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Adolescent Intermittent Alcohol Exposure: Dysregulation of thrombospondins and synapse formation are associated with decreased neuronal density in the adult hippocampus

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Abstract

Background—Adolescent intermittent alcohol exposure (AIE) has profound effects on neuronal function. We have previously shown that AIE causes aberrant hippocampal structure and function that persists into adulthood. However, the possible contributions of astrocytes and their signaling factors remain largely unexplored. We investigated the acute and enduring effects of AIE on astrocytic reactivity and signaling on synaptic expression in the hippocampus, including the impact of the thrombospondin (TSP) family of astrocyte-secreted synaptogenic factors and their neuronal receptor, alpha2delta-1 (α2δ-1). Our hypothesis is that some of the influences of AIE on neuronal function may be secondary to direct effects on astrocytes.

Methods—We conducted Western blot analysis on TSPs 1–4 and α2δ-1 from whole hippocampal lysates 24hrs after the 4th and 10th dose of AIE, then 24 days after the last dose (in adulthood). We used immunohistochemistry to assess astrocyte reactivity (i.e. morphology) and synaptogenesis (i.e. co-localization of pre- and post-synaptic puncta).

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Disclosure.

The authors declare no conflict of interest.

Results—AIE reduced α2δ-1 expression, and co-localized pre- and post-synaptic puncta after the 4th ethanol dose. By the 10th dose, increased TSP2 levels were accompanied by an increase in co-localized pre- and post-synaptic puncta, while α2δ-1 returned to control levels. 24 days after the last ethanol dose (i.e. adulthood), TSP2, TSP4, and α2δ-1 expression were all elevated. Astrocyte reactivity, indicated by increased astrocytic volume and area, was also observed at that time.

Conclusions—Repeated ethanol exposure during adolescence results in long-term changes in specific astrocyte signaling proteins and their neuronal synaptogenic receptor. Continued signaling by these traditionally developmental factors in adulthood may represent a compensatory mechanism whereby astrocytes reopen the synaptogenic window and repair lost connectivity, and consequently contribute to the enduring maladaptive structural and functional abnormalities previously observed in the hippocampus after AIE.

Keywords

hippocampus; thrombospondin; astrocytes; adolescence; ethanol

Introduction

Excessive alcohol use is common among individuals between 18–25 years of age and has been associated with an increased risk of developing an alcohol use disorder later in life (De Wit et al., 2000). Excessive alcohol use during this developmental period occurs when the brain is undergoing the final stages of neuronal maturation. This period is highlighted by the pruning of synaptic connections between neurons and the refinement of circuitry crucial for higher-order memory processing, planning, and inhibitory control (Williams et al., 1999, Casey et al., 2005, Paus, 2005). Given the increasing appreciation of the critical role that astrocytes play in synapse formation, connectivity, and function in the developing and injured central nervous system (CNS) it has become important to assess the possible direct effects of AIE on astrocytes (Ullian et al., 2004, Clarke and Barres, 2013, Sloan and Barres, 2014).

Growing evidence from studies in humans reveals that alcohol use during late brain maturation is associated with deficits across several domains of cognition, including verbal and non-verbal skills, attention, visuospatial function, and learning (Brown et al., 2000, Chin et al., 2010, Hanson et al., 2011). Furthermore, studies in both humans and rats have shown that adolescents and young adults are more vulnerable to acute alcohol-induced memory impairment than older subjects (Little et al., 1996, Markwiese et al., 1998). We have recently shown that adolescent rats (beginning postnatal day 30 (PND 30)) exposed to AIE have an increased number of immature dendritic spines, and an elevated propensity for induction of long-term potentiation in hippocampal area CA1 in adulthood (Risher et al., 2015). Both of those findings are indicative of increased neuronal plasticity consistent with our previous observation of greater synaptic plasticity in hippocampal area CA1 in slices from adolescent rats compared to those from adults (Swartzwelder et al., 1995). Importantly, these changes were observed well after the effects of ethanol withdrawal had dissipated, suggesting ongoing and/or persistent alterations of hippocampal neuronal structure and function. The onset of high alcohol exposure in this model occurs during late-stage brain maturation, highlighted by the end of excessive synaptogenesis, the onset of synaptic

pruning, and the refinement of circuitry, all requiring regulation by non-neuronal cells, including astrocytes (Stevens et al., 2007, Kucukdereli et al., 2011, Allen, 2013, Chung et al., 2013).

Recent studies have shown that a single acute exposure to ethanol can modulate astrocyte activity (Gonzalez et al., 2007, Pignataro et al., 2013). However, the long-term impact of repeated ethanol exposure on astrocyte reactivity and the release of astrocyte signaling factors involved in synaptogenesis are only beginning to be explored (Bray et al., 2013, Bull et al., 2015). Long-term alteration of astrocyte function could critically affect neuronal circuitry after AIE, not only because astrocytes greatly outnumber neurons and ensheathe multiple synaptic contacts, but also because their signaling factors play a critical role in synapse formation during development, synapse maintenance in adulthood, and synaptic recovery after injury (see Verkhratsky et al., (2015) for review). Well-known factors secreted by astrocytes include the thrombospondin (TSP) family. TSPs are found throughout the body and are involved in the regulation of cell-cell and cell-matrix interactions (Yang et al., 2000), acting through cell surface receptors and other extracellular matrix proteins (see Risher and Eroglu (2012) for review). A seminal study by Christopherson and colleagues (2005) showed that TSP is the major synaptogenic factor secreted by astrocytes, while Eroglu et al. (2009) found that these synaptic processes occur via interactions with the neuronal calcium channel subunit α2δ-1. Of the 5 known TSPs (1–5), TSP-1 & -2 expression peaks around postnatal day (PND)-10 during early rodent (rat and mouse) CNS development, when synaptogenesis is at its highest and competition for postsynaptic partners is similarly high. By PND-20, TSP-1 and -2 are declining as the synaptogenic period gives way to synaptic pruning and refinement of processes (Chamak et al., 1995, Ullian et al., 2004, Allen, 2013). Additional bursts of synaptogenesis (involving the release of TSPs) that recapitulate certain aspects of developmental synaptogenesis can occur later in life in response to disease and/or injury, potentially in an attempt to restore lost synaptic circuitry and thereby increase functional recovery (Chamak et al., 1994, Liauw et al., 2008). TSP upregulation can also occur in response to neuronal hyperexcitability (Andresen et al., 2014) and inflammation (Fairaq et al., 2015), events commonly observed during alcohol withdrawal. Upregulation of TSPs and increased synapse formation during AIE exposure, at a time when synaptic pruning would typically be occurring, may yield detrimental consequences to the local neuronal circuitry.

There is emerging evidence of the negative impact that unresolved (i.e. chronic) astrocyte reactivity, and unscheduled synaptogenesis, may have on normal neuronal function. For example during epileptogenesis a number of acute and chronic, cellular and molecular events result in brain remodeling through the formation of aberrant synaptic circuitry (Jacobs et al., 2009). Antagonizing the TSP-α2δ-1 interaction has been shown to attenuate the development of epileptiform activity (Andresen et al., 2014). Neuropathic pain is another condition in which TSPs are upregulated, promoting inappropriate synaptogenesis and neuronal hypersensitivity, resulting in chronic pain. (Kim et al., 2012, Crosby et al., 2015).

Given the importance of TSP regulation for the formation and maintenance of synaptic networks throughout life, as well as our previous findings that show long-term changes to hippocampal neuronal circuitry after AIE (Risher et al., 2015), in this study we investigated

the effects of AIE on the release of TSPs. We show that complex and divergent adaptive changes occur in TSP and α2δ-1 regulation during and immediately following AIE. Intriguingly, protracted dysregulation of α2δ-1 and TSPs are also present in adulthood. This inappropriate astrocyte signaling that persists into adulthood is consistent with a maladaptive rewiring of hippocampal circuitry, therefore representing a potential mechanism underlying the enduring neuronal plasticity-related changes we have previously observed after AIE.

Materials and Methods

All procedures were conducted in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care and the National Research Council's Guide for Care and Use of Laboratory Animals, and were approved by the Durham VA Medical Center and the Duke University IACUCs.

Male PND-25 Sprague-Dawley rats (Charles River, USA) were double-housed and maintained in a temperature- and humidity-controlled room with *ad libitum* access to food and water. Animals were dosed as previously described (Risher et al., 2015). Briefly, animals were allowed to acclimatize for 5 days on a reverse 12:12-hr light:dark cycle (lights off at 9:00 am) prior to beginning AIE or saline administration. All animals were exposed to AIE or saline beginning on PND-30 and consisting of 10 doses of 5 g/kg ethanol (35% v/v in saline at 18.12 mL/kg, VWR, Suwanee, GA, USA) or isovolumetric saline administered by intragastric gavage (i.g.) using a 2 days on, 1 day off, 2 days on, 2 days off intermittent schedule for 16 days. Animals were euthanized 24hr after the 4^{th} dose, 24hr after the 10^{th}/ last dose, or following a 24 day washout period. The last time point was chosen in order to allow animals to reach adulthood prior to sacrifice.

Ethanol doses were selected to produce blood ethanol concentrations (BECs) consistent with our previous studies and adolescent human BECs that are achieved during binge drinking episodes (199.7 ± 19.9 − 172.8 ± 13.3 mg/dl, measured sixty minutes after the first and last dose (Risher et al., 2015)). The animals in this study were dosed in parallel with cohorts from the Risher et al., (2015) manuscript.

Blood ethanol concentrations (BECs) were determined from a parallel group of animals, blood (approximately 300uL) was collected from the lateral saphenous vein 60 minutes after the first dose. BECs were determined using the Analox GL5 (Analox Instruments, Lunenburg, MA, USA). Doses were selected to produce blood ethanol concentrations (BECs) consistent with previous studies and adolescent human BECs that are achieved during binge drinking episodes (199.7 ± 19.9 − 172.8 ± 13.3 mg/dl, measured sixty minutes after the first and last dose (Livy et al., 2003, Vetreno and Crews, 2012, Risher et al., 2015).

Western Blot

Tissue Preparation—Rats were deeply anesthetized using isoflurane. The brains were removed and rinsed very briefly in cooled phosphate buffered solution (PBS, 4°C). The hippocampus was placed dorsal side up and the cerebellum was removed, then a second cut was made sagittally down the midline. The hippocampus was gently dissected out (see Zapala et al., (2005) for more details) and placed in homogenization buffer consisting of

25mL ddH2O: 250uL 1M Tris, 25uL 1M CaCl2 1, 50uL 0.5M MgCl2, protease inhibitor (Complete EDTA-free, cat#04693132001, Roche, Sigma-Aldrich, Saint Louis, MI, USA) and homogenized using a Kontes glass tissue grinder at 4°C. Samples were spun for 5 min. at 300g at 4°C, then the supernatant was collected and spun for 20min at 19,000g at 4°C. The resulting pellet was resuspended in solubilization buffer from 25mL ddH2O: 625uL 1M Tris ; 750uL 5M NaCl; 25uL 1M CaCl2; 50uL 0.5M MgCl2; 2.14g sucrose; 125uL NP-40; protease inhibitor (Complete EDTA-free, Roche). Samples were incubated while rotating for 15min at 4°C, then spun for 10min at 19,0700g at 4°C. The supernatant was collected, aliquoted, flash frozen in liquid nitrogen, and stored at −80 °C. Protein concentrations were determined using the BCA protein assay kit using the standard 96 well plate microassay protocol (cat#353072, Life Technologies, Grand Island, NY, USA).

Immunoblot Analysis—40ug of protein was loaded onto a 4–12% SDS-PAGE gradient gel (Bio-Rad, Hercules, CA, USA and Novex, Life Technologies, Grand Island, NY, USA) and resolved. Proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and blocked with Li-cor blocking buffer (Li-cor, Lincoln, NE, USA) for 1 hr at room temperature. Membranes were probed with primary antibody overnight at 4°C (mouse anti-TSP-1 1:250 (Abcam, Cambridge, MA, USA), mouse anti-TSP-2 1:250 (BD, San Jose, CA, USA), goat anti-TSP-4 1:500 (R&D Systems, Minneapolis, MN, USA), mouse anti-α2δ-1 1:500 (Sigma-Aldrich), and the next day probed with secondary antibody 1:20k (Li-cor) for 1 hr at room temperature. Membranes were dried for 2 hrs and scanned using Li-cor Odyssey Fc. Membranes were rehydrated in tris buffered saline (TBS) (50mM Tris, 150mM NaCl, pH 7.4) for 2 hours at room temperature. Membranes were then re-probed with mouse anti-tubulin 1:1000 (Abcam, Cambridge, MA, USA). The optical densities of all bands were quantified with Li-cor Image Studio and adjusted to within lane background subtraction using the standard Li-cor Image Studio-defined parameters. Bands of interest (1 lane per independent brain sample) were adjusted to the tubulin loading control values. n=6–8/treatment group.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described in Ippolito and Eroglu (2010) with modifications. Briefly, rats were deeply anesthetized with isoflurane and transcardially perfused with phosphate buffered saline (PBS), pH 7.4, containing 25 U/ml heparin, followed by 4% paraformaldehyde in PBS. Brains were removed and post-fixed for 24 hrs at 4°C in 4% paraformaldehyde in PBS. The brains were rinsed in PBS and placed in 30% sucrose in PBS. Upon sinking, brains were removed and frozen in tissue freezing medium (Electron Microscopy Sciences, Hatfield, PA, USA) and stored at −80°C. Sagittal sections (20μm) were cut using the Leica CM 3000 (Leica Microsystems, Buffalo Grove, IL, USA) and placed in glycerol:tris buffered saline solution. When necessary, adjacent sections were used for the following analyses.

Astrocyte Staining (GFAP)—Staining for glial fibrillary acidic protein (GFAP), an intermediate filament within astrocytes, was used to detect changes in astrocyte morphology, indicating astrocyte reactivity. Glycerol was removed and slices were blocked with 5% BSA, 0.5% triton 100-X in TBS for 1 hr at room temperature. Slices were incubated in goat

anti-GFAP, 1:200 (Abcam) prepared in 5% BSA and 0.5% triton 100-X in tris buffered saline for 3 days at 4°C. Slices were rinsed and incubated in donkey anti-goat IgG 488 Alexa Fluor 1:200 (Life Technologies, Grand Island, NY, USA) prepared in 5% BSA and 0.5% triton 100-X in tris buffered saline solution for 2 hrs at room temperature. Sections were rinsed and coverslipped with Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA).

Synaptic Staining (PSD-95/VGlut1)—Glycerol was removed and slices were blocked with 5% normal goat serum, 0.2% triton in tris buffered saline for 1 hr at room temperature. Slices were incubated for 3 days at 4°C in guinea pig anti-VGlut1 1:3500 (EMD Millipore, Billerica, MA, USA) and post-synaptic density protein-95 1:450 (rabbit anti-PSD-95, Invitrogen, Life Technologies, Grand Island, NY, USA) to identify pre- and post-synaptic partners, respectively. Slices were then rinsed and incubated in goat anti-guinea pig IgG 488 and goat anti-rabbit IgG 594 (Alexa Fluor, Life Technologies) prepared in 5% normal goat serum, 0.2% triton in tris buffered saline solution for 2 hours at room temperature. Sections were rinsed and coverslipped with Vectashield mounting media.

Neuronal Staining (NeuN)—Glycerol was removed and slices were blocked with 5% normal donkey serum, 0.2% triton for 1 hour at room temperature. Slices were incubated for 24 hours at 4°C in mouse anti-neuronal nuclei 1:1000 (NeuN, EMD Millipore). Slices were then rinsed and incubated in donkey anti-mouse pig IgG 594 (Alexa Fluor, Life Technologies) prepared in 5% normal donkey serum, 0.2% triton in tris buffered saline solution for 2 hours at room temperature. Sections were rinsed and coverslipped with Vectashield mounting media.

Imaging and Analysis (GFAP)—Confocal z-stacks (5μm thick, optical section depth 0.33μm, 15 sections/z-stack, 1024×1024 image size) of the synaptic zone in area CA1 were imaged at 63× magnification on a Leica SP5 confocal laser-scanning microscope to correspond with electrophysiological studies conducted within our laboratory. Individual image stacks were imported into Imaris software version 7.6.4 (Bitplane, Concord, MA, USA) in order to generate 3-D reconstructed images. Astrocyte volume, area, and branch sholl analysis were analyzed using the filament tracer method. Cell body thresholds were manually adjusted to capture astrocyte cell bodies within the region of interest. Automatic tracing was then conducted using a set seed point selected for use across all images from the same data set. All parameters used for the threshold algorithms were automatically generated with no input from the operator to limit bias. Measurement accuracy was visually confirmed by comparing traces generated by Imaris to actual Z projection images. Maximum projections were generated using the snapshot function in Imaris and ImageJ (National Institutes of Health, Bethesda, Maryland, USA) to produce high power representative images. Low power representative images were collected with 10× magnification using the Zeiss Axiomager M1 (20 μm thick, optical section depth 1 μm). Three image stacks/animal and n=4 animals/treatment group were used for analyses.

Imaging and Analysis (PSD-95/VGlut1)—Confocal z-stacks (5 μm thick, optical section depth 0.33 μm, 15 sections/z-stack, 1024×1024 image size) of the synaptic zone in

area CA1 were imaged at 63× magnification on a Leica SP5 confocal laser-scanning microscope. Maximum projections of 3 consecutive optical sections (corresponding to 1 μm total depth) were generated from the original z-stack. The Puncta Analyzer plugin (available upon request from c.eroglu@cellbio.duke.edu) for ImageJ was used to count the number of co-localized, pre- and postsynaptic puncta (Ippolito and Eroglu, 2010). Three image stacks/animal and n=5 animals/treatment group were used for this analysis.

Imaging and Analysis (NeuN)—Confocal z-stacks were collected under the same conditions as the synaptic IHC. Maximum z projections were generated for analysis using the ImageJ. Cell counts were presented as cell body count/area and cell body count/um. Three image stacks/animal and n=4 animals/treatment group were used for analyses.

Statistical Analysis

Comparisons between treatments were made using independent-samples Student's *t*-tests for Western blot and immunohistochemistry. All analyses were conducted using SPSS (v.21/22; Chicago, IL, USA). Statistical significance was assessed using an alpha level of 0.05. All data are presented in figures as the mean +/− S.E.M.

Results

AIE induces early reduction of TSPs synaptogenic neuronal receptor and synaptic number

Given the importance of TSPs in the formation and maintenance of synaptic networks, and their upregulation in response to a wide range of neuronal insults, we sought to determine whether modulation of TSPs and their neuronal synaptogenic receptor (α2δ-1) occurs during AIE. We assessed TSPs 1–4, α2δ-1 expression, astrocyte reactivity, and synaptogenesis 24 hrs after the 4th dose. There was no effect of AIE on TSP-1, -2, or -4 expression at that time point during AIE (p=0.22, 0.47, 0.38, respectively, Fig.1a–c); however, α2δ-1 expression was significantly reduced (p=0.02, Fig.1d). The reduction in α2δ-1 was accompanied by a reduction in pre- and post-synaptic co-localization (p=0.001, Fig.2a&d). The decrease in co-localized puncta was driven by a reduction in both pre-(VGlut1) and post-(PSD-95) synaptic puncta (p=0.044, p=0.031, respectively, Fig.2b–c,d–f), indicative of a reduction in synapses. AIE GFAP staining was indistinguishable from saline controls at this time point (all p's≥ 0.185, data not shown). These data show that AIE initially leads to loss of TSP-receptor α2δ-1 and concurrent reduction in synapses.

AIE promotes selective upregulation of TSPs 24 hours after the last ethanol dose

In order to determine whether modulation of TSP expression occurs late within the AIE paradigm, we assessed TSP and α2δ-1 expression 24 hours after the 10th (i.e. last) AIE dose. At that time point, TSP-1 and TSP-4 levels in tissue from AIE-exposed animals did not differ significantly from control (p=0.21, p=0.38, respectively, Fig 3a,c). However, TSP-2 expression was significantly increased (p=0.044, Fig.3b). Interestingly, despite the reduction in α2δ-1 early in the AIE paradigm, by the end of the paradigm α2δ-1 expression returned to control levels (p=0.17).

Since increases in TSP-1 and -2 are known to promote excitatory synaptogenesis through interactions with the neuronal α2δ-1 receptor (Christopherson et al., 2005, Eroglu et al., 2009), we assessed the co-localization of pre- (VGlut1) and post-synaptic markers (PSD95) using IHC. We found that AIE increased the number of co-localized pre- and post-synaptic puncta (p=0.05, Fig.4a,d), indicative of increased synapse formation. This was driven by an increase in PSD-95 puncta and a trend towards increased VGlut1 puncta (p=0.031, p=0.077, respectively, Fig.4b–f). Interestingly, despite the upregulation of TSP-2, astrocyte morphology remained comparable to saline-treated controls at this time point. No significant change in astrocyte volume, area, branch number or branch length was detected (all p's≥ 0.086, data not shown), suggesting that upregulation of astrocyte signaling factors occurs without the gross morphological signs of astrocyte reactivity typically observed in more aggressive injury models (Kelso et al., 2011). Taken together, these data show that TSP-2 upregulation is an early feature of AIE that triggers synaptic rewiring 24hrs post last dose of AIE.

AIE upregulates TSPs and their synaptogenic neuronal receptor in adulthood

24 days after the last dose of AIE or vehicle (in adulthood) we assessed TSPs 1–4, α2δ-1 expression, astrocyte reactivity, and synaptogenesis to determine the impact of AIE on TSP dysregulation and synaptic rewiring. We found that following AIE, TSP-1 levels were indistinguishable from controls (p=0.44) while TSP-2, TSP-4 and TSP's synaptogenic neuronal receptor, α2δ-1, were upregulated (p=0.012, p=0.002, p=0.045, respectively, Fig. 5b–e), showing a differential response to AIE from the various members of the TSP family. We then assessed whether TSP upregulation was associated with morphological indicators of astrocyte reactivity. The data revealed a significant increase in astrocyte volume and astrocyte area in the AIE group (p=0.031 and p=0.017, respectively, Fig.6a–c) with a trend toward an increase in branch length (p=0.065, Fig.6d). There was also an increase in the mean number of branches at the first branch level (12.5±2.01 vs. 8.4±1.12, p=0.041, data not shown), but the overall number of branches collapsed across level was not affected by AIE (p=0.12). NeuN positive cell counts revealed a significant decrease in neuronal density in the same region of the hippocampus (CA1), i.e, a reduction in the number of NeuN-positive neurons/μm^2, suggesting AIE-induced cell loss within this region (p=0.019, Fig.7a–b). These results show that AIE produces long-term changes in astrocyte morphology, indicative of astrocyte reactivity; this is accompanied by TSP dysregulation and neuronal loss.

Discussion

We investigated how repeated ethanol exposure alters the regulation of a family of synaptogenic astrocyte signaling factors, the TSPs, their neuronal synaptogenic receptor, α2δ-1, and the subsequent effect on synapse number in the hippocampus. We also assessed whether robust astrocyte reactivity was necessary for the upregulation of these signaling factors. We found that AIE decreased α2δ-1 expression and the number of co-localized synaptic puncta early in the binge paradigm (24 hours after the 4^{th} dose). This was followed by upregulation of TSP-2 and an increase in co-localized pre- and post-synaptic puncta 24 hours after last dose, while α2δ-1 receptor expression returned to control levels at this same

time point. In adulthood (24 days after the last ethanol dose), TSP-2 remained elevated and was accompanied by an increase in TSP-4 and α2δ-1 expression. Furthermore, we found that the number of neurons within CA1 was decreased at this same time point after AIE, correlating with the reduction in co-localized synaptic puncta we previously reported (Risher et al., 2015). Astrocyte reactivity was not apparent in AIE animals until adulthood, indicating that TSP upregulation precedes robust signs of astrocyte reactivity (these results are summarized in Figure 8).

Our observation that AIE reduced the number of co-localized synaptic puncta after the 4th dose is consistent with findings from Spiga and colleagues (2014), who showed that withdrawal of chronic ethanol exposure reduced synaptic number and the number of dendritic spines within the nucleus accumbens shell. These findings suggest that synaptic pruning may occur as a compensatory response to the neuronal hyperexcitability of intermittent ethanol withdrawal during the binge cycle. Interestingly, at the 4th dose time point we also showed a reduction in α2δ-1. Since α2δ-1 is known to drive excitatory synaptic formation (Eroglu et al., 2009) and is located in the tips of synaptic terminals (Dolphin, 2012), it follows that α2δ-1 would also be decreased. Whether the reduction in α2δ-1 is due to the pruning process or as a consequence of an independent event, which occurs to systematically limit synapse formation during this period of hyperexcitation, is yet to be determined.

TSP-1 is typically the first member of the TSP family to be upregulated in response to a wide range of neuronal insults (Liauw et al., 2008). It was therefore surprising to find that TSP-1 expression after AIE was indistinguishable from controls 24 hours after the last dose. Instead TSP-2, which is usually upregulated a few days after TSP-1 in more aggressive injury models, was significantly elevated at this time point, suggesting that repeated ethanol exposure produces a unique and selective TSP signaling response. Interestingly, TSP-2 upregulation was not associated with signs of astrocyte reactivity. One explanation is that TSP-1 upregulation is more closely tied to the activation of inflammatory cascades that likely precede TSP upregulation. For example, within hours of acute brain injury (e.g., stroke, see Liauw et al., 2008), an inflammatory response is mounted and microglia become activated. In the days after this response, TSP-1 is upregulated followed by the upregulation of TSP-2. These events are assumed to occur after the initiation of astrocyte reactivity. However, in the AIE paradigm, TSP-1 upregulation and astrocyte reactivity fail to occur acutely. This suggests that TSP-1 upregulation relys on upstream astrocyte activation whereas TSP-2 may be regulated by alternative signaling cascades. Such differential regulation would be consistent with previous studies in which TSPs, despite the high homology between proteins and shared cell surface receptors, show unique responses to the various physiological and pathological processes encountered (see Stenina-Adognravi (2014) for further discussion).

Given the roles of TSP-2 and α2δ-1 in synaptogenesis, it was not surprising to observe an increase in co-localized pre- and post-synaptic puncta coinciding with the recovery of α2δ-1 expression and the upregulation of TSP-2 after the last dose. This compensatory synaptogenesis may represent a compensation to replace the synapses that were pruned earlier in the binge cycle, though further work is required to determine the mechanism by

which this pruning occurs. Taken together, these results indicate that multiple events occur after AIE: an acute response, resulting in the removal of synaptic connections, and a period of compensatory synaptogenesis, occurring only after adaptation to the repeated intermittent cycling of ethanol.

Despite those interesting findings during, and immediately after AIE exposure, our main goal was to determine whether dysregulation of astrocyte signaling molecules contribute to the long-term effects of AIE on hippocampal structure and function that we have previously reported (see Risher et al., (2015)). At 24 days after the last dose, after animals had reached adulthood, we made the unexpected finding that TSP-2 upregulation persisted. It has previously been reported that, after neuronal insult, TSP-1 and -2 are upregulated in order to restore the synapses and circuitry within the affected area before returning to baseline levels (Liauw et al., 2008). In addition to TSP-2 elevation, we also found that TSP-4 and α2δ-1 were upregulated in adulthood despite being indistinguishable from controls immediately following AIE. Of the TSPs, TSP-4 is most often associated with injury or disease in which long-term aberrant synaptogenic pathology exists (see Crosby et al., (2015)), so its upregulation in adulthood in AIE-exposed rats is consistent with what is known. Interestingly, α2δ-1 expression is increased in rats genetically selected for alcohol self-administration when compared to alcohol avoidant rats, and in nicotine and methamphetamine abuse models (Hayashida et al., 2005, Worst et al., 2005, Kurokawa et al., 2011). Taken together, these findings implicate α2δ-1 as a candidate therapeutic target for the treatment of alcohol use disorders (AUD) as well as addiction to certain other substances of abuse.

Previously, we have reported that AIE lowers the induction threshold for long-term potentiation (indicative of hyperplasticity) in the CA1 region of the hippocampus in adulthood (Risher et al., 2015), which coincides with the upregulation of TSPs and α2δ-1 found in the present study. Interestingly, recent studies have shown that gabapentin, which antagonizes the TSP-α2δ-1 interaction (Eroglu et al., 2009), can actually prevent injury-induced astrocyte reactivity, excitatory synaptogenesis, and neuronal hyperexcitability (Lo et al., 2011, Li et al., 2012, Andresen et al., 2014). These properties of gabapentin, in combination with the previously demonstrated role for TSPs and α2δ-1 in synaptogenesis (Eroglu et al., 2009), could suggest that this signaling mechanism is involved in the hippocampal hyperplasticity previously observed. Whether TSPs are increasing the rate of synaptogenesis or are simply driving aberrant synaptic connectivity is yet to be determined. Increased synaptogenesis driving hyperplasticity is unlikely, because during adulthood there was actually a decrease in the overall number of co-localized synaptic puncta. However, this may be explained by the decrease in neuronal number (also see Reynolds et al., (2015)), suggesting that the reduction in synaptic puncta in adulthood may be a consequence of cell loss. The chronic upregulation of synaptogenic factors (such as TSPs) may represent a failed attempt to replenish the synaptic connections lost as a consequence of decreased neuronal density. This type of synaptogenic burst is not uncommon and is frequently observed after injuries that result in neuronal death (Lin et al., 2003, Zhou et al., 2010, Lo et al., 2011). Further work is required to determine whether, as compensation for AIE-induced cell loss, synaptic number is actually increased on a per cell basis due to the presence of chronic TSP upregulation.

We did not observe the classic signs of astrocyte reactivity after the last AIE dose despite the upregulation of TSPs. However, whether astrocyte reactivity is required for the release of signaling factors under moderate environmental stress is unclear. The level of induced astrocyte reactivity is typically a reflection of the severity of the environmental challenge/insult and presents with a profile that is dependent on the type of insult. Our model of AIE results in moderate BECs (173–200 mg/dl) that are not comparable to more severe models of ethanol consumption (447 mg/dl, see Collins et al., (1996)), which typically elicit high levels of cell death and withdrawal seizures (Collins et al., 1996, Livy et al., 2003) and therefore are much more likely to induce astrocyte reactivity. Interestingly, a moderate binge paradigm similar to the one used here can result in chronic inflammation in adulthood (Vetreno and Crews, 2012, Vetreno and Crews, 2013) although see Kane et al., (2014), and can cause the persistence of chronically elevated astrocyte reactivity and glial swelling even if the challenge was moderate (Tenorio et al., 2013, Collins et al., 2014). Whether the observed astrocyte reactivity in adulthood is simply a sign of astrocytic dysfunction, or whether it is driven by the presence of these inflammatory signaling molecules that persist into adulthood, remains unexplored. However, the presence of chronic reactivity may not only limit the astrocytes ability to respond to further insult but may also promote chronic and inappropriate release of signaling factors, as we have observed after AIE. These factors, typically expressed during developmental synaptogenesis, may promote neuronal remodeling in the injured brain (Kim et al., 2012, Crosby et al., 2015) and therefore contribute to the enduring structural and functional abnormalities previously observed in the hippocampus (Risher et al., 2015).

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References

Allen NJ. Role of glia in developmental synapse formation. Current opinion in neurobiology. 2013; 23:1027–1033. [PubMed: 23871217]

Andresen L, Hampton D, Taylor-Weiner A, Morel L, Yang Y, Maguire J, Dulla CG. Gabapentin attenuates hyperexcitability in the freeze-lesion model of developmental cortical malformation. Neurobiology of disease. 2014; 71:305–316. [PubMed: 25158291]

Bray JG, Reyes KC, Roberts AJ, Ransohoff RM, Gruol DL. Synaptic plasticity in the hippocampus shows resistance to acute ethanol exposure in transgenic mice with astrocyte-targeted enhanced CCL2 expression. Neuropharmacology. 2013; 67:115–125. [PubMed: 23164616]

Brown S, Tapert S, Granholm E, Delis D. Neurocognitive functioning of adolescents: effects of protracted alcohol use. Alcoholism, clinical and experimental research. 2000; 24:164–171.

Bull C, Syed WA, Minter SC, Bowers MS. Differential response of glial fibrillary acidic protein-positive astrocytes in the rat prefrontal cortex following ethanol self-administration. Alcoholism, clinical and experimental research. 2015; 39:650–658.

Casey B, Tottenham N, Liston C, Durston S. Imaging the developing brain: what have we learned about cognitive development? Trends in cognitive sciences. 2005; 9:104–110. [PubMed: 15737818]

Chamak B, Dobbertin A, Mallat M. Immunohistochemical detection of thrombospondin in microglia in the developing rat brain. Neuroscience. 1995; 69:177–187. [PubMed: 8637616]

Chamak B, Morandi V, Mallat M. Brain macrophages stimulate neurite growth and regeneration by secreting thrombospondin. Journal of neuroscience research. 1994; 38:221–233. [PubMed: 8078107]

Chin V, Van Skike C, Matthews D. Effects of ethanol on hippocampal function during adolescence: a look at the past and thoughts on the future. Alcohol (Fayetteville, NY). 2010; 44:3–14.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell. 2005; 120:421–433. [PubMed: 15707899]

Chung WS, Clarke LE, Wang GX, Stafford BK, Sher A, Chakraborty C, Joung J, Foo LC, Thompson A, Chen C, Smith SJ, Barres BA. Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. Nature. 2013; 504:394–400. [PubMed: 24270812]

Clarke L, Barres B. Emerging roles of astrocytes in neural circuit development. Nature reviews Neuroscience. 2013; 14:311–321. [PubMed: 23595014]

Collins MA, Corso TD, Neafsey EJ. Neuronal degeneration in rat cerebrocortical and olfactory regions during subchronic "binge" intoxication with ethanol: possible explanation for olfactory deficits in alcoholics. Alcoholism, clinical and experimental research. 1996; 20:284–292.

Collins MA, Tajuddin N, Moon KH, Kim HY, Nixon K, Neafsey EJ. Alcohol, phospholipase A2-associated neuroinflammation, and omega3 docosahexaenoic acid protection. Molecular neurobiology. 2014; 50:239–245. [PubMed: 24705861]

Crosby ND, Zaucke F, Kras JV, Dong L, Luo ZD, Winkelstein BA. Thrombospondin-4 and excitatory synaptogenesis promote spinal sensitization after painful mechanical joint injury. Experimental neurology. 2015; 264:111–120. [PubMed: 25483397]

Dolphin AC. Calcium channel auxiliary alpha2delta and beta subunits: trafficking and one step beyond. Nature reviews Neuroscience. 2012; 13:542–555. [PubMed: 22805911]

Eroglu C, Allen N, Susman M, O'Rourke N, Park C, Ozkan E, Chakraborty C, Mulinyawe S, Annis D, Huberman A, Green E, Lawler J, Dolmetsch R, Garcia K, Smith S, Luo Z, Rosenthal A, Mosher D, Barres B. Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell. 2009; 139:380–392. [PubMed: 19818485]

Fairaq A, Goc A, Artham S, Sabbineni H, Somanath P. TNFα induces inflammatory stress response in microvascular endothelial cells via Akt- and P38 MAP kinase-mediated thrombospondin-1 expression. Mol Cell Biochem. 2015; 406:227–236. [PubMed: 25963668]

Gonzalez A, Pariente JA, Salido GM. Ethanol stimulates ROS generation by mitochondria through Ca2+ mobilization and increases GFAP content in rat hippocampal astrocytes. Brain research. 2007; 1178:28–37. [PubMed: 17888892]

Hanson K, Medina K, Padula C, Tapert S, Brown S. Impact of Adolescent Alcohol and Drug Use on Neuropsychological Functioning in Young Adulthood: 10-Year Outcomes. Journal of child & adolescent substance abuse. 2011; 20:135–154. [PubMed: 21532924]

Hayashida S, Katsura M, Torigoe F, Tsujimura A, Ohkuma S. Increased expression of L-type high voltage-gated calcium channel alpha1 and alpha2/delta subunits in mouse brain after chronic nicotine administration. Brain research Molecular brain research. 2005; 135:280–284. [PubMed: 15857690]

Ippolito D, Eroglu C. Quantifying synapses: an immunocytochemistry-based assay to quantify synapse number. Journal of visualized experiments : JoVE. 2010; 45:1–8.

Jacobs MP, Leblanc GG, Brooks-Kayal A, Jensen FE, Lowenstein DH, Noebels JL, Spencer DD, Swann JW. Curing epilepsy: progress and future directions. Epilepsy & behavior : E&B. 2009; 14:438–445.

Kane C, Phelan K, Douglas J, Wagoner G, Johnson J, Xu J, Phelan P, Drew P. Effects of ethanol on immune response in the brain: region-specific changes in adolescent versus adult mice. Alcoholism, clinical and experimental research. 2014; 38:384–391.

Kelso M, Liput D, Eaves D, Nixon K. Upregulated vimentin suggests new areas of neurodegeneration in a model of an alcohol use disorder. Neuroscience. 2011; 197:381–393. [PubMed: 21958862]

Kim DS, Li KW, Boroujerdi A, Peter Yu Y, Zhou CY, Deng P, Park J, Zhang X, Lee J, Corpe M, Sharp K, Steward O, Eroglu C, Barres B, Zaucke F, Xu ZC, Luo ZD. Thrombospondin-4 contributes to spinal sensitization and neuropathic pain states. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2012; 32:8977–8987. [PubMed: 22745497]

Kucukdereli H, Allen N, Lee A, Feng A, Ozlu M, Conatser L, Chakraborty C, Workman G, Weaver M, Sage E, Barres B, Eroglu C. Control of excitatory CNS synaptogenesis by astrocyte-secreted proteins Hevin and SPARC. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108:440–449.

Kurokawa K, Shibasaki M, Mizuno K, Ohkuma S. Gabapentin blocks methamphetamine-induced sensitization and conditioned place preference via inhibition of alpha(2)/delta-1 subunits of the voltage-gated calcium channels. Neuroscience. 2011; 176:328–335. [PubMed: 21182903]

Li H, Graber KD, Jin S, McDonald W, Barres BA, Prince DA. Gabapentin decreases epileptiform discharges in a chronic model of neocortical trauma. Neurobiology of disease. 2012; 48:429–438. [PubMed: 22766033]

Liauw J, Hoang S, Choi M, Eroglu C, Choi M, Sun G-h, Percy M, Wildman-Tobriner B, Bliss T, Guzman R, Barres B, Steinberg G. Thrombospondins 1 and 2 are necessary for synaptic plasticity and functional recovery after stroke. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2008; 28:1722–1732.

Lin, T-n; Kim, G-M.; Chen, J-J.; Cheung, W-M.; He, Y.; Hsu, C. Differential regulation of thrombospondin-1 and thrombospondin-2 after focal cerebral ischemia/reperfusion. Stroke; a journal of cerebral circulation. 2003; 34:177–186.

Little P, Kuhn C, Wilson W, Swartzwelder H. Differential effects of ethanol in adolescent and adult rats. Alcoholism, clinical and experimental research. 1996; 20:1346–1351.

Livy D, Parnell S, West J. Blood ethanol concentration profiles: a comparison between rats and mice. Alcohol (Fayetteville, NY). 2003; 29:165–171.

Lo FS, Zhao S, Erzurumlu RS. Astrocytes promote peripheral nerve injury-induced reactive synaptogenesis in the neonatal CNS. Journal of neurophysiology. 2011; 106:2876–2887. [PubMed: 21900512]

Markwiese B, Acheson S, Levin E, Wilson W, Swartzwelder H. Differential effects of ethanol on memory in adolescent and adult rats. Alcoholism, clinical and experimental research. 1998; 22:416–421.

Paus T. Mapping brain maturation and cognitive development during adolescence. Trends in cognitive sciences. 2005; 9:60–68. [PubMed: 15668098]

Pignataro L, Varodayan F, Tannenholz L, Protiva P, Harrison N. Brief alcohol exposure alters transcription in astrocytes via the heat shock pathway. Brain and behavior. 2013; 3:114–133. [PubMed: 23533150]

Reynolds AR, Berry JN, Sharrett-Field L, Prendergast MA. Ethanol withdrawal is required to produce persisting N-methyl-d-aspartate receptor-dependent hippocampal cytotoxicity during chronic intermittent ethanol exposure. Alcohol. 2015; 49:219–227. [PubMed: 25746220]

Risher ML, Fleming RL, Risher WC, Miller KM, Klein RC, Wills T, Acheson SK, Moore SD, Wilson WA, Eroglu C, Swartzwelder HS. Adolescent intermittent alcohol exposure: persistence of structural and functional hippocampal abnormalities into adulthood. Alcoholism, clinical and experimental research. 2015; 39:989–997.

Risher W, Eroglu C. Thrombospondins as key regulators of synaptogenesis in the central nervous system. Matrix biology : journal of the International Society for Matrix Biology. 2012; 31:170–177. [PubMed: 22285841]

Sloan SA, Barres BA. Mechanisms of astrocyte development and their contributions to neurodevelopmental disorders. Current opinion in neurobiology. 2014; 27:75–81. [PubMed: 24694749]

Spiga S, Talani G, Mulas G, Licheri V, Fois GR, Muggironi G, Masala N, Cannizzaro C, Biggio G, Sanna E, Diana M. Hampered long-term depression and thin spine loss in the nucleus accumbens of ethanol-dependent rats. Proceedings of the National Academy of Sciences of the United States of America. 2014; 111:E3745–E3754. [PubMed: 25122682]

Stenina-Adognravi O. Invoking the power of thrombospondins: regulation of thrombospondins expression. Matrix biology : journal of the International Society for Matrix Biology. 2014; 37:69–82. [PubMed: 24582666]

Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B, Sher A, Litke AM, Lambris JD, Smith SJ, John SW, Barres BA. The classical complement cascade mediates CNS synapse elimination. Cell. 2007; 131:1164–1178. [PubMed: 18083105]

Swartzwelder HS, Wilson WA, Tayyeb MI. Age-dependent inhibition of long-term potentiation by ethanol in immature versus mature hippocampus. Alcoholism, clinical and experimental research. 1995; 19:1480–1485.

Tenorio G, Kulkarni A, Kerr BJ. Resident glial cell activation in response to perispinal inflammation leads to acute changes in nociceptive sensitivity: implications for the generation of neuropathic pain. Pain. 2013; 154:71–81. [PubMed: 23103436]

Ullian EM, Christopherson KS, Barres BA. Role for glia in synaptogenesis. Glia. 2004; 47:209–216. [PubMed: 15252809]

Verkhratsky A, Nedergaard M, Hertz L. Why are astrocytes important? Neurochem Res. 2015; 40:389–401. [PubMed: 25113122]

Vetreno RP, Crews FT. Adolescent binge drinking increases expression of the danger signal receptor agonist HMGB1 and Toll-like receptors in the adult prefrontal cortex. Neuroscience. 2012; 226:475–488. [PubMed: 22986167]

Vetreno, RP.; Crews, FT. Innate Immune Signaling and Alcoholism. Springer; 2013. p. 251-278.

Williams B, Ponesse J, Schachar R, Logan G, Tannock R. Development of inhibitory control across the life span. Developmental psychology. 1999; 35:205–213. [PubMed: 9923475]

Worst TJ, Tan JC, Robertson DJ, Freeman WM, Hyytia P, Kiianmaa K, Vrana KE. Transcriptome analysis of frontal cortex in alcohol-preferring and nonpreferring rats. J Neurosci Res. 2005; 80:529–538. [PubMed: 15846778]

Yang Z, Kyriakides T, Bornstein P. Matricellular proteins as modulators of cell-matrix interactions: adhesive defect in thrombospondin 2-null fibroblasts is a consequence of increased levels of matrix metalloproteinase-2. Molecular biology of the cell. 2000; 11:3353–3364. [PubMed: 11029041]

Zapala MA, Hovatta I, Ellison JA, Wodicka L, Del Rio JA, Tennant R, Tynan W, Broide RS, Helton R, Stoveken BS, Winrow C, Lockhart DJ, Reilly JF, Young WG, Bloom FE, Lockhart DJ, Barlow C. Adult mouse brain gene expression patterns bear an embryologic imprint. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102:10357–10362. [PubMed: 16002470]

Zhou HJ, Zhang HN, Tang T, Zhong JH, Qi Y, Luo JK, Lin Y, Yang QD, Li XQ. Alteration of thrombospondin-1 and-2 in rat brains following experimental intracerebral hemorrhage. Laboratory investigation. Journal of neurosurgery. 2010; 113:820–825. [PubMed: 20136391]

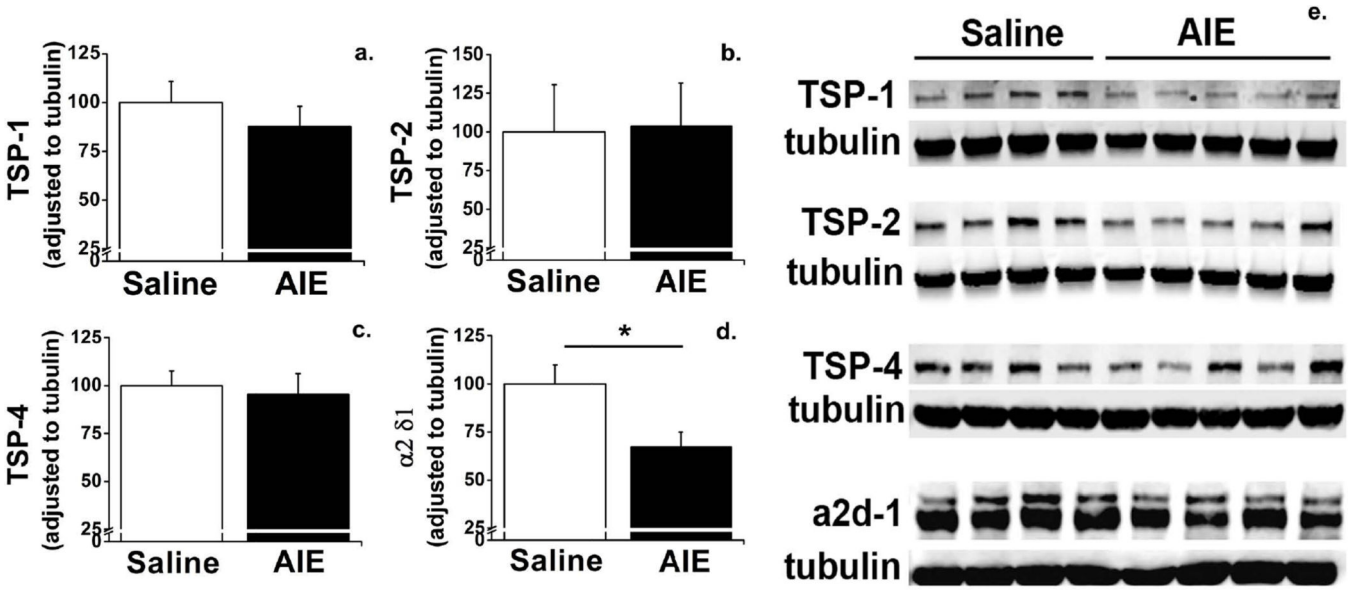

Figure 1. AIE does not upregulate TSPs 24 hours after the 4th ethanol dose

Quantification of Western blot analysis revealed that AIE had no effect on TSP-1 (a), TSP-2 (b), or TSP-4 (c) levels early in the AIE paradigm. However, α2δ-1 (d) expression was significantly reduced in AIE hippocampal lysates. Representative blots including loading control (e). n=4 and 5 animals/treatment (control and AIE, respectively), $p<0.05$*.

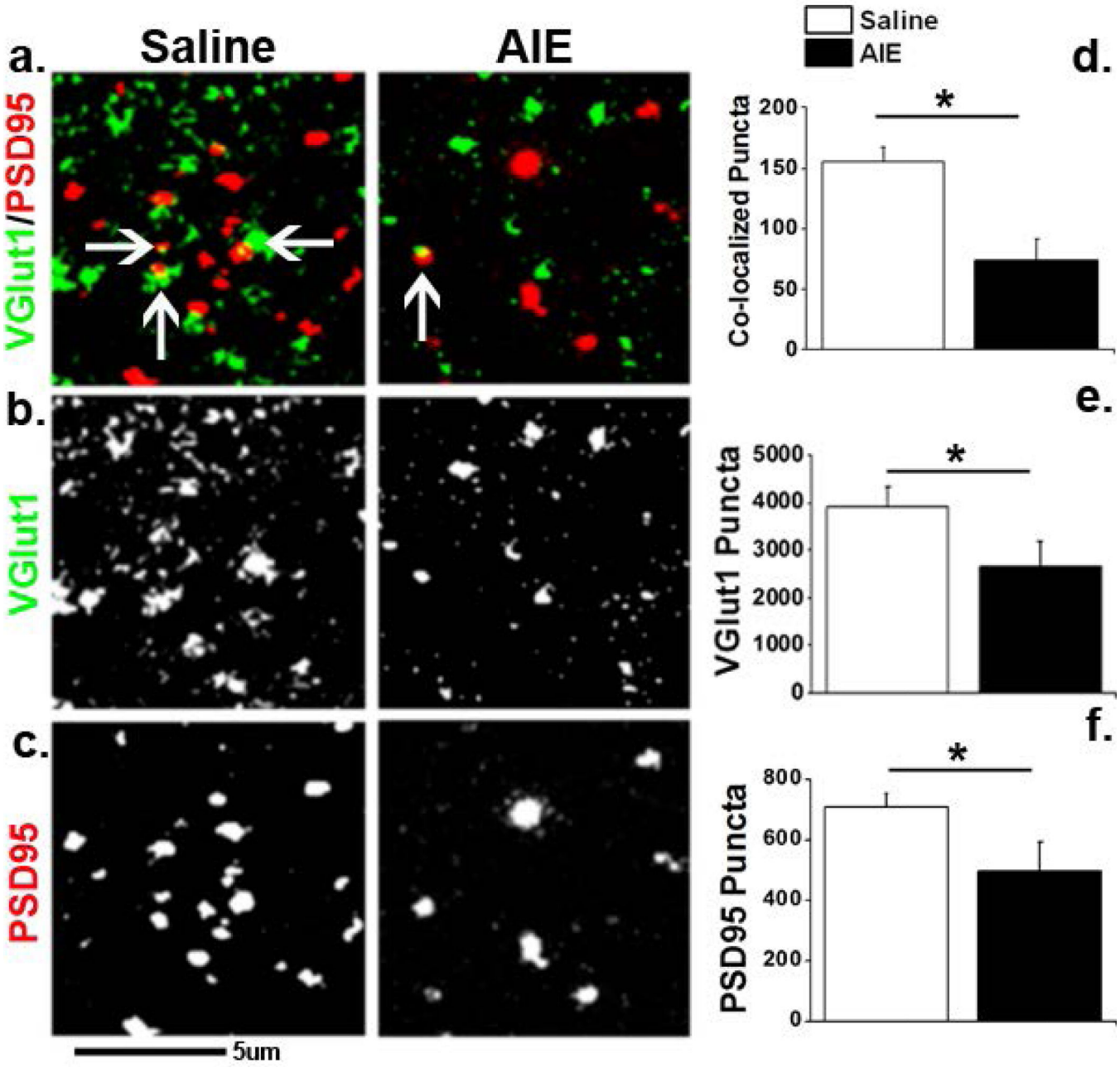

Figure 2. Reduced synapse number 24 hours after the 4th AIE dose

Representative images of merged (a), pre- (VGlut1) (b), and post- (PSD-95) (c) synaptic markers 24 hours after the last ethanol dose. Arrows indicate yellow co-localized puncta, i.e. synapses (a). Quantitative analysis reveals that AIE significantly decreased the number of co-localized pre- and post-synaptic puncta (d); this decrease was driven by a reduction in both presynaptic VGlut1 (e) and post-synaptic PSD-95 (f). Three image stacks per animal and n=5 animals/treatment, $p<0.05$*.

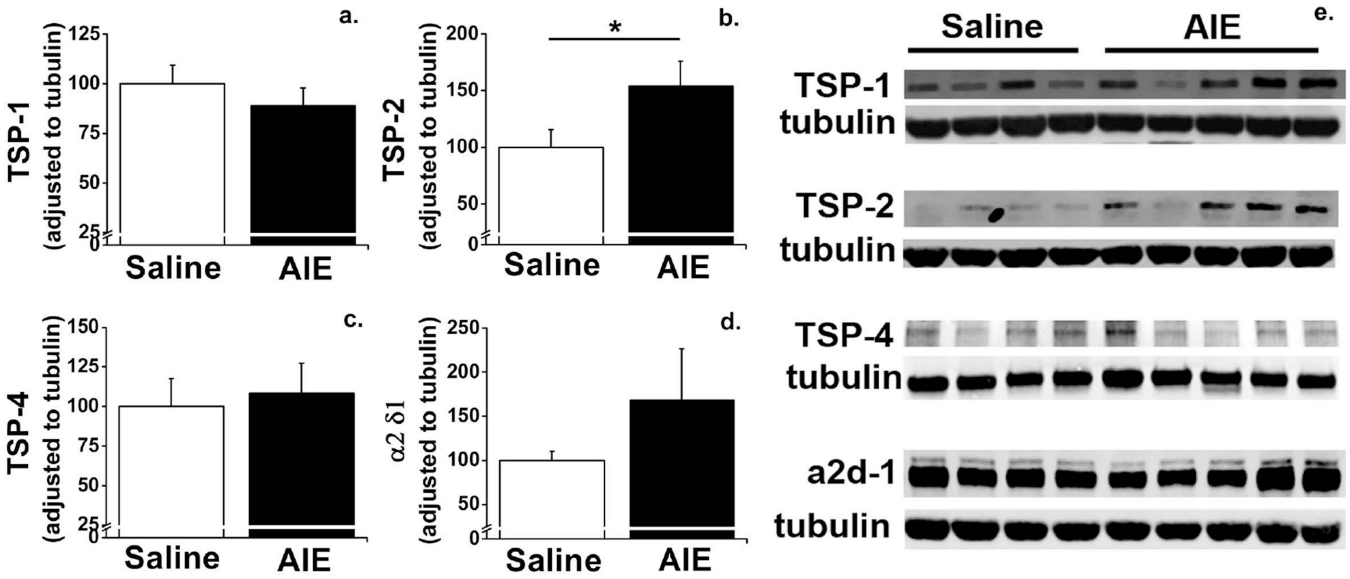

Figure 3. AIE promotes the upregulation of TSP-2 24 hours after the last ethanol dose

Western blot quantification of hippocampal lysates revealed that AIE had no effect on TSP-1 (a) levels, while TSP-2 was significantly upregulated (b). TSP-4 (c) and α2δ-1 (d) expression remained indistinguishable from saline controls. Representative blots including loading control (e). n=4 and 5 animals/treatment (control and AIE, respectively), $p<0.05$*.

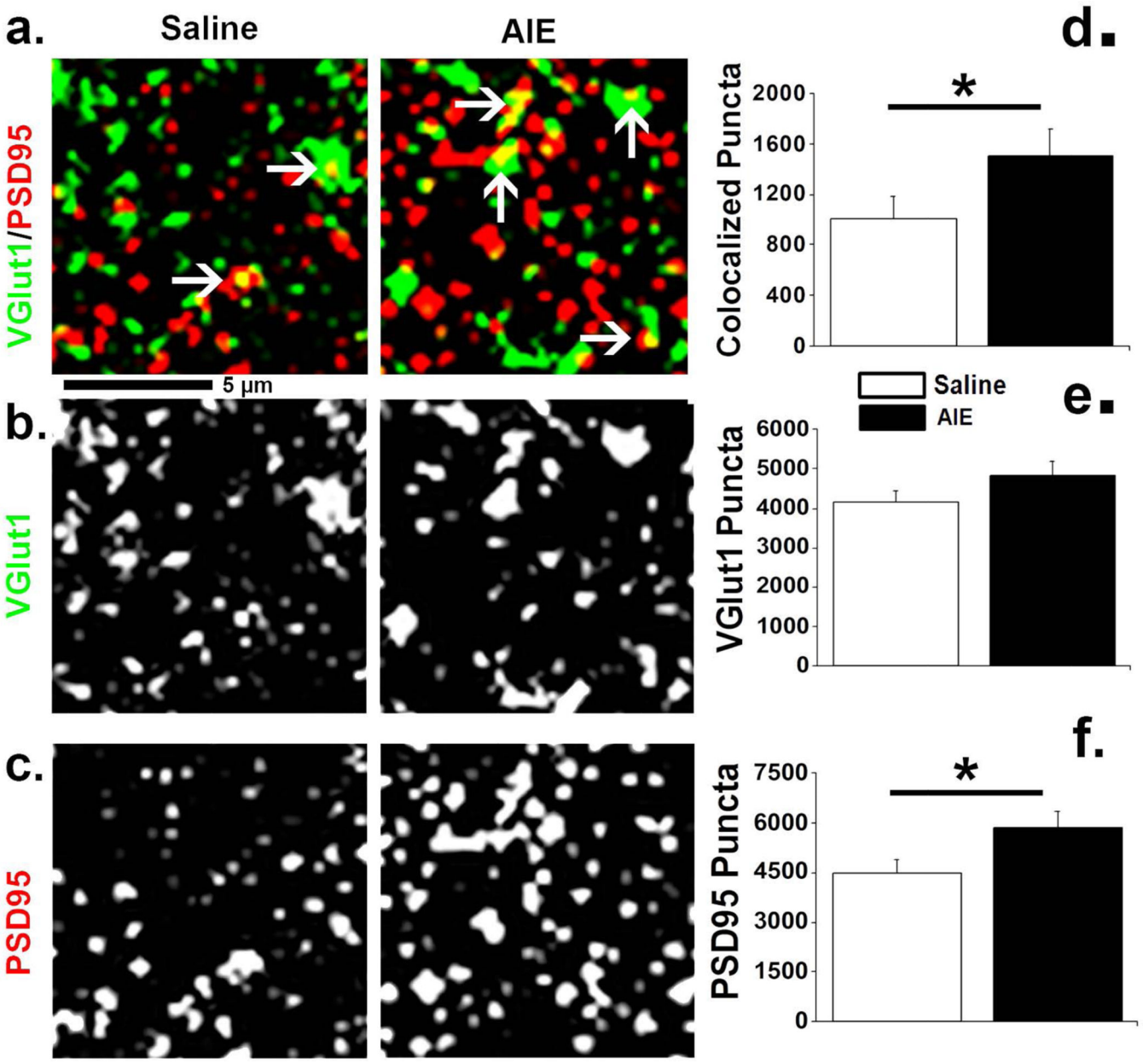

Figure 4. Excitatory synapse formation 24 hours after AIE

Representative images of merged (a), pre- (VGlut1) (b), and post- (PSD-95) (c) synaptic markers 24 hours after the last ethanol dose. Arrows indicate yellow co-localized puncta, i.e. synapses (a). Quantitative analysis reveals that AIE significantly increased the number of co-localized pre- and post-synaptic puncta (d); this increase was not driven by increases in presynaptic VGlut1 (e) but by increased PSD-95 (f). Three image stacks per animal and n=5 animals/treatment, $p<0.05$*.

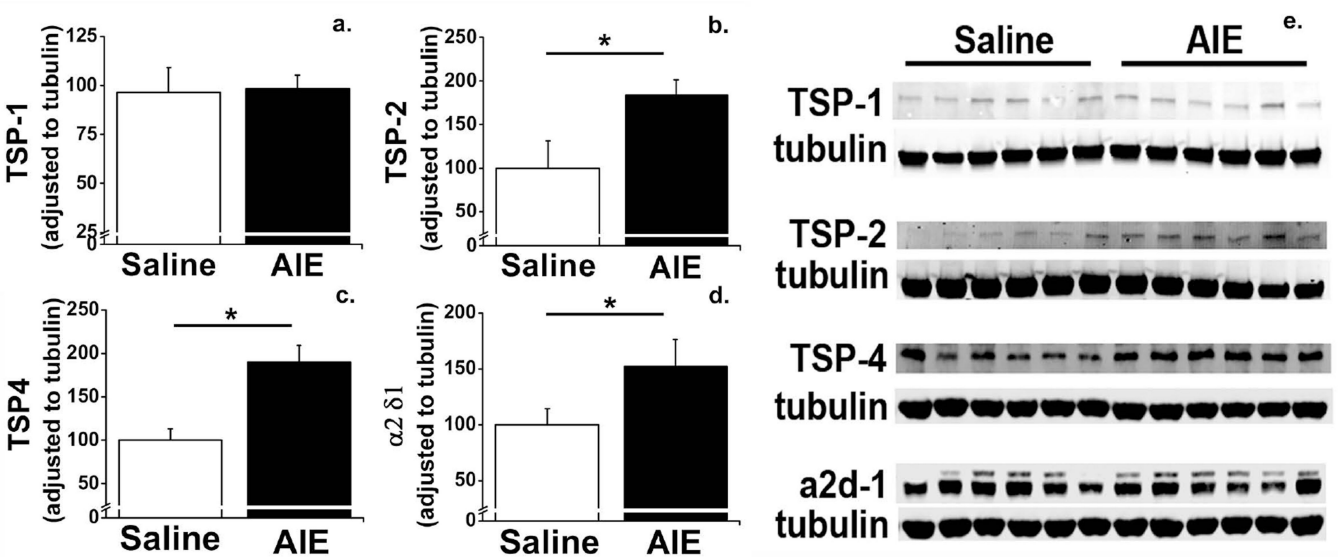

Figure 5. AIE promotes the upregulation of multiple TSPs and their neuronal receptor in adulthood

Western blot quantification of hippocampal lysates revealed that AIE had no effect on TSP-1 (a) levels, while TSP-2 (b), TSP-4 (c), and α2δ-1 (d) were significantly upregulated in adulthood. Representative blots including loading control (e). n=6 animals/treatment, $p<0.05$*.

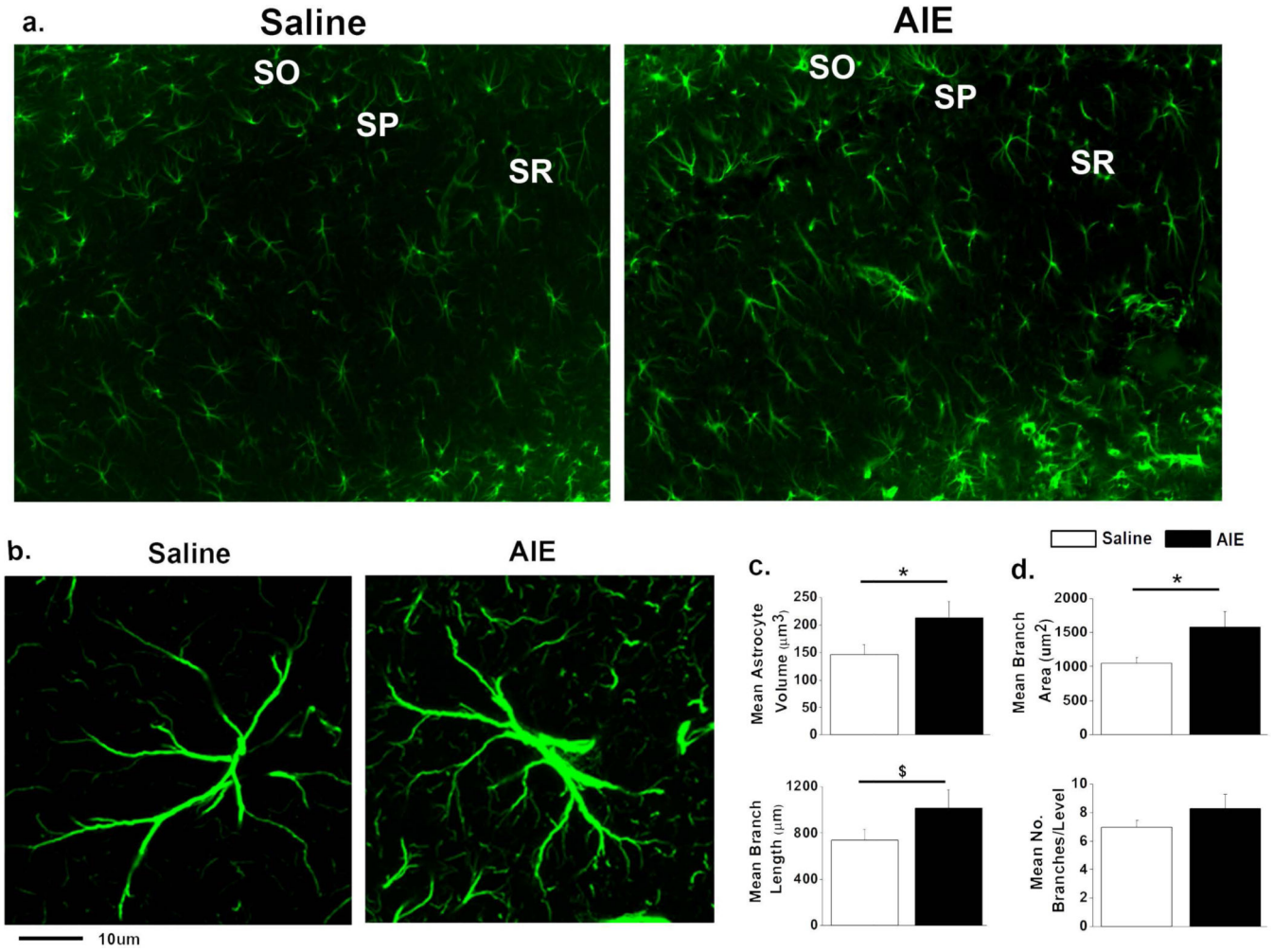

Figure 6. AIE results in protracted upregulation of astrocyte reactivity in adulthood

Representative images of immunohistochemistry for glial fibrillary acidic protein (GFAP) in CA1 hippocampus, SO = stratum oriens, SP = stratum pyramidale, SR = stratum radiatum (a) and higher magnification image of SR (b). AIE results in delayed upregulation of astrocyte reactivity indicated by an increase in astrocyte volume (c), and an increase in astrocyte branch area (d), along with a trend towards an increase in branch length (e). The overall number of branches remained similar to saline control animals (f). Three image stacks per animal and n=4 animals/treatment, $p<0.05$*, $p=0.065^{\$}$.

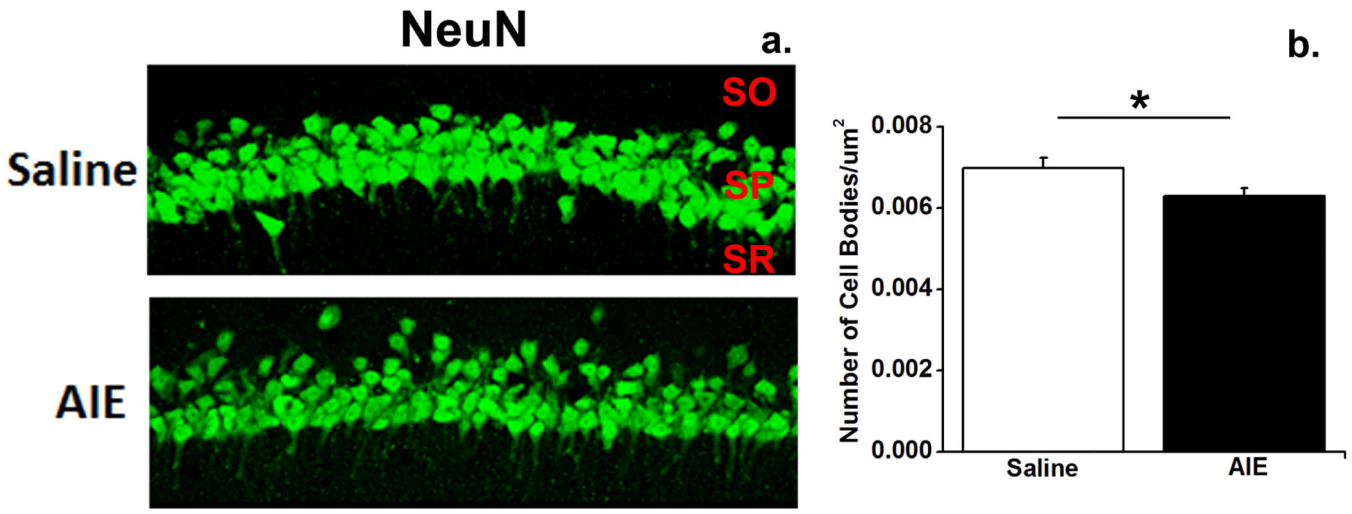

Figure 7. AIE reduces the number of NeuN-positive cells within hippocampal CA1 in adulthood

Immunohistochemistry for the neuronal nuclear antigen (NeuN) within the stratum pyramidale (SP) zone, located between the stratum oriens (SO) and the stratum radiatum (SR), 24 days after the last ethanol dose. Representative images for saline- and AIE-treated animals (a). Quantification revealed a significant decrease in the number of NeuN-positive cells (b) in adulthood within the hippocampal CA1 SP layer. Three image stacks per animal and n=4 animals/treatment, $p<0.05$*.

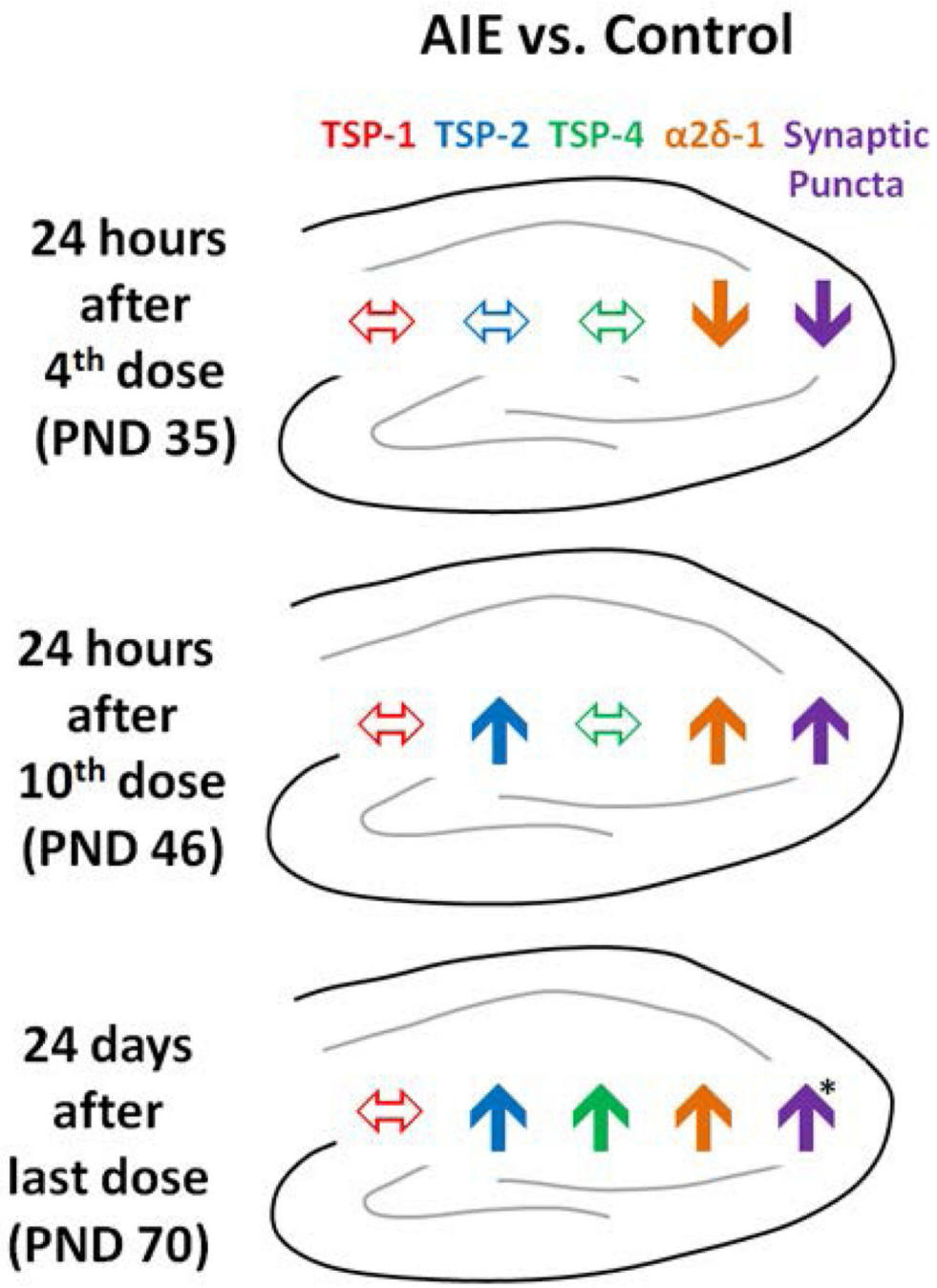

Figure 8. Summary of Results

TSP expression, α2δ-1 expression, and associated changes in co-localized synaptic puncta in the hippocampus at all three time points. ⇩ Indicates a decrease, ⇧ indicates an increase, ⇔ indicates no change at that particular time point for each measure when compared to control. * indicates one previously published data point from Risher et al., (2015).