SYNAPTOPODIN DRIVES HOMEOSTATIC UPSCALING THROUGH REGULATION OF AMPARS AND PHOSPHORYLATION OF CAMKII

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ABSTRACT

Synaptopodin (SP) is a plasticity related protein found in a subset of dendritic spines. It is essential for the formation of endoplasmic reticulum (ER) like structure, the spine apparatus found in spines. SP regulates calcium dependent accumulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), functions in local protein synthesis and its loss impairs learning and memory. Reductions in SP are implicated in many neurodegenerative diseases including Alzheimer's and Parkinson's Disease.

Interestingly, SP deficient neurons do not undergo synaptic scaling (SS), a type of plasticity that maintains the stability of neuronal activity during perturbations. They are unable to upregulate synaptic strength when challenged with chronic activity deprivation or denervation. Induction of SS by chronic inactivity typically causes the inflammatory cytokine, tumor necrosis factor α (TNFα), to stimulate the upregulation of AMPARs to the surface. In the absence of SP, TNFα does not have a stimulatory effect on SS. Despite being a critical mediator of SS, the mechanism through which SP operates in this plasticity is not well understood. Thus, we sought to understand the mechanistic role of SP in SS induced by chronic inactivity. We induced scaling by treating wildtype (WT) and synaptopodin knockout (SPKO) organotypic hippocampal cultures (*DIV* 11-14) with tetrodotoxin (TTX, 1μM) for 2-3 days or TNFα (1μg/ml) for 1-2 hours *(DIV* 11-18). TNFα receptor 1 and members of the TNFα transcriptional pathway were evaluated using western blot and nuclear fractionation. The functionality of the transcriptional pathway led us to investigate the mammalian target of rapamycin (mTOR) translational pathway which is also activated by TNFα. Results from qRT-PCR and western blots showed that loss of SP does not impair TNFα's ability to stimulate mTOR dependent translation. Despite TNFα's ability to stimulate general

translation, total levels of GluA1 and GluA2 AMPARs were not elevated in SPKO neurons. Phosphorylation of serine residues 845 and 831 on GluA1, which are implicated in the trafficking of AMPARS, were unaffected by absence of SP. The kinase involved in anchoring GluA1 AMPARs, Ca^{2+}/calmodulin dependent kinase II (CAMKII), had reduced phosphorylation at threonine 286/287 in SPKO neurons during SS. The reductions in CAMKII phosphorylation were coupled with impairments in the surface accumulation of GluA1 AMPARs. Taken together, the results center SP in the regulation of AMPARs and CAMKII in SS. They suggest that SP operates in scaling through phosphorylation of CAMKII and modulation of AMPARs production. The findings from this study contribute to the evolving literature on synaptic scaling. They unveil the role of SP in SS and expand our understanding of the mechanisms underlying homeostatic plasticity.

RÉSUMÉ

La synaptopodine (SP) est une protéine liée à la plasticité, située dans un sous-ensemble d'épines dendritiques. Elle est essentielle pour la formation d'une structure de type réticulum endoplasmique (RE), l'appareil de la colonne vertébrale trouvée dans les épines dendritiques. Cette protéine, SP, est impliqué dans l'accumulation des récepteurs de l'acide α-amino-3-hydroxy-5- méthyl-4-isoxazolepropionique (AMPAR), la synthèse locale des protéines et sa perte mène à des troubles d'apprentissage et à de la mémoire. La réduction de la SP est également impliquée dans les maladies neurodégénératives, notamment Alzheimer et Parkinson.

Fait intéressant, les neurones déficients en SP ont un déficit en plasticité synaptique homéostatique (SH), un type de plasticité qui maintient la stabilité de l'activité neuronale pendant les perturbations. Ils sont incapables de réguler positivement la force synaptique lorsqu'ils sont confrontés à un manque d'activité ou une dénervation d'activité chronique. L'induction de SH par un manque d'activité mené généralement la cytokine inflammatoire, facteur de nécrose tumorale α (TNFα), à stimuler la régulation positive des AMPAR à la surface. En l'absence de SP, le TNFα n'a pas d'effet stimulant sur les SH. Bien qu'il soit un médiateur critique de SH, nous en savons peux sur le mécanisme par lequel la SP opère dans cette plasticité. Ainsi, nous avons cherché à comprendre le rôle de SP dans les SH induits par la privation d'activité. Nous avons induit une inactivation des neurones en traitant des cultures hippocampes organotypiques contrôle (WT) et synaptopodine knockout (SPKO) (*DIV* 11-14) avec de la tétrodotoxine (TTX, 1μM) pendant 2-3 jours ou du TNFα (1μg / ml) pendant 1-2 heures (*DIV* 11-18). Le récepteur 1 du TNFα et les membres sous-jacents de la voie de transcription de TNFα ont été évalués en utilisant un transfert Western et un fractionnement nucléaire. La voie de transcription étant nous a amenés à cibler la voie de traduction génétique et la protéine mammifère la rapamycine (mTOR) qui est également

activée par le TNFα. Les résultats de la qRT-PCR et des western blots ont montré que la perte de SP n'altère pas la capacité du TNFα à stimuler la traduction génétique qui dépendante de mTOR. Malgré la capacité du TNFα à stimuler la traduction génétique globalement, les niveaux d'expression des facteurs GluA1 et GluA2 AMPARs n'étaient pas élevés dans les neurones SPKO. La phosphorylation des résidus de sérine 845 et 831 impliqués dans le trafic de GluA1 n'a pas été affectée par l'absence de SP. La kinase impliquée dans l'ancrage des GluA1 AMPAR, Ca^{2+} / calmoduline kinase II dépendante (CAMKII), avait réduit la phosphorylation à la thréonine 286/287 dans les neurones SPKO pendant la SH. Les réductions de la phosphorylation de CAMKII ont été couplées à des altérations de l'accumulation de surface des GluA1 AMPAR. L'ensemble de ces résultats suggère un rôle de la SP dans la régulation des AMPAR et CAMKII dans SH. Ils suggèrent que la SP opère dans la plasticité synaptique homéostatique par la phosphorylation de CAMKII et la modulation de la production d'AMPARs. Les résultats de cette étude contribuent à l'évolution de la littérature sur la plasticité synaptique homéostatique. Ils dévoilent le rôle de la SP et élargissent notre compréhension des mécanismes sous-jacents à la plasticité synaptique homéostatique.

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AUTHOR CONTRIBUTIONS

The experiments presented in this thesis were devised by Dr. McKinney and myself. Experiments and figures presented in the Results and Discussion sections were produced by me. Jelena Popic guided me in the early stages of western blotting and Pei You Wu assisted me with western blots of phosphorylated GluA1. Electrophysiological results were generated Melanie Chan and Philip K.Y. Chang. All organotypic hippocampal cultures used for experimentation were prepared by Francois Charron.

TABLE OF ABBREVIATIONS

APV	(2R)-amino-5-phosphonovaleric acid
AMPARs	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
Ca^{2+}/CaM	Calcium Calmodulin
CaM	Calmodulin
CAMKII	Calcium/calmodulin dependent kinase II
CAMKIV	Calcium/calmodulin dependent kinase IV
CAMKK	Calcium/calmodulin-dependent protein kinase kinase
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
CNS	Central nervous system
CNQX	Cyanquixaline (6-cyano-7-nitroquinoxaline-2,3-dione)
EK1	Ethanolamine kinase 1
IκBα	Inhibitor of kappa-light-chain-enhancer of activated B cells α
IP3	Inositol 1,4,5-triphosphate
LTP	Long term potentiation
LTD	Long term depression
mEPSC	Miniature excitatory postsynaptic currents
mGluR	Metabotropic glutamate receptor
MD	Monocular deprivation
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NMDARs	N-methyl-D-aspartate receptors
PhTx	Philathotoxin-433
PI3K	Phosphoinositide-3-kinase
PKA	Protein Kinase A
PSD	Post synaptic density
RA	All-trans retinoic acid
RARα	Retinoic acid receptor α
RIP	Protein receptor interacting protein
SA	Spine apparatus
sER	Smooth endoplasmic reticulum
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SP	Synaptopodin
SPKO	Synaptopodin knockout
SS	Synaptic Scaling
SRF	Serum Response Factor
sTNFR1	Soluble TNFα receptor 1
TACE	Tumor necrosis factor-α-converting enzyme
TARPS	Transmembrane AMPAR regulatory protein subunits
TBS	Theta burst stimulation
TNFα	Tumour necrosis factor α
TRAF2	TNF receptor associated factor 2
TTX	Tetrodotoxin

CHAPTER 1

1.1 Dendritic Spines

The primary recipients of excitatory input in the central nervous system (CNS) are dendritic spines (Bourne and Harris, 2008, Bosch and Hayashi, 2012). Dendritic spines are micro-sized protrusions on the dendritic tree of excitatory neurons (Bourne and Harris, 2008, Bosch and Hayashi, 2012). They possess a head and a constricted neck that allows them to compartmentalize chemical and electrical signalling at individual synapses (Bourne and Harris, 2008, McKinney, 2010, Bosch and Hayashi, 2012). Dendritic spines are heterogenous in shape and size with the head to neck ratio the key classifier of the different subtypes (**Fig 1**). Mushroom spines possess large heads with wide necks; long, thin spines have small heads with thin necks and stubby spines have equal head to neck ratio (**Fig 1**; McKinney et al., 1999, Bourne and Harris, 2008, McKinney, 2010, Bosch and Hayashi, 2012). Long, thin spines predominate during development, but stabilization and maturity evolve them into mushroom type spines (Bourne and Harris, 2008, McKinney, 2010)

Mushroom spines have larger post synaptic densities (PSD) which are electron dense, disc-like structures located in a region of the spine head apposed to the presynaptic active zone (Harris et al., 1992, Matsuzaki et al., 2001, Bourne and Harris, 2008, Bosch and Hayashi, 2012,). The PSD is a protein matrix composed of a variety of molecules including neurotransmitter receptors, cytoskeletal scaffolding proteins, ion channels and signalling proteins (Kennedy, 1997, Bourne and Harris, 2008, McKinney, 2010). Majority of the proteins in the PSD like α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), N-methyl-D-aspartate receptors (NMDARs), and Ca^{2+}/calmodulin dependent kinase II (CAMKII) are involved in synaptic

transmission and activity dependent plasticity (McKinney, 2010, Nicoll and Roche, 2013, Fox and Stryker, 2017). The size of the PSD correlates with the size of the spine head (Harris et al., 1992, Matsuzaki et al., 2001, Bourne and Harris, 2008, McKinney, 2010, Bosch and Hayashi, 2012). Spines with big head size have larger PSDs and contain more functional, AMPA type glutamate receptors (Harris et al., 1992, Matsuzaki et al., 2001, Bourne and Harris, 2008, Kasai et al., 2010). As AMPARs are ionotropic glutamate receptors that mediate majority of fast excitatory synaptic currents (Chater and Goda, 2014, Fox and Stryker, 2017), large spines result in functionally stronger synapses (Harris et al., 1992, Matsuzaki et al., 2001). Thus, spine morphology is tightly associated with synaptic strength and function (Bourne and Harris, 2008, Bosch and Hayashi, 2012).

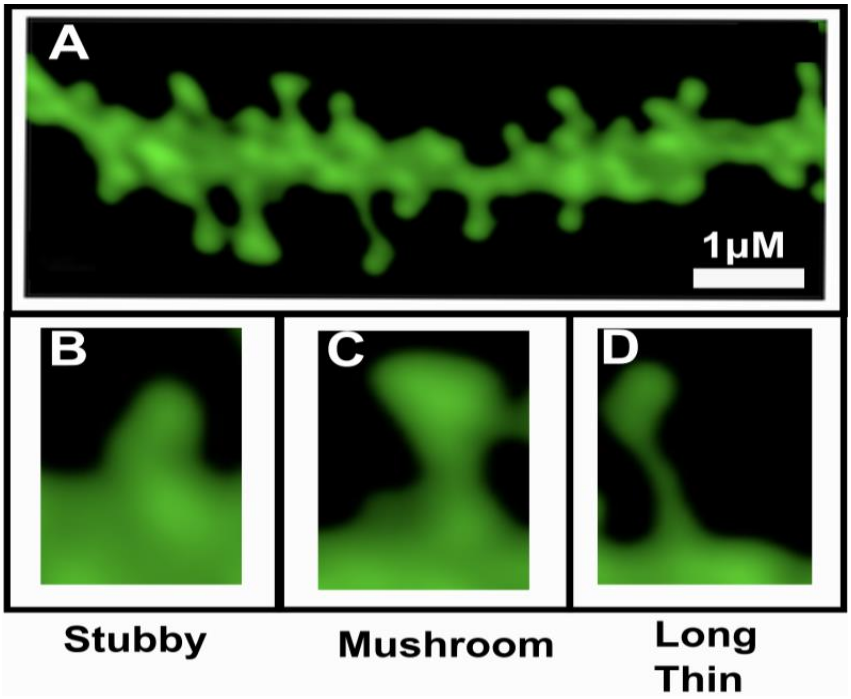

Figure 1. *Dendritic spines are heterogenous in shape and size.* A. A membrane tagged EGFP labelled CA1 pyramidal cell dendritic tree expressing a diversityof spine shapes. There are three main types of spines: B. Stubby spines have equal head to neck ratio C. Mushroom spines have large heads with wide necks D. Thin long spines have small heads and thin necks.

1.1.1 Dendritic spine remodelling of during Hebbian plasticity

Changes in synaptic strength is thought to underlie the remarkable ability of the brain to learn, form memories and store information (Nicoll and Roche, 2013, Fox and Stryker, 2017). The idea was first proposed by Ramon Cajal then later developed into a concrete synaptic model by Donald Hebb (Nicoll and Roche, 2013). In his model, Hebb postulated that coincident presynaptic and postsynaptic activity leads to synaptic strengthening while desynchrony in presynaptic and postsynaptic activity result in synaptic weakening (Hebb 1949, Nicoll and Roche, 2013, Fox and Stryker, 2017). Thus, Hebbian plasticity, long-term potentiation (LTP) and long-term depression (LTD), refers to persistent changes in synaptic transmission due to synchronous or dyssynchronous activity between proximal cells (Malinow and Malenka, 2002, Nicoll and Roche, 2013, Fox and Stryker, 2017). The discovery that brief high frequency stimulation leads to long term potentiation of glutamatergic synapses by Bliss and Lomo was the first empirical evidence in support of Hebb's theory (Bliss and Lomo, 1973). Since Bliss and Lomo, substantial evidence has accrued in support of Hebbian plasticity and its link to memory formation, making it the most compelling model for learning and memory to date (Malinow and Malenka, 2002, Nicoll and Roche, 2013, Fox and Stryker, 2017).

At the synaptic level, Hebbian plasticity results in the structural remodelling of dendritic spines (Matsuzaki et al., 2004, Kasai et al., 2010, Nicoll and Roche, 2013). Hebbian plasticity alters the size, shape and number of dendritic spines (Bourne and Harris, 2008, Patterson and Yasuda, 2011, Okamoto et al., 2004). Enlargement of spines is induced by LTP paradigms while shrinkage of spines is associated with LTD (Matsuzaki et al., 2004, Zhou et al., 2004, Okamoto et al., 2004, Bosch and Hayashi, 2012, Kasai et al., 2010, Patterson and Yasuda, 2011). The

emergence of new spines as well as an increase in the number of bifurcated spines has also been reported to occur with LTP (Engert and Bonhoeffer, 1999, Bourne and Harris, 2008). Hebbian plasticity is synapse specific and using glutamate uncaging, Matsuzaki and colleagues (2004) revealed that structural plasticity also occurs at individual synapses. They found that uncaging glutamate at individual spines led to a transient enlargement in mushroom spines and persistent increase in thin spines (Matsuzaki et al., 2004). The enhancement of spine volume exhibited properties of N-methyl-D-aspartate (NMDA) dependent LTP including a dependency on NMDAR and CAMKII (Matsuzaki et al., 2004). The rapid time course of the enlargement also corresponded with LTP induction, leading structural plasticity to be considered the morphological correlate of memory (Matsuzaki et al., 2004, Kasai et al., 2010, Patterson and Yasuda, 2011).

1.1.2 Actin Dynamics in Spines

Structural plasticity of spines is impaired by compounds that disrupt actin (Engert and Bonhoeffer, 1999, Star et al., 2002). Spines contain high levels of F-actin which function as both a structural framework and a regulator of spine dynamics (Harris et al., 1992, Matus, 2000, Okamoto et al., 2004). Actin filaments can be stable with a specified length or dynamic where it undergoes constant elongation and shrinkage (Star et al., 2002). This duality of actin enables spines to be dynamic, motile structures that can persist for hours or undergo morphological changes in a matter of minutes (Harris et al., 1992, Matus, 2000, Star et al., 2002, Okamoto et al., 2004). Polymerization of actin filaments result in enlargement of spine head size while depolymerization lead to its shrinkage (Kim and Lisman, 1999, Matus, 2000, Star et al., 2002, Fukazawa et al., 2003, Zhou et al., 2004). The actin cytoskeleton is regulated by actin binding and actin associated proteins (Ethell and Pasquale, 2005, Bourne and Harris, 2008). *If the dynamism of spines is*

impacted by actin binding and actin associated proteins, then is there a population of these proteins unique to mature, stable spines?

1.2 Synaptopodin

Synaptopodin (SP) is an actin associated protein found in kidney podocytes and dendritic spines in the brain (Mundel et al., 1997, Deller et al., 2000). SP is present in a subset of dendritic spines in the olfactory bulb, striatum, cortex and hippocampus (Mundel et al., 1997, Deller et al., 2000, Bas Orth et al., 2005, Deller et al., 2007). The expression of SP occurs later in development (day 15) where it coincides with maturation of dendritic spines (Mundel et al., 1997, Deller et al., 2000, Bas Orth et al., 2005, Deller et al., 2007). SP is located in the necks of large, stable, mature spines (Deller et al., 2000, Bas Orth et al., 2005, Deller et al., 2007). This strategic positioning in spine necks allow SP to crosslink with the scaffolding protein, alpha-actinin, in order to elongate actin filaments and form parallel actin bundles (Asanuma et al., 2005, Kremerskothen et al., 2005). SP plays a role in stabilizing F-actin and drugs that depolymerize actin filaments also disrupt the distribution of SP *in-vivo* (Asanuma et al., 2005, Kremerskothen et al., 2005, Deller et al., 2007). Interestingly, SP containing spines are resistant to morphological changes caused by drugs that disrupt actin polymerization (Okubo-Suzuki et al., 2008). Through its interaction with actin, SP contributes to the maintenance of spine structure and links the actin cytoskeleton to synaptic membrane proteins.

SP links actin to intracellular calcium stores through its association with the spine apparatus (SA) (Segal et al., 2010, Korkotian and Segal, 2011, Korkotian et al., 2014). The SA is an enigmatic structure first discovered in cortical neurons in 1959 (Gray, 1959) by electron microscopy then later in hippocampal neurons (Hamlyn, 1962). It's a structure composed of stacks

of smooth endoplasmic reticulum (sER) with electron dense plates (Gray, 1959, Harris et al., 1992). The SA contains cisterns of calcium making it a source and regulator of intracellular calcium stores (Fifkova et al 1983, Jedlicka and Deller, 2017). It is also hypothesized to be involved in local protein synthesis and post translational modifications of proteins (Pierce et al., 2000; Steward and Schuman, 2001). Just like SP, SA is found in larger, mature, mushroom type spines (Deller et al 2000; 2003, Segal et al., 2010, Vlachos, 2012). In fact, the distribution of SA corresponds to the distribution of SP in the brain (Deller et al 2000, Bas Orth et al., 2005). In the cerebellum where SP is not expressed, SA is also absent (Deller et al 2003). The two proteins colocalize together in hippocampal dendritic spines and the absence of SP results in the loss of SA (Deller et al 2000, Bas Orth et al., 2005). Although the expression of SA is dependent on SP, SP can be found without SA (Deller et al 2000). In the axon initial segment, SP associates with the cisternal organelle, a structure comprised of stacks of sER with interdigitating plates of electron-dense material (Benedeczky et al., 1994, Bas Orth et al., 2007). The cisternal organelle is structurally similar to SA and its expression is also dependent on SP (Bas Orth et al., 2007). The functions of the cisternal organelle are still under investigation however its localization with sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) type calcium pumps argue for its role in calcium regulation like SA (Benedeczky et al., 1994).

1.2.1 Synaptopodin in structural plasticity

SP's strategic localization with SA, a source and regulator of intracellular calcium stores, suggest it functions in plasticity. Indeed, SP is a critical mediator of structural plasticity in spines (Okubo-Suzuki et al., 2008; Vlachos et al., 2009; Verbich et al., 2016). SP containing spines are able to increase spine volume in response to local release of glutamate from flash photolysis

(Vlachos et al., 2009). Overexpressing SP maintains the activity induced enlargement of spine volume (Okubo-Suzuki et al., 2008) and enhances reactivity to glutamate (Vlachos et al., 2009). Activating NMDARs also results in increased spine volume and a rise in GluA1 in SP positive spine heads (Okubo-Suzuki et al., 2008; Vlachos et al., 2009; Zhang et al., 2013). Using short hairpin RNA (shRNA) to knockdown SP abolishes both the increase in spine volume and GluA1's presence in spine heads (Vlachos et al., 2009).

SP's role in structural plasticity in spines has not only been attributed to its ability to regulate actin dynamics but it has also been linked with its capacity modulate intracellular calcium (Okubo-Suzuki et al., 2008; Vlachos et al., 2009; Korkotian et al., 2014). SP colocalizes with ryanodine receptors, one of the receptors that regulate the release of calcium from intracellular stores (Vlachos et al., 2009; Segal et al., 2010). Ryanodine receptors exist in dendrites and spines with the primary function of amplifying calcium transients through calcium induced calcium release (Vlachos et al., 2009, Korkotian et al., 2014). Loss of SP elicits reductions in the accumulation of ryanodine in spines. Unsurprisingly, intracellular calcium transients in SP lacking spines are smaller and do not exhibit the prolonged calcium decay present in SP positive spines (Korkotian and Segal, 2011; Korkotian et al., 2014; Jedlicka and Deller, 2017). Spines with large calcium transients increase spine volume and produce greater postsynaptic responses to glutamate (Korkotian et al., 2014). They are also able to induce an upregulation of AMPARs in spines (Vlachos et al., 2009), making them more plastic than their SP lacking counterparts. SP's regulation of spine dynamics is dependent on its association with internal stores as depleting internal calcium stores abolish volume expansion in spines endowed with SP (Vlachos et al., 2009; Korkotian and Segal, 2011; Korkotian et al., 2014). It also impairs the plastic properties associated with SP positive spines. Delivery of GluA1 AMPARs is supressed by inhibition of ryanodine

receptors and abolished by blockade of intracellular calcium stores (Vlachos et al., 2009, Korkotian et al., 2014). *SP is evidently necessary for calcium dependent accumulation of AMPARs at individual spines, but does it influence plasticity on a global, network level?*

1.2.2 Synaptopodin in functional plasticity: Hebbian plasticity

Although present in only a subset of spines, the presence of SP is important for learning and memory (Deller et al 2003, Jedlicka et al., 2009, Zhang et al. 2013). Cellular models of learning induce the upregulation of SP mRNA in the hippocampus (Yamazaki et al., 2001; Fukazawa et al., 2003). Likewise, novel environments which stimulate learning and memory mechanisms enhance SP mRNA expression in mature dentate gyrus' granule neurons (Paul et al. 2019). The absence of SP impairs learning at both cellular and behavioral levels. SP deficient animals have increased error rates when performing spatial learning tasks (Deller et al 2003). They also have a reduced rate of LTP induced *in-vitro* by both theta burst stimulation (TBS) and tetanic stimulation at CA3-CA1 synapses (Deller et al 2003). *In-vivo* at dentate gyrus granule neurons, LTP impairment is only observed with TBS (Jedlicka et al., 2009). Loss of SP does not impact the expression of tetanus induced LTP (Jedlicka et al., 2009). In comparison to tetanic stimulation, TBS is a stronger stimulation that evokes physiological calcium dynamics (Jedlicka et al., 2009). TBS is sensitive to disruptions in actin dynamics and it mimics hippocampal activity during exploratory behavior in animals (Deller et al 2003, Jedlicka et al., 2009). Thus, the finding that TBS LTP is impaired in SPKO animals corresponds with SP's involvement in actin dynamics and calcium regulation. It also supports behavioral findings that SPKO animals have defective spatial learning (Jedlicka et al., 2009). Another puzzling observation is that LTP (TBS LTP) is defective in young but not adult animals (Zhang et al. 2013). This observation does not only contrast

previous findings that SP is necessary for LTP in adult animals (Deller et al 2003, Jedlicka et al., 2009), but it also brings to light our lack of knowledge about SP's role in LTP

In comparison to LTP, less is known about the necessity of SP for LTD. While one study has shown that SP is not required for NMDA dependent LTD (Zhang et al. 2013), evidence suggests that it may play a role in metabotropic glutamate receptor (mGluR) dependent LTD (Holbro et al., 2009). In a comparative study of ER containing spines against non-ER spines, it was discovered that 78% of ER containing spines possessed SP (Holbro et al., 2009). This group of ER containing spines were able to elicit mGluR LTD. The mGluR LTD could be blocked by inhibitors of inositol 1,4,5-triphosphate (IP3) receptors indicating the necessity of calcium release from IP3 receptors (Holbro et al., 2009). Although the relationship between SP and IP3 receptors is unknown, the fact that majority of spines that mediated this type of plasticity possess SP, a calcium regulator, suggests a potential role for SP in mGluR dependent LTD. The fact that SP is involved in NMDA dependent LTP and potentially has a role in mGluR LTD brings into question SP's role in other forms of plasticity such as synaptic scaling (SS). *Is SP involved in SS and if it is, does it regulate it in a calcium dependent manner?*

1.3 Synaptic Scaling

SP is an essential component of machinery that elicits synaptic plasticity. It regulates calcium dependent accumulation of AMPARs (Vlachos et al., 2009; Korkotian et al., 2014), and its loss impairs learning and memory (Deller et al 2003, Jedlicka et al., 2009, Zhang et al. 2013). Despite being an important component of the plasticity machinery, the mechanism through which SP causes its effects is not well understood. Thus, examining its involvement in homeostatic

plasticity, another type of synaptic plasticity that is dependent on AMPARs, presents as a critical avenue to understanding SP's role in plasticity.

Homeostatic plasticity is a set of mechanisms that maintains neuronal activity at a dynamic range during perturbations (Turrigiano et al., 1998). It has been postulated to counter the destabilizing effects that can occur if Hebbian mechanisms are left uncontrolled (Turrigiano et al., 1998;Turrigiano, 2008, 2017; Fox and Stryker, 2017). Neural circuits can be stabilized during perturbations by modulating synapse number, synapse strength, excitability, neurotransmitter release probability, and neurotransmitter receptor content (Fernandes and Carvalho, 2016; Chowdhury and Hell, 2018). An extensively studied form of homeostatic plasticity is synaptic scaling (SS), a mechanism for regulating neuronal firing rate at a functional range during perturbations. Synaptic scaling maintains overall firing rates of neurons by making compensatory changes to synaptic strength (Turrigiano et al., 1998). Reductions in activity trigger SS to induce increases in excitatory drive while hyperactivity leads to decreases in synaptic strength (Turrigiano et al., 1998). Synaptic scaling adjusts synapses multiplicatively by a common factor in order to regulate total synaptic drive while maintaining the relative strength of individual synapses and synaptic inputs (Turrigiano et al., 1998, Fernandes and Carvalho, 2016; Chowdhury and Hell, 2018). The timescale of detection, integration and modification of neuronal activity by SS is slow; typically occurring over the course of a day to several days (Leslie et al., 2001).

Dr Turriagiano's group was the first to report SS. They treated neocortical neurons with the Na^+ channel blocker tetrodotoxin (TTX) for 48 hours to reduce action potential firing and observed an increase in the amplitude of AMPA mediated miniature excitatory postsynaptic currents (mEPSCs) after the treatment (Turrigiano et al., 1998). Conversely, increasing neuronal firing by blocking $GABA_A$ receptors resulted in a reduction in the amplitude of mEPSCs

(Turrigiano et al., 1998). Miniature excitatory postsynaptic currents are caused by spontaneous release of glutamatergic vesicles at individual synapses, therefore, increases in mEPSC represent strengthening of a synapse while reductions of mEPSC are indicative of a weakening synapse (Stellwagen, 2011). Chronic inactivity followed by compensatory increases in synaptic strength has subsequently been termed homeostatic upscaling and hyperactivity followed by a synaptic depression is called homeostatic downscaling. The following body of work will be focused primarily on homeostatic upscaling.

1.3.1 AMPAR Composition in Synaptic Scaling

The changes in postsynaptic strength stimulated by SS is due to the accumulation or depletion of AMPARs at excitatory synapses (Turrigiano et al., 1998). AMPARs are ionotropic glutamate receptors that mediate majority of fast excitatory synaptic currents (Harris, 1999; Shepherd and Huganir, 2007). They exist in a tetrameric complex composed of GluA1-4 subunits (Shepherd and Huganir, 2007; Chater and Goda, 2014). The adult brain expresses GluA1-3 while GluA4 is expressed primarily during development (Shepherd and Huganir, 2007). In the hippocampus, majority of AMPARs are heteromers of GluA1/GluA2 or GluA2/GluA3(Harris, 1999; Lu et al., 2014). The subunit composition of AMPARs determines trafficking dynamics, channel properties, gating kinetics as well as calcium permeability (Greger et al., 2003; Lu et al., 2014; Chowdhury and Hell, 2018). While GluA1 flux calcium and dominates during activity dependent exocytosis, GluA2 containing AMPARs prevent calcium permeability and predominate during activity-dependent endocytosis (Greger et al., 2003; Lu et al., 2014; Pozo and Goda, 2010; Pick and Ziff, 2018).

Synaptic scaling triggers changes in subunit composition and abundance of AMPARs at the synapse. Several *in-vitro* (Aoto et al., 2008; Soares et al., 2013; Stellwagen et al., 2005) and *in-vivo* studies (Goel et al., 2006; Goel and Lee, 2007) have reported the exclusive involvement of calcium permeable GluA1 AMPARs with little change to GluA2 containing AMPARs in inactivity induced SS. These reports are challenged by other studies that have observed changes in only GluA2 during inactivity induced SS or concurrent changes in both subunits (Gainey et al., 2009; Hou et al., 2008; Sutton et al., 2006). One study using a knockdown approach has also observed that there are no subunit requirements during scaling (Altimimi and Stellwagen, 2013). The discrepancy in results can be attributed to different experimental conditions and diversity in scaling paradigms (Fernandes and Carvalho, 2016). Inactivity induced SS can be developed in an *in-vitro* system through 48 hours treatment with TTX, competitive AMPAR antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), entorhinal cortex lesion or 24 hours treatment with TTX in conjunction with competitive NMDAR antagonist, (2R)-amino-5-phosphonovaleric acid (APV; Turrigiano et al., 1998; Vlachos et al., 2013; Chen et al., 2014; Fernandes and Carvalho, 2016). *In-vivo* scaling has been studied predominately in the visual cortex using several days of monocular (MD) or binocular activity deprivation (Desai et al., 2002; Kaneko et al., 2008; Gainey et al., 2009). The different scaling paradigms can trigger distinct types of SS that result in differential regulation of AMPAR subunits (Fernandes and Carvalho, 2016). In fact, the induction protocol involving 24 hours of TTX with APV has been shown to operate through local translation and synaptic insertion of homomeric GluA1 AMPARs (Aoto et al., 2008; Maghsoodi et al., 2008). Alternatively, the different results have been proposed to reflect an LTP like process where GluA1 containing AMPARs are inserted first then later replaced by GluA2 containing AMPARs (Fernandes and Carvalho, 2016).

1.3.2 AMPAR Trafficking in Synaptic Scaling

Newly synthesized AMPARs get to the surface through phosphorylation and dephosphorylation of key serine residues. Synaptic trafficking of GluA1 AMPARs is facilitated by phosphorylation of S845 and S831 by protein kinase A (PKA) and CAMKII respectively (Louros et al., 2018a; Malinow and Malenka, 2002; Shepherd and Huganir, 2007). Dephosphorylation of these key residues on the other hand promote AMPAR internalization (Louros et al., 2018a; Malinow and Malenka, 2002; Shepherd and Huganir, 2007). In homeostatic upscaling, while phosphorylation of GluA1 S845 is necessary, phosphorylation of GluA1 S831 is not required (Diering et al., 2014, Louros et al., 2014, 2018, Sanderson et al., 2018). Silencing phosphorylation using GluA1 S845A mutant prevents the rise in synaptic AMPARs during TTX treatment (Diering et al., 2014; Sanderson et al., 2018). In contrast, GluA1 S831A mutant successfully undergoes synaptic upscaling, indicating that S831 phosphorylation is not a requirement for this process. (Diering et al., 2014)

CAMKII, the multifunctional kinase that phosphorylates S831 plays a pivotal role in SS even though there is no requirement for S831 phosphorylation (Groth et al., 2011; Thiagarajan et al., 2002). CAMKII is a dodecameric protein consisting of various subunits of which the α and β subunits are predominant in the brain (Okamoto et al., 2007; Kim et al., 2016). The β subunit stabilizes the actin cytoskeleton while the α subunit modulates neurotransmission through its kinase activity (Okamoto et al., 2007). Activation of CAMKII occurs through the binding of calcium to calmodulin (Ca^{2+}/CaM) which leads to its autophosphorylation at Thr286 in the α subunit and Thr287 in β subunit (Okamoto et al., 2007; Kim et al., 2016). Autophosphorylation of the α and β subunits allow activation of the kinase to outlast the calcium signal (Okamoto et al.,

2007; Kim et al., 2016) and also stimulate translocation of CAMKII into the PSD (Strack et al., 1997). CAMKII's response to calcium transients is dependent on the ratio of α/β subunits in the complex (Groth et al., 2011; Thiagarajan et al., 2002). The β subunit is attuned to lower levels of calcium therefore; it is not surprising that reduced activity triggers higher expression of CAMKIIβ while hyperactivity upregulates CAMKIIα (Groth et al., 2011; Thiagarajan et al., 2002). Blocking CAMKII using selective inhibitor, KN93 prevents the changes in AMPARs that occur during upscaling and downscaling (Groth et al., 2011). Knocking down CAMKIIβ using lentivirus also elicits the same results indicating that β subunit dominates during SS (Groth et al., 2011).

AMPARs at the surface diffuse freely between synaptic and extrasynaptic sites; they become stabilized at the synapse during processes that require enhanced AMPA mediated neurotransmission (Opazo et al., 2010; Wang et al., 2012; Lu et al., 2014). AMPARs are anchored to the synapse through interactions with transmembrane AMPAR regulatory protein subunits (TARPS) and various scaffolding proteins including postsynaptic density 95 (PSD95) (Bats et al., 2007; Schnell et al., 2002; Sun and Turrigiano, 2011). PSD95 is a member of the membrane associated guanylate kinase family that acts as a slot protein for AMPARs (Schnell et al., 2002; Bats et al., 2007; Sun and Turrigiano, 2011). PSD95 promotes the clustering of AMPARs through binding with stargazin (Schnell et al., 2002; Bats et al., 2007). Stargazin is a TARP that regulates the diffusion of AMPARs to synaptic sites (Louros et al., 2014, 2018b). Stargazin is phosphorylated by oligomeric CAMKII, leading to its binding to PSD95 and the synaptic trapping of AMPARs (Chater and Goda, 2014; Kim et al., 2016; Louros et al., 2018). In SS stargazin is the dominant TARP; phosphorylation of stargazin by CAMKII is increased by activity deprivation and its dephosphorylation is induced by hyperexcitability (Louros et al., 2014, 2018b). The dephosphorylation of stargazin results in a reduction in its association with PSD95 and

subsequently greater mobility of synaptic AMPARs (Louros et al., 2018) Abundance of PSD95 is also increased by activity blockade and reduced by hyperactivity (Sun and Turrigiano, 2011).

1.3.3 The role of Calcium in Synaptic Scaling

Neuronal activity levels are highly correlated with intracellular calcium levels (Harris, 1999; Turrigiano et al. 1998); therefore, it has been proposed that SS is facilitated by calcium-dependent sensors that can detect changes in calcium levels and trigger series of events that lead to AMPAR trafficking (Chater and Goda, 2014). Indeed, several studies have reported drops in calcium levels following inactivity induced scaling (Ibata et al., 2008; Thiagarajan et al., 2005). Declines in calcium influx are observed when neurons are perfused with TTX and likewise, blockade of calcium transients result in accumulation of AMPARs similar to TTX treatment (Ibata et al., 2008). The decline in calcium influx after 4 hours of TTX treatment is followed by a reduction in activated Ca^{2+}/calmodulin dependent kinase IV (CAMKIV), one of the calcium dependent kinases (Ibata et al., 2008). CAMKIV is a serine threonine kinase that stimulates transcription through CREB phosphorylation (Ibata et al., 2008; Goold and Nicoll, 2010). In the cytoplasm, CAMKIV exists as an inactivated kinase that becomes active through rise of intracellular calcium and phosphorylation by Ca^{2+}/calmodulin kinase kinase (CaMKK) in the nucleus (Ibata et al., 2008; Goold and Nicoll, 2010). Inhibiting activity of CAMKK or using dominant negative CAMKIV (dnCAMKIV) for 24 hours recapitulates the effects of TTX on mEPSC amplitude (Ibata et al., 2008) . Conversely, transfecting a dnCAMKIV into neurons prevent homeostatic downscaling. Thus, CAMKIV is one of the few factors implicated in bidirectional regulation of scaling (Goold and Nicoll, 2010). The findings regarding CAMKIV are paradoxical given that activated CAMKIV leads to transcription, a requirement for SS. Most

likely, the decline in CAMKIV during chronic inactivity precedes transcription; functioning more as a calcium sensor during the earlier stages of scaling.

1.3.4 Transcription and Translation in Synaptic Scaling

In addition to modulating AMPAR trafficking, SS utilizes transcription and translation to regulate synthesis of new receptors. Receptor accumulation typical of activity blockade is abolished when transcription inhibitor, actinomycin or translation inhibitor, anisomycin, is used (Ibata et al 2008, Aoto et al 2008). The transcriptional machinery underlying SS is still unclear, although it has been proposed to be mediated by a CAMKIV dependent mechanism (Ibata et al 2008). Another group has also shown that the transcription factor SRF, and its cofactor EK1, are responsible for upregulating neuropaxin 1, a protein involved in clustering AMPARs at cell surface and whose absence prevents TTX induced scaling (Schaukowitch et al., 2017).

Unlike transcription, extensive work by the Chen lab has uncovered a mechanism through which SS induced by dual activity blockade leads to local translation of GluA2 lacking AMPARs (Aoto et al., 2008; Maghsoodi et al., 2008; Chen et al., 2014). In their experiments, a rapid form of SS is induced by simultaneous blockade of activity with TTX and APV. The dual activity blockade for 24 hours results in synthesis of all-trans retinoic acid (RA) in addition to the increase in AMPA mEPSC amplitude that occurs with prolonged TTX treatment alone (Aoto et al., 2008; Maghsoodi et al., 2008). RA induced SS is inhibited by cycloheximide and anisomycin, two protein synthesis inhibitors (Aoto et al., 2008; Maghsoodi et al., 2008; Chen et al., 2014). Production of RA leads to local translation of GluA1 AMPARs in a mammalian target of rapamycin (mTOR) independent manner (Aoto et al., 2008; Maghsoodi et al., 2008; Chen et al., 2014). Translation of GluA2 receptors were not investigated in the study however, the ability of philathotoxin-433 (PhTx), a blocker of GluA2 lacking AMPARs,

in reversing the increase in AMPA mediated synaptic activity after RA treatment indicates that RA stimulates the synthesis of only calcium permeable GluA1 AMPARs (Aoto et al., 2008).

RA is the critical mediator of this local translation mechanism, however, not all scaling paradigms are dependent on RA. Synaptic upscaling stimulated by TTX alone is not impaired when RA synthesis is blocked (Aoto et al., 2008). Scaling mediated by RA also does not utilize GluA2 containing AMPARs which have been found by several studies to be important for SS (Aoto et al., 2008). The discrepancy between RA dependent scaling and other scaling paradigms indicates that multiple mechanisms underlie SS. Synthesis of RA and local translation of GluA1 is stimulated to induce a rapid form of SS. The slower form of SS using TTX alone may employ other factors such as synaptopodin to facilitate local translation or may utilize a global translation mechanism. Further research is required to tease apart the different mechanisms and how they intersect.

1.4 Glia Derived TNFα in Excitatory Synaptic Scaling

Neurons were proposed to sense changes in their activity level in a cell autonomous manner, but non-autonomous modes (Beattie et al., 2002; Stellwagen and Malenka, 2006) have been discovered to be utilized during scaling. Cell non-autonomous modes utilize glia cells which have largely been viewed as the support cells of the CNS (Beattie et al., 2002; Stellwagen and Malenka, 2006). Glia cells can detect changes in neural activity and release gliotransmitters in response, making them ideal mediators of SS (Perea et al., 2014). A classic study by Beattie and colleagues (2002) found that chronic inactivity induces the release of tumour necrosis factor α (TNFα) from glia cells. The addition of exogenous TNFα elicited a rapid doubling of surface AMPAR receptors and a rise in mEPSC amplitude reminiscent of synaptic upscaling. The effect of exogenous TNFα

on surface AMPAR receptors was mimicked by incubation of neurons with astrocyte conditioned media, indicating that glia maybe the source of endogenous TNFα. Elimination of the effect of the conditioned media upon treatment with several TNFα inhibitors showed that it was TNFα in the media that was mediating the effect (Beattie et al., 2002). Neurons also produce TNFα, so to rule out the involvement of neuronal derived TNFα in the upregulation of AMPAR, a follow up study induced upscaling in neurons plated on glia with TNFα knocked out (Tnf$^{-/-}$) (Stellwagen and Malenka, 2006). Wildtype (WT) neurons plated on Tnf$^{-/-}$ glia were not able to undergo scaling with TTX treatment but Tnf$^{-/-}$ neurons plated on wildtype glia exhibited scaling with the same treatment (Stellwagen and Malenka, 2006). Glia derived TNFα is therefore not only sufficient to induce synaptic upscaling but it is also necessary for this process to occur. Interestingly, Tnf$^{-/-}$ slice cultures which do not have TNFα in either neurons or glia can reduce neuronal activity upon chronic hyperexcitability with picrotoxin, ($GABA_A$ receptor antagonist) indicating that other factors are needed to oppose hyperexcitability (Stellwagen and Malenka, 2006). A requirement for TNFα has also been found for SS in the visual cortex (Kaneko et al., 2008). Occluding visual input to one eye, monocular deprivation (MD), causes decreased responsiveness in the deprived eye and increased acuity in the open eye in control animals (Desai et al., 2002; Kaneko et al., 2008). TNFα knock out animals fail to undergo the subsequent increase in responsiveness in the open eye after MD (Kaneko et al., 2008). Even wildtype (WT) animals that exhibit this activity dependent plasticity cannot upregulate responsiveness in the open eye if TNFα signalling is abolished using the sTNFR1 during MD (Kaneko et al., 2008). Therefore, TNFα is not only necessary for upscaling in *in-vitro* but it is also relevant in *in-vivo* mechanisms of SS.

1.4.1 TNFα Signalling in Synaptic Scaling

How exactly does TNFα, an inflammatory cytokine known primarily for its role in systemic inflammation, modulate AMPAR trafficking? TNFα is a 17kDa transmembrane protein that is proteolytically cleaved by ADMA17/TACE into soluble form (MacEwan, 2002; Wajant et al., 2003). The soluble form is the biologically active form through which TNFα exerts most of its effects (Black et al., 1997). TNFα signals through TNFα receptor 1(TNFR1) and TNFα receptor 2 (TNFR2), the latter being widely expressed in the CNS and former limited to expression in endothelial and immune cells (Wajant et al., 2003). In homeostatic upscaling TNFR2 does not play a role since modulating its activity does not abolish TNFα's effect on AMPAR accumulation (Stellwagen et al., 2005; Stellwagen and Malenka, 2006). Stimulating TNFR1 activity on the other hand sufficiently induces AMPAR accumulation while inhibiting its activity eliminates synaptic upscaling (Stellwagen et al., 2005). The binding of TNFα to TNFR1 leads to recruitment of TNFR1 associated death domain protein (TRADD) and its binding partners adapter protein receptor interacting protein (RIP) and TNF receptor associated factor 2 (TRAF2) (Ozes et al., 1999; MacEwan, 2002). Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is subsequently activated leading to cytokine production, cell survival and proliferation depending on the cell type (Ozes et al., 1999; MacEwan, 2002). An apoptotic pathway can also be triggered if the TNFα-TNFR1 complex is internalized (MacEwan, 2002). TNFα does not operate through this classic pathway in homeostatic upscaling rather, it is dependent on the activation of phosphoinostide-3-kinase (PI3K) pathway (Stellwagen et al., 2005). PI3K is a signal transducer that phosphorylates many targets including itself. Activation of PI3K leads to AKT phosphorylation, degradation of inhibitory IκBα and stimulation of NFκB mediated transcription (**Fig.2**; Ozes et al., 1999). AKT phosphorylation also stimulates the mTOR translational pathway

(**Fig. 2**; Plaisance et al., 2008). In addition to phosphorylating AKT, PI3K can phosphorylate kinases involved in AMPAR surface trafficking including CAMKII (Joyal et al., 1997). Since treatment with PKA and CAMKII inhibitors did not impact TNFα dependent accumulation of AMPARs (Stellwagen and Malenka, 2006), TNFα likely leads to AMPAR surface trafficking through either the transcriptional or translational pathway. Although, the mechanism through which TNFα's mediates scaling has been partly elucidated, there remains questions about how TNFα interacts and cooperates with other scaling factors to evoke homeostasis. The finding that SP is required for TNFα to have a stimulatory effect on LTP (Maggio and Vlachos, 2018) suggest that TNFα's enhancing effects in SS is also SP dependent.

1.5 Synaptopodin in Synaptic Scaling

As an essential component of the machinery that control calcium dependent accumulation of AMPARs, SP is necessary for inactivity induced scaling. SP deficient CA1 neurons do not experience the increase in AMPA mediated mEPSC amplitude that occurs with chronic TTX treatment (**Fig 3**; unpublished data, McKinney lab). Likewise, deafferented dentate gyrus neurons which normally respond with an increase in postsynaptic strength 3-4 days after lesioning do not elicit this response if SP is knocked out (Vlachos et al., 2013). Transfecting SP back into SPKO neurons rescue the response to denervation, providing further evidence for the necessity of SP in this type of plasticity (Vlachos et al., 2013). Surprisingly, addition of exogenous TNFα does not rescue scaling in CA1 neurons (**Fig 4a-d**; unpublished data, McKinney lab) although a requirement for TNFα has been established in both *in-vivo* and *in-vitro* forms of scaling (Stellwagen et al., 2005; Stellwagen and Malenka, 2006; Kaneko et al., 2008). The findings suggest that SP is

necessary for TNFα to induce AMPAR accumulation during synaptic upscaling but the exact mechanism through which SP regulates synaptic upscaling is unknown. How SP modulates TNFα's signalling and orchestrates synaptic upscaling has not been examined and therefore the impetus for this thesis.

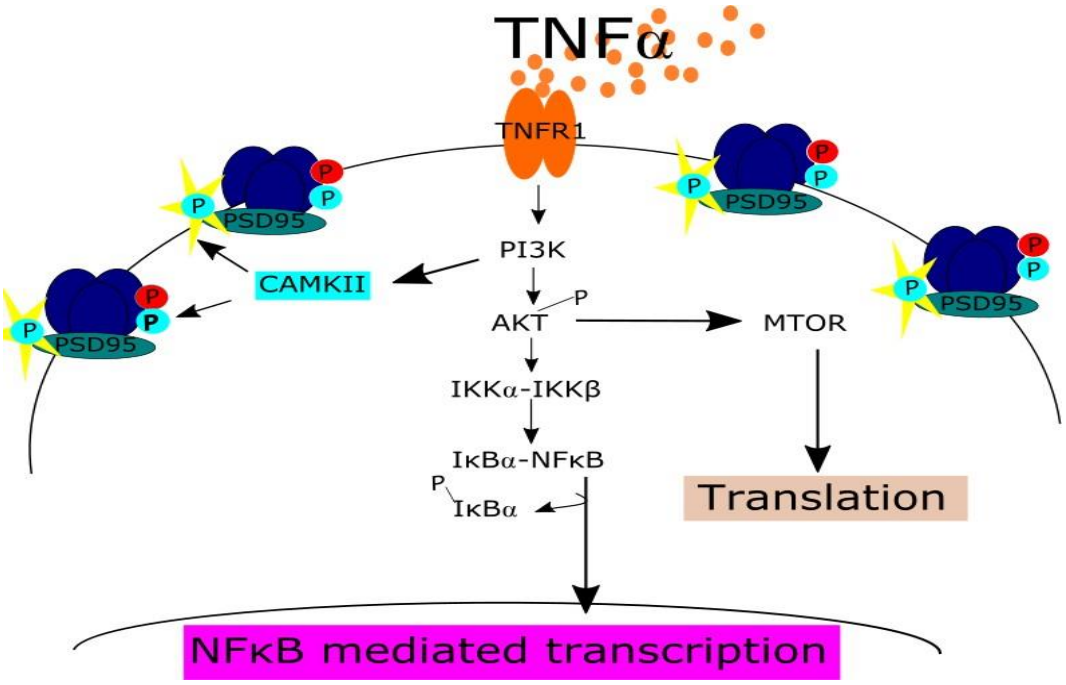

Figure 2. *TNFα signalling through TNFR1*. TNFα induces phosphorylation of AKT leading to translocation of transcription factor, NFκB, in the nucleus and subsequent transcription. TNFα also activates translation through the mTOR pathway

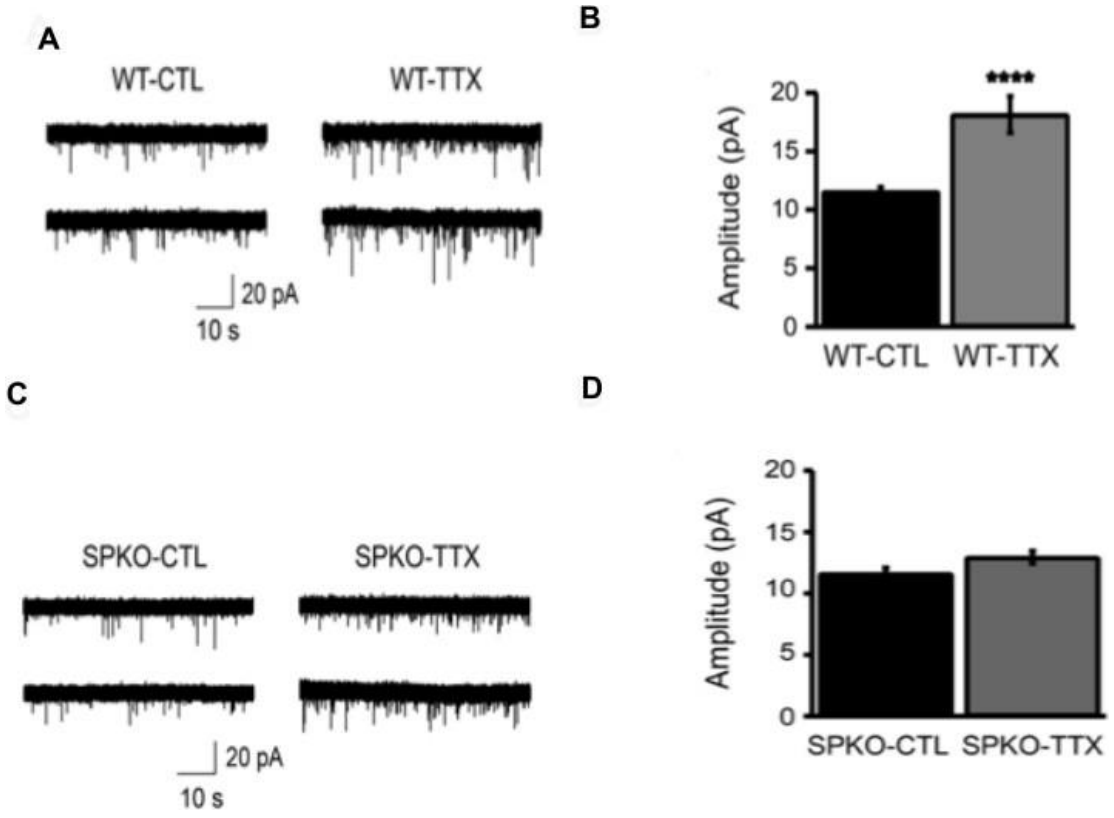

Figure 3. *Loss of synaptopodin prevents CA1 pyramidal neurons from scaling after 3 days of TTX treatment.* (A) Example current traces (membrane potential -60mV) of AMPA mediated mEPSC of WT control and TTX treated CA1 neurons. (B) Mean AMPA-mEPSCs amplitude from WT control (n=18, 11.57±0.38pA, *n* represents number of cells patched) and WT-TTX-treated (n=14, 18.14 ± 1.57pA) CA1 hippocampal neurons. There is a significant increase in mEPSC amplitude after TTX treatment in WT CA1 pyramidal neurons (***p<0.0001, unpaired two tailed t-test) (C) Example current traces (membrane potential -60mV) of AMPA mediated mEPSC of SPKO control and TTX treated CA1 neurons. (D) Mean AMPA-mEPSCs amplitude from SPKO (n=20, 11.59±0.50pA) and SPKO TTX-treated (n=15, 12.93 ± 0.50pA) CA1 hippocampal neurons. There is no significant difference between treated and untreated SPKO neurons. Reproduced from (Chan, 2016)

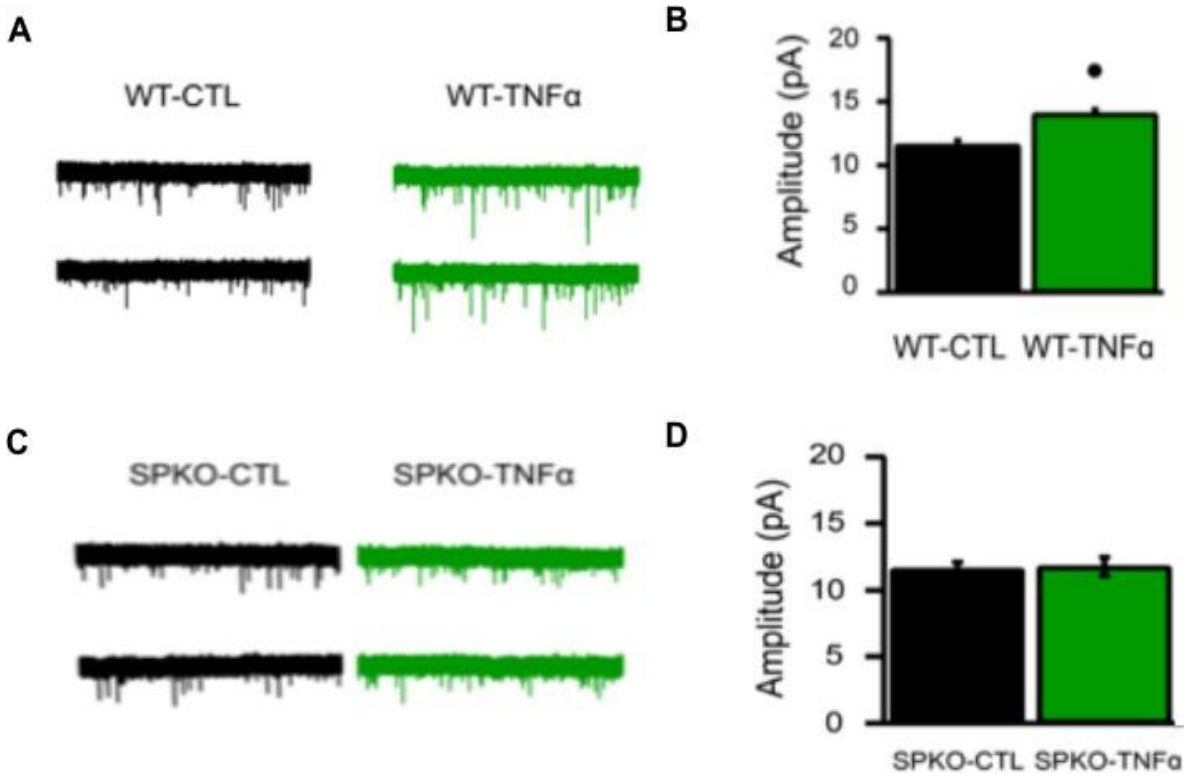

Figure 4. *Exogenous TNFα is not sufficient to induce scaling in synaptopodin knockout (SPKO) CA1 neurons.* (A) Example current traces (membrane potential -60mV) of AMPA mediated mEPSC of WT control and TNFα treated CA1 neurons. (B) Mean AMPA mEPSCs from WT control (n=20, 11.57±0.38pA, *n* represents number of cells patched) and TNFα-treated (n=6, 14.15±0.58pA) CA1 hippocampal neurons. Treatment with TNFα increased amplitude of mEPSC in WT neurons (*p<0.05, paired two tailed t-test). (C) Example current traces (membrane potential -60mV) of AMPA mediated mEPSC of SPKO control and TNFα-treated CA1 hippocampal neurons. (D) Mean AMPA mEPSCs of SPKO control (n=20, 11.59±0.50pA, *n* represents number of cells patched), and TNFα-treated (n=6, 11.75±0.71pA) CA1 hippocampal neurons. There was no significant difference in the amplitude of AMPAR mEPSC between SPKO control and TNFα-treated neurons. Reproduced from (Chan, 2016)

CHAPTER 2

2.1 Rationale

SS is fundamental to the stability of neuronal networks. It maintains the balance between excitation and inhibition during Hebbian plasticity which has the potential to cause overexcitation in the case of LTP or quiescence during LTD. Numerous factors have been discovered to be involved in SS and a few mechanisms have been proposed, yet SS remains incompletely understood. A prominent gap in our knowledge remains the lack of understanding on how different scaling factors cooperate to elicit homeostasis. TNFα is one of the scaling factors whose mechanism has been partly elucidated. TNFα's effect on plasticity has been found to operate through SP. SP regulates AMPAR trafficking and functions in both Hebbian and non-Hebbian plasticity. SP is required for homeostatic upscaling in deafferented dentate gyrus neurons and our lab has found that SP is also necessary for SS at CA3-CA1 synapses during inactivity induced scaling. Adding exogenous TNFα to SPKO neurons does not rescue scaling suggesting that SP is necessary for TNFα to have an effect on homeostatic upscaling. Thus, the focus of my research is to investigate the mechanism through which SP functions in SS and elucidate why its loss abolishes TNFα's stimulatory effect on SS. SP may regulate SS by modulating TNFα signalling downstream of TNFα release or by stimulating trafficking and anchoring of AMPARs through calcium dependent kinases.

Hence the aims of the project are to:

1. Determine whether SP regulates AMPAR accumulation during SS through TNFα-dependent transcriptional signaling cascade

2. Determine whether SP regulates SS through TNFα-dependent translational signaling cascade
3. Determine if SP plays a role in regulation of calcium dependent kinases in AMPAR trafficking during SS

2.1.1 Hypothesis

I hypothesize that SP will function as a regulator of calcium in homeostatic upscaling. It will stimulate AMPAR trafficking and anchoring through its modulation of calcium/calmodulin dependent kinases.

2.1.2 Experimental Outline

I will test my hypothesis by using TNFα to induce SS. I will then perform western blot and quantitative real time PCR (qRT-PCR) to examine the downstream effectors of the TNFα signalling through TNFR1. Nuclear fractionation will be conducted to investigate transcriptional activation and the mTOR pathway will be analyzed for translational activation. The amount and phosphorylation of CAMKII, the calcium dependent kinase necessary for trafficking and anchoring of AMPARs will be examined using western blot. Lastly, surface biotinylation will then be utilized to study the surface trafficking of AMPARs.

2.2 Methods

Organotypic Hippocampal Slice Culture: Organotypic hippocampal slices were prepared from postnatal (P) 6-8 wildtype (WT) and synaptopodin knockout mice (SPKO) mice of L15 and L15S background (Deller et al. 2003). Animals of L15 background express low but consistent levels of membrane tagged EGFP in hippocampal cells (De Paola et al 2003). L15S animals are generated by crossing L15 mice with mice that have SP knocked out. Hippocampal slices, 400μM thick, were created via vibratome, deposited on glass coverslips and placed into flat bottom test tubes with culture media (50% Eagle's basal medium, 25% HBSS, and 25% horse serum). The cultures were incubated in an air dry, roller drum incubator at 36 °C (Gahwiler et al 1997).

Pharmacological Treatment. Hippocampal slice cultures (wild-type and SPKO) were maintained for 11-14 days *in-vitro* (DIV) before treatment for 1-2 hours with either fresh regular media (as previously described) or media containing 1μg/ml of TNFα or 2-3 days treatment with 1μM TTX.

Western Blotting. Twenty control or twenty TNFα-treated (1-2 hours) organotypic hippocampal wild-type and SPKO slices at 11-14 DIV were removed from their chicken plasma clot and pooled together for each condition. Pooled slices for each condition were then lysed in ice-cold RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 50 mM Tris-HCl (pH 8.0)) with protease and phosphatase inhibitors (1X Roche Complete Mini, 5mM NaF, 1mM sodium orthovandate, 1mM PMSF). Protein concentrations were determined with the bicinchoninic acid (BCA) dye-binding assay (Thomson Scientific) with bovine serum albumin (BSA) as a standard, completed as per the instructions of the manufacturer. 30 μgs of total protein were isolated via sodium dodecylsulfate polyacrylamide gel electrophoresis (10% resolving/5% stacking), and afterwards immediately transferred onto 0.20 μm-pore polyvinylidene

difluoride membranes (Millipore). Membranes were blocked for 1 hour at room temperature with 5% BSA (BSA, Bio-Rad Laboratories) in Tris-buffered saline with 0.01% Tween-20 (TBS-T). Proteinaceous membranes were then kept in incubation at 4ₒC overnight with rabbit anti-TNFR1 (abcam; 1:1000), p-AKT Serine 473(CST 9271;1:500), AKT (CST 4691; 1:1000), IκBα (abcam;1:1000) and mouse anti-β-actin (Sigma; 1:5000; internal loading control) diluted in 5% BSA in TBS-T. Primary antibodies were detected via membrane incubation in horseradish peroxidase-conjugated secondary antibodies goat anti-rabbit HRP (abcam; 1:5000) and goat anti-mouse (Bio-Rad Laboratories; 1:10,000) diluted in 5% BSA in TBS-T for 1 hour at room temperature. Immunoreactive bands were then detected with enhanced chemiluminescence (ECL; Perkin Elmer) following the manufacturer's protocol and identified with ImageQuant LAS500 (GE Healthcare). Blots were ultimately analyzed with ImageJ software for mean pixel intensity. *n* values for these experiments represents number of separate culture preparations tested.

Nuclear Fractionation. Thirty control or thirty TNFα-treated (1-2 hours) organotypic hippocampal wild-type and SPKO slices at 11-14 DIV were removed from their chicken plasma clot and pooled together for each condition. Nuclear fractionation was conducted with Nuclei EZ prep kit (Sigma N3408) according to manufacturer's instructions. Briefly, pooled slices were lysed with 500µl of ice-cold Nuclei EZ prep lysis buffer. Nuclei was collected by centrifugation at 500 x g for 5 minutes at 4ₒC and washed with EZ prep lysis buffer. Supernatant containing cytoplasmic fraction was collected for later analysis. Washed nuclei were resuspended in 100µl of ice-cold Nuclei EZ prep storage buffer with protease and phosphatase inhibitors (1X Roche Complete Mini, 5mM NaF, 1mM sodium orthovandate, 1mM PMSF). Protein concentrations were determined with BCA dye-binding assay (Thomson Scientific). 10 µgs of nuclei were run on SDS (12.5%

resolving/5% stacking) and transferred to nitrocellulose membranes (Millipore). The resulting blot was kept in incubation at 4$_{o}$C overnight with rabbit anti- NFκB (CST 4764; 1:500), acetyl NFκB lysine 310 (CST 3045; 1:1000), CAMKIV (CST 4032; 1:1000) p-CREB Serine 133 (CST 9198; 1:1000), CREB (CST 9197; 1:1000), CAMKIIα/β (phosphosolutions; 1:1000), p-CAMKIIα/β Threonine 286/287 (phosphosolutions; 1:1000), GluA1 (abcam; 1:1000), p-GluA1 Ser 831(phosphosolutions; 1:1000), p-GluA1 Ser 845 (phosphosolutions; 1:1000) and mouse anti-ß-actin (Sigma; 1:5000; internal loading control) diluted in 5% BSA in TBS-T. Primary antibodies were detected via membrane incubation in horseradish peroxidase-conjugated secondary antibodies goat anti-rabbit HRP (abcam; 1:5000) and goat anti-mouse (Bio-Rad Laboratories; 1:10,000) diluted in 5% BSA in TBS-T for 1 hour at room temperature. Immunoreactive bands were then detected with ECL (Perkin Elmer) and identified with ImageQuant LAS500 (GE Healthcare). Blots were ultimately analyzed with ImageJ software for mean pixel intensity. *n* values for these experiments represents number of separate culture preparations tested.

Quantitative Real Time PCR. Hippocampal RNA was isolated from 2-2.5 months old wildtype and SPKO male mice of the L15 background using the Nucleospin RNA kit (Macherey-Nagel, 740955.250). Tissues were homogenized using the Ultra-Turrax T8 homogenizer (IKA-Werke, Staufen, Germany) The quantity and quality of the isolated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies). The 260/280 nm ratio of all our samples was between 1.8 and 2.1. Gene sequences from Akt1 (NM_001165894.1), Akt2 (NM_007434.3), Akt3 (NM_011785.3) and Arc (NP_061260.1) were accessed from GenBank and primers were designed using the Harvard Primer Bank. Total RNA (500 ng) was reverse transcribed into cDNA with high-capacity cDNA reverse transcription kit (ThermoFisher) according to supplier's instructions. The

amplification reaction took place in CFX 384 Real Time System (Biorad) using a Fast Start Universal SYBR Green Master (Rox) (Roche Applied Biosciences). The primer sequences were: Akt1 forward, 5′-tcgtgtggcaggatgtgtat-3′, Akt1 reverse 5′-acctggtgtcagtctcagagg-3′, Akt3 forward, 5′-tggaccactgttatagagagaacattt-3′, Akt3 reverse 5′-tggatagcttccgtccactc-3′ , Arc forward 5′-caacaggctcggtgaagaac-3′, Arc reverse 5′-ctcctcagcgtccacatacagt-3′ (Invitrogen, ThermoFisher Scientific). Each reaction contained 300 nm of primer. The cycling parameters were 3 minutes at 95°C, then 40 cycles of 15 seconds at 95° C, 15 seconds at 60° C and 20 seconds at 68 ° C. We calculated relative quantities of specifically amplified cDNA with the comparative threshold cycle method. GAPDH, Actin and UBC (Invitrogen, ThermoFisher Scientific) were used as reference genes but GAPDH acted as an endogenous reference used for quantification. No-template and no-reverse-transcription controls ensured that nonspecific amplification and DNA contamination could be excluded.

Surface biotinylation. Thirty control and thirty treated (TTX and TNFα) wild-type and SPKO cultures were chilled on ice and washed once with cold ACSF. Slice cultures were incubated for 45 minutes with 1 mg/ml EZ-Link Sulfo-NHS-SS-biotin (Thermo Fisher Scientific) 4°C. The reaction was stopped by washing the cultures with ACSF for 10 minutes and two times for 25 minutes with chilled 100mM glycine in ACSF. Cultures were removed from their chicken plasma clot and pooled together for each condition. Pooled slices for each condition were lysed with RIPA lysis buffer with protease and phosphatase inhibitors (1X Roche Complete Mini, 5mM NaF, 1mM sodium orthovandate, 1mM PMSF). Pooled slices were allowed to rotate at 4°C for 1 hour, and then spun for 5 mins at 10 000 x g in a tabletop microcentrifuge. Lysate (5 µl) was removed and used to determine protein concentration by BCA assay. 200µg of supernatant was

immunoprecipitated with streptavidin beads (Thermo Fisher Scientific) overnight at 4°C with rotation. Beads were washed with lysis buffer, eluted in SDS sample buffer with 100mM DTT, run out using PAGE, and transferred to nitrocellulose. The resulting Western blot was probed for GluA1 (abcam; 1:1000). Immunoreactive bands were then detected with enhanced chemiluminescence (Perkin Elmer) following the manufacturer's protocol and identified with ImageQuant LAS500 (GE Healthcare). Blots were analyzed with ImageJ software for mean pixel intensity. *n* values for these experiments represents number of separate culture preparations tested.

Reagents. All reagents were purchased from Tocris Bioscience (Ellisville, MO), except TTX, which was purchased from Alomone Labs (Jerusalem, Israel), soluble TNF receptor I (R&D Systems, Minneapolis, USA) and TNFα (Kamiya Biomedical, Tukwila, WA).

Statistics. Statistical analysis was performed in Graphpad Prism (GraphPad Software, La Jolla California USA). All values are shown as the mean ± SEM. Statistical comparisons for western blots were calculated via two-tailed, two-sample t-test. P-values for multiple comparisons were adjusted according to Bonferroni's method. Statistical comparisons for qRT-PCR were calculated via non-parametric Wilcoxon test. $P<0.05$ was considered as significant for all statistical comparisons.

CHAPTER 3

3.1 Results

TNFα Receptor 1 is present and functional in SPKO neurons

Synaptic upscaling is impaired in excitatory hippocampal neurons that lack SP (**Fig 3**; unpublished data, McKinney lab; Vlachos et al., 2013). TNFα, an inflammatory cytokine that typically induces scaling in WT neurons does not restore scaling in SPKO neurons (**Fig 4**; unpublished data, McKinney lab). The fact that exogenous TNFα does not rescue scaling phenotype in SPKO neurons led us to question the functionality of the receptor, TNFR1. As SP is an actin associated protein that can act as a scaffold to anchor various receptors, its loss can affect the localization and function of receptors. To test whether SP affects the quantity and presence of TNFR1 during scaling, we performed a western blot on control and TTX treated WT and SPKO organotypic cultures. Protein levels of TNFR1 was unchanged between WT and SPKO hippocampal neurons before and after SS (**Fig 5a, b**; n=3 sets of separate culture preparations, 20 cultures per treatment group, ANOVA p=0.234). If TNFR1 is present yet unable to induce scaling, then its function may be compromised by the lack of SP. To test this hypothesis, we examined downstream effectors of the TNFα-TNFR1 signaling cascade. It has been shown that the binding of TNFα to TNFR1 triggers the activation of PI3K during synaptic upscaling (**Fig 2**; Ozes et al., 1999; Stellwagen et al., 2005). Activation of PI3K results in the degradation of IκBα, a protein that sequesters the transcription factor NFκB in the cytoplasm (**Fig 2**). After treatment with TNFα for 1-2 hours, there was significant reduction in the protein levels of IκBα in both WT (**Fig 5c,d**; n=5 sets of separate culture preparations, 20 cultures per treatment group; WT=100±4.14%, WT-TNFα=53±

6.06 %, paired two tailed t-test p=0.003) and SPKO (n=5; SPKO= 113± 8.56%, SPKO-TNFα=63± 7.55%, paired two tailed t-test p=0.018) demonstrating the functionality of the receptor.

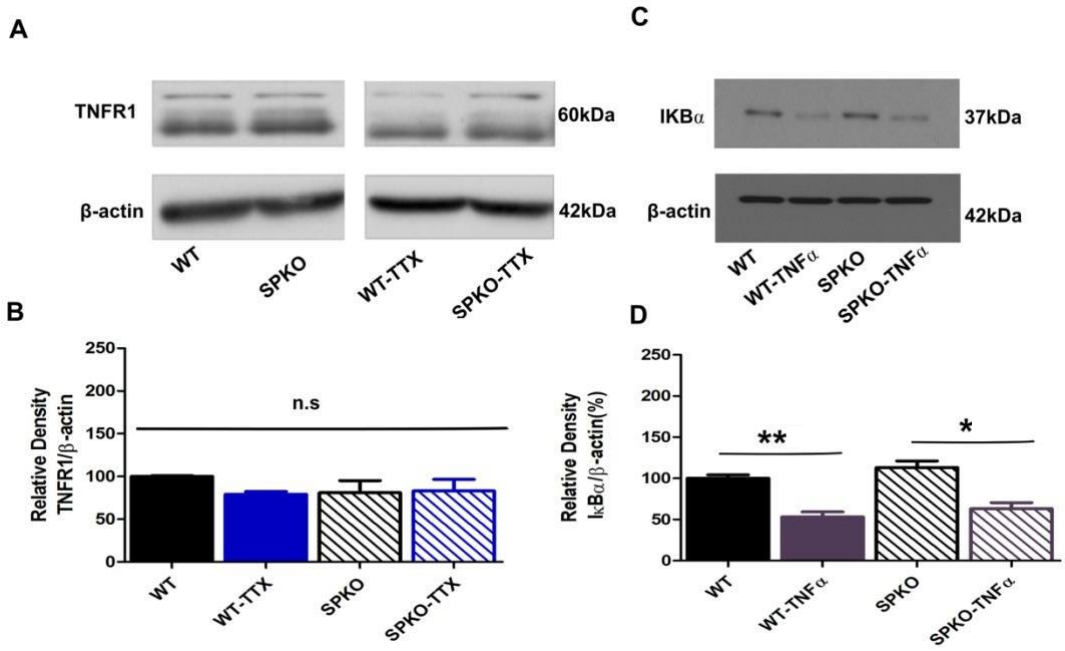

Figure 5. *TNFα Receptor 1 is present and functional in SPKO neurons.* (A) Representative western blot of WT and SPKO hippocampal cultures treated with 1μM TTX for 2-3days. Blots were probed for TNFR1 and β-actin (B) Quantification of TNFR1 expression normalized to β-actin. There is no significant difference in TNFR1 expression between treatment groups (n=3, ANOVA p=0.234). (C) Representative western blot of WT and SPKO hippocampal cultures treated with 1μg/ml of TNFα. Blot was probed for downstream effector of TNFR1, IκBα. (D) Quantification of IκBα expression normalized to β-actin. TNFα treatment resulted in a dramatic reduction in IκBα in WT (n=5; WT=100±4.14%, WT-TNFα=53± 6.06%, paired two tailed t-test p=0.003) and SPKO hippocampal neurons (n=5; SPKO= 113± 8.56%, SPKO-TNFα=63± 7.55%, paired two tailed t-test p=0.018). *n* values for these experiments represents number of separate batches of culture preparations tested, 20 cultures per treatment group. *p<0.05, **p<0.01

Loss of synaptopodin does not impair transcriptional activation of NFκB

A functional TNFα-TNFR1 signaling cascade can lead to upregulation of AMPAR through 3 different pathways: (1) NFκB dependent transcription, (2) AKT dependent activation of mTOR translational pathway, and (3) activation of kinases (CAMKII) that stimulate the trafficking of AMPAR through phosphorylation of key serine residues (**Fig 2**; Joyal et al., 1997; Ozes et al., 1999; Stellwagen et al., 2005). The reduction in levels of IκBα after TNFα treatment indicates that NFκB is active and should translocate to the nucleus. To verify that NFκB does translocate to the nucleus to facilitate transcription, we performed nuclear fractionation on WT and SPKO cultures after treatment with TNFα. Indeed, TNFα treatment resulted in a significant increase in nuclear NFκB in both WT (**Fig 6a,b**; n=3 sets of separate culture preparations, 30 cultures per treatment group; WT=100±13.36%, WT-TNFα=206± 9.44%, two tailed t-test $p<0.0001$) and SPKO neurons (n=3; SPKO= 107± 1.912%, SPKO-TNFα=197± 23.63%, two tailed t-test $p=0.04$). The rise in acetylation of NFκB on lysine 310 upon TNFα treatment demonstrated that nuclear NFκB is transcriptionally active (Chen et al., 2002; Huang et al., 2010) in both WT (**Fig 6c,d**; n=3 sets of separate culture preparations, 30 cultures per treatment group; WT=100±9.02%, WT-TNFα=148± 2.44%, two tailed t-test $p=0.0006$) and SPKO neurons (n=3; SPKO= 94± 8.24%, SPKO-TNFα=143± 16.92%, two tailed t-test $p=0.03$).

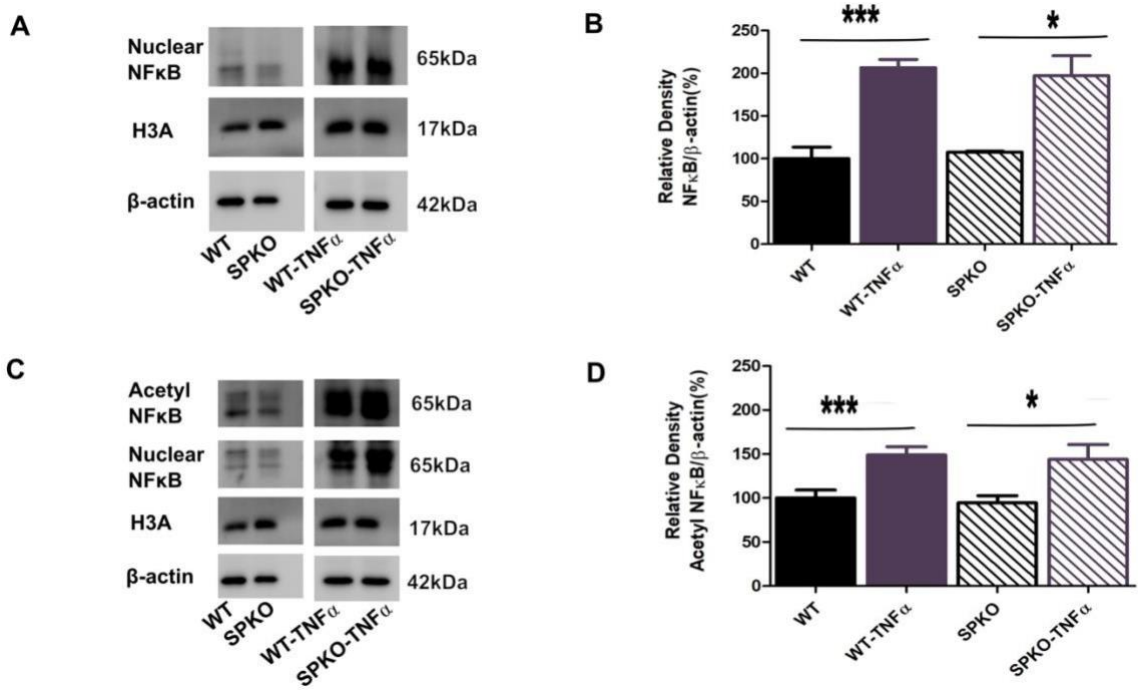

Figure 6. *Exogenous TNFα induces translocation of NFκB to the nucleus and stimulates NFκB's transcriptional activity.* (A) Nuclear fractionation was performed on control and TNFα treated WT and SPKO hippocampal cultures. Nuclear fractions were run and probed for NFκB, β-actin and nuclear marker, H3A. Presence of H3A indicated that pure nuclear fractions had been obtained. (B) Quantification of NFκB expression normalized to β-actin. A dramatic rise in NFκB was observed upon treatment with TNFα in both WT and SPKO neurons. (n=3; WT=100±13.36%, WT-TNFα=206± 9.44%, two tailed t-test $p<0.0001$) and SPKO hippocampal neurons (n=3; SPKO= 107± 1.912%, SPKO-TNFα=197± 23.63%, two tailed t-test $p=0.04$). (C) Representative western blot of nuclear fractions probed for acetyl NFκB lysine 310, β-actin and nuclear marker, H3A. (D) Quantification of acetyl NFκB expression normalized to β-actin. TNFα treatment resulted in an increase in acetyl NFκB WT (n=3; WT=100±9.02%, WT-TNFα=148± 2.44%, two tailed t-test $p=0.0006$) and SPKO hippocampal neurons (n=3; SPKO= 94± 8.24%, SPKO-TNFα=143± 16.92%, paired two tailed t-test $p=0.03$). *n* values for these experiments represents number of separate batches of culture preparations tested, 30 cultures per treatment group. *$p<0.05$, **$p<0.01$, *** $p<0.001$

mTOR translational pathway is functional in the absence of synaptopodin

In addition to stimulating transcription, TNFα can also activate the mTOR translational pathway. Unlike the transcriptional pathway, which is not dependent on AKT (Ozes et al., 1999), TNFα triggers translation through PI3K's phosphorylation of AKT. As AKT is a critical mediator of this pathway, we decided to examine it in naïve untreated animals (2-2.5 months old). Using western blot, we observed that the hippocampi of SPKO animals had high levels of AKT at the protein level (**Fig 7a,b**; n=6; WT=100±3.54%, SPKO=116± 3.02%, unpaired two tailed t-test p=0.01. *n* represents number of animals). This surprising increase led us to use qRT-PCR to determine whether the increase was at the transcriptional or translational level. Probing for the two isoforms of AKT found in the brain, AKT1 and AKT3 (Brand et al., 2015; Dummler et al., 2006) we observed that mRNA of both isoforms was upregulated in SPKO animals (**Fig 7c**; n=6, $-\Delta\Delta C_t$ AKT1 WT=1.35±0.74, SPKO=11.97± 3.29, Wilcoxon p<0.0001. $-\Delta\Delta C_t$ AKT3 WT=2.16±0.46, SPKO=11.12± 2.43, Wilcoxon p<0.0029. *n* represents number of animals). Phosphorylation of AKT occurs in the presence of TNFα (**Fig 8a**), therefore, we treated organotypic hippocampal cultures with TNFα (1μg/ml for 1-2 hours) to determine phosphorylation levels. In accordance with previous findings, AKT was phosphorylated in WT cultures treated with TNFα (**Fig 8b**,c; n=6 sets of separate culture preparations, 20 cultures per treatment group; WT=100±10.42%, WT-TNFα=140± 14.89%, paired two tailed t-test p=0.02). Exogenous TNFα did not induce an increase in AKT phosphorylation in SPKO cultures however, this was due to the fact that SPKO cultures maintained high levels of phosphorylated AKT prior to treatment (**Fig 8b,c** n=6 sets of separate culture preparations, 20 cultures per treatment group; SPKO=130±9.27%, SPKO-TNFα=139± 14.58%). To validate the activation of the mTOR pathway in SPKO cultures, we assessed phosphorylation of ribosomal protein S6 (rpS6) which is phosphorylated by S6 kinase (S6K) when

mTOR is activated (**Fig 8a**; Ruvinsky and Meyuhas, 2006). Treatment with TNFα resulted in S6 phosphorylation in both WT and SPKO cultures (Fig 8d,e; n=3 sets of separate culture preparations, 20 cultures per treatment group; WT=100±7.68%, WT-TNFα=161± 6.34, paired two tailed t-test p=0.010, SPKO=114±7.30%, SPKO-TNFα=166± 5.27%, paired two tailed t-test p=0.010) leading to the conclusion that absence of SP does not impair TNFα mediated translation.

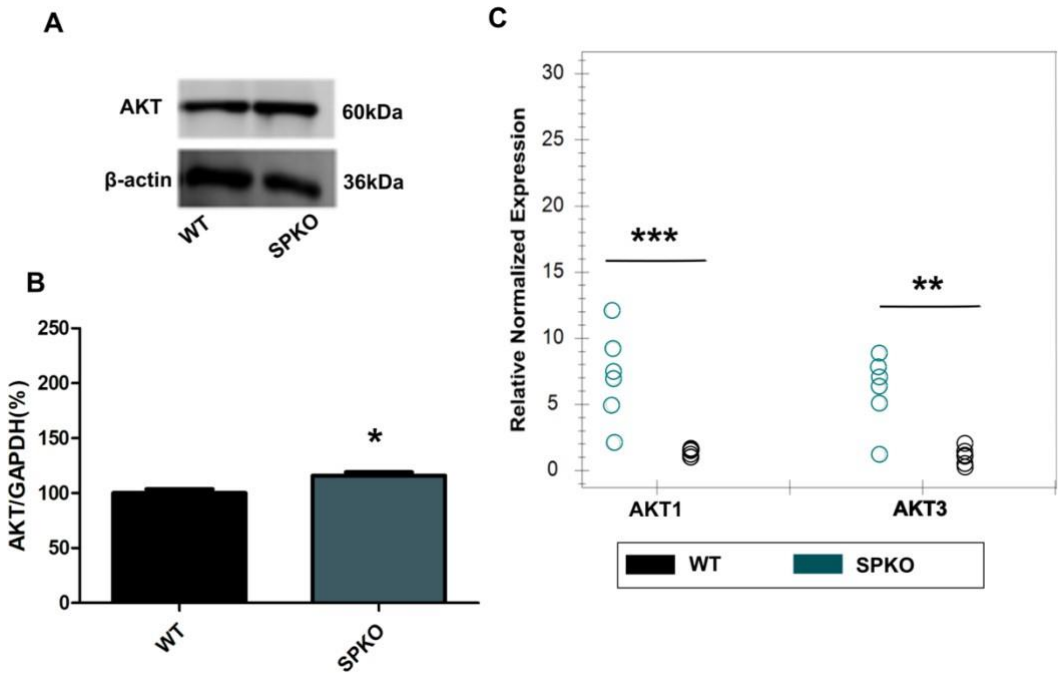

Figure 7. *Loss of synaptopodin results in high levels of AKT at both mRNA and protein levels.* (A,B) Representative western blot and quantification of AKT from the hippocampi of WT and SPKO naïve untreated animals (2-2.5 months). Blot was normalized to GAPDH. SPKO animals exhibited high levels of AKT in comparison to WT (n=6; WT=100±3.54%, SPKO=116± 3.02%, unpaired two tailed t-test p=0.01). (C) mRNA expression levels of two isoforms of AKT, AKT1 and AKT3, normalized to GAPDH. There are increased levels of both AKT isoforms in SPKO animals (n=6, -ΔΔCt AKT1 WT=1.35±0.74, SPKO=11.97± 3.29, Wilcoxon p<0.0001. -ΔΔCt AKT3 WT=2.16±0.46, SPKO=11.12± 2.43, Wilcoxon p<0.0029. *n* represents number of animals Wilcoxon p<0.0029). *p<0.05, **p<0.01, *** p<0.001

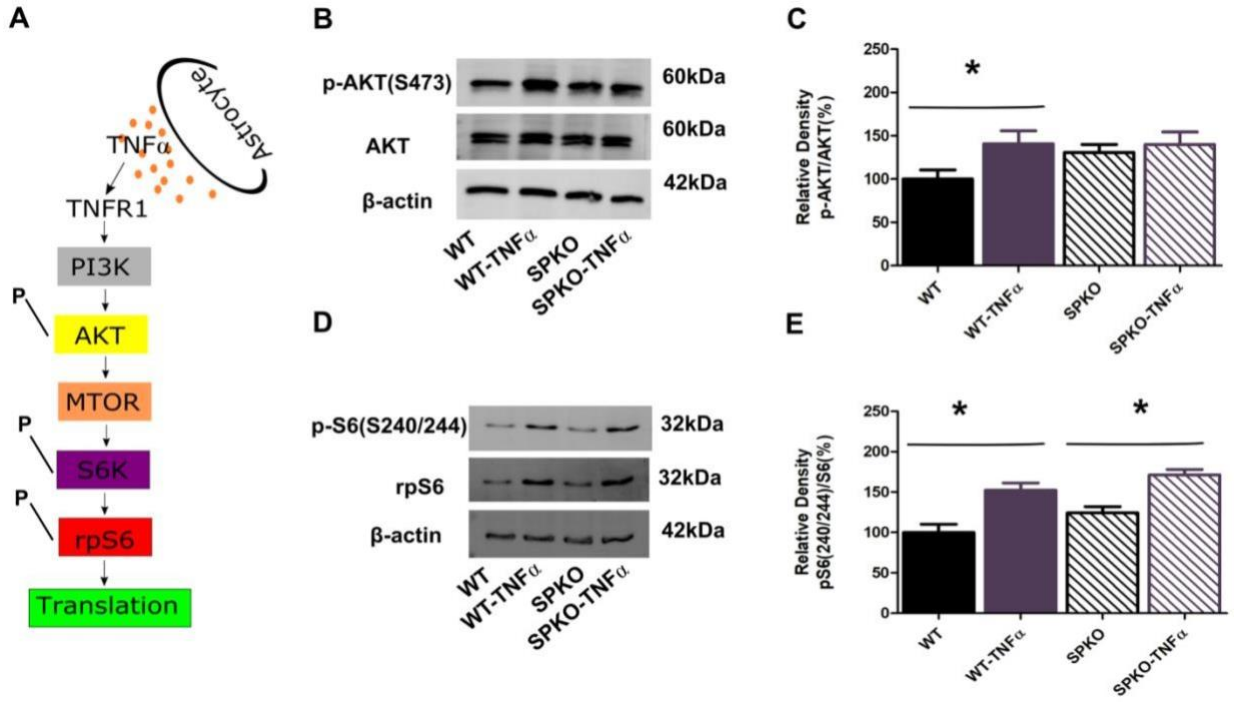

Figure 8. *Exogenous TNFα stimulates phosphorylation of AKT and mTOR translational pathway.* (A) Figure depicting TNFα's stimulation of AKT-mTOR pathway. (B, C) Representative western blot and of p-AKT (S473) normalized to total AKT. TNFα treatment resulted in a rise in p-AKT WT (n=6; WT=100±10.42%, WT-TNFα=140± 14.89%, paired two tailed t-test p=0.02) but not SPKO (n=6; SPKO=130±9.27%, SPKO-TNFα=139± 14.58%). (D) Representativewestern blot of p-rpS6 (S240/244), rpS6 and β-actin in TNFα treated WT and SPKO neurons. (E) Quantification of p-rpS6 normalized to total rpS6. TNFα treatment resulted in an increase in p-rpS6 in both WT and SPKO (n=3; WT=100±7.68%, WT-TNFα=161± 6.34%, paired two tailed t-test p=0.010, SPKO=114±7.30%, SPKO-TNFα=166± 5.27%, paired two tailed t-test p=0.010, *n* values for these experiments represents number of separate batches of culture preparations tested, 20 cultures per treatment group) *p<0.05

Loss of synaptopodin results in alterations of CAMKII

SP is a source of intracellular calcium stores through its association with the spine apparatus and ryanodine receptors (Deller et al., 2007; Vlachos et al., 2009; Korkotian et al., 2014). The absence

of SP results in the loss of the spine apparatus and a decrease of ryanodine receptors culminating in an overall reduction in the ability to induce intracellular calcium release (Segal et al., 2010, Korkotian and Segal, 2011, Korkotian et al., 2014). As an influx of calcium is necessary for the activation of kinases that regulate AMPAR trafficking, loss of SP can severely impair synaptic upscaling. To test whether calcium dependent kinases are affected by lack of SP, we blotted for the two isoforms of CAMKII dominant in the brain (α and β) and their phosphorylated states. While expression levels of CAMKIIα did not differ between WT and SPKO neurons during TNFα treatment (data not shown), the degree of phosphorylation was significantly reduced in SPKO neurons. In WT neurons, phosphorylation of CAMKIIα was unchanged during TNFα induced scaling (**Fig 9a,c**; n=3 sets of separate culture preparations, 20 cultures per treatment group; WT=100±6.33%, WT-TNFα=91±4.85%) however, in SPKO neurons, TNFα treatment induced a reduction in CAMKIIα phosphorylation (**Fig 9a,c;** SPKO=85± 3.67 SPKO-TNFα=60± 7.50%, paired two tailed t-test p=0.003; p-value adjusted using Bonferroni method). Similar to CAMKIIα, phosphorylation of CAMKIIβ in SPKO neurons also decreased upon TNFα treatment (**Fig 9a,d**; (WT=100±8.18%, WT-TNFα=87±3.04%, SPKO=76± 7.49%, SPKO-TNFα=68± 15.76% unpaired two tailed t-test p=0.010; p-value adjusted using Bonferroni method). Previous studies have observed alterations in the ratio of CAMKIIα to CAMKIIβ during SS therefore, we decided to examine the α/β ratio. Control WT neurons exhibited a reduction in the ratio of CAMKIIα to CAMKIIβ after scaling (**Fig 9a,b**; n=3 sets of separate culture preparations, 20 cultures per treatment group; WT=100±7.59%, WT-TNFα=81± 3.29%, two tailed t-test p=0.009; p-value adjusted using Bonferroni method) but the α/β ratio remained unchanged in SPKO neurons (n=3 sets of separate culture preparations, 20 cultures per treatment group; SPKO=84± 2.22%, SPKO-TNFα=79± 3.57%,). Interestingly, SPKO neurons had a lower ratio of CAMKIIα to CAMKIIβ prior to TNFα treatment (**Fig 9a,b**; n=same as above; WT-SPKO, unpaired two tailed t-test p=0.037; p-value adjusted using

Bonferroni method). We did not observe a significant difference in the expression levels of CAMKIIα or CAMKIIβ however, a decrease in the ratio of CAMKIIα to CAMKIIβ during SS could be due to either a decrease in CAMKIIα or an increase in CAMKIIβ. A switch to greater CAMKIIβ has been previously shown to be important for upscaling and as SS does not occur in SPKO neurons, SP maybe necessary for CAMKIIβ to exert its effects.

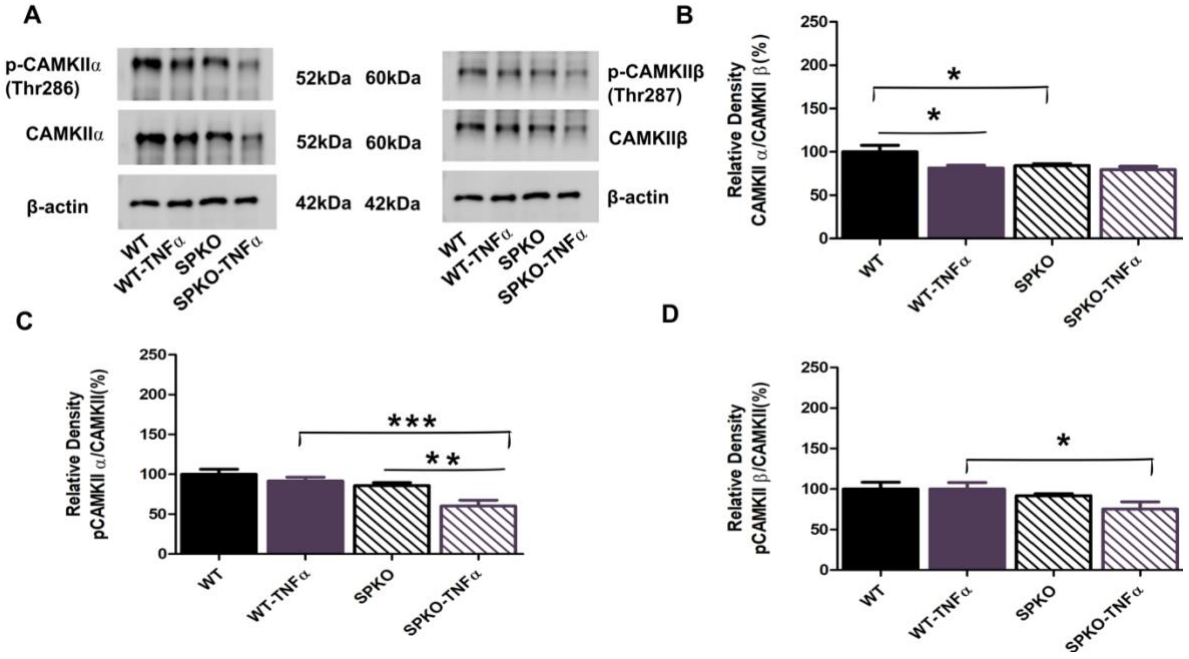

Figure 9. *Loss of synaptopodin impairs phosphorylation of CAMKIIα/β.* (A) Representative western blot of WT and SPKO hippocampal cultures treated with 1μg/ml of TNFα. Blots were probed with antibody that recognizes p-CAMKIIα/β, CAMKIIα/β and β-actin (B) Quantification of the ratio of CAMKIIα to CAMKIIβ. Ratio of CAMKIIα/β after TNFα treatment resulted in decrease in ratio of CAMKIIα/β in WT neurons (n=3; WT=100±7.59%, WT-TNFα=81± 3.29%, paired two tailed t-test p=0.009; p-value adjusted using Bonferroni method). SPKO neurons had a lower ratio of CAMKIIα/β prior to TNFα treatment (n=3; WT-SPKO, paired two tailed t-test p=0.037; p-value adjusted using Bonferroni method) and remain unaffected after treatment (n=3; SPKO=84± 2.22%, SPKO-TNFα=79± 3.57%) (C) Quantification of p-CAMKIIα expression normalized to CAMKIIα. Phosphorylation of CAMKIIα is unchanged in WT neurons during TNFα treatment (n=3, WT=100±6.33%, WT-TNFα=91±4.85%). In SPKO neurons, TNFα treatment induces a decrease in CAMKIIα phosphorylation (n=3, SPKO=85± 3.67%, SPKO-TNFα=60± 7.50%, paired two tailed t-test p=0.003; p-value adjusted using Bonferroni method) (D) Quantification of p-CAMKIIβ expression normalized to CAMKIIβ. SPKO neurons also have lower levels of p-CAMKIIβ upon treatment with TNFα (WT=100±8.18%, WT-TNFα=87±3.04%, SPKO=76± 7.49%, SPKO-TNFα=68± 15.76% unpaired two tailed t-test p=0.010; p-value adjusted using Bonferroni method). *n* values for these experiments represents number of separate batches of culture preparations tested, 20 cultures per treatment group). *p<0.05, **p<0.01, ***p<0.001

Loss of synaptopodin does not impair phosphorylation of GluA1

Phosphorylation allows CAMKII to operate long (up to an hour) after the removal of calcium transients which activated the kinase (Groth et al., 2011; Kim et al., 2016). A reduction in the phosphorylation of CAMKII shortens the duration of the kinase's activity and can diminish the ability of the kinase to perform its functions which include phosphorylating S831 on GluA1 and stargazin, the TARP that synaptically traps GluA1 AMPARs. In order to evaluate how decreased phosphorylation of CAMKIIα and β contributes to lack of scaling in SPKO neurons, we decided to examine phosphorylation of S831 on GluA1. Phosphorylation of S845 was also investigated since it has been shown that defective phosphorylation of this site prevents surface accumulation of AMPARs during SS (Diering et al., 2014, Sanderson et al., 2018). Consistent with previous findings that phosphorylation of S831 is not implicated in SS (Diering et al., 2014), treatment with TNFα did not impact phosphorylation of S831 in either WT or SPKO neurons (**Fig 10a,b**; n=4 sets of separate culture preparations, 20 cultures per treatment group; WT=100±10.37%, WT- TNFα=81± 11.15%, paired two tailed t-test p=0.185, SPKO=84± 15.53%, SPKO-TNFα=91± 11.88%,, paired two tailed t-test p=0.194). In contrast to S831, phosphorylation of S845 was upregulated with exogenous TNFα in WT neurons (**Fig 10c,d**; n=4 sets of separate culture preparations, 20 cultures per treatment group; WT=100±10.91%, WT-TNFα=135± 6.05%, paired two tailed t-test p=0.02). SP deficient neurons also had increased S845 phosphorylation when challenged with TNFα (**Fig 10c,d**; n=4; SPKO=105± 7.96%, SPKO-TNFα=133± 12.95% , paired two tailed t-test p=0.02) suggesting that SP does not modulate PKA's phosphorylation of S845.

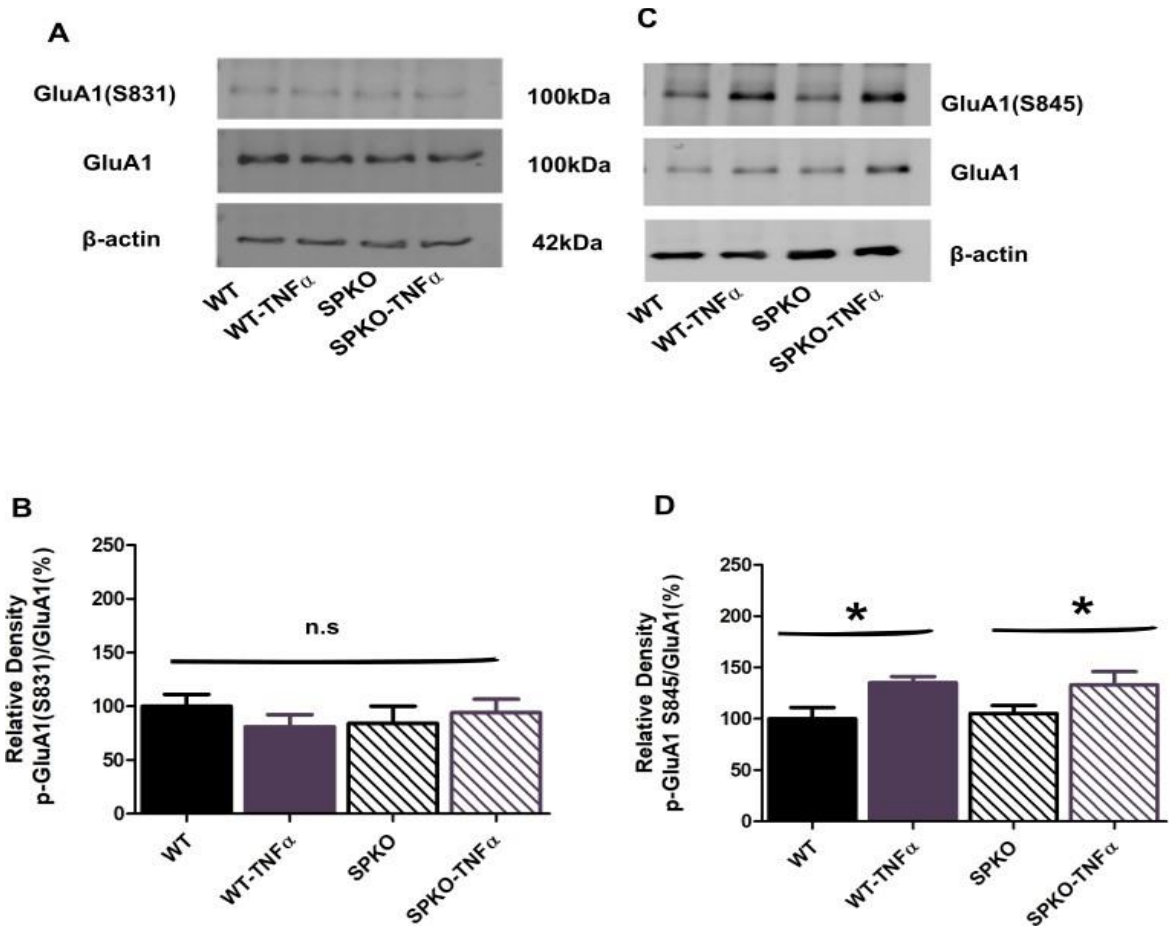

Figure 10. *GluA1 phosphorylation is not impaired by loss of synaptopodin.* (A) Representative western blot of WT and SPKO hippocampal cultures treated with 1μg/ml of TNFα. Blot was probed for p-S831 and GluA1. (B) Quantification of p-S831 expression normalized to GluA1. GluA1 phosphorylation at S831 is unaffected by loss of SP and scaling. (n=4, WT=100±10.37%, WT-TNFα=81± 11.15%, paired two tailed t-test p=0.185, SPKO=84± 15.53%, SPKO-TNFα=91± 11.88%, paired two tailed t-test p=0.194). (C) Representative western blot of WT and SPKO hippocampal cultures treated with 1μg/ml of TNFα. Blot was probed for p-S845 and GluA1. (D) Quantification of p-S845 expression normalized to GluA1. Treatment with TNFα resulted in an increase in WT neurons (n=4, WT=100±10.91%, WT-TNFα=135± 6.05%, paired two tailed t-test p=0.02) and SPKO neurons (n=4; SPKO=105± 7.96%, SPKO-TNFα=133± 12.95%, paired two tailed t-test p=0.02). *n* values for these experiments represents number of separate batches of culture preparations tested, 20 cultures per treatment group) *p<0.05

Loss of synaptopodin reduces total levels GluA1 and impairs upregulation of GluA2 in the hippocampus during scaling

Total amounts of AMPARs does not significantly change in homeostatic scaling but given the necessity of transcription and translation in this phenomenon, we decided to analyze the total amounts of GluA1 and GluA2 AMPARs. Absence of SP decreased the total amounts of GluA1 (**Fig 11a,b**; n=6 sets of separate culture preparations, 20 cultures per treatment group; WT=100±11.10%, SPKO=69± 7.27%, unpaired two tailed t-test p=0.006, WT-TNFα=86± 9.08%, SPKO-TNFα=76± 9.72%) and treatment with TNFα did not ameliorate the levels. Total GluA2 levels were unaffected by loss of SP (**Fig 11c,d**; n=3, SPKO=103± 19.35%, SPKO-TNFα=83± 13.67%) but TNFα induced scaling upregulated GluA2 in WT neurons but not SPKO neurons (**Fig 11c,d**; n=3, WT=100±9.97%, WT-TNFα=149± 5.74%,, paired two tailed t-test p=0.05). Trafficking and anchoring of AMPARs were assessed using surface biotinylation to detect surface levels of GluA1 AMPARs. Preliminary datashows that TNFα induced scaling increased levels of surface AMPARs in WT neurons (**Fig 12a,b**; n=2, WT=100±23.72%, WT-TNFα=315± 79.58%) but not SPKO neurons (**Fig 12a,b**; n=2, SPKO=119± 65.99%, SPKO-TNFα=62± 57.05%). This finding is consistent with electrophysiological results (**Fig 4a-d**) showing that SPKO neurons do not undergo TNFα induced scaling. It also aligns with observations in our lab that PSD95 volume increases during scaling in WT neurons but not SPKO neurons (data not shown).

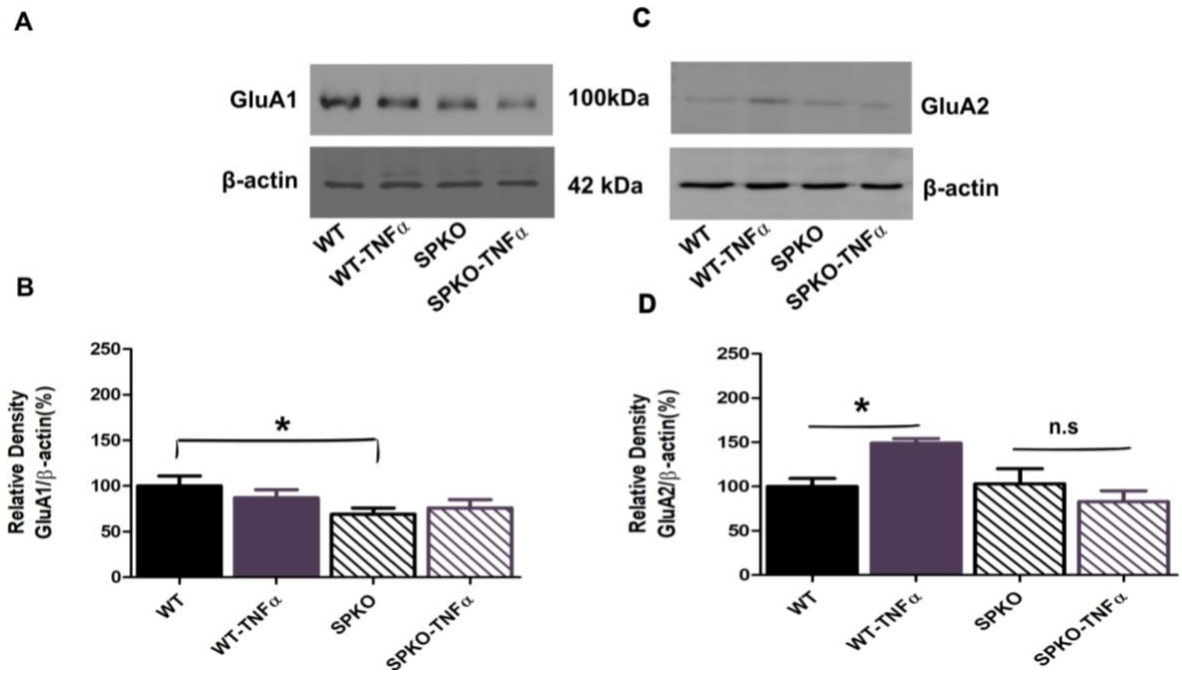

Figure 11. *Total AMPARs levels are not upregulated in the absence of synaptopodin during scaling.* (A) Representative western blot of WT and SPKO hippocampal cultures treated with 1μg/ml of TNFα. Blot was probed for total GluA1 (B) Quantification of GluA1 expression normalized to β-actin. SPKO neurons have reduced total GluA1 (n=6, WT=100±11.10%, SPKO=69± 7.27%, unpaired two tailed t-test p=0.006) and TNFα treatment does not significantly increase it back up (WT-TNFα=86± 9.08%, SPKO-TNFα=76± 9.72%). (C) Representative western blot of WT and SPKO hippocampal cultures treated with 1μg/ml of TNFα. Blot was probed for total GluA2. (D) Quantification of GluA2 expression normalized to β-actin. Treatment with TNFα resulted in an increase in total GluA2 in WT neurons (n=3, WT=100±9.97%, WT-TNFα=149± 5.74%, paired two tailed t-test p=0.05) but not SPKO neurons (n=3, SPKO=103± 19.35%, SPKO-TNFα=83± 13.67%). *n* values for these experiments represent number of separate batches of culture preparations tested, 20 cultures per treatment group) *p<0.05

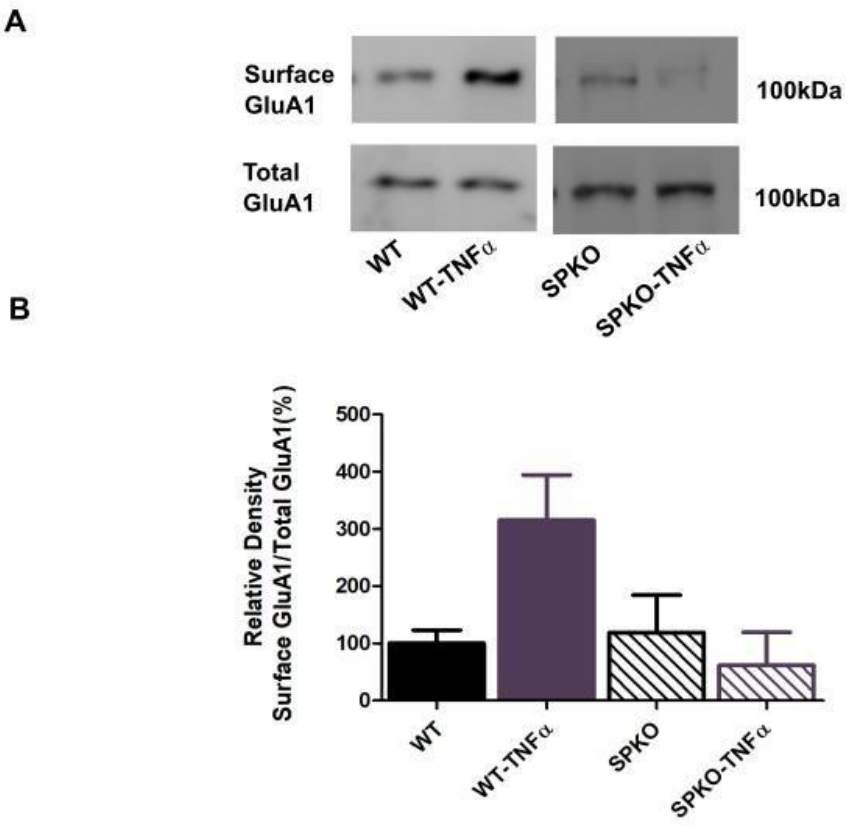

Figure 12. *Exogenous TNFα is not sufficient to induce upregulation of surface GluA1 in SPKO neurons*

(A) Representative western blot from surface biotinylation of WT and SPKO hippocampal cultures treated with 1μg/ml of TNFα. Blots were probed for GluA1. (B) Quantification of surface GluA1. Surface GluA1 was determined by normalizing biotinylated GluA1 to total GluA1. There was a trend towards increased surface GluA1 in WT (n=2, WT=100±23.72%, WT-TNFα=315± 79.58%) after TNFα treatment. SPKO neurons in comparison showed a trend towards decrease surface GluA1 after TNFα treatment (n=2, SPKO=119± 65.99%, SPKO-TNFα=62± 57.05)

CHAPTER 4

4.1 Discussion

SP is an established mediator of synaptic plasticity at excitatory synapses. It is necessary for homeostatic upscaling (**Fig 3**; unpublished data, McKinney lab; Vlachos et al., 2013) and its loss hinders SS even in the presence of required scaling factor, TNFα (**Fig 4** unpublished data, McKinney lab). SP also contributes to the maintenance of cognitive function as its levels are downregulated in Alzheimer's, Parkinson's and other mild dementia (Aloni et al., 2019). Despite its importance, how SP functions and the role it plays in SS remains unknown. In this study, we investigated the role of SP in homeostatic upscaling. We found that SP does not modulate TNFα signaling rather, it plays a central role in the regulation of AMPARs and CAMKII during SS. In the absence of SP, exogenous TNFα sufficiently detaches NFκB from cytoplasmic inhibitor, IκBα and prompts its translocation to the nucleus (**Fig 5c,d, 6a,b**). Transcriptional activation of NFκB (**Fig 6c,d**; Chen et al., 2002; Huang et al., 2010) and stimulation of the mTOR translational pathway (**Fig 7-8**) are not hindered by the loss of SP. Irrespective of the functionality of TNFα's transcriptional and translational pathways, total AMPARs are not upregulated by exogenous TNFα in SPKO neurons. In fact, loss of SP cause decreases in GluA1 levels and abolish rise in GluA2 upon addition of TNFα (**Fig 10**). Phosphorylation of CAMKIIα/β, an indicator of the kinase's activity, is also reduced in SPKO neurons during TNFα induced scaling (**Fig 9**). Altogether, our results suggest that SP operates in SS through regulation of AMPARs and phosphorylation of CAMKII.

Neurons possess a repertoire of mechanisms to maintain homeostasis of neural activity. Transcription and translation are two key mechanisms used to restore neuronal function when

activity is chronically reduced (Aoto et al., 2008; Ibata et al., 2008). Inhibitors of transcription and translation, actinomycin and anisomycin, respectively prevent homeostasis of neural activity. Transcription during SS is hypothesized to occur through CAMKIV and CREB. This hypothesis is based on the evidence that activated CAMKIV levels decrease upon 24 hours of activity deprivation (Ibata et al., 2008; Goold and Nicoll, 2010). While this finding implicates CAMKIV in SS, it is paradoxical with CAMKIV's role in transcription. A rise in activated CAMKIV is required for the stimulation of CREB dependent transcription (Ibata et al., 2008; Goold and Nicoll, 2010) therefore, a decline in activated CAMKIV will dampen rather than stimulate transcription. The importance of AMPARs in SS make them the expected gene targets of transcription but to date, there is no evidence of direct transcription of AMPARs during SS. Gene products that mediate adaptive responses including N-cadherin, stargazin and neuropaxin 1, have been shown to be transcribed during scaling (Schaukowitch et al., 2017). Transcription of neuropaxin 1, a protein that clusters AMPARs at the synapse, was found to occur through transcription factor SRF, and its cofactor EK1(Schaukowitch et al., 2017).

In comparison to transcription, there is evidence that translation leads to synthesis of AMPARs. The AKT-mTOR pathway is one of the primary signaling cascades for translational activation. It is also a signaling cascade stimulated by TNFα. In our investigations, we found that SP deficient neurons exhibited high levels of AKT and increased activation of AKT. Despite the implication of enhanced protein synthesis through mTOR, we did not find an elevation in the total levels of GluA1 and GluA2 AMPARs in SPKO cultures during scaling. Our results suggest two possibilities: either AMPAR production is not dependent on TNFα's stimulation of mTOR or AMPAR production is dependent on local rather than global translation.

Our investigations into protein synthesis were conducted on a global level due to the fact that SS is thought to reflect a global cellular response (Turrigiano, 2012). However, there is an emerging view that local mechanisms can induce changes in synaptic strength that lead to homeostasis (Ju et al., 2004, Sutton et al., 2006, Aoto et al., 2008, Maghsoodi et al., 2008). Specifically, local protein synthesis has been found to facilitate the rapid induction of SS (Aoto et al., 2008, Maghsoodi et al., 2008). Local protein synthesis is an alternative approach utilized by neurons to efficiently deliver proteins necessary for plasticity (Steward and Falk, 1986, Steward and Schuman, 2001). Dendrites possess polyribosomes, ER and Golgi, core elements of the synthesis and transport machinery (Ju et al., 2004, Kennedy and Ehlers, 2006, Maghsoodi et al., 2008). They also house mRNAs of select synaptic proteins including CAMKII, GluA1 and GluA2 AMPARs (Ju et al., 2004, Kennedy and Ehlers, 2006, Maghsoodi et al., 2008). The presence of the synthesis machinery in dendrites enables local translation of select proteins and averts the time delay that occurs with transporting proteins from the soma (Steward and Falk, 1986, Steward and Schuman, 2001). Dendritically produced AMPARs are functional and effective in inducing changes in synaptic strength (Ju et al., 2004, Sutton et al., 2006, Maghsoodi et al., 2008). In SS, local translation of GluA1 is observed when activity is reduced by NMDAR blockade or a combination of TTX and APV (Sutton et al., 2006, Aoto et al., 2008, Maghsoodi et al., 2008). Chronic activity deprivation by TTX alone increases dendritic levels of AMPARs, and this synthesis occurs even in dendrites transected from the soma (Ju et al., 2004). Our investigations into protein synthesis were conducted on a global level and does not provide information on local mechanisms. However, as an essential component of SA, an intricate ER like structure, SP has been linked to local protein synthesis (Pierce et al., 2000, Steward and Schuman, 2001). How SP promotes local protein synthesis is unknown but the fact that GluA1 levels drop in the absence of

SP make it plausible that SP stimulates local synthesis of AMPARs. The finding that GluA2 levels are upregulated by TNFα in WT neurons but not SPKO neurons lend support to this hypothesis. The concept is further substantiated by observations that local translation of GluA1 in SS occurs in an mTOR independent manner (Aoto et al., 2008, Maghsoodi et al., 2008). In our study, we found an intact mTOR pathway in SPKO neurons however, there was no increase in AMPARs.

In addition to an involvement in local protein synthesis, SP is known to operate in plasticity through its regulation of intracellular calcium. Calcium is a ubiquitous second messenger necessary for the activation of kinases and signaling cascades. During high levels of activity, calcium flux inside the cell through extracellular sources however, chronic activity deprivation limits the entry of extracellular calcium (Turrigiano, 2008, Kim and Ziff, 2014; Vlachos et al., 2013). The drop in somatic calcium upon chronic activity suppression is coupled with alterations in the signaling of calcium dependent kinases. CAMKIV's activation is reduced and CAMKII undergoes a change in the ratio of the α to β subunits (Ibata et al., 2008, Goold and Nicoll, 2010, Thiagarajan et al., 2002; Groth et al., 2011). Our study further explored the effect of TNFα induced scaling and SP loss on CAMKII, the calcium kinase that facilitates synaptic trapping and increases synaptic transmission of GluA1 AMPARs. We examined the ratio of CAMKIIα to CAMKIIβ and congruent with previous findings, we observed a reduction in the ratio during scaling in WT neurons. A decrease in the α/β ratio denotes an upregulation of the β isoform (Thiagarajan et al., 2002; Groth et al., 2011). The dominance of the β subunit during reduced activity has been associated with the ability of the β subunit to respond to low levels of calcium (Thiagarajan et al., 2002; Groth et al., 2011). The β isoform is eightfold more responsive to calcium, therefore, in the event of chronic activity deprivation where somatic calcium levels drop, making a switch to greater CAMKIIβ is advantageous to inducing SS (Thiagarajan et al., 2002).

SP deficient neurons make the switch to CAMKIIβ even before being subjected to scaling conditions. The switch can be understood in the context of SP's role as a regulator of intracellular calcium. The formation of the spine apparatus, an intracellular calcium store is dependent on SP (Deller et al., 2003). Ryanodine receptors which amplify calcium influx through calcium induced calcium release are associated with SP and their levels decrease when SP is absent (Vlachos et al., 2009; Korkotian et al., 2014). The absence of the spine apparatus and the reduction in ryanodine receptors limits the calcium transients produced in SPKO neurons. SP deficient neurons generate smaller calcium transients and these transients decay faster than those in SP positive spines. A system without SP is a low calcium system; it is a system that requires sensors attuned to low levels of calcium. Decreasing the ratio of CAMKIIα to CAMKIIβ is an effective way for SPKO neurons to enhance responsiveness to calcium.

It is also plausible that the reduction in the ratio of CAMKIIα to CAMKIIβ is a response to the decrease in GluA1 AMPARs found in SPKO neurons. The dominance of CAMKIIβ is inversely correlated with AMPARs (Thiagarajan et al., 2002). A reduction in CAMKIIβ is found when AMPAR activity is high while a rise in the β isoform persists during decreased AMPAR activity (Thiagarajan et al., 2002). In our study, we discovered that in addition to having a decreased ratio of CAMKIIα to CAMKIIβ, SPKO neurons also exhibit reduced levels of GluA1 AMPARs. The idea that the switch to greater CAMKIIβ in SP deficient neurons is a response to reductions in AMPARs can be tested in WT neurons by reducing AMPARs levels then evaluating the ratio of CAMKIIα to CAMKIIβ. If the results replicate our findings in SPKO neurons, then it will indicate that the switch to greater CAMKIIβ is due to reductions in AMPARs.

An upregulation of the β isoform, although pivotal, is not sufficient to drive SS. SP deficient neurons, which have reductions in the ratio of CAMKIIα to CAMKIIβ, do not undergo SS. These neurons also experience declines in the phosphorylation of CAMKIIα and β at Thr286/287 during scaling conditions. Activation and phosphorylation of CAMKIIα and β is dependent on Ca^{2+}/CaM (Strack et al., 1997; Abdul Majeed et al., 2014). Calcium mobilizes CaM to bind to CAMKII, activating the kinase and stimulating its autophosphorylation (Strack et al., 1997; Abdul Majeed et al., 2014). Phosphorylation of CAMKII at Thr286/287 facilitates translocation of CAMKII into the PSD (Strack et al., 1997; Abdul Majeed et al., 2014) where it is well positioned to bind PSD95 and phosphorylate its substrates. It also renders the kinase autonomous and allows its activation to outlast calcium transients (Taha et al., 2002; Kimura et al., 2008).

Autophosphorylation at Thr286/287 extends the activation of CAMKII, therefore, reductions in its phosphorylation is likely to limit the ability of the kinase to perform its functions. CAMKII performs many functions including increasing single channel conductance (Derkach et al., 1999) and facilitating the immobilization of surface GluA1 AMPARs (Kim et al., 2016; Opazo et al., 2010). Although CAMKII manipulates single channel conductance of AMPARs by phosphorylating S831(Derkach et al., 1999; Malinow and Malenka, 2002), we did not find any significant changes in the phosphorylation of S831 in the absence of SP or during scaling. This finding suggests that SP does not regulate SS through CAMKII's phosphorylation of S831. SP may however be modulating the immobilization of AMPARs through CAMKII because preliminary results show that SPKO neurons have low levels of GluA1 AMPARs at the surface. (**Fig 12**). While WT neurons upregulate levels of surface GluA1 AMPARs upon treatment with TNFα, SPKO neurons do not (**Fig 12)**. These preliminary findings are consistent with

electrophysiological results that show that TNFα mediated scaling does not occur in SPKO neurons (**Fig 4**). They also corroborate findings in our lab that PSD95 volume is not enhanced in SP deficient neurons during scaling (data not shown). Altogether, the results suggest that reduction in phosphorylation of CAMKII by loss of SP impairs surface accumulation of GluA1 AMPARs.

The autophosphorylation of CAMKII at Thr286/287 is integral to different forms of plasticity. CAMKIIα mutants that cannot induce autophosphorylation at Thr286 do not elicit NMDA dependent LTP or have spatial memory (Giese et al., 1998; Kimura et al., 2008). These mutants also do not experience an enhancement of activity after monocular deprivation, an *in-vivo* model of homeostatic plasticity (Taha et al., 2002). Similarly, we have found that SPKO neurons which have decreased phosphorylation of CAMKIIα/β at Thr286/287 have impaired plasticity. In our study, the decline in CAMKIIα/β phosphorylation is mediated by the loss of SP. Absence of SP decreases ryanodine receptors (Vlachos et al.,2009) and release of calcium from ryanodine receptors have been shown to stimulate activation of CAMKII (Kouzu et al., 2000, Arias-Cavieres et al., 2018). Deficits in the functioning of CAMKII and contextual fear memory occurs in the absence of calcium induced calcium release by ryanodine receptors (Kouzu et al., 2000). Our observation that loss of SP elicits defects in plasticity similar to those observed in animals that have impaired CAMKII phosphorylation emphasizes the necessity of SP as a calcium regulator in plasticity.

4.2 Future Directions

Altogether, our results center SP in the regulation of AMPARs and CAMKII in homeostatic upscaling. Synaptopodin is an important regulator of calcium through its association with the spine apparatus and ryanodine receptors. During scaling where extracellular sources of

calcium are limited, SP facilitates ample calcium release from SA, ryanodine receptors, and IP3Rs, triggering signaling cascades involved in the upregulation of AMPARs. When SP is lost, total GluA1 levels drop and total GluA2 levels do not elevate in the presence of TNFα. Activity of calcium dependent kinase, CAMKII, is reduced as evidenced by the decrease in its phosphorylation. The reduction in CAMKII phosphorylation is correlated with impairments in the surface trapping of GluA1 AMPARs leading to the conclusion that SP operates in SS through the production and synaptic trapping of AMPARs (**Fig 13**). Synaptopodin is likely regulating the production of AMPARs through local translation since global mTOR translation was found to be functional in SPKO neurons. Moreover, SP is present in only a subset of spines and therefore more likely to control translation at a local rather than global level. The defects in synaptic trapping is hypothesized to be the result of the decrease in CAMKII phosphorylation which places limitations on the kinase's ability to perform its functions (**Fig 13**). The finding that loss of SP causes reductions in GluA1 is not surprising since SP has been found to play a role in calcium dependent accumulation of AMPARs (Vlachos et al., 2009). It is however puzzling that AMPARs do not increase in SPKO neurons even though global translation by mTOR is functional. Synaptopodin is thought to have a role in local protein synthesis therefore, the next step of this project is to investigate SP's involvement in local synthesis of AMPARs during SS. To tease this apart, we will treat WT and SPKO organotypic hippocampal cultures with TNFα for 1-2 hours, then perform qRT-PCR to examine mRNA levels of AMPARs. Synaptoneurosomes which are enriched with presynaptic and postsynaptic structures will be extracted and a western blot of GluA1 and GluA2 will be used to analyze local translation of AMPARs. If levels of GluA1 and GluA2 in synaptoneurosomes increase with TNFα treatment in WT neurons but not SPKO neurons, then it will suggest that SP plays a role in local translation of AMPARs. Once we have determined the influence of SP in local translation of AMPARs, we will need to evaluate whether SP also plays a

role in the trafficking of AMPARs. We can evaluate this by transfecting WT and SPKO hippocampal neurons with AMPARs tagged with super ecliptic pHluorin (SEP). SEP is a pH sensitive variation of GFP which has diminished fluorescence in areas with low pH and intense fluorescence in areas with high pH. Membrane proteins tagged with SEP will fluorescence intensely when inserted into the cell membrane. Transfected neurons will be treated with TNFα to induce SS and trigger the trafficking of AMPARs. An assessment of fluorescence intensity will allow us to determine whether trafficking of AMPARs during SS is impacted by loss of SP.

In our study we found reductions in the phosphorylation of CAMKII and impairments in the surface trafficking of GluA1 AMPARs in SPKO neurons during scaling. CAMKII mediates the synaptic trapping of GluA1 AMPARs therefore to determine whether lack of surface GluA1 is due to declines in CAMKII phosphorylation, we must examine the ability of the kinase to function. Since autophosphorylation causes CAMKII to translocate into the PSD, we should evaluate translocation of CAMKII to PSD using immunohistochemistry. We can examine colocalization of CAMKII with PSD95, a prominent PSD protein. We would expect greater colocalization between CAMKII and PSD95 in WT neurons after scaling is induced by TNFα. If TNFα treated SPKO neurons have less colocalization between CAMKII and PSD95, it would suggest that CAMKII is not translocating into the PSD where it can access its substrate, stargazin, the TARP which synaptically traps GluA1 AMPARs upon phosphorylation by CAMKII. If translocation of CAMKII into the PSD is intact, then western blot must be used to analyze the phosphorylation of stargazin. We can also try to rescue the deficits in synaptic trapping by enhancing the phosphorylation of CAMKII in SPKO neurons. This can be done by using a drug that stimulates the phosphorylation of CAMKII such as the cell-permeant protein phosphatase inhibitor, calyculin A (Strack et al., 1997). Calyculin A causes phosphorylation of Thr286 and given that Thr286 is implicated in numerous plasticity mechanisms, it may rescue the impairments in synaptic trapping during SS. Transfecting a

constitutively active CAMKII into SPKO neurons is an alternative rescue method that can be used. A constitutively active CAMKII will be able to increase autophosphorylation at both Thr286 and Thr287 leading to a rescue of impairments in synaptic trapping.

Lastly, we must examine surface accumulation of GluA2 AMPARs using surface biotinylation. We evaluated surface trafficking of GluA1 in this study but did not investigate surface GluA2. The finding that total GluA2 levels are not elevated in SPKO neurons during scaling suggests that surface accumulation of GluA2 may also be impaired. Moreover, there is evidence showing that CAMKII activated from internal calcium stores stimulate the trafficking of GluA2 AMPARs (Lu et al., 2014). Thus, the reduction in CAMKII in SPKO neurons during scaling may not just affect surface GluA1 but also surface GluA2.

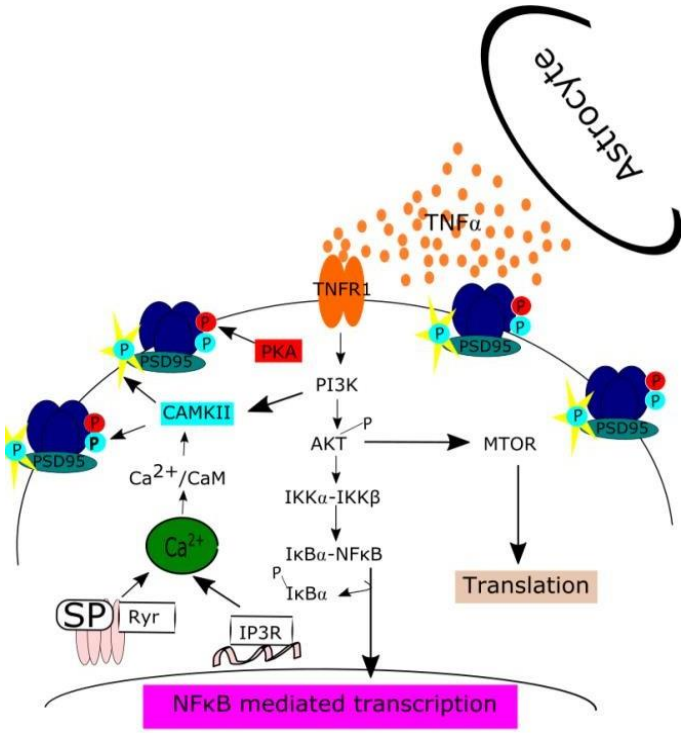

Figure 13. *Synaptopodin regulates anchoring of GluA1 AMPARs through phosphorylation of CAMKII.* In the presence of synaptopodin, calcium released from both ryanodine and IP3Rs activate Ca^{2+}/CAM. Activated Ca^{2+}/CAM stimulates CAMKII activity and its autophosphorylation. Upon autophosphorylation, CAMKII translocate into the PSD to phosphorylate stargazin and mediate the anchoring of GluA1 AMPARs at the synapse. In the absence of synaptopodin, intracellular calcium release is reduced leading to declines in CAMKII phosphorylation. Translocation of CAMKII to the PSD and its phosphorylation of stargazin are impaired leading to defective scaling in synaptopodin deficient neurons.

4.3 Conclusion

The necessity of SP in synaptic plasticity has been well established but the mechanism through which SP exerts its effects are unknown. In this study we explored SP's role in SS and investigated how it interacted with TNFα signaling. We found that SP does not modulate TNFα signaling; instead, its absence impairs production of AMPARs and reduces the phosphorylation of CAMKII during scaling. We also have preliminary evidence in support of our electrophysiological data that loss of SP impairs the upregulation of GluA1 AMPARs to the surface. Overall, our results suggest that SP regulates SS through phosphorylation of CAMKII and modulation of AMPAR production. They show that SP plays crucial role in SS such that its absence hinders scaling even when other scaling factors are functional. The findings of this study unveil a potential mechanism through which SP, an intracellular calcium regulator, exerts its effects. They expand our knowledge of SP and the dynamic way it functions in plasticity. The results contribute and support the emerging view of SP as a critical, plasticity protein. Understanding how SP operates does not only extend our knowledge of synaptic plasticity, but it also contributes to our understanding of neurological diseases. A downregulation of SP is found in Alzheimer's, Parkinson's Diseases, and mild dementia (Aloni et al., 2019); therefore, knowledge of SP contributes to the greater work of understanding how proper cognitive function is maintained.

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