Modulation of the Astrocytic Influence on Synapse Structure and Function by Ethanol and Cholinergic Stimulation

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Abstract

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It is increasingly becoming clear that astrocytes release numerous factors that contribute to the process of synaptogenesis. The fact that astrocytes express a wide range of neurotransmitter receptors suggest that they are capable of responding to environmental clues from surrounding neurons, yet knowledge about the signals controlling the release of factors from astrocytes is relatively sparse. Understanding how astrocytes influence typical synapse formation may provide insight as to their role in mediating disease. Using an *in vitro* co-culture system, the present study investigates how an important physiological neurotransmitter, acetylcholine, and a known developmental neurotoxicant, ethanol, each independently and differently act on astrocytes to modulate synapse formation and function.

Here we report that primary rat hippocampal neurons (E21) grown in culture for 13 days and co-cultured for 24 hours with astrocytes pre-treated with either carbachol, an acetylcholine receptor agonist (0.010, 0.100, 1 mM) or ethanol (25, 50, 75 mM) for 24 hours, show increased expression of the pre- and post-synaptic proteins, synaptophysin and PSD-95, with carbachol pre-treatment inducing a greater effect than ethanol. Immunocytochemical labeling of the same proteins, followed by confocal imaging and 3-dimensional object analysis shows that carbachol (1 mM) pre-treatment of astrocytes resulted in a 3.2-fold potentiation of synaptic structure formation, an effect mediated by cholinergic actions on the M3 muscarinic and nicotinic ACh receptors. Ethanol pre-treatment of

astrocytes induced a bi-phasic increase in the number of synapses, with 50 mM ethanol inducing a 4.5-fold increase, an effect greater than 75 mM pre-treatment (2.6-fold).

To corroborate that the observed increase in synaptic structures is reflected in an increase in functionality, whole cell patch clamp techniques were used to measure spontaneous miniature excitatory post-synaptic currents in neurons after co-culture with pre-treated astrocytes. A higher frequency of events was observed in neurons of both treatment groups, suggesting more functional synapses, however, a second population of neurons in the ethanol (50 mM) pre-treated group showed a decreased frequency of events.

Both astrocyte-released thrombospondin (TSP) and cholesterol have been shown to potentiate synapse formation in retinal ganglion cells. Our laboratory has previously shown that treatment of astrocytes with carbachol induces increased TSP1 release, while ethanol causes increased efflux of cholesterol-containing lipoproteins from astrocytes. Investigations of these two factors as potential candidates in the observed effects showed that both TSP1 and cholesterol-containing lipoproteins (CCL) are sufficient to induce synapse formation in hippocampal neurons. Pharmacologically blocking the neuronal receptors which these factors act upon showed that the release of TSP1 from carbachol-treated astrocytes and the release of cholesterol-containing lipoproteins from ethanol pre-treated astrocytes are responsible, at least in part, for the observed potentiation of synaptic structures in the co-culture system.

Together these data confirm the astrocytic role in synapse formation and development, and clearly shows that they may mediate portions of the important physiological process of cholinergic stimulation during brain development. Additionally, the effects observed after astrocyte pre-treatment with ethanol suggests that astrocytes also may play a role in influencing some of the cognitive and learning disabilities observed in children exposed *in utero* to alcohol. While both cholinergic stimulation of astrocytes and pre-treatment with ethanol show broadly similar effects, the differences suggest that cholinergic stimulation may move the process of synapse formation forward in a regulated manner, while ethanol may be acting in a way that dysregulates the typical process.

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Glossary

22HC	22R-hydroxycholesterol
ACh	Acetylcholine
ABCA1	ATP-binding cassette A1
ABCG1	ATP-binding cassette G1
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ApoER2	Apo-E Receptor 2
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CARB	Carbachol
C-HDL	Cholesterol-containing High Density Lipoprotein
CICR	Calcium-induced, Calcium-release
CNS	Central Nervous System
DIC	Days in Culture
DMEM	Dulbecco's Modified Eagle's Medium
EtOH	Ethanol
FAS	Fetal Alcohol Syndrome
FASD	Fetal Alcohol Spectrum Disorders
GABA	γ-Aminobutyric acid
HBSS	Hank's Balanced Salt Solution
ICC	Immunocytochemistry
IEI	Inter-Event Interval
IP_3	Inositol 1, 4, 5 - trisphosphate
LDS	Lithium Dodecyl sulfate

LXR	Liver X Receptor
mEPSC	miniature Excitatory post-synaptic current
nAChR	Nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartic acid
NR1	NMDA receptor subunit NR1
NR2A	NMDA receptor subunit NR2A
NR2B	NMDA receptor subunit NR2B
PDL	Poly-d-lysine
PLC	Phospholipase C
PLD	Phospholipase D
PLO	Poly-L-ornithine
PFA	Paraformaldehyde
PVDF	Polyvinylidene difluoride
PSD-95	Post-synaptic density protein-95
RA	9-cis-retinoic acid
RAP	Receptor associated protein
RXR	Retinoid X Receptor
SDS	Sodium dodecyl sulfate
TBST	Tris-buffered saline with Tween-20
TSP	Thrombospondin
TTX	Tetrodotoxin

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Nothing is ever accomplished alone. There are always many who contribute, in numerous ways, and in ways often unknown to them. I'd first like to thank my Dissertation Committee. I would like to express my great appreciation to Lucio G. Costa, for giving me this opportunity to investigate the intriguing area of neuron-glia interactions, for helping me reign in my, at times, grandiose ideas with the constant reminder of the power of simple, focused experiments, for pushing me further than I thought I could go, and for patiently supporting me, especially when the need came. I'd also like to thank Marina Guizzetti, who was a constant in the early years, providing daily guidance and training during the development of the project and for her friendship over the years. Her example, scientific interest and approach will always be valued. Of course, I am also extremely grateful Andrés Barria, who opened up his laboratory to take on the challenge of training me in whole cell patch clamping, and who consistently showed patience throughout my incessant repeated questions, always interacting with respect and encouragement and a clear desire for me to understand. Not only has he provided me with solid scientific training, and a second laboratory full of wonderful and supportive people, but his support and words consistently offered a sense of perspective, which was definitely appreciated. I will also always appreciate the contributions of Terry Kavanagh, which were consistent throughout all of my years in graduate school. Terry is capable of answering any scientific question with depth and nuance, but also was always willing to give of his time, regardless of his schedule, to offer advice, both scientific, but more importantly, personal, with real interest, understanding, truth and sincerity. I definitely valued that and am sure others after me will. Thanks are due to Zhengui Xia, who not only offered consistent opportunity and training while I was her TA, but her participation on my committee was definitely appreciated.

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Sociology and ultimately, during the one year spent as a Student Fellow in the Louise W. and Edmund J. Kahn Liberal Arts Institute. Here I was able to meet weekly with a small, interdisciplinary group of students and faculty, to explore, discuss and debate, from our unique perspectives, one topic: Form and Function. Together this experience informed the development of our independent research projects and was an incredible exercise in intellectual exchange and growth. I will forever be grateful for my experience as an Ada Comstock Scholar at Smith, for my time in the Kahn Institute, and for the intellectual generosity and example that my colleagues provided during that time. I truly was given a unique and rare gift.

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Chapter 1:

Introduction

1.1 Fetal Alcohol Syndrome: The effect of ethanol on the developing brain

In utero ethanol exposure causes permanent and devastating effects on the developing brain. The most serious outcome after maternal consumption of alcohol during pregnancy results in a diagnosis of Fetal Alcohol Syndrome (FAS), which was first named as such, in 1973 (Jones and Smith, 1973). Children born with FAS present with a unique cluster of minor facial abnormalities, including a smooth philtrum, thin vermillion border, and short palpebral fissures; growth deficiencies, which may be evident pre- and/or postnatally; and finally, either structural or functional central nervous system abnormalities, or both (Astley, 2011; CDC, 2005). Within each potential area of effect, there is great variability in the manifestation when comparing those exposed.

In addition to the more overt outcomes that lead to a diagnosis of FAS, there are a range of equally variable effects associated with alcohol exposure that are less easily recognized and are classified as Fetal Alcohol Spectrum Disorder (FASD) (Sokol et al., 2003). As a non-diagnostic term, FASD incorporates the range and diversity of effects, from severe to mild, caused by prenatal ethanol exposure, which includes the diagnosis of full blown FAS, as well as the classifications of Partial FAS, and Alcohol-related Neurodevelopmental Disorder (Astley, 2011). The most debilitating and long-lasting consequences are the cognitive and neurobehavioral effects. Even moderate maternal drinking has been shown to be associated with attention and memory deficits, hyperactivity and learning disabilities, which result in social and academic difficulties in children and problems later in life with executive functioning and antisocial behavior (Sokol, et al., 2003; Warren and Foudin, 2001). For all outcomes under the umbrella of FASD, the severity of effects depends on multiple

factors, including the pattern and amount of ethanol exposure, the time during development that exposure occurs, as well as genetic and socioeconomic factors, maternal stress and nutritional status (Guerri et al., 2009; May and Gossage, 2011; Warren and Li, 2005).

Recognized as the leading preventable cause of intellectual disability in the United States (Bailey and Sokol, 2008; Warren et al., 2011), obtaining accurate prevalence data is difficult. Estimates of the prevalence of FAS in the United States range from 0.2 to 1.5 children per 1,000 live births (CDC, 2005), and in some studies reach as high as 2 to 7 cases per 1,000. In certain areas of the world, or in high risk populations, those estimates can be even higher. Additionally, the number of people affected by *in utero* ethanol exposure increases if cases of FASD are included in estimates, making prenatal exposure an important public health problem (May et al., 2009). The neurological complexity of the disease, difficulties in relating self-reported alcohol consumption with developmental endpoints, and the need for a team of professionals to diagnosis the condition, makes accurate and early diagnosis, effective treatment and clear scientific understanding of the condition difficult (Astley and Clarren, 2000; May, et al., 2009; Warren, et al., 2011).

Understanding the mechanisms of prenatal alcohol exposure's effect is necessary and important for the treatment or amelioration of this condition. While much work in this regard has been completed and consensus on some aspects of the mechanisms of ethanol on the developing brain has been reached, the variety of effects that are evident in the differential outcomes in children exposed to *in utero* alcohol are also clearly reflected even in the controlled scientific work that attempts to understand the disease. Variable outcomes observed across studies are due to a number of factors, including differences in the amount of exposure, the dosing regimens or patterns of exposure (acute, chronic, or binge like), clear differences due to exposure occurring at varying developmental time points; and differences seen when endpoints are measured at varied times after exposure. The variability of results due to these factors highlights the complexity of the mechanisms by which ethanol exerts its harmful effects.

1.2 Ethanol and Synapse Formation

Multiple mechanisms have been proposed to explain the neurodevelopmental effects of ethanol, including the induction of neuronal apoptosis, oxidative stress, inhibition of neuronal precursor and glial cell proliferation and alterations in the process of neuronal differentiation and migration (Cartwright et al., 1998; Henderson et al., 1995; Kotch et al., 1995; Miller, 1996; Miller and Robertson, 1993; Warren and Foudin, 2001). Relevant to the scope of this proposal are the effects of ethanol on one aspect of neuronal development, synaptogenesis. The learning and memory disabilities observed in those exposed *in utero* to ethanol suggest that one possible mechanism of ethanol's effects is interference with the process of synapse formation in the hippocampus of the developing brain. Several studies have reported that ethanol inhibits synapse formation and function (Bellinger et al., 1999; Kuge et al., 1993; Whitcher and Klintsova, 2008; Yanni and Lindsley, 2000). Hippocampal slices prepared from rats exposed to ethanol vapors during the brain growth spurt displayed a decreased efficacy of synapses, suggesting that ethanol treatment either caused a reduction in synapse number, in transmitter release or in the number of post-synaptic receptors (Bellinger, et al., 1999). A decrease in CA1 hippocampal synapse density, measured in electron micrographs, was observed in rats exposed to ethanol throughout embryonic and neonatal development (Kuge, et al., 1993). In hippocampal neurons incubated with ethanol for six days, the number of PSD95 puncta was reduced, suggesting a reduction in synaptogenesis, although this effect was a consequence of decreased dendritic arborization (Yanni and Lindsley, 2000). In a binge-like model of exposure, spine density was decreased in the pre-frontal cortex of rats postnatally exposed to ethanol without a change in dendritic morphology (Whitcher and Klintsova, 2008).

However, in contrast, some studies suggest alternative effects on synapse formation and function. In rat hippocampal slices from P3-P4 animals, but not P6, exposure to concentrations of ethanol as low as 15mM potently accelerated the stabilization of excitatory synapses, an effect mediated by pregnenolone-sulfate, a neurosteroid derived from cholesterol (Mameli and Valenzuela, 2006). Additionally, chronic exposure to 50 mM ethanol in primary hippocampal cultures increased the clustering of the NMDA receptor scaffolding protein, PSD-95 (Carpenter-Hyland and Chandler,

2006). More recently it was found that exposure of hippocampal neurons *in vitro* for 7 days to 80 mM ethanol increased synaptic localization and clustering of NMDA receptors measured by immunocytochemistry, an effect attributed to an adaptive homeostatic increase as a consequence of acute ethanol-induced NMDAR channel activity inhibition (Clapp et al., 2010). Furthermore, adult mice, exposed prenatally to moderate levels of alcohol showed impaired NMDAR-dependent synaptic plasticity, with changes in the expression of NMDA receptor subunits. Synaptic protein levels of the obligatory NR1 subunit were increased, NR2B subunits were decreased, and levels of NR2A subunits did not change. Interestingly, levels of NR3A, a subunit whose insertion lowers calcium conductance, and may protect from overstimulation, were increased (Brady et al., 2013). Clearly, the effects of ethanol on synapse formation and plasticity are quite varied depending on numerous factors, and one clear mechanism of action most likely does not exist.

1.3 Astrocytes and Neuronal Development

Increasing evidence shows that astrocytes, the most abundant cells in the brain, are not passive players providing only metabolic and structural support to neurons, as previously believed, but are important contributors to central nervous system development and function. While their roles in forming the blood brain barrier, maintaining ion homeostasis, delivering glucose, and recycling glutamate from the synaptic cleft are well established (Barker and Ullian, 2010; He and Sun, 2007; Nedergaard et al., 2003), recent work has shown them to be actively involved in neuritogenesis (Giordano et al., 2011; Guizzetti et al., 2010), neuronal differentiation, and synaptogenesis (Christopherson et al., 2005; Mauch et al., 2001; Pfrieger and Barres, 1997; Ullian et al., 2001).

Astrocyte processes extend into the synaptic space and are in close connection with neuronal synapses, making them ideally located to interact with neurons and influence synapse formation (Nedergaard, et al., 2003). It is estimated that a single CA1 adult rat astrocyte may be in contact with approximately 140,000 synapses (Bushong et al., 2002). It has been shown that the ability of

neurons to form synapses in the absence of glial cells is limited, but their presence causes an increase in the number of mature and functional synapses (Pfrieger and Barres, 1996). The findings of this study are in support of this and show that hippocampal neurons grown in the presence of astrocytes cause a 1.7 fold increase in the number of synaptic structures when compared to neurons grown alone (Figure 1.1). These facts suggest that astrocytes may be necessary to maintain synaptic stability and to facilitate the maturation of newly formed, highly plastic synapses (Nagler et al., 2001; Pfrieger and Barres, 1996, 1997; Ullian et al., 2004; Ullian, et al., 2001).

Many of the roles ascribed to astrocytes are initiated by the secretion of factors that influence neuronal development (Theodosis et al., 2008), some of which have been shown to both accelerate neuronal maturation and directly induce synapse formation (Waites et al., 2005). Although the precise mechanism is currently being actively investigated and debated, astrocytes release neuroactive "gliotransmitters," such as glutamate, ATP and the NMDA receptor co-agonist, d-serine, all of which are capable of modulating synaptic transmission (Araque et al., 1998a; Araque et al., 1998b; Nedergaard, et al., 2003; Newman, 2003; Oberheim et al., 2006; Panatier et al., 2011; Parri and Crunelli, 2007; Volterra and Meldolesi, 2005).

Both astrocyte-released cholesterol and astrocyte-released thrombospondin-1 (TSP-1) have been shown to contribute to astrocyte-induced synaptogenesis (Christopherson, et al., 2005; Mauch, et al., 2001; Pfrieger, 2003). In addition, pregnenolone-sulfate and estradiol, two neurosteroids derived from cholesterol and produced by both neurons and astrocytes (Zwain and Yen, 1999), also play a role in synapse induction and stabilization (Mameli and Valenzuela, 2006), and neurosteroid levels have been shown to be altered by ethanol (Caldeira et al., 2004). Clearly, over the past 15 years, the understanding of the role of astrocytes has evolved from that of simply being seen as neuronal structural support cells, to being active players in synaptogenesis and participants in what is now termed the 'tripartate synapse' (Araque et al., 1999).

Equally compelling is the observation that synaptogenesis occurs during the same time that astrocytes are proliferating (Dobbing and Sands, 1979), suggesting that astrocytes may be involved in

synaptic maturation (Stevens, 2008). The fact that astrocytes express a wide range of receptors, including most neurotransmitter receptors (Araque, et al., 1999; Lalo et al., 2006; Lee et al., 2010; Porter and McCarthy, 1997; Schipke et al., 2001; Sharma and Vijayaraghavan, 2001; Verkhratsky and Kirchhoff, 2007; Verkhratsky and Steinhauser, 2000) suggests that they are capable of responding to environmental cues from surrounding neurons (Araque et al., 2002; Lalo, et al., 2006; Stevens, 2008; Theodosis, et al., 2008), yet knowledge about the signals controlling the release of factors from astrocytes is relatively sparse.

1.4 Astrocytes and Cholinergic Stimulation

Work in our laboratory has been focused for several years on the role played by acetylcholine (ACh), a major excitatory neurotransmitter, in modulating the astrocytic influence on neuronal development. That acetylcholine has an important influence on early brain development is well supported. Most components of the cholinergic system are present in the fetus prenatally (Abreu-Villaca et al., 2011; Lauder and Schambra, 1999); both choline acetyltranferase and acetylcholinesterase are found as early as gastrulation (Mansvelder et al., 2006), and neuronal precursor cells and glial cells proliferate after cholinergic-stimulation (Guizzetti et al., 1996; Ma et al., 2000). The release of ACh from growth cones during axonal path finding influences not only proliferation, but neuronal differentiation, migration and growth (Lauder and Schambra, 1999; Yao et al., 2000). Additionally, spontaneous activity is also thought to influence synapse formation during early development (Garaschuk et al., 1998; Moody and Bosma, 2005) and developmental cholinergic modulation of excitatory spontaneous activity is temporally regulated and diminishes with time, suggesting that ACh release may play a role in regulating synapse formation (Garaschuk, et al., 1998; Wong et al., 2000).

That astrocytes have been shown to be integral to neuronal development and that they express receptors for and respond to synaptic release of ACh (Araque, et al., 2002) has led our laboratory to hypothesize that stimulation of astrocyte acetylcholine receptors may promote

neuronal development by causing the expression and release of factors that create an environment conducive to it. Recent work in our laboratory, to this end, has shown that neurite outgrowth is potentiated in neurons after co-culture with astrocytes that have been pre-treated with a cholinergic agonist, as well as in hippocampal slices after cholinergic stimulation. This effect is mediated by the astrocytic- M3 muscarinic ACh receptor and is due in part to increased release of the astrocyte secreted factors, fibronectin and laminin (Giordano, et al., 2011; Guizzetti et al., 2008). Additionally, we have shown that ethanol, a strong inhibitor of muscarinic signaling, inhibits carbachol-stimulated, astrocyte-induced neuritogenesis in both systems, as well as the carbachol-induced release of fibronectin and laminin from astrocytes by interfering with M3 muscarinic receptor signaling. In these studies, without cholinergic stimulation, ethanol alone, had no effect on astrocyte-induced neuritogenesis (Guizzetti, et al., 2010).

The knowledge that cholinergic stimulation of astrocytes induces the release of factors that promote neurite outgrowth and that ethanol interferes with that effect, led us to hypothesize that this role may extend to the next stage of neuronal development, that of synapse formation. The fact that ethanol is a strong inhibitor of ACh, muscarinic receptor signaling, suggested that it may have the potential to hinder any positive astrocytic influence on synaptic development, making this an important area of investigation. This is the focus of the following work.

Early on in this project, initial experiments were performed to assess the effect of carbachol stimulation and ethanol treatment on synaptic structure formation. Initial analysis showed that cholinergic stimulation of astrocytes did indeed potentiate synapse formation, as expected, but unlike the findings in the neurite outgrowth experiments, treatment of astrocytes with ethanol alone also robustly increased synaptogenesis, as will be subsequently shown. Our preliminary findings were surprising and indicated that ethanol seemed to be acting directly on astrocytes to influence hippocampal synapse formation. These intriguing findings lead us to modify our initial investigatory path to focus instead on characterizing how cholinergic stimulation of astrocytes and how ethanol pre-treatment of astrocytes, independently, and perhaps differently, modulate the astrocytic effect

on synapse formation.

The work presented here will more broadly investigate how the factors that astrocytes release influence synapse formation in hippocampal neurons and how both cholinergic stimulation and ethanol treatment modulate that effect. To do this I have optimized and modified an *in vitro* primary astrocyte-neuron co-culture system used in our laboratory for the determination of the effect of astrocytes on neuritogenesis. Because we are investigating effects on synapse formation, and are interested in how astrocytes modulate the synaptogenic effect, this necessitated a primary hippocampal neuron culture system that would support long-term growth, with neurons that differentiate and develop a neuronal network and synaptic structures, without the influence of astrocytes. Modification and optimization of the culturing method was necessary, and a method to quantify synaptic structure formation was developed. These optimized methods have been previously described in detail (Roque et al., 2011).

Using these methods, I will show that astrocytes pre-treated with the cholinergic agonist, carbachol, or physiologically relevant concentrations of ethanol, release factors that potentiate synapse formation in hippocampal neurons, and I will characterize the structural, as well as the functional effect of each more completely. Additionally, these results will show that it is the release of thrombospondin-1 from carbachol-treated astrocytes and cholesterol-containing lipoproteins from ethanol pre-treated astrocytes that are, at least in part, responsible for the observed effects.

These results add to the growing body of knowledge regarding the mechanisms by which astrocytes contribute to synaptogenesis. Moreover, they suggest the involvement of astrocytes in mediating some of the effects of ethanol on the developing brain, and may provide insight into the varying degrees of cognitive and learning deficits that are found in children affected by FASD. The fact that astrocytes are integral to neuronal development suggests that they play an important, but often overlooked role in neurological disorders and disease (Ransom et al., 2003).

Chapter 2:

Materials and Methods

2.1 Hippocampal Neuron Preparation and Culture

Primary hippocampus neurons from E21 Sprague Dawley rats were prepared as previously described (Roque, et al., 2011). Briefly, cells were plated in 6-well plates at a density of 750,000 cells/mL or 1.5 x 10^6/well for protein expression experiments, or 80,000 cells/mL on glass coverslips with paraffin spacers adhered for neuronal co-culture experiments, maintained in Neurobasal-A medium containing B-27 neuronal survival and growth factors (1%), 3 mM GlutaMax, 30 mM D—(+) glucose solution, 0.5% fungizone, and 100 μg/mL gentamicin. Neurons were grown at 37˚C for 12-13 days prior to co-culture. Both plates and coverslips were coated with poly l-ornithine hydrobromide (Sigma, P3655, 15 μg/mL). To hinder astrocyte proliferation, cytosine D-arabinofuranoside hydrochloride (Sigma, C6654) was added at a final concentration of 2.5uM to each well to each well after three days in culture. One-third of the medium was changed every three to four days.

2.2 Primary Astrocyte Preparation and Culture

Primary cortical astrocytes were dissected and prepared from E21 Sprague Dawley rat pups, as previously described (Guizzetti, et al., 1996; Roque, et al., 2011) and cultured in 75 cm^2 flasks coated with PDL (40 μg/mL) for one to two weeks prior to passage for experimental use. Medium was changed every 2-3 days. To increase astrocyte purity, flasks were rocked by hand to remove non-adherent, non-astrocytic cells at each medium change. Astrocytes were plated at low density to limit survival of contaminating cell types (2.5-3.0 x 10^6 cells per flask).

2.3 Astrocyte Treatments

Primary cortical astrocytes were sub-cultured in 24 well plates (250,000 cells/mL) for synaptogenesis and electrophysiology experiments or on the underside of 6-well plate mesh inserts for protein expression experiments. Wells and inserts were coated with poly-d-lysine (40 μg/mL). Forty-eight hours prior to co-culture with neurons, astrocytes were serum deprived (DMEM + 0.1% BSA) for 24 hours, and then treated with or without carbachol (0.01, 0.10, 1 mM) or ethanol (25, 50, or 75 mM) for an additional 24 hours. Following treatment, astrocytes were washed twice in PBS to remove the treatment compounds and incubated in serum-free medium for three hours prior to the addition of the neuronal coverslips to allow the release of factors supporting neuronal survival.

For experiments requiring treatment with ethanol, sealed chambers (Billups-Rothernberg Inc., Del Mar, CA) were used to reduce ethanol evaporation during treatment. Ethanol-treated astrocytes and their matched controls were incubated in separate sealed chambers with a reservoir of water containing the same concentration of ethanol. The concentration of ethanol was verified periodically at the start and end of some experiments.

Acetylcholine Receptor Studies: One half hour prior to astrocyte pre-treatment with carbachol (1 mM), astrocytes were pre-treated with 10 uM of the acetylcholine receptor antagonists, atropine, mecamylamine, gallamine, or 4-DAMP, prepared in BSA medium. At pre-treatment end, carbachol (1 mM) was added. Cells were incubated for 24 hours and at treatment end, washed 2X with PBS. The medium was replaced (DMEM/BSA) 2.5-3 hours prior to neuronal co-culture.

LXR/RXR Agonists: After 24 hours of serum deprivation, astrocytes were co-treated with retinoic acid (1 uM) and 22-hydroxycholesterol (1 uM) for 24 hours. Astrocytes were washed 2X with PBS at treatment end and the medium replaced with DMEM-BSA 2.5-3 hours prior to co-culture with neuronal coverslips.

2.4 Astrocyte-Neuron Co-Cultures

Prior to the co-culturing of neurons with astrocytes, neurons were washed once with warmed HBSS. For synaptogenesis immunocytochemistry experiments, neurons plated on coverslips with paraffin spacers were inverted to sit above but separated from the astrocyte monolayer. For Western blot experiments astrocytes were plated on the underside of porous inserts and the inserts and their medium were transferred to 6-well plates containing neurons. Astrocytes and neurons were co-cultured for 24 hours, after which neurons were either washed twice in HBSS and lysed in 1% SDS lysis buffer for Western Blotting, fixed in paraformaldehyde (PFA) (4%) for 20 minutes at 37˚C, for imaging experiments, or subjected to whole cell patch clamping to record activity.

2.5 Immunocytochemistry

Synaptic Structures: Neurons were fixed in 4% paraformaldehyde and co-labeled with antibodies against synaptophysin (1:250, Abcam, ab52636) and PSD-95 (1:200, Affinity Bioreagents, Inc., MA1-046), or synaptotagmin (Abcam, 1:250) and the NR2B or NR1 subunit of the NMDA receptor (Neuromab, 1:200) overnight. Coverslips were then incubated with fluorescent secondary antibodies (1:500, donkey anti-rabbit Alexa 488, donkey anti-mouse Alexa 555) and the nuclear dye Hoechst 33342 (1 μg/mL). After labeling, the coverslips were mounted with Vectashield on glass slides, topped with a cover glass, and sealed with nail polish.

Astrocyte Surface TSP-1: Astrocytes were plated on PDL (40 μg/mL) coated glass coverslips at a density of 250,000 cells per coverslip and grown as previously described. Astrocytes were treated with carbachol (1 mM), or not, in triplicate, for twenty-four hours and were fixed in PFA (4%) immediately at treatment end, and at 6, 12, and 24 hours post-treatment washout. Without membrane permeabilization, astrocytes were labeled for goat anti-mouse TSP1 (Calbiochem, A6), fluorescent secondary antibody, and membrane permeable Hoechst 33342 (1 μg/mL). After labeling, the coverslips were mounted with Vectashield on glass slides, topped with a cover glass, and sealed with nail polish.

2.6 Image Acquisition and Analysis

Synaptic Structures: Healthy neurons, identified with Hoechst stain and located at least two cell bodies apart, were imaged using confocal microscopy (Olympus Fluoview-1000) at the Center for Human Development and Disability at the University of Washington, Seattle. Confocal images were acquired at a 1024 x 1024 image size with a 2X zoom, using a 60X oil immersion objective, yielding a 0.103 μm/pixel resolution. An average of 12 -18 planes per cell were acquired using a step size of 0.30μm from the bottom of the coverslip. Confocal settings remained constant between treatment groups and channel images were taken sequentially. Images were deconvolved using Huygens Professional software (Scientific Volume Imaging). Parameters were set for a maximum of 40 iterations and included settings for the numerical aperture of the objective, and the refractive indices of the mounting and immersion medium. The threshold for 3-dimensional object analysis, which includes both a size and intensity parameter, was determined for each experiment using Image J, by obtaining the mean intensity of 350-450 manually selected, size appropriate puncta from 15 neuronal fields, of each channel. During analysis, thresholds were held constant for each channel and treatment group within the same experiment. The number of individual pre- and post-synaptic objects and those overlapping between channels were automatically calculated and recorded using Huygens Object Analysis software.

Astrocyte Surface TSP1: Confocal images were acquired using a 40X oil immersion lens, at a step-size of 0.50μm for a total of 21 planes per field. A total of ten fields were imaged per treatment group from two coverslips. For each field, the integrated optical density per plane was determined using Metamorph software (Molecular Devices), totaled, normalized to cell number and averaged for each treatment group.

2.7 Direct Neuronal Treatment

Primary hippocampal neurons were grown as described above. On day 12 in culture, neurons were treated directly with either human thrombospondin-1 (0, 5, 10 μg/mL), cholesterol-containing high density lipoproteins (0, 10, 20 ug/mL cholesterol) or low-density lipoproteins for 24 hours. At

treatment end, neurons were washed 2X with HBSS and fixed in PFA (4%) for 20 minutes at 37˚C. Neurons were immunocytochemically labeled for pre and post synaptic proteins, imaged and synaptic structures were quantified as previously described.

2.8 Treatment of Astrocyte/Neuron co-cultures

Gabapentin Treatment: Astrocytes were pre-treated with carbachol (1 mM) or not treated for 24 hours. Treatment was washed out and the medium was replaced 2.5-3 hours prior to co-culture. One-half hour prior to the astrocyte co-culture with neurons, gabapentin (15 or 30 uM) was added to the appropriate astrocyte wells and the corresponding neuronal wells. At pre-treatment end, unwashed neurons were inverted over corresponding astrocyte wells and incubated together for 24 hours. At co-culture end, neuronal coverslips were washed, fixed in PFA, immunocytochemically (ICC) labeled and imaged, as previously described.

Lipoprotein Receptor Inhibition using Receptor-associated protein Treatment: RAP (50 nM) was added to the appropriate untreated and ethanol (50 mM) pre-treated astrocyte wells and directly to the corresponding hippocampal neuron wells ½ hour prior to the co-incubation of neurons and astrocytes in the co-culture system. At the time of co-culture, hippocampal neurons were inverted over the astrocyte monolayers without washing. Astrocytes were co-cultured for 24 hours. At co-culture end, neuronal coverslips were washed, fixed in PFA, ICC labeled and imaged, as previously described.

2.9 Protein Expression: Western Blotting

Whole cell protein levels: After co-culture with treated or untreated astrocytes, neurons were lysed in 1% SDS lysis buffer, sonicated twice at 3.5 power for 5 seconds and the protein quantified using the BCA method.

Synaptophysin and PSD-95 Western Blot: 10% Bis-Trisacrylamide gels were cast and equal amounts of protein were loaded into wells. Proteins were separated by gel electrophoresis and transferred to PVDF membranes. Membranes were blocked in TBST (5% milk) for 1 hour, then probed overnight at

4˚C with primary antibodies for synaptophysin (Abcam, ab52636, 1:500), or PSD-95 (Neuromab, 1:500). Membranes were incubated for 1 hour with secondary antibodies conjugated to horse-radish peroxidase (1:1000) and developed. Band densitometry was determined using Image J software, with total protein after Coomassie blue protein stain used to verify equal loading and to normalize the signal.

Thrombospondin-1 Time Course:

Astrocyte Culture and Treatment: Astrocytes were plated on 100 mm plates coated with PDL (40 μg/mL) at a density of 2.5 x 10^6 per plate and cultured for 4 days. Astrocytes were treated with carbachol (1 mM) for 24-hours. Medium was collected and cells were lysed in 1% SDS lysis buffer from treated and untreated cells at treatment end (time 0), and at 6, 12, and 24 hours post treatment washout.

Thrombospondin-1: Cytosol: Protein amounts were quantified from lysate samples using the BCA method. Equal amounts of protein were loaded into 3-8% Tris-Acetate gels and the proteins separated by gel electrophoresis. After transfer, PDVF membranes were blocked in TBST (3% BSA) for 1 hour and incubated overnight in goat anti-mouse TSP-1 (Calbiochem, A.6, 1:1000). Membranes were incubated for 1 hour with secondary antibodies conjugated to horse-radish peroxidase (1:1000) and developed. Membranes were normalized to β-actin levels.

Thrombospondin-1: Medium: At each time point, 7 mLs of media was concentrated to 200 μLs using Pierce concentrators (4000 x g for 25 minutes at 25^0C). Protease inhibitors (1:10), LDS sample buffer (4X) and reducing agent (10X) were added to the 200 μL concentrate. Samples were heated at 70°C for 10 minutes and equal volumes of concentrated sample were loaded into 3-8% Tris-Acetate pre-cast gels. After transfer, PDVF membranes were blocked in TBST (3% BSA) for 1-3 hours and incubated overnight in goat anti-mouse TSP-1 (Calbiochem, A.6, 1:500). Band densitometry was determined using Image J software. Samples were not normalized for equal protein loading.

2.10 Electrophysiology

Spontaneous miniature excitatory post-synaptic currents (mEPSCs) were recorded from hippocampal neurons after co-culture with carbachol (1 mM) or ethanol (50 mM) pre-treated astrocytes, or untreated astrocyte controls using whole cell patch clamp techniques. Cells were recorded at room temperature (21.5-23.5˚C). Currents were recorded using a MultiClamp 700B by Axon Instruments; cells were voltage clamped at a holding potential of -70 mV. Patch pipettes were pulled from borosilicate capillary glass (2.4-5.9 MΩ). Dissociated hippocampal neurons were bathed in artificial cerebral spinal fluid (ACSF) (119 mM NaCl, 2.5 mM KCl, 4 mM CaCl2, 4 mM MgCl2, 26 mM NaHCO3, 1 mM NaH2PO4, 11 mM glucose at pH of 7.4 and gassed with 5% CO2 and 95% O2). Tetrodotoxin (1 uM) was added to the circulating bath to isolate spontaneous miniature post-synaptic currents. Internal solution contained: 115 mM $CsMeSO_4$, 20 mM CsCl, 2.5 mM $MgCl_2$, 10 mM HEPES, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 10 mM Na-phosphocreatine, and ~0.6 mM EGTA, pH 7.25 (CsOH). Activity was recorded for up to ten minutes and events were manually selected using Synaptosoft Mini Analysis software. The frequency of events, as measured by inter-event-intervals, was used to represent the relative number of functional synapses. The amplitude of events was used as a measure of the number of post-synaptic receptors and the time to decay of events compared receptor kinetics between treatment groups and potential changes in subunit composition. Statistical analysis was performed using Kolmogorov-Smirnov Two-Sample test to compare the cumulative distributions of arrayed data. All other analysis used Student's t test and mean ± SEM. Significance was set at $p \leq 0.05$.

2.11 Statistical Analysis

Graph Pad Prism was used for graphing and statistical analysis. For imaging experiments, cells were plated on triplicate coverslips and 5 cells were imaged from 2-3 coverslips (10-15 cells per treatment group). Individual pre- and post-synaptic puncta and overlapping puncta per individual neurons from 2-3 independent experiments were averaged. Student's t-test was used for the comparison of experiments containing two treatment groups and One-way ANOVA with Dunnett's Multiple Comparison test was used where multiple concentrations were assessed relative to control. To

compare multiple treatments, one-way ANOVA followed by Bonferroni's Multiple Comparison tests was performed. Electrophysiology analysis was performed using Synaptosoft Mini Analysis software. Kolmogorov-Smirnov Two Sample Analysis was used to compare the cumulative distributions from arrayed recordings, and the student's t-test was used to compare means of two treatment groups. All tests used $p = 0.05$ for statistical significance.

Chapter 3:

Astrocytes stimulated with a cholinergic agonist increase synaptic structures and function in hippocampal neurons

3.1 Introduction

As mentioned previously, acetylcholine, a major neurotransmitter, is known to affect various aspects of brain development, including neuronal and glial proliferation, neuronal differentiation, and synaptic formation and function (Garaschuk, et al., 1998; Guizzetti, et al., 1996; Lauder and Schambra, 1999; Ma, et al., 2000; Wong, et al., 2000; Yakel, 2012; Yao, et al., 2000). The cholinergic system broadly innervates the CNS, and the primary hippocampal inputs derive from the medial septum of the basal forebrain (Dani and Bertrand, 2007).

Acetylcholine receptors are of two types, nicotinic and muscarinic. Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels formed by various combinations of 5 unique subunits. The combination of subunits determines the receptors relative permeability to calcium and sodium (Dani and Bertrand, 2007; Felder, 1995). Activation of neuronal pre-synaptic nAChRs enhances neurotransmitter release, while activation of post-synaptic receptors modulates neuronal depolarization; both neurotransmitter release and depolarization after receptor activation are facilitated by the influx of calcium through the receptor (Dani and Bertrand, 2007; Sharma and Vijayaraghavan, 2001). Astrocytes in culture have been shown to express nACh receptors containing the α4. α7, β2 and β3 subunits (Xiu et al., 2005).

Muscarinic acetylcholine receptors are G-protein coupled receptors, and there are five types (M1-M5). M2 and M4 receptors are tied to inhibitory signaling processes and inhibit adenyl cyclase, while the M1, M3, and M5 type receptors are classified by their ability to activate phospholipase C

and mobilize intracellular calcium (Felder, 1995). Astrocytes express both M2 and M3 muscarinic receptors (Guizzetti, et al., 1996). M3 muscarinic signaling stimulates canonical pathways, like the phospholipase C (PLC), resulting in the IP_3 receptor mediated release of intracellular calcium stores from the endoplasmic reticulum, as well as PLD and tyrosine kinase pathways (Agulhon et al., 2008; Felder, 1995; Sharma and Vijayaraghavan, 2001; Verkhratsky et al., 1998).

Astrocytes extend processes into the synaptic space and are in close contact with the synapse. From this position, astrocytes are capable of responding to synaptic release of neurotransmitters (Nedergaard, et al., 2003). ACh receptor activation in astrocytes has been shown to result in calcium elevations (Araque, et al., 2002; Panatier, et al., 2011) and those elevations have been shown to be localized to the astrocyte processes (Panatier, et al., 2011). The rise of intracellular calcium in astrocytes has been tied to the release of gliotransmitters (Araque, et al., 1998b; Newman, 2003; Oberheim, et al., 2006; Parri and Crunelli, 2007; Volterra and Meldolesi, 2005) and has been observed to influence downstream signaling events like vesicular release, cell growth, and gene expression (Araque, et al., 1998b; Sharma and Vijayaraghavan, 2001).

Previous work in our laboratory has shown that cholinergic stimulation of astrocytes with carbachol causes the release of factors that potentiate neurite outgrowth in both a co-culture system of hippocampal neurons and astrocytes, and in hippocampal slices. This effect occurs through the M3 muscarinic receptor pathway and is mediated by the increased release of the extracellular matrix proteins, fibronectin and laminin (Giordano, et al., 2011; Guizzetti, et al., 2008). Additionally, proteomic studies investigating the effects of carbachol stimulation (1 mM) on the astrocyte secretome after 24 hours, clearly shows the increased release of numerous factors (Moore et al., 2009), confirming the fact that cholinergic signaling can mediate the release of factors from astrocytes that modulate neuronal development.

The physiological importance of ACh, and the knowledge that cholinergic-induced, astrocyte-secreted factors promote neurite outgrowth, led us to expect that it would also positively influence synapse formation. In the experiments that follow, I have characterized the effect of cholinergic-

stimulation of astrocytes on hippocampal neuron synapse formation and function. The results show that during the 24 hours post-cholinergic stimulation of astrocytes, astrocytes release factors that potentiate the formation of excitatory pre- and post-synaptic structures and their overlap, and that this effect is mediated by M3 muscarinic receptor and ionotropic nicotinic receptor stimulation. Additionally, the increase in observed structural synapses is also reflected in an increase in functionality.

3.2 Results

To investigate the effect of cholinergic-stimulation of astrocytes on hippocampal synapse formation, astrocytes were treated for 24 hours with increasing concentrations of the cholinergic agonist, carbachol (0.010, 0.100, or 1 mM) or left untreated. At treatment end, carbachol was removed by washing, and hippocampal neurons (13 DIC), grown on glass coverslips with wax spacers, were inverted over the pre-treated astrocytes. In this system, the neurons were never exposed to carbachol nor were they ever in direct contact with the astrocytes. Neurons and astrocytes were co-cultured for 24 hours, after which the neurons were fixed, immunocytochemically labeled for the presynaptic protein, synaptophysin, and the post-synaptic protein, PSD-95, and imaged using confocal microscopy.

Initial observations showed an increase in puncta size and intensity in the confocal images, relative to control (Figure 3.1), suggesting that carbachol-stimulated astrocytes either acted to promote the localization of synaptic proteins into pre- and post-synaptic structures, increased the expression of the synaptic proteins, or both. To quantify this effect, three-dimensional surface renderings of the clustered protein puncta from deconvolved images were generated. To compare differences between treatment groups, the number of pre- and post-synaptic specializations, as well as the number of overlapping pre- and post-synaptic puncta, or synapses, were automatically obtained for each neuronal field, and averaged. Because synapses are structures consisting of aligned pre- and post-synaptic specializations, the number of overlapping synaptophysin and PSD-

95 puncta are used here to represent the number of structural synapses in each treatment group.

Astrocytes pre-treated with carbachol increased the number of synaptophysin and PSD-95 puncta in neurons, relative to control (Figure 3.2.A). An almost two-fold increase ($p < 0.001$) in synaptophysin puncta and a 2.2 fold increase in PSD-95 puncta ($p < 0.01$) was observed after co-culture with astrocytes pre-treated with 100 μM carbachol. Neurons co-cultured with astrocytes pre-treated with 1 mM carbachol showed a 1.6 ($p < 0.05$) and 2.3 ($p < 0.001$) fold increase in the number of individual synaptophysin and PSD-95 puncta, respectively (Figure 3.2.A).

While there was an increasing trend in the number of synapses with increasing concentrations of carbachol, only pre-treatment of astrocytes with 1 mM carbachol induced a 3.2 fold ($p < 0.0001$) increase in the number of aligned pre- and post-synaptic puncta (Figure 3.2.B). These results suggest that cholinergic-stimulation of astrocytes potentiates the formation of synapses in hippocampal neurons.

To determine the acetylcholine receptor involved in the astrocytic-cholinergic effect on synaptic structure formation, astrocytes were pre-treated with carbachol (1 mM) in the presence of a series of acetylcholine receptor antagonists for 24 h. After treatment washout, astrocytes were co-cultured with neurons and synaptic structure formation was quantified as previously described. Astrocyte pre-treatment with carbachol alone induced an almost 3-fold increase ($p < 0.001$) in the number of synaptophysin/PSD-95 synapses in hippocampal neurons after the 24-hour co-culture (Figure 3.3). Atropine (10 μM), a non-selective muscarinic acetylcholine receptor antagonist, attenuated the carbachol effect slightly (27%), although this effect was not significant. Gallamine (10 uM), a M2 receptor specific antagonist had no effect, while the M3 muscarinic receptor antagonist, 4-DAMP (10 μM) completely attenuated the effect of carbachol ($p < 0.001$). Unexpectedly, the ionotropic, nicotinic receptor antagonist, mecamylamine (10 μM) also completely attenuated ($p < 0.001$) the effect of carbachol on synapse formation. These results suggest that carbachol-activation of both astrocyte acetylcholine M3 muscarinic receptors and nicotinic receptors are responsible for

the observed increase in hippocampal neuron synapse number after co-culture with carbachol-treated astrocytes.

Next, Western blotting techniques were used to examine whether carbachol pre-treatment of astrocytes affected the expression of the synaptic proteins, synaptophysin and PSD-95, in neurons. Neurons co-cultured with carbachol (1 mM) pre-treated astrocytes showed a significant increase in synaptophysin (2.34 fold) ($p < 0.001$) and PSD-95 levels (1.97-fold) ($p < 0.001$). Pre-treatment of astrocytes with concentrations of carbachol lower than 1 mM did not significantly change the levels of either protein (Figure 3.5.A and 3.5.B).

To verify that the effect on synapse formation in neurons after co-culture with carbachol pre-treated astrocytes is not limited to specific effects on synaptophysin and PSD-95, but indicative of changes in synaptogenesis more broadly, synaptic structure experiments were replicated. Neurons were immunocytochemically labeled with alternative markers of pre- and post-synaptic structures, the pre-synaptic protein, synaptotagmin, and the post-synaptic NR2B subunit of the excitatory NMDA receptor.

Analysis of the synaptic structures showed a 2.3 fold increase in synaptotagmin puncta ($p < 0.0001$) and a 2.8 fold increase in the number of NR2B subunit labeled post-synaptic puncta ($p < 0.0001$) (Figure 3.4.A) in those neurons co-cultured with carbachol (1 mM) pre-treated astrocytes, relative to control astrocytes. The mean number of synaptotagmin/NR2B-containing synapses increased 4.6 times ($p < 0.0001$) after incubation of neurons with carbachol pre-treated astrocytes (Figure 3.4.B). Together, these data suggest that cholinergic-simulation of astrocytes causes a broad increase in excitatory synaptic structure formation.

An important question that arises from these findings is the extent to which these synaptic structures are functional. To assess this, hippocampal neurons were co-cultured for 24 hours with carbachol (1 mM) pre-treated or un-treated astrocytes. At treatment end, spontaneous, miniature excitatory post-synaptic currents (mEPSCs) were recorded from the neurons in the presence of tetrodotoxin (TTX) (1 μM), and were clamped at a holding voltage of -70 mV, using whole cell patch

clamp techniques. Analysis of the frequency of events, as measured by the inter-event interval (IEI), was used to determine differences in the relative number of functional synapses in neurons between treatment groups. Combined arrayed analysis of recordings from 5 neurons co-cultured with untreated astrocytes and 3 neurons co-cultured with carbachol pre-treated astrocytes, from 2 experiments, showed a significantly greater frequency of events in those neurons co-cultured with carbachol astrocytes (Figure 3.6.A) with a median inter-event interval of 387.25 msec for the carbachol group, and 503.1 msecs for the control group, representing a 1.3 times increase (Figure 3.6A). No differences were observed in the median amplitude (control group, 17.7 pA; carbachol group 18.9 pA)(Figure 3.6B), suggesting no change in the number of post synaptic receptors between groups and no difference in the event time to decay (Figure 3.6.C) were observed, suggesting no difference in the receptor subunit composition. These results suggest that cholinergic stimulation of astrocytes increases the number of functional synapses relative to control, and thus modulates hippocampal neuron synaptic function.

3.3 Discussion

The results indicate that cholinergic stimulation of astrocytes increases synaptic structure formation, synaptic protein expression and potentiates the number of functional synapses. The finding that cholinergic-stimulation of astrocytes has a potentiating effect on the number and function of synapses, was not unexpected. We have previously shown that activation of astrocytic M3 muscarinic receptors has a positive effect on neuronal development, specifically, neurite outgrowth (Giordano, et al., 2011; Guizzetti, et al., 2008), and we were expecting that the observed increase in synaptic structure formation would be mediated through the same signaling pathway. Additionally, because carbachol is not present during the 24 hours post-treatment washout, any effect would be expected to be mediated through a signaling cascade that was relatively long-acting and broad. This would implicate a G-protein mediated effect, and this is confirmed by the attenuation of the carbachol effect by the M3 muscarinic receptor antagonist, 4-DAMP (Figure3.3)

The finding that mecamylamine, a nicotinic receptor antagonist, also attenuated the carbachol effect (Figure 3.3), was surprising, as it suggests that carbachol is also acting through the ionotropic, nicotinic receptors. Signaling through ionotropic receptors is usually rapid and short-lived and so it would not be expected to induce changes in the 24-hours post-treatment washout, when carbachol is no longer present to activate these receptors. However, it has been shown that astrocytes in culture express nicotinic receptors containing the α7 subunit, and these receptors have functionality that seems to be unique to astrocytes. Activation of α7-containing nicotinic receptors in astrocytes results in an initial influx of calcium through the receptors, which results in the calcium-induced, calcium release (CICR) from internal, endoplasmic reticulum stores, a response shown to be mediated by the ryanodine receptor (Sharma and Vijayaraghavan, 2001). The large and long-lasting increase in calcium due to CICR raises the possibility that signaling through this ionotropic receptor may result in longer-term down-stream signaling, and so may result in broader effects than that seen after activation of typical ionotropic receptor channels. It should be noted however, that the expression of the α7 receptor, and the described function was observed in cultured astrocytes, and there is debate as to whether these receptors are expressed *in situ* (Agulhon, et al., 2008; Araque, et al., 2002). The use of a specific α7- nicotinic AChR antagonist would be necessary to confirm that they are in fact expressed in this system, and before any effect on synapse function could be attributed to this specific nicotinic acetylcholine receptor.

Initial imaging of neurons after co-culture with carbachol (1 mM) pre-treated astrocytes showed an overall increase in the size and intensity of pre- and post-synaptic puncta relative to control (Figure 3.1), suggesting either an effect on the localization of the proteins into synaptic structures, and/or an increase in protein expression. Western blotting results show that carbachol (1 mM) pre-treatment of astrocytes induced a robust increase in whole cell levels of the neuronal synaptic proteins, synaptophysin and PSD-95 (Figure 3.5). The observed increase suggests that the increased size and intensity of synaptic protein puncta may be due, at least in part, to an effect of carbachol stimulation of astrocytes on the expression of synaptophysin and PSD-95.

In addition to the increase in synaptic structures and protein levels, the electrophysiology data indicate that cholinergic stimulation of astrocytes increases the number of functional synapses and is thus capable of modulating hippocampal neuron synaptic function. It is possible that the increased frequency of events observed after treatment could be mediated by changes in the probability of vesicular release. Additional studies using the styryl dye FM1-43 to measure vesicular recycling or measuring paired-pulse facilitation in a more complex system, may add greater mechanistic understanding of how cholinergic-stimulation of astrocytes influence synaptic function.

Taken together, these data indicate that the activation of astrocyte acetylcholine receptors results in the release of factors that potentiate synaptogenesis, causing an increase in the formation of pre-synaptic (synaptophysin and synaptotagmin) and post-synaptic (PSD-95 and NR2B subunit of the NMDAR) structures and their assembly into functional synapses, an effect mediated by the activation of both M3 muscarinic and nicotinic receptors. Cholinergic activation also induces increase synaptic protein expression, and an increase in the number of functional synapses. The next chapter will investigate astrocyte-released thrombospondin-1 as a potential factor responsible for some of these observed effects.

Chapter 4:

Increased TSP1 release from astrocytes after cholinergic stimulation induces synaptogenesis

4.1 Introduction

Having shown that cholinergic-stimulation of astrocytes modulates synapse structure and function I next attempted to investigate a potential astrocyte-released factor responsible for the observed effects. Recent work has identified thrombospondin (TSP), an astrocyte-secreted extracellular matrix protein, as a component of astrocyte-conditioned medium that is a strong potentiator of synapse formation (Christopherson, et al., 2005).

Thrombospondin is a large, multimeric, 450 kDa, glycoprotein, that was first identified in blood platelets (Lawler, 1986) and is now known to be expressed in numerous cell types, including monocytes, endothelial cells, aortic smooth muscle, fibroblasts, and macrophages (Lawler, 1986). In addition to its role in synapse formation, TSP has been shown to play roles in cell migration, wound healing, neurogenesis, the inhibition of angiogenesis and the promotion of cell adhesion (Asch et al., 1986; Eroglu et al., 2009; Lu and Kipnis, 2010).

Unlike many extracellular matrix proteins that are thought to function more narrowly, as scaffolding components, TSP is seen as an important signaling molecule within the extracellular matrix. With five distinct binding domains, it is able to interact with numerous factors, including, heparin, endothelial growth factor, fibronectin, collagen, lipoprotein receptor 1 (LPR1), ApoER2 and calcium, (Asch, et al., 1986; Frazier, 1991; Lawler, 1986; Risher and Eroglu, 2012). These multiple and diverse binding domains facilitate interactions between cells, as well as between cells and various substrates (Asch, et al., 1986).

In the brain, TSP1 and TSP2 are expressed in astrocytes and are localized to the fine astrocytic processes (Asch, et al., 1986; Christopherson, et al., 2005). Expression is developmentally regulated, with levels of the protein high during early post-natal time points, and much lower during adulthood, although after insult, TSP expression is up-regulated (Christopherson, et al., 2005; Eroglu, et al., 2009). That thrombospondin is expressed during the time of synapse formation and astrocyte proliferation, and that is located in the fine processes of the astrocytes in, close proximity to the synapses, supports its role in synaptogenesis.

In retinal ganglion cells (RGC), astrocyte released-thrombospondin has been shown to strongly induce synapse formation. While TSP- induced synapses are ultrastructurally normal and pre-synaptically active, they are post-synaptically silent (Christopherson, et al., 2005). Despite the fact that TSP is both necessary and sufficient to induce synapse formation in retinal ganglion cells, additional astrocyte-secreted factors may contribute to the maturation of functional synapses (Christopherson, et al., 2005; Eroglu, et al., 2009).

Recent work has identified the neuronal receptor through which astrocyte-released thrombospondin acts to induce its synaptogenic effect. Binding of the EGF domain of thrombospondin to the α2δ1 subunit of the voltage gated calcium channel is both necessary and sufficient to induce synapse formation. The analgesic, anti-epileptic drug, gabapentin, has been shown to block the effect of TSP1 receptor binding. While the mechanism is not yet completely elucidated, it has been hypothesized that binding of TSP to this receptor induces a conformational change, resulting in the activation of what has been termed a "synaptogenic signaling complex." This may allow for the subsequent recruitment of various cell adhesion and scaffolding proteins that act to promote and stabilize synapses (Risher and Eroglu, 2012).

In our laboratory, a proteomic study investigating how cholinergic stimulation modulates the astrocyte secretome has shown that TSP-1 release is increased after 24 hour treatment with carbachol (Moore, et al., 2009). Because of its role in synapse formation, and because carbachol treatment of astrocytes has been observed to increase the release of TSP1 from astrocytes, it is

possible that thrombospondin may be a factor responsible for the increased synaptic structure formation observed after carbachol pre-treatment of astrocytes.

The following investigations show that thrombospondin-1 is sufficient to induce synapse formation in hippocampal neurons, that cholinergic-stimulation of astrocytes more than likely increases thrombospondin release from astrocytes in the 24-hours after carbachol pre-treatment, and that carbachol-induced, astrocyte-released TSP1 is necessary for the increase in synapses observed in hippocampal neurons after 24-hours of co-culture with hippocampal neurons.

4.2 Results

Thrombospondin has been shown to increase synapse number in retinal ganglion cells (Christopherson, et al., 2005). To show that TSP1 is sufficient to increase synapses in the hippocampal neuron culture used here, neurons (13 DIC) were directly treated with human thrombospondin-1 (TSP-1) (5, or 10 μg/mL) for 24 hours. At incubation end, neurons were fixed, immunocytochemically labeled for synaptophysin and PSD-95, imaged and analyzed, as previously described. An increasing trend in the number of synaptophysin/PSD-95 synapses was observed after direct treatment with increasing concentrations of TSP-1, with 10 μg/mL thrombospondin inducing a 2-fold increase in synaptic structure formation, relative to control ($p < 0.05$) (Figure 4.1.B). Interestingly, this increase in synapse number occurred without changes in the number of synaptophysin or PSD-95 puncta, at either concentration of thrombospondin (Figure 4.1.A), a finding that is different than that observed when neurons were co-cultured in the presence of carbachol-stimulated astrocytes (Figure3.2.A).

Previous proteomic studies in our laboratory investigating the effect of cholinergic-stimulation on the astrocyte secretome showed that TSP1 release to the medium was increased relative to control, at the end of a 24-hour treatment with carbachol (1 mM) (Moore, et al., 2009). However, the synaptic endpoints investigated here were measured 24-hours after carbachol treatment had been washed out. If TSP-1 is indeed a factor responsible for the observed increase in

synaptic structures, cholinergic-stimulation must induce a continued, long-term response that lasts after carbachol has been removed. To confirm that increased TSP-1 release continues during the 24-hours post carbachol treatment and washout, a time-course experiment was performed. Medium and cytosolic proteins from carbachol (1 mM) pre-treated and untreated astrocytes were collected immediately at the end of the 24 hour treatment (time 0), and at 6, 12 and 24 hours post-treatment washout. Because the amount of overall protein released to the medium is much less than that obtained from the cytosol of cells, detecting released protein in the medium is challenging, and so the medium at each time point was concentrated. Equal volumes of concentrated medium were loaded into pre-cast gels and protein levels of TSP-1 released to the medium were analyzed using Western blotting techniques.

Comparison of the mean optical densities after equal loading of concentrated medium showed that TSP-1 release to the medium is doubled in those astrocytes treated with 1 mM carbachol at the end of 24-hour carbachol treatment. (Figure 4.2, time 0) ($p = 0.023$). As would be expected, the amount of TSP-1 in the medium in both control and treated astrocytes drops significantly after treatment washout, as evidenced by the dramatic reduction in the optical densities of both groups (Figure 4.2, Time 0 vs. Time 6 hours). However, the two-fold increase of TSP-1 is maintained at 6 hours post-washout ($p = 0.027$) relative to its time-matched control. Over time, levels of TSP-1 released to the medium increase in both the control and carbachol pre-treated astrocyte groups relative to the proceeding time point, which would be expected, as even basal release would accumulate in the medium, yet the magnitude of the carbachol effect on TSP1 decreases. No significant increase in TSP-1 release was observed at either the 12 hour or 24 hour time points.

Because of variability within time points, the lack of a proper loading control, and concerns with equal loading of highly concentrated medium, an additional, confirmatory experiment was undertaken to strengthen the body of evidence that TSP1 release is indeed increased in the 24 hours after carbachol treatment. Immunocytochemical techniques were used to label and compare the surface levels of TSP-1 on carbachol (1 mM) pre-treated or untreated astrocytes. Initial experiments

indicate a 3.3-fold increase, relative to control, at 24 hours post-treatment washout ($p = 0.0002$) (Figure 4.3). This difference determined by confocal analysis is higher than expected if compared to the released TSP1 levels in the medium at the 24 hour time point measured by Western Blotting (Figure 4.2). However, there was great inter-experiment variability in the measure of TSP1 released to the medium at the 24-hour post-treatment washout.

To determine the effect of carbachol treatment on thrombospondin intracellular expression, cells were collected in lysis buffer at each time point. After protein quantification, equal amounts of protein were loaded into gels and subjected to electrophoresis. After transfer, the membranes were probed for TSP1 and β-actin and levels were compared after chemiluminsence detection and normalization to β-actin levels using densitometric analysis. These data show a slight (25%) but not significant increase in levels of TSP1 relative to control immediately at the end of the 24-hour carbachol treatment (Figure 4.4, time 0). At 6 hours post-treatment washout, levels of intracellular TSP1 in both treated and control cells were almost equal. Over the 24-hours, there is a general decrease in levels of TSP1 in carbachol treated cells relative to matched time point controls, although no significant differences were observed at any time point or across time points (Figure 4.4). These results would suggest that there is a trend of a decreased intracellular levels in astrocytes pre-treated with carbachol.

TSP has been shown to exert its synaptogenic effects through interactions with the α2δ1 subunit of the voltage gated calcium channel, and the anti-epileptic drug, gabapentin, has been shown to block this effect in retinal ganglion cells (Eroglu, et al., 2009). To determine whether TSP release from astrocytes pre-treated with carbachol is necessary for the increase in synaptic structures observed after co-culture, gabapentin was used to block the effect of TSP on neurons.

The addition of gabapentin (15 or 30 μM) to the co-culture system containing both neurons and carbachol (1 mM) pre-treated astrocytes significantly attenuated the astrocytic-carbachol influence on synapse formation in a concentration-dependent manner (Figure 4.5). Incubation of neurons and carbachol pre-treated astrocytes with 15 μM gabapentin attenuated the carbachol effect

on synapse number by slightly more than 50% ($p < 0.01$). Adding 30 μM gabapentin to the carbachol pre-treated co-culture system reduced the effect by more than 75% ($p < 0.001$). Gabapentin alone (30 μM) decreased the mean number of synapses in neurons incubated with control astrocytes by approximately half (Figure 4.5), though this effect was not statistically significant.

Together these results indicate that TSP1 is sufficient to increase synapse formation in hippocampal neurons, and is a necessary factor released from astrocytes after carbachol stimulation, that is responsible, at least in part, for the increase in synapse formation.

4.3 Discussion

Thrombospondin-1 has been shown to be an astrocyte-secreted factor important in synapse formation (Christopherson, et al., 2005; Eroglu, et al., 2009). My findings are in strong support of this. Direct treatment of hippocampal neurons with TSP-1 caused an increase of synaptophysin/PSD-95 synaptic structures, and increased TSP-1 release from astrocytes is necessary for the increase in synapse formation observed in neurons after co-culture with carbachol pre-stimulated astrocytes.

The finding that the direct treatment of neurons with TSP1, in the absence of astrocytes, increases the number of synapses in hippocampal neurons (Figure 4.1.B) was not unexpected, as it has been shown to do so in other cell types (Christopherson, et al., 2005). However, the fact that there is an increase in the number of synapses after direct treatment, without a change in the number of pre- and post-synaptic puncta (Figure 4.1.A) is interesting. Because this is different than the increase in puncta observed in those neurons co-cultured in the presence of carbachol-stimulated astrocytes (Figure 3.2.A), it suggests that while TSP1 may be sufficient to induce synaptogenesis, it may not be the only factor released by carbachol-stimulated astrocytes that positively influences synaptic structure formation. Additionally, because more synapses were formed without a change to the number of pre- and post-synaptic structures, TSP1 may be functioning to facilitate the alignment

of those pre- and post-synaptic specializations already present. This finding would support the role of TSP1 as activating the "synaptogenic signaling complex" proposed by the Eroglu group (Risher and Eroglu, 2012). This is a new area of research; future studies will further the understanding of the function of TSP1.

In an attempt to show that TSP-1 is released to the co-culture system in the 24 hours after carbachol pre-treatment washout, Western blotting experiments were performed. Analysis of TSP1 released to the medium appears to show increased release of TSP1 from astrocytes after treatment with carbachol, at least through the 6-hour time point (Figure 4.2). The general increase in levels of TSP1 over time is expected, as TSP-1 should accumulate at each time point within in the time frame. That fact that the magnitude of the carbachol effect on TSP1 release lessens over time (6 hours vs. 12 hours) is not completely unexpected, as the astrocyte response to cholinergic stimulation may decrease when carbachol is no longer present. These data, however, must be approached with caution, has they have not been normalized to a loading control.

Determining the levels of protein released to medium was complicated due to a number of factors, and required the loading of equal volumes of medium, as opposed to equal amounts of protein. Attempts were made to quantify protein amounts using various methods, without success. The loading of equal volumes of medium presents with difficulties as carbachol-stimulation increases the release of numerous proteins, relative to control. Additionally, any slight changes in volume when loading medium to be concentrated, could magnify any loading errors and confound the data. However, the fact that levels of TSP1 released to the medium at treatment end (time 0) have increased, as was found in our previous proteomic study (Moore, et al., 2009), is reassuring. Additionally, the trend of decreasing levels of intracellular TSP-1, although not significant, may also suggest that carbachol pre-treatment mediates the release of TSP1, with little to no effect on its expression. Alternative studies could be performed to further strengthen and confirm these findings.

Despite the methodological difficulties in definitively showing increased TSP-1 release from astrocytes in the 24-hours post carbachol treatment, the attenuation of the carbachol effect on

synapse number by gabapentin, the inhibitor of the TSP1 synaptogenic effect (Figure 4.5), adds confidence to the fact that carbachol-stimulation induces increased TSP1 release that remains elevated in the co-culture system during the 24-hours post-treatment washout.

That gabapentin blocked the effect of carbachol-stimulation on synapse number was not unexpected, as it has been shown to do so in retinal ganglion cells (Eroglu, et al., 2009). However, it is unclear why gabapentin completely attenuated the carbachol effect (Figure 4.3), given that direct treatment of neurons with TSP1 seems to suggest that TSP1 may not the only factor involved (Figure 4.1).

Together, these results show that astrocytes release factors in response to cholinergic-stimulation that are capable of potentiating synapse formation and function in hippocampal neurons. Additionally, the studies investigation TSP1 as a potential factor in promoting structural synapse formation show that TSP1 is sufficient to potentiate formation in hippocampal neurons and is a necessary factor for the increase in the number of synapses observed after co-culture with astrocytes pre-stimulated with a cholinergic agonist.

Chapter 5:

Ethanol modulates the astrocyte influence on synapse formation and function

5.1 Introduction

The developing fetus exposed to ethanol presents with numerous deficits, including a range of cognitive disabilities, such as attention and memory deficits, hyperactivity and learning disabilities (Sokol, et al., 2003; Warren and Foudin, 2001; Warren, et al., 2011). Problems with learning and memory in children with Fetal Alcohol Spectrum Disorder suggest the involvement of the hippocampus and possible effects on synapse formation and function.

Pre-natal ethanol exposure has been shown to inhibit synapse formation and function in numerous studies. Decreases in and spine density, synapse number, and the release of neurotransmitter have been reported (Bellinger, et al., 1999; Kuge, et al., 1993; Whitcher and Klintsova, 2008). More recently, it has been shown that prenatal exposure to moderate levels of alcohol impairs NMDA receptor synaptic plasticity and alters NMDA receptor subunits expression and composition (Brady, et al., 2013).

In contrast, some studies suggest that ethanol may accelerate the stabilization of synapses (Mameli and Valenzuela, 2006). Increases in the clustering of NMDA receptors (Clapp, et al., 2010), as well as increases in the NMDA receptor scaffolding protein, PSD-95 (Carpenter-Hyland and Chandler, 2006) have been reported. Ethanol inhibits excitatory NMDARs (Lovinger et al., 1989), and some of these effects were attributed to compensatory mechanisms due to receptor inhibition (Clapp, et al., 2010). Ethanol has also been shown to potentiate inhibitory GABA release and GABA receptor conductance, and studies investigating ethanol's effects on the GABAergic system after *in utero* ethanol exposure are equally varied (Valenzuela et al., 2011).

Clearly, the effects of pre-natal ethanol exposure on synapse formation and neurotransmitter systems are complex and some of that complexity can be attributed to differences in the time during development that the exposure occurs, the amount and pattern of exposure, when the endpoint is measured and the region of the brain studied (Valenzuela, et al., 2011). One clear mechanism of action does not exist. Despite the differential effects on synaptic components and processes, a disruption of typical synapse formation and function, at any time in development can be detrimental.

How astrocytes modulate the ethanol effect on synaptogenesis has not been sufficiently investigated. Here I used the same methods and approach as was used previously to characterize the effect of ethanol pre-treatment of astrocytes on hippocampal synapse formation and function and show that astrocytes pre-treated with physiologically relevant concentrations of ethanol potentiate synapse formation and dysregulate typical synapse function after co-culture with hippocampal neurons.

5.2 Results

To investigate the effect of ethanol pre-treatment of astrocytes on hippocampal synapse formation, astrocytes were treated for 24 hours with increasing concentrations of ethanol (25, 50, 75 mM) or left untreated. These levels of ethanol are physiologically relevant concentrations and reflect a blood alcohol content of 0.12, 0.23, and 0.35%, respectively. Even the highest concentration used here could be found in someone who is alcohol dependent, without significantly evident outward behavioral effects due to tolerance. At treatment end, ethanol was removed by washing and hippocampal neurons (13 DIC), grown on glass coverslips with wax spacers, were inverted over the pre-treated astrocytes. Neurons and astrocytes were incubated together for 24 hours. At the end of the co-culture period, the neurons were fixed, immunocytochemically labeled for the presynaptic protein, synaptophysin, and the post-synaptic protein, PSD-95, and imaged using confocal microscopy. In this system, the neurons were never exposed to ethanol, nor were they ever in direct contact with the astrocytes.

The increase in puncta size and intensity initially observed in the confocal images (Figure 5.1) suggests that ethanol-pre-treated astrocytes either acted to promote the localization of synaptic proteins into pre- and post-synaptic structures, increased the expression of the synaptic proteins, or both. To quantify this effect, three-dimensional surface renderings of the clustered protein puncta from deconvolved images were generated and the number of pre- and post-synaptic specializations, as well as the number of synaptophysin/PSD-95 synapses were automatically obtained for each neuronal field, and averaged.

Incubation of neurons with increasing concentrations of ethanol (25, 50, and 75 mM) induced a bi-phasic increase in both pre- and post-synaptic puncta, as well as the number of aligned synaptic structures (Figure 5.2.A and 5.2.B); astrocytes treated with 50 mM ethanol showed the greatest effect in all cases. Pre-treatment of astrocytes with 50 mM ethanol induced a 2.9-fold increase in synaptophysin puncta, an effect greater than that observed after pre-treatment with 25 mM (2.1-fold increase) and 75 mM (1.7-fold increase). While all concentrations of ethanol induced significant increases in the mean number of pre-synaptic puncta ($p < 0.001$ for each concentration), this was not the case for the post-synaptic puncta. While increases in PSD-95 trended toward a similar bi-phasic effect, only the 50 mM ethanol pre-treatment was significant, resulting in a 2.2-fold increase ($p < 0.01$). Astrocytes treated with 50 mM ethanol showed a dramatic 4.5-fold increase in overlapping puncta ($p < 0.001$), an effect greater than that observed after pre-treatment with 75 mM ethanol (2.6- fold; $p < 0.001$) (Figure 5.2B). Together, these data show that astrocytes pre-exposed to ethanol modulate the development of synaptic structures.

To assess whether ethanol-treated astrocytes affect the protein levels of synaptophysin and PSD-95, Western blotting was performed. Pre-treatment of astrocytes with increasing concentrations of ethanol (25, 50, and 75 mM) induced an increase in synaptophysin and PSD-95 protein levels in neurons (Figure 5.3), but to a lesser extent than that seen after carbachol-pre-treatment (Figure 3.5). Significant increases in the pre-synaptic protein, synaptophysin, were observed after co-culture with 50 and 75 mM ethanol (1.47 and 1.58 fold, respectively; $p< 0.01$ and p

< 0.001). Increases in PSD-95 protein levels were also significantly different than control at the same ethanol concentrations, with both 50 mM and 75 mM inducing an approximate 1.4 fold increase ($p < 0.01$ and $p < 0.001$, respectively).

To verify that the effect in neurons after co-culture with ethanol pre-treated astrocytes is not limited to effects on synaptophysin and PSD-95, but indicative of a larger effect on the process of synaptogenesis, hippocampal neurons, after co-culture with ethanol pre-treated astrocytes, were labeled for alternative pre- and post-synaptic markers, synaptotagmin and the NR2B subunit of the excitatory NMDA receptor. Unlike the increase in pre- and post-synaptic puncta observed after neurons were incubated with carbachol-pre-treated astrocytes (Figure 3.4), ethanol (75 mM) pre-treated astrocytes had no effect on the mean number of neuronal synaptotagmin puncta, and actually induced a 19% decrease in the number of NR2B subunit puncta ($p < 0.023$) (Figure 5.4.A). Additionally, the mean number of synaptotagmin/NR2B synapses trended toward a 33% reduction relative to control neurons; however this effect was not significant (Figure 5.4.B).

PSD-95 is a scaffolding protein that plays a role in NMDAR trafficking and anchors NMDARs at the post-synaptic cleft (Cousins et al., 2008; Kornau et al., 1995; Okabe, 2002). Using it as a marker of post-synaptic structures and a component of the synapses being quantified would suggest that the increases in synapse number observed after co-culture with ethanol pre-treated astrocytes would reflect an increase in excitatory NMDARs containing synapses. However, since the NR2B subunit is one of many subunits that could be differentially expressed in the heteromeric receptor complex at the synapse, I next tested the effect of ethanol pre-treated astrocytes on neuronal synaptic structure formation after labeling with the obligatory NMDAR subunit, NR1. Ethanol pre-treatment of astrocytes (50 mM) induced a 1.3 fold increase in the number of post-synaptic, NR1 subunit puncta ($p = 0.0018$) with no change in pre-synaptic, synaptotagmin puncta, relative to control (Figure 5.5.A). The mean number of synaptotagmin/NR1 synapses increased almost two-fold ($p = 0.0016$) (Figure 5.5.B). These data confirm that pre-treatment of astrocytes with ethanol causes a potentiation of synapse formation that is not limited to effects on synaptophysin or PSD-95, but is

indicative of a broader effect on excitatory synaptogenesis. They also indicate that the factors that astrocytes release after pre-treatment with ethanol may modulate NMDAR subunit synaptic incorporation, and as a result, function.

To determine whether the increase in synaptic structures is reflected in an increase in functionality, whole cell patch clamp techniques were used to record spontaneous miniature excitatory post-synaptic currents from hippocampal neurons after co-culture for 24 hours with ethanol (50 mM) pre-treated astrocytes or control astrocytes. Analysis of the frequency of events, as measured by the inter-event interval (IEI), was used to determine relative differences in the number of functional synapses between treatment groups. Events were recorded from 12 neurons co-cultured with control astrocytes and 13 neurons co-cultured with ethanol (50 mM) astrocytes, from 6 independent experiments.

Interestingly, in neurons co-cultured with ethanol pre-treated astrocytes, a percentage of neurons expressed a high frequency of events, while the remaining neurons showed a limited number of events, suggesting that ethanol has differential effects on two distinct populations of neurons. This is clearly evidenced when the cumulative probability curves of the individual recordings are plotted individually (Figure 5.6.A). Because of these findings, the two ethanol groups were treated as distinct and analyzed as such. When recordings from each group were combined in arrays for analysis, eight of the 13 neurons from the ethanol-pre-treated group were quite active (EtOH-Group A: median IEI = 244.7 msecs), while events occurring in the remaining 5 neurons, or 38% of the ethanol group (EtOH-Group B) were significantly less frequent (median IEI = 1508 msecs) (Figure 5.6.B, Table 5.1). These results clearly indicate a difference in the number of functional synapses between two populations of neurons co-cultured with ethanol pre-treated astrocytes. It is of interest that two populations of neurons are also evident in the structural synapse data at the same ethanol concentration (50 mM), which is made clear when the distribution of the number of structural synapses per neuron are plotted (Figure 5.7 A & B). This is unique to the 50

mM ethanol group, as a second population of neurons is not observed after pre-treatment with either 25 or 75 mM ethanol (data not shown).

When compared to the arrayed recordings from the 12 neurons co-cultured with untreated astrocytes, the more active neurons co-cultured with ethanol pre-treated astrocytes (EtOH – Group A), show a significantly greater frequency of events when the cumulative probability curves of arrayed data are compared (Figure 5.6.B, Table 5.1, $p = 0$ by the Kolmogorov-Smirnov Two Sample test, $Z = 3.30$), with a shorter median IEI interval for the ethanol group (244.7 msec) than the control group (274.6 msecs). While the differences in the medians are slight, the mean IEI of the ethanol and control groups (435.8 ± 7.0 SEM and 592.6 ± 11.6 SEM msecs, respectively), show that events in the ethanol group occur 27% more frequently than those in the control group ($p < 0.0001$). The second, less frequent ethanol group was also significantly different than both, with a median IEI of 1508 msec (mean IEI: 2359 ± 100.9 msecs; $p < 0.001$) (Figure 5.6B, Table 5.1).

Because there were no differences in the amplitude and time to decay data between the two populations of neurons co-cultured with ethanol when analyzed separately, these recordings were combined for arrayed analysis and compared to those neurons co-cultured with control neurons. No differences were observed in the median amplitudes (control group, 18.9 pA; ethanol group A+B, 18.3 pA) (Figure 5.6C, Table 5.1), suggesting no difference in the number of post-synaptic receptors. Interestingly, there was a significant difference in the time to decay when the cumulative probability distributions were compared ($p = 0$; K-S $Z = 9.06$ as measured by the Kolmogarov-Smirnov Two Sample test). Events from neurons co-cultured with ethanol pre-treated astrocytes had a 1.3-fold longer median time to decay (median, 8.2 msecs; mean, 9.2 ± 0.07 SEM msecs) compared to those neurons co-cultured with untreated astrocytes (median, 6.3 msecs; mean, 7.7 ± 0.07 SEM msecs) (Figure 5.6D, Table 5.1). These results suggest that astrocytes pre-treated with ethanol release factors that potentiate functional synapses in some cells, but may also slow synapse formation in a second population. Additionally, the slight change in event time to decay suggests that ethanol pre-

treated astrocytes may induce a change in the composition of the neuronal post-synaptic receptor subunit.

5.3 Discussion

I have characterized how astrocytes modulate hippocampal neuron synapse formation and function in the 24-hours post-ethanol treatment. The results show that ethanol pre-treated astrocytes stimulate synaptic structure formation in a bi-phasic manner (Figure 5.2). That ethanol induces this bi-phasic effect in neurons is not unusual (Moghaddam and Bolinao, 1994; Pohorecky, 1977; Stancampiano et al., 2004). It is most evident in studies of the effect of increasing concentrations of ethanol on behavior, where alcohol, consumed in small amounts, acts as a stimulant, but with increasing consumption, it acts as a central nervous system depressant. In addition, to the biphasic effect typically observed with increasing concentrations of ethanol, as shown in this study, it is also seen after exposure to one concentration of ethanol over time. While the mechanisms are not clearly elucidated, it is thought to be due to ethanol's interaction with neurotransmitter receptors and their adaptation to its presence, or tolerance (Pohorecky, 1977). However, what is interesting here is that the neurons in this system are never exposed to ethanol, suggesting that the bi-phasic effect observed in this model system is mediated by astrocyte secreted factors.

Ethanol pre-treatment of astrocytes also induced the expression of synaptophysin and PSD-95 protein in hippocampal neurons, however, compared to the robust potentiation of synaptic structures (Figure 5.2), the increase was slight and the biphasic effect was no longer evident (1.4-1.5 fold; Figure 5.3). The fact that the changes in protein levels are not reflective of the magnitude of the increase observed in the synaptic structure experiments suggests that the pre-treatment of astrocytes with ethanol may have a greater influence on the localization of those synaptic proteins already present, as opposed to an effect on protein expression.

To show that the observed increase in synaptic structures in neurons after co-culture with ethanol pre-treated astrocytes is not limited to an effect on synaptophysin and PSD-95, neurons were also labeled with the pre-synaptic protein, synaptotagmin, and both the NR2B and NR1 subunit of the NMDA receptor. The fact that the number of NR1 labeled puncta and NR1-containing synapses increased while the number of NR2B puncta decreased suggests that ethanol pre-treated astrocytes release factors that may modulate the subunit composition of the NMDAR. The NMDAR is a heteromeric glutamate receptor with great subunit diversity. In addition to the obligatory NR1 subunit, NMDARs may contain at least one or more of the four NR2 subunits (A, B, C or D), or a combined NR2 and NR3 (A or B) subunit, and subunit composition affects receptor calcium conductance (Nagy, 2008). Further studies identifying the composition of NMDA receptors subunits in neurons co-cultured with ethanol pre-treated astrocytes would be of interest, as pre-mature changes in subunit composition may indicate the pre-mature stabilization of synapses, and may influence synaptic plasticity in neurons (Barria and Malinow, 2002, 2005; Gambrill and Barria, 2011; Nagy, 2008).

The electrophysiology data indicate that ethanol pre-treatment of astrocytes causes the release of factors that increase the number of functional synapses relative to control in one population of neurons (EtOH A, Figure 5.6.B) and this would support the observed increase in synaptic structures (Figure 5.2). Interestingly, the results also show a second population of neurons (EtOH – B, Figure 5.6B) with significantly fewer functional synapses than both the control and the more active ethanol group. It is possible that ethanol pre-treated astrocytes may release factors that promote the formation of structural synapses in one population of neurons, but that are ineffective in inducing post-synaptic maturation in others, resulting in silent synapses.

Additionally, the fact that two populations of neurons are also observed in the structural synapse data after pre-treatment of astrocytes with 50 mM ethanol (Figure 5.7), and that the effect on synaptic structures is greater at 50 mM and decreases in those neurons co-cultured with astrocytes pre-treated with 75 mM ethanol (Figure 5.2), suggests that we may have captured

transitional phase in the astrocyte-mediated ethanol effect on synapse formation, synapse stabilization or synaptic pruning. It may be possible that those neurons of the 75 mM ethanol group, which show fewer synaptic structures on average compared to those neurons of the 50 mM ethanol group, have passed through a similar phase at an earlier time point, within the 24 hours. Additional structural and electrophysiology experiments could be performed at time points prior to 24 hours to further elucidate temporal changes. Differences in the frequency of events may also be a result of an increase in the probability of neurotransmitter release, and this has not been assessed here. Additional studies, such as vesicular recycling studies, or paired pulse facilitation studies could be undertaken to investigate this possibility.

It cannot be ruled out that that the differences in the number of functional synapses between these two ethanol populations may be related to the inherent variation that comes from recording from dissociated neuronal cultures. Differences in neuronal location on the coverslip may lead to differences in the amount of innervation a neuron receives. However, no distinct second population was observed in those neurons co-cultured with control astrocytes, which would be expected if the presence of the two populations were simply due to coverslip location, as the methods used to select neurons for recording are replicated between treatment groups.

The finding of a slight, but significant difference in the event time to decay in those neurons co-cultured with ethanol treated astrocytes may also indicate differences in AMPAR subunit composition, and may indicate differences in the amount of excitatory synaptic activity experienced by the neurons of the ethanol group compared to the control group. Additional studies would be necessary to make any conclusions in this regard.

Taken together, these results indicate that astrocytes pre-treated with physiological relevant concentrations of ethanol, release factors in the time after ethanol exposure that increase pre- and post-synaptic synaptic structures and their apposition, alters NMDAR subunit composition, and potentiates synaptogenesis in one population of neurons, while reduces the number of functional

synapses in another. Together, these data suggest that astrocytes are capable of mediating a dysregulation of synaptogenesis in the 24-hours post-ethanol pre-treatment.

Chapter 6:

Astrocyte-released HDL after ethanol exposure is a candidate for observed effects

6.1 Introduction

Having shown that ethanol pre-treatment of astrocytes causes the release of factors that potentiate synaptic structure formation and modify synaptic functionality in hippocampal neurons, I next attempted to investigate a potential factor responsible for the observed effect. Extensive work in our laboratory investigating ethanol and cholesterol homeostasis has shown that the treatment of astrocytes with increasing concentrations of ethanol results in increased efflux of cholesterol-containing lipoproteins from astrocytes (Chen et al., 2013; Guizzetti et al., 2007). Additionally, cholesterol, and astrocyte-released cholesterol-containing lipoproteins have been shown to contribute to astrocyte-induced synaptogenic effects both in retinal ganglion cells and cortical neurons (Eroglu and Barres, 2010; Goritz et al., 2005; Hu et al., 2007; Mauch, et al., 2001) .

Cholesterol in the circulation does not cross the blood brain barrier, but it is endogenously synthesized, and astrocytes provide an important source of cholesterol, especially during development. At the time of synaptogenesis, expanding membranes and the generation of synaptic vesicles make the demand for cholesterol extremely high. Additionally, synaptic activity, which is energetically costly for neurons, increases during this time. While both neurons and astrocytes are able to synthesize cholesterol, it is thought that during synaptogenesis neurons will decrease their synthesis and rely on the astrocytic pool of cholesterol (Pfrieger and Ungerer, 2011).

Cholesterol plays a role as both a source of material for the formation of synaptic vesicles, as well as the pre-cursor for endogenously produced neurosteroids, which have many functions,

including an influence on the formation of synapses (Fester et al., 2009). In retinal ganglion cells, cholesterol, and glial-derived cholesterol containing lipoproteins (Goritz, et al., 2005; Mauch, et al., 2001; Pfrieger, 2003), but not apo-E alone (Christopherson, et al., 2005; Mauch, et al., 2001), have been shown to increase synapse formation. Additionally astrocyte- derived cholesterol and its derivative, estrogen, cause an increase in synapses in cortical neurons (Hu, et al., 2007). In our laboratory, a project investigating the role of ethanol in cholesterol homeostasis has shown that ethanol causes the efflux of cholesterol from astrocytes in a concentration-dependent manner (Chen, et al., 2013; Guizzetti, et al., 2007). Since astrocytes release more cholesterol to the extracellular space after ethanol exposure, it is possible that the additional substrate available to neurons may influence the formation of synapses.

A series of experiments were performed that show that cholesterol-containing high density lipoproteins (C-HDL) are sufficient to increase synapse formation in hippocampal neurons, that pharmacologically increasing astrocytic cholesterol-containing lipoprotein release is sufficient to increase synapse number in hippocampal neurons after co-culture, and that cholesterol-containing lipoproteins, released from astrocytes after ethanol pre-treatment, are necessary for the observed increase in synaptic structure formation.

6.2 Results

While it has been shown that cholesterol-containing lipoproteins are capable of increasing synapse formation in retinal ganglion cells and cortical neurons (Hu, et al., 2007; Mauch, et al., 2001), I tested whether the same effect could be seen in hippocampal neurons. Direct treatment of hippocampal neurons (13 DIC) with cholesterol-containing high density lipoprotein particles (C-HDL, 20 μg/mL cholesterol) for 24 hours induced a 3.8- fold increase in the number of synaptophysin/PSD-95 synapses ($p < 0.05$) (Figure 6.1.B). Analysis of the effect on the pre-synaptic, synaptophysin, puncta shows an increasing trend, with 20 μg/mL final cholesterol causing a 2.8-fold increase relative to control ($p < 0.001$). However, cholesterol-containing lipoproteins had no effect

on the number of post-synaptic PSD-95 puncta (Figure 6.1.A). Direct treatment of neurons with low-density lipoproteins at the same concentrations did not have an effect on the number of pre- or post-synaptic puncta, nor their overlap (data not shown). These results indicate that cholesterol-containing, high-density lipoproteins are sufficient to potentiate synapse formation in hippocampal neurons.

In our laboratory, ethanol has been shown to increase the levels of the ATP-binding cassette cholesterol transporters, ABCA1 and ABCG1 in astrocytes, resulting in increased efflux of cholesterol and cholesterol-containing lipoproteins from astrocytes (Chen, et al., 2013; Guizzetti, et al., 2007). To test whether increased-HDL release from astrocytes is sufficient to induce increased synapse formation in neurons after co-culture, ABCA1 and ABCG1 transporters in astrocytes were pharmacologically up-regulated by treating astrocytes with the nuclear liver X receptor (LXR) agonist, 22R-hydroxycholesterol (22HC) and the retinoid X receptor (RXR) agonist, 9-cis-retinoic acid (RA). Activation of the LXR/RXR nuclear transcription dimer with both agonists has been shown to increase expression of the ABCA1 and ABCG1 transporters in astrocytes (Guizzetti, et al., 2007; Koldamova et al., 2003), and we have previously shown that this results in the induction of lipoprotein release (Chen, et al., 2013; Guizzetti, et al., 2007).

Pre-treatment of astrocytes with the LXR/RXR agonists, RA and 22HC (1 μM each) for 24 hours induced a 1.7-fold increase in both the number of pre-synaptic ($p = 0.028$) and post-synaptic puncta ($p = 0.011$) (Figure 6.2.A), and caused a 2.6-fold increase in the number of aligned synaptophysin/PSD-95 synapses ($p = 0.005$) (Figure 6.2.B). These data suggest that astrocyte-released lipoproteins are sufficient to cause an increase in synaptic structure formation in hippocampal neurons.

Finally, to test whether astrocyte-released C-HDLs are necessary for the increase in synapse number observed in hippocampal neurons after ethanol pre-treatment (Figure 5.2), neuronal lipoprotein receptors were blocked during the co-culturing of neurons with ethanol pre-treated astrocytes. Using the same approach as in previous experiments, astrocytes were pre-treated with 50

mM ethanol for 24 hours. After treatment washout, neurons and astrocytes were co-cultured, in the presence, or absence of 50 nM of receptor-associated protein (RAP). RAP inhibits ligand binding to members of the low-density lipoprotein receptor family; blocking low density lipoprotein receptors has been shown to inhibit the uptake HDL (Medh et al., 1995; Posse de Chaves et al., 2000) and RAP has been shown to inhibit cholesterol-induced synapse formation in retinal ganglion cells (Mauch, et al., 2001).

In neurons co-cultured with ethanol pre-treated astrocytes, synapse number increased, as expected ($p < 0.001$, relative to control). Blocking neuronal lipoprotein receptors with the addition of RAP (50 nM) attenuated the effect of ethanol on synapse number, by 43% ($p < 0.05$ relative to ethanol). In co-cultures of neurons with un-treated astrocytes, the number of synapses formed in the presence of RAP was no different than control (Figure 6.3). These data indicate that cholesterol-containing HDL is one factor released from astrocytes after pre-treatment with ethanol that is necessary for increased synapse formation.

6.3 Discussion

The role of astrocyte-released cholesterol in synapse formation is well substantiated and the results presented here are in support of that. I have shown that ethanol-induced release of cholesterol, in the form of cholesterol-containing-HDL is necessary for an increase in synaptic structure formation.

That direct treatment of neurons with C-HDL potentiated the number of synaptic structures (Figure 6.1B) was not unexpected, as cholesterol and lipoproteins have been shown to be sufficient to do so in retinal ganglion cells and in cortical neurons (Hu, et al., 2007; Mauch, et al., 2001). The fact that increasing concentrations of C-HDL in neurons grown in the absence of astrocytes had no effect on the number of post-synaptic puncta, but had a clear effect on pre-synaptic structure formation (Figure 6.1.A) is interesting and suggests two things. First, because this finding is different than that observed after neurons were co-cultured with ethanol pre-treated astrocytes (Figure 5.2.A), C-HDL

may not be the only factor released by astrocytes capable of increasing synapse number. Secondly, it indicates that the effect of C-HDL may be mediated through a pre-synaptic mechanism. This is in agreement with studies in RGCs that also show increased pre-synaptic structures after cholesterol incubation, and those that show an increased frequency of events, related to increased probability of pre-synaptic vesicular release (Eroglu and Barres, 2010; Goritz, et al., 2005; Mauch, et al., 2001).

Because cholesterol has been shown to act through pre-synaptic mechanisms, causing an increase in the probability of presynaptic vesicular release (Goritz, et al., 2005; Mauch, et al., 2001), and because the data shown here suggest that the effect of C-HDL on neuronal synaptic structure is pre-synaptic (Figure 6.1.A), the increased frequency of events observed in neurons co-cultured with ethanol pre-treated astrocytes (EtOH A; Figure 5.5.B) may also be a result of increased pre-synaptic vesicular release. However, this has not been tested here. Experiments should be performed to examine changes in pre-synaptic function, with the use of fluorescent styryl dyes to examine vesicular recycling, or the use of paired pulse facilitation to investigate changes in the probability of vesicular release and the effect on functionality.

While the addition of exogenous C-HDL is capable of inducing increased synaptic structures in hippocampal neurons, to show that astrocyte-derived lipoprotein is a factor sufficient to induce an effect on synaptic structure formation that is comparable to what is seen in neurons after co-culture with ethanol pre-treated astrocytes, LXR/RXR agonists were used to up-regulate ABCA1 and ABCG1 cholesterol transporters in astrocytes. Because astrocytes pharmacologically treated with inducers of cholesterol transporter expression have previously been shown to cause increased lipoprotein efflux from astrocytes (Chen et al., 2011; Guizzetti, et al., 2007), the finding that the LXR/RXR agonists, HC and RA potentiated synaptic structure formation after co-culture with hippocampal neurons was not unexpected.

The fact that blocking the interaction of ethanol-induced, astrocyte released C-HDL with neuronal lipoprotein receptors using RAP in the co-culture system attenuated the ethanol effect on synapse structure formation, clearly indicates that C-HDL is an astrocyte secreted-factor that is

necessary for potentiating synapse formation in this co-culture system. At this time, it is unclear whether the effects observed here are mediated by apo-lipoprotein-E signaling at the lipoprotein receptor, or whether it is the uptake and use of cholesterol by the neurons that is responsible for the increase in synapses induced by astrocyte released C-HDL after ethanol pre-treatment. At the present time, the precise mechanisms are unclear, however, in retinal ganglion cells, it has been shown that neither direct incubation with Apo-E itself (Mauch, et al., 2001), nor immunodepletion of apo-E containing compounds from astrocyte conditioned medium (Christopherson, et al., 2005), had an effect on synapse formation, while treatment with cholesterol did (Mauch, et al., 2001). Additional studies could be undertaken to further elucidate the mechanism involved in the effect of increased C-HDL efflux on neurons. Also, it should be noted, that although treatment with ethanol induces increased cholesterol efflux from astrocytes, there is no immediate concomitant increase in cholesterol synthesis by astrocytes, which may lead to astrocyte cholesterol depletion (Chen, et al., 2013; Guizzetti, et al., 2007), and so additional deficits in synapse formation and function may occur at time points later than the one studied here.

While there is confidence in the findings that increased cholesterol-HDL release from astrocytes after ethanol exposure is playing a role in potentiating synapses at the time point investigated here, it is also clear that it may not be the only factor. The observation that cholesterol-containing lipoproteins do not induce structural post-synaptic changes (Figure 6.1 A), while the co-culture of neurons with ethanol-treated astrocytes do (Figure 5.2 A), suggests that this may be the case. The fact that blocking cholesterol uptake only partially attenuates synaptic structure formation in the co-culture system, suggests this as well (Figure 6.3).

While there are numerous potential candidates, a number of the findings presented here suggest that as a next step it would be interesting to investigate an astrocyte secreted factor that more directly influences synaptic activity and plasticity. As stated previously, astrocytes express neurotransmitter receptors and are capable of responding to their synaptic environment and releasing neuroactive “gliotransmitters” such as glutamate, ATP, d-serine, all of which can modulate

synaptic activity (Araque, et al., 1998b; Nedergaard, et al., 2003; Newman, 2003; Oberheim, et al., 2006; Panatier, et al., 2011; Parri and Crunelli, 2007; Volterra and Meldolesi, 2005). During acute ethanol exposure, ethanol inhibits excitatory NMDA receptors (Lovinger, et al., 1989). The result of that inhibition is a compensatory upregulation of the receptor during the time of exposure (Crews et al., 1996; Grant and Lovinger, 1995), which then leads to receptor overstimulation during the period of withdrawal. This is clinically evident in the symptoms of withdrawal, particularly in alcoholics, such as cognitive issues, shakes and at times, seizures. Excessive stimulation of NMDARs cause high levels of calcium to enter the cell and can result in excitotoxic cell death (Clements et al., 2012; Idrus and Thomas, 2011; Thomas et al., 2001).

Excitotoxicity occurring through NMDARs during withdrawal has been proposed as an outcome of third-trimester exposure to alcohol. In an *in vivo*, binge-like model of pre-natal alcohol exposure, behavioral deficits were attenuated when MK-801, and inhibitor of NMDARs was administered during alcohol withdrawal, but showed increased deficits when administered during ethanol exposure, suggesting that NMDAR up-regulation was mediating the negative effects of ethanol during the withdrawal period (Thomas, et al., 2001). A more recent study investigating the same time of exposure identified increases in NMDARs, as well as increased evidence of apoptotic cell death during withdrawal (Clements, et al., 2012).

The results presented here point to the ability of astrocytes to modulate ethanol's effects on synapse formation and function, and suggest a potential area of future investigation. Here I have shown an increase in excitatory synaptic structures (Figure 5.2 A, B). The fact that NR1 receptor puncta increase, while there is a decrease in NR2B subunits suggests that NMDARs subunits are replaced with alternative subunits. Additionally, the slight, but significant difference in the time to decay of events in neurons co-cultured with ethanol pre-treated astrocytes (Figure 5.6.D) suggests that there may also be a change in AMPAR subunits. Although this has not been tested and is not clear at this time, it is reasonable to assume that changes in these receptor subunits may be in response to a need to limit calcium conductance, and excitotoxicity.

While it has been debated for a while (Porter and McCarthy, 1997), astrocytes have been shown to express NMDARs (Lalo, et al., 2006; Lee, et al., 2010; Schipke, et al., 2001). It may be possible that pre-treatment with ethanol inhibits these astrocyte receptors, which may result in an up-regulation. If so, after ethanol washout, and during co-culture, when astrocytes are introduced to the neuronal milieu, including the neurotransmitter receptors released by neurons, the activation of the astrocytic NMDARs may feed back to the neurons, facilitating a change in their synaptic activity. While this is conjecture at this point, the first phases, the changes in the number and response of astrocytic NMDARs in astrocytes after ethanol exposure are easily testable.

Research in this area of NMDAR mediated-excitotoxicity has been expanding greatly since the time the idea was first proposed, however, the contributions of astrocytes have not been investigated. It is clear, through the work presented here, that their influence, although understudied, and often overlooked, may be important to our understanding of FASD and disease in general.

Chapter 7:

Conclusions

This project set out to investigate how astrocytes influence synapse formation and how cholinergic-stimulation -- a physiological process important for development -- and ethanol -- a known teratogen -- may modulate the astrocytic effect. While there are broad similarities in their effects, it is also clear that each has a distinct influence on synapse formation and I could argue that one proceeds in a seemingly more regulated progression, a progression that gently moves forward the process of synaptic maturation, while the other has a larger, dysregulating effect.

Both cholinergic-stimulation and ethanol pre-treatment of astrocytes cause the release of factors that potentiate synaptic structure formation, increasing both the number of individual pre- and post-synaptic puncta, as well as increasing their apposition, or synapse number. However, unlike the concentration-dependent effect of carbachol (Figure 3.2) the effect of ethanol is bi-phasic (Figure 5.2).

The effect on synapse number by cholinergic-stimulation was mediated through activation of both M3 muscarinic ACh and nicotinic receptors (Figure 3.3). While not tested, signaling through one or both of these regulated pathways most likely influenced the strong increase in the expression of the synaptic proteins, synaptophysin and PSD-95 (2-2.3-fold, Figure 3.5). Ethanol also induced expression of these proteins, however to a lesser extent than that seen by the regulated signaling of carbachol (1.4-1.5-fold, Figure 5.3), despite the fact that the increase in pre and post-synaptic puncta and synapse number was quite robust. Additionally, while both 50 mM and 75 mM ethanol caused an increase in protein levels, the bi-phasic effect was no longer present. Together these results suggest that the astrocyte-mediated ethanol potentiation of synaptic structures may be due more to

an effect on the localization of synaptic proteins already present into synaptic structures, as opposed to increased expression of the synaptic proteins.

The analysis of synaptic structures after labeling for alternative pre- and post-synaptic markers also yields interesting differences between the carbachol-stimulated and ethanol pre-treated astrocyte groups. While neurons co-cultured with carbachol-stimulated astrocytes showed increases in synaptotagmin and NR2B-NMDAR subunit puncta (Figure 3.4 A), those co-cultured with ethanol pre-treated astrocytes showed no change in synaptotagmin puncta, and a 19% decrease in the number of NR2B puncta (Figure 5.4 A). This decrease in NR2B puncta was not, however, reflected in a decrease in the number of excitatory NMDA receptor synapses, as neurons co-cultured with ethanol treated astrocytes showed increases in post-synaptic puncta containing the obligatory NR1 subunit of the NMDA receptor and a 2-fold increase in synaptotagmin/NR1 synapses (Figure 5.6). These results suggest that astrocytes pre-treated with ethanol mediated a shift in NMDAR subunit composition. While it is unclear which subunit is replacing the NR2B subunit in the neurons of the ethanol group, it has been shown that early interference with a normal developmental shift from NR2B-containing NMDARs at the synapse to NR2A-containing receptors results in the pre-mature stabilization of synapses, as NR2B is required for the motility of spines and facilitates normal synapse formation. Early expression of NR2A results in decreased spine motility and a decrease in the number of functional synapses, as measured by mEPSCs (Gambrill and Barria, 2011).

In the analysis of the frequency of spontaneous miniature post-synaptic currents, carbachol-stimulated astrocytes induced an increase in the number of functional synapses (Figure 3.6. Ethanol pre-treated astrocytes (50 mM) also increased the number of functional synapses in one population of neurons, but resulted in a large decrease in the frequency of events in a second group of neurons (Figure 5.6). Additionally, the time to decay in those neurons co-cultured with ethanol pre-treated astrocytes was longer, relative to control, suggesting that ethanol pre-treated astrocytes may influence a change in post-synaptic receptor subunit incorporation.

The results indicated that astrocytic TSP-1 was necessary for the potentiation of synaptic

structures in neurons co-cultured with cholinergic-stimulated astrocytes. In those neurons co-cultured with ethanol pre-treated astrocytes, cholesterol-containing high-density lipoproteins were found to be a necessary factor. While C-HDL has a potentiating effect on synaptic structure formation, ethanol increases lipoprotein efflux from astrocytes without a concomitant increase in cholesterol synthesis, so a disruption of cholesterol homeostasis occurs (Guizzetti, 2007). This may result in further detrimental effects at a later developmental time point.

During development, synapse formation is a highly-regulated process (McAllister, 2007) and the results presented here, as a whole, indicate that astrocytes are capable of positively influencing, as well as causing a disruption to typical synaptogenesis. While there are broadly similar effects between the two compounds studied here, distinct and important differences have clearly been observed. Whether the astrocytic influence is mediating a physiological process, like cholinergic-stimulation, or playing a role in mediating impairment, as in conditions like FASD, understanding the ways they do so is certainly important so that we can come to understand how better to protect the developing brain.

Figures

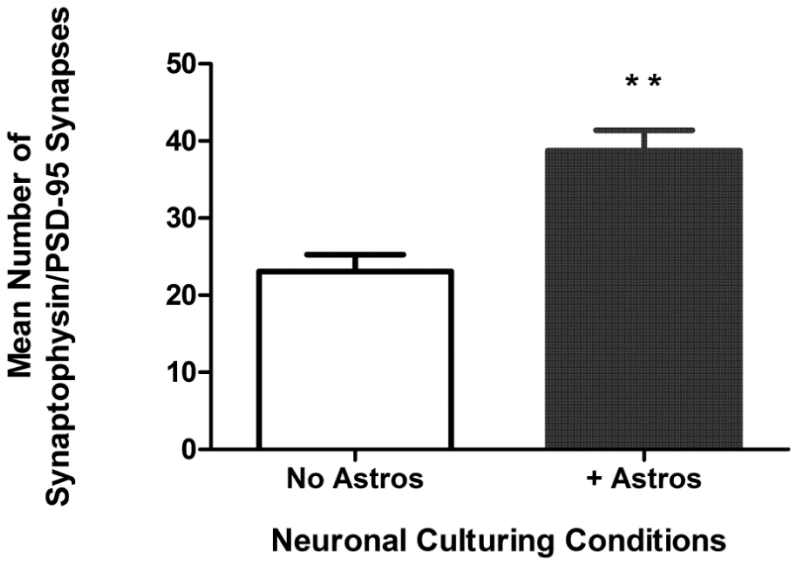


Figure 1.1: The effect of astrocytes on the mean number of synaptophysin/PSD-95 synapses in hippocampal neurons.

The mean number of synapses from control groups of those neurons that were directly treated and had no exposure to astrocytes or astrocyte conditioned medium is compared to the control group of those experiments where neurons were co-cultured for 24 hours with control neurons. The presence of astrocytes in co-culture with neurons increase synapse number by 1.7 times. (No astrocytes: n = 3 experimental groups; + astrocytes: n = 5 experimental groups). Shown are means ± SEM. Statistical significance relative to control was performed using Student's t-test * * $p < 0.0069$, relative to no astrocytes/

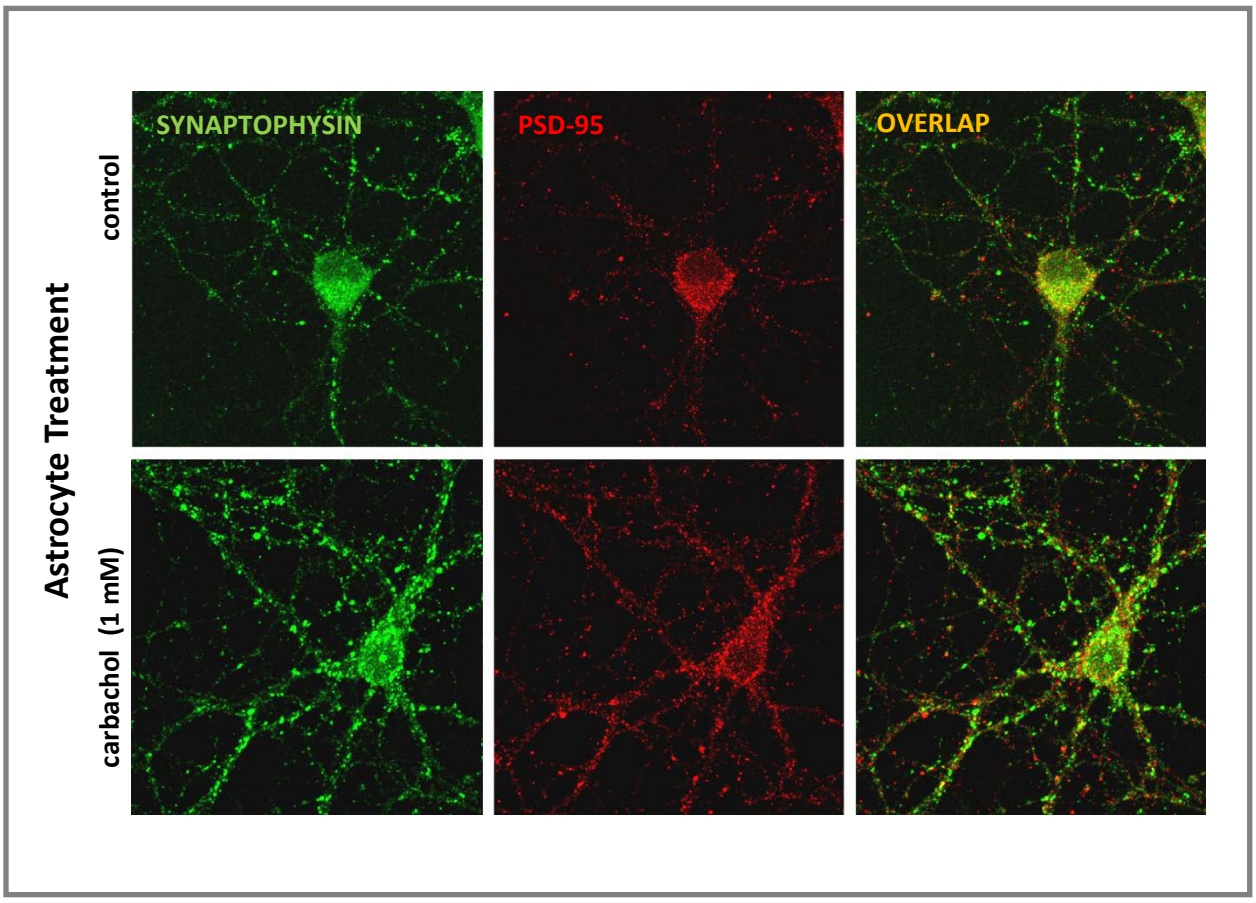
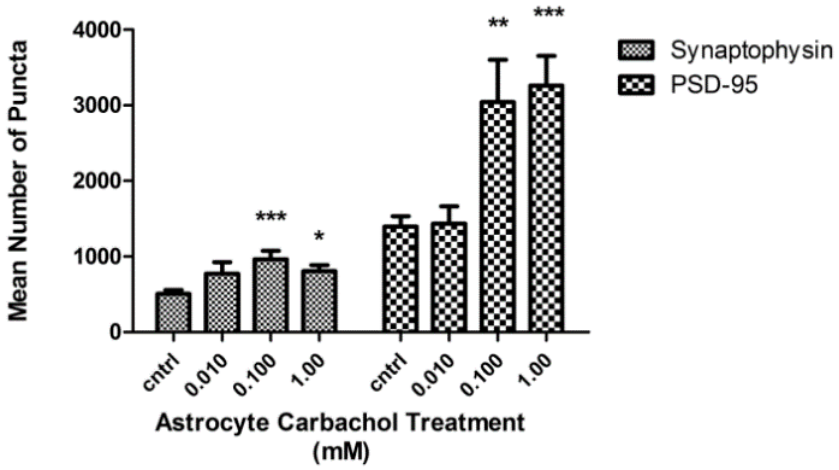


Figure 3.1: Representative images of the effect of carbachol-stimulated astrocytes on the localization of pre-and post-synaptic proteins.
Shown are representative single slice, deconvolved images of hippocampal neurons (14DIC) after co-culture for 24 hours with carbachol (1 mM) pre-treated astrocytes or untreated astrocytes. Synaptophysin = green; PSD-95 = red).

A.



B.

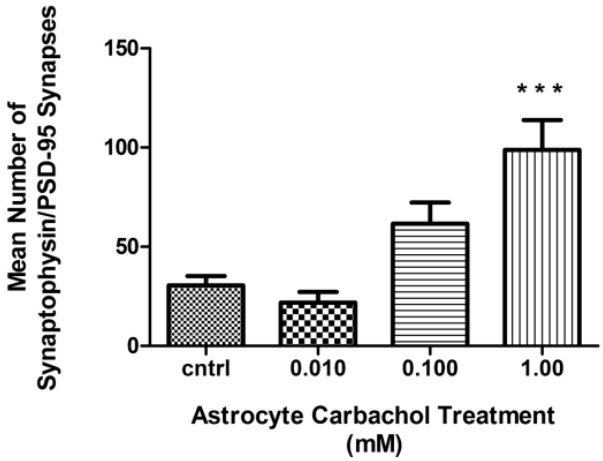


Figure 3.2: Quantification of synaptophysin and PSD-95 puncta(A) and their overlap (B) in hippocampal neurons exposed for 24 hours to control astrocytes or astrocytes pre-treated with increasing concentrations of the cholinergic agonist, carbachol.
Astrocytes were treated with carbachol (0.010, 0.100, and 1 mM) or not treated, for 24-hours. After treatment washout, hippocampal neurons grown on glass coverslips (12-13 DIC) were inverted over the astrocytes and co-cultured for 24-hours. Pre-and post-synaptic puncta and their overlap were quantified using three-dimensional analysis of confocal images after immunocytochemical labeling for synaptophysin and PSD-95. (control and 1 mM treatments: n= 55-66 neurons from 6 independent experiments; 0.010 and 0.100 mM treatments: n = 21-31 neurons from 3 independent experiments). Shown are means ± SEM. Statistical significance relative to control was performed using ANOVA analysis followed by Dunnett's Multiple Comparison test: * $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$.

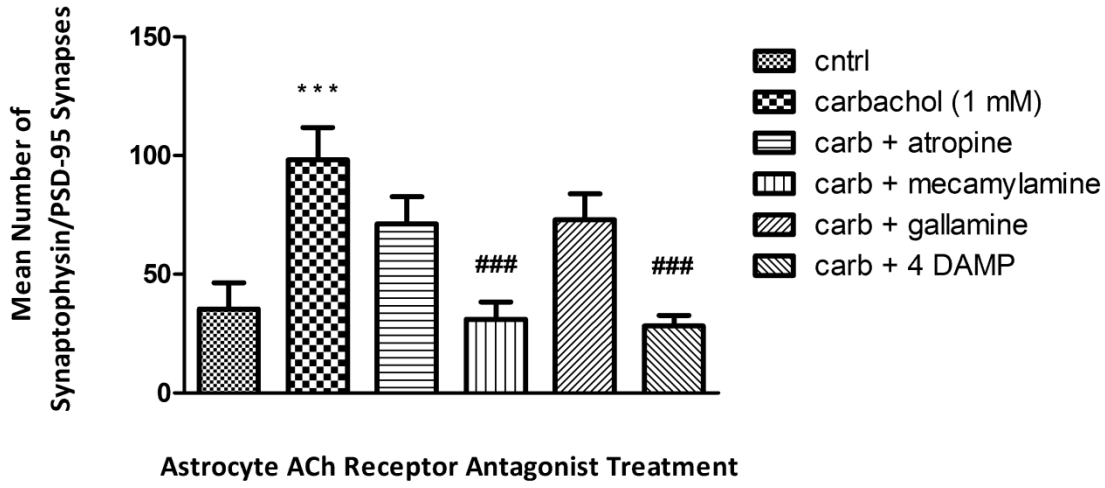
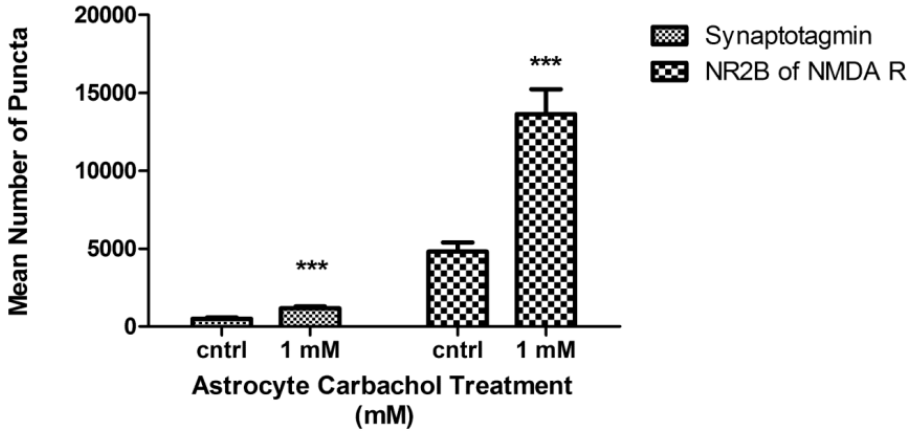


Figure 3.3: Quantification of the number of synaptophysin and PSD-95 aligned synapses in hippocampal neurons after co-culture with astrocytes co-incubated with carbachol and acetylcholine receptor (AChR) antagonists. Astrocyte AChRs were blocked with muscarinic (atropine), nicotinic (mecamylamine), M1/M2-muscarinic AChR (gallamine) and M3-muscarinic (4-DAMP) receptor antagonists for 24 hours in the presence of carbachol (1 mM). After treatment washout, hippocampal neurons were co-cultured with astrocytes for 24 hours. Pre-and post-synaptic puncta and their overlap were as quantified as previously described. (n= 25-33 neurons from 3 independent experiments). Shown are means ± SEM. Statistical significance relative to control (*) or carbachol (#) was performed using ANOVA analysis followed by Bonferroni's Multiple Comparison test. ***, $p < 0.001$ vs. control; ###, $p < 0.001$ vs. carbachol.

A.



B.

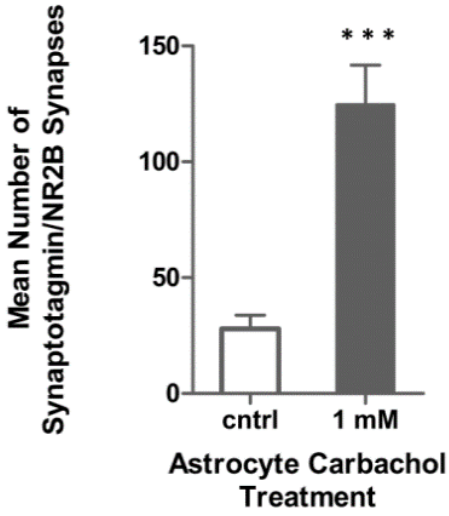


Figure 3.4: Effect of carbachol-stimulation of astrocytes on the overlap of synaptotagmin and NR2B pre-and post-synaptic puncta (A) and their overlap (B). Hippocampal neurons (13 DIC) were co-cultured for 24 hours with astrocytes pre-treated with carbachol (1 mM). Neurons were immunolabeled for the pre-synaptic protein, synaptotagmin, and the post-synaptic NR2B subunit of the NMDA receptor. The number of synaptic puncta and their overlap were quantified by three-dimensional analysis, as previously described. (n = 19-24 neurons from 2 independent experiments. Shown are means ± SEM. Statistical analysis was performed using the Student's t-test. * * * $p < 0.0001$ vs. control.

A. **B.**

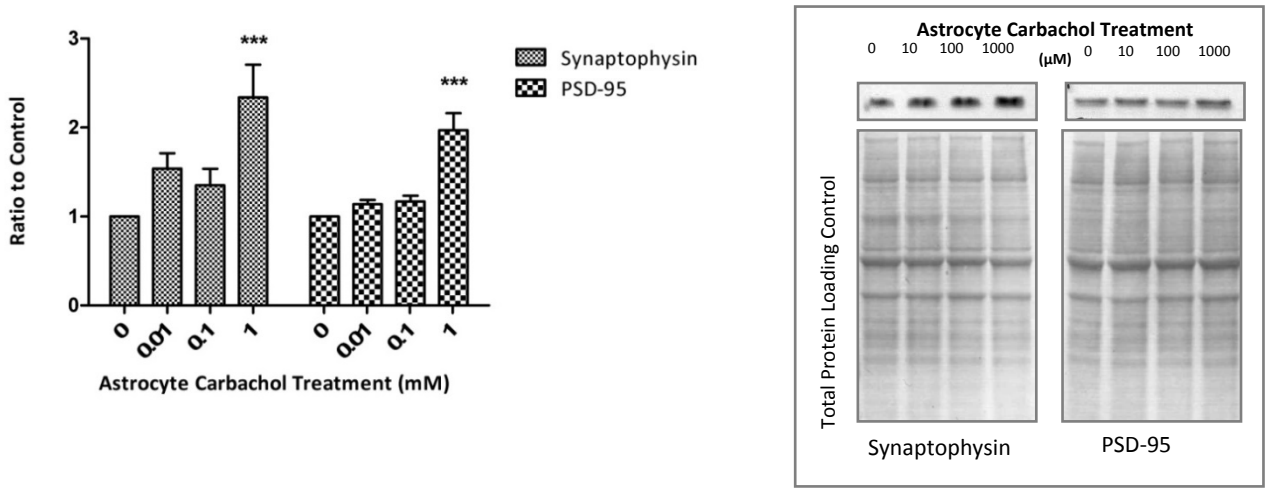
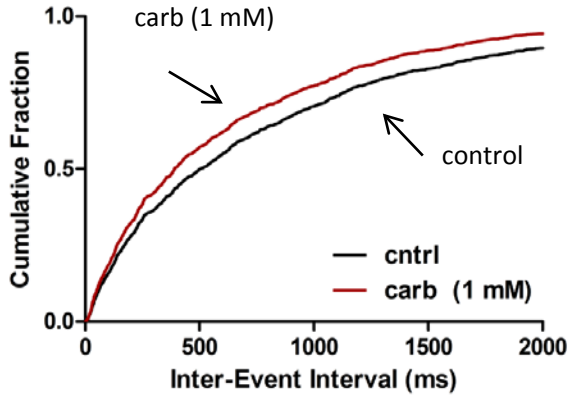


Figure 3.5: Effect of carbachol-stimulated astrocytes on levels of synaptophysin and PSD-95 protein in hippocampal neuron cell lysate. Western blot densitometry indicates increases in levels of synaptophysin and PSD-95 in neurons after co-culture for 24 hours with astrocytes pre-stimulated with increasing concentrations of carbachol(0.01, 0.10, and 1 mM) Protein levels of synaptophysin increased (2.3-fold) to a greater extent than PSD-95 (2-fold) after co-culture with astrocytes pre-treated with 1 mM carbachol. Lower concentrations did not have an effect (A). Representative images of synaptophysin and PSD-95 immunostained blots and Coomassie Blue stained total protein loading control (B). Shown are means ± SEM. Statistical analysis was performed using a one-way ANOVA analysis followed by a Dunnett's ad hoc test (* * *, $p < 0.001$); n = 5 experiments for 10 and 100 uM carbachol; n = 15 experiments for control and 1 mM carbachol).

A.



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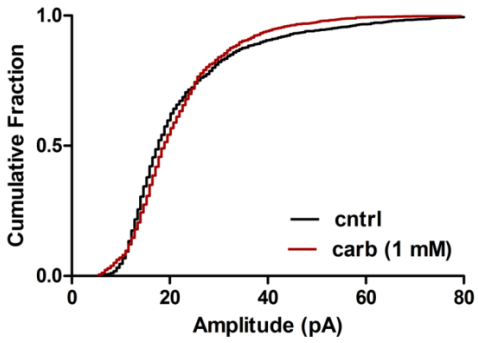
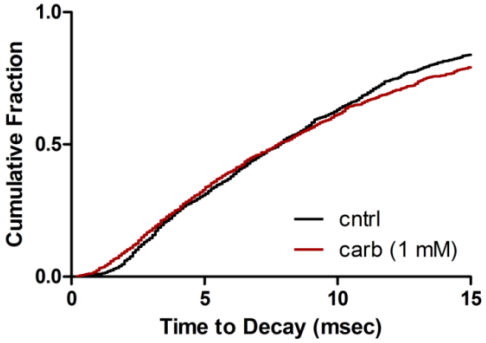


C.

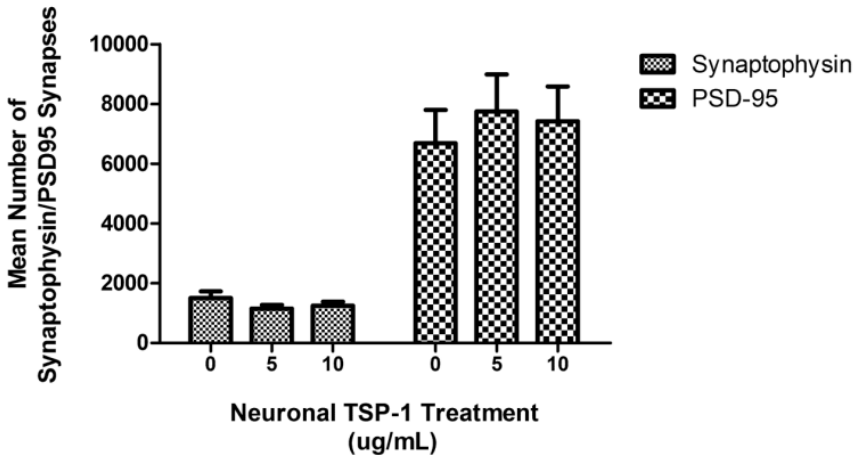


D.

	Control (n = 5)		**Carbachol (1 mM)** (n = 3)	
	Median	***Mean***	***Median***	***Mean***
IEI	503.1	847.2 ± 29.7	387.3	687.5 ± 27.3 ***
Amplitude	17.7	22.3 ± 0.36	18.9	21.4 ± 0.30
Time to Decay	7.9	9.07 ± 0.17	7.7	8.9 ± 0.14

Figure 3.6: Electrophysiological analysis of arrayed spontaneous excitatory miniature post-synaptic current (mEPSC) inter-event interval (A), amplitude (B) and time to decay (C) in neurons after co-culture with astrocytes pre-treated with carbachol (1 mM) or not treated. mEPSCs were recorded at RT in the presence of TTX (1 µM) with cells held constant at -70 mV using whole cell patch clamp techniques. IEI, amplitude and time to decay statistics (D). (cntrl n = 5, carb: n = 3 neurons from 2 independent experiments). Statistical significance of arrayed cumulative distributions was performed using the Kolmogorov-Smirnov test. Student's t-test was performed to compare mean IEI (***, $p < 0.0001$)

A.



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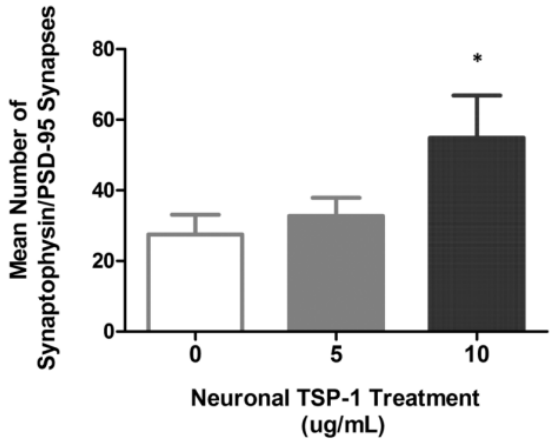
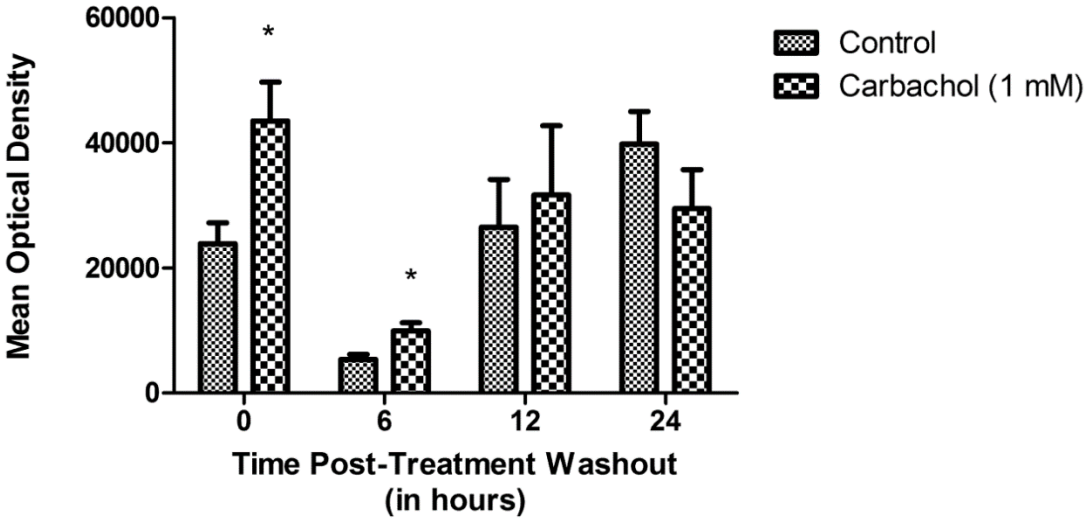


Figure 4.1: Quantification of synaptophysin and PSD-95 puncta (A) and their overlap (B) in hippocampal neurons after direct treatment with TSP-1. Primary hippocampal neurons (13 DIC) were treated with TSP-1 (5, 10 μg/mL), or not treated for 24 hours. Immunocytochemical labeling of the pre- and post-synaptic proteins, synaptophysin and PSD-95 was performed and three-dimensional analysis of confocal images was used to quantify synaptic structures overlap. (n= 19-20 neurons from 2 independent experiments). Shown are means ± SEM. Statistical significance relative to control was performed using ANOVA analysis followed by Dunnett's ad hoc test: * $p < 0.05$.

A.



B.

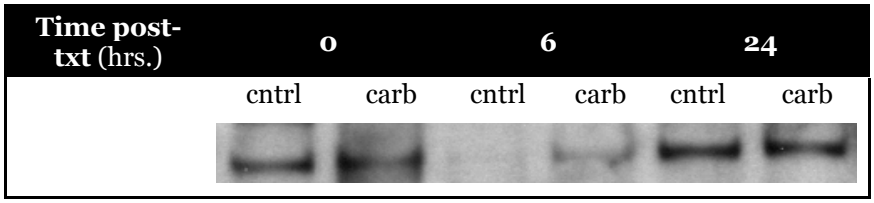
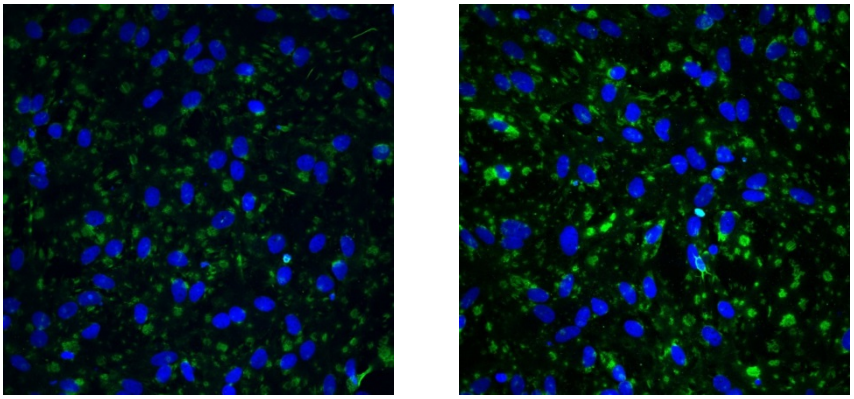


Figure 4.2: Western blot analysis of TSP1 levels released by astrocytes to the medium immediately after 24-hour carbachol (1 mM) treatment (time 0) and at 6, 12, and 24 hours post-treatment washout (A). Astrocytes, plated at a density of 2.5 x 10^6 cells per 100 mm plate were maintained in culture as previously described. Cells were treated with carbachol (1 mM) for 24 hours. At each time point, medium was collected from control and treated astrocytes then concentrated approximately 35-fold. Equal volumes of concentrated medium from each time point were subjected to gel electrophoresis and TSP1 levels were immunochemically probed and detected by chemiluninescence. Mean optical density of TSP1 bands without loading control normalization are reported here (A). Representative blots of TSP1 levels (B). Shown are means ± SEM. Statistical analysis was performed using the Student's t-test at each time point relative to control (time 0: * = 0.023; time 6: * = 0.027) n = 3-5 experiments per time point from 6 independent experiments.

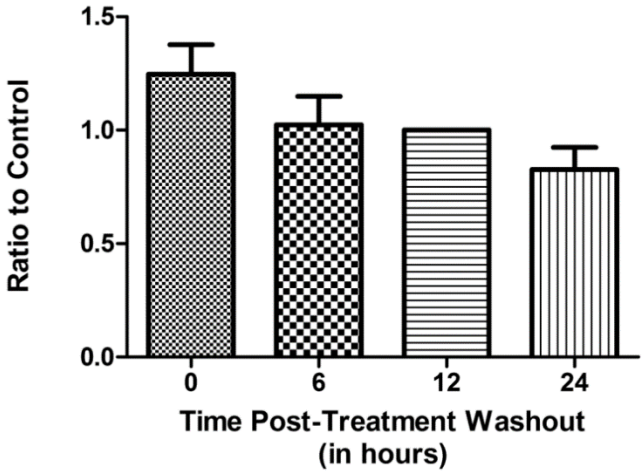


Control Carbachol (1 mM)

ASTROCYTE TREATMENT

Figure 4.3: Astrocyte TSP1 surface expression 24-hours post carbachol (1 mM) pre-treatment washout. Astrocytes were plated on 12mm glass coverslips at a density of 250,000 cells per coverslip and maintained as in previous experiments. Cells were treated with carbachol (1 mM) for 24-hours, after which treatment was washed out and the medium replaced. Cells were fixed and immunolabeled without membrane permeablization, using antibodies to TSP1 and a nuclear stain at 24-hours post treatment washout. Shown are representative images of 9-10 fields per treatment group from 1 experiment. Blue = nuclei; green = TSP1.

A.



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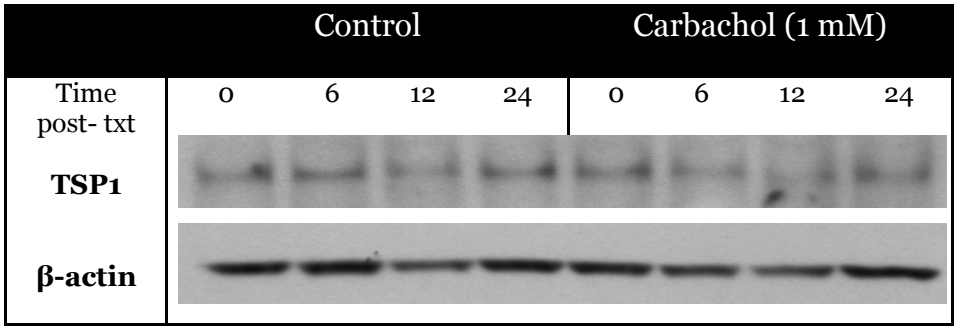

Figure 4.4: Whole-cell levels of thrombosponin-1 protein levels over time in cortical astrocytes immediately after 24-hour carbachol (1 mM) treatment (time 0) and at 6, 12, and 24 hours post-treatment washout relative to control (A). Astrocytes, plated at a density of 2.5 x 10^6 cells per 100 mm plate were maintained in culture as previously described. Cells were treated with carbachol (1 mM) for 24 hours. Cells were collected in 1% SDS lysis buffer at treatment end (time 0) and at 6, 12, and 24 hour post-treatment washout Equal amounts of protein per treatment group were subjected to gel electrophoresis. Densitometric analysis of TSP1 immunolabeled bands were normalized to β-actin loading control. (A). Representative images of TSP-1 immunostained blots and β-actin loading control (B). Shown are means ± SEM. Statistical analysis was performed using the student's t-test at each time point. n = 3 for time 0, 6, and 24 from 3 independent experiments; n = 1 for time 12).

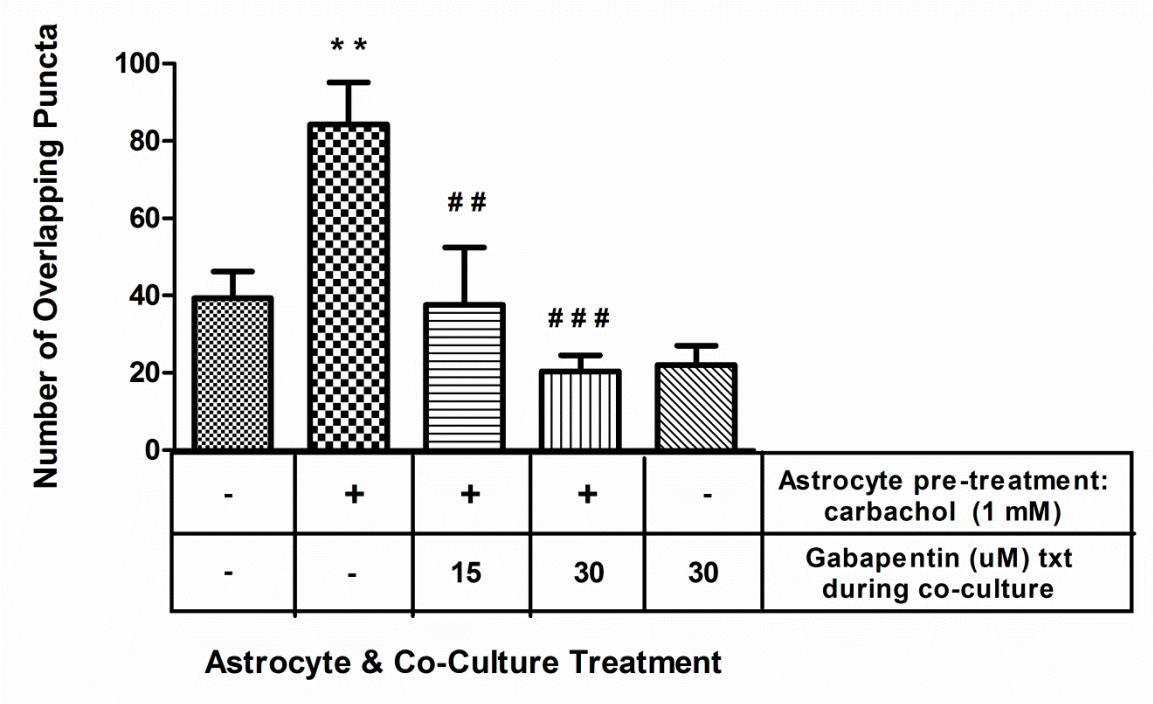

Figure 4.5: Quantification of levels of synaptophysin and PSD-95 synapses in hippocampal neurons co-cultured with carbachol pre-treated or untreated astrocytes, in the presence of gabapentin, an inhibitor of TSP1's synaptogenic effect. Astrocytes were pre-treated for 24 hours with carbachol (1 mM) or untreated. After treatment washout, astrocytes were co-cultured with hippocampal neurons for 24 hours. Gabapentin (15, 30 μM) was added to appropriate wells of the co-culture system to block TSP1 function in neurons. Pre-and post-synaptic overlap was quantified as previously described. (n= 25-43 neurons from 4 independent experiments). Shown are means ± SEM. Statistical significance relative to control was performed using ANOVA analysis followed by Bonferroni's Multiple Comparison test. * * $p < 0.01$, vs. control; ## $p < 0.01$, ### $p < 0.001$ vs. carbachol.

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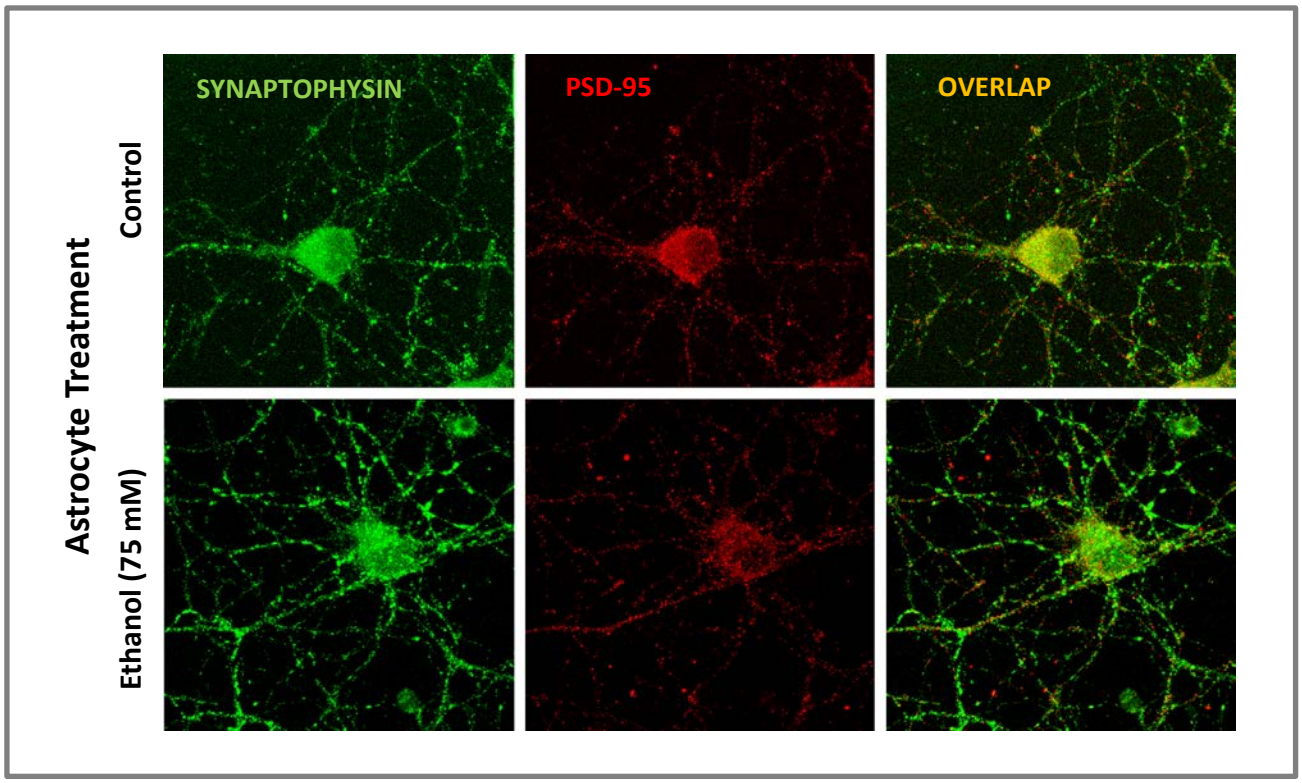
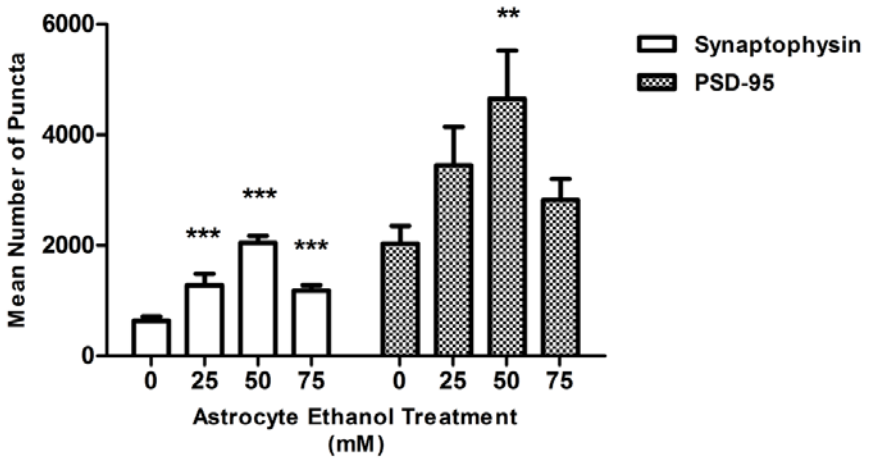


Figure 5.1: Representative images of the effect of ethanol- treated astrocytes on the localization of pre-and post-synaptic proteins. Shown are representative single slice deconvolved images of hippocampal neurons (13DIC) after co-culture for 24 hours with ethanol (75 mM)-treated astrocytes or untreated astrocytes. Synaptophysin = green; PSD-95 = red).

A.



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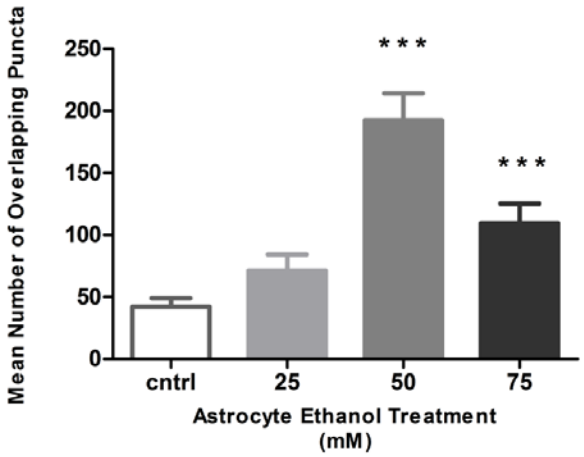


Figure 5.2: Quantification of synaptophysin and PSD-95 puncta (A) and their overlap (B) in hippocampal neurons exposed for 24 hours to control astrocytes or astrocytes pre-treated with increasing concentrations of ethanol. Pre-and post-synaptic puncta and their overlap were quantified using three-dimensional analysis of confocal images after immunocytochemical staining. Ethanol pre-treatment of astrocytes (25 mM, 50 mM, and 75 mM) induces a bi-phasic increase in the number pre- and post-synaptic puncta in hippocampal neurons after co-culturing (A). Treatment of astrocytes with 50 mM ethanol induced a 4.5 fold increase in overlapping puncta (B) (n= 70-80 fields from 6 independent experiments for control, 75 mM ethanol; n = 39-43 fields taken from 3 independent experiments for 25 and 50 mM treatment). Shown are means ± SEM. Statistical significance relative to control was performed using ANOVA analysis followed by Dunnett's ad hoc test: * $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$.

A. **B.**

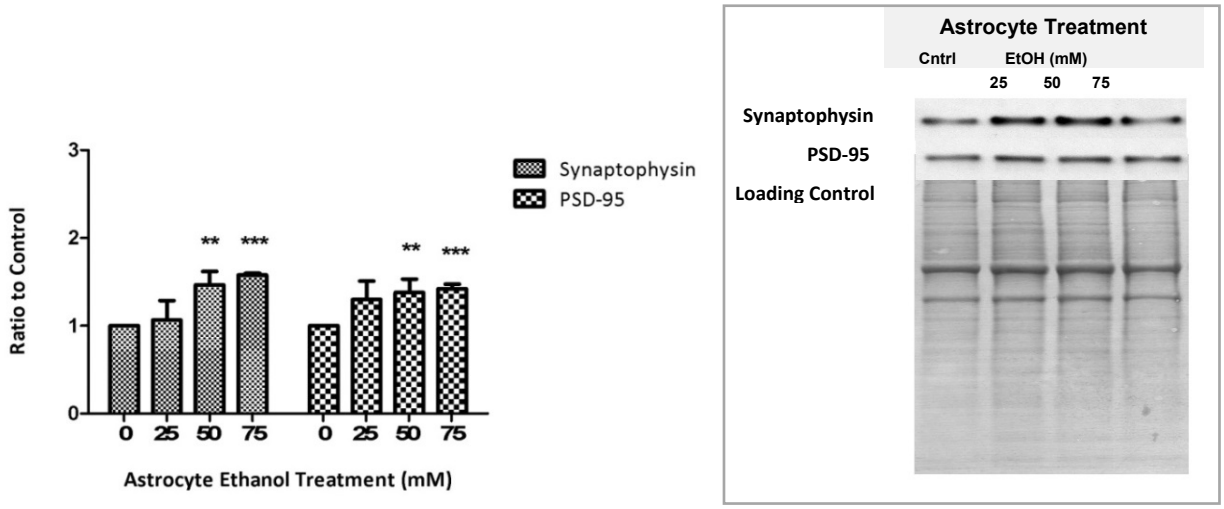
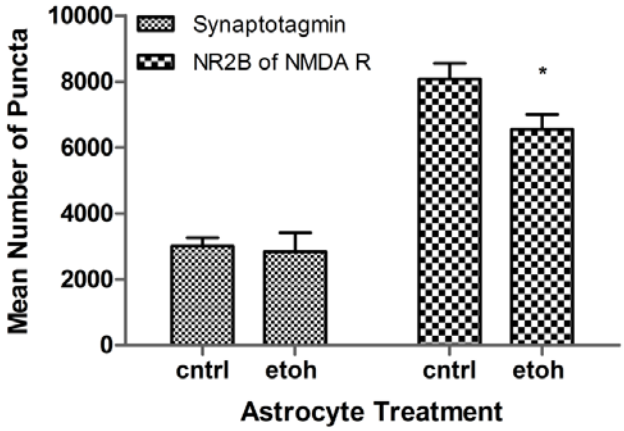


Figure 5.3: Effect of ethanol pre-treated astrocytes on levels of synaptophysin and PSD-95 protein in hippocampal neuron cytosol. Western blot densitometry indicates increases in levels of synaptophysin and PSD-95 in neurons after co-culture for 24 hours with astrocytes pre-stimulated with increasing concentrations of ethanol (25, 50, and 75 mM). Pre-treatment of astrocytes with 50 and 75 mM ethanol induced increased expression of synaptophysin protein levels relative to control to a similar extent (1.5-1.6). Both concentrations induced a 1.4-fold increase in PSD-95 protein levels (A) (n = 3 – 5 experiments per concentration). Representative images of synaptophysin and PSD-95 immunostained blots and Coomassie Blue stained total protein loading control (B). Shown are means ± SEM. Statistical analysis was performed using a one-way ANOVA analysis followed by a Dunnett's ad hoc test (* * , $p < 0.01$; * * *, $p < 0.001$).

A.



B.

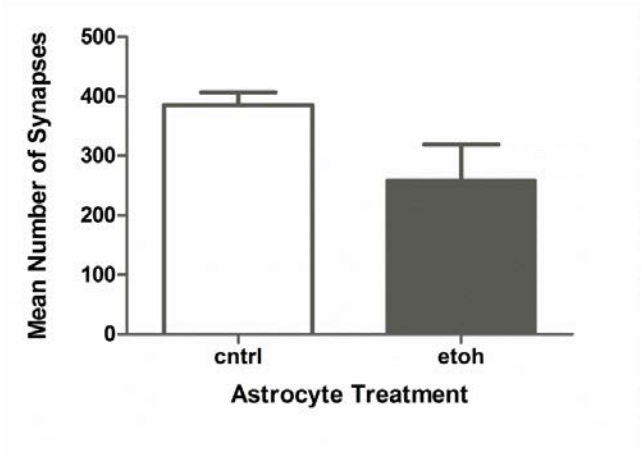
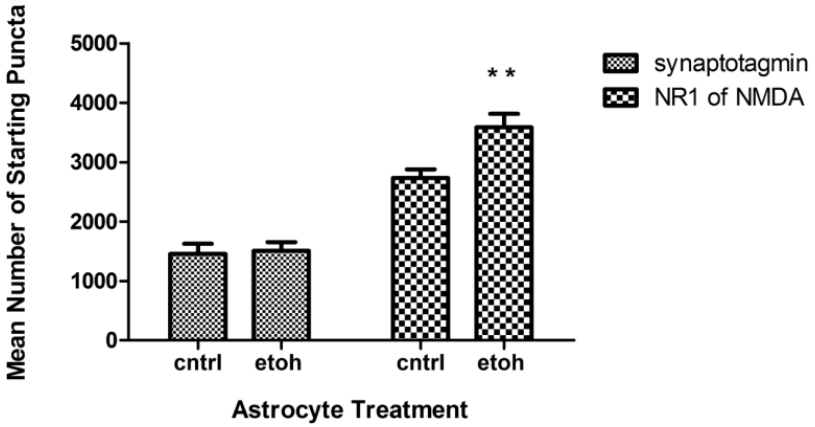


Figure 5.4: Quantification of synaptotagmin and the NR2B subunit of the NMDA receptor puncta (A) and their overlap (B) in hippocampal neurons exposed for 24 hours to control astrocytes or astrocytes pre-treated with 75 mM of ethanol. Pre-and post-synaptic puncta and their overlap were quantified using three-dimensional analysis of confocal images after immunocytochemical staining. Ethanol pre-treatment of astrocytes (75 mM) reduced the number of the number post-synaptic NMDA receptor NR1 subunit puncta by 19% relative to control ($p = 0.023$), with no change in the mean number of pre-synaptic, synaptotagmin puncta (A). Treatment of astrocytes with 75mM trended toward a 33% decrease in synaptotagmin/NR2B synapses, although this decrease was not significant (B) (n= 20-28 neurons from 2 independent experiments). Shown are means ± SEM. Statistical significance relative to control was performed using the student's t-test. *$p = 0.023$.

A.



B.

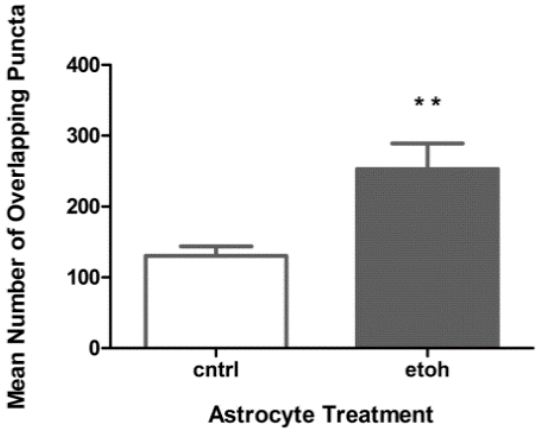
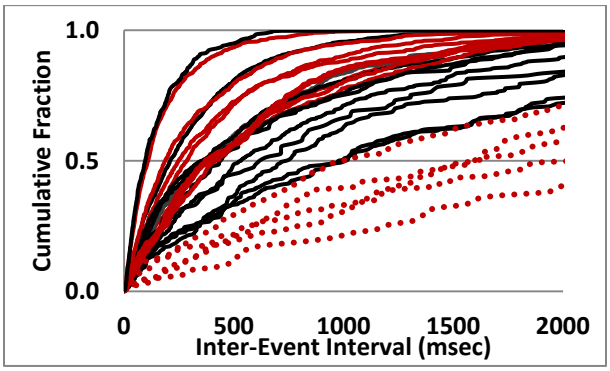
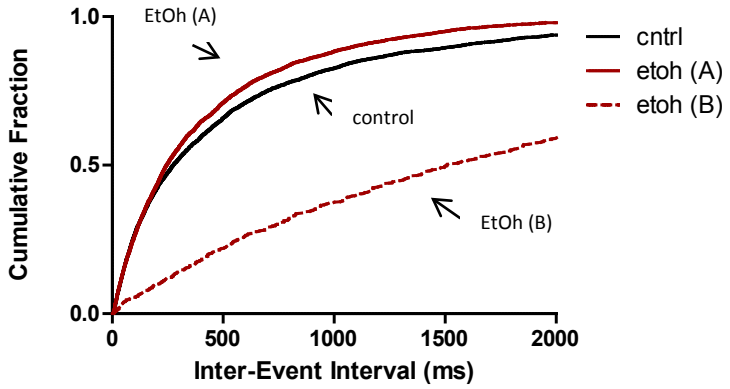


Figure 5.5: Quantification of synaptotagmin and the NR1 subunit of the NMDA receptor puncta (A) and their overlap (B) in hippocampal neurons exposed for 24 hours to control astrocytes or astrocytes pre-treated with 50 mM of ethanol. Pre-and post-synaptic puncta and their overlap were quantified using three-dimensional analysis of confocal images after immunocytochemical staining. Ethanol pre-treatment of astrocytes (50 mM) induced 1.3 fold increase in the number post-synaptic NMDA receptor NR1 subunit puncta ($p = 0.018$), with no change in the mean number of pre-synaptic, synaptotagmin puncta (A). Treatment of astrocytes with 50 mM ethanol induced a 1.9 fold increase in overlapping puncta ($p = 0.016$) (B) (n= 31-35 neurons from 3 independent experiments). Shown are means ± SEM. Statistical significance relative to control was performed using the student's t-test.

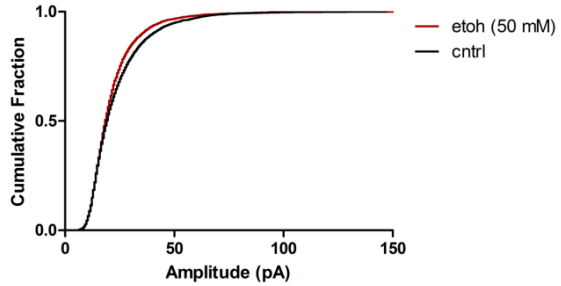
A.



B.



C.



D.

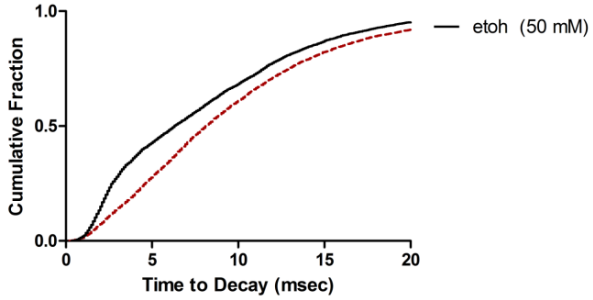
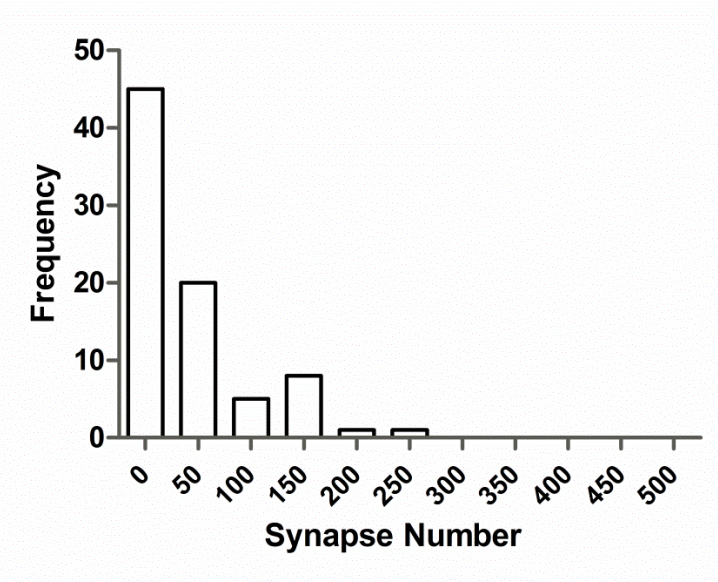


Figure 5.6: Frequency (A and B), amplitude (C) and time to decay (D) electrophysiological analysis of spontaneous miniature post-synaptic currents (mEPSCs) in neurons after co-culture with astrocytes pre-treated with ethanol (50 mM) or not treated. mEPSCs were recorded at RT in the presence of TTX (1 μM) with cells held constant at -70 mV using whole cell patch clamp techniques. Inter-event interval cumulative probability arrays (B): cntrl: n = 12 neurons; etoh (A) = 8 neurons; etoh (B): n = 5 neurons from 5 independent experiments. Amplitude (C) and Time to Decay (D) arrayed data combines both ethanol (A) with ethanol (B) groups. (cntrl: n = 12 neurons; etoh (A+B): n = 13 neurons from 5 experiments. Black = neurons co-cultured with control astrocytes; red = neurons co-cultured with ethanol (50 mM) pre-treated astrocytes. Statistical significance relative to control was performed using the Kolmogorov-Smirnov test for cumulative distributions and ANOVA followed by Dunnett's ad hoc test for comparison of means.

Table 5.1: Descriptive Statistics of mEPSC frequency (IEI), amplitude and time to decay in neurons after co-culture with ethanol (50 mM) pre-treated astrocytes (See Figure 5.6). mEPSCs were recorded at RT in the presence of TTX (1 μM) with cells held constant at -70 mV using whole cell patch clamp techniques. Inter-event interval cumulative probability arrays (B): cntrl: n = 12 neurons; etoh (A) = 8 neurons; etoh, from 5 independent experiments. Amplitude (C) and Time to Decay (D) arrayed data combines both ethanol (A) with ethanol (B) groups. (cntrl: n = 12 neurons; etoh (A+B): n = 13 neurons from 5 experiments. Black = neurons co-cultured with control astrocytes; red = neurons co-cultured with ethanol (50 mM) pre-treated astrocytes. Statistical significance relative to control was performed using the Kolmogorov-Smirnov test for cumulative distributions and ANOVA followed by Dunnett's ad hoc test for comparison of means.

	Control (n = 12)		**EtOH (A)** (n = 8)		**EtOH (B) (n = 5)**	
	Median	***Mean***	***Median***	***Mean***	***Median***	***Mean***
IEI	274.6	592.6 ± 11.6	244.7	435.8 ± 7.0 ***	1507.6	2359.1 ± 100.9 ***
	Control (n = 12)		**EtOH (A + B) (n = 13)**			
	Median	***Mean***	***Median***		***Mean***	
Amplitude	18.9	23.2 ± 0.2	18.3		21.8 ± 0.2	
Time to Decay	6.3	7.7 ± 0.07	8.2		9.2 ± 0.07 *	

A.



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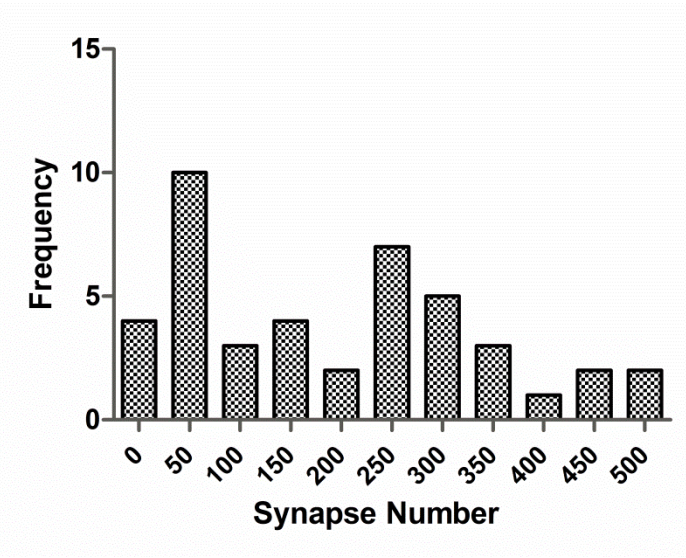
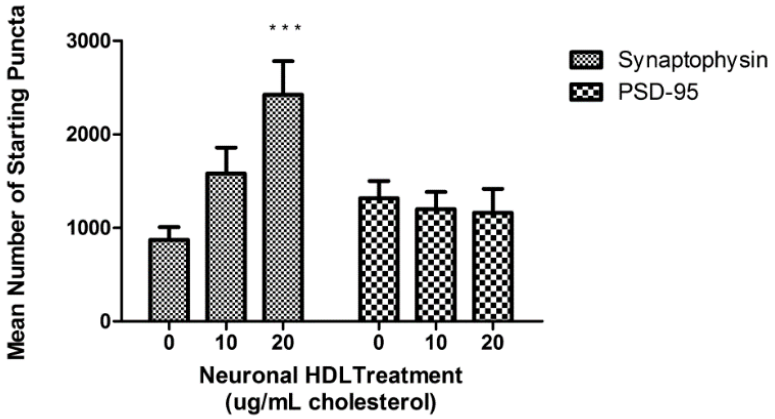


Figure 5.7: Distribution of the number of structural synapses in neurons co-cultured with control astrocytes (A) and ethanol (50 mM) pre-treated astrocytes (B). Distribution of the mean number of synapses in neurons co-cultured for 24-hours with untreated (A) or astrocytes pre-treated with 50 mM ethanol (B) (See figure 5.2B).

A.



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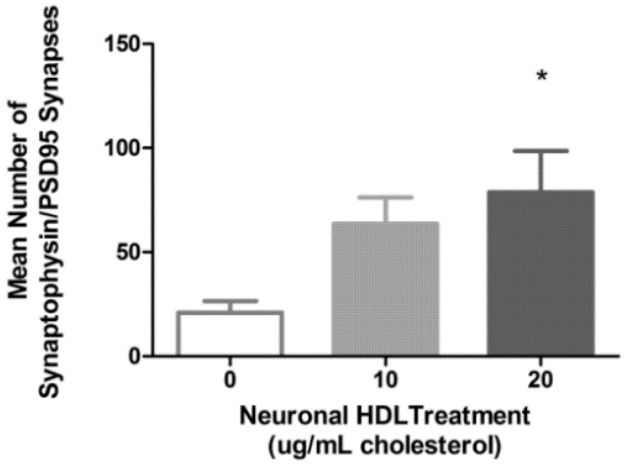
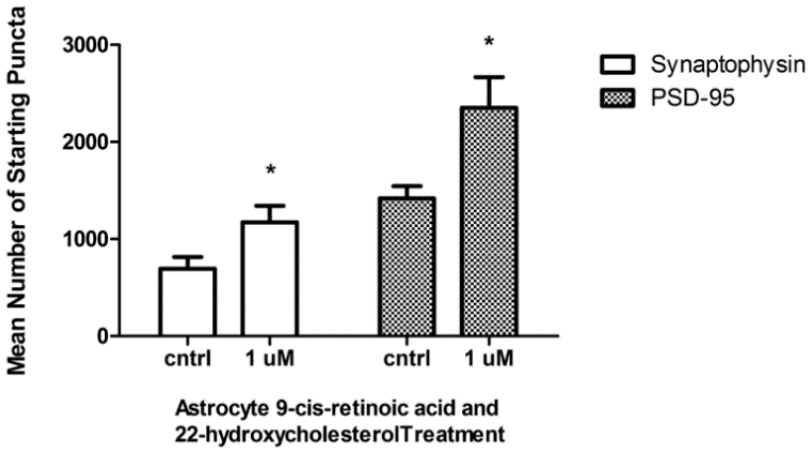


Figure 6.1: Quantification of synaptophysin and PSD-95 puncta (A) and their overlap (B) in hippocampal neurons after direct treatment with cholesterol- containing high density lipoproteins (C-HDL). Neurons (13 DIC) were treated directly with C-HDL (10 or 20 μg/mL of cholesterol), or not treated, for 24 hours. Immunocytochemical labeling of the pre- and post-synaptic proteins, synaptophysin and PSD-95 was performed Pre- and post-synaptic puncta and their overlap were quantified as previously described. Cholesterol-containing high-density lipoprotein (20 μg/mL) induced a 2.8-fold increase in pre-synaptic puncta, with no effect on post-synaptic puncta (A). The number of overlapping puncta was increased 3.8-fold (20 ug/mL HDL) (B) (n = 19-20 neurons per treatment group taken from duplicate coverslips from 2 independent experiments). Shown are means ± SEM. Statistical significance relative to control was performed using ANOVA analysis followed by Dunnett's ad hoc test: * $p < 0.05$; * * * $p < 0.001$.

A.



B.

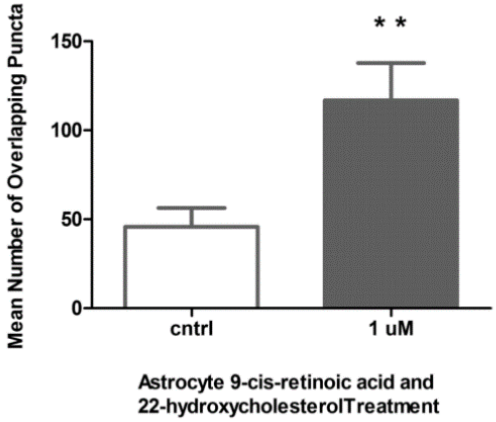


Figure 6.2: Quantification of synaptophysin and PSD-95 puncta (A) and their overlap (B) in hippocampal neurons after co-culture with astrocytes pre-treated with RXR and LXR agonists to induce increased efflux of cholesterol-containing lipoproteins. RXR and LXR agonists, 9-cis-retinoic acid (9-cis-RA) and 22-hydroxycholesterol (22-HC) induce the expression of cholesterol transporters (ABCA1 & ABCG1) in astrocytes, resulting in increased cholesterol efflux. Astrocytes were co-treated with 9-cis RA (1 uM) and 22-HC (1 uM) for 24 hours. After treatment washout, astrocytes were co-cultured with hippocampal neurons for 24 hours. Pre- and post-synaptic puncta and their overlap were as quantified as previously described. Astrocyte treatment with LXR/RXR agonists induced a 1.7-fold increase in the number of pre-synaptic puncta ($p = 0.028$) and post-synaptic puncta ($p = 0.011$). The number synaptophysin/PSD-95 synapses increased 2.6 times in neurons co-cultured with pre-treated astrocytes ($p = 0.005$) (n= 28-31 neurons from 3 independent experiments). Shown are means ± SEM. Statistical significance relative to control was performed using Student's t-test.

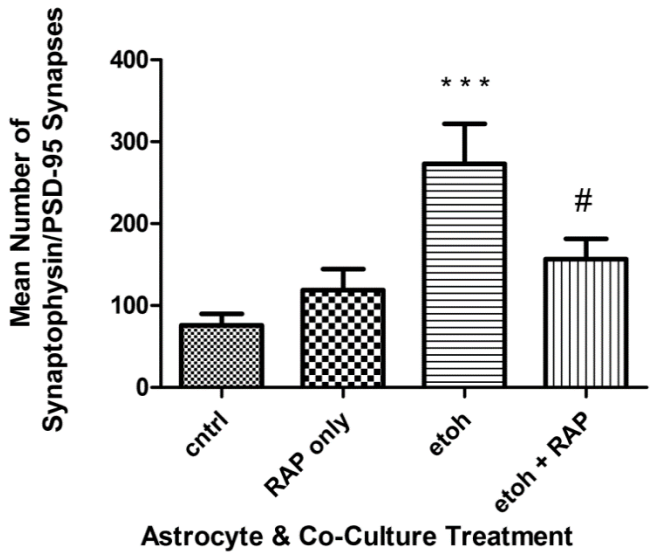


Figure 6.3: Quantification of levels of synaptophysin /PSD-95 synapses in hippocampal neurons co-cultured with ethanol (50 µM) pre-treated or untreated astrocytes, in the presence of receptor associated protein (RAP), an inhibitor of lipoprotein receptor binding. Astrocytes were pre-treated for 24 hours with ethanol (50 mM) or untreated for 24 hours. After treatment washout, astrocytes were co-cultured with hippocampal neurons for 24 hours. RAP (50 nM) was added to untreated and ethanol pre-treated treatment groups during co-culture system to block the neuronal response to cholesterol-containing high density lipoproteins. Pre- and post-synaptic overlap was quantified as previously described (n= 25-32 neurons from 2 independent experiments). Shown are means ± SEM. Statistical significance relative to control was performed using ANOVA analysis followed by Bonferroni's Multiple Comparison test. * * * $p < 0.001$, relative to control; # $p < 0.05$, relative to ethanol pre-treatment.

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