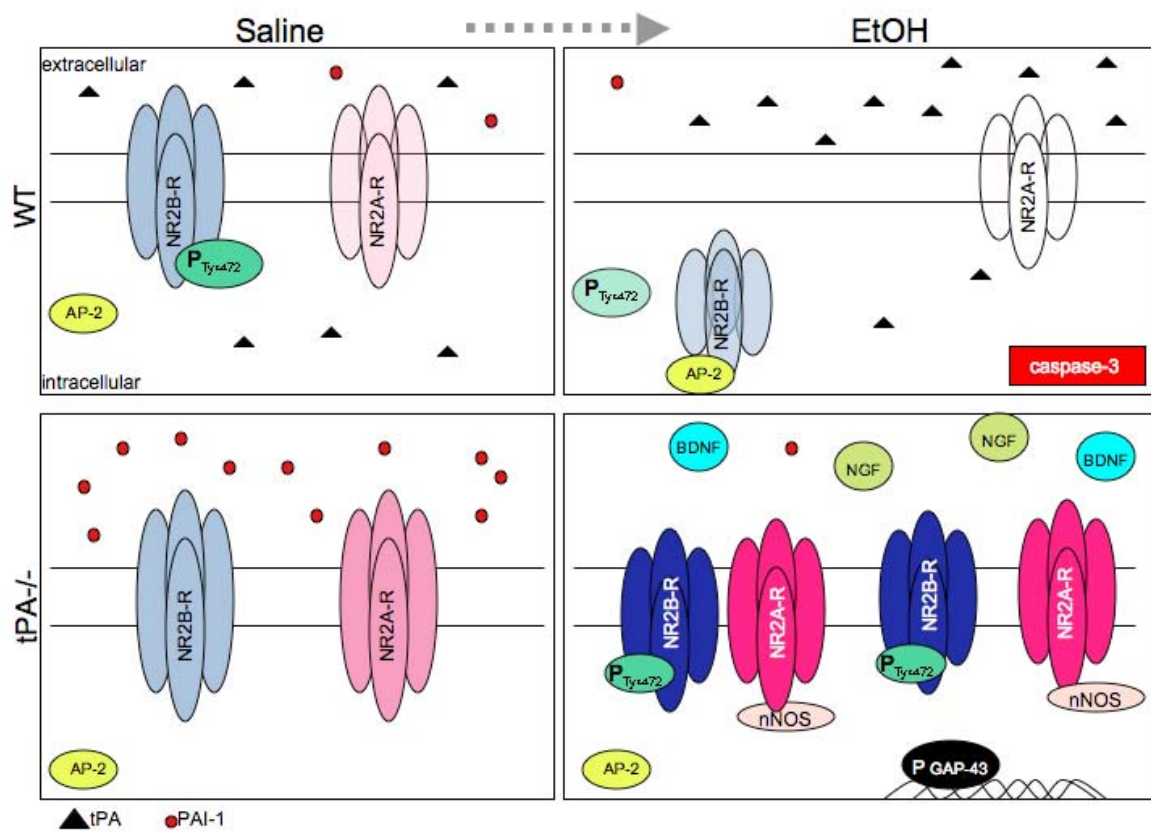
#### **4.13 PAI-1 promotes neuronal survival and tPA promotes neuronal death after ethanol treatment**

In order to further dissect the individual contributions tPA and PAI-1 have in neuronal death and survival after ethanol exposure, we created tPA<sup>-/-</sup>:PAI-1<sup>+/-</sup> mice by crossing double knock outs with tPA<sup>-/-</sup> mice. We found that ethanol-induced neurodegeneration in these mice was above what is observed in the tPA<sup>-/-</sup>, but below WT levels. These mice do not make tPA which makes them more resistant to neuronal death, but losing one allele of the PAI-1 gene, which likely results in reduced expression of PAI-1 protein, is sufficient to significantly increase neuronal death after ethanol. It is possible the elevated levels of PAI-1 in the tPA<sup>-/-</sup> mice, in conjunction to the lack of tPA, serve as an advantage and help protect neurons when exposed to ethanol. Further research into the trophic properties of PAI-1 during neonatal brain development might lead open new therapeutic avenues for the treatment of FAS-related pathologies.

**Figure 29. Model representing ethanol modulation of tPA and PAI-1 in the neonatal brain.** Early exposure of the brain to ethanol leads to changes in tPA and PAI-1 that result in neurotoxicity. tPA-deficient animals are resistant to ethanol-induced neurotoxicity, implicating tPA as a key regulator of the effects of ethanol in the immature brain. We hypothesize that abnormal differences in NMDA-R composition and function between WT and tPA<sup>-/-</sup> mice underlies the changes observed in neurodegeneration. The basal composition of the NMDA-R differs between WT and tPA-deficient animals, with higher NR2A expression observed in the tPA<sup>-/-</sup> brain, possibly serving as an advantageous starting point that protects against ethanol toxicity. NR2A is further increased after ethanol in these mice but decreased in the WT mice. Furthermore, ethanol increased the association of AP-2 to NR2B in the WT, suggesting ethanol promoted the internalization of these receptors in WT mice. Ethanol also increased neurotrophic factor expression in the tPA<sup>-/-</sup>, suggesting these molecules might serve as a source of neuroprotection in the presence of ethanol. Lastly, PAI-1<sup>-/-</sup> mice were more vulnerable to neurodegeneration, consistent with the observed increased basal PAI-1 expression in the tPA<sup>-/-</sup> brain, suggesting increased PAI-1 in the tPA<sup>-/-</sup> mice might preclude ethanol-induced neurodegeneration in these mice.



**Figure 29**



#### **4.14 Ethanol exposure of the immature murine brain can lead to learning deficits that are characteristic of FAS in humans**

Prenatal exposure to ethanol leads to developmental defects in the brain that often result in long-lasting behavioral complications, as is the case in FAS. Neonatal P7 rodents that are exposed to binge-like doses of ethanol, as we have done in our studies, leads to hyper-activity in the elevated plus maze and open field, and impairment of contextual fear conditioning (Ieraci and Herrera, 2006). Our results show that adult WT mice that had been treated with ethanol at P7 have decreased contextual, but not cued, fear conditioning. Adult tPA-deficient mice that had been treated with the same dose of ethanol did not show differences in fear conditioning when compared to saline-treated tPA<sup>-/-</sup>. These results are consistent with previous reports, and support a causal link between ethanol-induced neuronal death with the cognitive impairments that are observed in patients with FAS. Furthermore, these results suggest that therapeutic agents that block, or attenuate ethanol-induced neurodegeneration, might also be able to prevent the cognitive deficits and possibly the development of other behavioral disturbances that stem from ethanol exposure.

#### **4.15 Chronic stress regulates hippocampal-dependent behavior via modulation of the tPA/PAI-1 system**

tPA has multiple effects in the physiology of the CNS. In particular, tPA modulates synaptic plasticity events that underlie several forms of learning and memory of spatial memory tasks, LTP, and addiction. Furthermore, tPA is modulated by stress in the hippocampus and amygdala (Pawlak et al, 2003 and 2005). The stress-induced increase observed in tPA activity in the amygdala is mediated by the stress neuromodulator corticotropin releasing factor (CRF) acting via corticotropin releasing factor receptor type 1 (CRF-1) (Matys et al, 2004). tPA-deficient mice exhibit reduced anxiety-like behavior to CRF but undergo a sustained corticosterone response after CRF administration. These studies establish tPA as an important mediator of cellular, behavioral, and hormonal responses to CRF, and consequently stress.

We focused on the effects of stress on the hippocampal formation. Previous reports from our laboratory showed chronic stress differentially affected hippocampal NMDA-R expression, dendritic arborization, and Morris water maze learning in WT and tPA<sup>-/-</sup> mice (Pawlak et al, 2005). We found 21 days of restraint stress significantly elevated extracellular tPA activity in WT and PAI-1<sup>-/-</sup>. Simultaneously, chronic stress had the opposite

effect on PAI-1 levels, reducing it below the baseline in WT mice. Furthermore, these changes observed in tPA and PAI-1 were reflected behaviorally, as WT and PAI-1<sup>-/-</sup> animals that had been chronically stressed showed decreased contextual, but not cued, fear conditioning. Chronically stressed tPA<sup>-/-</sup> animals did not undergo changes in either contextual or cued fear conditioning. These data are consistent with the lack of hippocampal atrophy observed in the tPA<sup>-/-</sup> mice. Furthermore, they implicate PAI-1 as a modulator of the stress response in the hippocampus. It is likely that decreased hippocampal levels of PAI-1 in the WT, and the lack of PAI-1 in the PAI-1<sup>-/-</sup> mice, underlie the observed increase in tPA activity. It might be possible the prolonged and elevated levels of tPA due to chronic stress lead to detrimental changes in the molecular composition of the extracellular matrix that contribute to neuronal atrophy. Increased tPA might also result in degradation of the NMDA-R. Plasminogen-deficient mice show a phenotype similar to that observed in tPA<sup>-/-</sup> animals (Pawlak et al, 2005), suggesting tPA acts via plasmin activation after chronic stress. Since tPA-derived plasmin can cleave the NR1 subunit of the NMDA-R (Matys and Strickland, 2003) we can speculate this could be an attractive mechanism for tPA action after stress. Increased NR1 cleavage by plasmin could underlie the reduction in NMDA-R observed at the end of chronic stress (Pawlak et

al, 2005). Furthermore, cleavage of NR1 could result in increased calcium influx and stimulate excitotoxicity and neuronal death. Overall, these data support a role for the tPA/PAI-1 system as key regulators of the cellular and behavioral response to chronic stress stimuli.

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