Synaptopodin is necessary for the enlargement and maintenance of dendritic spines after chemLTP through the regulation of RhoA-GTPase

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Abstract

Dendritic spines are the post-synaptic compartments of the majority of excitatory neurons in the brain, and these dynamic structures can be modulated by neuronal activity. We have previously shown that a subset of innervated dendritic spines can extend a spine head protrusion (SHP) to a neighbouring terminal as a reaction to receiving insufficient activity from their presynaptic bouton. The formation and stability of SHPs is regulated by the presence of synaptopodin, an actin-associated protein expressed within kidney podocytes and a subset of spines in the brain. Interestingly, when synaptopodin is absent in mice, *in vivo* deficits in spatial learning and impairments in long-term potentiation (LTP) in hippocampal acute slice are present.

To elucidate synaptopodin's role in synaptic plasticity, we sought to examine its cellular mechanism in structural remodelling after enhanced activity. Previous work in kidney podocytes demonstrated that the renal long-form of synaptopodin can regulate the actin cytoskeleton by competitively binding to RhoA, a member of the RhoGTPase family of small signalling G-proteins, and preventing it from being targeted for degradation by Smurf-1, an E3 ubiquitin ligase. For this reason, we investigated whether the neuronal short-form of synaptopodin regulated RhoA in a similar manner as the renal isoform. Secondly, we examined whether synaptopodin could stabilize spine volumes enlargements after LTP by influencing Ca^{2+} influx. Since synaptopodin has been shown to crosslink with α-actinin-2, which is involved in the anchoring of the GluN2B subunit of the NMDA receptors, glutamate-gated cation channels, to the PSD, we decided to examine whether synaptopodin influences GluN2B subunit protein levels. **We hypothesized that (1) synaptopodin is necessary for the enlargement and stability of dendritic spines from tertiary dendrites of CA1 pyramidal neurons, (2) the brain isoform of synaptopodin can regulate the actin cytoskeleton through competitively binding to RhoA to**

prevent its degradation by Smurf-1, and (3) synaptopodin can regulate the GluN2B subunit, known to be involved in enhanced LTP expression within the hippocampus.

Using organotypic hippocampal slice cultures, we first induced chemical-LTP (chemLTP) to examine structural plasticity of dendritic spines from tertiary dendrites of CA1 pyramidal neurons globally. We found that (1) wild-type (WT) dendritic spines underwent and maintained a spine enlargement for 45 minutes after chemLTP, (2) spines with synaptopodin exhibited a significant enlargement after chemLTP induction compared spines without synaptopodin in WT cultures, as revealed by *post hoc* analyses, and (3) spines from SPKO cultures exhibited significantly less spine volume increases post-chemLTP compared to WT. To ascertain whether synaptopodin's regulation of spine enlargement was due regulation of RhoA expression, we used both immunoblot analysis and immunohistochemistry, and found a significant decrease in RhoA and a significant increase in Smurf-1 expression along the tertiary dendrites of CA1 pyramidal neurons in SPKO cultures compared to WT. Finally, we found that there was a significant reduction in GluN2B expression levels within adult hippocampi of SPKO mice compared to WT.

Our findings show that synaptopodin is a necessary component of dendritic spine stability within CA1 pyramidal neurons after LTP. Moreover, the decrease in RhoA expression and increase in Smurf-1 expression observed within dendrites of CA1 pyramidal neurons suggest that synaptopodin may regulate spine stability through the stabilization of the actin cytoskeleton. Finally, the decrease of GluN2B levels in SPKO hippocampi compared to WT suggests that synaptopodin may regulate the presence of GluN2B subunit in the hippocampus, which could result in reduced activity-dependent remodelling and learning deficits in SPKO mice.

Résumé

Les épinées dendritiques sont les compartiments postsynaptiques de la plupart des neurones excitateurs dans le cerveau, et ces structures dynamiques peuvent être modulés par l'activité neuronale. Nous avons déjà montré qu'une partie des épinées dendritiques innervés peuvent élargir une Protrusions de têtes des épinées (spine head protrusion, *SHP* en anglais) à une terminale voisine comme une réaction à une activité insuffisant reçue de ses boutons présynaptiques. La formation et la stabilité des SHP sont régulés par la présence de synaptopodin, une protéine qui est associé à actine et dont expression a lieu dans les podocytes du rein et une partie des épinées dendritiques du cerveau. Intéressement, lorsque synaptopodin est absente dans les souris, il y a des déficits *in vivo* au niveau de l'apprentissage spatial et déficiences dans la potentialisons à long terme (PLT) dans des tranches d'hippocampe.

Pour explorer la fonction de synaptopodin dans la plasticité synaptique, on a décidé d'examiner son mécanisme cellulaire au remodelage structural après une augmentation d'activité, et comme synaptopodin pourrait réguler l'élargissement des épinées dendritiques après PLT. Des recherches précédentes sur les podocytes des reins ont montré que la longue forme rénal de synaptopodin peut réguler le cytosquelette d'actine par une reliure compétitive à RhoA, un membre de la famille de RhoGTPase des petites Protéines-G de signalisation, et en prévenant que RhoA soit ciblée pour la dégradation par Smurf-1, une ligase ubiquitine E3. Pour cette raison, on a décidé d'examiner si la petite forme de synaptopodin participe à la régulation de RhoA dans une façon similaire à l'isoforme rénale. Ensuite, on a décidé d'examiner si synaptopodin pourrait stabiliser les agrandissements des volumes des épinées après PLT, à travers une influence sur les influx de calcium. Puisque des autres études ont déjà montré que synaptopodin établisse une liaison transversale avec alpha-actinin-2, laquelle est impliqué dans l'ancrage de la sous-unité

GluN2B des récepteurs NMDA des canaux de cations régis par glutamate, à la densité postsynaptique, on a décidé d'examiner si synaptopodin influence les niveaux protéiques de la sous-unité GluN2B.

Nous avons formulé les hypothèses suivants, (1) synaptopodin est nécessaire pour l'élargissement et la stabilité des épinées dendritiques des dendrites tertiaires des neurones pyramidaux en CA1; (2) l'isoforme de synaptopodin au cerveau peut réguler la sous-unité GluN2B, laquelle a été impliqué dans une augmentation de la PLT à l'hippocampe.

Avec des cultures des tranches organotypiques d'hippocampe, pour commencer nous avons utilisé un protocole de PLT-chimie (chem-LTP) afin d'examiner la plasticité structurelle des épinées dendritiques sur des dendrites tertiaires de neurones pyramidales. On a trouvé que les épinées dendritiques du type sauvage (WT) ont suivi et maintenu un élargissement pour 45 minutes après le PLT-chimie par rapport aux épinées sans synaptopodin dans les cultures WT, comme les analyses *post-hoc* ont révélé, et (3) les épinées dans les cultures SPKO montrent une mineur augmentation du volume de l'épinée après PLT-chimie par rapport au WT. Pour évaluer si la régulation de l'élargissement des épinées par synaptopodin est due à la régulation de l'expression de RhoA, on a utilisé des analyses d'immunoblot et immunochimie, et on a trouvé une réduction significative de RhoA et une augmentation significative de l'expression de Smurf-1 sur les dendrites tertiaires de neurones pyramidales de CA1 dans les cultures SPKO en relation aux WT. Finalement, on a aussi trouvé une réduction significative des niveaux d'expression de GluN2B dans les hippocampes adultes de SPKO en relation à WT.

Nos résultats montrent que synaptopodin est un component nécessaire pour la stabilité des épinées dendritiques dans les neurones pyramidales à CA1 après PLT. D'ailleurs, la réduction de l'expression de RhoA et l'augmentation de l'expression de Smurf-1 que l'on a

observé dans les dendrites des neurones pyramidales de CA1 suggèrent que synaptopodin peut réguler cette stabilité à travers la déstabilisation de la cytosquelette d'actine. Finalement, la réduction des niveaux GluN2B dans les hippocampes SPKO en relation aux WT indique qu'il se peut que synaptopodin régule la présence de la sous-unité GluN2B dans l'hippocampe, et cela pourrait entraîner une réduction du remodelage qui dépende de l'activité et déficits d'apprentissages en souris SPKO.

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Introduction and Statement of the Problem

Neural connections in an adult brain were once thought to be stable structures maintained throughout the course of a lifetime. With the development of live imaging techniques, we now know that neural circuits are dynamic structures with synaptic connections that can be either maintained or modified during development, and this plasticity persists even after maturation (Lendvai *et al.*, 2000; Trachtenberg *et al.*, 2002; Holtmaat *et al.*, 2006). In the brain, dendritic spines form the post-synaptic compartments of the majority of excitatory neurons, and are morphologically distinct electrical and biochemical compartments that protrude off the dendrite (Bourne & Harris, 2008). These highly dynamic structures utilize the actin cytoskeleton to undergo activity-dependent morphological changes (Bourne & Harris, 2007; Rochefort & Konnerth, 2012). We have previously shown that a subset of innervated spines can extend a spine head protrusion (SHP) to a neighbouring terminal as a reaction to receiving insufficient activity from their presynaptic bouton (Richards *et al.*, 2005). The formation and stability of SHPs appears to be regulated by the presence of synaptopodin, an actin-associated protein expressed within kidney podocytes and a subset of spines in the brain (Deller *et al.*, 2000; Richards *et al.*, 2005). Synaptopodin has been shown to be involved in calcium dynamics, synaptic plasticity, and learning and memory within the hippocampus (Deller *et al.*, 2003; Jedlicka *et al.*, 2008; Jedlicka *et al.*, 2009; Vlachos *et al.*, 2009; Vlachos *et al.*, 2013). However, synaptopodin's mechanism in learning has yet to be elucidated. Therefore, we decided to examine synaptopodin's role in structural remodelling after a learning stimulus. Previously, the kidney isoform of synaptopodin has been shown to regulate the actin cytoskeleton by competitively binding to RhoA-GTPase, and preventing RhoA from being targeted for degradation by Smurf-1, an E3 ubiquitin ligase (Asanuma *et al.*, 2006). So, we decided to

examine whether the brain isoform of synaptopodin is involved in the regulation of Rho-GTPase in a similar manner as the kidney isoform.

An alternative mechanism to the stabilization of the actin cytoskeleton could be through the modulation of Ca^{2+} present within a dendritic spine after enhanced activity. Previous work done in our lab has shown that synaptopodin can influence the stability of spine head protrusions (SHPs) through calcium-induced calcium release (CICR) from ryanodine-sensitive Ca^{2+} stores (Verbich, 2013). Moreover, we have previously shown that SHP formation partially depends on NMDA receptor activation (Richards *et al.*, 2005). As NMDA receptors can trigger CICR important for SHP stability through Ca^{2+} influx, we investigated whether synaptopodin could stabilize spine volumes enlargements after enhanced activity through the regulation of Ca^{2+} influx. Since synaptopodin has been shown to crosslink with α-actinin-2, which is involved in the anchoring of the GluN2B subunit of NMDA receptors to the PSD, we decided to examine whether synaptopodin influences the expression of the GluN2B subunit.

Background

1.1 General Introduction

The astonishing complexity of the human brain enables us to explore surrounding environments, integrate information, and make decisions. The human brain is able to accomplish this task via communication of 100 billion neurons through trillions of specialized connections between neurons called synapses. Synapses were once thought to be static structures, but with the advent of real-time imaging techniques, we now know that synapses are highly dynamic even in the adult brain, both at a morphological and functional level (Dailey & Smith, 1996a; Bonhoeffer & Yuste, 2002; Trachtenberg *et al.*, 2002; Yoshihara *et al.*, 2009). Since synapses maintain an important role for neuronal function, considerable research has been done to understand their normal development, maintenance, and dynamic plasticity, as well as the disruption of synaptic structures in order to better understand neuronal communication and brain functioning, and how it may be altered by disease (Holtmaat & Svoboda, 2009; van Spronsen & Hoogenraad, 2010; Audrain *et al.*, 2016).

A significant portion of our perception of how central excitatory synapses function has been done within the hippocampus, a brain region involved in learning and memory, spatial memory, and navigation (Scoville & Milner, 1957; O'Keefe & Dostrovsky, 1971; Moser *et al.*, 2008). Over the past 50 years, work fulfilled on the central excitatory synapses in the hippocampus has led to the discovery that modifying synapses and neural circuits underlies learning, memory, and behaviour (O'Keefe & Dostrovsky, 1971; Bliss & Lømo, 1973; Thompson, 1983). Interestingly, synapses experience a heterogeneous response to activity. In fact, there are subtypes of synapses that are more susceptible to structurally respond (Harris *et al.*, 1992); however, the mechanism behind the heterogeneous response has yet to be elucidated.

Therefore, my MSc thesis focused on how spine heterogeneity is involved in spine structural remodelling after learning paradigms in the hippocampus, especially focusing on the CA3-CA1 synapse, as it is a highly studied brain region that is affected by many disorders, such as epilepsy, stroke, and Alzheimer's disease.

1.2 The Hippocampus

The hippocampus is a brain structure located in the medial temporal lobes that is important for the formation and consolidation of new memories (Scoville & Milner, 1957; Smith & Milner, 1981). When cut transversely, the unique structural and neuronal organization of the hippocampus is evident even at the anatomical level.

Although the hippocampal structure served to intrigue anatomists, its function in cognition was unknown until its importance in learning and memory was characterized in the 1950s by Dr. Wilder Penfield (Penfield & Baldwin, 1952; Penfield & Milner, 1958). After neurosurgeon Dr. Penfield performed unilateral medial temporal lobe resections to treat epilepsy, he noticed that two of his patients developed anterograde amnesia, an unexpected complication (Penfield & Baldwin, 1952; Penfield & Milner, 1958). This phenomenon was observed by Dr. Brenda Milner, who was later invited to study H. M. (Henry Gustave Molaison), a patient of Dr. William Scoville, that received an experimental bilateral medial temporal lobe resection to treat his intractable epilepsy (Scoville & Milner, 1957). Drs. Scoville and Milner had observed that this surgery resulted in a more severe form of anterograde amnesia than Dr. Milner had seen in previous patients with a unilateral medial temporal lobe resection (Scoville & Milner, 1957; Smith & Milner, 1981), thereby indicating that the hippocampus is an important structure for memory formation.

The hippocampus is one of the most studied regions of the brain not only for its role in memory, but also due to its cellular organization that has proven advantageous for the studying synaptic connections between neurons. The hippocampus is organized in distinct layers and has a unidirectional synaptic projection. Moreover, the Schaffer collateral synapse that forms between the CA3-CA1 pyramidal neurons is the best-characterized excitatory synapse in the brain (Bourne & Harris, 2008; Yuste, 2011; Frotscher *et al.*, 2014). These properties of the hippocampus offer many advantages to study structural remodelling at central synapses.

1.1 Comparison of *in vitro* and *ex vivo* techniques

The role of the hippocampus in learning and memory, as well as its unique well-structured thoroughly characterized anatomy allows it to be an apt model for studying synaptic plasticity. However, given that the hippocampus is a deep-lying brain structure found within the medial temporal lobes under the inferior horn of the lateral ventricles, it is difficult to functional and morphologically study these synapses with *in vivo* models.

Therefore, since the 1960s neuroscientists have been removing rodent brains and creating tissues preparations in order to record electrical responses and image neuronal morphology (Yamamoto & McIlwain, 1966; Madison & Edson, 2001). The most widely used methods are acute slices, organotypic hippocampal slice cultures, and primary dissociated neuronal cultures.

Acute slices allow synapses to be accessible to manipulations for electrophysiological or morphological study, however, within acute slices synaptic structures experience rapid, transient changes compared to fixed slices from rats (Reid *et al.*, 1988; Kirov & Harris, 1999). This level of structural modification proves to be a challenge as we wish to study structural synaptic plasticity, therefore, we need a stable preparation that is not undergoing structural changes because of damage.

An alternative method widely used to study neuronal function is the production of dissociated neuronal cultures, which involves mechanically disrupting the isolated hippocampus, and plating them in culture dishes. Since dissociated cultures have a lower neuronal population density, this allows for the studying and manipulation of synaptic proteins (Goslin *et al.*, 1998). However, the low density of neurons and their lack of organized synaptic network makes it more difficult to study electrophysiological activity (Papa *et al.*, 1995). Moreover, the neuronal morphology in dissociated cultures does not resemble that of *in vivo* tissue (Cornell-Bell *et al.*, 1990). Therefore, in order to study synapse structural plasticity and what underlies synapse remodelling, we need a preparation that maintains neuronal morphology and its synaptic network as close to *in vivo* tissue as possible.

Since we wished to study synapse structural remodelling in an intact network that resembled the *in vivo* hippocampus, we utilized organotypic slice cultures, which are more amenable to studying structural remodifications. Organotypic slice cultures were allowed to mature for 3 weeks before experimentation (Mateos *et al.*, 2007; Verbich *et al.*, 2012). Immediately following explanation, neurons undergo active synaptogenesis for the first week (Dailey & Smith, 1996b; Fiala *et al.*, 1998). Post-synaptogenesis there is a critical period of refinement whereby the overproduction of synapses on dendritic shafts and immature protrusions are either eliminated or stabilized. During the following two weeks the total number of excitatory synapses decreases as some synapses are pruned, and by week three the density of synaptic connections reaches an equilibrium that is maintained for the duration of the culture lifespan of several months (McKinney, 2010). The maturation period of organotypic cultures enables the slice culture to maintain intrinsic connectivity, neuronal organization and physiological characteristics of neurons *in vivo* similar to what has been seen in acute slices (Gähwiler *et al.*,

1997; Gähwiler *et al.*, 1998). After their maturation, organotypic slices maintain an active neuronal network where cellular models of learning and memory, such as long-term potentiation (LTP) and long-term depression (LTD) can be induced similar to acute hippocampal slices (Bonhoeffer *et al.*, 1989; Debanne *et al.*, 1994). Moreover, the density and specificity of synaptic connections is similar to *in vivo* postnatal day 15 rodents, which suggests that mature orgnaotypic slices are able to form stable synapses that can undergo synaptic plasticity (McKinney *et al.*, 1999; De Simoni *et al.*, 2003). Therefore, in order to study structural synaptic remodeling with both high temporal and spatial resolutions that are representative of the *in vivo* hippocampus, we used organotypic slice cultures.

1.4 Dendritic Spines

1.4.1 Dendritic spine formation

Dendritic spines are the post-synaptic compartments of the majority of excitatory glutamatergic neurons in the brain (Bourne & Harris, 2008; Yuste, 2011; Frotscher *et al.*, 2014). They are protrusions off the dendritic shaft that form synapses with the presynaptic terminal of another neuron. The formation and maturation of excitatory synapses begins during the first postnatal week in rodents (Harris, 1999). This process has been postulated to be one of two main models: (1) axons travel in the neuropil attracting filopodia extensions from nearby dendrites (Jontes & Smith, 2000; Yuste & Bonhoeffer, 2004) or (2) that during development filopodia are randomly sent off dendritic shafts into the neuropil where they may come into contact with a presynaptic terminal (Dailey & Smith, 1996b; Ziv & Smith, 1996; Lohmann & Bonhoeffer, 2008). Filopodial extensions are believed to then form stable synapses once they are in contact with presynaptic zones that can trigger local Ca^{2+} transients (Ziv & Smith, 1996; Lohmann *et al.*, 2005; Lohmann & Bonhoeffer, 2008; Kanjhan *et al.*, 2016).

1.4.2 Dendritic Spines as Postsynaptic Sites for Excitatory Synaptic Transmission

Dendritic spines are composed of a spine head and neck that allows for the separation of the active zone and the dendrite. This enables spines to serve as biochemical and electrical compartments that isolates incoming neuronal information to maintain synapse specificity (Harris & Stevens, 1989; Harris *et al.*, 1992; Bloodgood & Sabatini, 2005; Yuste, 2011; 2013; Tonnesen *et al.*, 2014). The tip of spine head is composed of the post-synaptic density (PSD), a thickening of the postsynaptic membrane at the synaptic junction due to a high density of proteins (Gray, 1959; Harris *et al.*, 1992). The size of the PSD correlates to the size of presynaptic active zone, thus larger synapses should be functionally stronger than smaller synapses (Harris & Stevens, 1989; Shepherd & Harris, 1998; Sheng & Hoogenraad, 2007; Meyer *et al.*, 2014). The proteins found within the PSD are involved in synaptic transmission and also synaptic plasticity (Sheng & Hoogenraad, 2007; Colgan & Yasuda, 2014). Scaffolding proteins such as, PSD-95, act to anchor all of the required neurotransmission machineries including, neurotransmitter receptors and signalling proteins to the PSD (Cho *et al.*, 1992; Müller *et al.*, 1996; Bourne & Harris, 2008; Harris & Weinberg, 2012; Colgan & Yasuda, 2014). These receptors range from AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors), the main type of glutamate receptor that mediates fast, excitatory synaptic transmission; NMDAR (N-methyl-D-aspartate receptors), coincidence detectors for activity; and metabotropic glutamate receptors (mGluR), that activate biochemical cascades, leading to the modification of other proteins (Malenka & Nicoll, 1999; Kerchner & Nicoll, 2008; Niswender & Conn, 2010; Colgan & Yasuda, 2014) .

The size of the PSD correlates with the number of AMPA receptors present, with larger spines containing more AMPA receptors in the PSD are more sensitive to glutamate (Nusser *et al.*, 1998; Bourne & Harris, 2008; Harris & Weinberg, 2012; Colgan & Yasuda, 2014). As

previously mentioned, AMPA receptors are the main type of glutamate receptor that mediates fast, excitatory synaptic transmission (Ozawa *et al.*, 1998; Dingledine *et al.*, 1999; Traynelis *et al.*, 2010). They are composed of heterotetramers from GluA1-4 subunits that come together to form the receptor (Ozawa *et al.*, 1998; Dingledine *et al.*, 1999; Collingridge *et al.*, 2009). Once glutamate binding activates AMPA receptors, its ion channel opens to allow for the influx of sodium and/or calcium ions. The type of cation that enters through the AMPA receptor is dependent on the type of subunit it is composed of, the most important of which being GluR2 (Ozawa *et al.*, 1998; Dingledine *et al.*, 1999; Greger *et al.*, 2003). AMPA receptors lacking GluR2 subunit allow for the influx of Ca^{2+} into neurons (Sommer *et al.*, 1991; Bowie, 2012). However, AMPA receptors with GluR2 have low Ca^{2+} permeability, which is determined by a single amino acid residue in the GluR2 M2 pore loop segment (Sommer *et al.*, 1991; Seeburg, 1996). The primary transcript of GluR2 undergoes selective RNA editing that changes the glutamine (Q) into an arginine (R) once translated, also known as Q/R site editing (Sommer *et al.*, 1991; Seeburg *et al.*, 2001). This editing occurs in all mature transcripts and is essential for normal brain function as this type of AMPA receptor is predominant in mature neurons (Seeburg *et al.*, 2001; Lu *et al.*, 2009). Therefore, extracellular Ca^{2+} entry in mature neurons at Schaffer collateral synapses relies predominantly on voltage-gated Ca^{2+} channels and NMDA receptors (Mainen *et al.*, 1999; Kovalchuk *et al.*, 2000; Sabatini *et al.*, 2002; Ngo-Anh *et al.*, 2005; Bloodgood & Sabatini, 2007a; b). Thus, for AMPA receptors at the CA3 – CA1 synapse, ion influx will cause depolarization of the postsynaptic membrane, and sufficient membrane depolarization will activate the NMDA receptor to open for the entry of Ca^{2+} into the neuron (Collingridge *et al.*, 1983; Malinow & Miller, 1986; Wigström *et al.*, 1986; Colgan & Yasuda, 2014). Moreover, previous work done in our laboratory has demonstrated that these AMPA-

mediated miniature excitatory postsynaptic currents (mEPSCs) are required to maintain dendritic spines (McKinney *et al.*, 1999).

NMDA receptors have a voltage-dependent Mg^{2+} block of the channel pore, high permeability to Ca^{2+} ions, and a high affinity for glutamate (Nowak *et al.*, 1984; Paoletti *et al.*, 2013; Glasgow *et al.*, 2015). These features allow NMDA receptors to act as coincidence detectors for presynaptic activity depending on glutamate release and postsynaptic activity depending on sufficient depolarization of the postsynaptic membrane. The Mg^{2+} blockade of the NMDA receptor occurs at resting membrane potential, -70 mV, and can be unblocked through adequate depolarization of the membrane through either AMPA receptor activation or voltage gated Na^{+} or Ca^{2+} channel opening (Nowak *et al.*, 1984; Emptage *et al.*, 1999; Yuste *et al.*, 1999; Bloodgood & Sabatini, 2007a; Grunditz *et al.*, 2008). NMDA receptors are composed of two subunits of GluN1, and two other subunits of GluN2 (A to D) and/or GluN3 (A and B) (Collingridge *et al.*, 2009; Paoletti *et al.*, 2013). The GluN2 receptor subunit type can determine the electrophysiological and pharmacological profile of the receptor since it can affect its opening time, channel conductance, and Mg^{2+} sensitivity. Moreover, GluN2A/GluN2B ratio increases during postnatal development, thus suggesting that GluN2B plays an important role during early postnatal development (Petralia *et al.*, 1994a; Petralia *et al.*, 1994b; Racca *et al.*, 2000; Paoletti *et al.*, 2013), yet there is evidence that the NMDA receptors containing the GluN2B subunit do appear at the periphery of mature spines and are thought to have implications in detecting glutamate spillover (Scimemi *et al.*, 2004; Newpher & Ehlers, 2009; Petralia, 2012). Interestingly, the GluN2B receptor subunit is known to be sufficient for adult hippocampal LTP and spatial learning (Sakimura *et al.*, 1995; Kiyama *et al.*, 1998), and when the GluN2B subunit is overexpressed in the forebrain this has been shown to increase LTP at the CA3 to CA1

synapse and enhance associative learning and memory (Tang *et al.*, 1999). This enhancement is the result of increased opening time of the GluN2B subunit and overall enhanced coincidence detection by the NMDA receptors (Tang *et al.*, 1999). NMDA receptors regulate dendrite spine morphology and stabilization, and thus, alteration of the NMDA receptor subunit composition can lead to deficits in learning and memory, and neurological disorders (Malinow, 2012; Bellot *et al.*, 2014).

1.4.3 Dendritic spine morphology

Once formed, dendritic spines can experience dynamic morphological changes through an actin-dependent mechanism in response to various patterns of neurotransmission (McKinney, 2005; Alvarez & Sabatini, 2007; Holtmaat & Svoboda, 2009; Bergami *et al.*, 2015). Dendritic spines are believed to be the sites of long-term memory as they can alter their synaptic connections and morphology in response to activity (Bourne & Harris, 2007; Rochefort & Konnerth, 2012; Sanders *et al.*, 2012; Hayashi-Takagi *et al.*, 2015).

Dendritic spines are heterogeneous in size and shape, and have been traditionally classified into three categories of thin, mushroom, and stubby spines based on morphology (Harris *et al.*, 1992). Short stubby spines lack a distinct head and neck; long thin spines have elongated necks with a small spine head; and mushroom spines, believed to be the most mature spine type, have a small neck with a very large spine head (Harris *et al.*, 1992; McKinney & Thompson, 2009; McKinney, 2010; Sala & Segal, 2014). The heterogeneity of dendritic spine morphology can modulate how a spine will respond to plasticity-inducing stimuli, and can translate to a spine's distinct intracellular molecular machinery and protein composition (Bourne & Harris, 2008; Colgan & Yasuda, 2014; Sala & Segal, 2014). For example, within larger, mature mushroom spines the spine apparatus (SA) is an important organelle proposed to act as

an internal calcium store or the site of local protein synthesis (Gray & Guillery, 1963; Fifková *et al.*, 1983; Spacek, 1985; Spacek & Harris, 1997; Pierce *et al.*, 2000). *Does the underlying variation of proteins translate to a varied response from a similar plasticity inducing stimuli?*

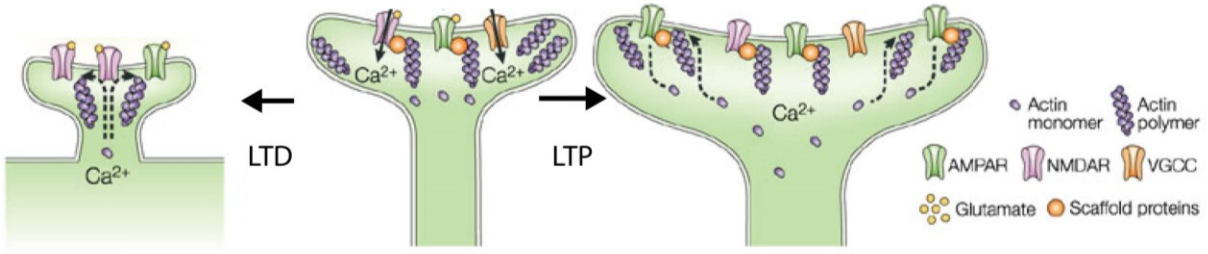
1.5 Activity-Dependent Synaptic Plasticity: Long-term Potentiation and Long-term Depression

The theory of synaptic plasticity was first described by Donald Hebb whereby he posited that cellular learning depends how reliably one neuron activates another (Hebb, 1949). However, it was not demonstrated experimentally until Bliss and Lømo showed that high frequency stimulation of presynaptic fibres could elicit a robust increase in the response of the postsynaptic cell (Bliss & Lømo, 1973; Lømo, 2003).

Since then, the mechanism for long-term potentiation (LTP) has been elucidated at CA3-CA1 synapses, and it typically follows an NMDAR-dependent LTP. Simply put, NMDAR-dependent LTP follows these steps: (1) NMDA receptor activation (Collingridge *et al.*, 1983; Kauer *et al.*, 1988; Schiller *et al.*, 1998), (2) influx of Ca^{2+} into the postsynaptic compartment (Lynch *et al.*, 1983; Kauer *et al.*, 1988; Malenka, 1991; Schiller *et al.*, 1998), (3) concurrent postsynaptic depolarization (Malinow & Miller, 1986; Wigström *et al.*, 1986), (4) activation of CAMKII and signalling pathways (Malenka & Bear, 2004; Vlachos *et al.*, 2009; Murakoshi & Yasuda, 2012), and (5) AMPA receptor insertion in the postsynaptic membrane resulting in enlargement in spine volume (Matsuzaki *et al.*, 2004; Kopec *et al.*, 2006; Kopec *et al.*, 2007; Kessels & Malinow, 2009; Makino & Malinow, 2009).

In contrast, long-term depression (LTD) represents the inverse of LTP and is the result of: (1) low frequency stimulation that minimally activates NMDARs or mGluRs (Dudek & Bear, 1992; Bolshakov & Siegelbaum, 1994), (2) a modest influx of Ca^{2+} (Mulkey & Malenka, 1992) (3) calcium-calmodulin unit preferentially activates the phosphatase calcinuerin

Figure I. Dendritic spine structural modifications after LTP and LTD



Schematic depicting the structural modifications that occur during LTD and LTP. After LTP induction, dendritic spines experience an influx of calcium that leads to spine enlargement through activation downstream signaling cascades, which include the insertion of AMPA receptors into the membrane, and an increase in F-actin filaments. LTD, on the other hand, receives a modest influx of calcium into the cell and that leads to the removal of AMPA receptors from the membrane, depolymerization of F-actin filaments, and spine shrinkage. Modified from (Lamprecht & LeDoux, 2004).

(Malenka & Bear, 2004; Kessels & Malinow, 2009), and (4) results in the expression of LTD in the dendritic spine through receptor internalization, and expression of depression-related proteins, and leads to spine shrinkage (Malenka & Bear, 2004; Kessels & Malinow, 2009; Lüscher & Malenka, 2012).

At Schaffer collateral synapses, synaptic plasticity is associated with structural changes of dendritic spines. LTP or LTD manifests itself through either spine enlargement or shrinkage, respectively, caused by a combination of spatiotemporal post-synaptic processes from the reorganization of the actin cytoskeleton (Kim & Lisman, 1999; Krucker *et al.*, 2000; Fukazawa *et al.*, 2003; Cingolani & Goda, 2008). Since the AMPA receptors found in the PSD are anchored by scaffolding proteins that link them to the actin cytoskeleton, the insertion or deletion of receptors in the post-synaptic membrane is likely to affect the structure of the synapse (Matsuzaki *et al.*, 2004; Kopec *et al.*, 2006; Kopec *et al.*, 2007; Kessels & Malinow, 2009; Makino & Malinow, 2009; Lüscher & Malenka, 2012).

In order to study synaptic structural remodification and its underlying plasticity after LTP, we decided to use a chemical LTP (chemLTP) protocol instead of the more commonly used electrical LTP protocol in order to globally induce LTP throughout the hippocampal slice (Kopec *et al.*, 2006; Kopec *et al.*, 2007; Makino & Malinow, 2009; Chang *et al.*, 2014). Although the chemLTP protocol is advantageous to ensure that all neurons in the hippocampal slice are equally exposed to an 'LTP'-like stimulation, including the dendrites from neurons that are being imaged for structural remodifications, this protocol is a harsh stimulus that would not be seen naturally after normal synaptic signaling, or an electrical LTP protocol (Kopec *et al.*, 2006; Kopec *et al.*, 2007; Makino & Malinow, 2009; Chang *et al.*, 2014).

Interestingly, the structural changes observed in dendritic spines after a stimulus are not uniform. Thus, structural changes of different spines will be varied depending on the spine's potential, and development. Nevertheless, the changes in the post-synaptic membrane involve a multitude of signalling cascades that affect many different aspects of the enlargement or shrinkage of a spine, an important aspect would be the reorganization of actin described in section 1.7.

1. 5. 1 Calcium-induced calcium release

As previously described, after an activity-dependent stimulus depolarizes a postsynaptic membrane and there is an influx of Ca^{2+} into the cell that leads to a downstream signaling cascade involved in LTP (Lynch *et al.*, 1983; Kauer *et al.*, 1988; Malenka, 1991; Schiller *et al.*, 1998). This influx of Ca^{2+} can trigger a Ca^{2+} induced- Ca^{2+} release (CICR) from the calcium stores within dendritic spines for the generation and maintenance of LTP (Malenka, 1991; Verkhratsky & Shmigol, 1996; Berridge, 1998; Emptage *et al.*, 1999; Roderick *et al.*, 2003; Korkotian *et al.*, 2014; Segal & Korkotian, 2015).

Within neurons, the main calcium store is the smooth endoplasmic reticulum (ER) found throughout the dendritic tree, and in some cases enters dendritic spines (Malenka, 1991; Verkhratsky & Shmigol, 1996; Berridge, 1998; Emptage *et al.*, 1999; Roderick *et al.*, 2003; Korkotian *et al.*, 2014; Segal & Korkotian, 2015). In large dendritic spines, however, the smooth ER can become a specialized structured called the spine apparatus (SA), a derivative of the smooth ER with electron dense material between cisternal sacs (Spacek & Harris, 1997; Ostroff *et al.*, 2010). These calcium stores are activated by two types of receptors: (1) the inositol 1,4,5 triphosphate receptor (IP3R), present at high concentration in dendritic shafts and cell bodies of hippocampal neurons (Berridge & Taylor, 1988; Mayer & Miller, 1990; Sharp *et al.*, 1993; Segal

& Korkotian, 2015), and (2) the ryanodine receptor (RyR) found within dendritic spines and axons of hippocampal neurons (Mayer & Miller, 1990; Korkotian & Segal, 1999; Segal & Korkotian, 2015). Within spines, the influx of Ca^{2+} can activate RyRs present on calcium stores which allows for the release of Ca^{2+} into the cytosol (Verkhratsky & Shmigol, 1996; Berridge, 1998; Emptage *et al.*, 1999; Roderick *et al.*, 2003; Korkotian *et al.*, 2014; Segal & Korkotian, 2015). CICR in the spine can influence the delivery of AMPA receptors to the synaptic site, as well as the regulation of calcium-dependent processes within the spine (Vlachos *et al.*, 2009; Segal & Korkotian, 2015). Moreover, the smooth ER in some spines can mediate mGluR-dependent LTD synaptic responses through the regulation of IP3R induced Ca^{2+} release (Holbro *et al.*, 2009). Therefore, the role of calcium stores and CICR within spines can have a profound influence on how a spine will respond to synaptic transmission through the generation of LTP or LTD.

1.6 Morphological Changes in Excitatory Synapses

Actin is highly enriched in dendritic spines (Matus *et al.*, 1982; Landis & Reese, 1983), whereas the dendrite is primarily structured by microtubules (Matus *et al.*, 1983). However, recent live-imaging experiments have demonstrated that microtubules are able to transiently enter spines (Hu *et al.*, 2008; Jaworski *et al.*, 2009; Schätzle *et al.*, 2016). The transient entry of microtubules into spines may allow them to interact with actin in spines to enact changes in spine morphology (Dent *et al.*, 2011; Schatzle *et al.*, 2011), or be involved in the delivery of actin-binding proteins that reorganize the actin cytoskeleton (Hoogenraad & Akhmanova, 2010). Nevertheless, actin cytoskeleton reorganization appears to be the underlying process that controls dendritic spine morphologies during basal conditions or in response to changes in synaptic transmission (Kim & Lisman, 1999; Krucker *et al.*, 2000; Matus, 2000).

The actin cytoskeleton is ubiquitously found throughout nearly all cells in all organisms, and is important for many cellular processes from cellular motility, division, intracellular transport, and morphogenesis (Matus *et al.*, 1982; Landis & Reese, 1983; Kaech *et al.*, 1997; Duan *et al.*, 1999; Matus, 2000; Luo, 2002; Zito *et al.*, 2004; Lamprecht, 2014). Within the brain, actin is found as β or γ cytosolic forms (Kaech *et al.*, 1997). Actin can exists as either monomeric, globular (G)-actin, or polymerize into filamentous (F)-actin (Luo, 2002; Salbreux *et al.*, 2012). The ability actin has to alter spine morphology after synaptic transmission lies in its ability to undergo rapid actin assembly and disassembly, a phenomenon termed treadmilling (Luo, 2002; Star *et al.*, 2002; Honkura *et al.*, 2008; Frost *et al.*, 2010a; Lamprecht, 2014). Actin treadmilling is a tightly-controlled process whereby monomeric G-actin is added to the barbed ends, and actin disassembly occurs at the pointed ends of F-actin (Ridley, 2006; Cingolani & Goda, 2008; Murakoshi & Yasuda, 2012).

When actin monomers are added to F-actin, there is a net flow of actin monomers from the head of the spine towards the neck of the spine (Fukazawa *et al.*, 2003; Honkura *et al.*, 2008). Within the spine there are three factors that promote actin filament assembly: actin-related proteins 2 and 3 (Arp2/3), formins, and profilin (Harris & Higgs, 2004; Rotty *et al.*, 2013; Spence & Soderling, 2015). Formins are involved in the polymerization of linear actin filaments and they are enriched within spine projections, as well as dendritic filopodia during spine development (Harris & Higgs, 2004; Hotulainen *et al.*, 2009; Chazeau *et al.*, 2014; Spence & Soderling, 2015). Profilins, on the other hand, are involved in ADP to ATP nucleotide exchange, which provides a pool of available monomers for the polymerization of actin at the barbed end of the growing filament (Miki *et al.*, 1998; Harris & Higgs, 2004; Spence & Soderling, 2015). This G-actin binding protein is an important contributor to dendritic spine development and

stabilization, and is recruited to spines following activity (Miki *et al.*, 1998; Ackermann & Matus, 2003; Romero *et al.*, 2004). The most important protein in actin filament assembly is Arp2/3 that binds to F-actin at a 70° angle to begin the nucleation of a new actin filament from the existing filament (Rotty *et al.*, 2013; Spence & Soderling, 2015). This pattern of generating actin filaments at an angle off the existing filament allows actin to exist in the dendritic spine as a dense branched network of actin that is important for its stabilization. Arp2/3 is activated by nucleation promoting factors such as N-WASP, WAVE1, or WASH, and by removing the ability for Arp2/3 to be activated can lead to alterations in spine morphology in addition to behavioural abnormalities (Kim *et al.*, 2006; Soderling *et al.*, 2007; Wegner *et al.*, 2008; Burianek & Soderling, 2013; Kim *et al.*, 2013; Rotty *et al.*, 2013; Kim *et al.*, 2015). Thus, the role Arp2/3 has in the regulation of the actin cytoskeleton enables this complex to be an important component for dendritic spine stability and function.

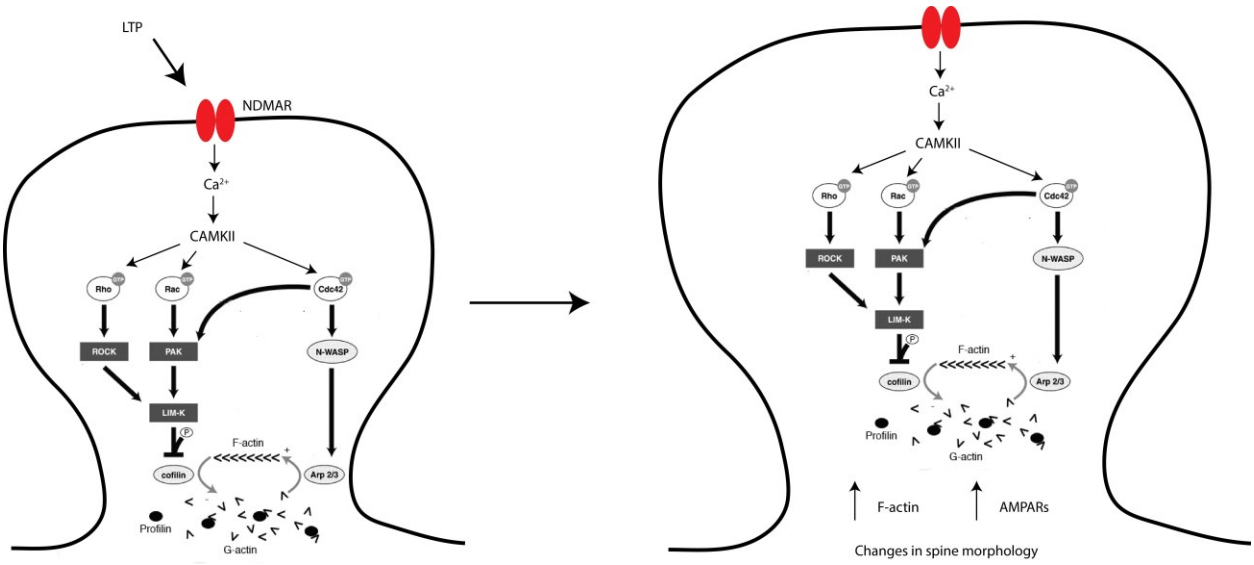
During the actin treadmilling process, actin polymerization is counterbalanced by actin filament disassembly that occurs at the pointed ends of F-actin that is primarily controlled by (ADF)/cofilins (Luo, 2002; Andrianantoandro & Pollard, 2006; Bernstein & Bamburg, 2010; Spence & Soderling, 2015) (**Fig** II). This family of actin depolymerizing factors severs actin filaments leading to F-actin disassembly. Part of the cofilin family, cofilin-1 is localized to the PSD, where actin is highly enriched and highly dynamic (Racz & Weinberg, 2006; Bernstein & Bamburg, 2010; Frost *et al.*, 2010b). The regulation of cofilin occurs through its inactivation when it is phosphorylated at serine-3 by LIM kinase 1 (LIMK-1) that is downstream of the RhoGTPase signalling cascade, and this prevents the disassembly of the actin filament (Yang *et al.*, 1998; Bernstein & Bamburg, 2010; Lamprecht, 2014; Spence & Soderling, 2015) (**Fig** II). This newly formed actin filament is then stabilized by a capping protein (CapZ) that binds to the

barbed end of F-actin filaments and prevents their further elongation (Fan *et al.*, 2011; Menna *et al.*, 2013; Edwards *et al.*, 2014). Therefore, this rapid addition of actin monomers and process of treadmilling allows the actin to gain enough momentum and mechanical force to push out the membrane for spine enlargement (Fukazawa *et al.*, 2003; Honkura *et al.*, 2008).

1.6.1 Dendritic Spine Actin Regulation through RhoGTPases

The RhoGTPases, a subgroup of the Ras small GTPase family including RhoA, Rac1, and Cdc42, are involved in the regulation of the actin cytoskeleton (Luo, 2002; Dillon & Goda, 2005; Newey *et al.*, 2005; Heasman & Ridley, 2008; Tashiro & Yuste, 2008; Hall & Lalli, 2010; Briz *et al.*, 2015). The activation of these RhoGTPases can trigger downstream signalling cascades that lead to actin treadmilling and cytoskeleton remodelling that results in structural changes of dendritic spines (Bourne *et al.*, 1990; Dillon & Goda, 2005; Newey *et al.*, 2005; Scheffzek & Ahmadian, 2005; Honkura *et al.*, 2008; Frost *et al.*, 2010b; Hall & Lalli, 2010; Briz *et al.*, 2015). As their name suggests, the RhoGTPases can bind to guanosine triphosphate (GTP) and hydrolyze GTP to guanosine diphosphate (GDP) with their intrinsic enzymatic hydrolase activity (Hall & Nobes, 2000; Luo, 2002; Scheffzek & Ahmadian, 2005) (**Fig** III). RhoGTPases use this switch between GTP-bound and GDP-bound state as molecular on/off switches, which hold an important role for the induction and regulation of a variety of signalling cascades including actin remodelling (Bourne *et al.*, 1990; Hall & Nobes, 2000). RhoGTPases are further regulated by guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP to GTP and GTPase activating proteins (GAPs) that promote the hydrolysis of GTP to GDP (Bourne *et al.*, 1990; Vetter & Wittinghofer, 2001; Itoh *et al.*, 2002; Rossman *et al.*, 2005; Scheffzek & Ahmadian, 2005; García‐Mata *et al.*, 2006) (**Fig** III). The RhoGTPases act as actin regulators that facilitate actin polymerization, disassembly, or stabilization

Figure II. RhoGTPase signalling pathways.



Schematic depicting the regulation of RhoA by synaptopodin and the downstream signalling pathways for the RhoGTPases, RhoA, Rac-1, and Cdc42. Synaptopodin competitively binds to RhoA, and thereby prevents the targeting of RhoA for proteasomal degradation by Smurf-1-mediated ubiquitination. The downstream signalling pathways for RhoA, Rac-1, and Cdc42 regulate actin cytoskeleton remodelling through treadmilling of actin between polymerized F-actin to monomeric G-actin at the barbed (+) end versus the pointed end (-). Modified from (Luo, 2002; Asanuma et al., 2006).

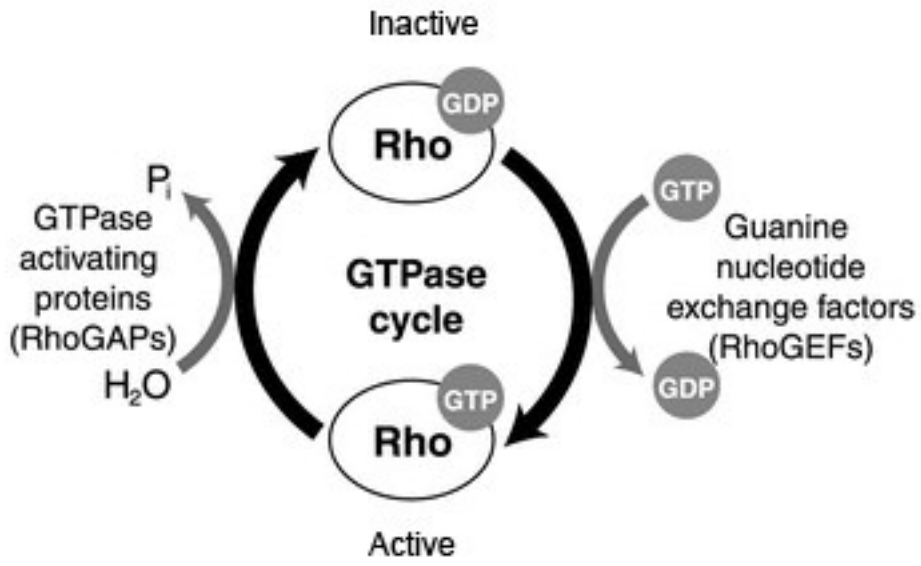
(Newey *et al.*, 2005; Scheffzek & Ahmadian, 2005; Hall & Lalli, 2010; Briz *et al.*, 2015) (**Fig** II). Through actin regulation, the RhoGTPases can modulate dendritic spine morphology and stability; however, the precise contribution and mechanism has yet to be elucidated because of their overlapping downstream targets, such as cofilin. The RhoGTPases Rac1 and Cdc42 share a common part of the downstream signaling cascade by both activating Arp2/3 and p21-activated kinase (Pak) (Hall, 1998; Luo, 2002; Burianek & Soderling, 2013; Spence & Soderling, 2015). As previously mentioned, Arp2/3 stimulates nucleation of actin polymerization and causes *de novo* F-actin assembly (Rotty *et al.*, 2013; Spence & Soderling, 2015). When Pak is activated, it uses its kinase activity to (1) activate itself, (2) activate LIM-domain-containing protein kinase (LIMK), and (3) activate myosin light chain kinase (MLCK) (Daniels & Bokoch, 1999; Luo, 2000; 2002; Zhang *et al.*, 2005; Kreis & Barnier, 2009; Lamprecht, 2014). LIMK then goes on to phosphorylate cofilin and, thus, inhibit the actin depolymerization activity of cofilin (Luo, 2002; Nishita *et al.*, 2005; Andrianantoandro & Pollard, 2006; Bernard, 2007; Sit & Manser, 2011). MLCK acts to phosphorylate the regulatory myosin light chains of myosin II in order to facilitate myosin binding to actin and aid in motility (Bagrodia & Cerione, 1999; Zhang *et al.*, 2005). However, active MLCK inhibits PAK and causes decreased myosin-regulatory light chain phosphorylation, and therefore a decrease in myosin motor activity (Bagrodia & Cerione, 1999; Luo, 2000; Zhang *et al.*, 2005). However, RhoA activates (1) Rho-associated, coiled-coil-containing protein kinase (ROCK) and (2) the formin mDia (Luo, 2000; 2002; Hotulainen *et al.*, 2009; Lamprecht, 2014; Spence & Soderling, 2015). The downstream effectors of ROCK include (1) LIMK, which leads to the inhibition of cofilin, and prevent the depolymerization of F-actin (Nishita *et al.*, 2005; Andrianantoandro & Pollard, 2006; Bernard, 2007; Sit & Manser, 2011), and (2) the regulatory light chain of myosin II that promotes ATPase and motor activity

of myosin II, as well as inhibit the MRLC phosphatase (Bagrodia & Cerione, 1999; Luo, 2000; Zhang *et al.*, 2005). In the second part of the pathway, RhoA activation of mDia leads to the activation of profilin, involved in ADP to ATP nucleotide exchange on G-actin monomers, which provides a pool of available monomers for the polymerization of actin at the barbed end of the growing filament (Miki *et al.*, 1998; Ackermann & Matus, 2003; Harris & Higgs, 2004; Romero *et al.*, 2004; Lamprecht, 2014). Although the precise contribution of individual RhoGTPases is not fully understood, together they act to modulate the actin cytoskeleton within neurons.

Nevertheless, the RhoGTPases have been shown to vary effects on spine structural modulation. Constitutively active RhoA decreases both spine density and length; whereas Rac1 increases the density of small spines (Nakayama *et al.*, 2000; Tashiro & Yuste, 2004; Schubert *et al.*, 2006). Furthermore, after glutamate uncaging at the stimulated spine, there was a rapid activation of RhoA and Cdc42 (Murakoshi *et al.*, 2011). In this model, inhibition of RhoA or ROCK preferentially inhibited the transient initial phase of structural plasticity, whereas inhibition of Cdc42 or Pak inhibited the long-term stabilization and maintenance of spine enlargement (Murakoshi *et al.*, 2011). Hence, the spatiotemporal activation of the RhoGTPases and downstream signalling cascades implicates their role in structural plasticity through actin remodelling.

Moreover, in addition to their activity being modulated by a wide variety of incoming signals, the degradation of the RhoGTPases is tightly regulated within cells. Ubiquitin-dependent proteolysis is a central cellular degradation mechanism whereby ubiquitin-tagged intracellular and membrane proteins are targeted for degradation by the proteasome or lysosome, respectively (Klimaschewski, 2003; DiAntonio & Hicke, 2004; Cao & Zhang, 2012). The enzymatic cascade

Figure III. Activation and inactivation of Rho Family GTPase.



RhoGTPases bind to guanosine triphosphate (GTP) (active state) and hydrolyze GTP to guanosine diphosphate (GDP) (inactive state) with their intrinsic enzymatic hydrolase activity. Guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP to GTP and GTPase activating proteins (GAPs) that promote the hydrolysis of GTP to GDP. Modified from (Luo, 2002).

to target ubiquitin to proteins involves an E1-ubiquitin-activating enzyme that transfers ubiquitin to an E2-ubiqutin-conjugating enzyme, which can use adaptors to add ubiquitin to protein targets, or move the ubiquitin to E3 ligases (Klimaschewski, 2003; Bryan *et al.*, 2005; Cao & Zhang, 2012). E3 ligases, then transfer ubiquitin to the protein targeted for degradation. In this study, we will focus on Smurf1, an E3 ubiquitin ligase that traditionally targeted bone morphogenetic protein (BMP) receptor SMAD proteins, and was involved in the regulation of osteoblast differentiation, bone formation, and myogenic differentiation (Izzi & Attisano, 0000; Murakami *et al.*, 2003; Ying *et al.*, 2003; Zhao *et al.*, 2004). More recently, Smurf-1 has been demonstrated to target RhoA for ubiquitination and subsequent proteasomal degradation within a variety of neuronal cell types (Wang *et al.*, 2003; Zhang *et al.*, 2004; Bryan *et al.*, 2005; Wang *et al.*, 2006; Cheng *et al.*, 2011; Stiess & Bradke, 2011; Kannan *et al.*, 2012). Thus, in this study we will focus on how proteasomal degradation and regulation of RhoA protein levels within neurons can alter dendritic spine enlargement and stability after LTP.

1.6.2 Modulation of the RhoGTPases through Sex Hormones

The regulation of the RhoGTPases within the hippocampus maintains an important role in structural plasticity after a stimulus. However, the regulation of the RhoGTPases and the actin cytoskeleton is complex with a variety of different extracellular and intracellular signals modulating their regulation. Estrogen, a steroid hormone, has been shown to enhance synaptic plasticity, enhance LTP, and induce structural remodifications, such as spine formation (Spencer *et al.*, 2008; Foy, 2011; Fester *et al.*, 2013). Along with circulating estrogen, the hippocampal neurons can synthesis endogenous estradiol *de novo* from cholesterol, which has been shown to be essential for synaptic plasticity (Prange-Kiel *et al.*, 2003; Kretz *et al.*, 2004; Vierk *et al.*, 2012). Within the hippocampus, estradiol's role in the enhancement of LTP has been shown to

be dependent on its ability to modulate the actin cytoskeleton filament assembly (Kramár *et al.*, 2013). The role estradiol has on the modulation of the actin cytoskeleton appears to be through the regulation of RhoA, as an antagonist of ROCK has been shown to block estradiol's synaptic effects (Kramár *et al.*, 2013). Moreover, the inhibition of local estradiol synthesis in hippocampal neurons in female mice not only lead to the impairment of LTP and synapse loss, but also to the dephosphorylation of cofilin, and thus, the destabilization of dendritic spines (Vierk *et al.*, 2012; Vierk *et al.*, 2014). Therefore, taken together the role of estradiol in the regulation of the actin cytoskeleton appears to be through the regulation of RhoA, and thus, the inactivation of cofilin to prevent the breakdown of the filamentous actin within neurons.

1. 7 Dendritic Spine Maintenance

An adapting neuronal circuitry enables the brain to engage in learning and memory. Thus, the nervous system must not only maintain synapse specificity developed during development, but also be flexible throughout an organism's lifetime. Once dendritic spines are formed, they require AMPA-mediated miniature excitatory postsynaptic currents (mEPSCs) to be maintained (McKinney *et al.*, 1999).

However, what happens when a spine is not receiving sufficient activity from its associated bouton? In these circumstances, there are a subset of innervated spines that can extend a protrusion from their spine head that contacts a neighbouring presynaptic bouton (Richards *et al.*, 2005). These spine head protrusions (SHPs) form after a few minutes and mature rapidly over 20 minutes so that they can become stable and persist for hours. Their formation is enhanced after action potential elimination with tetrodotoxin (TTX), and they extend directionally toward boutons. Thus, suggesting that they are a reaction to glutamate spillover,

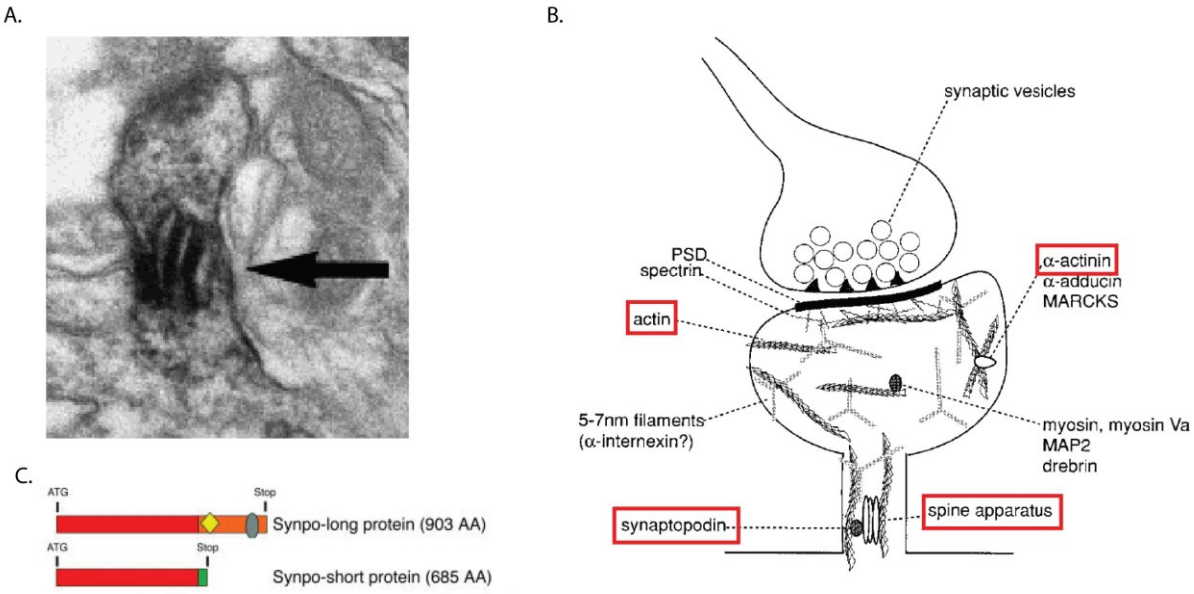
which can activate receptors on spines receiving insufficient activity from their associated bouton, and produce a SHP to a neighbouring terminal (Richards *et al.*, 2005).

Since SHPs are formed off a subset of spines, recent work in our lab aimed to understand the mechanism behind SHP formation and stability by examining at the role of synaptopodin, an actin-binding protein associated found within a subset of dendritic spines. Interestingly, ~70% of spines forming SHPs have synaptopodin, and SHPs formed from spines in synaptopodin-knockout hippocampus are unable (Verbich, 2013). Hence, synaptopodin stabilizes the formation and maintenance of SHPs (Verbich, 2013). Therefore, the heterogeneity of structural spine type translates to a varied intracellular protein composition, and this has been shown to alter the responses of spines in a structural manner. Could synaptopodin, a protein expressed heterogeneously within dendritic spines later during development, be said protein that gives rise to an altered response within spines?

1. 8 Synaptopodin

Within a subset of dendritic spines lies the spine apparatus (SA), an organelle first described by Gray (1959) . This organelle is composed of stacked smooth endoplasmic reticulum (sER) with electron dense material between cisternal sacs (Spacek & Harris, 1997; Ostroff *et al.*, 2010) (**Fig** IVa). The sER extends throughout the dendritic tree and can enter dendritic spines; where in large spines, the sER can then become the SA. Although the precise function of the SA is unknown, it has been proposed to act as an internal calcium store in spines or be the site of local protein synthesis (Fifková *et al.*, 1983; Pierce *et al.*, 2000). Within the SA, there is an essential protein called synaptopodin, an actin-binding protein found in both telencephalic synapses and the processes of kidney podocytes (Mundel *et al.*, 1997). Synaptopodin is required for the

Figure IV. Synaptopodin



A. Electron microscope (EM) image of a synaptopodin positive spine with the spine apparatus present at the base of the neck of the spine. B. Schematic depicting synaptopodin's interaction with the spine apparatus, the actin cytoskeleton, and some cytoskeletal-related proteins, such as α-actinin within a dendritic spine. C. Schematic of the two synaptopodin isoforms present, the synaptopodin-long protein found in the kidney, and the synaptopodin-short protein found in the brain. *Modified from (Deller et al., 2000; Zhang & Benson, 2000; Asanuma et al., 2005)*

formation of the SA; when knocked out, these mice no longer possess the SA (Deller *et al.*, 2003).

Synaptopodin exists as two different isoforms, a renal podocyte synaptopodin-long form with a molecular weight of 110 kDa and 4 α-actinin interacting sites, and a neuronal synaptopodin-short form at 100 kDa and only 2 α-actinin interacting sites (Mundel *et al.*, 1997; Asanuma *et al.*, 2005) (**Fig** IVc). Within the brain, synaptopodin is first detected at post-natal day 15 and reaches maximum expression in the adult animal (Mundel *et al.*, 1997). Although synaptopodin is not involved in development, synaptopodin-knockout mice demonstrate deficits in functional and structural LTP expression, as well as defects in spatial learning (Deller *et al.*, 2003; Jedlicka *et al.*, 2009; Zhang *et al.*, 2013). Thus, suggesting that the spine apparatus and synaptopodin have a role in synaptic plasticity, and are important for learning and memory (Deller *et al.*, 2007; Jedlicka *et al.*, 2008; Segal, 2010; Vlachos, 2012).

Synaptopodin localizes in larger, more mature, and functionally stronger spines (Vlachos *et al.*, 2009). The molecular mechanism that underlies the role synaptopodin has in synaptic plasticity has yet to be elucidated. Synaptopodin has been shown to elongate and bundle F-actin through interactions with α-actinin (Asanuma *et al.*, 2005) (**Fig** IVb). In renal podocytes, synaptopodin has also been shown to interact with the cytoskeleton to induce stress fibre formation through F-actin elongation by regulating RhoA signalling (Asanuma *et al.*, 2006). Where synaptopodin can competitively bind to RhoA-GDP, preventing its ubiquitination and subsequent degradation by Smurf-1, an E3 ubiquitin ligase (Asanuma *et al.*, 2006). Interestingly, both synaptopodin long form and short forms were both shown to bind with RhoA (Asanuma *et al.*, 2006). However, it is unknown whether synaptopodin is directly involved in regulation of the actin cytoskeleton within dendritic spines. Thus, in the present study we examined how spine

heterogeneity, specifically the actin-associated protein, synaptopodin, is involved in spine structural remodelling and actin cytoskeletal reorganization.

Research Rationale

We have previously shown that a subset of innervated spines can extend a SHP to a neighbouring terminal as a reaction to spines receiving insufficient activity from their presynaptic bouton. The formation and stability of SHPs appears to be regulated by the presence of synaptopodin, an actin-associated protein expressed within a subset of larger, more mature spines. Moreover, studies have shown that when synaptopodin is absent in mice they have *in vivo* deficits in spatial learning and impairments in LTP in hippocampal acute slice. Since synaptopodin is expressed heterogeneously throughout spines, we wanted to examine whether it contributes to the altered response different spines can exhibit to the same stimulus. Previous work in kidney podocytes has shown that the kidney isoform of synaptopodin can regulate the actin cytoskeleton by competitively binding to RhoA-GTPase and preventing it from being targeted for degradation by Smurf-1, an E3 ubiquitin ligase. We decided to examine whether the brain isoform of synaptopodin is involved in the regulation of Rho-GTPase in a similar manner as the kidney isoform. Furthermore, as synaptopodin has been shown to be involved in learning, we wanted to determine whether synaptopodin is involved in other aspects of synaptic plasticity, such as calcium influx. Since synaptopodin has been shown to crosslink with α-actinin-2, which is involved in the anchoring of NMDA receptors to the PSD, we decided to examine whether synaptopodin influences the expression of the GluN2B subunit, an NMDA receptor subunit known to be involved in enhanced LTP expression within the hippocampus. Therefore, in this study we investigated if synaptopodin is involved in the enlargement and stability of dendritic spines after enhanced activity, such as chemLTP, through the regulation of the RhoGTPases, and whether synaptopodin can regulate GluN2B subunit expression in the hippocampus.

Hypotheses

We hypothesize that (1) synaptopodin is necessary for the enlargement and stability of dendritic spines from tertiary dendrites of CA1 pyramidal neurons, (2) the brain isoform of synaptopodin can regulate the actin cytoskeleton through competitively binding to RhoA to prevent its degradation by Smurf-1, and (3) synaptopodin can regulate the GluN2B subunit expression within the hippocampus.

Research Objectives and Specific Aims

Aim 1: To determine whether synaptopodin is necessary for the maintenance of spine volume increases observed after learning stimulus.

Aim 2: To determine whether the brain isoform of synaptopodin regulates the actin cytoskeleton through the regulation of RhoA.

Aim 3: To determine whether synaptopodin is involved in the expression of the NMDA receptor subunit GluN2B within the hippocampus.

Materials and Methods

Organotypic Roller Drum mouse hippocampal slice cultures

Organotypic hippocampal slice cultures (Gähwiler *et al.*, 1997; Gähwiler *et al.*, 1998) were prepared from either P 6-8 L15 transgenic mice that express membrane-tagged eGFP under the Thy-1 promoter sparsely within CA1 pyramidal cells (De Paola *et al.*, 2003) or synaptopodin-knockout mice crossed with L15 mice to establish homozygous synaptopodin null mice expressing mGFP (L15-S). The mice were sacrificed by cervical dislocation, their brains removed, and hippocampi dissected out in an aseptic environment. Transverse slices (400 μm thick) were made using a McIlwain tissue chopper (Lafayette Instrument), and adhered onto glass coverslips with chicken plasma clot (Cocalico Biologicals; Reamstown, PA, USA). The coverslipped slices were transferred to flat bottom tubes with 1 ml of medium (50% Eagle's basal medium (Gibco), 25% HBSS (Gibco) and 25% New Zealand horse serum (Gibco)) and placed in a roller-drum dry-air incubator (36 °C), which rotates at approximately 10 revolutions/h. The slow rotation enables the cultures to be immersed in media for only half a turn and allows for an oxygen-nutrient interchange. Slice cultures were allowed to mature for 3 weeks *in vitro* before experimentation and media were changed weekly (Mateos *et al.*, 2007; Verbich *et al.*, 2012).

Chemical LTP (chemLTP) Induction and Time-Lapse Confocal Microscopy

Slices were transferred to a temperature-controlled chamber (30 °C) mounted on an upright microscope (DM LFSA, Leica Microsystems) equipped with a Leica TCS SP2 scanhead. Tyrode solution consisting of (in mM): NaCl, 137; KCl, 2.7; $CaCl_2$, 2.5; $MgCl_2$, 2; $NaHCO_3$, 11.6; NaH_2PO_4, 0.4; and glucose, 5.6 (pH 7.4) continuously perfused into the chamber. Image stacks were collected at 1-minute intervals for the entire duration of the experiments using HCX APO 63× 0.9 NA (Leica) water immersion long working distance lens at Z intervals = 0.25 μm. To determine normal motility of the spines, baseline images of apical or basal secondary and

tertiary dendritic branches from CA1 pyramidal cells were collected for 20 minutes prior to perfusion of the chemical long-term potentiation (chemLTP) solution consisting of $MgCl_2$-lacking Tyrode with 100 nM rolipram (selective phosphodiesterase-4 inhibitor that blocks the degradation of cyclic adenosine monophosphate (cAMP)), 50 μM forskolin (adenylyl cyclase activator and increases intracellular levels of cAMP), and 100 μM picrotoxin (competitive agonist for $GABA_A$ receptor chloride channels) for exactly 16 minutes, as previously reported (Kopec *et al.*, 2006; Kopec *et al.*, 2007; Makino & Malinow, 2009). After chemLTP solution perfusion, control tyrode was perfused for the remainder of the time-lapsed imaging (approximately 45 minutes). Slices were then fixed in in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 1h, and processed for synaptopodin (see section *Immunostaining*). Secondary and tertiary dendritic branches from either apical or basal dendrites were imaged for the duration of the experiment. No difference between basal or apical dendrites was detected, thus data was then pooled for analysis. Image stacks collected were deconvolved using Huygens Essential software (Scientific Volume Imaging, Hilversum, The Netherlands), and then analyzed blind in Imaris software (Bitplane AG).

Spine Volume Quantification

Time-lapse confocal image stacks were imported into Imaris and displayed with the Surpass mode. Individual spines were demarcated with a 3-dimensional cube that covered the entire spine from the head (large bulbous structure at the end of the spine) to the base of the neck (the point of contact between the dendritic spine structure and the dendrite) at each time point. Under the Surface function in Surpass mode, a 3D surface was rendered based on the voxel intensity of the marked spine, also known as isosurface. The isosurface was used to measure spine volume in μm^3 at each time point. Spine image reconstruction used the same parameters

throughout all the time points of an experiment for both L15 and L15-S cultures. We measured volume changes in thin and mushroom spine subtypes, but excluded stubby spines as they are too close to the dendrite to be properly resolved by light microscopy and thus prevented volume analysis. In order to ensure photobleaching did not alter the measured spine volume over time, a low laser power and a fast image acquisition speed were used. In addition, multiple locations on the dendrite were monitored for fluctuations in fluorescence intensity over the course of imaging and any dendrite with changes greater than 20% were removed from analysis.

Immunoblotting

Immunoblotting experiments were performed to determine protein levels using either adult hippocampi or organotypic slice cultures. Ten L15 or L15-S slices from the same batch of cultures were removed from the plasma clot, and pooled together for each group (defined as one sample group). Tissue from either whole adult hippocampi or pooled slices was lysed in 1 mL or 100 µL ice-cold RIPA (radioimmunoprecipitation assay) buffer, respectively. The RIPA buffer consisted of: 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl and completeMini protease inhibitors (Roche). The lysate was incubated at 4 °C rotating for 1 hour, then sonicated in a water sonicator for 5 seconds, and this was repeated twice more. The lysate was centrifuged for 5 mins at 13,200 RPM, the supernatant was extracted, and the pellet was discarded. In order to ensure uniform protein loading, the protein concentration of the supernatant was determined using a bicinchoninic acid dye-binding assay (Thermo Scientific) compared against bovine serum albumin (BSA) as a standard (re protocol of manufacturer). Briefly, a working solution was created and added to both the standard samples and supernatant in equal quantity to a microplate prior to being heated at 37 °C for 30 mins. The plate reader (Synergy H1 Hybrid Reader, BioTek) measured the absorbance at

562 nm. A standard curve was created from the absorbance of the BSA standards, which was used to compare the absorbance of the unknown samples to determine protein concentrations.

Adjusted and equal levels of total protein were resolved using SDS-PAGE gel electrophoresis (15% resolving), and then transferred to 0.45 μm PVDF membrane (Biorad) for immunoblotting. Post-transfer, membranes were blocked for 1h at room temperature with 5% BDA in 0.05% Tween-20 in Tris-buffered saline (TBST). Membranes were then incubated at 4 °C overnight with anti-RhoA (Santa Cruz Biotechology; 1:250), anti-Rac1 (Santa Cruz Biotechology; 1:250), anti-Cdc42 (Santa Cruz Biotechology; 1:250), anti-GluN2B (Millipore; 1:250), or anti β-tubulin (Sigma-Aldrich; 1:10,000; internal loading control) diluted in 5% BSA in 0.05% TBST. Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies diluted in 5% BSA in 0.05% TBST for 1h at room temperature to reveal the primary antibody binding. Immunoreactive bands were detected using ECL™ Western blotting detection reagents (GE Healthcare) and Kodak BioMax Light Film (Sigma-Aldrich). Blots were analyzed with Adobe Photoshop software for mean pixel intensity.

Immunostaining

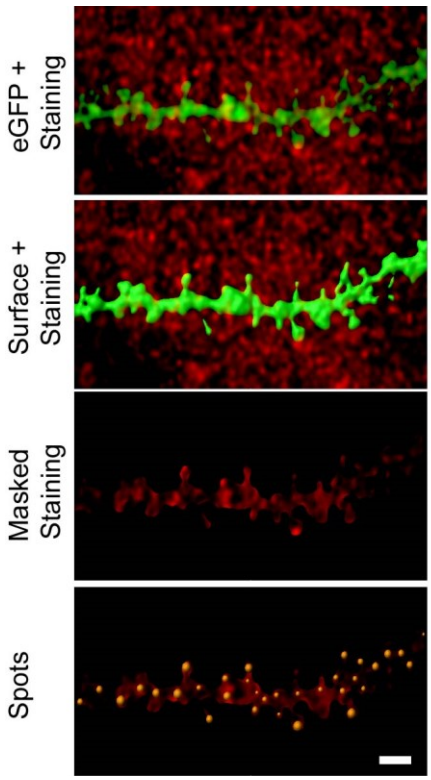
Slices were fixed for 1h at room temperature in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). Slices were then washed with 0.1 M PB, and then incubated a blocking and permeabilizing solution (0.1 M PB with 1.5% heat-inactivated horse serum (HIHS) and 0.4% Triton-X-100) overnight at 4 °C. Slices were incubated with either anti-RhoA (Santa Cruz Biotechology; 1:100), anti-Smurf-1 (Thermo Scientific; 1:50), or anti-Synaptopodin (Sigma Aldrich; 1:500) in the blocking/permeabilizing solution and covered for 5 days at 4 °C with agitation. After 5-day incubation, slices were washed 5 times for 15 minutes each wash with 0.1M PB with 1.5% HIHS. Slices were then incubated with a 1:250 dilution of species-matched

secondary antibodies (anti-rabbit Alexa Fluor 594, Molecular Probes, Eugene; or anti-mouse DyLight 649; Jackson ImmunoResearch, West Grove, PA) diluted in 0.1 M PB containing 1.5% HIHS and covered for 3h at room temperature with agitation. Slices were washed in 0.1 M PB with 1.5% HIHS overnight at 4 °C, and then mounted with Dako Fluorescent Mounting medium (Dako Canada) onto microscope slides. Immunostained cultures were imaged imaged with voxel dimensions of ~46 x 46 x 300-350 nm (512 x 512) or with a Leica TCS SP2 scanhead (Leica Microsystems) on an upright microscope (DM 6000 B) using a 63x oil objective (HCX PL APO 1.40 NA, Leica). Image stacks were collected at Z = 0.3 μm, and frame averaged (2 – 4 times) to improve the signal-to-noise ratio. Secondary and tertiary dendritic branches from either apical or basal dendrites were imaged with voxel dimensions of ~46 x 46 x 300-350 nm (512 x 512). No difference between basal or apical dendrites was detected, thus data was then pooled for analysis. Images were then deconvolved with Huygens Essentials software, and processed as described below (see Three Dimension Image Reconstruction and Analysis).

Three Dimension Image Reconstruction and Analysis

Image stacks collected were deconvolved using Huygens Essential software (Scientific Volume Imaging) using the maximum likely extrapolation, and analyzed blind in Imaris x64 software (Bitplane AG). To determine the spatial localization of the protein of interest within our dendrite, we masked and retained only puncta from the immunostained red or far red channel that colocalized within the green mGFP dendrite channel. A colocalization channel that contained only voxels representing colocalization results i.e. 3D pixel overlap. The colocalization was analyzed using the spot detection mode in Imaris that allowed us to determine the number of puncta localized to the spine and dendrite. We manually traced the length of the dendrite using the Filament Tracer function to calculate the number of puncta localized per micron of dendrite.

Figure V. Spatial localization of protein of interest within a dendrite



To determine the spatial localization of the protein of interest within our dendrite, we created a surface based on the fluorescence intensity of the dendrite with the Surpass function in Imaris. We then masked the immunostained channel within that surface and retained only puncta from the immunostained channel that colocalized within the green mGFP dendrite channel. The colocalization channel contained only voxels representing colocalization results i.e. 3D pixel overlap. The colocalization was analyzed using the spot detection mode in Imaris that allowed us to determine the number of puncta localized to the spine and dendrite. Scale bar, 2 μm

Statistical Analysis

Unless otherwise stated, error bars represent the standard error of the mean and statistical analysis was performed with two-tailed, two sample, paired Student's t-test, or chi squared test, and $P < 0.05$ considered as significant for all statistical comparison.

Results

In this study, we investigated the mechanisms underlying structural stability in dendritic spines of secondary or tertiary dendrites in CA1 pyramidal neurons following enhanced activity. Previously, it has been shown that synaptopodin is necessary for synaptic plasticity and dendritic spine structural changes in hippocampal pyramidal neurons. Furthermore, synaptopodin has been shown to regulate the actin cytoskeleton via regulation of RhoA-GTPase in kidney podocytes. Thus, we wished to determine whether post-synaptic structural plasticity in CA1 pyramidal neurons was due by synaptopodin regulation of RhoA-GTPase.

Lack of synaptopodin does not affect distribution of dendritic spines

Previous work by Deller *et al.* (2000) has shown that synaptopodin is expressed within the dendrites and spines of hippocampal CA1 pyramidal cells. Thus, we first we looked at the localization of immunostained synaptopodin in mature hippocampal CA1 pyramidal cells of our L15 organotypic culture system (**Fig** 1 a). Consistent with those previous findings (Deller *et al.*, 2000), synaptopodin in our culture system was localized to dendritic shafts, and predominantly to larger, more mature dendritic spines of secondary and tertiary CA1 pyramidal neurons (**Fig** 1 a).

Prior to starting our experiments, we first wanted to examine whether in our preparation there were any gross morphology and dendritic spines changes observed in the hippocampus of the SPKO mice compared to WT. First, we examined the overall hippocampal cytoarchitecture with nissl stain. We found that L15-S slices had a comparable gross morphology throughout the hippocampus to L15 slices (**Fig** 1 b). In addition, the cell morphology and spine density of mGFP CA1 pyramidal neurons was comparable to both SPKO and WT cultures (**Fig** 1 c). All spine subtypes were present with a similar density (Mushroom: WT, 0.45 ± 0.05 spines per μm of dendrite, SPKO 0.46 ± 0.10 spines per μm of dendrite; Thin: WT, 0.37 ± 0.04 spines per μm

of dendrite, SPKO 0.37 ± 0.06 spines per μm of dendrite; Stubby: WT, 0.42 ± 0.01 spines per μm of dendrite, SPKO 0.40 ± 0.03 spines per μm of dendrite; Total spines: WT, 1.24 ± 0.08 spines per μm of dendrite, SPKO 1.23 ± 0.12 spines per μm of dendrite) (**Fig** 1 c,d). Thus, even in the absence of synaptopodin, the dendritic arborization (Verbich, 2013), as well as the distribution of spines is comparable to WT, indicating that the learning deficits experienced when synaptopodin is absent is not the result of a basal structural abnormality.

Spine head enlargement after chemical-LTP is unstable in synaptopodin-KO slices

To determine whether the loss of sustained LTP was due to lack of synaptopodin, we examined the effect synaptopodin has on the structural modifications of dendritic spines after a LTP. In order to accomplish this, we first examined how dendritic spines responded structurally to a learning stimulus through the postsynaptic structural changes induced by LTP through time-lapsed imaging of mGFP dendritic spines from secondary and tertiary dendrites of CA1 pyramidal neurons. We used a forskolin-induced chemical LTP (chemLTP) protocol that induces global potentiation through an NMDAR-dependent mechanism, as previously reported (Kopec *et al.*, 2006; Kopec *et al.*, 2007; Makino & Malinow, 2009; Chang *et al.*, 2013). We used spine volumes as a marker for structural plasticity after LTP, as it has been previously reported that structural changes in spines that occur, as well as AMPAR insertion (Matsuzaki *et al.*, 2004; Hayashi-Takagi *et al.*, 2015). Thus, we measured these volume changes of thin and mushroom subtypes, yet excluded stubby spine volume analysis because they are too close to the dendrite for to be properly resolved for volume measurements. We performed baseline imaging for the first 20 minutes prior to chemLTP treatment to determine the transient volume changes that normally occur in dendritic spines. When we examined the changes of mushroom, thin, and combined all spines, we found that spines from CA1 pyramidal neurons in WT cultures on

average were significantly larger from 35 to 45 minutes following chemLTP induction compared to the first 10 minutes of baseline imaging (**Fig** 2 a-d). This indicated that the chemLTP induction was successful in increasing spine volume in secondary and tertiary dendrites from WT CA1 pyramidal neurons in our culture system.

Next, we wanted to determine whether spines that demonstrated and maintained a volume increase in CA1 pyramidal neurons of L15 cultures contained synaptopodin. Thus, after the chemLTP protocol reported above, we immediately fixed the culture and proceeded with immunostaining for synaptopodin. We first quantified the volume changes of the dendritic spines post chemLTP, and then checked for colocalization in spines with synaptopodin. We observed that synaptopodin positive spines from dendrites of CA1 pyramidal neurons had a significantly larger volume from 36 to 45 minutes post chemLTP compared to the first 9 minutes of baseline imaging (First 9 minutes 1.00 ± 0.02; last 9 minutes 1.33 ± 0.1) (**Fig** 3 a,b). Interestingly, spines without synaptopodin experienced a significant volume decrease comparing the 36 to 45 minutes post chemLTP compared to the first 9 minutes of baseline imaging (First 9 minutes 1.00 ± 0.03; last 9 minutes 0.88 ± 0.05) (**Fig** 3 a,b). Moreover, from 5 to 45 minutes post chemLTP spines containing synaptopodin were significantly larger than spines without synaptopodin and maintained that enlargement until 46 minutes post-chemLTP (**Fig** 3 a,b). Thus, we concluded that synaptopodin in dendritic spines enabled and maintained a spine volume enlargement in response to chemLTP. Hence, we anticipated that in slices where synaptopodin was removed, there would not be sustained structural plasticity after chemLTP.

Accordingly, we wanted to determine what effect the spines would have if synaptopodin were removed. We hypothesized that if synaptopodin were involved in the stabilization of dendritic spines after a learning stimulus, then removing synaptopodin from our cultures would

cause spines to be unable to maintain a spine enlargement. We examined the dendritic spine volume changes post-chemLTP for both WT and SPKO cultures. As mentioned previously, WT cultures became significantly larger during the last 10 minutes imagining (35 to 45 minutes following chemLTP induction) compared to the first 10 minutes of baseline imaging (**Fig** 4 a,c-e; **Table** 1.1). However, when SPKO spines of the same subtype were examined, there was no change in dendritic spine volume towards the end of chemLTP induction compared to baseline (**Fig** 4 b,c-e; **Table** 1.1).

Moreover, when we compared normalized volume changes of mushroom spines from secondary and tertiary dendrites of CA1 pyramidal neurons, SPKO mushroom spines were comparable to WT mushroom spines during the first 20 minutes of baseline imaging, and for approximately the first 7 minutes after chemLTP induction (**Fig** 4 a,b,c). From 7 minutes until 45 minutes after chemLTP induction, WT mushroom spines exhibited a spine volume increase that was not observed in SPKO mushroom spines, and WT mushroom spines were significantly larger than their SPKO counterparts during these time points (**Fig** 4 a,b,c). When all subtypes were combined together and compared, the spine volume changes were similar to those experienced by mushroom spines (**Fig** 4 a,b,e). Thin spines, on the other hand, from SPKO and WT dendrites of CA1 pyramidal neurons were comparable during baseline imaging, and for approximately the first 15 minutes after chemLTP induction as both SPKO and WT spines exhibited a spine volume increase (**Fig** 4 a,b,d). This volume increase in WT thin spines was maintained from 13 to 45 minutes after chemLTP (**Fig** 4 a,b,d). Whereas, SPKO thin spines experienced a decrease in spine volume, and we significantly smaller than WT thin spines at these time points, with the exception of 25 minutes after chemLTP (**Fig** 4 a,b,d), suggesting that SPKO spines were unable to maintain their spine enlargement after chemLTP. Moreover, when

overall spine volume changes were examined, we observed that there was a significantly greater proportion of WT spines that increased in size compared to SPKO spines (WT: 65.6%; SPKO: 34.4%), and there was a significantly greater proportion of SPKO pines that decreased in size compared to WT (WT: 44. 8%; SPKO: 55.1%) ($p<0.05$)(**Table** 1.2). We concluded that lack of synaptopodin significantly decreased dendritic spine structural stability in response to LTP. Hence, synaptopodin appears to be a necessary component in the structural stability of dendritic spines after LTP. How can synaptopodin be regulating spine stability after LTP enlargement?

RhoA is down-regulated in SPKO slice cultures

Interestingly, in the kidney podocyte, synaptopodin can regulate RhoA, a member of the RhoGTPase family involved in regulation of actin dynamics (Asanuma *et al.*, 2006). However, the synaptopodin isoform present in the kidney is 110 kDa, whereas in the brain there is a short isoform of synaptopodin that is 100 kDa. So, we wanted to determine whether the lack of stability observed in dendritic spines could be due to a general phenomenon of synaptopodin to regulate RhoA, or if it was specific to the kidney. Therefore, we investigated the levels of RhoA, and in SPKO mice compared to WT. We used immunoblot analysis to determine the expression levels of RhoA, Rac1, and Cdc42, members of the RhoGTPase family that are linked with spine stability, between WT and SPKO mice. When we first began looking at the expression of the Rho-GTPases, including Rac-1, RhoA, and Cdc42, in adult hippocampi of SPKO and WT mice, we found inconsistent results in the expression levels of RhoA and Cdc42, whereby there would be a decrease or increase or no change in the protein expression when comparing SPKO and WT mice. When we scrutinized the blots, we found that the ratio of male or female used within each blot seemed to make a difference in the overall expression seen. After examining the literature, we found that estrogen has the ability to regulate the levels of the Rho-GTPases. So we divided

the blots into either male or female hippocampi, and examined the Rho-GTPase protein expression levels between WT and SPKO mice.

We found a significant difference in the expression of the RhoGTPases between the hippocampi of adult male and female mice. There was a significant increase in RhoA and Cdc42 expression in SPKO adult male hippocampi compared to WT (RhoA- $p< 0.05$; Cdc42 - $p< 0.01$) (**Fig** 5 a,b). Surprisingly, with adult female hippocampi from WT and SPKO, we observed a decrease in RhoA and Cdc42 expression in SPKO female hippocampi compared to WT (RhoA - $p< 0.05$; Cdc42 - $p< 0.01$) (**Fig** 6 a,b). There was no significant change in Rac1 expression when either adult male WT and SPKO, or adult female WT and SPKO were compared (**Fig** 5 a,b; **Fig** 6 a,b). In order to control that the observed differences between SPKO and WT seen within male or female hippocampi was not due to a difference in the WT protein expression level, we compared WT male and female mouse hippocampi. We found that there was no change in RhoA and Rac1 expression between WT male and WT female mice (**Fig** 7 a,b,d), yet Cdc42 was significantly elevated in WT female hippocampi compared to WT male hippocampi ($p<0.001$) (**Fig** 7 a,c). As we detected a significant change in Cdc42 between WT female and WT male, we concluded that the Cdc42 levels detected are independent of the presence of synaptopodin, and instead may be regulated by sex hormones. Thus, for further studies we only concentrated on RhoA and Rac1. Next, we wanted to determine the expression of RhoA and Rac1 in our organotypic slice cultures, where we did our chemLTP experiments. Using immunoblot analysis we observed a significant decrease in RhoA expression in SPKO slice culture compared to WT ($p<0.01$) (**Fig** 8 a,b), and no change in Rac1 (**Fig** 8 c,d). As we could see a decrease in RhoA protein expression levels through our hippocampal slice cultures, we decided to examine where these changes in RhoA can occur, so we then decided to focus on the expression and localization

of RhoA in our cultures using immunohistochemistry. We observed a significant decrease in the number of RhoA puncta per micron of secondary and tertiary dendrites of CA1 pyramidal neurons in SPKO slices (1.37 ± 0.05 puncta/μm of dendrite; n = 16 dendrites from 4 CA1 cells) compared to dendrites of CA1 pyramidal neurons in WT slices (1.75 ± 0.06 puncta/μm of dendrite; n = 18 dendrites from 4 CA1 cells; $p<0.0001$) (**Fig** 9 a,b). These findings indicate that synaptopodin is involved in the regulation of RhoA in the brain as well as previously reported in the kidney. This decrease of RhoA expression within SPKO hippocampi could lead to the change in spine stability through the destabilization of the actin cytoskeleton.

Increased Smurf-1 in SPKO dendrites of CA1 pyramidal neurons

In the kidney, synaptopodin has been shown to competitively bind to RhoA and as such prevent Smurf1, and E3 ubiquitin ligase, from binding to RhoA and targeting it for degradation (Asanuma et al., 2006). Since synaptopodin within CA1 pyramidal neurons of the hippocampus appears to regulate the expression of RhoA, we wanted to determine whether synaptopodin's absence affects Smurf-1 levels in secondary and tertiary dendrites of CA1 pyramidal neurons in our SPKO cultures compared to WT. We observed a significant increase in the number of smurf1 puncta per micron of dendrite in SPKO cultures (0.14 ± 0.04 puncta/μm of dendrite; n = 19 dendrites from 8 CA1 cells) compared to WT cultures (0.07 ± puncta/μm of dendrite; n = 23 dendrites from 8 CA1 cells; $p<0.05$) (**Fig** 10 a,b). This increase in Smurf1 density within the dendrite of CA1 pyramidal neurons in SPKO cultures suggests that the absence of synaptopodin can regulate the expression of Smurf1. Since Smurf1 targets RhoA for degradation (Asanuma et al., 2006), an increase in Smurf1 density along the dendrite could be a potential mechanism that is leading to the decrease in RhoA, and thus the spine instability observed in our cultures after learning.

GluN2B receptor subunit is down regulated in adult SPKO hippocampi

Next, we wanted to determine whether a change in calcium influx, which triggers LTP induction, could be altered when synaptopodin is absent, as this could be a potential mechanism leading to the decrease in potentiation seen after LTP (Deller *et al.*, 2003), as well as the change in spine stabilization after chemLTP we observed in our cultures. Since synaptopodin cross-links with α-actinin-2, which is involved in the anchoring of the GluN2B subunit of the NMDA receptor to the PSD, we decided to look at the GluN2B subunit within our SPKO mice. In particular, we examined the protein levels of the GluN2B subunit because it is known to play an important role in learning and memory, especially induction (Sakimura *et al.*, 1995; Kiyama *et al.*, 1998; Tang *et al.*, 1999). Therefore, we wanted to examine whether there was a difference in protein expression levels between in SPKO and WT hippocampi of adult mice (between 4 to 9 months of age). We observed that there was a significant decreased in GluN2B expression in the SPKO adult mouse hippocampus compared to WT (WT: 0.44 ± 0.03; SPKO: 0.20 ± 0.06, $p<0.05$) (**Fig** 11 a,b). This decrease of GluN2B protein expression could lead to less calcium influx after LTP, which is a potential mechanism contributing to the decrease in potentiation observed after LTP. Thus, this could be an alternative mechanism that enables synaptopodin to maintain dendritic spine stabilization after LTP.

Discussion and Future Directions

My thesis examined the role of synaptopodin in the structural stability of dendritic spines from tertiary dendrites in CA1 pyramidal neurons following enhanced activity. Synaptopodin is an actin-binding protein that exists as two different isoforms, a renal podocyte synaptopodin-long form, and a neuronal synaptopodin-short form (Mundel *et al.*, 1997). Within the hippocampus, previous work has been shown synaptopodin to be necessary for *in vivo* and *in vitro* models of learning (Deller *et al.*, 2003), as well as spine structural changes in hippocampal pyramidal neurons (Zhang *et al.*, 2013). Nevertheless, how synaptopodin exerts control over the learning and memory process remains to be determined. Evidence from research in the kidney podocytes has shown that the long isoform of synaptopodin regulates the actin cytoskeleton via the regulation of RhoA (Asanuma *et al.*, 2006). Therefore, we investigated whether post-synaptic structural plasticity in CA1 pyramidal neurons was also due to the regulation of RhoA by synaptopodin.

SPKO cultures have comparable dendritic spine distribution and baseline synaptic transmission as WT cultures

We found that synaptopodin in our culture system was predominantly localized to dendritic shafts and within larger, more mature spines of secondary and tertiary dendrites of CA1 pyramidal neurons, consistent with Deller *et al.* (2000). Moreover, we found that slice cultures derived from SPKO mice had comparable gross cytoarchitecture, CA1 pyramidal cell morphology, and dendritic spine density to WT slices. Upon examination of basal synaptic transmission, our lab found that excitatory AMPA-mediated mEPSCs from CA1 pyramidal neurons of SPKO slices were not significantly different from WT (Verbich, 2013). These findings are consistent with what was previously reported by Deller *et al.* (2000). However,

SPKO mice exhibited spatial learning deficits *in vivo* and reduced LTP response *ex vivo* (Deller *et al.*, 2003). These findings indicate that the learning deficits experienced by the knockout animals are not the result of a gross structural morphology or reduction in dendritic spine morphology. *Therefore, how can the learning deficits exhibited by these animals be accounted for?*

Synaptopodin sustains spine enlargement after LTP

As previously mentioned, dendritic spines undergo structural plasticity following neuronal activity, and these modifications can affect synaptic transmission and plasticity. Since there were functional deficits seen in SPKO mice, we decided to look at how synaptopodin influences synaptic structures following LTP. We found, synaptopodin appears to be necessary for improving the stability of the spine volume increase after chemLTP. First, we demonstrated that in our WT cultures dendritic spines from CA1 pyramidal neurons were able to undergo and maintain spine enlargement for 45 minutes after chemLTP induction. Moreover, spines with synaptopodin from WT cultures exhibited a significant enlargement after chemLTP induction compared to both baseline levels and spines without synaptopodin, as revealed by *post hoc* analyses. However, since the immunostaining for synaptopodin was performed after the chemLTP experiment was completed, we could not determine whether the spine volume increases were predisposed to spines that already contained synaptopodin at the beginning of induction or whether synaptopodin entered those spines in an activity-dependent manner. We expect that synaptopodin would enter spines after LTP induction to aid with spine enlargement and stability, consistent with previous work that has shown that synaptopodin is essential for activity-dependent regulation of dendritic spine volume increases (Suzuki *et al.*, 2008; Zhang *et al.*, 2013; Korkotian *et al.*, 2014).

To further demonstrate the necessity of synaptopodin in spine enlargement and maintenance after LTP induction, we examined cultures derived from SPKO mice. We found that dendritic mushroom spines that did not contain synaptopodin were significantly smaller than WT mushroom spines for the entire 45 minutes after chemLTP induction. Thin spines, on the other hand, exhibited an initial increase in spine volumes following chemLTP, yet spine volumes quickly returned to baseline levels about 20 minutes after chemLTP induction compared to WT thin spines. Although mushroom spines do not typically exhibit enlargements as they are the more mature and stabilized spines (Harris *et al.*, 1992; Bourne & Harris, 2007; McKinney & Thompson, 2009; McKinney, 2010; Sala & Segal, 2014), we did observe a significant increase in volume in the WT mushroom spines follow chemLTP. This increase, however, was not as prominent as the volume change experienced by the thin spines. Instead, thin spines were able to exhibit an immediate spine volume enlargement after chemLTP, yet without synaptopodin this spine volume enlargement was not maintained. Therefore, synaptopodin appears to be an important mediator of sustained spine volume increase and stability after a learning stimulus, such as chemLTP. This inability to change spine shape and size following potentiation may account for the LTP functional reduction of the hippocampal Schaffer Collateral pathway in SPKO mice (Deller *et al.*, 2003). We concluded that synaptopodin is a required component in the structural stability of dendritic spines after LTP.

In the future, it would be interesting to see how synaptopodin is regulated within dendritic spines and whether synaptopodin is sufficient for structural stability following LTP. We can reintroduce synaptopodin into CA1 neurons of our SPKO cultures with biolistic transfection of a tdTomato-labeled synaptopodin under a CMV promoter. Then, we can live confocal image to visualize whether the tdTomato-labeled synaptopodin traffics into spines that

exhibit enlargements after chemLTP, or if spines that already had tdTomato-labeled synaptopodin were the spines with volume increases after chemLTP. Since synaptopodin is essential for activity-dependent regulation of spine increases (Suzuki *et al.*, 2008; Zhang *et al.*, 2013), we would expect that synaptopodin would enter spines after LTP induction to enable and stabilize spine enlargements. In addition, through the biolistic transfection of synaptopodin into our SPKO cultures, we will be able to determine whether reintroduction of synaptopodin is sufficient for spine enlargement and maintenance following LTP.

How does synaptopodin maintain spine stability after enhanced activity?

Next, we wanted to determine the mechanism for regulating spine stability and enlargement by synaptopodin after LTP. Synaptopodin is an actin-binding protein (Mundel *et al.*, 1997), and since spine motility is driven by actin dynamics (Fischer *et al.*, 1998), we hypothesized that synaptopodin may act with other actin-binding proteins, as well as actin regulatory proteins in order to stabilize the actin filaments within the spine. Actin regulatory proteins, such as the RhoGTPases have been shown to vary effects on spine structural modulation through the regulation of the actin cytoskeleton. Previous work has demonstrated that RhoA and its downstream effectors are involved in the initial phase of structural plasticity, and inhibition of this pathway led to blocked spine enlargement at individual spines (Murakoshi *et al.*, 2011). Moreover, in the kidney podocytes, the long-form of synaptopodin has been shown regulates the actin cytoskeleton through the regulation of small GTPase, RhoA (Asanuma *et al.*, 2006). Thus, we tested whether the neuronal synaptopodin-short form could regulate the actin cytoskeleton in a similar manner as the kidney in order to stabilize the actin filaments within the spine.

Therefore, we next investigated the levels of the RhoGTPases in SPKO mice compared to

WT. We found that the levels of RhoA and Cdc42, when compared to WT, were significantly upregulated in male SPKO mice when compared to wildtype, whereas significant downregulation was observed in female SPKO mice. When we compared WT male to WT female mice, we found no significant change in Rac1 levels, but RhoA levels showed an increasing trend while Cdc42 levels were significantly higher in WT female hippocampi.

The differential levels of sex hormones within the hippocampus may have resulted in the discrepancy of RhoGTPase regulation observed by us. Estradiol has been shown to be involved in the enhancement of LTP by modulating the assembly of actin cytoskeleton filaments (Kramár *et al.*, 2013). In addition, the inhibition of local estradiol synthesis in hippocampal neurons in female mice not only lead to the impairment of LTP and synapse loss, but also to the dephosphorylation of cofilin, and thus, the destabilization of dendritic spines (Vierk *et al.*, 2012; Vierk *et al.*, 2014). This phenomenon appears to occur through the regulation of RhoA as been shown when an antagonist of ROCK, a major downstream effector of RhoA, blocks estradiol's synaptic effects (Kramár *et al.*, 2013), and, importantly, act as a regulator of cofilin phosphorylation (Nishita *et al.*, 2005; Andrianantoandro & Pollard, 2006; Bernard, 2007; Sit & Manser, 2011). Moreover, when combined, estradiol and progesterone have been shown to increase phosphorylation of WAVE1, a downstream effector of Cdc42 (Hansberg-Pastor *et al.*, 2015). Although sex hormones have not been shown to directly regulate Cdc42 within the hippocampus, work done in neuroblastoma cells has shown that estradiol increases Rac1 and Cdc42 and decreases RhoA activity, in order to promote neurite outgrowth (Takahashi *et al.*, 2011).

Interestingly, synaptopodin has been shown be downregulated both when estradiol is increased with dissociated hippocampal neurons (Fester *et al.*, 2009), as well as when estradiol is

either inhibited in aromatase knock-out mice or with an aromatase inhibitor, letrozole, in hippocampal cultures (Chang *et al.*, 2013; Fester *et al.*, 2013). Thus, synaptopodin levels appear to be differentially regulated by the varying presence of estradiol. Since estradiol inhibition leads to a downregulation in synaptopodin levels (Chang *et al.*, 2013; Fester *et al.*, 2013), the impairment of LTP through the RhoA pathway under this condition (Vierk *et al.*, 2012; Vierk *et al.*, 2014) may be due to synaptopodin's regulation of RhoA pathway in the hippocampus (Asanuma *et al.*, 2006). Thus, estrogen's regulation of RhoA can result from the downregulation of synaptopodin within the hippocampus. In addition, when examining WT male and female mice, there is a significant increase in Cdc42 levels in female compared to male mice, yet no significant change in RhoA levels, thus suggesting that the effects estrogen on the regulation of Cdc42 are independent of synaptopodin, and may be regulated by estrogen's regulation of another pathway.

For this reason, we concentrated on RhoA and Rac1 protein levels in WT and SPKO cultures made from P5-P7 mice of either sex for the next set of studies. Since P5-P7 mice have not yet reached sexual development, circulating sex hormones do not interfere with the regulation of proteins at this age. Using immunoblot analysis, we found that there was no change in Rac1, but a significant decrease in RhoA levels in SPKO organotypic hippocampal slices compared to WT. Using immunohistochemistry, we observed the protein level and localization of RhoA within secondary and tertiary dendrites of CA1 pyramidal neurons to be significantly decreased in SPKO compared to WT slice cultures. This diminished RhoA level may account for the anomalies in spine enlargement and stability after LTP through the actin cytoskeleton destabilization.

We hypothesized, how synaptopodin could regulate RhoA protein levels within the

dendrites of SPKO CA1 pyramidal neurons? In the kidney podocyte, synaptopodin competitively binds to RhoA and prevents its degradation by Smurf-1, an E3 ubiquitin ligase, which has been shown to target RhoA for protein degradation (Asanuma *et al.*, 2006). In our cultures, we observed a significant increase in Smurf-1 protein levels along the secondary and tertiary dendrites of CA1 pyramidal neurons. This increase in Smurf-1 levels when synaptopodin is absent may account for the loss of RhoA. Synaptopodin competitively binds to RhoA and prevents its interaction with Smurf-1. Therefore, in the absence of synaptopodin, RhoA may be actively targeted for degradation by Smurf-1.

During LTP, structural modifications of the dendritic spines rely on actin filament assembly, a necessary component of the stability of the actin cytoskeleton. Therefore, by protecting actin filaments from disassembly, synaptopodin is also important for the maintenance of the dendritic spines structural modification during LTP (Suzuki *et al.*, 2008; Zhang *et al.*, 2013). As mentioned, our work shows that when synaptopodin is absent, RhoA protein levels within the hippocampus are downregulated. RhoA regulates the actin cytoskeleton by inactivating cofilin through phosphorylation (Nishita *et al.*, 2005; Andrianantoandro & Pollard, 2006; Bernard, 2007; Sit & Manser, 2011). Cofilin is an actin depolymerization factor (ADF) that depolymerizes the minus end of the actin filament (Luo, 2002; Andrianantoandro & Pollard, 2006; Bernstein & Bamburg, 2010; Spence & Soderling, 2015). Thus, RhoA prevents the depolymerization of the F-actin filaments by cofilin in order to stabilize dendritic spine structures (Yang *et al.*, 1998; Bernstein & Bamburg, 2010; Lamprecht, 2014; Spence & Soderling, 2015). Our observed decrease in RhoA could lead to an overall increase in active cofilin, which depolymerizes F-actin filaments and prevents the stabilization of the spine structure following LTP-mediated enlargement in SPKO cultures.

Future work is needed to fully elucidate the precise mechanism for the downstream signalling cascade of synaptopodin in dendritic spines after LTP. We hypothesize that RhoA is acting through the cofilin pathway; thus, with immunoblotting we could to look at the ratio between unphosphorylated and phosphorylated levels of cofilin to determine if there is an increase cofilin activity in SPKO cultures compared to WT. We expect that there would be an overall increase in unphosphorylated active cofilin compared to phosphorylated inactive cofilin, and, thus, lead to increased depolymerization of F-actin filaments that would prevent of spine enlargement and stability after chemLTP. In addition, prospective work should examine whether the spine instability seen in SPKO cultures after LTP can be recapitulated by modulated the levels of RhoA within our cultures. This can be done through biolistic transfection of a tdTomato-labeled RhoA under the CMV promoter into our SPKO cultures in order to increase levels of RhoA within spines. We would expect to see a rescue in the spine instability after LTP, if in fact synaptopodin's role in spine enlargement is through regulating the actin cytoskeleton via RhoA. In turn, within our WT cultures we want to determine whether we can obtain our spine instability phenotype by either reducing the protein levels of RhoA through biolistic transfection of shRNA targeted against RhoA, or by using a ROCK blocker in order to inhibit the downstream signaling cascade of RhoA.

In order to determine if the decrease in RhoA levels is due to an increase in degradation, we could examine the colocalization of RhoA with the proteasome within WT and SPKO cultures using immunohistochemistry, and we expect that there would be an increase amount of RhoA targeted, and thus, colocalized with the proteasome for degradation. Moreover, to clarify the role of Smurf-1 in targeting RhoA for degradation, modulation of Smurf-1 levels with biolistic transfection of dominant negative Smurf-1 in our SPKO cultures can be completed, we

anticipate that there would be an increase in RhoA, and accordingly, led to a rescue in the spine instability phenotype observed. Therefore, through the modulation of the downstream signaling components of the RhoA pathway, this future work will further clarify synaptopdin's role in spine enlargement and stability after LTP.

In addition to synaptopodin's role in stabilizing actin through the regulation of RhoA, synaptopodin can also modulate local Ca^{2+} signalling. Synaptopodin is required for the formation of the SA, a calcium sequestering organelle within dendritic spines (Deller *et al.*, 2003). The SA has been suggested to function as a local Ca^{2+} store that could regulate actin-based spine motility by releasing or sequestering Ca^{2+} (Fifková *et al.*, 1983; Holbro *et al.*, 2009; Vlachos *et al.*, 2009; Korkotian & Segal, 2011). Thus, posing a potential alternative mechanism for synaptopodin stabilization of the actin cytoskeleton through the modulation dendritic spine Ca^{2+} levels after enhanced activity.

Previous work from our lab has shown that synaptopodin can influence the stability of SHPs, a form of activity-dependent structural modification of dendritic spines, through CICR from RyR-sensitive Ca^{2+} stores (Verbich, 2013). Moreover, we have also shown that SHP formation partially depends on NMDA receptor activation (Richards *et al.*, 2005). Since, activation of the CICR requires calcium entry through NMDA or voltage-gated calcium channels (VGCC) (Verkhratsky & Shmigol, 1996; Berridge, 1998; Emptage *et al.*, 1999; Roderick *et al.*, 2003; Korkotian *et al.*, 2014; Segal & Korkotian, 2015), we decided to examine whether synaptopodin could stabilize spine volumes enlargements after LTP by influencing Ca^{2+} influx. Previously, it has been shown that synaptopodin also binds to α-actinin-2 (Asanuma *et al.*, 2005; Kremerskothen *et al.*, 2005) that crosslinks and bundles actin (Sjöblom *et al.*, 2008). α-actinin-2 tethers GluN1 and GluN2B receptor subunits to the PSD (Sheng & Pak, 2000). Since

synaptopodin cross links with α-actinin-2, it remains to be determined that whether synaptopodin is involved in the anchoring of the GluN2B subunit to the PSD. The GluN2B subunit plays an important role in learning and memory (Sakimura et al., 1995; Kiyama et al., 1998) as it increases the channel opening time of the GluN2B subunit and enhances coincidence detection by the NMDA receptors (Tang *et al.*, 1999). Interestingly, during postnatal development there is an increase in the GluN2A/GluN2B ratio (Williams *et al.*, 1993; Petralia *et al.*, 1994a; Petralia *et al.*, 1994b; Racca *et al.*, 2000; Paoletti *et al.*, 2013), thus enabling an easier LTP induction in younger animals because of the presence of the GluN2B subunit (Barria & Malinow, 2005; Yashiro & Philpot, 2008; Müller *et al.*, 2013).

Our results showed that there was a significant reduction in GluN2B protein levels within adult hippocampi of SPKO mice compared to WT. The change of GluN2B levels may offer an explanation for the reduction in LTP *in vivo* (Deller *et al.*, 2003) and the aberrant spine enlargement after chemLTP in our organotypic hippocampal slice cultures. The tethering capabilities of α-actinin-2 of GluN2B subunit to the membrane may be decreased and account for the decline in GluN2B subunit from SPKO mice. The reduction of GluN2B subunit could cause a decrease in the Ca^{2+} influx, and cause a reduction of CICR within a spine, effectively dampening the typical synaptic and structural plasticity responses. However, it remains to be determined whether the reduction in GluN2B protein levels observed in SPKO mice is due to a rapid developmental switch from GluN2A to GluN2B, or to a decrease in GluN2B protein levels initially during development.

Although previous work done examining the fEPSPs of the CA1 *stratum radiatum* showed that the ratio of NMDA receptors to AMPA receptors were normal in SPKO mice (Deller *et al.*, 2003), more work needs to be done to determine whether the GluN2B subunit was

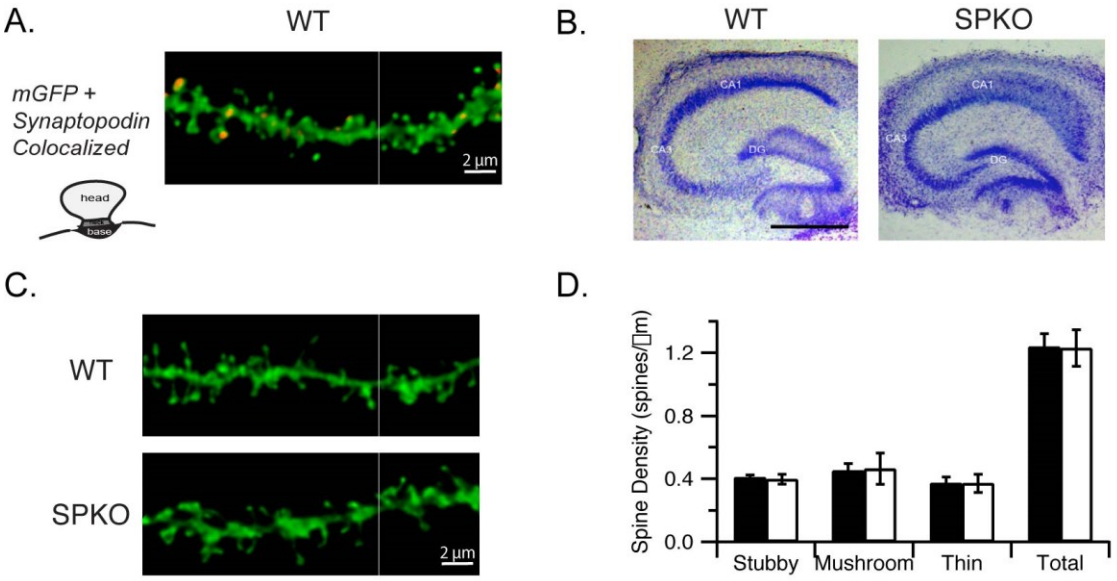
exchanged for the GluN2A subunit, a subunit tethered to the membrane with spectrin. Immunoblotting could be performed in our cultures to examine the ratio of the GluN2B to the GluN2A subunit present within our SPKO cultures compared to WT. Furthermore, the ratio of GluN2B to GluN2A subunits can be observed with electrophysiology by examining NMDA mEPSCs in WT and SPKO cultures, and then pharmacologically inhibiting the GluN2B subunit with Ro 25-6981, a highly potent and selective blocker of the GluN2B subunit. Thus, when the GluN2B subunit is blocked in our SPKO cultures, if there is a switch from GluN2B to GluN2A subunits, we anticipate less of a reduction in NMDA currents compared to WT. This work would begin to explain whether synaptopodin's role in modifying GluN2B levels also modulates Ca^{2+} influx after LTP.

Concluding Remarks

Within CA1 pyramidal neurons, our findings show that synaptopodin is a necessary component of dendritic spine enlargement and stability after LTP. In this study, we investigated how synaptopodin could regulate spine structural changes after enhanced activity. We hypothesized that the neuronal short-form of synaptopodin could regulate RhoA proteins levels by competitively binding to RhoA and preventing its degradation from Smurf-1 within SPKO CA1 pyramidal neurons, in a similar manner as in kidney podocytes. From our experiments, we observed a decrease in RhoA and increase in Smurf-1 protein levels within dendrites of SPKO CA1 pyramidal neurons. This suggests that the presence of synaptopodin regulates RhoA protein levels within dendrites. Our observed decrease in RhoA could lead to an overall increase in active cofilin, which depolymerizes F-actin filaments and prevents the enlargement and stabilization of the spine structure following LTP in SPKO cultures. Further investigation directly linking the neuronal synaptopodin-short form of synaptopodin, RhoA, and Smurf-1 within the hippocampus is needed. Moreover, the precise effects of synaptopodin's regulation of the RhoA signaling cascade on the actin cytoskeleton have yet to be elucidated. Finally, the decrease of GluN2B expression within SPKO hippocampi compared to WT suggests that synaptopodin may also be regulating the presence of GluN2B subunit within the hippocampus, and after enhanced activity this can lead to a reduction in sufficient Ca^{2+} influx into the cell to induce LTP. In conclusion, the present work begins to clarify synaptopodin's role in dendritic spine enlargement after LTP by determining its necessity in spine enlargement and maintenance, it's role in the regulation of RhoA within the brain, and its regulation of the GluN2B subunit involved in calcium influx.

Figures

Figure 1. Organotypic slice cultures from synaptopodin-KO slices are comparable structurally and functionally to wild-type slices.



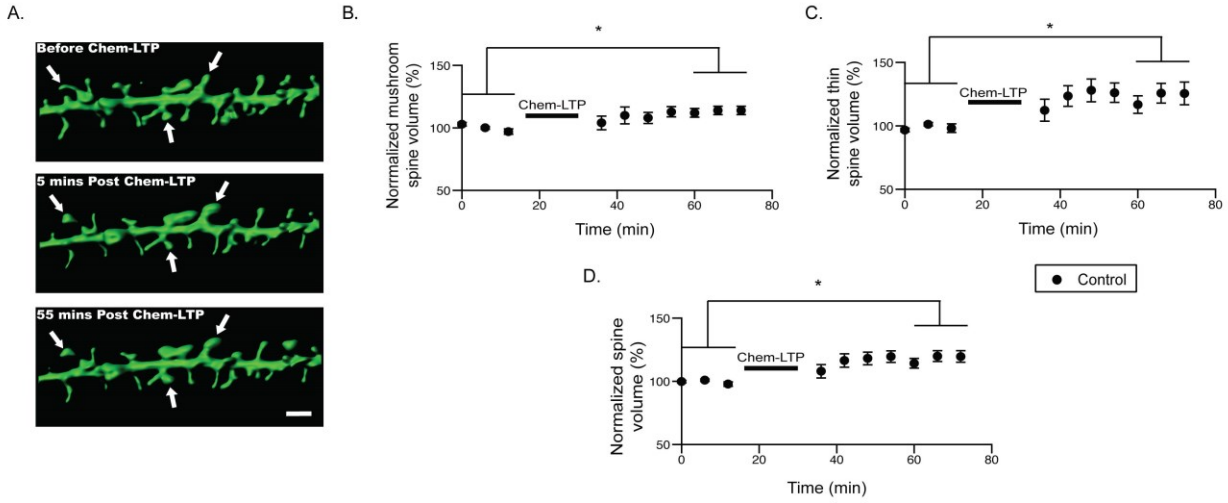
A. Immunostained synaptopodin (red) was localized to dendritic shafts and predominantly to larger, more mature dendritic spines of secondary and tertiary CA1 pyramidal neurons expressing membrane GFP (mGFP). The green channel was used to mask the immunostained red channel in order to colocalize the synaptopodin-positive puncta found within the dendrite.

B. Nissl-stained slice cultures (21 DIV) from wild type (WT) (*left*) and synaptopodin knock out (SPKO) (*right*) mice reveal comparable gross anatomy. DG, dentate gyrus. Scale, 500 µm.

C. Examples of tertiary dendrites from WT and SPKO CA1 pyramidal neurons showing that the overall presence of dendritic spines of SPKO CA1 pyramidal neurons is comparable to WT.

D. Spine densities, lengths, and volume quantification from WT and SPKO CA1 dendrites expressed as a percent that was normalized to WT. Note: averages are mean ± SEM

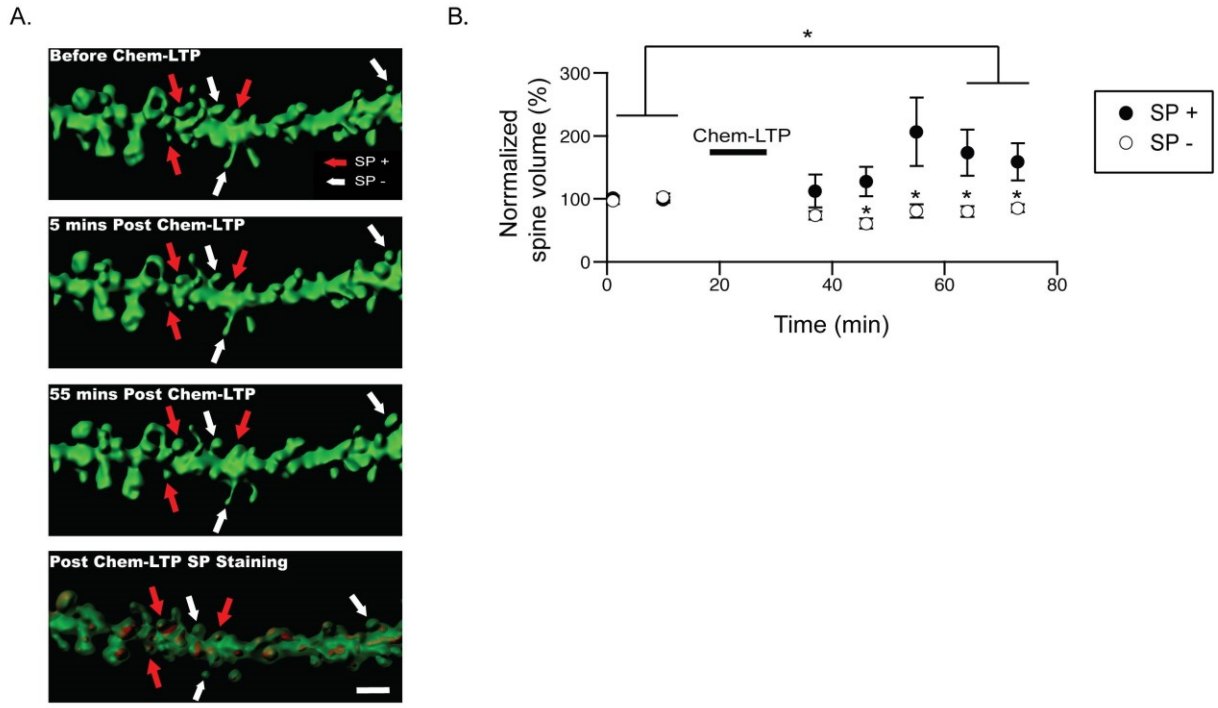
Figure 2. Spine head enlargement occurs in our WT slice cultures after chem-LTP.



A. Example 3D images of a CA1 tertiary dendrite from WT slices before chemLTP, 5 mins post chemLTP, and 55 mins post chemLTP. Arrows represent spines that have a volume increase that has been maintained post-chemLTP. Scale bar 2 µm.

B. Normalized spine volume of mushroom, **C.** thin, and **D.** all spines before, during, and after chemLTP. Black bar indicates chemLTP induction. n = 68 spines from 5 dendrites in 5 slices (5 control); *$p < 0.05$. Note: averages are mean ± SEM

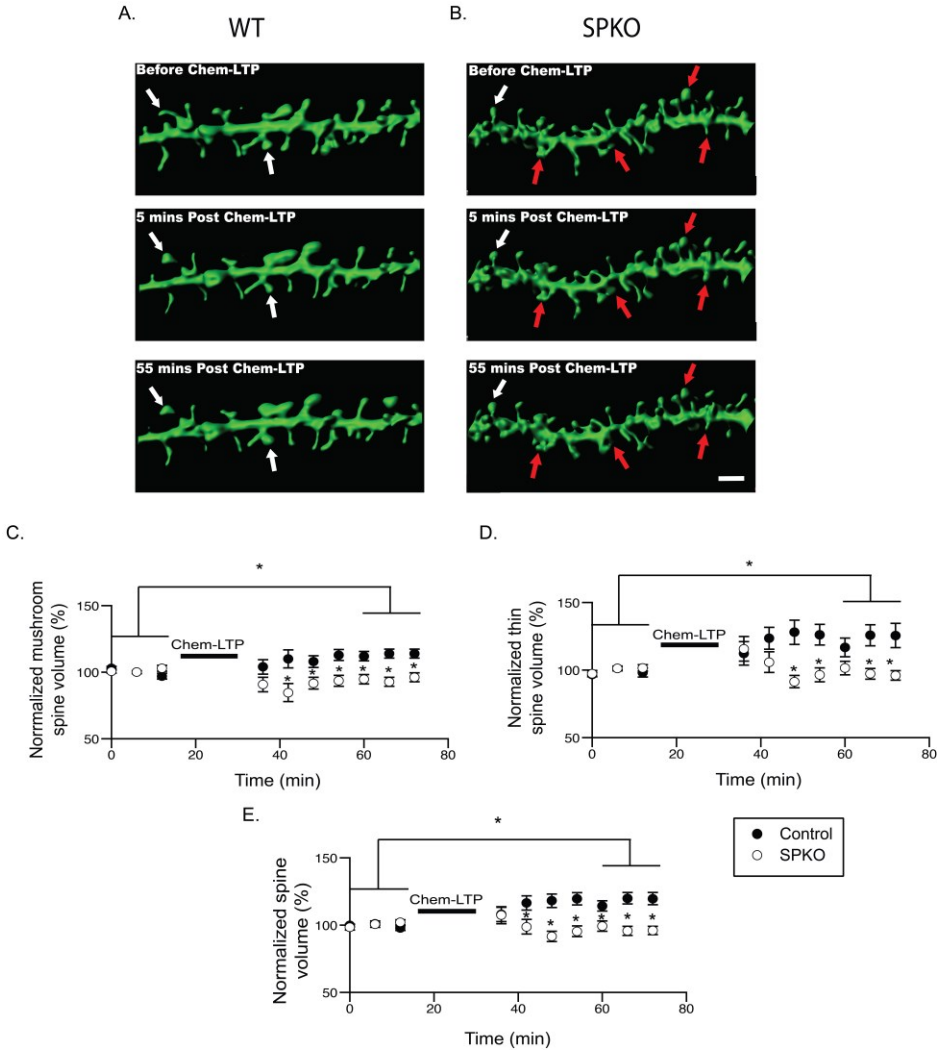
Figure 3. Synaptopodin is localized to dendritic spines that experienced a spine volume increase



A. 3D image of dendrites from control and SPKO slices. Enlargement of spine heads can be observed at various locations along the dendrite following chemLTP. White arrows represent spines that do not have synaptopodin; red arrows represent spines that do have synaptopodin. Scale bar 2 µm.

B. Normalized spine volume of dendritic spines with and without synaptopodin before, during, and after chemLTP. Black bar indicates chemLTP induction. n = 15 spines from 2 dendrites; * $p < 0.05$. Note: averages are mean ± SEM

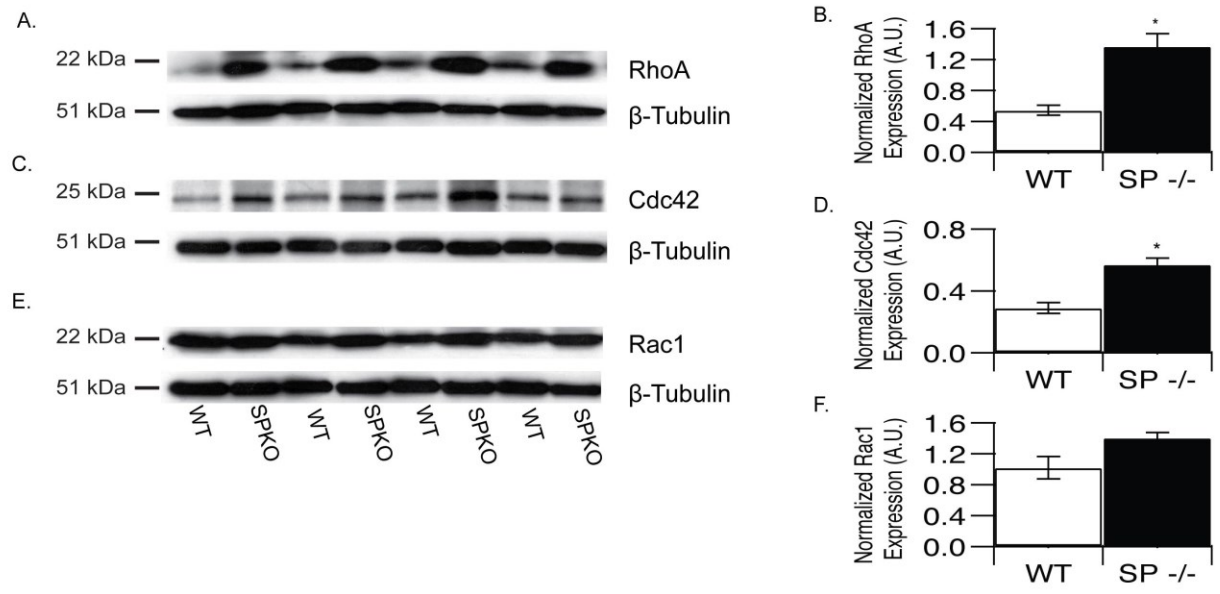
Figure 4. Spine head enlargement is instable in synaptopodin-KO slices after chemical-LTP.



A. Example 3D images of a CA1 tertiary dendrite from WT and SPKO slices before chemLTP, 5 mins post chemLTP, and 55 mins post chemLTP. Arrows indicate spine heads that have undergone structural remodification. White arrows represent spines that have a volume increase that has been maintained post-chemLTP; red arrows represent spines that have undergone a volume increase, yet were unable to maintain that structural remodification. Scale bar 2 µm.

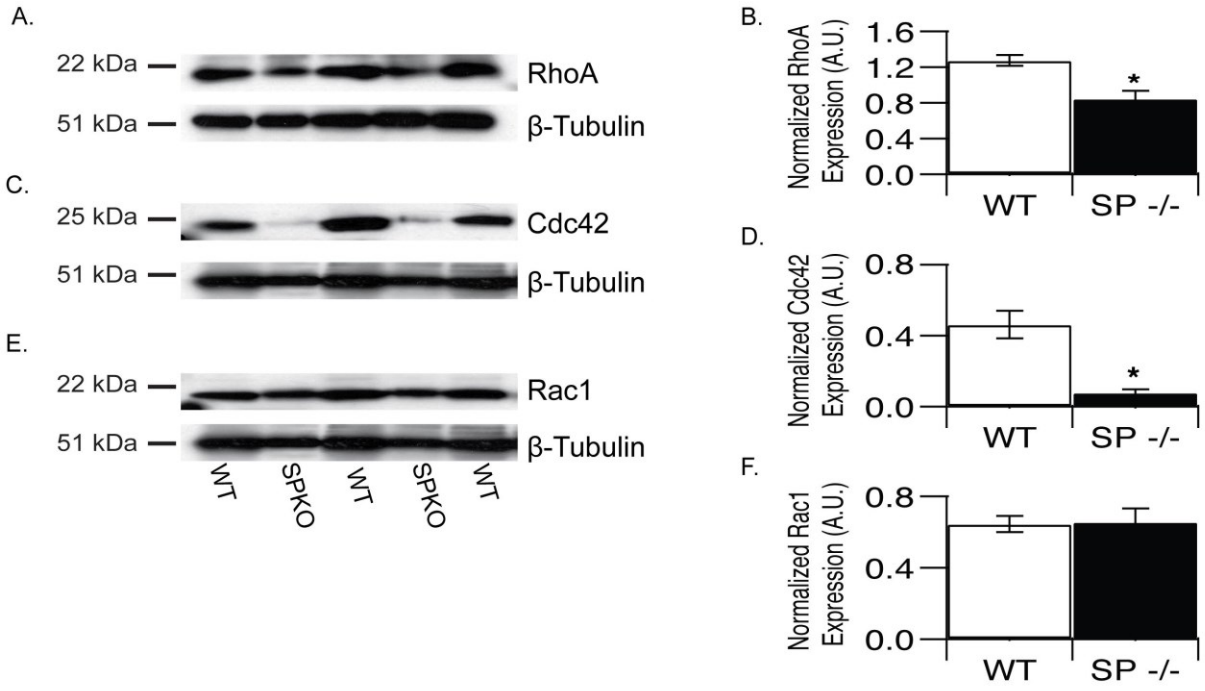
B. Normalized spine volume of mushroom, thin, and all spines before, during, and after chemLTP. Black bar indicates chemLTP induction. n = 120 spines from 10 dendrites in 10 slices (5 control and 5 SPKO); *$p < 0.05$. Note: averages are mean ± SEM

Figure 5. Elevated RhoA and Cdc42 expression in synaptopodin-KO adult male hippocampi compared to WT



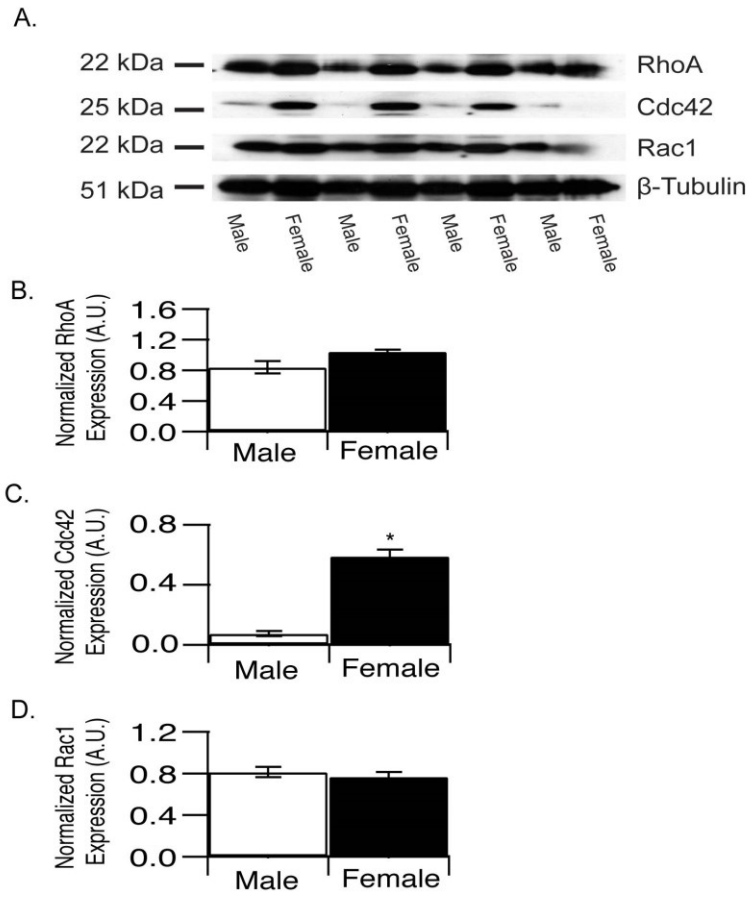
Western blot of RhoA, Cdc42, and Rac1 expression in male SPKO mice compared to WT. **A – B.** Quantification reveals that there is a significant increase in RhoA expression in the SPKO hippocampi compared to WT (WT: 0.55 ± 0.06 A.U.; SPKO: 0.183 ± 0.16 A.U., $p< 0.05$). **C –D.** There is a significant increase in Cdc42 expression in SPKO hippocampi compared to WT (WT: 0.29 ± 0.04 A.U.; SPKO: 0.57 ± 0.04 A.U., $p< 0.01$). **E –F.** No change is observed in Rac-1 (WT: 1.02 ± 0.15 A.U.; SPKO: 1.41 ± 0.07 A.U.). β-tubulin was used as a total protein loading control; *$p<0.05$. Note: averages are mean ± SEM

Figure 6. Decreased RhoA and Cdc42 expression in synaptopodin-KO adult female hippocampi compared to WT



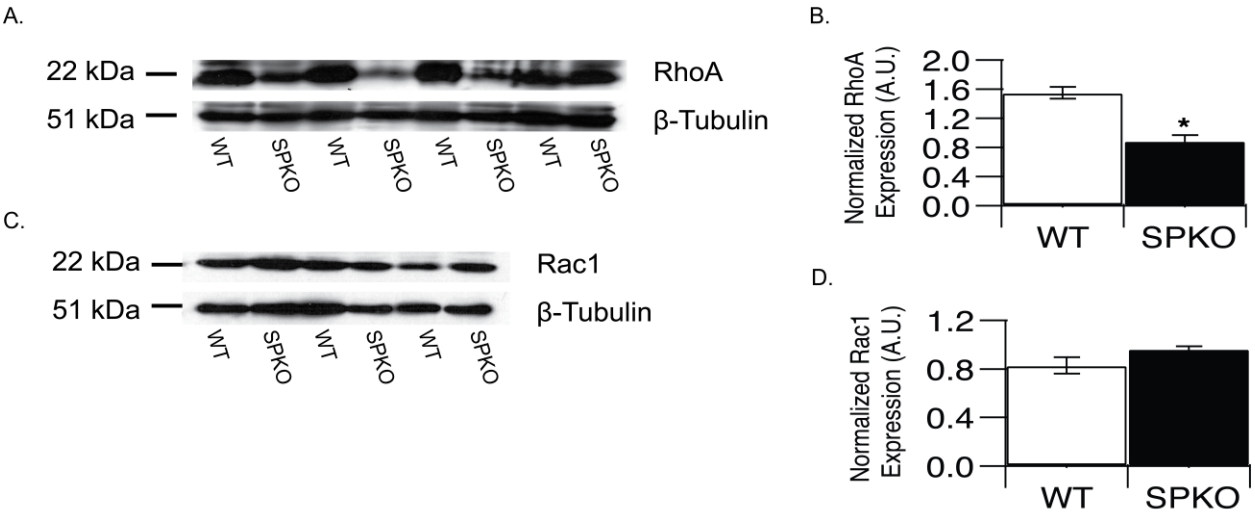
Western blot of RhoA, Cdc42, and Rac1 expression in female SPKO mice compared to WT. **A – B.** Quantification reveals that there is a significant decrease in RhoA expression in the SPKO hippocampi compared to WT (RhoA - WT: 1.28 ± 0.062 A.U.; SPKO: 0.85 ± 0.09 A.U., $p< 0.05$). **C – D.** There is a significant decrease in Cdc42 expression in the SPKO hippocampi compared to WT (Cdc42 - WT: 0.51 ± 0.06 A.U.; SPKO: 0.10 ± 0.03 A.U., $p< 0.01$). **E – F.** No change is observed in Rac-1 (WT: 0.55 ± 0.03 A.U.; SPKO: 0.57 ± 0.08 A.U.). β-tubulin was used as a total protein loading control; *$p<0.05$. Note: averages are mean ± SEM

Figure 7. Elevated Cdc42 expression in WT adult female hippocampi compared to adult male hippocampi



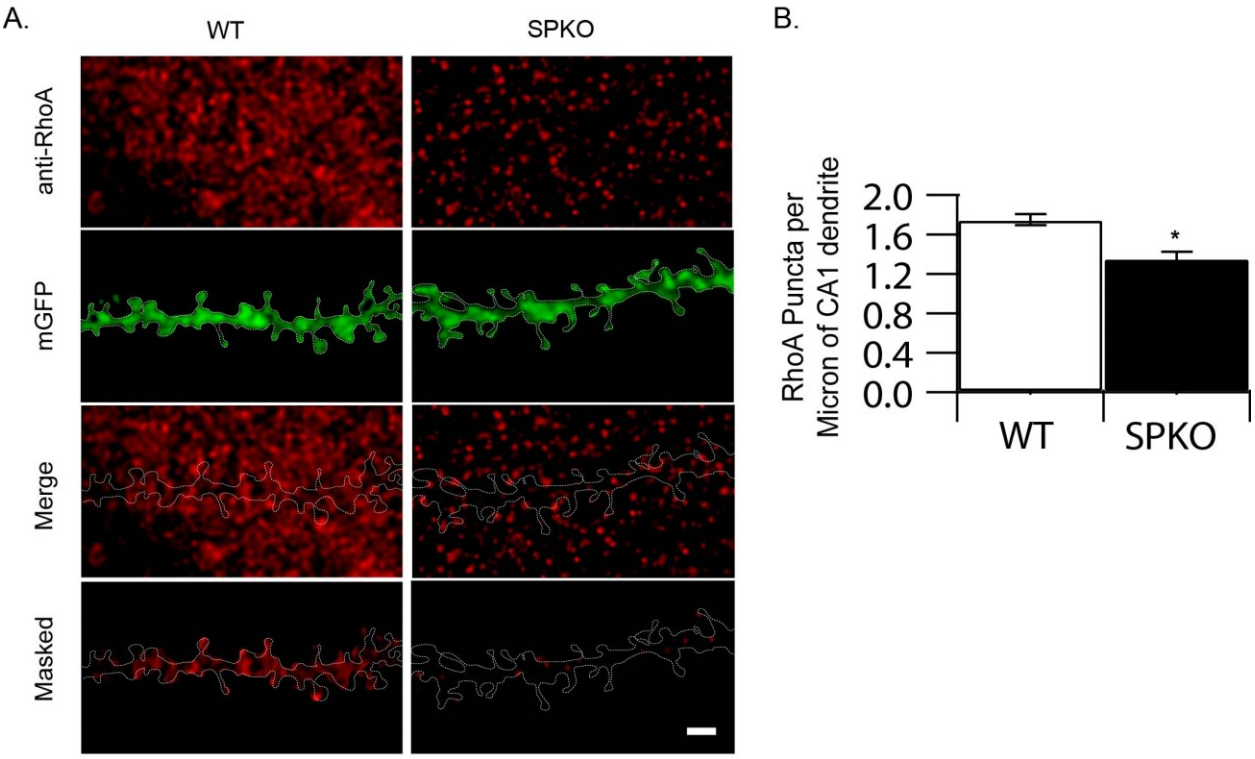
A. Western blot of RhoA, Cdc42, and Rac1 expression in WT female mice compared to male. **B.** There is no significant change in RhoA expression in wild type male and female hippocampi (Male: 1.04 ± 0.09 A.U.; Female: 1.25 ± 0.02 A.U.). **C.** There is a significant increase in Cdc42 expression in WT female hippocampi compared to WT male hippocampi (Male: 0.10 ± 0.02 A.U.; Female: 0.78 ± 0.08 A.U., $p<0.001$). **D.** No change is observed in Rac-1 (Male: 0.87 ± 0.07 A.U.; Female: 0.82 ± 0.16 A.U.). β-tubulin was used as a total protein loading control; *$p<0.05$. Note: averages are mean ± SEM

Figure 8. Decreased RhoA expression in Synaptopodin-KO organotypic slice cultures compared to WT



Western blot of RhoA and Rac1 expression in SPKO organotypic slice cultures compared to WT. **A – B.** Quantification reveals that there is a significant decrease in RhoA expression in the SPKO hippocampi compared to WT WT: 1.55 ± 0.08 A.U.; SPKO: 0.86 ± 0.08 A.U., $p<0.01$). **C – D.** No change is observed in Rac-1 (WT: 0.83 ± 0.07 A.U.; SPKO: 0.96 ± 0.02 A.U.). β-tubulin was used as a total protein loading control; *$p<0.05$. Note: averages are mean ± SEM

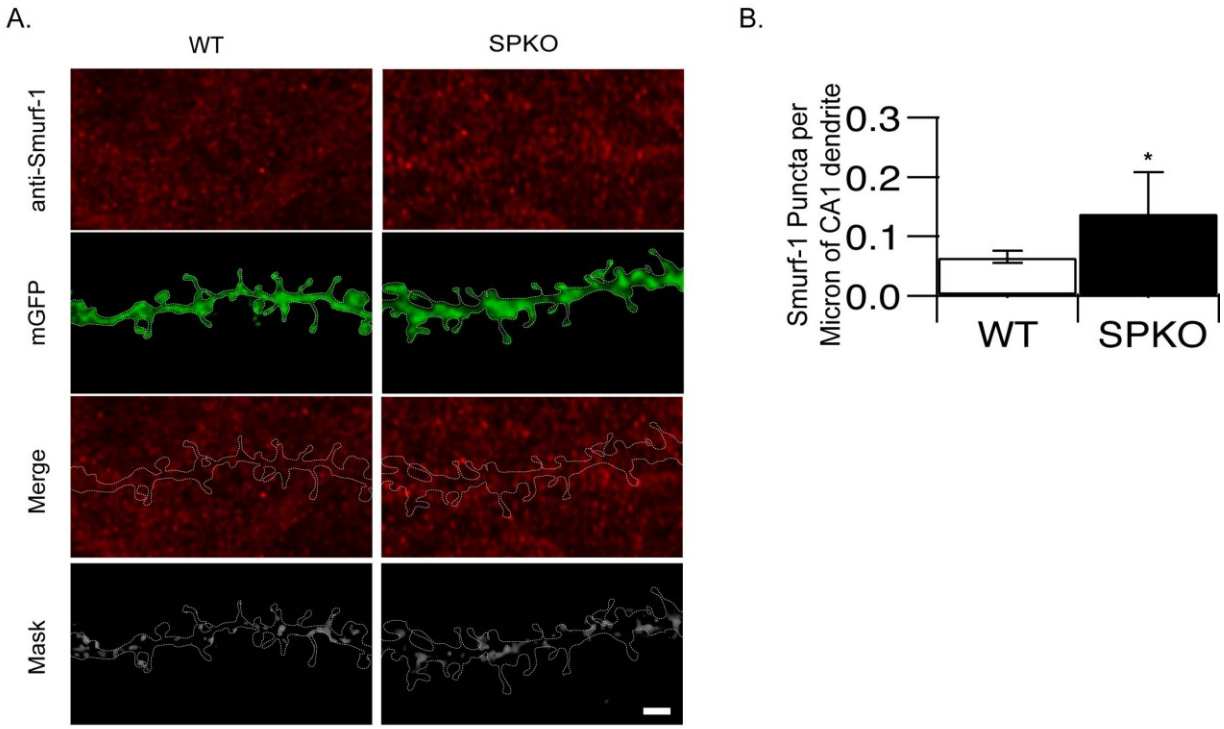
Figure 9. Reduced number of immunostained RhoA puncta in synaptopodin-KO organotypic slice cultures



A. Immunostaining and image analysis for RhoA in WT and SPKO slice cultures. Example images of WT and SPKO immunostained RhoA within GFP expressing CA1 tertiary dendrites in organotypic slice cultures. The green channel was used to mask the immunostained red channel in order to colocalize the RhoA-positive puncta found within the dendrite (Masked).

B. Quantification revealed that there was a reduction in RhoA in SPKO cultures (1.3659 ± 0.045 puncta/µm of dendrite; n = 16 dendrites from 4 CA1 cells) when compared to WT (1.750 ± 0.058 puncta/µm of dendrite; n = 18 dendrites from 4 CA1 cells). ** $p<0.001$, independent students' t-test, two tailed. Note: averages are mean ± SEM

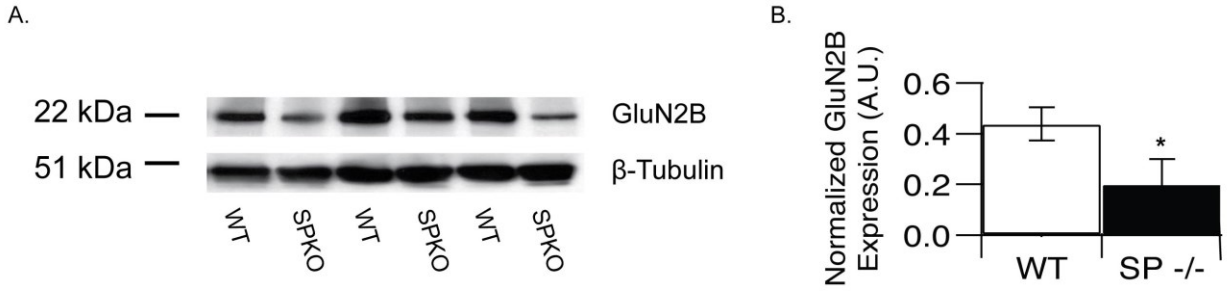
Figure 10. Smurf1 shows elevated expression in synaptopodin-KO organotypic slice cultures



A. Immunostaining and image analysis for Smurf1 in L15 slice cultures. Example images of wild type and SPKO immunostained Smurf1 and CA1 tertiary dendrites in organotypic slice cultures. The green channel was used to mask the immunostained red channel in order to colocalize the Smurf1-positive puncta found within the dendrite (Masked).

B. Quantification revealed that there was an elevation in Smurf1 in SPKO cultures (0.140 ± 0.038 puncta/µm of dendrite; n = 19 dendrites from 8 CA1 cells) when compared to WT (0.066 puncta/µm of dendrite; n = 23 dendrites from 8 CA1 cells). * $p<0.05$, independent students' t-test, two tailed. Note averages are mean ± SEM

Figure 11. Reduced GluN2B expression in synaptopodin-KO adult mouse hippocampi compared to WT



A. Western blot of GluN2B expression in adult SPKO mouse hippocampi slice cultures compared to WT. B. Quantification reveals that there is a significant decrease in GluN2B expression in the SPKO hippocampi compared to WT. β-tubulin was used as a total protein loading control; *$p<0.05$

Table 1.1 Normalized dendritic spine volume from CA1 pyramidal neurons in WT or SPKO slices during the first 10 minutes of baseline imaging and after between 35 and 45 minutes post-chemLTP induction. (*n= number of spines; l = number of CA1 pyramidal neurons; s = number of slices)*

	WT			SPKO		
	Mushroom Spines	**Thin Spines**	**All Spines**	**Mushroom Spines**	**Thin Spines**	**All Spines**
Baseline	1.01 ± 0.01	0.98 ± 0.01	1.00 ± 0.01	1.00 ± 0.02	0.98 ± 0.02	0.99 ± 0.01
Post-chemLTP Induction	1.15 ± 0.03	1.27 ± 0.07	1.20 ± 0.04	0.94 ± 0.05	0.97 ± 0.02	0.96 ± 0.03
n	33	33	66	18	36	54
l	9	9	18	8	8	16
s	9	9	18	8	8	16

Table 1.2 Number of spines from WT or SPKO CA1 pyramidal neurons that either increased or decreased in volume from between 35 and 45 minutes post-chemLTP induction compared to the first 10 minutes of baseline imaging

	WT	SPKO
Number of increased spines	40	21
Number of decreased spines	26	32
Chi square statistic	5.1804	
P-value	p <0.05	

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