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MECHANISMS AND CONSEQUENCES OF REGULATING THE SPINOPHILIN/NMDA RECEPTOR INTERACTION

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To humanity which needs help more than ever

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LIST OF ABBREVITIONS

6-OHDA: 6-hydroxydopamine

Ala: Alanine

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

Asp: Aspartic acid (aspartate)

CAMKII: Calcium/calmodulin-dependent kinase II

CDK5: Cyclin-dependent kinase 5

DA: Dopamine

GPCR: G protein-coupled receptor

GST: glutathione *S*-transferase

HEK: Human embryonic kidney

IP: Immunoprecipitation

LTD: Long-term depression

LTP: Long-term potentiation

MSN: Medium spiny neuron

NMDAR: N-methyl-D-Aspartate Receptor

PD: Parkinson’s disease

PK: Protein Kinase

PKA: cAMP-dependent protein kinase

PKC: Protein Kinase C

PP: Protein phosphotase

PP1: Serine/threonine protein phosphatase 1

PSD: Post-synaptic density

Ser: Serine

SpAP: Spinophilin-associated protein

Spino: Spinophilin

ABSTRACT

Beiraghi Salek, Asma. M.S., Purdue University, August 2016. Mechanisms and Consequences of Regulating the Spinophilin/NMDA Receptor Interaction. Major Professor: A.J. Baucum.

Parkinson disease (PD) is the second most common neurodegenerative disease. It is characterized by loss of dopaminergic cells in the substantia nigra, which causes loss of dopaminergic synapses onto striatal medium spiny neurons (MSNs). Dendritic spines that are localized to these striatal MSNs receive synaptic inputs from both the nigral dopamine neurons and cortical glutamate neurons. Signaling downstream of excitatory, glutamatergic drive is modulated by dopamine. This tripartite connection: glutamate, dopamine, and MSN dendritic spine, is important for normal motor function. Glutamate released from presynaptic terminals binds to and activates two classes of inotropic glutamate receptors that are localized to dendritic spines on striatal MSNs: the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and the N-methyl-D-aspartate receptor (NMDAR). Once these receptors are activated, they allow for Ca^{2+} influx, which in turn activates Ca^{2+}-dependent processes that underlie neural plasticity, including long-term potentiation (LTP) and long-term depression (LTD). Proper machinery in the pre- and post-synaptic neurons is required for normal signal transduction. Moreover, this signal transduction requires proper organization of synaptic proteins, which is achieved by

specific protein-protein interactions. These protein-protein interactions are dynamic and can be modulated under various conditions, including pathological changes in the phosphorylation status of a specific protein. Catalytically active proteins called phosphatases and kinases specifically regulate the phosphorylation status of synaptic proteins. Pathologically, in PD there is increased autophosphorylation and activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). This increased phosphorylation may be due to changes in the activity of the serine/threonine protein phosphatase 1 (PP1), a highly conserved protein serine/threonine phosphatase that has a diverse set of functions in eukaryotes. Serine/threonine phosphatase substrate specificity is obtained via interactions with targeting and regulatory proteins. One such protein, spinophilin, is a scaffolding protein that targets PP1 to various synaptic substrates to regulate their phosphorylation. Interestingly, the association of PP1 with spinophilin is enhanced in a rat model of PD. The NMDAR is another protein that has altered phosphorylation in animal models of PD. We have found that there is a decrease in the NMDAR-spinophilin interaction in an animal model of PD. Here, we have found that spinophilin and the NMDAR interact in brain tissue and when overexpressed in a mammalian cell system. Moreover, we have identified novel mechanisms that regulate this interaction and have identified putative consequences of altering this association. These studies give us novel insight into mechanisms and consequences underlying pathological changes observed in an animal model of PD. Understanding these changes will inform novel therapeutic targets that may be useful in modulating striatal function.

1 INTRODUCTION

1.1 Parkinson Disease History and Pathology

Parkinson disease (PD) is a neurodegenerative disease that was first described about two centuries ago by James Parkinson. He described this disease with four main hallmarks including resting tremor, postural and gait instability, and hypokinesia (Parkinson, 2002). Friedrich Lewy was the first person who started to study this disease from a histological standpoint in the early 20th century and successfully characterized the major histopathological markers of the disease (Lewy, 1913) which were named Lewy Bodies later. Lewy bodies contain α-synuclein aggregates that interfere with normal cell function, resulting in the death of presynaptic cells (Spillantini et al., 1997).

The substantia nigra is a brain region that is most affected in PD, leading to a severe atrophy and a loss of pigment therein. Loss of approximately 80% of the dopaminergic neurons in the substantia nigra will lead to appearance of motor deficits (Rodriguez-Oroz et al., 2009). There are various forms of PD, but the sporadic form is the most common. Currently, the exact mechanisms linked to the sporadic form are not fully understood. However, environmental factors, gene-environment interactions, and gene-gene interactions may play a role.

Other forms of PD have familial or genetic roots. The most common known mutations leading to familial PD are observed in the *LRRK2* (leucine-rich repeat kinase 2)

and *SNCA* (synuclein) genes among others (Lesage et al., 2006; Polymeropoulos et al., 1997). These and other proteins are known to regulate mitochondrial function, protein aggregation, and/or protein degradation. This protein aggregation may underlie some of the PD pathology, such as Lewy body formation. Formation of Lewy bodies can then result in perturbations in synaptic communication and cell death (Gibb & Lees, 1988; Spillantini et al., 1997).

L-DOPA is the most commonly prescribed drug for treating PD. L-DOPA is a precursor of dopamine, and is used to replace the loss of dopamine released by nigral dopaminergic neurons (Dauer & Przedborski, 2003). In PD patients, L-DOPA is converted to DA in the remaining dopaminergic neurons and this acts to attenuate many of the motor symptoms associated with PD (Hornykiewicz, 1974). Despite L-DOPA's positive effects, long-term treatment with L-DOPA will lead to the appearance of side effects including an increase in involuntary movements known as dyskinesias (Cenci, Lee, & Bjorklund, 1998; Pearce, Jackson, Smith, Jenner, & Marsden, 1995). Moreover, the efficacy of L-DOPA also wanes over time, in part due to a further loss of dopamine neurons and changes in functional synaptic connectivity in the striatum. Deep-brain stimulation (DBS) of the subthalamic nucleus or the globus pallidus is a more recent treatment for PD. In this method of treatment, electrodes are implanted directly into the brain regions mentioned above, and pulses are generated at a specific frequency. This method has proven to be successful in attenuating the severity of PD motor symptoms (Kumar et al., 1998); Deep-Brain Stimulation for Parkinson's Disease Study Group 2001). While both of these treatments are proven to help PD patients, they only treat the symptoms of PD. Unfortunately there is no proven treatment for healing the underlying causes of the disease which has proven to be

much more difficult (Shulman, De Jager, & Feany, 2011). This is due, in part, to a lack of understanding of the changes in functional connectivity that occur in the striatum following loss of dopamine neurons.

1.2 Brain Function and Parkinson Disease

Proper synaptic connectivity is essential for normal brain function. One example of a neural circuit and connectivity in the brain is dopaminergic projections from the substantia nigra and glutamatergic projections from motor cortex that both synapse on small protrusions called dendritic spines that are localized to striatal medium spiny neurons (MSNs). Once the synapse is formed, proper machinery in the pre- and post-synaptic neurons is needed for normal signal transduction, which underlies appropriate synaptic connectivity. This machinery that is essential for signal transduction is a protein dense specialization in the tip of the spines referred as to the postsynaptic density (PSD) (Hausser, Spruston, & Stuart, 2000). MSN dendritic spines were first described by Ramón y Cajal (Cajal, 1888). These small protrusions play a significant role in proper synaptic connectivity since they serve as the main site to receive presynaptic input by significantly increasing the overall dendrite surface area (Gray, 1959).

Upon glutamate release from the pre-synaptic neuron, glutamate will bind to its receptors that reside on the dendritic spines of MSNs. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and N-methyl-D-aspartate receptor (NMDAR) are two inotropic glutamate receptors that are localized to dendritic spines on striatal MSNs. Following glutamate activation of postsynaptic receptors, Ca^{2+} fluxes into the cell, which in turn activates Ca^{2+}-dependent processes. This glutamate signal is modulated by

the release of dopamine (DA). Specifically, two classes of DA receptors reside on two unique populations of striatal MSNs: the DA D1R-containing, or direct pathway MSNs, and the DA D2R-containing, or indirect pathway MSNs (Strange, 1993). The D_1 family of receptors that activate the direct pathway are $G_{\alpha s}$-protein-coupled and upon ligand binding activate adenylyl cyclase which in turn will activate protein kinase A (PKA) signaling. Conversely, the D_2-family of DA receptors are found on indirect pathway striatal MSNs, are $G_{\alpha i}$-coupled, and inhibit adenylyl cyclase, which blocks downstream PKA signaling (Stoof & Kebabian, 1984). The balance between direct and indirect pathways is essential for normal motor control, with the direct pathway being linked to initiation of normal movement and the indirect pathway suppressing unintentional or inappropriate movements (Albin, Young, & Penney, 1989; Frank, Seeberger, & O'Reilly R, 2004).

As mentioned previously, loss of nigral dopaminergic projections causes alterations in the functionality of striatal MSNs (German, Manaye, Smith, Woodward, & Saper, 1989), such as modulation of normal PKA signaling (Nishi et al., 2008), as well as the appearance of motor deficits associated with PD (Albin et al., 1989; Rodriguez-Oroz et al., 2009; Starr, 1995). Molecularly, DA depletion alters the functionality of PSD proteins. Previous studies have shown that PSD proteins are critical for normal synaptic communication. Specifically, knocking out one or more of these synaptic proteins leads to changes in normal synaptic function and connectivity. One of the proteins that has altered functionality following DA depletion is the NMDAR. NMDARs are glutamate receptors that underlie long-term potentiation (LTP) and/or long-term depression (LTD), molecular correlates of learning and memory (Malenka & Bear, 2004). Moreover, DA depletion affects LTP and LTD in both PD patients and animal models of PD. Specifically, 6-hydroxydopamine (6-OHDA)-

lesion of the substantia nigra in rats leads to loss of LTD (Calabresi, Maj, Pisani, Mercuri, & Bernardi, 1992; Ingham, Hood, Taggart, & Arbuthnott, 1998). However, the specific changes that occur in NMDAR function that link to these pathological changes in learning and memory observed in animal models of PD are unclear.

1.3 NMDAR Function and Localization

Glutamatergic synapses regulate most of the excitatory neurotransmission in the mammalian brain and play a critical role in mediating functional neuronal connectivity. Glutamate released from presynaptic axons activates several glutamate-gated ion channels on postsynaptic cells including AMPARs, NMDARs, and kainate receptors, which get their names from their specific response to pharmacological agents (Dingledine, Borges, Bowie, & Traynelis, 1999; Hollmann & Heinemann, 1994). It has been shown that dysfunction of these receptors is associated with multiple neurological and psychiatric disorders, including Parkinson disease, Alzheimer disease, and schizophrenia (S. Cull-Candy, Brickley, & Farrant, 2001; Waxman & Lynch, 2005).

As mentioned above, NMDARs are a major class of glutamate receptors. These receptors have several subunits and three families of genes (*Grin1*, *Grin2* and *Grin3*) that encode three families of proteins (GluN1, GluN2, and GluN3) (S. Cull-Candy et al., 2001). Studies show that NMDARs are tetramers in which two GluN1 subunits assemble with two NR2 and/or one NR2 and one NR3 subunit. GluN1 subunits are obligatory subunits necessary for functional expression of the NMDA receptors (S. G. Cull-Candy & Leszkiewicz, 2004). According to biochemical, electrophysiological and crystallographic

analysis, a GluN1/ GluN2 heterodimer is the functional unit in tetrameric NMDARs (Furukawa, Singh, Mancusso, & Gouaux, 2005).

The NMDAR is a voltage-sensitive glutamate receptor, which is blocked by extracellular Mg^{2+} ion under resting membrane potential. The blocking Mg^{2+} ion acts as a switch that allows for Ca^{2+} influx upon membrane depolarization together with binding of glutamate to the GluN2 subunit and a co-agonist, such as glycine, to the GluN1 subunit (Erreger, Chen, Wyllie, & Traynelis, 2004).

Many studies have shown that GluN2 and GluN3 subunits connote specific electrophysiological properties to the NMDARs (S. G. Cull-Candy & Leszkiewicz, 2004). As a result, variability in NMDAR subunit composition is an important factor to regulate NMDAR function. According to previous studies, subunit composition of NMDARs is developmentally regulated (Monyer, Burnashev, Laurie, Sakmann, & Seeburg, 1994). The GluN1 subunit is the product of a single gene, which can be alternatively spliced, and normally is found ubiquitously throughout the brain. In contrast, GluN2 subunits (GluN2A-D) are encoded by four different genes and their expression patterns depend on the developmental stage and brain region. The expression patterns of GluN2A and GluN2B throughout the brain are relatively broad, with a parallel decrease in GluN2B and increase in GluN2A expression. However GluN2C and GluN2D have a more restricted expression, with GluN2C expression in cerebellum starting later in development and GluN2D being expressed early in development mostly in the brainstem and in thalamic and hypothalamic nuclei (Monyer et al., 1994). Endogenous NMDARs normally only contain GluN1 and GluN2 subunits, with GluN3 subunits only incorporated in a subpopulation of NMDARs

and exhibiting decreased channel conductance properties (S. G. Cull-Candy & Leszkiewicz, 2004).

From a structural standpoint, NMDAR subunits contain several domains including a long extracellular N-terminal domain, a membrane-spanning domain, a pore loop, and a subunit-dependent, variable length intracellular C-terminal domain. The C-terminal "Tail" domain is the most variable region when comparing the various NMDAR subunit sequences. The Tail region is known to regulate receptor interactions with various intracellular proteins. These protein-protein interactions are important for proper trafficking and localization of NMDARs to membranes. Additionally, different subunits of the NMDAR can couple receptors to various cytosolic signaling complexes. For instance, GluN2B interacts with various proteins such as SynGAP (Kim, Dunah, Wang, & Sheng, 2005) and an active form of CaMKII (Colbran et al., 1997), which leads to differing forms of synaptic plasticity (Barria & Malinow, 2005). Furthermore, the tail region of NMDARs is subject to various post-translational modifications such as phosphorylation, which can directly modulate protein activity, interactions, localization, and mobility.

1.4 Protein Kinases and Phosphatases

Protein kinases and phosphatases regulate substrate protein phosphorylation. These proteins are some of the most widely expressed enzymes in eukaryotes. Some studies estimate that the human genome encodes about 500 kinases (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002) and 150 phosphatases (P. T. Cohen, 2002). Moreover, it is known that these enzymes play very important roles in neuronal processes such as axon/dendrite formation and synaptic plasticity (Soderling, 2000).

Normally, protein phosphorylation is modulated by a balance between phosphatase and kinase activity. As a result, regulation of phosphatase or kinase activity and their localization can have significant effects on substrate phosphorylation (P. Cohen, 1992; Meiselbach, Sticht, & Enz, 2006). There are multiple classes of kinases and phosphatases. One specific class, the serine/threonine family of kinases and phosphotases, phosphorylates or dephosphorylates serine and/or threonine residues. Studies suggest that there are ~385 known serine/threonine kinases (Manning et al., 2002), while there are only ~40 known serine/threonine phosphatases (P. T. Cohen, 2002). Given the discrepancy in the number of kinases and phosphatases, serine/threonine phosphatases associate with specific targeting or regulatory proteins to obtain substrate specificity (P. T. Cohen, 2002; Janssens, Longin, & Goris, 2008; J. D. Scott & Pawson, 2009).

Protein kinase A (PKA) is a highly abundant serine/threonine kinase in the CNS and is known to play an integral role in modulating glutamate receptor phosphorylation (Tingley et al., 1997). Glutamate receptor phosphorylation underlies various neurological processes such as LTP and LTD (Raymond, Blackstone, & Huganir, 1993; Roche, Tingley, & Huganir, 1994). Moreover, PKA is important in cell development, where its activation increases neurite formation in developing cells (Vogt Weisenhorn, Roback, Kwon, & Wainer, 2001). Furthermore, PKA activity is altered in rat models of Parkinson disease and following long-term L-DOPA treatment (Oh, Del Dotto, & Chase, 1997). Linking PKA activity to PD, dopamine signaling modulates PKA activity. As stated above, D_1 dopamine receptor activation increases adenylyl cyclase activity (Herve et al., 2001; Sibley & Monsma, 1992; Stoof & Kebabian, 1984). Active adenylyl cyclase cleaves off the phosphate group from ATP and folds the molecule to form cAMP. cAMP, which is a

critical second messenger, will then bind to the PKA regulatory subunit, allowing for the release of the PKA catalytic subunit (PKAc) which will in turn phosphorylate specific substrates. Conversely, activation of D2 dopamine receptors inhibits adenylyl cyclase, resulting in PKA inhibition (Sibley & Monsma, 1992; Stoof & Kebabian, 1984). As mentioned previously, dopaminergic neurons synapse on striatal MSNs, which also receive excitatory glutamatergic inputs from cortex. As a result, activation or inhibition of PKA activity affects glutamate receptor phosphorylation (Tingley et al., 1997).

Cyclin-dependent kinase 5 (CDK5) is another important protein kinase that is believed to affect the cell cycle and play a role in cellular development. CDK5 is a misnomer, since it has been demonstrated that cyclins are not essential for its activity (Dhavan & Tsai, 2001). It is shown that removal of CDK5 expression in mice is fatal because of improper cortex formation. This effect was specific to central nervous system tissue since formation of other organs was unaffected (Ohshima et al., 1996). Moreover, studies suggest that CDK5 is known to regulate neurite growth in newly differentiated cells. CDK5 is also known to phosphorylate Tau, an important neuronal protein contributing to its aggregation in Alzheimer disease (Baumann, Mandelkow, Biernat, Piwnica-Worms, & Mandelkow, 1993).

Interestingly, CDK5 plays an important role in dopamine-signaling pathways. DARPP-32 phosphorylation by CDK5 inhibits PKA activity (Bibb et al., 1999). Protein phosphatase inhibitor 1 is also known to be phosphorylated by CDK5, which keeps PP1 in an active state and implicates CDK5 as a PKA antagonist (Bibb et al., 2001).

As stated before, phosphatases are diametrically opposed to kinases via their ability to dephosphorylate specific substrates. One of the well-known serine/threonine

phosphatases is PP1. This phosphatase is localized to dendritic spines in neurons and is an important regulator of synaptic function. PP1 catalytic subunits associate with >50 regulatory or targeting proteins (P. T. Cohen, 2002), usually via an R-V-x-F motif on the targeting protein (Ceulemans & Bollen, 2006; Meiselbach et al., 2006). Spinophilin is the major PP1 binding protein in the PSD (Colbran et al., 1997) that acts to target PP1 to myriad synaptic substrates (Allen, Ouimet, & Greengard, 1997). Moreover, PP1 is known to play a role in the down-regulation of AMPA receptors (Yan et al., 1999). The former study also shows that PP1-spinophilin dissociation leads to AMPAR dephosphorylation, allowing for decreases in channel activity. Interestingly, more recent studies have also shown that spinophilin can target PP1 to protein kinases such as CaMKII and this targeting increases in an age-dependent manner (Baucum, Strack, & Colbran, 2012). Together, these data suggest that proper synaptic formation and function depends on various kinase and phosphatase activity and the balance between their function.

1.5 NMDA Receptor Phosphorylation

Direct phosphorylation of ionotropic glutamate receptors plays a very integral role in regulating channel conductance, function, and receptor localization at synapses (Lee, 2006). NMDA receptor subunits are phosphorylated at serine/threonine residues by multiple kinases, including PKA, protein kinase B (PKB), protein kinase C (PKC), CDK5, CaMKII, and casein kinase II (CKII) (Mammen, Kamboj, & Huganir, 1999; Roche et al., 1994). In addition to kinases and phosphatases, *per se*, phosphorylation of synaptic proteins that modulate kinase or phosphatase targeting also regulate NMDAR phosphorylation (Lan

et al., 2001; Sigel, Baur, & Malherbe, 1994; Zheng, Zhang, Wang, Bennett, & Zukin, 1999).

PKC has multiple effects on NMDAR function, including increasing the opening rate and upregulating NMDAR surface expression, which in turn regulates NMDAR activity (Lan et al., 2001; W. Y. Lu et al., 1999). PKA also plays a role in mediating NMDAR function by enhancing the amplitude of NMDAR-mediated excitatory postsynaptic currents (EPSCs) (Raman, Tong, & Jahr, 1996). Consistently, PKA activation seems to increase synaptic targeting of NMDA receptors (Crump, Dillman, & Craig, 2001) along with increasing calcium permeability of NMDARs (Skeberdis et al., 2006).

GluN1, the obligate subunit of the NMDAR, is also phosphorylated by various protein kinases (PK). Studies suggest that phosphorylation of serine 890 disrupts GluN1 clustering (Tingley et al., 1997) while serine 896 phosphorylation by PKC has no effect on clustering of GluN1. However, phosphorylation of S896 together with PKA phosphorylation of S897 contributes to increase in NMDA receptor surface localization (D. B. Scott, Blanpied, Swanson, Zhang, & Ehlers, 2001). GluN2A can be phosphorylated by PKC, which leads to phosphorylation of S1291 and S1312 and potentiation of GluN2A-containing NMDARs (Grant, Guttmann, Seifert, & Lynch, 2001; Jones & Leonard, 2005). Phosphorylation of GluN2A at S1416 by PKC decreases the GluN2A binding affinity to CaMKII (Gardoni et al., 2001). CDK5 is another PK that also phosphorylates GluN2A, which contributes to an increase in NMDA receptor activity (B. S. Li et al., 2001).

GluN2B constitutes most of the NMDARs in most brain regions early in development (S. Cull-Candy et al., 2001). While GluN2B expression is attenuated as the animal matures, it remains in hippocampus, cortex, striatum and other brain regions into

adulthood. GluN2B containing NMDARs are located at both synaptic and extrasynaptic sites early in development. As development progresses, GluN2B becomes enriched at extrasynaptic sites (B. Li et al., 2002; Tovar & Westbrook, 1999). Moreover, GluN2B-containing NMDA receptors have higher surface mobility compared to GluN2A-containing NMDARs (Groc et al., 2006). Like GluN2A, GluN2B-containing receptors are also phosphorylated by PKC. Specifically, PKC phosphorylates GluN2B at S1303 and S1323 (Liao, Wagner, Hsu, & Leonard, 2001). Intriguingly, other studies show that S1303 of GluN2B is also a phosphorylation site for CaMKII (Omkumar, Kiely, Rosenstein, Min, & Kennedy, 1996). Phosphorylation of Serine 1303 by CaMKII modulates NMDAR function in a different way from phosphorylation of PKC of the same site. CKII is another PK that phosphorylates GluN2B on S1480, which is localized to the PDZ domain at the extreme C-terminus. Phosphorylation of this site disrupts the GluN2B/PSD95 interaction.

PKA also plays a role in GluN2B phosphorylation on S1166 (Murphy et al., 2014), which is critical in synaptic NMDAR function and Ca^{2+} signaling in spines. Along with this PKA site, Y1472 (Zhang, Edelmann, Liu, Crandall, & Morabito, 2008), S1116 (Plattner et al., 2014) and S1284 are recently characterized phosphorylation sites that are either indirectly or directly phosphorylated by CDK5 and can modulate NMDAR function (W. Lu et al., 2015). Together, these data suggest that phosphorylation of NMDA receptor subunits plays an integral role in proper signaling as well as normal synaptic connectivity.

1.6 Spinophilin Function and Localization

As mentioned above, many molecules, proteins, and enzymes including phosphatases and kinases underlie normal synaptic signaling in the PSD. One of highly conserved molecules

that has a wide range of activity in eukaryotes is PP1 (Mathieu Bollen, Peti, Ragusa, & Beullens, 2010). This ubiquitous protein phosphatase regulates a wide array of cellular processes through the association of its catalytic subunit with regulatory proteins (P. T. Cohen, 2002). The majority of these regulatory proteins are targeting or scaffolding proteins that can target PP1 to various substrates (P. T. Cohen, 2002). In 1997, a new protein was observed in PP1 immunoprecipitations of mouse brain lysates. This protein had a lot in common with another PP1 binding protein (Satoh et al., 1998) discovered before, referred to as "Neurabin" in their amino acid sequence and their PP1 binding ability (Egloff et al., 1997). The new PP1 binding protein known as Neurabin II got its new name, spinophilin, because of its abundance in neural spines (Allen et al., 1997). The spinophilin gene, PPP1R9B (gene ID 84687) is localized on chromosome 17, 17q21.33 and consists of 10 exons. Analysis of PP1 holoenzyme of rat brain shows that neurabin and spinophilin both associate with different isoforms of PP1 (MacMillan *et al*., 1999). Other studies show that this protein is not only enriched in synapses but also in cadherin cell-cell adhesion sites (Satoh et al., 1998).

This well characterized PP1 targeting protein has 817 amino acids and consists of multiple domains that mediate protein-protein interactions: including an N-terminal actin-binding domain, a PP1-binding domain, a PSD-95/discs large/zona occludens-1 (PDZ) domain, and a C-terminal coiled-coil region (Sarrouilhe, di Tommaso, Metaye, & Ladeveze, 2006). Looking closer at the PP1 binding domain reveals the fact that the primary binding site of PP1 on spinophilin is localized between amino acids 417-494, which contains a pentapeptide motif (R/K-R/K-V/I-X-F). This motif is between amino acids 447-451 and is conserved in other PP1 regulatory subunits (M. Bollen, 2001).

Spinophilin binding to F-actin anchors a pool of PP1 to the PSD, where it regulates glutamatergic neurotransmission and plasticity (Allen et al., 1997; Feng et al., 2000; Satoh et al., 1998; Yan et al., 1999). Spinophilin targets PP1 to specific neuronal substrates (Grossman et al., 2004; Ragusa et al., 2010; Sarrouilhe et al., 2006; Terry-Lorenzo et al., 2002). Spinophilin not only targets PP1 to dephosphorylate various substrates, but it can also inhibit PP1 activity towards certain substrates by binding tightly to PP1 and not allowing it to dissociate from spinohilin (Mathieu Bollen et al., 2010; Ragusa et al., 2010).

Various proteins have been shown to interact with spinophilin such as CaMKII. Spinophilin can interact with CaMKII and may play a role in targeting the CaMKIIα isoform to F-actin (Baucum et al., 2012; L. C. Carmody, A. J. Baucum, M. A. Bass, & R. J. Colbran, 2008; Terry-Lorenzo et al., 2005). Moreover, spinophilin can interact with F-actin and this interaction is modulated by spinophilin phosphorylation at the N-terminal, F-actin binding domain (Feng et al., 2000; Grossman et al., 2004; Hsieh-Wilson et al., 2003). Since both actin (Cingolani & Goda, 2008) and spinophilin are enriched in dendritic spines (Bordelon et al., 2005), their interaction may affect spine structure. Specifically, their interaction has been shown to affect spine maturation, synaptic plasticity, and spine maintenance (Feng et al., 2000; Nakanishi et al., 1997; Zito, Knott, Shepherd, Shenolikar, & Svoboda, 2004). One other study supports this and suggests that spinophilin aids in binding F-actin to the cell membrane (Satoh et al., 1998). Additionally, ion channels and various receptors such as NMDA and AMPA receptors are known to interact with spinophilin. The GluR1 subunit of the AMPAR (Yan et al., 1999) and GluN2B subunit of the NMDAR interact with spinophilin (Baucum et al., 2012). Moreover it is shown that

spinophilin can modulate glutamate receptor function through its PP1 targeting role (Hu et al., 2015; D. W. Li et al., 2006; Yan et al., 1999)

As stated above, spinophilin interacts with multiple synaptic proteins such as PP1, F-actin, NMDAR, AMPAR, and CaMKII. These interactions may affect normal synaptic function and spine morphology. Consistent with this hypothesis, studies using spinophilin knockout mice report that spinophilin is essential to normal spine structure and function (Allen et al., 1997) since a significant increase in spine density and alteration in filopodia formation is observed in spinophilin KO mice (Feng et al., 2000). Contrarily, spinophilin knockdown in hippocampal cultures causes decreases in dendritic spine density (Evans, Robinson, Shi, & Webb, 2015). Furthermore, spinophilin KO mice experience altered LTD (Allen et al., 2006), which, along with LTP, is considered to be essential for synaptic plasticity.

A decrease in PP1 activity is observed as a result of 6-OHDA lesion of the substantia nigra, an animal model of PD. This attenuation in activity can possibly be a result of an altered spinophilin interaction with PP1 (Ragusa et al., 2010). Interestingly, the PP1-spinophilin association is increased in a rat model of PD (Brown, Deutch, & Colbran, 2005). From these studies, it can be inferred that spinophilin interacts with multiple synaptic proteins in MSN dendrites, potentially playing a role in synaptic connectivity, plasticity and spine formation.

1.7 Hypotheses

Taken together, these data suggest that NMDAR/spinophilin association may be important in proper synaptic formation, functionality, and post-synaptic signaling.

Previous data, discussed above, (Baucum et al., 2012) show that NMDAR and spinophilin associate both in hippocampal and striatal lysates.

Phosphorylation of the NMDAR is very important in modulating its channel conductance and activity, which in turn affects post-synaptic signaling events, and brain function. Also, there are changes in phosphatase activity in animal models of PD. Our preliminary data demonstrate that spinophilin phosphorylation at a PKA and CDK5 site are enhanced in 6-OHDA lesioned animals, while spinophilin's association with both GluN1 and GluN2B subunit of NMDAR is decreased. Furthermore, NMDAR phosphorylation in a rat model of PD is decreased. Given the above background, *we hypothesize that kinase activity may modulate the spinophilin/NMDAR interaction and that spinophilin targets PP1 to the NMDAR to regulate NMDAR phosphorylation and function*. Given spinophilin's role as a synaptic scaffolding protein, it may also regulate NMDAR localization at synaptic and extrasynaptic sites. Consequently, alterations in spinophilin interactions may underlie pathologies associated with various neurodegenerative diseases, including PD. Overall, in this thesis, we have begun to characterize the spinophilin/NMDAR interaction and elucidate mechanisms that regulate this association.

2 METHODS

2.1 Generating DNA Constructs

2.2 Templates

In order to generate epitope-tagged forms of the synaptic proteins used in these studies, we first amplified cDNAs for spinophilin, GluN1, GluN2B, PKAc, CDK5, the CDK5 activator, p35, and the γ1 isoform of PP1 (PP1γ1). Templates used were: human spinophilin (a gift from Dr. Maria Vivo, University of Naples "Federico II"), mouse GluN1 (pCS6(BC039157); Transomic Technologies, Huntsville, AL), human GluN2B (BC113618; Transomic Technologies), human PKAc - pDONR223-PRKACA, human CDK5 - pDONR223-CDK5, p35 - pDONR223-CDK5SR1 (PKAc, CDK5, and p35 were gifts from William Hahn & David Root (Johannessen et al., 2010) (Addgene plasmid #s 23495, 23699, and 23779), and rat PP1γ1 (L. C. Carmody, A. J. Baucum, M. A. Bass, & R. J. Colbran, 2008).

2.3 PCR Reactions

PCR primers for the above cDNAs containing attB sites and either Shine-Dalgarno and Kozak sequences (for production of C-terminal tagged proteins), or a stop codon (for N-terminal tagged proteins) were synthesized. To create PCR products, PCR amplification

was performed using either Q5 DNA polymerase (New England Biolabs, Ipswich, MA) or VAPRase DNA polymerase (Vanderbilt University Medical Center, Nashville, TN), using manufacturers' recommendations. The PCR conditions were as follows: 1) a 2-minute initial denaturation at 98°C, 2) a 30-second denaturation at 98°C, 3) a 10-second annealing reaction at a primer-specific gradient temperature (see below), and 4) a 4-minute extension period at 72°C. Steps 2-4 were repeated 30 times for standard reactions. Annealing temperature was varied using a gradient with multiple ranges according to specific melting points of oligonucleotides (gradient usually ranged from 60-75°C). A final elongation step of 12 minutes was performed at 72°C. PCR products were mixed with 6x DNA loading dye (New England Biolabs) and separated on 1% agarose gels containing SYBR Safe (Life Technologies, Carlsbad, CA). Electrophoresis was performed for ~40-45 minutes at 80V. Amplification of the correct size DNA was confirmed by the appearance of a band of the appropriate size (e.g. 2445 base pairs for full-length spinophilin). Bands were subsequently excised from the gel and DNA was isolated using a DNA gel extraction kit (Zymo Research, Irvine, CA or ThermoFisher Scientific, Waltham, MA). DNA was generally eluted in molecular biology grade deionized water or Tris-EDTA (TE) buffer. The concentration was then quantified using the BioTek Cytation 3 system (BioTek Instruments, Inc. Winooski, VT). All vectors were then sequence verified (GENEWIZ, Inc., South Plainfield, NJ).

2.4 Gateway BP Cloning

The PCR product for each DNA construct was combined with donor vector (pDONR 221) using BP Gateway cloning technology from Life Technologies. The PCR product (20-

50 fmol) was used in each reaction along with 150 ng of plasmid vector and BP Clonase II enzyme mixture. Reactions were performed for 1 hour to overnight at 25°C according to manufacturer's protocol. Mixtures were then treated with proteinase K at 37°C for 10 minutes. Transformation was then carried out by use of 10 µL of reaction mixture to transform DH5α competent *E. coli* from New England Biolabs. Cells were plated on Luria broth (LB) agar with kanamycin antibiotic and incubated overnight at 37°C. Individual bacterial colonies were picked and placed into 8mL liquid LB cultures with kanamycin and incubated overnight at 37°C with shaking.

The next day, cells were centrifuged for 15 minutes at 4000 x g and lysed. DNA purification from the lysate was performed using miniprep purification kits (Zymo Research or Thermo Scientific). The concentration of DNA was then quantified using the BioTek Cytation 3 system (BioTek Instruments, Inc.). A diagnostic digestion was then performed on 0.5-2 µg of the resulting DNA using appropriate restriction enzymes and then separated on a 1% agarose gel for validation of proper insertion. Gels were imaged on a Bio-Rad Gel Doc EZ (Bio-Rad Laboratories, Inc. Hercules, CA). Successful BP recombination was confirmed by appearance of appropriate bands and samples were then further validated by sequencing (GENEWIZ, Inc). Sequence verified samples were used for LR recombination.

2.5 Gateway LR Cloning

LR recombination was used to generate proteins with different epitope tags. For mammalian protein expression, pcDNA3.1 destination vectors with either HA, V5, myc, or FLAG tags were used. LR reactions were performed using manufacturer's

recommendation. Specifically, 150ng of donor vector containing the intended DNA was incubated with 150 ng of the appropriate destination vector, at 25°C for 1 hour. LR reaction was then followed by Proteinase K digestion at 37°C for 10 minutes. Competent DH5α *E. coli* were transformed with 1-5 µL of the reaction mixture. The cells were then plated on LB-containing plates in the presence of ampicillin and incubated at 37°C overnight.

Bacterial colonies were excised after overnight growth and cultured in 8mL liquid LB cultures with ampicillin. DNA was then extracted as described above and confirmed via restriction digest. If appropriate DNA banding patterns were present, additional bacterial colonies were selected from the plate and were cultured in larger (50-250 mL) cultures for maxipreps (Zymo Research or Thermo Scientific). DNA was re-screened via restriction digestion. All original empty DNA vectors used in BP or LR cloning were obtained from Life Technologies.

2.6 Mutagenesis PCR

In order to generate point mutations, mutagenesis reactions were performed using QuikChange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA). Reactions were carried out using Q5 DNA buffer and 1µl DNA polymerase in the presence of 5 µM DNTPs and 10 ng of template DNA. The following reaction protocol was performed: 1) an initial denaturation of 98°C for 2 minutes, 2) a 45-second denaturation at 98°C, 3) a 1-minute annealing reaction at a primer-specific temperature, 4) a 15-minute elongation at 68°C. Steps 2-4 were repeated 18 times. To eliminate template DNA, 10 µl of each reaction mixture was digested using 1µL of DpnI for ~2 hours at 37°C. Each mixture was then incubated with 6X loading dye and separated on a 1% agarose gel. If a high-intensity (i.e.

equal to or greater than the DNA ladder), proper molecular weight band was visualized, then 1 µL of the PCR product was transformed in competent DH5α-derived *E. coli*. Vectors were then sequence verified (GENEWIZ, Inc.) for the mutation.

2.7 Mammalian Protein Expression

Human embryonic kidney cells (HEK293) were used for mammalian protein expression. Cells were typically stored long-term in liquid nitrogen and thawed at 37°C when needed. Cell incubation and growth was performed in Dulbecco's modified Eagle's medium (DMEM) that contained 10% FBS, 584 mg/L L-glutamine, 1mM Sodium Pyruvate, 100 U/mL penicillin and 100µg/mL streptomycin. 50mm culture flasks were incubated at a constant 37°C and 5% CO_2 (Panasonic Healthcare; Secaucus, NJ).

Cells were counted and approximately 1,000,000 cells were plated into 25 mm flat-bottomed culture flasks and left for overnight growth. Typically, cells were transfected the next day at ~70-80% confluency. Confluency was measured by estimating cell coverage on the bottom of the flask. Depending on expression level of each protein, an appropriate amount of DNA was transfected (0.5 - 5 µg per DNA). The appropriate amount of DNA was added to 250 µL of serum-free DMEM in a 1.7mL microcentrifuge tube. In a separate microfuge tube, transfection reagent (Lipofectamine ,Life Technologies or PolyJet reagent SignaGen Laboratories Rockville, MD) was added to an additional 250 µL of serum-free DMEM. Polyjet and Lipofectamine were both used in a 3:1 volume:mass ratio (e.g. 18 µL of Polyjet was used with 6 µg DNA). For each experiment, DNA concentrations were equalized using an empty DNA vector, so that each condition in the same experiment had an equal mass of DNA and equal amount of transfection reagent. The transfection reagent

containing mixture was then added to the tube containing DNA and incubated at room temperature for 15 minutes. The entire mixture was then added to the proper flask and cells were incubated overnight.

Following overnight incubation, cell adherence to flask bottoms was examined. If there was little to no disruption of adherence of the cells, the DMEM was aspirated off and cells were washed with 6mL of cold phosphate-buffered saline (PBS). PBS was aspirated off and cells were lysed in 1.5 mL KCl lysis buffer then transferred into 2 mL microcentrifuge tubes. If a high percentage of cells were unattached, they were re-suspended in DMEM, then transferred to 15 mL centrifuge tubes and centrifuged at 250 x g for 5 minutes. After aspiration of media, 6 ml of cold PBS was added to cells and the pellet triterated, which was followed by an additional centrifugation. PBS was then aspirated and cells were lysed in KCl lysis buffer (150 mM KCl, 1 mM DTT, 2 mM EDTA, 50 mM Tris-HCl pH 7.5, 1% (v/v) Triton X-100, 20 mM NaF, 20 mM β-glycerophosphate, 20 mM $NaVO_3$, 10 mM Na pyrophosphate, 1X Halt protease inhibitor cocktail; Thermo Scientific Waltham, MA). Cells were sonicated at 25% amplitude for 15 seconds at 4°C using a probe sonicator (Thermo Scientific) and centrifuged (4°C for 10 minutes at 14,000 x g). Cell lysates were then used for immunoprecipitations.

2.8 Tissue Homogenization

Male or female, WT, C57Bl6 or spinophilin knockout mouse (Jackson laboratories, Bar Harbor, ME) brains were dissected. Forebrain tissue was flash-frozen in liquid nitrogen. Half of frontal cortex was homogenized in 2mL of isotonic RIPA buffer containing 1% Triton X-100 buffer using fifteen up-and-down movements of a pestle in a

2 mL tight-fitting glass homogenizer. Tissue homogenate was then transferred to a 2 mL microcentrifuge tube and processed in the same manner as HEK293 cells described above. Tissue was then brought up to 4 mL using the RIPA buffer. IP pulldowns were then performed as described below.

2.9 Immunoprecipitations (IPs)

HEK293 cell lysate or brain homogenate were transferred to a microcentrifuge tube for IPs (400-500 µl) or for a total input (75 µl). For the input, 25 µL of 4X sample buffer (0.2 M Tris HCl pH 6.8, 40% glycerol, 0.1 M DTT, 8% SDS w/v, 0.04% bromophenol blue w/v in water) was added to each input sample, vortexed and stored at -20°C. For the IPs, the appropriate IP antibody was added and incubated at 4°C for approximately 1 hour. Antibodies used for IPs were: rabbit monoclonal anti- spinophilin (E1E7R, 14136, Cell Signaling technology, INC.), goat polyclonal anti-Neurabin II (A-20, SC14774, Santa Cruz Biotechnology, INC.), rabbit monoclonal anti-NMDAR2B (D15B3, 4212, Cell Signaling technology, INC.) goat polyclonal anti-V5 tag (A190-119A, Bethyl Laboratories, Inc.), rabbit polyclonal anti V5 tag (D3H8Q, 13202, Cell Signaling technology, INC.), goat polyclonal anti-HA tag (A190-107A, Bethyl Laboratories, Inc. Montgomery, TX), goat polyclonal anti-Myc tag (A190-104A, Bethyl Laboratories, Inc. Montgomery, TX), and mouse polyclonal anti-PP1 (E-9, sc-7482, Santa Cruz Biotechnology, INC.). After 1-hour incubation of IP antibodies with samples, 30 µL of protein G magnetic beads that had been previously washed in IP buffer (50 mM Tris HCl, 150 mM NaCl, 0.5% Triton X-100) was added to each sample and incubated rotating overnight at 4°C.

Following incubation, samples were magnetically separated and washed three times with IP wash buffer. Then 40 µL of 2x sample buffer (4x buffer diluted 1:2 with Milli-Q water) was added to each of the samples, vortexed and stored at -20°C until they were analyzed by western blot.

2.10 SDS-PAGE and Western Blotting

Cell lysates or protein IPs were then used for western blotting. All samples were heated at 70°C for 10 minutes, then IP samples were placed on a magnet prior to loading in order to separate magnetic beads out of suspension. 5 µL of each input and 10 µL of each sample was loaded onto a 26-well, pre-cast Criterion 4-15% polyacrylamide gradient gel (Bio-Rad), a 15 well 4-15% Mini-Protein TGX polyacrylamide gradient gel (Bio-Rad), or a 1.5 mm hand-cast 10% polyacrylamide gel. Hand-cast gels were generally run at 75 V for 15 minutes and 175 V for approximately 1 hour and the precast gels were run typically at 165 V for 1 hour. Proteins were transferred to nitrocellulose membranes using one of two transfer methods.

For a full wet transfer, proteins were transferred to a nitrocellulose membrane using an N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) transfer buffer (10% MeOH, 0.01 M CAPS pH 11). The transfer was performed in a transfer tank attached to a water-cooling unit set at 2.5-4°C and transfer occurred at a constant 1.0 Amps for 1.5 hours.

For semi-dry transfer, a Trans-Blot Turbo was used. Gels were transferred to nitrocellulose using a cold TransBlot Turbo transfer buffer with 20% ethanol. Transfers were performed using the TransBlot Turbo system (Bio-Rad) at a voltage of 9V for 30 minutes.

After transferring, membranes were placed in blotting boxes. An optional Ponceau S stain for 10 minutes was often used to confirm protein transfer and to evaluate equal loading. Following staining, membranes were washed with deionized water and scanned. Membranes were blocked using 5% (w/v) nonfat dry milk in 0.1% (v/v) Tween-20 in 1 M Tris-buffered saline pH 7.5 (TBST). Blocking was performed 3 times, 10 minutes each, for total of 30 minutes. After the final blocking step, the TBST-milk was replaced with primary antibodies diluted in 5% milk in TBST and incubated overnight at 4°C with shaking. Primary antibodies used were: a rabbit polyclonal anti-V5 (G-14, sc-83849, Santa Cruz Biotechnology, INC), a goat polyclonal anti-HA (A190-107A, Bethyl Laboratories, Inc. Montgomery, TX), a goat polyclonal anti-PP1γ (sc-6108 Santa Cruz Biotechnology), a mouse monoclonal anti-PP1 α (E-9, sc-7482, Santa Cruz Technology, INC), a rabbit polyclonal anti phosphor-NMDAR2B-Ser1284 (5355, Cell Signaling Technology) and a mouse monoclonal anti-Myc (9E10, sc-40, santa Cruz Technology, INC). After incubation, membranes were washed 3 times for 10 minutes per wash with TBST containing 5% milk. Following the washes, appropriate secondary antibodies were added to the membranes. Secondary antibodies used were: Alexa Fluor 790-conjugated AffiniPure Donkey Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, INC.), Alexa Fluor 790-conjugated AffiniPure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, INC.), Alexa Fluor 790-conjugated AffiniPure Donkey Anti-Goat IgG (Jackson ImmunoResearch Laboratories, INC.), Alexa Fluor 680-conjugated donkey anti-Goat (Molecular Probes, Invitrogen detection technologies) and Alexa Fluor 680-conjugated donkey anti-Rabbit (Molecular Probes, Invitrogen detection technologies). Jackson ImmunoResearch antibodies were typically diluted 1:50000 in 5% milk and Invitrogen antibodies were

generally diluted 1:10000 in 5% milk. Secondary antibodies were incubated with membranes for 60 minutes at room temperature in darkness with shaking. After proper incubation, secondary antibodies were discarded and membranes were washed three times with Tris-Buffered saline without Tween for 10 minutes in each wash. Fluorescence scans were performed using the Odyssey imaging system and data analysis was done using Image Studio software (LiCor, Lincoln, NE).

2.11 In-vitro Kinase Activation

To activate endogenous PKA in HEK293 cells, 500 µM forskolin in dimethylsulfoxide (DMSO), and 5 mM 3-isobutyl-1-methylxanthine (IBMX) were used. After transfection and overnight incubation, DMEM was removed and replaced with DMEM containing forskolin and IBMX (1:1000 dilution) or vehicle alone. Cells were then incubated for 1 hour for short-term activation or for 24 hours for long-term activation. At the end of incubation, cells were processed immediately as described above.

2.12 Mass Spectrometry

Samples collected from SDS-PAGE were digested using trypsin and analyzed by mass spectrometry. All the samples were analyzed in the laboratory of Dr. Lisa Jones, Department of Chemistry and Chemical Biology, IUPUI. Raw data from mass spectrometry were searched against the human database using Mascot algorithm and Proteome Discoverer (Thermo Scientific). Magellan Storage output files (MSF) were imported into Scaffold 4 (Proteome Software, Portland, OR). MS/MS spectra of tryptic fragments matching specific phosphorylation sites were validated and the area under the curve (AUC) of the extracted ion chromatogram (XIC) was calculated for the

phosphorylated peptide. These AUCs of the XICs were normalized to a control, non-phosphorylated peptide AUC of the XIC to create a phosphorylation ratio. The generated ratios were compared across different groups, as previously described (Baucum, Shonesy, Rose, & Colbran, 2015).

2.13 Statistical Analyses

Image Studio software was used for quantification of the integrated fluorescence intensities detected in the western blots. To calculate associations, we divided the integrated fluorescence intensity for the co-immunoprecipitated protein by the integrated fluorescence intensity for the immunoprecipitated protein. In order to normalize for any differences in protein expression, we took the above normalized value and divided it by the input value for the co-immunoprecipitated protein. To compare different conditions across gels, we normalized the above ratio from the experimental condition by the ratio generated on the same gel for a control condition. As an example, to determine if PKA overexpression modulates the association between spinophilin and $GluN2B_{Tail}$, we performed the following measurements and calculations. First, we divided the fluorescence intensity of the V5-tagged GluN2B protein that is present in the HA-tagged spinophilin immunoprecipitates by the fluorescence intensity of the HA-tagged spinophilin in these same IPs. We would then normalize this value to the fluorescence intensity of the V5-tagged GluN2B protein that is expressed in the input sample. This calculation would be performed for both the sample without PKA overexpression and the sample with PKA overexpression. The value from the PKA-containing sample would then be divided by the PKA-absent sample to generate a ratio. The formula for this ratio is

EXPERIMENTAL((Intensity co-IP $protein_{Precipitate}$/Intensity IP $protein_{Precipitate}$)/(Intensity co-IP $protein_{input}$))/CONTROL((Intensity co-IP $protein_{Precipitate}$/Intensity IP $protein_{Precipitate}$)/(Intensity co-IP $protein_{input}$)). This ratio was averaged across multiple transfections, with each transfection corresponding to a unique biological replicate. The N values for each individual experiment correspond to the number of unique biological replicates. To compare between groups, a one-column t-test was performed to compare the experimental to a theoretical value of 1. If more than two groups were compared, a one-way ANOVA was used to determine significance. This was followed by a Tukey-posthoc test. Where appropriate, a two-way ANOVA was used to compare across two different treatment conditions (e.g. Figure 10)

3 RESULTS

3.1 Spinophilin Associates with GluN1 in a Heterologous Cell System

Studies show that GluN1 subunits are obligatory subunits necessary for functional expression of the NMDA receptors (S. Cull-Candy et al., 2001). The PDZ domain of spinophilin can associate with multiple subunits of the NMDA receptor such as GluN1 and GluN2B (Baucum, Brown, & Colbran, 2013; Kelker et al., 2007). A recent proteomics study identified changes in multiple synaptic proteins isolated from spinophilin immunoprecipitates from mice with a 6-OHDA lesion of the substantia nigra (Hiday and Edler et al., Manuscript in preparation). Specifically, the association of GluN1 and GluN2B following 6-OHDA lesion is decreased following lesion. However the mechanisms that modulate the spinophilin/NMDAR interaction are unclear. To further study the GluN1/spinophilin interaction, we aimed to use a heterologous cell system. Consequently, we overexpressed V5-tagged GluN1 in a HEK293 cell system alongside HA-tagged spinophilin. 48-hours post transfection, cells were lysed using KCl buffer. Immunoblot results show an interaction between spinophilin and GluN1 in HEK293 cells in both GluN1 and spinophilin immunoprecipitates (Figure 1).

3.2 Spinophilin Associates with the GluN2B subunit of NMDARs in mouse brain lysates and with C-terminal tail of GluN2B Subunit of NMDAR in HEK293 Cells

Recent data have shown that spinophilin and GluN2B subunit of NMDAR co-immunoprecipitate from striatal lysates (Baucum et al., 2013). To further probe the interaction between spinophilin and GluN2B, we immunoprecipitated spinophilin or GluN2B from brain lysates. Our results show that spinophilin pulls down GluN2B subunit of NMDAR along with it. Moreover, we were able to detect spinophilin in GluN2B IPs (Figure 2A). To further investigate the specificity of this interaction, we used spinophilin knockout mice and performed spinophilin and GluN2B IPs from spinophilin KO mice brain. Two different spinophilin antibodies (Goat polyclonal and rabbit monoclonal) were used for spinophilin IP. Results show no spinophilin band in inputs suggesting a successful spinophilin knockout. Additionally, IP of spinophilin from mice brain, did not bring down any GluN2B along with it, suggesting that the interaction observed between spinophilin and GluN2B in previous experiment is specific (Figure 2B). Furthermore, since it is easier to manipulate, we used heterologous cell system to further study of the interaction in HEK293 cells. For this purpose, we first wanted to determine where spinophilin binds to the GluN2B subunit of the NMDAR. GluN2B alone cannot traffic to the membrane and tends to become trapped in the ER (Das et al., 1998); however, the C-terminal intracellular tail domain of human GluN2B ($GluN2B_{Tail}$; amino acids 839-1484) is not trapped in the ER and is localized to the cytosol. To determine if spinophilin interacts with the $GluN2B_{Tail}$, HA-tagged spinophilin and V5-tagged $GluN2B_{Tail}$ were overexpressed in a heterologous cell line (HEK293FT cells) and subsequently immunoprecipitated. Both inputs and immunoprecipitates were separated by SDS-PAGE and immnoblotted for

GluN2B$_{Tail}$ and spinophilin. We detected GluN2B$_{Tail}$ in spinophilin immunoprecipitates and spinophilin in GluN2B$_{Tail}$ immunoprecipitates (Figure 3).

3.3 Spinophilin Interacts with GluN2B Residues 839-1088

Given the robust interaction between spinophilin and the C-terminal tail of GluN2B, we wanted to specify the motif on the GluN2B$_{Tail}$ that interacts with spinophilin. We generated V5-tagged GluN2B$_{Tail}$ fragments containing amino acids 839-1088, 1038-1484 and 1268-1484. HEK293 cells were transfected with V5-tagged GluN2B$_{Tail}$ fragments and HA-tagged spinophilin. Since the expression level of the fragments was not robust, we also transfected 1μg of Myc-tagged PKA in all the conditions (see below). Cells were lysed 24-hours post transfection and samples were submitted to SDS-PAGE. Qualitative data show a robust interaction of HA-tagged spinophilin with the first fragment of GluN2B$_{Tail}$, 839-1088 (Figure 4). We also intended to identify the domains of spinophilin that interact with GluN2B$_{Tail}$. For that purpose we generated different spinophilin fragments; however, we could not identify specific fragments that associate with GluN2B$_{Tail}$ suggesting that a larger piece or the full-length spinophilin protein is required for interaction (Data not shown).

3.4 Overexpression of PKA in HEK293 Cells Increases Spinophilin and NMDAR Interaction

Previous studies from the laboratory have demonstrated that dopamine depletion decreases the association between spinophilin and the NMDAR (Hiday and Edler et al., Manuscript in preparation). Dopamine depletion modulates protein kinase A (PKA) activity in striatal MSNs. As mentioned previously, phosphorylation of PKA sites on spinophilin are increased in an animal model of PD (Hiday and Edler et al., Manuscript in

preparation). Therefore, we aimed to determine if the catalytic subunit of PKA (PKAc) can directly regulate the interaction between spinophilin and the NMDAR. V5-tagged GluN1 or V5-tagged GluN2B$_{Tail}$ and HA-tagged spinophilin were co-expressed in HEK293 cells alone or alongside myc-tagged PKAc. Qualitatively, results show a robust increase in the association between spinophilin and GluN1 in the presence of PKAc (Figure 5A). There was a trend for an increase in spinophilin isolated from the GluN1 IPs, P value = 0.07 (Figure 5C). Moreover, GluN1 levels were significantly increased in spinophilin IPs (Figure 5B).

Overexpression of PKAc also increased the association of spinophilin and GluN2B$_{Tail}$ (Figure 6A). Quantified data show a significant increase of GluN2B$_{Tail}$ in the spinophilin IP (Figure 6B) as well as spinophilin in the GluN2B$_{Tail}$ IP (Figure 6C) in the presence compared to the absence of overexpressed PKAc.

3.5 Long-term Activation of Endogenous PKA Increases the Interaction between Spinophilin and the NMDAR

To further validate a catalytic role of PKA in modulating the spinophilin/NMDAR association, we measured the interaction of spinophilin and the NMDAR when endogenous PKA was activated. IBMX and forskolin were used to pharmacologically activate endogenously expressed PKA in HEK293 cells over a period of 16-20 hours. V5-tagged GluN1 or V5-tagged GluN2B$_{Tail}$ were transfected alone or together with HA-tagged spinophilin and cells were incubated for 24 hours. Then the cells were treated with IBMX/forskolin in DMSO or in DMSO alone for another 24 hours to activate PKA. Qualitatively, long-term activation of PKA increased the spinophilin-GluN1 interaction

(Figure 7A). Quantified data show a significant increase in levels of GluN1 in HA IPs when normalized to GluN1 in the inputs (Figure 7B). V5 IPs were not quantified due to background fluorescence. As with GluN1, activation of endogenous PKA increased the association of spinophilin with $GluN2B_{Tail}$ (Figure 8A). Quantitatively, $GluN2B_{Tail}$ and spinophilin levels were significantly increased in the HA and V5 IPs, respectively (Figure 8B, 8C). All the values were normalized to levels of non-immunoprecipitated inputs (See methods for description of quantification). Normally, the NMDAR is organized with two GluN1 and two GluN2 subunits. In order to have a better understanding of the effect of PKA on the association of spinophilin with a functional NMDAR, V5-tagged GluN1, Myc-tagged GluN2B and HA-tagged spinophilin were co-expressed in a heterologous cell system with or without endogenous PKA 24-hour activation. Results suggest an increase in the association of GluN1-GluN2B complex with spinophilin when accompanied with PKA overexpression (Figure 9).

3.6 Overexpression of CDK5 in HEK293 Cells Decreases Spinophilin and GluN2BTail Interaction

In addition to PKA, CDK5 activity is known to be regulated in animal models of PD. We found that spinophilin phosphorylation at Ser17, a CDK5 site (Futter et al., 2005), was increased in an animal model of PD (Hiday and Edler et al., manuscript in preparation). To determine the effect of CDK5 on modulating the spinophilin/$GluN2B_{tail}$ association, V5-tagged $GluN2B_{Tail}$ and HA-tagged spinophilin were co-expressed in HEK293 cells alone or alongside FLAG-tagged CDK5 and the CDK5 activator, p35. Quantitatively, there was a robust decrease in the association between spinophilin and $GluN2B_{tail}$ in the presence

of CDK5 (Figure 10A-B). V5 IPs were not quantified due to low expression levels in the co-IP.

3.7 Phosphorylation of Ser17 on Spinophilin is not Responsible for the Decrease in Spinophilin- GluN2BTail Interaction

As previously mentioned, CDK5 decreases spinophilin-GluN2B$_{Tail}$ interaction. Studies show that CDK5 can phosphorylate multiple sites on spinophilin and GluN2B. For instance, Serine 17 on spinophilin (Futter et al., 2005) and Serine 1284 on GluN2B are CDK5 phosphorylation sites (W. Lu et al., 2015). Together, these data suggest that phosphorylation on either S17 or spinophilin or S1284 on GluN2B regulate spinophilin-GluN2B interaction. To begin to test this hypothesis, we generated phospho-mimetic (S17D) and phospho-deficient (S17A) mutants of spinophilin. Ser to Ala and Ser to Asp mutants cannot be phosphorylated (Ala) or mimic a charged (i.e. phosphorylated) form (Asp) of a protein (Bornancin & Parker, 1997). In order to investigate the effect of S17 mutation on spinophilin-GluN2B$_{Tail}$ association, S17A and S17D mutants along with GluN2B$_{Tail}$ were overexpressed in HEK293 cells in presence or absence of over expressed CDK5. Results suggest that, S17 phosphorylation alone does not have an effect on the association. Moreover, the S17A mutant when accompanied with CDK5 overexpression still has a decreased association with GluN2B. Two-way ANOVA shows a significant effect of CDK5 overexpression ($P<0.0001$) while no significant effect of S17 genotype was observed (Figure 11A, B).

3.8 Overexpression of CDK5 in HEK293 Cells Increases Spinophilin and PP1 Interaction

Given the PP1 targeting role of spinophilin, modulating the spinophilin/PP1 association may also have implications in substrate phosphorylation. Recent studies indicate that PP1 can be phosphorylated by CDK5 on Thr320, which is known to have an inhibitory role on PP1 activity (Hou et al., 2013). As indicated previously, spinophilin is also known to be phosphorylated by CDK5 (Futter et al., 2005). As a consequence we intended to look at the effect of CDK5 on the PP1/spinophilin association. We overexpressed HA-tagged spinophilin in HEK293 cells along with FLAG-tagged CDK5 and Myc-tagged P35. We did not overexpress PP1 since PP1 is robustly expressed in HEK293 cells. Lysates were immunoprecipitated for HA (spinophilin). IPs were blotted for PP1 or spinophilin. Qualitatively, there was a robust increase in the association between spinophilin and PP1 in the presence of CDK5 (Figure 12A). Quantitative measurements showed a three-fold increase in the PP1-spinophilin association in presence of CDK5 (Figure 12B).

3.9 Spinophilin Decreases the Association of PP1 with GluN2BTail

Spinophilin is known to regulate NMDAR function as spinophilin KO mice have altered NMDAR kinetics (Allen et al., 2006; Feng et al., 2000). Moreover, spinophilin is known to bind to PP1 to alter its targeting to specific substrates. To identify potential mechanisms by which spinophilin modulates the PP1/NMDAR interaction, we evaluated PP1 levels in $GluN2B_{Tail}$ immunoprecipitates. V5-tagged $GluN2B_{Tail}$ and Myc-tagged PP1γ1 were co-overexpressed in HEK293 cells in presence and absence of overexpressed

HA-tagged spinophilin. Lysates were immunoprecipitated with the appropriate antibody (see methods). Results revealed that PP1γ1 associated with GluN2B$_{Tail}$ in the absence of spinophilin, suggesting that PP1 can associate with GluN2B$_{Tail}$ directly or via a HEK-cell expressed targeting protein (Figure 13A). In contrast to a putative role for spinophilin targeting PP1 to the NMDAR, overexpression of spinophilin dramatically decreased the abundance of PP1 bound to GluN2B$_{Tail}$ (Figure 13A). This suggests that spinophilin traffics PP1 away from GluN2B$_{Tail}$ or that spinophilin can displace PP1 directly from GluN2B$_{Tail}$. While spinophilin can associate with GluN2B$_{Tail}$, only a very small fraction is probably bound. Quantified data demonstrate a ~84% decrease in the levels of PP1γ1 in the GluN2B$_{Tail}$ immunoprecipitates (Figure 13B). Consistent with this, there was a ~75% decrease in the levels of GluN2B$_{Tail}$ in the PP1γ1 immunoprecipitates (Figure 13C).

3.10 Overexpression of PP1 Binding Deficient Mutant Spinophilin (F451A) Does Not Affect PP1 Binding to GluN2BTail

The spinophilin-dependent regulation of GluN2B$_{Tail}$ binding to PP1γ1 may be due to either spinophilin binding to the same location on GluN2B$_{Tail}$ where PP1γ1 is bound, or it could be that unbound spinophilin is competing for PP1. To test this, we utilized a PP1-binding deficient mutant (F451A). Studies have shown that the PP1 binding motif on spinophilin is located between residues 447-451 and an F451A mutation of spinophilin abolishes the spinophilin-PP1 interaction (Hsieh-Wilson, Allen, Watanabe, Nairn, & Greengard, 1999). We overexpressed WT and F451A mutant spinophilin with or without PP1γ1 overexpression. According to our results, spinophilin association with HEK293 cell endogenous PP1 is completely abrogated when spinophilin in mutated at 451 residue

(Figure 14A), while interaction of this mutant to PP1γ1 is attenuated, but not completely abrogated (Figure 14B). Subsequently, HEK293 cells were transfected with V5-tagged $GluN2B_{Tail}$ and Myc-tagged PP1γ1 with either HA tagged WT spinophilin or MU spinophilin. As in Figure 13, WT spinophilin decreased PP1 bound to $GluN2B_{Tail}$; however, F451A mutant spinophilin had no effect on the association of PP1γ1 with $GluN2B_{Tail}$ (Figure 15 B). Quantitatively, there was a significant effect of spinophilin on the PP1γ1 association with GluN2B tail (One-way ANOVA $p < 0.05$). Specifically, the WT, but not the mutant, spinophilin caused a decreased association of $GluN2B_{Tail}$. This altered association between PP1 was not due to differences in binding of WT and F451A spinophilin, as if anything, the mutant form of spinophilin bound more $GluN2B_{Tail}$.

3.11 Spinophilin Rescues PP1-dependent Dephosphorylation of S1284 on GluN2BTail

To determine functional implications of modulating PP1 targeting to $GluN2B_{Tail}$, we transfected V5-tagged $GluN2B_{Tail}$ along with Myc-tagged PP1γ1 with and without HA-tagged spinophilin. We also overexpressed Myc-tagged PKAc in order to enhance PKA phosphorylation of $GluN2B_{Tail}$. HEK293 cells were incubated 16-24 hours after transfection and were lysed using KCl buffer followed by immunoprecipitation using appropriate antibodies (see methods). SDS-PAGE was performed and the gel was stained with Coomassie Brilliant Blue protein stain. Samples were then prepared for mass spectrometry following the procedure explained in section 2.7. According to MS/MS results, several PKA phosphorylation sites were observed, some of which were previously known like Serine 1303 (Figure 16D) and some were identified during this experiment such as Serine 929/930 (Figure 16A), Serine 940 (Figure 16B), and Serine 1050 (Figure

16C). In addition to these sites, we identified serine 1284 on GluN2B$_{Tail}$, which is highly phosphorylated endogenously and is not sensitive to PKA expression. Interestingly, overexpression of PP1γ1 decreased S1284 phosphorylation on GluN2B$_{Tail}$ (Figure 17A). Overexpression of PKA along with PP1γ1 does not rescue this decreased phosphorylation, suggesting that this is not a PKA sensitive site, consistent with other data suggesting it is a CDK5 site (W. Lu et al., 2015). Interestingly, co-expression of spinophilin along with PKA and PP1γ1 attenuated the PP1-dependent decrease in S1284 phosphorylation (Figure 17A). To validate that spinophilin can modulate S1284 phosphorylation, we used phosphorylation-specific antibodies. HEK293 cells were transfected with V5-tagged GluN2B$_{Tail}$ and Myc tagged PP1γ1 in presence or absence of HA-tagged spinophilin. Cells were lysed 24-hours post transfection, and were separated using SDS-PAGE. Phospho-NMDAR2B-Ser1284 antibody was used for blotting. The phospho signal in the GluN2B$_{tail}$ IP was normalized to the total NMDAR level in the GluN2B$_{tail}$ IP. Preliminary data (N = 2) suggest a decrease of GluN2B$_{Tail}$ phosphorylation at S1284 in presence of PP1. Moreover, spinophilin expression rescues this decrease in phosphorylation (Figure 17 B, C)

4 DISCUSSION

4.1 Spinophilin Interacts with GluN1 and GluN2B Subunit of NMDARs and This Interaction is Decreased in 6-OHDA Treated Mice

NMDARs are tetramers in which two essential GluN1 subunits with two GluN2 and/or one GluN2 and one GluN3 subunit assemble together. GluN1 subunits are essential for expression of a functional channel (S. Cull-Candy et al., 2001). Studies suggest that the GluN1/GluN2 heterodimer is the functional unit in NMDARs. As mentioned previously, spinophilin can affect the phosphorylation state of various substrates by targeting PP1 to them (Grossman et al., 2004; Ragusa et al., 2010; Terry-Lorenzo et al., 2002) or inhibiting PP1 activity towards certain substrates by binding tightly to PP1 (Mathieu Bollen et al., 2010; Ragusa et al., 2010). Therefore, we evaluated the association between spinophilin and various subunits of NMDARs. Previous studies have shown that spinophilin can interact with GluN1 and GluN2B subunit of NMDARs both in vivo (Baucum et al., 2013) and in vitro (Kelker et al., 2007). Preliminary data from the laboratory shows spinophilin/GluN1 and spinophilin/GluN2B interaction in mouse brain lysate. Moreover spinophilin/GluN1, spinophilin/GluN2B and spinophilin/GluN1/GluN2B interactions

were observed in HEK293 cells where an isotonic KCl-containing buffer was used to mimic an isotonic brain environment.

6-OHDA is a chemical compound known to lesion nigral projections to striatum and mimic PD (Simola, Morelli, & Carta, 2007). Preliminary data from our lab suggest a decrease in the spinophilin/GluN1 and spinophilin/GluN2B interaction in 6-OHDA treated mice (Hiday and Edler et al., Manuscript in preparation). This change in interaction may be due to alterations in phosphorylation of spinophilin and/or the NMDAR. Previous studies show that application of 6-OHDA reduces NMDAR phosphorylation at some residues and increases its phosphorylation at other amino acids; however, the overall phosphorylation of GluN1 and GluN2B is strikingly increased (Dunah et al., 2000; Koutsokera, Kafkalias, Giompres, Kouvelas, & Mitsacos, 2014). Furthermore, spinophilin phosphorylation on Ser17 and Ser100 is increased in an animal model of PD (Hiday and Edler et al., Manuscript in preparation). All in all, DA depletion alters NMDAR and spinophilin phosphorylation status, which can possibly regulate the spinophilin/NMDAR interaction. Given the role of spinophilin in targeting PP1 to myriad substrates, the change in NMDAR/spinophilin interaction may have implications in the phosphorylation status of the NMDAR.

4.2 Spinophilin Interacts with Residues 839-1088 of C-Terminal Tail of GluN2B Subunit

In order to further study the spinophilin/NMDAR association, we aimed to look at domains of the subunits that interact with spinophilin. Structurally, NMDAR subunits contain a number of domains including an extracellular N-terminal domain, a transmembrane domain, a pore loop, and a variable length, subunit-dependent, intracellular C-terminal tail domain. The tail region is known to regulate receptor interactions with

various intracellular proteins. As a result, it is a good candidate to interact with intracellular proteins. For this purpose, we generated a DNA construct of the tail region of GluN2B, which is known to have 650 amino acids and transfected it along with spinophilin. Our results show a robust interaction between C-terminal tail of GluN2B and spinophilin. To further identify the interacting domain on $GluN2B_{Tail}$, we generated DNA constructs matching portions of the Tail region. Due to low expression level of tail fragments, PKAc was overexpressed as we found that overexpression of PKAc enhances the association between the proteins. Our results suggest that there was a very robust basal association between spinophilin and amino acids 839-1088 of $GluN2B_{Tail}$ in HEK293 cells.

4.3 PKA Enhances the Interaction of GluN1 and GluN2BTail with Spinophilin

In order to identify the mechanisms that affect the spinophilin-NMDAR association, we evaluated the role of kinases on modulating the spinophilin/NMDAR interaction. Spinophilin has been previously shown to target PP1 to specific substrates (Allen et al., 1997; Ragusa et al., 2010). Furthermore, 6-OHDA lesioned mice, which is used as a model of Parkinson disease, show an increased spinophilin phosphorylation on Serine 100 and Serine 17 (Hiday and Edler et al., Manuscript in preparation) which are PKA (Hsieh-Wilson et al., 2003) and CDK5 (Futter et al., 2005) sites, respectively. These changes occured along with a decrease in the association of GluN1 and GluN2B with spinophilin. Thus, we hypothesized that enhanced phosphorylation with PKA and CDK5 may contribute to this decreased association. PKA has been shown in previous studies to phosphorylate spinophilin (Hsieh-Wilson et al., 2003), GluN1 (D. B. Scott et al., 2001) and GluN2B (Murphy et al., 2014). According to results, spinophilin-GluN1 and $GluN2B_{Tail}$ -

spinophilin interactions were increased in presence of overexpressed PKAc. However, details of this interaction (i.e. if PKA phosphorylation causes this increase or it has a kind of bridging role) are not entirely clear. In order to clarify this interaction, endogenous PKA was activated for 24hrs using IBMX, a phosphodiesterase inhibitor (Francis, Turko, & Corbin, 2001) and forskolin, an adenylyl cyclase activator (Seamon & Daly, 1981). Results suggest that long-term activation of PKA has the same influence on spinophilin-GluN1 and $GluN2B_{Tail}$ interaction. From these results, we infer that, phosphorylation caused by PKA increases the interaction of GluN1 and $GluN2B_{Tail}$ with spinophilin. However we cannot be certain if PKAc also has a bridging role in enhancement of this association. Taken together, our data demonstrate a PKA-dependent modulation of the spinophilin/NMDAR interaction and suggest that phosphorylation is a critical regulator of the GluN1-spinophilin and GluN2B-spinophilin association. Moreover, as stated previously, the decreased association of spinophilin and GluN1 and GluN2B in 6-OHDA treated mice, is probably not because of phosphorylation by PKA as, if anything, PKA actually increases the interaction between spinophilin and the NMDAR.

4.4 CDK5 Decreases the Interaction Between GluN2BTail and Spinophilin by Phosphorylating GluN2BTail and/or Spinophilin on a Non-Ser17 Residue

As mentioned above, 6-OHDA lesioned mice have greater phosphorylation of spinophilin at Serine 17 (Hiday and Edler et al., Manuscript in preparation), a CDK5 site (Futter et al., 2005). In addition, GluN2B is also phosphorylated by CDK5 (W. Lu et al., 2015). Therefore, we tested whether CDK5 activity modulates the association between spinophilin and GluN2B. Overexpression of CDK5 in HEK293 cells causes an overall

decrease in protein expression in the cells which may be due to aggregation of CDK5 phosphorylated proteins and targeting them to ubiquitin-proteasome pathway for degradation. While having low expression levels of proteins, overexpression of CDK5 decreased the association between spinophilin and GluN2B. We hypothesize that phosphorylation of CDK5 sensitive sites on either spinophilin and/or GluN2B are responsible for this result. To investigate this hypothesis, we generated S17A and S17D phospho- mutant isoforms of spinophilin. Results show that S17A mutant which is unable to be phosphorylated and S17D mutant which mimics phosphorylation (Bornancin & Parker, 1997), does not have any effect on the interaction. Furthermore, the S17A mutant when accompanied with CDK5 overexpression still has a decreased association with GluN2B. These results suggest that either phosphorylation of $GluN2B_{Tail}$ or phosphorylation of a different site on spinophilin by CDK5 may be responsible for decreased interaction between spinophilin and $GluN2B_{Tail}$. Moreover, we cannot rule out that CDK5 binding to either spinophilin or the $GluN2B_{Tail}$ may modulate their association by changing the conformation of either spinophilin or GluN2B.

4.5 Spinophilin, Through its PP1-Targeting Role, Attenuates Abundance of PP1 on GluN2Btail

PP1 interacts directly with spinophilin (Allen et al., 1997; Colbran et al., 1997). Our results also showed that PP1 also interacts with $GluN2B_{Tail}$ (Figure 13). As a consequence spinophilin-$GluN2B_{Tail}$ interaction could potentially alter the abundance of PP1 on each of these proteins. Therefore, we determined the association of PP1 with

$GluN2B_{Tail}$ in the presence and absence of spinophilin. Results show that overexpression of spinophilin attenuated PP1 bound to $GluN2B_{Tail}$.

Since spinophilin has a PP1 targeting role (Allen et al., 1997; Ragusa et al., 2010) it should increase the PP1 bound to $GluN2B_{Tail}$ unless the domains of spinophilin which bind to $GluN2B_{Tail}$ and PP1 are overlapping. In order to test this idea, we used a PP1-binding deficient mutant of spinophilin (F451A) (Hsieh-Wilson et al., 1999). The mutation had little to no effect of spinophilin binding to $GluN2B_{Tail}$ and if anything actually increased the association between these proteins (Figure 15). However, in contrast to WT spinophilin, mutant spinophilin had no effect on the association between PP1 and $GluN2B_{Tail}$. We predict that only a small portion of spinophilin and $GluN2B_{Tail}$ are associated with each other in HEK cells. The low intensity of the Co-IP signal that is detected in these studies supports this prediction. Therefore, when spinophilin is overexpressed, it may displace PP1 from the tail of GluN2B. While a small amount of spinophilin-targeted PP1 may still associate with GluN2B, unbound spinophilin sequesters a majority of the PP1 away from GluN2B. Previous studies have found that spinophilin binding to PP1 can attenuate PP1 activity towards certain substrates (Mathieu Bollen et al., 2010; Ragusa et al., 2010). Our data demonstrate a different mechanism by which spinophilin may compete away PP1 from other interacting proteins.

4.6 Novel Mechanisms Regulating the Spinophilin/PP1 Interaction

Given that spinophilin may sequester PP1 from GluN2B, we next wanted to determine mechanisms that modulate the association between PP1 and spinophilin. Interestingly, we found that CDK5 activity enhances the association between spinophilin

and PP1. PP1 binds to a central domain on spinophilin, containing residues 447-451 (Ragusa reference, Hsieh Wilson references). PP1α is also known to be phosphorylated by CDK5 at Thr320 (Hou et al., 2013). Therefore future studies will need to determine if phosphorylation at this site plays a role. Moreover, multiple isoforms of PP1 are known to associate with spinophilin, with both the γ1 and α isoforms being predominant interactors. However, PP1γ1 does not contain the same Thr320 site. Previous studies have identified additional spinophilin binding sites that can enhance spinophilin association with PP1γ1(L. C. Carmody, A. J. Baucum, 2nd, M. A. Bass, & R. J. Colbran, 2008). Moreover, while F451A mutation of spinophilin completely abrogates binding to the endogenous PP1 isoform in HEK293 cells (presumably α), it only partially attenuates the association with overexpressed PP1γ1. The effect of CDK5 on regulating spinophilin binding with different isoforms of PP1 is not known and future studies will determine isoform-specific differences between the CDK5-dependent changes in the interaction of spinophilin and PP1.

4.7 Functional Implications of the Spinophilin/NMDAR Interaction

To determine the functional consequences of modulating the spinophilin/GluN2B interaction and/or spinophilin expression, we utilized MS/MS-based approaches to identify and ratiometrically quantify various phosphorylation sites on $GluN2B_{Tail}$ in the absence or presence of overexpressed spinophilin and PP1. Interestingly, our MS/MS results show a significant decrease in Ser1284 phosphorylation caused by overexpression of PP1. This decrease was rescued when spinophilin was also overexpressed. Preliminary data immunoblotting with a phospho-specific antibody also suggests that Ser1284

phosphorylation is attenuated in presence of PP1 and is recovered by overexpression of spinophilin.

Ser1284 is a CDK5 phosphorylation site. Previous studies show that CDK5 is involved in synaptic plasticity, memory and learning (Cheung & Ip, 2007; Hawasli et al., 2007; Plattner et al., 2014). Moreover, it is notable that CDK5 phosphorylation of NMDARs, especially GluN2B-containing NMDARs, is critical in normal learning and memory and synaptic plasticity either through direct phosphorylation of NMDARs (B. S. Li et al., 2001; Plattner et al., 2014) or modulation of its partners (Morabito, Sheng, & Tsai, 2004; Zhang et al., 2008). Consequently, it is intriguing to explore alterations of CDK5 phosphorylation sites and the molecules responsible for this alteration. Ser1284 (W. Lu et al., 2015) and Ser1116 (Plattner et al., 2014) are two CDK5 phosphorylation sites on GluN2B. Ser1284 is known to have decreased phosphorylation in ischemic conditions while showing no change during LTP simulation or fear condition (W. Lu et al., 2015). All in all these data suggest that Ser1284 is a CDK5 phosphorylation site that can be modulated by spinophilin-dependent redistribution of PP1.

4.8 Summary

Data presented here suggest that the interaction between spinophilin and NMDARs is attenuated by CDK5 overexpression and enhanced by PKA overexpression and activity. Our preliminary data showed an interaction between spinophilin and GluN1 as well as spinophilin and GluN2B in mouse brain. We validated that spinophilin and NMDAR interact in heterologous cell lines. We also established that spinophilin interacts with amino acids 839-1088 on the C-terminal tail region of GluN2B. We also found that point

mutations that mimic phosphorylation of spinophilin at CDK5 site do not change the association of $GluN2B_{Tail}$ and spinophilin. However co-expression of CDK5 along with these point mutant species still attenuates the interaction, suggesting another phosphorylation site on either of GluN2B or spinophilin is responsible for the observed change in the association. Taken together, these data suggest the modulatory effect of two PKs in spinophilin-NMDAR association.

Functionally, we determined that PP1 overexpression decreases Ser1284 phosphorylation on GluN2B (Figure 18A). Interestingly, spinophilin can compete off PP1 from GluN2B and this competition attenuates the PP1-induced decreases in GluN2B phosphorylation at Ser1284 of GluN2B (Figure 18B). Consequently, overexpression of F451A mutant spinophilin does not have significant effect on modulating PP1 level in GluN2B IP (18C).

4.9 Conclusion and Future Directions

Parkinson disease is one of the most common neurodegenerative diseases and is characterized by the loss of nigrostriatal dopaminergic projections to MSNs in striatum. Dopamine depletion has various consequences on MSNs, such as PKA disinhibition of D2 receptor containing population of neurons and rearrangement of the PSD family members on MSN spines. As suggested by an animal model of PD, spinophilin and NMDA receptors are two members of PSD that have altered interaction as a result of PD. Possibly, spinophilin may play a role in proper localization of NMDARs as well as regulating their phosphorylation status. As a result, alterations of the association of spinophilin and NMDAR may lead to perturbations in normal synaptic function.

Our data suggest GluN1 and GluN2B subunits of NMDARs interact with spinophilin. In our future studies we will investigate the possible interaction of GluN2A subunit with spinophilin using brain IPs and a heterologous cell system. Moreover, immunofluorescence imaging studies using confocal microscopy could help elucidate further questions about the co-localization of the various proteins discussed herein.

While we identified one site on GluN2B that is modulated by spinophilin expression in a heterologous cell system, the roles of spinophilin on *in vivo* NMDAR phosphorylation are less clear. Moreover, implications of regulating the spinophilin/PP1 interaction on regulating substrate phosphorylation and PP1 targeting to the NMDAR and other synaptic proteins are not known. Therefore, future studies will utilize WT and spinophilin KO mice to evaluate PP1 binding to, and the phosphorylation of, GluN2B. Moreover, we aim to investigate the role of modulating GluN2B phosphorylation at Ser1284 on NMDAR localization and function. Additionally, we have reported that Ser1284 phosphorylation of GluN2B subunit is increased in the presence of spinophilin. Since Ser1284 phosphorylation is strikingly decreased in ischemic conditions, we will also look at spinophilin's role in rescuing the dephosphorylation of NMDAR in oxygen deprived conditions.

We have also shown that PKAc and CDK5 regulate the association between spinophilin and NMDARs. However, the exact phosphorylation sites that modulate the association are unclear. To further test this, we plan to generate phospho-mimic mutations of GluN1, GluN2B and spinophilin on specific PKA or CDK5 sites and try to uncover the exact phosphorylation sites that regulate the association. Furthermore, we intend to investigate the effect of other protein kinases such as CaMKII, PKC, Tyrosine kinases and Casein kinases on the interaction.

Here we also showed that NMDAR/spinophilin interaction is altered in an animal model of PD. Further studies utilizing animal models of PD could be useful in assessing increases and/or decreases in either kinase or phosphatase levels and/or activity that occur concurrently with these altered interactions. Additionally, understanding the pathological consequences of these altered interactions on the symptoms of the disease will be critical.

All in all, the data presented here reveal changes in the interaction between two important neuronal proteins. Furthermore, it seems that phosphorylation via PKA and CDK5 have a significant effect on spinophilin's ability to interact with the NMDAR. Our exciting data give a novel insight into mechanisms that regulate the interaction between these critical synaptic proteins; however, the potential consequences of these changes has yet to be fully explored.

FIGURES

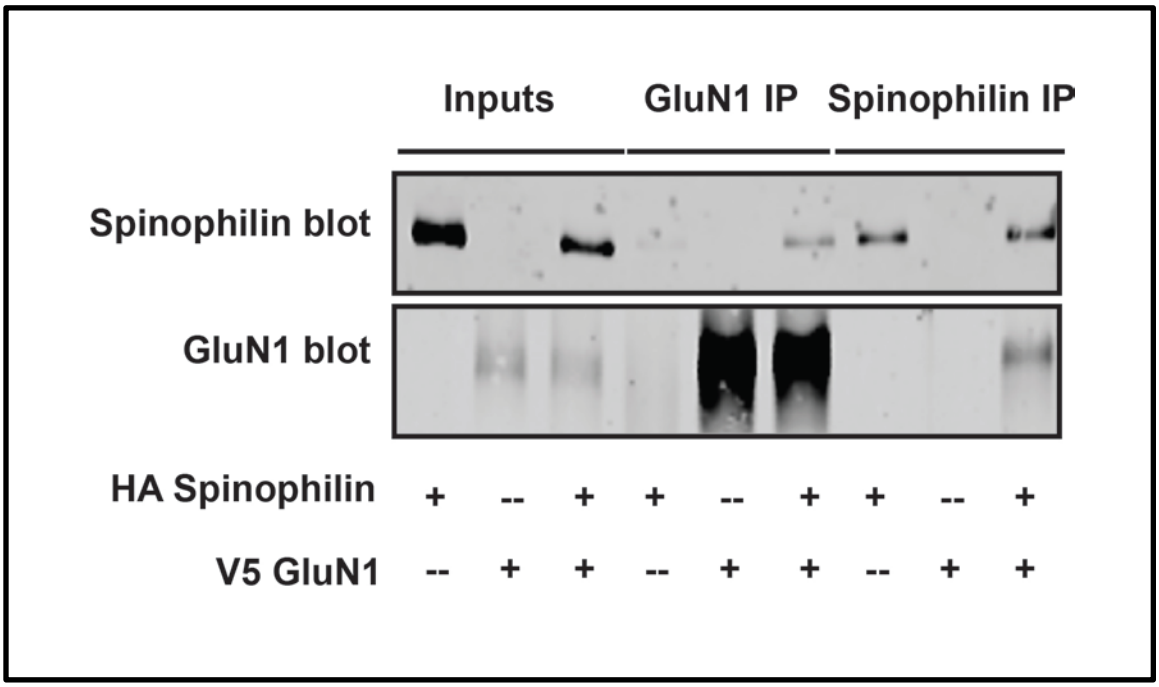

Figure 1: Spinophilin and GluN1 interact in HEK293 cells.
HEK293 cells were transfected with HA-spinophilin and/or V5-GluN1. Immunoprecipitations and immunoblots were performed with antibodies raised against either the HA or the V5 tag. Western blot results show an association between these two proteins. Image is representative of 3 independent experiments.

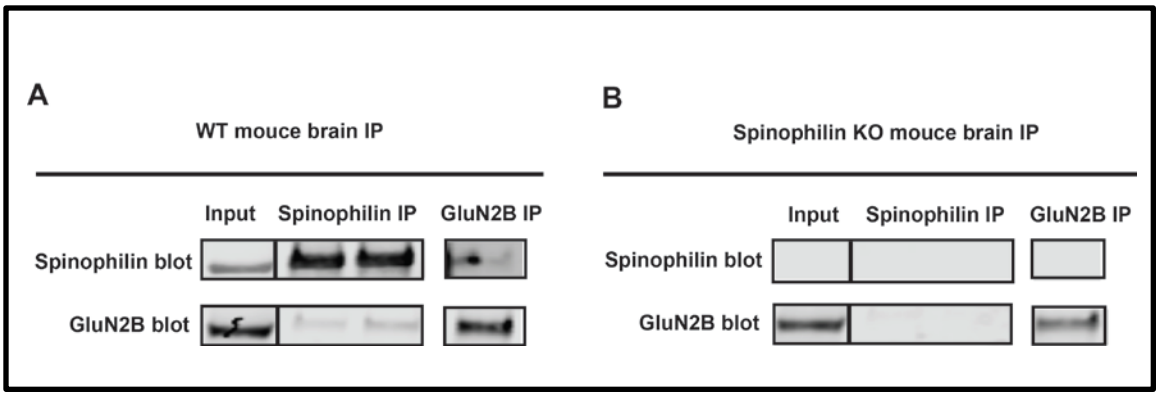

Figure 2: Spinophilin associates with GluN2B in mouse brain.
Immunoprecipitations performed using mouse cortical tissue homogenized in RIPA lysis buffer. Immunoprecipitates were blotted using antibodies raised against spinophilin or GluN2B. A) Immunoprecipitations and immunoblots were performed with antibodies raised against either the spinophilin or GluN2B. **B)** No GluN2B is co-immunoprecipitated with spinophilin in spinophilin KO mouse.

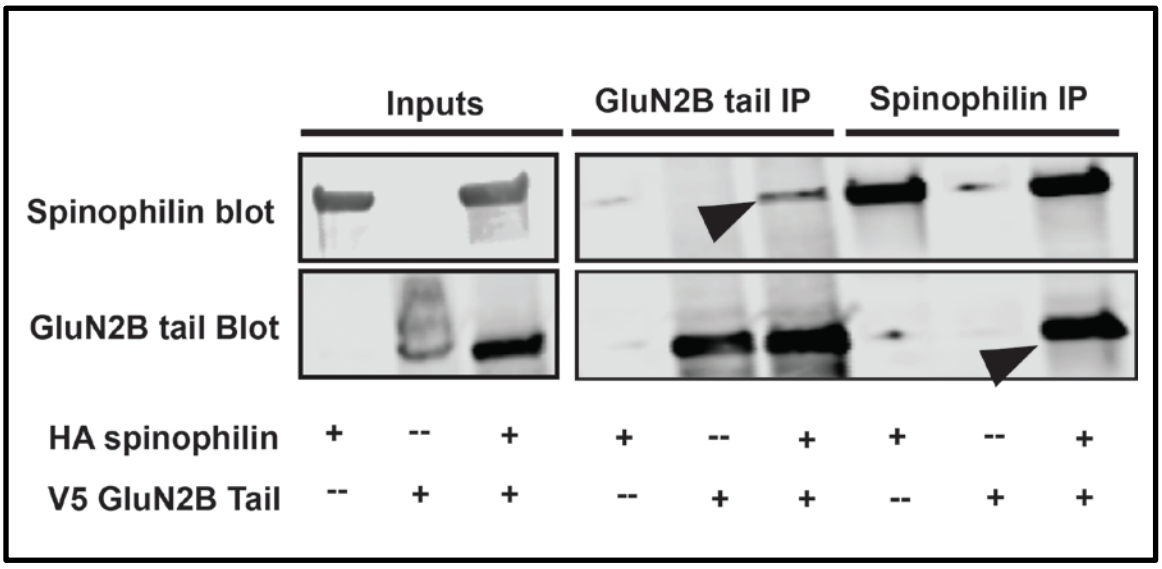

Figure 3: Spinophilin associates with the C-terminal domain of GluN2B.
HEK293 cells were transfected with spinophilin and/or $GluN2B_{Tail}$ (HA and V5 tags respectively). Immunoprecipitations were performed with antibodies raised against either the HA or the V5 tag. Western blots were performed using antibodies raised against either $GluN2B_{Tail}$ or spinophilin. Western blot results show an association between these two proteins. Image is representative of 3 independent experiments.

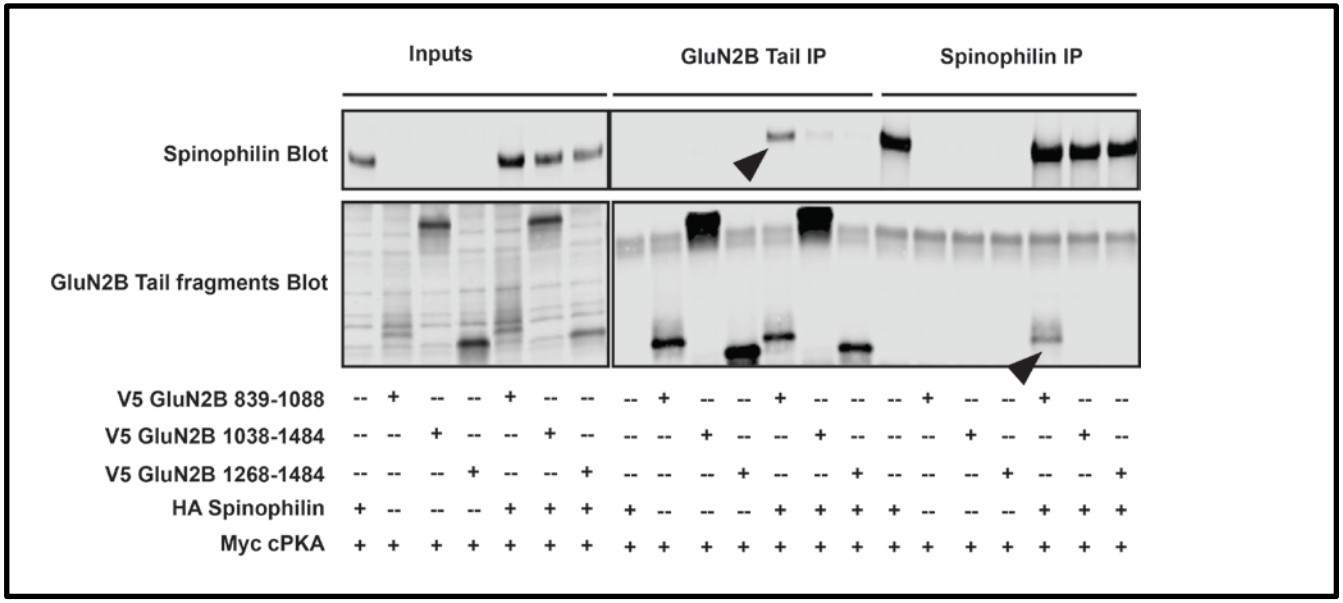

Figure 4: Spinophilin associates with residues 839-1088 of GluN2BTail.
HEK293 cells were transfected with V5-tagged GluN2BTail fragments in the absence or presence of HA-tagged spinophilin. Spinophilin and GluN2B fragment IPs and immunoblots were performed using antibodies raised against HA and V5. Image is representative of 5 independent experiments.

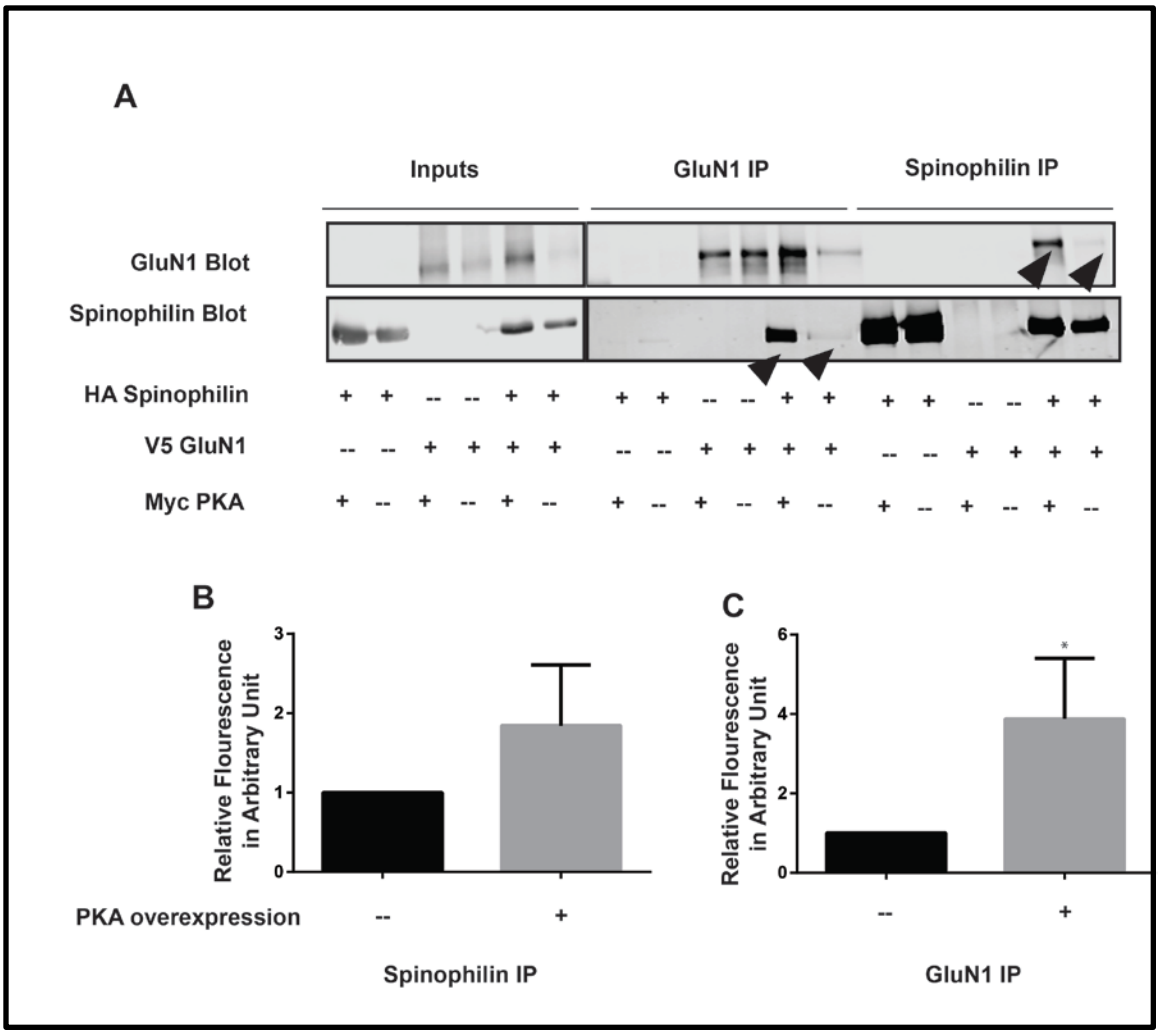

Figure 5: Overexpression of the catalytic subunit of PKA increases spinophilin-GluN1 association.
HEK293 cells were transfected with HA-spinophilin and/or V5-GluN1 with and without Myc tagged PKAc. **A)** Immunoprecipitations and immunoblots were performed with antibodies raised against either the HA or the V5 tag. **B)** There was a trend for an increase in the amount of GluN1 in the HA IPs in the presence of PKA. $p = 0.07$. **C)** There was an increase in the amount of spinophilin in V5 IPs in the presence of PKA. $*p < 0.05$. N=9. Graphs show the mean + the standard error of the mean.

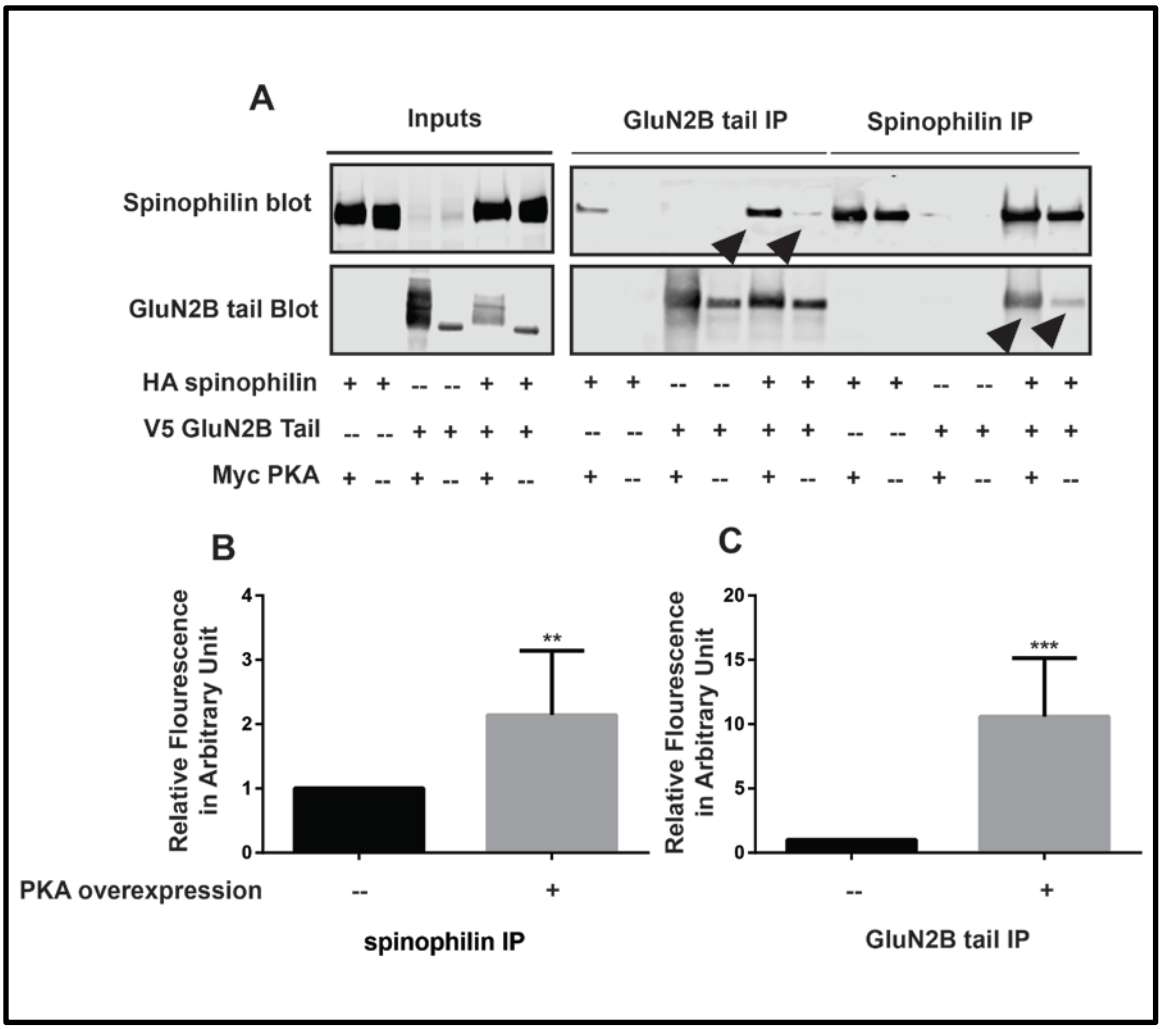

Figure 6: Overexpression of catalytic subunit of PKA increases spinophilin- GluN2BTail association.

HEK293 cells were transfected with HA-spinophilin and/or V5- GluN2B$_{Tail}$ with and without Myc tagged PKA. **A)** Immunoprecipitations and immunoblots were performed with antibodies raised against either the HA or the V5 tag. **B)** There was a trend for an increase in the amount of GluN2B in Spinophilin (HA) IPs when PKA was overexpressed. **p < 0.01. **C)** There was a significant increase in the amount of spinophilin in GluN2B (V5) IPs when PKA was overexpressed. ***p < 0.001. N=10. Graphs show the mean + the standard error of the mean.

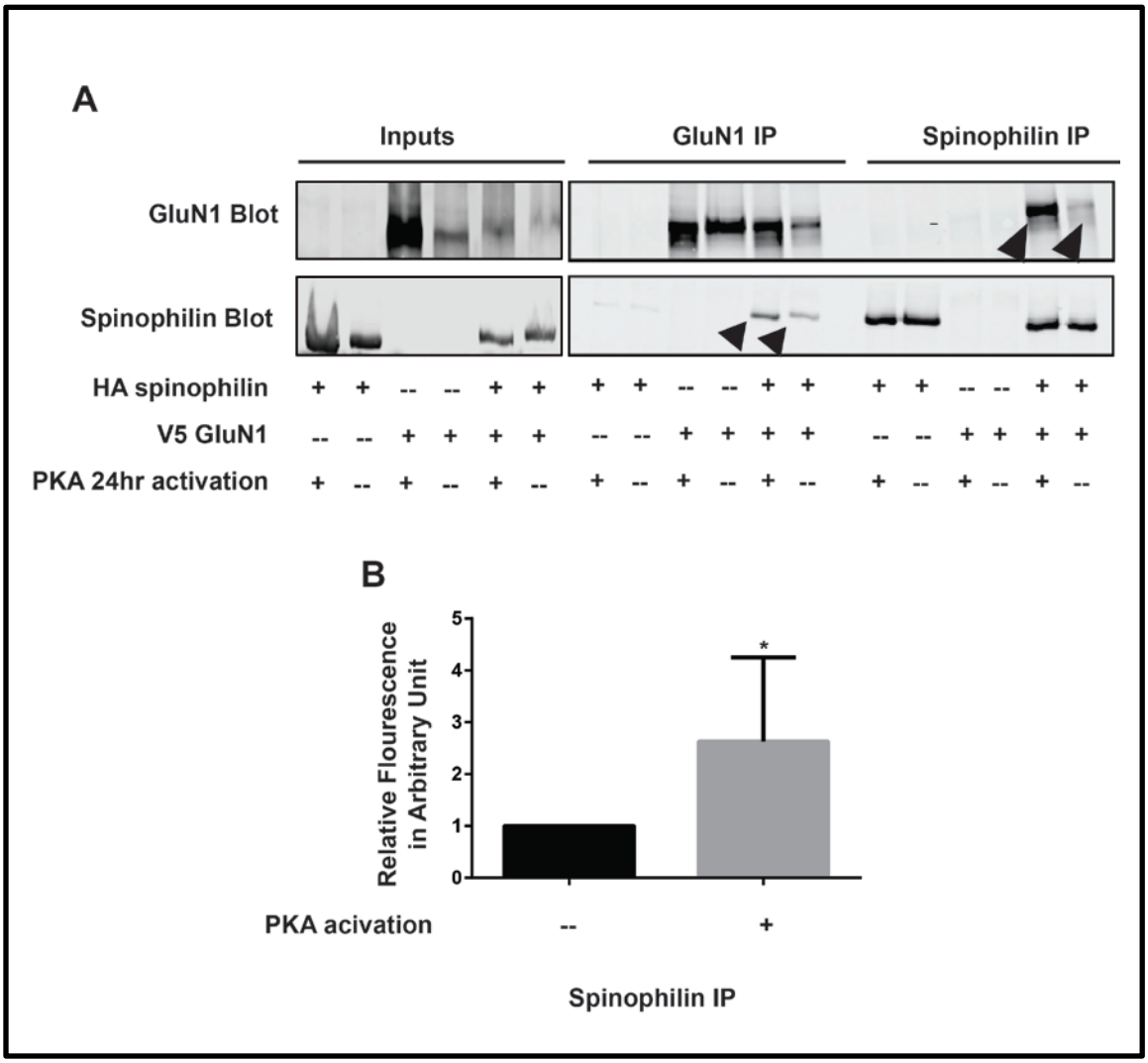

Figure 7: Activation of endogenous PKA increases spinophilin-GluN1 association.
HEK293 cells were transfected with HA-spinophilin and/or V5-GluN1. Endogenous PKA was activated through the use of forskolin and IBMX. **A)** Immunoprecipitations and immunoblots were performed with antibodies raised against either the HA or the V5 tag. **B)** There was a significant increase in the amount of GluN1 in Spinophilin (HA) IPs following activation with PKA. *$p < 0.05$ N=8. Graph shows the mean + the standard error of the mean.

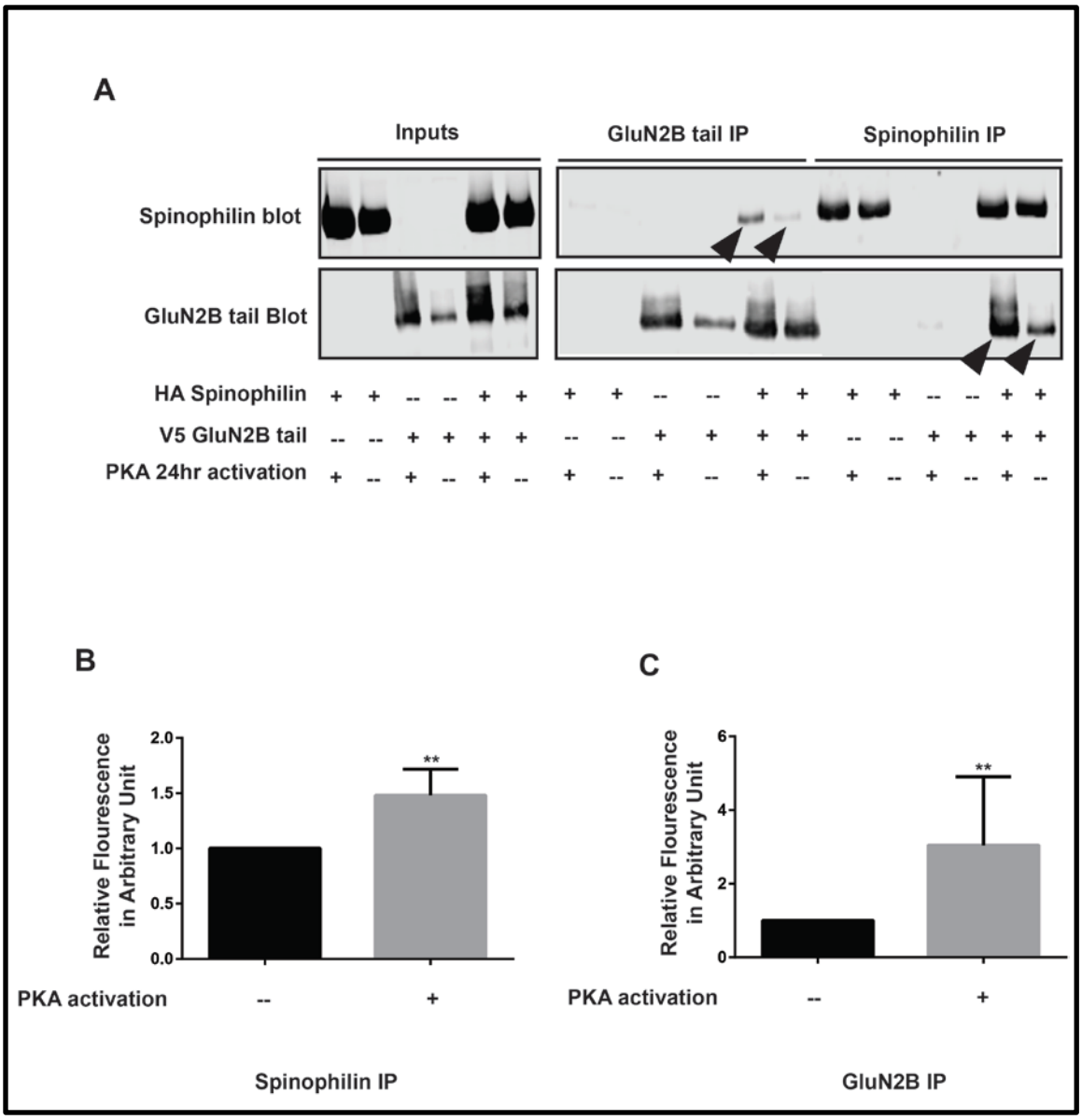

Figure 8: Activation of endogenous PKA increases spinophilin- GluN2BTail association. HEK293 cells were transfected with HA-spinophilin and/or V5- GluN2B$_{Tail}$. Endogenous PKA was activated through the use of forskolin and IBMX. **A)** Immunoprecipitations and immunoblots were performed with antibodies raised against either the HA or the V5 tag. **B)** There was a significant increase in the amount of GluN2B$_{Tail}$ in Spinophilin (HA) IPs following activation of PKA. **p < 0.01. **C)** There was a significant increase in the amount of Spinophilin in the GluN2B$_{Tail}$ (V5) IPs following activation of PKA. **p < 0.01. N=6. Graphs show the mean + the standard error of the mean.

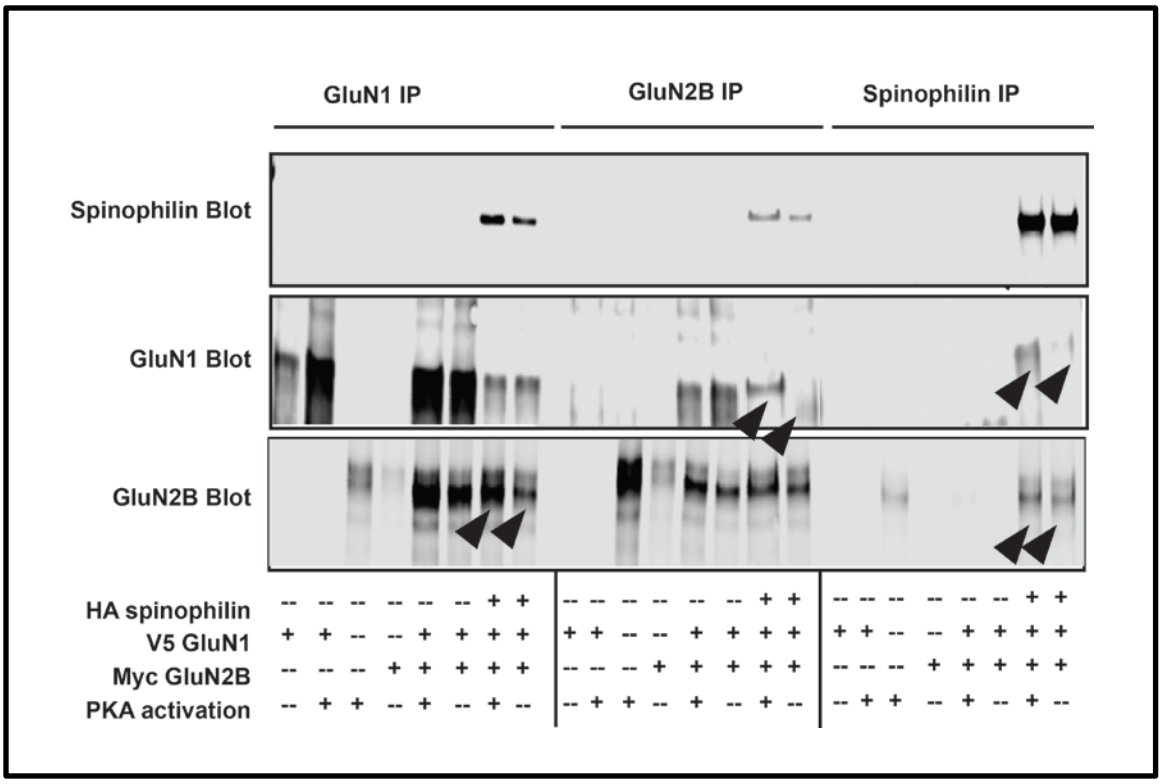

Figure 9: Long-term activation of endogenous PKA increases spinophilin-GluN1-GluN2B association.

HEK293 cells were transfected with spinophilin and/or GluN1-GluN2B. Endogenous PKA was activated through the use of forskolin and IBMX. Immunoprecipitations were performed with antibodies raised against either the HA or the V5 or Myc tag. Western blots were performed using antibodies raised against either Spinophilin or GluN1 or GluN2B. Preliminary data suggest an increase in the association of NMDAR and spinophilin in presence of activated PKA. Data are representative of 3 independent experiments.

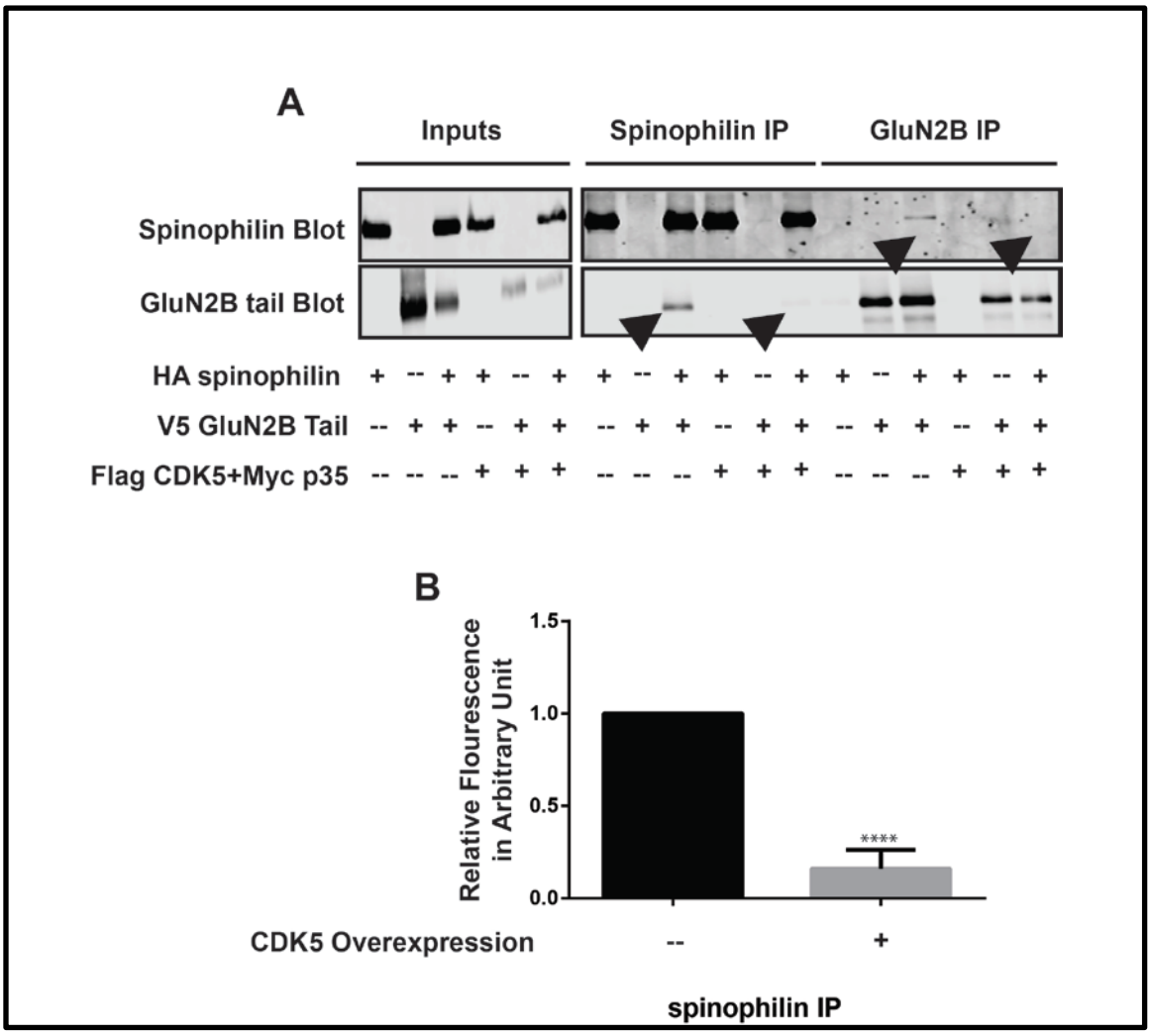

Figure 10: Spinophilin/GluN2BTail association is decreased upon CDK5/p35 overexpression in HEK293 cells.
HEK293 cells were transfected with HA-spinophilin and/or V5- $GluN2B_{Tail}$. **A)** Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or the V5 epitpoe tags. **B)** There was a significant decrease in the amount of $GluN2B_{Tail}$ in Spinophilin (HA) IPs when CDK5 was overexpresssed. ****$p < 0.0001$. N=8. Graphs show the mean + the standard error of the mean.

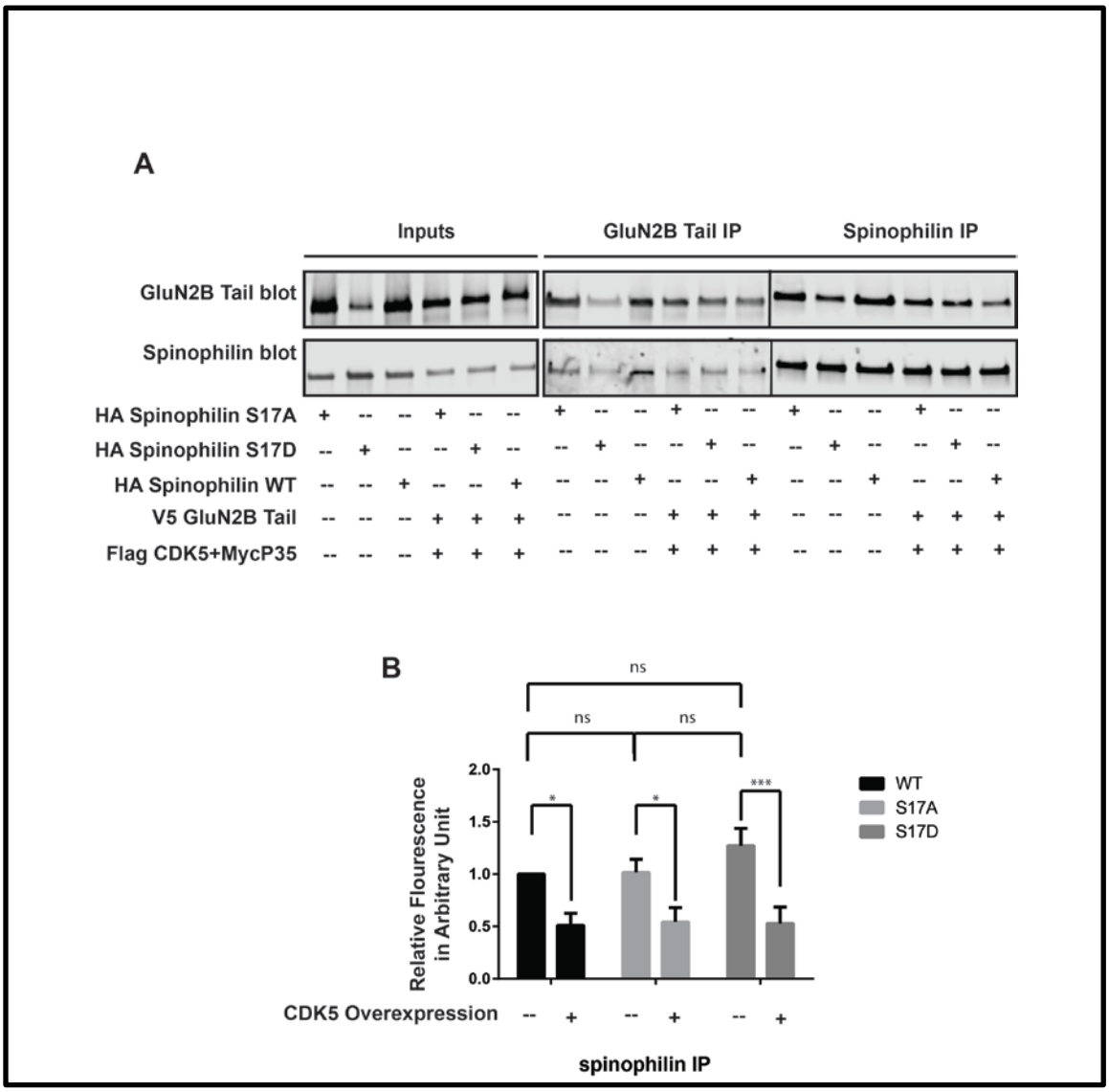

Figure 11: Phosphorylation of Ser17 on spinophilin is not responsible for the decrease in spinophilin-GluN2BTail interaction.
HEK293 cells were transfected with WT, S17A, or S17D HA-spinophilin and/or V5 $GluN2B_{Tail}$. **A)** Immunoprecipitations and immunoblots were performed with antibodies raised against either the HA or the V5 tag. **B)** Quantified data show that S17A/D mutation does not have effect on Spinophilin- $GluN2B_{Tail}$ association while CDK5 presence still decreases the association. *$p < 0.05$, ***$p < 0.001$. N=6. Graphs show the mean + the standard error of the mean.

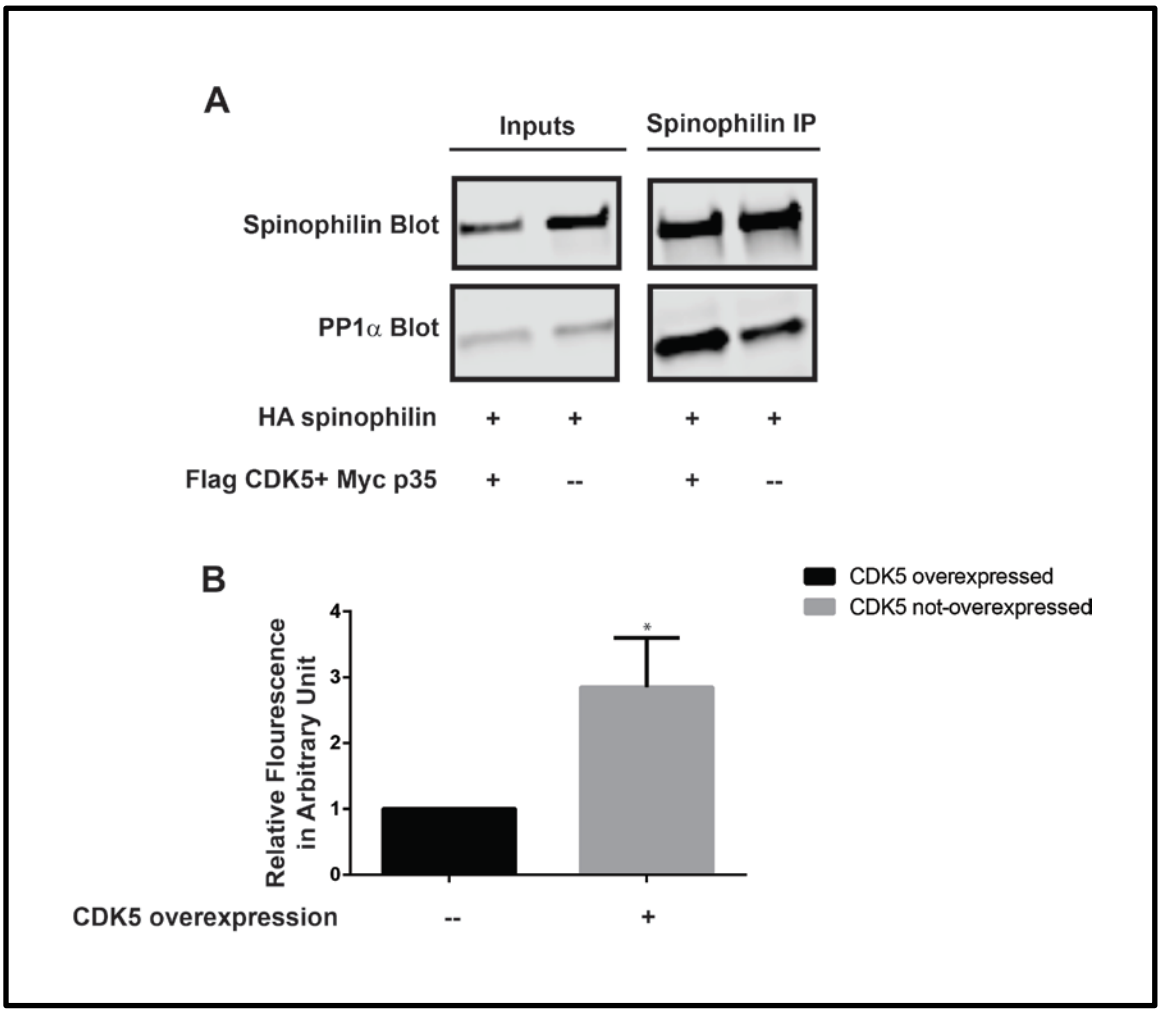

Figure 12: Overexpression of CDK5 increases spinophilin-PP1□ association.
HEK293 cells were transfected with HA-tagged spinophilin with or without CDK5-p35 overexpression. Spinophilin IPs were performed using antibodies raised against HA tag. **A)** Immunoblots were performed using antibodies against HA tag and endogenous PP1. **B)** These data show a significant increase in the amount of PP1 in Spinophilin (HA) IPs when CDK5 was overexpresssed.*$p < 0.05$. N=6. Graphs show the mean + the standard error of the mean.

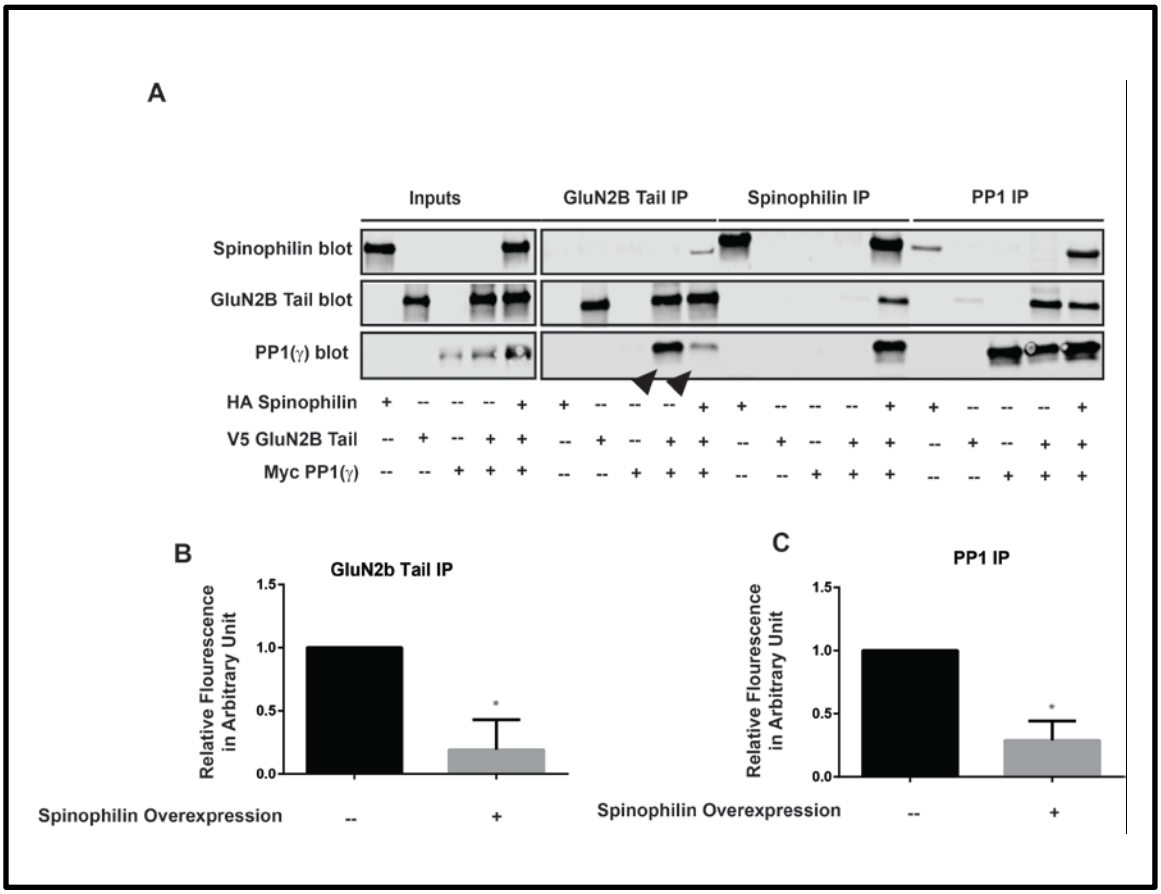

Figure 13: Spinophilin decreases the amount of PP1γ1 bound to the C-terminal domain of the GluN2B**.**

HEK293 cells were transfected with PP1γ1 and/or $GluN2B_{Tail}$ (Myc and V5 tags respectively) with and without HA tagged spinophilin overexpression. Immunoprecipitations were performed with antibodies raised against either the Myc or the V5 tag. **A)** Immunoblots were performed using antibodies raised against either the $GluN2B_{Tail}$ or PP1. **B and C)** There was a decrease in the amount of PP1 and $GluN2B_{Tail}$ in $GluN2B_{Tail}$ (V5) and PP1 IPs respectively when spinophilin is overexpressed. *$p < 0.05$ N=7. Graphs show the mean + the standard error of the mean.

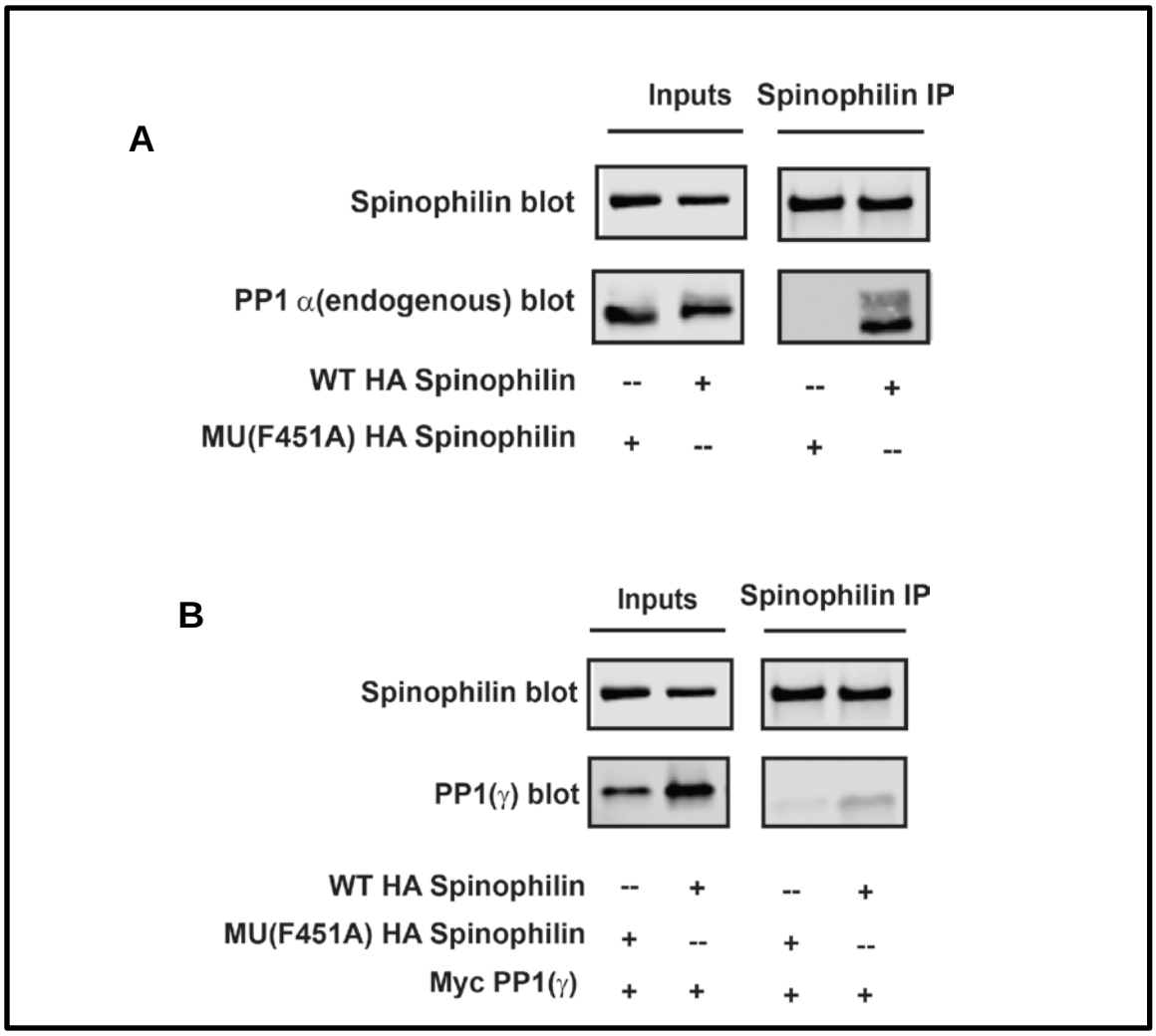

Figure 14: F451A mutation on spinophilin attenuates its ability to bind to PP1.
HEK293 cells were transfected with F451A mutant (Mu) spinophilin and/or WT spinophilin in the absence or presence of Myc-tagged PP1. Immunoprecipitations were performed with antibodies raised against either the HA tag. Immunoprecipitates were immunoblotted for endogenous PP1 **(A)** or overexpressed PP1γ1 **(B)**. Graphs show the mean + the standard error of the mean.

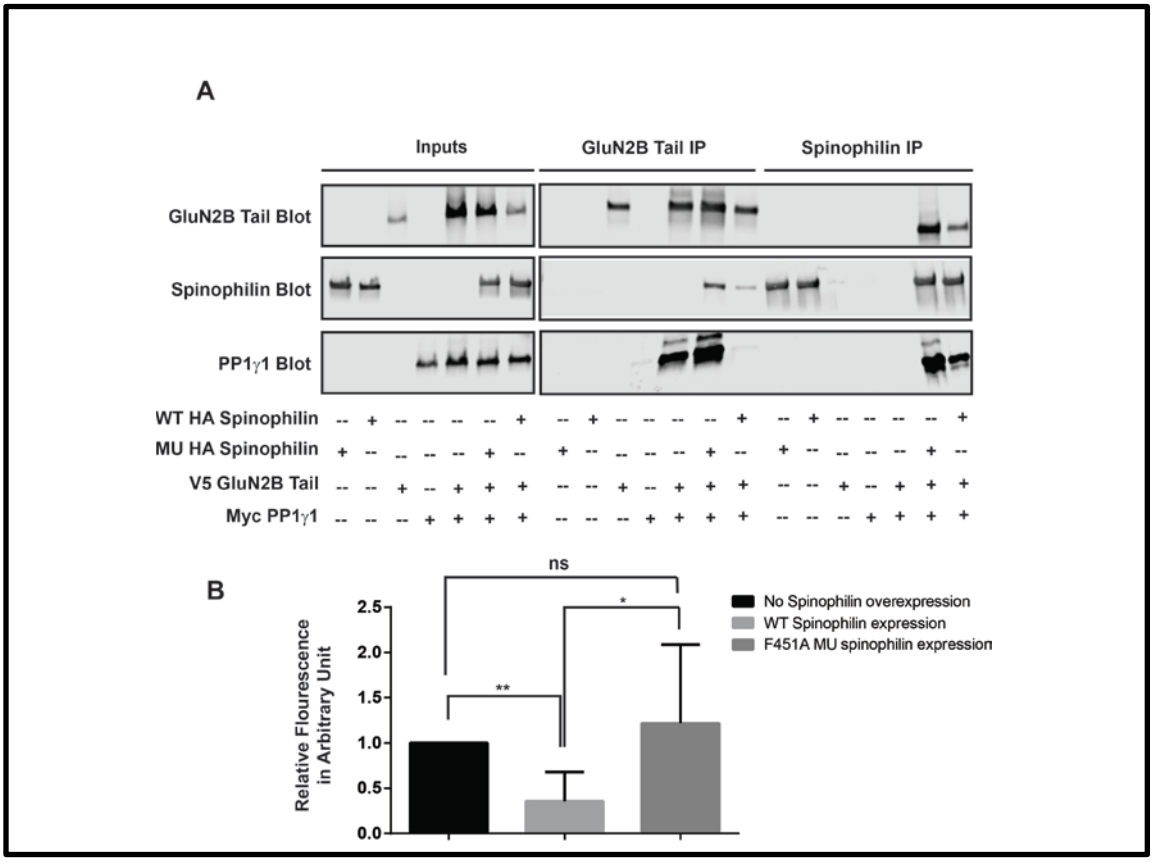

Figure 15: WT, but not F451A mutant spinophilin alters the PP1 association with the C-terminal domain of GluN2B.

HEK293 cells were transfected with PP1γ1 and/or $GluN2B_{Tail}$ (Myc and V5 tags respectively) with and without HA tagged wildtype or mutant (F451A) spinophilin. Immunoprecipitations were performed with antibodies raised against either the Myc or the V5 tag. Western blots were performed using antibodies raised against either V5 tag, HA tag, or PP1γ1. B) Quantified data show an overall significant ANOVA p-value ($p < 0.05$). A Tukey post hoc test revealed a significant decrease of PP1 association with $GluN2B_{Tail}$ (**$p < 0.01$) when WT spinophilin is present. However, there was no significant difference ($p > 0.05$) in the interaction between PP1 and $GluN2B_{Tail}$ in presence or absence of F451A spinophilin. N=8. Graphs show the mean + the standard error of the mean.

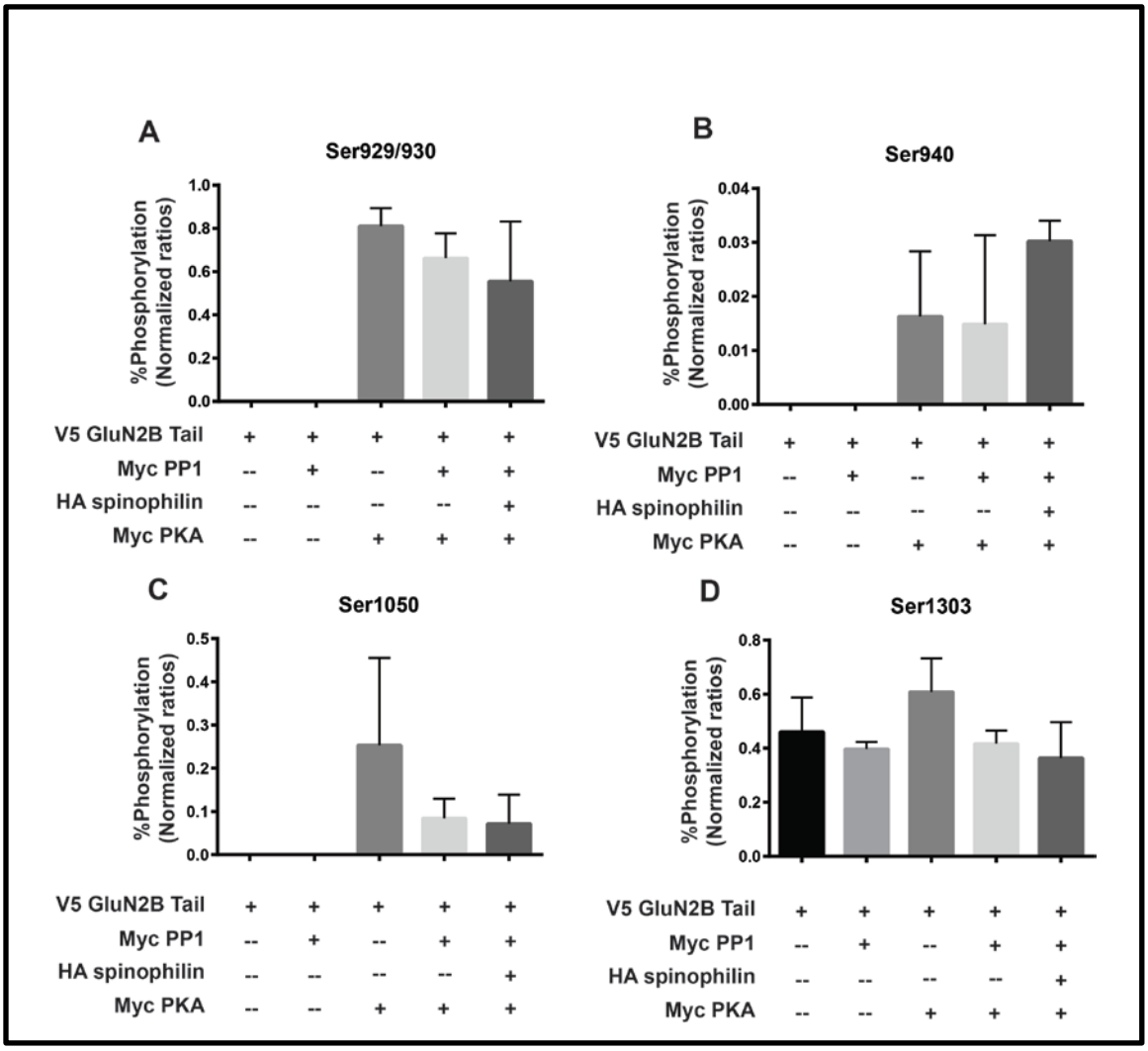

Figure 16: PKA phosphorylates multiple serine residues on GluN2B.
HEK293 cells were transfected as indicated. Immunoprecipitations were performed with antibodies for the V5 tag ($GluN2B_{Tail}$) and samples were separated by SDS-PAGE then stained with Imperial stain. Phosphorylation at Ser929/930 **(A)**, Ser940 **(B)**, Ser1050 **(C)**, and Ser1303 **(D)** was ratiometrically quantified as described in methods in the absence (-) or presence (+) of PKA. N=3. Graphs show the mean + the standard error of the mean.

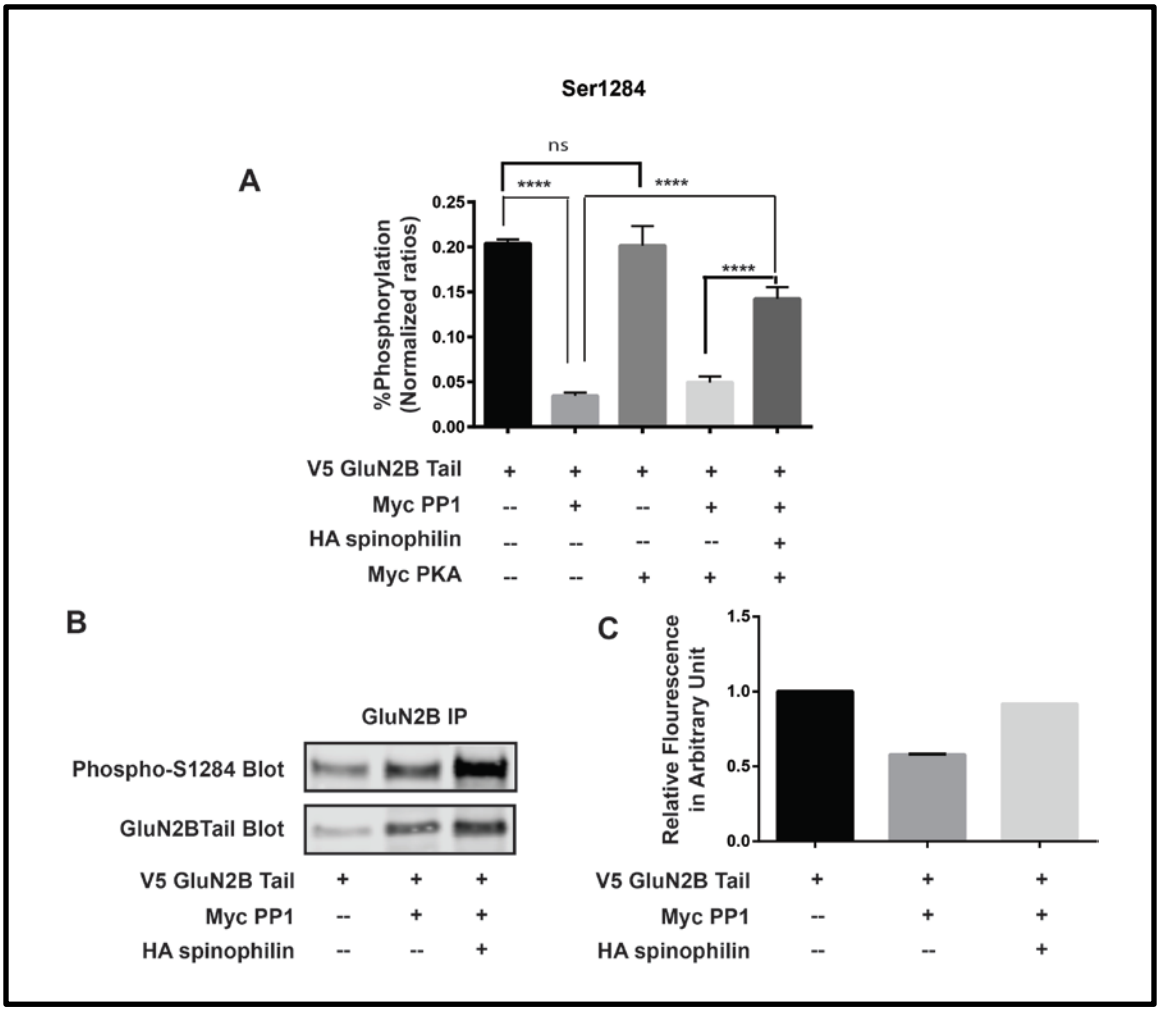

Figure 17: Spinophilin rescues PP1-induced dephosphorylation of S1284 on GluN2B. HEK293 cells were transfected as indicated. Immunoprecipitations were performed with antibodies for the V5 tag ($GluN2B_{Tail}$) and samples were separated by SDS-PAGE then stained with Imperial stain. **A)** A one-way ANOVA revealed a significant treatment effect ($p < 0.0001$). A Tukey post-hoc test revealed a significant decrease in S1284 phosphorylation when PP1 was added ****$p < 0.0001$. This decrease was attenuated by overexpression of spinophilin ****$p<0.0001$. N = 3. **B)** Western blot showing the increase in phosphorylation of S1284 when spinophilin is overexpressed compared to decrease in the phosphorylation caused by PP1 alone. Immunoprecipitations were conducted using antibodies for the V5 (GluNBTail) tag. Western blots were obtained using antibodies for phospho-S1284 and total V5 tag. **C)** Preliminary data suggest that PP1 decreases the S1284 phosphorylation signal and that this decrease is rescued by overexpression of spinophilin. N = 2. Graphs show the mean + the standard error of the mean

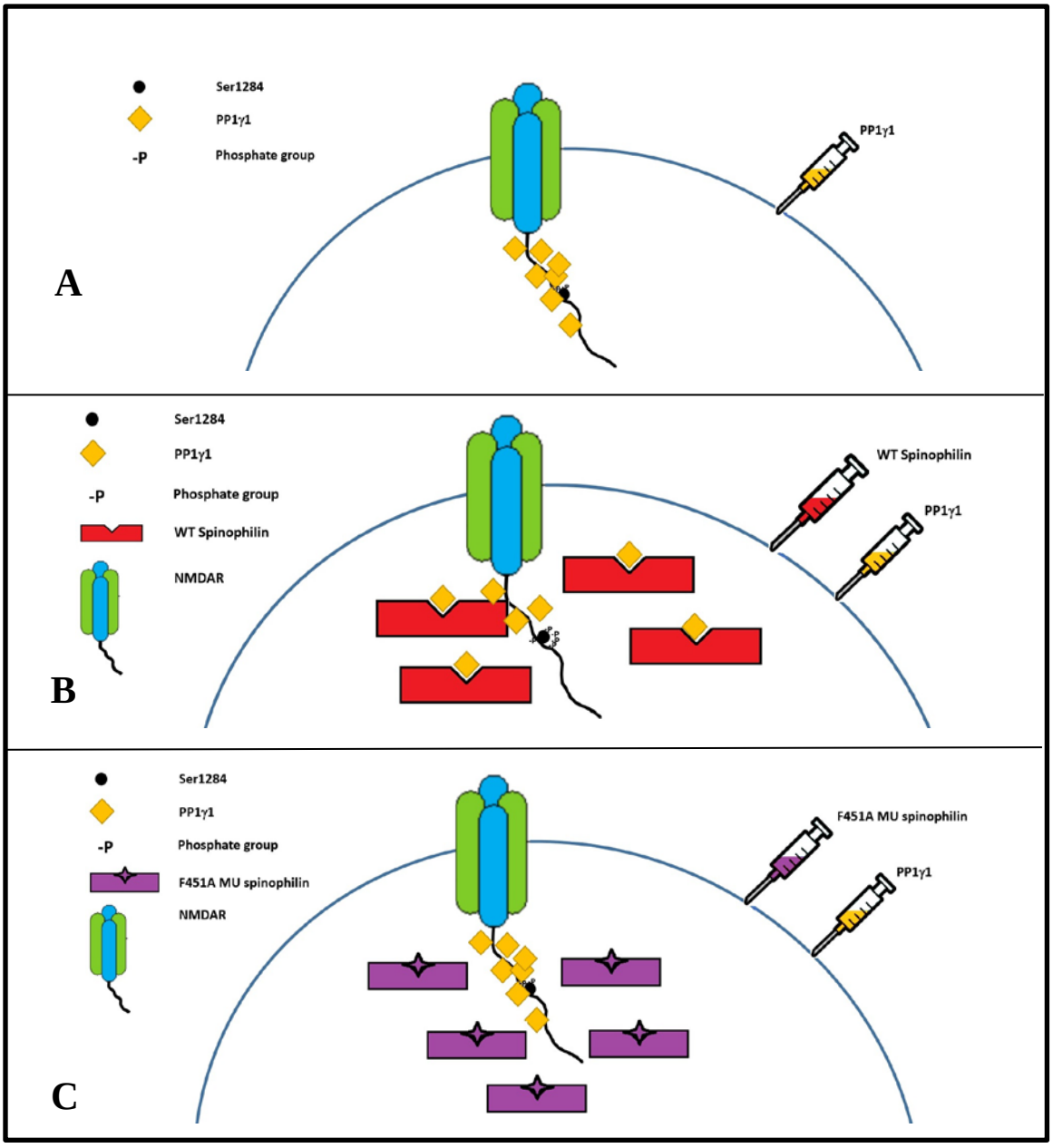

Figure 18: PP1 binding to GluN2B is modulated by spinophilin.
A) PP1 binds to GluN2Btail and dephosphorylates GluN2B at Ser1284. WT **(B)**, but not F451A **(C)** spinophilin displaces PP1 from GluN2Btail and rescues PP1 dephosphorylation at GluN2B at Ser1284

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