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Función de la proteína NSPA en la regulación de los receptores de NMDA en hipocampo

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FUNCIÓN DE LA PROTEÍNA NSPA EN LA REGULACIÓN DE LOS RECEPTORES DE NMDA EN HIPOCAMPO

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ABREVIATIONS

ACSF	Artificial Cerebrospinal Fluid
aCaMKII	a-Calmodulin Kinase II
AMPAR	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Anti-P	Anti-ribosomal P antibodies
APC10	Anaphase Promoter Complex 10
APC/C	Anaphase Promotor Complex
APV	D-2-amino-5-phosphonovalerate
BBB	Blood-brain Barrier
b-gal	b-galactosidase
BLAST	Basic Local Alignment Search Tool
BrdU	5-Bromo-2′-deoxyuridine
CKII	Casein Kinase II
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DCX	Doublecortin
fEPSP	Field Excitatory Post-synaptic Potential
HECT	Homologous to E6AP C-Terminus
HFS	High-frecuency Stimulation
ICV	Intracerebroventricular
LTD	Long-term Depression
LTP	Long-term Potentiation
MPP	Medial Perforant Pathway
NMDAR	*N*-methyl-D-aspartate receptor
NPSLE	Neuropsychiatric Systemic Lupus Erythematosus
NSPA	Neuronal Surface P Antigen
PBS	Phosphate buffered saline
PKC	Protein Kinase C
PSD	Postsynaptic Density
PSD95	Postsynaptic density protein 95
PTPN4	Protein tyrosine phosphatase, non-receptor type 4
PTX	Picrotoxin
RBR	RING-in-between-RING
RING	Really Interesting New Gene
SAP102	Synapse associated protein 102
SC	Schaffer collateral
SDS	Sodium dodecyl sulfate
SGZ	Subgranular Zone

SLE	Systemic Lupus Erythematosus
$STEP_{61}$	Striatal-enriched protein tyrosine phosphatase
Syn-1	Synapsin-1
UPS	Ubiquitin Proteasome System

RESUMEN

El lupus eritematoso sistémico, prototipo de enfermedad autoinmune, frecuentemente manifiesta disfunciones cerebrales difusas que incluyen deficiencias cognitivas y psicosis en casos más extremos. Estas manifestaciones son parte del espectro de manifestaciones del lupus neuropsiquiátrico (LES-NP) cuyos mecanismos todavía no están bien dilucidados. Autoanticuerpos anti-proteína P ribosomal (Anti-P) se han asociado a psicosis lúpica y déficit cognitivo en pacientes con LES-NP. Nuestro laboratorio introdujo el concepto de que las manifestaciones cerebrales difusas del LES-NP involucrarían una reacción cruzada de los anticuerpos anti-P con una proteína de la superficie celular de función desconocida que llamamos NSPA (neuronal surface P antigen). En cerebro, la NSPA se expresa exclusivamente en neuronas y en regiones específicas tales como corteza e hipocampo. Para entender la patogenia de los anticuerpos anti-P en el LES-NP es necesario dilucidar la función de la NSPA en el cerebro. La estructura primaria de la NSPA muestra un dominio APC10 que sólo se ha descrito en E3 ubiquitin ligasas, sugiriendo que la NSPA podría ser una E3 ubiquitin ligasa. En base al fenotipo de un ratón que expresa una versión truncada de la NSPA que carece del dominio APC10 postulamos la siguiente hipótesis: La NSPA es una ubiquitin ligasa que regula los niveles del receptor de NMDA en la región post-sináptica. Para esto caracterizamos un ratón carente de NSPA evaluando si la NSPA es necesaria para la función del hipocampo y la transmisión sináptica, particularmente en procesos dependientes del receptor de NMDA, también evaluamos si la NSPA posee características de algún tipo de E3 ubiquitin ligasa. Encontramos que la NSPA se requiere para la transmisión glutamatérgica y la plasticidad sináptica mediada por los NMDARs, lo que se traduce en alteraciones en procesos de memoria espacial y de reconocimiento. La falta de NSPA lleva a una disminución de las subunidades GluN2 del NMDAR en la densidad post-sináptica. También se asocia a una reducción en la fosforilación de la subunidad GluN2B en la tirosina 1472, residuo que modula la endocitosis del NMDAR. Encontramos que la tirosina fosfatasa PTPN4 se ubiquitina y sus niveles de ubiquitinación se encuentran disminuidos en ratones que carecen de NSPA. Esto lleva a un aumento de la masa de PTPN4 en la región sináptica, resultando en una menor fosforilación del residuo de tirosina 1472 de la subunidad GluN2B del NMDAR involucrado en endocitosis. Además, la NSPA se requiere para la neurogénesis adulta en el giro dentado del hipocampo, proceso que también se ha asociado a función de los NMDARs. Por otra parte, vimos que la NSPA posee algunas características E3 ligasas del tipo RBR. Por lo tanto, nuestros resultados indican que la NSPA podría ser o formar parte de una E3 ubiquitin ligasa, que tiene como sustrato a la PTPN4, que a su vez regula los niveles de NMDAR en la región post-sináptica y en consecuencia regula las funciones del hipocampo que dependen de estos receptores, incluyendo la memoria.

ABSTRACT

Systemic lupus erythematosus, a prototype of autoimmune disease, frequently manifests diffuse cerebral dysfunctions that include cognitive deficits and psychosis in more extreme cases. These manifestations are part of the spectrum of manifestations of neuropsychiatric lupus (NPSLE) whose mechanisms are still not well understood. Anti-ribosomal P autoantibodies (Anti-P) have been associated with lupus psychosis and cognitive impairment in patients with NPSLE. Our laboratory introduced the concept that diffuse brain manifestations of NPSLE would involve a cross reaction of anti-P antibodies with a cell surface protein of unknown function that we call NSPA (neuronal surface P antigen). In the brain, NSPA is only expressed by neurons of specific regions, including cortex and hippocampus. To understand the pathogenesis of anti-P antibodies in NPSLE, it is necessary to elucidate the function of NSPA in the brain. NSPA primary structure shows the presence of an APC10 domain, this domain has been found only in E3 ubiquitin ligases, suggesting that NSPA could be an E3 ligase. Based on the phenotype of a mouse that expresses a truncated version of NSPA that lacks the APC10 domain, we postulate the following hypothesis: NSPA is an ubiquitin ligase that regulates the levels of NMDA receptor in the postsynaptic region. We characterized a NSPA knockout mice to evaluate if NSPA is necessary for hippocampal function and synaptic plasticity, particularly in NMDAR-dependent processes, also we evaluate whether NSPA possesses E3 ubiquitin ligases characteristics. We found that NSPA is required for glutamatergic transmission and NMDARs-mediated synaptic plasticity, which results in alteration of spatial and recognition memory. NSPA absence resulted in decreased levels of NMDAR GluN2 subunits in the postsynaptic density. It is also associated with a reduction in the phosphorylation of GluN2B in tyrosine 1472, residue that mediates NMDAR endocytosis. We found that PTPN4 tyrosine phosphatase is ubiquitinated and there is decreased ubiquitination in NSPA knockout mice. This leads to an increase in the mass of PTPN4 in the synaptic region, resulting in less phosphorylation of tyrosine 1472 of GluN2B subunit of NMDAR that is involved in endocytosis. Moreover, NSPA is required for adult neurogenesis in the dentate gyrus of the hippocampus, process that also has been associated with NMDARs function. On the other hand, we saw that NSPA has some characteristics of RBR E3 ligases. Therefore, our results indicate that NSPA could be or be part of an E3 ubiquitin ligase, that has PTPN4 as a substrate, which in turn regulates NMDAR levels in the postsynaptic region and consequently regulates hippocampal functions that depend on these receptors, including memory.

INTRODUCTION

Statement of the problem

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by the generation of a wide variety of autoantibodies and the compromise of several organs including the brain (Mok & Lau 2003, Sherer et al 2004, Yaniv et al 2015). Neuropsychiatric systemic lupus erythematosus (NPSLE) syndromes include diffuse brain dysfunctions like cognitive dysfunction, acute confusional state, anxiety, mood disorder and psychosis (Hanly 2004, Hanly 2014). NPSLE has been estimated to occur in >20 % of SLE patients, being a major source of morbidity and second cause of mortality after lupus nephritis (Schwartz et al 2019). The pathogenic mechanisms of NPSLE are likely diverse, considering the variety of neuropsychiatric syndromes developed by these patients. However, increasing interest has been focused on the possibility that certain autoantibodies might be neuropathogenic and therefore their recognized antigens and functional consequences in neurons are the center of attention (Schwartz et al 2019).

Two autoantibodies have been extensively studied as potential pathogenic mediators of NPSLE. One is a subset of anti-double stranded DNA antibodies found to cross-react with an epitope located in GluN2 subunits of NMDAR (Diamond et al 2013). The other corresponds to anti-ribosomal P (Anti-P) antibodies that have been associated with lupus psychosis and cognitive impairment in patients with SLE (Bonfa et al 1987, Gonzalez & Massardo 2018, Massardo et al 2015).

Anti-P antibodies induce calcium influx leading to apoptosis in cortical neurons, both in primary culture and *in situ* (Matus et al 2007). In mice, anti-P autoantibodies induce depression-like manifestations (Katzav et al 2008), and memory impairment without signs of apoptosis in hippocampus (Bravo-Zehnder et al 2015). Electrophysiology measuring shows that anti-P antibodies enhance glutamatergic postsynaptic transmission in the hippocampus, involving both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and *N*-methyl-D-aspartate (NMDA) receptor activation, leading to suppression of synaptic plasticity measured by LTP (Segovia-Miranda et al 2015). Thus anti-P antibodies reaching the hippocampus can produce perturbations of synaptic transmission causing impairment of hippocampal-dependent memory.

Our laboratory has contributed to this field identifying a cross-reacting P antigen found in a cell surface protein of unknown function, which was named neuronal surface P antigen (NSPA) (Matus et al 2007). NSPA expression in brain includes areas involved in cognition, memory and emotion like hippocampus, cortex and amygdala (Matus et al 2007). Analysis of NSPA amino acidic sequence shows some conserved protein domains, an EF-hand domain, one anaphase promoter complex 10 (APC10) domain and two ZZ-type zinc finger domains (Segovia-Miranda et al 2015). The presence of an APC10 domain in NSPA points to a possible role as an E3 ubiquitin ligase as this domain is only present in these enzymes (Boratyn et al 2012). ZZ-type finger domains are also found in some E3 ubiquitin ligases (Garcia-Gonzalo & Rosa 2005, Hwang & Murray 1997, Kaustov et al 2007). E3 ligases are enzymes that catalyzes ubiquitin transfer onto a specific protein in the process of

protein ubiquitination (Glickman & Ciechanover 2002). E3 ligases are important components of the machinery that regulates synaptic transmission and plasticity (Fu et al 2011, Mabb & Ehlers 2010).

NSPA knock-in mice ($NSPA^{tr/tr}$), that express a truncated NSPA lacking the N-terminal region bearing the APC10 domain, are characterized by depressed NMDAR-dependent transmission, impaired LTP and poor performance in the Morris water maze and flexible memory tests (Segovia-Miranda et al 2015). Also anti-P antibodies do not increase the intracellular calcium levels or enhance the postsynaptic potentials in $NSPA^{tr/tr}$ neurons (Segovia-Miranda et al 2015).

All these data indicate that NSPA is the mediator of the neuronal function-interfering effects of anti-P and that the APC10 domain is required for NSPA function. However, how NSPA regulates NMDAR function in synaptic transmission and its related processes remains unknown. Actually, the function of NSPA in the brain is not clear and this uncertainty includes whether NSPA effectively is or is part of an E3 ubiquitin ligase.

Glutamatergic ionotropic NMDA receptors are widely distributed and mediate most of the excitatory neurotransmission in the brain (Rao & Finkbeiner 2007). NMDAR play a critical role in excitatory synaptic transmission, plasticity and excitotoxicity in the central nervous system (CNS) (Cull-Candy & Leszkiewicz 2004). Synaptic plasticity underlies memory processes (Citri & Malenka 2008). Thus, NMDARs play an important role in learning and memory (Cercato et al 2014). Posttranslational modifications of NMDAR, such as phosphorylation and ubiquitination, alter both the activity and properties of NMDARs (Goebel-Goody et al

2009, Mao et al 2009). Particularly phosphorylation of cytoplasmic C-tails of GluN2A and GluN2B subunits of NMDAR has emerged as a key regulatory mechanism to regulate NMDAR function, trafficking and interactions with cytosolic proteins (Chen & Roche 2007, Lussier et al 2015).

This thesis studies the mechanism by which NSPA might modulate NMDAR function. We use both NSPA knock-in and NSPA knockout mice to analyze if NSPA absence affects NMDAR expression levels, posttranslational modifications and synaptic abundance, all modulators of NMDAR function. We also evaluate if NSPA absence could affect NMDAR function in hippocampus, mainly synaptic transmission and plasticity dependent of NMDAR activity (Li et al 2007), as well as hippocampal-dependent processes that have been related to NMDAR function like spatial and recognition memory (Yamada et al 2015) and adult neurogenesis in the subgranular zone of the dentate gyrus (Taylor et al 2014). We also performed biochemical experiments aimed to obtain experimental evidence of NSPA as an E3 ubiquitin ligase.

The results of this thesis provide evidence of a role of NSPA as an E3 ubiquitin ligase that regulates NMDAR function involving an ubiquitinated substrate such as the tyrosine phosphatase PTPN4. We show data suggesting that PTPN4 mediates NMDAR tyrosine phosphorylation with consequences on NMDAR synaptic abundance and function. The phenotype of NSPA knockout mice indicates that NSPA is not only required for glutamatergic synaptic plasticity in hippocampus but also for adult neurogenesis in the dentate gyrus, both explainable through a determinant

regulation of NMDAR function. PTPN4 emerged as the first molecular link between NSPA and NMDAR function.

Literature Review

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic and multisystemic autoimmune disease that predominantly affects women of childbearing age, characterized by the generation of autoantibodies and the compromise of several organs (Mok & Lau 2003, Tsokos 2011, Yaniv et al 2015). The great diversity of clinical manifestations is accompanied by a large number of autoantibodies, some correlating with disease activity and clinical manifestations (Sherer et al 2004). Within this diversity of clinical manifestations, cerebral manifestations are called neuropsychiatric systemic lupus erythematosus (NPSLE), NPSLE frequency is unknown as different studies report among 12% to 95% in adult SLE (AlE'ed et al 2017, Schwartz et al 2019), but the most likely figure stands as >20% (Schwartz et al 2019). Central nervous system disease predominates in NPSLE and may take the form of either focal or diffuse brain dysfunctions (Hanly & Harrison 2005, Sciascia et al 2014). Diffuse brain dysfunctions includes headaches, cognitive dysfunction, acute confusional state, anxiety, mood disorder and psychosis, being cognitive dysfunction the most frequent manifestation (Hanly 2004, Harboe et al 2009, Schwartz et al 2019). The pathogenic mechanism responsible for these manifestations remains incompletely understood, but accumulated evidence suggests that function-perturbing autoantibodies against neuronal cell surface components might contribute (Diamond

et al 2013, Hanly 2014, Hanly & Harrison 2005). Several autoantibodies have been associated with NPSLE, but mainly there are two autoantibodies found to cross-react with intracellular components and neuronal surface protein-targets providing mechanistic insights for direct neuropathogenic actions (Diamond et al 2013, Diamond et al 2009). One is a subset of anti-double-stranded DNA antibodies that cross-react with *N*-methyl-D-aspartate receptor (NMDAR) (DeGiorgio et al 2001), the other are anti-ribosomal P antibodies that cross-react with a cell surface protein of unknown function called neuronal surface P antigen (NSPA) (Massardo et al 2015, Matus et al 2007). Anti-NMDAR and anti-P antibodies are present in the cerebrospinal fluid (CSF) of patients with SLE who experienced diffuse NPSLE syndromes (Arinuma et al 2008, Hanly et al 2011, Hirohata et al 2007, Lapteva et al 2006). Anti-NMDAR antibodies induce neurotoxicity and cognitive alterations in mice (DeGiorgio et al 2001, Huerta et al 2006, Kowal et al 2004). These antibodies recognize a consensus amino acid sequence (DWEYS) in the GluN2 subunits (GluN2A and GluN2B) of NMDA receptor, bound to the active/open configuration of the receptor enhancing its activity (Faust et al 2010) and providing a pathogenic mechanism for the anti-NMDAR in NPSLE.

Antiribosomal P antibodies (Anti-P)

Anti-P antibodies have been associated mostly with lupus psychosis and cognitive impairment in patients with SLE (Bonfa et al 1987, Massardo et al 2015, Sciascia et al 2014). Studies in vitro have shown that anti-P antibodies can bind to the surface of different human cells (Koren et al 1992, Stafford et al 1997), penetrating into live cells and causing cellular dysfunctions like inhibition of protein synthesis and

apoptosis (Koscec et al 1997, Reichlin 1998, Sun et al 2001). In cortical neurons, anti-P antibodies have been reported to induce calcium influx leading to apoptosis both *in vitro* and *in vivo* (Matus et al 2007), more over, passive-transfer experiments in mice showed that anti-P autoantibodies induced smell alterations (Katzav et al 2008), depression-like manifestations when injected intracerebroventricular (ICV) (Katzav et al 2007), and memory impairment when injected intravenously and the blood-brain barrier (BBB) is disrupted (Bravo-Zehnder et al 2015). In the later case anti-P antibodies driven memory impairment occur without signs of apoptosis in hippocampus, thus anti-P antibodies reaching the hippocampus may be producing synaptic alterations enough to perturb memory. Electrophysiology measuring field excitatory postsynaptic potentials in hippocampal slices and whole-cell voltage-clamp in spinal cord neurons shows that anti-P antibodies enhance glutamatergic postsynaptic transmission in the hippocampus, involving both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and NMDA receptor activation, which leads to suppression of synaptic plasticity measured by LTP (Segovia-Miranda et al 2015). These effects can explain the impairment of hippocampal-dependent memory caused by circulating anti-P autoantibodies in mice.

Anti-P antibodies recognize an 11 amino acid epitope (P-epitope) contained in the C-terminal of three highly conserved phosphoproteins, P0 (38 kDa), P1 (19 kDa) and P2 (17 kDa) of the large ribosomal subunit (Elkon et al 1988, Elkon et al 1986). The early demonstration of interaction of anti-P antibodies with a cell surface component, originally attributed to a ribosomal like P0 (Koren et al 1992, Stafford et al 1997), led to the identification of a new cross-reacting antigen that shares and

exposes a P-epitope to the cell surface in neurons, called neuronal surface P antigen (NSPA) (Matus et al 2007).

Neuronal Surface P Antigen (NSPA)

NSPA is a high mass integral plasma membrane protein of unknown function, in brain is expressed exclusively by neurons and at particular regions, including areas involved in cognition, memory and emotion like hippocampus, cortex and amygdala (Matus et al 2007). We analyzed the amino acidic sequence of NSPA with BLAST (Basic Local Alignment Search Tool), and we did not found homology with other known family of proteins. However, NSPA has some conserved protein domains that could help us to elucidate its function. These domains include an EF-hand domain, one anaphase promoter complex 10 (APC10) domain and two ZZ-type zinc finger domains (Segovia-Miranda et al 2015). EF-hand domains are Ca^{2+} binding sites found in more than 100 proteins, many of which function as Ca^{2+} sensors or Ca^{2+} buffers (Ikura 1996). Using Delta-Blast we found that APC10 domain is only present in E3 ubiquitin ligase proteins (Boratyn et al 2012). The APC10 domain was formerly described in the anaphase promotor complex (APC/C) (Hwang & Murray 1997), a 1,5 MDa E3 ubiquitin ligase conformed by at least 12 subunits, and it likely mediates substrate interaction and recognition (Buschhorn et al 2011, Nourry et al 2004, Passmore et al 2003). ZZ-type zinc finger domains, named because of their ability to bind two zinc ions (Ponting et al 1996), contain 4-6 Cysteine residues that participate in zinc binding, these zinc fingers are thought to be involved in protein-protein interactions. The structure of the ZZ domain shows that it belongs to the family of cross-brace zinc finger motifs that include the PHD, RING, and FYVE domains

(Legge et al 2004). ZZ-type zinc finger domains are found in transcription factors, chromatin-remodeling complex, Dystrophin and its homologues and E3 ubiquitin ligases, among others (Gamsjaeger et al 2007, Hnia et al 2007, Nishito et al 2006, Wu et al 2010). Interestingly there are some E3 ubiquitin ligases like Cullin9/PARC and HERC2 that contain an APC10 domain and one or more Zinc finger domains (Garcia-Gonzalo & Rosa 2005, Kaustov et al 2007).

NSPA distribution in hippocampus includes the postsynaptic region and partial colocalization with NMDAR at the surface of neurons (Segovia-Miranda et al 2015). By using an NSPA knock-in mouse ($NSPA^{tr/tr}$), that express NSPA lacking both EH-hand and APC10 domains, we determined that NSPA mediates the neuronal effects of anti-P, and that it is required for NMDAR function, LTP, and memory tasks. $NSPA^{tr/tr}$ mice perform poorly in the Morris water maze and memory flexibility tests reflecting an impaired hippocampal function. Furthermore, electrophysiology experiments showed that hippocampus of $NSPA^{tr/tr}$ mice has depressed NMDAR-dependent transmission and impaired LTP (Segovia-Miranda et al 2015). This evidence suggests that NSPA may be an E3 ubiquitin ligase or a subunit of one that regulates NMDAR in glutamatergic synaptic transmission.

N-methyl-D-aspartate receptor (NMDAR)

Excitatory synapses in the mammalian brain occur mostly on dendritic spines, where receptors and downstream signaling enzymes are clustered in the postsynaptic density (PSD). The PSD is a cytoskeletal web beneath the plasma membrane that contains five classes of proteins: neurotransmitter receptors, cell-adhesion proteins, adaptor molecules, signaling enzymes and cytoskeletal proteins (Kennedy 2000).

The vast majority of synapses in the central nervous system (CNS) uses glutamate as neurotransmitter to produce rapid neuronal excitation (Nakanishi 1992). Glutamate released from presynaptic terminals activates several types of glutamate-gated ion channels on postsynaptic membranes, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and *N*-methyl-D-aspartate (NMDA) receptors (Rao & Finkbeiner 2007).

AMPA receptors mediate most of the rapid excitatory transmission in the mature brain, while NMDA receptors require membrane depolarization to open because of a voltage-dependent Mg^{2+} block. During bouts of synaptic activity, AMPA receptor-mediated depolarization of the postsynaptic membrane facilitates activation of NMDA receptors, which mediates Ca^{2+} influx (Perez-Otano & Ehlers 2004, Rao & Finkbeiner 2007). This influx is critical for activity-dependent synaptic plasticity (Monyer et al 1992) and initiate Ca^{2+}-dependent signaling pathways that modulate the surface presence of AMPA receptors (Ehlers 2000). AMPA receptors are composed of various combinations of four subunits (GluA1-GluA4), and only AMPA receptors that lack the GluA2 subunit are permeable to Ca^{2+} (Cull-Candy et al 2006), by contrast permeability to Ca^{2+} is a feature of all NMDA receptors (Rao & Finkbeiner 2007).

NMDA receptors are heterotetrameric complexes incorporating seven different subunits within a repertoire of three subtypes: the GluN1 subunit, four distinct GluN2 subunits (GluN2A, GluN2B, GluN2C and GluN2D) and two GluN3 subunits (GluN3A and GluN3B) (Paoletti et al 2013, Paoletti & Neyton 2007), with two obligatory GluN1 subunits (Laube et al 1998). Subunit composition of NMDA receptors changes during

development and varies in different regions of the mature brain (Paoletti et al 2013, Petralia et al 2005), for instance GluN1 subunit is expressed ubiquitously throughout the brain regions from embryonic stages to adulthood, GluN2A expression starts shortly after birth and rises to become widely and abundantly expressed in virtually every CNS area in the adult. GluN2B expression is maintained at high levels following birth, peaks around the first postnatal week and becomes progressively restricted to the forebrain, mainly to the hippocampus and cortex (Monyer et al 1994, Monyer et al 1992). Different levels of synaptic NMDA receptor activation with corresponding degrees of calcium influx can lead to multiple effects, thus the number and composition of synaptic NMDAR must be under careful control and can be modulated by several factors (Barria & Malinow 2002, Wenthold et al 2003).

Posttranslational regulation of NMDA receptors

The cytoplasmic carboxyl termini of synaptic receptors have been shown to play critical roles in directing the trafficking to and stabilization at synaptic sites, particularly the cytoplasmic C-tails of GluN2A and GluN2B subunits of NMDAR contain distinct motifs that control their trafficking (Sanz-Clemente et al 2013). GluN2A and GluN2B are subjected to differential regulation by several posttranslational mechanisms in their C-tails, including ubiquitination (Jurd et al 2008, Yin et al 2011) and palmitoylation (Mattison et al 2012). However, the best-characterized example is the modulation of NMDAR by phosphorylation, emerging as a key regulatory mechanism controlling NMDAR function, trafficking and interactions with cytosolic proteins (Chen & Roche 2007, Goebel-Goody et al 2009). Phosphorylation regulates the surface and synaptic expression of NMDARs in a

subunit-specific manner, providing a highly plastic and precise mechanism to accurately control different subunits in response to stimuli (Lussier et al 2015).

Regulation of NMDAR by phosphorylation

There is ample evidence that NMDAR function is regulated by a variety of protein kinases, including serine/threonine and tyrosine kinases (Chen & Roche 2007). GluN2A and GluN2B subunits have been found to undergo active phosphorylation in serine/threonine residues, for instance PKC-mediated phosphorylation on Ser1416 residue of GluN2A decreases its binding affinity for aCaMKII (Gardoni et al 2001), Ser1480 residue of GluN2B is directly phosphorylated by casein kinase II (CKII), this residue is present in the PDZ ligand ($ESDV_{COOH}$) of GluN2B, when phosphorylated it reduces GluN2B interaction with PSD95 and synapse associated protein 102 (SAP102) regulating endocytosis of NMDARs (Chung et al 2004, Lee 2006). NMDARs are tightly and finely modulated by the counterbalanced activity of protein-tyrosine kinases and tyrosine phosphatases (Salter & Kalia 2004), GluN2 subunits contain several tyrosine phosphorylation sites that can be phosphorylated by non-receptor tyrosine kinases, Src and Fyn (Chen & Roche 2007, Kalia et al 2004, Kohr & Seeburg 1996). Internalization of GluN2A is regulated by phosphorylation of Tyr842 residue, which is part of a consensus endocytic motif (YXXØ) that is recognized by the clathrin adaptor AP-2, phosphorylation by Src prevents AP-2 interaction blocking the receptor internalization (Vissel et al 2001). In a similar manner, GluN2B phosphorylation at Tyr1472 prevents endocytosis enhancing NMDAR surface expression (Prybylowski et al 2005), and has been associated with synaptic enrichment of NMDARs (Goebel-Goody et al 2009).

This residue is also contained within an endocytic motif (Roche et al 2001), Tyr1472 phosphorylation blocks AP-2 binding thus preventing endocytosis of the receptor and, therefore, increasing its surface expression (Lavezzari et al 2003, Prybylowski et al 2005). Tyr1472 of GluN2B is specifically phosphorylated by the Src family tyrosine kinase Fyn (Nakazawa et al 2001) and dephosphorylated by Striatal-enriched protein tyrosine phosphatase (STEP), leading to internalization of NMDAR complexes (Kurup et al 2010, Venkitaramani et al 2011).

NMDA receptors role in synaptic plasticity and memory

NMDARs play a critical role in synaptogenesis, synaptic plasticity including experience-dependent synaptic remodeling and long-lasting changes in synaptic efficacy (Collingridge et al 2004, Wang et al 2006). For over a century, synaptic plasticity has been proposed to play a central role in the capacity of the brain to incorporate transient experiences into persistent memory traces (Citri & Malenka 2008). The most extensively studied and therefore prototypic forms of synaptic plasticity are the long term-potentiation (LTP) and long-term depression (LTD) observed in the CA1 region of the hippocampus, which are triggered by activation of NMDARs during postsynaptic depolarization (Citri & Malenka 2008, Malenka & Bear 2004). LTP and LTD are forms of activity-dependent synaptic plasticity believed to play important roles in learning and memory processes (Barria & Malinow 2005). Thus hippocampal NMDARs play an important role in learning and memory, especially in spatial memory (Cercato et al 2014, Iwamura et al 2016, Morris 1989).

NMDA receptors and adult neurogenesis

Other process that is regulated by hippocampal neuronal activity levels is adult neurogenesis in the dentate gyrus subgranular zone (Deisseroth et al 2004). The dentate gyrus of the mammalian hippocampus continuously generates new neurons during adulthood, and they functionally integrate into the hippocampal circuitry (Goncalves et al 2016, Mu et al 2015), moreover it has been observed a positive correlation between hippocampal neurogenesis, LTP in dentate gyrus, learning and memory in mice (Singer et al 2011, Snyder et al 2001, Zhao et al 2008). The roles of NMDAR in regulating neurogenesis have been elusive, some studies have demonstrated that stimulation of NMDAR increases neurogenesis (Arvidsson et al 2001, Deisseroth et al 2004)., others strongly suggest an important role of NMDARs in the survival of new neurons in the dentate gyrus, but not in the general mechanism of neuronal maturation (Tashiro et al 2006). Whereas other reports indicated that neurogenesis in the dentate gyrus is down-regulated by NMDAR activation, or up-regulated by NMDAR inhibition (Cameron et al 1995, Hu et al 2008, Nacher & McEwen 2006). The contradictory results may be explained by the experimental conditions used, and also by the NMDAR subunit composition (Kheirbek et al 2012, Mu et al 2015), among other variables.

Ubiquitin-proteasome system and synaptic function

The covalent addition of ubiquitin to target proteins has been shown to mediate protein degradation, signal transduction, and membrane trafficking (Haglund & Dikic 2005). The ubiquitin-proteasome system (UPS) is a complex proteolytic pathway that degrades proteins and has emerged as an important mechanism controlling normal

brain functions, including synapse maintenance, regulation and organization, as well as brain disorders (Hegde & DiAntonio 2002, Patrick 2006, Yi & Ehlers 2007). Postsynaptic plasticity can be modulated by the remodeling of the postsynaptic density (PSD) (Mabb & Ehlers 2010). It has been shown that synaptic activity regulates postsynaptic composition and signaling through degradation of postsynaptic proteins by the UPS (Ehlers 2003). UPS activity is required for LTP maintenance (Karpova et al 2006) and also for the formation of long-term memory in animals (Lopez-Salon et al 2001).

The process of protein ubiquitination involves the covalent tethering of a small 76 amino acid protein called ubiquitin to target proteins and occurs in a sequential, ATP-dependent reaction involving three enzymes: an E1 activating enzyme, an E2 conjugating enzyme and an E3 ubiquitin ligase which catalyzes ubiquitination of specific targets (Glickman & Ciechanover 2002, Pickart 2001). Monoubiquitination usually marks the protein substrates in the plasma membrane for endocytosis; poliubiquitination marks proteins for degradation by the proteasome (Glickman & Ciechanover 2002, Hegde 2004).

E3 ubiquitin ligases

E3 ubiquitin ligases binds to both the E2-Ub thioester and the substrate and promote ubiquitin transfer onto a specific protein, playing a crucial role in target specificity (Berndsen & Wolberger 2014). In human these ligases comprise over 600 different proteins, in contrast with the two E1 and the estimated 40 E2, consistent with their role in conferring specificity and regulation to ubiquitination (Li et al 2008, Metzger et al 2014). A few E3 ligases have emerged as regulators of diverse

processes in neurons (Yamada et al 2013b), a prominent example is the APC/C, this RING E3 ubiquitin ligase has been implicated in axon growth regulation (Stegmuller et al 2006), neurogenesis (Delgado-Esteban et al 2013) and synaptic transmission and plasticity (Fu et al 2011). There are three major classes of eukaryotic E3 ligases: Really Interesting New Gene (RING)-type E3s, Homologous to E6-AP C-Terminus (HECT)-type E3s, and RING-in-between-RING (RBR) E3s (Dove et al 2016).

RING E3 ligases

RING ligases are defined by the presence of a RING (or RING-like) domain, a zinc finger type domain and constitute the vast majority of known E3s (Deshaies & Joazeiro 2009). RING domains consists of seven cysteine residues and one histidine residue forming a single folded domain that coordinate two Zn^{2+} ions in a cross-braced arrangement to create a platform for binding of E2s (Borden et al 1995). RING ligases serves as scaffolds that facilitate direct transfer of ubiquitin from the E2 to the target protein (de Bie & Ciechanover 2011) (Figure 1). Within the family of RING ligases there are single-chain enzymes, homodimers and heterodimers, and also there are RING ligases that exist as multi-subunits assemblies (Berndsen & Wolberger 2014, Deshaies & Joazeiro 2009).

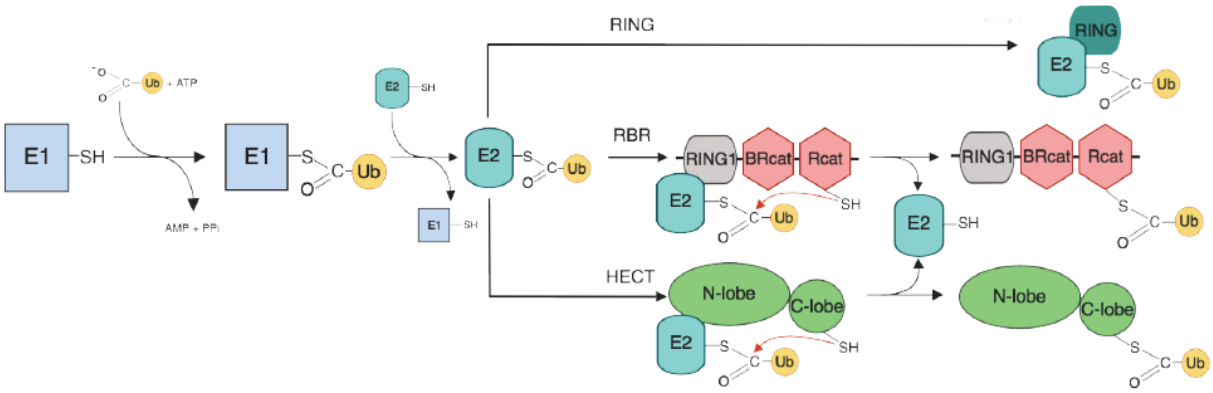
HECT E3 ligases

HECT E3 ligases are characterized by the presence of a ~350-residue region, called HECT domain (homologous to E6-AP C-Terminus), which is a strictly conserved cysteine residue positioned ~35 residues upstream of the C terminus (Zhang et al 2009). The HECT domain contains an active cysteine residue to which ubiquitin binds prior to its transfer to the substrate, forming a thioester with ubiquitin

(Huibregtse et al 1995) (Figure 1). This ubiquitin is subsequently transferred to a lysine residue of a substrate molecule (Al-Hakim et al 2012).

RING-in-between-RING (RBR) E3 ligases

RBR E3 ligases contain a highly conserved catalytic unit consisting of a RING1, an in-between RING (IBR) also called BRcat, and a RING2 domain also known as Rcat (Spratt et al 2014, Wenzel et al 2011). RBR E3 ligases share some features with both RING and HECT E3 ligases family, the ubiquitin transfer is initiated by the interaction of an E2-Ub with the RBR, similar to the interaction between E2s and classical RING E3-ligases, this interaction is used to facilitate the formation of a HECT-like thioester intermediate between the C-terminus of the ubiquitin and an active cysteine on RING2 before it is coupled to its substrate (Marin et al 2004, Smit et al 2012) (Figure 1). An intact RBR domain is necessary for efficient E3-ligase functioning, however Parkin IBR-RING2 can mediate the formation of ubiquitin linkages in the absence of RING1 (Chew et al 2011, Matsuda et al 2006).



(Adapted from *Spratt et al. Biochem J 2014*)

Figure 1. Ubiquitination pathways by different E3 ubiquitin ligases. The ubiquitin activating enzyme (E1) activates ubiquitin through an ATP-dependent mechanism to form a thioester bond between ubiquitin and the catalytic cysteine in the E1. The ubiquitin is then transferred to an ubiquitin conjugating enzyme (E2) to form a thioester bond between ubiquitin and the conserved catalytic cysteine residue of the E2. RING E3 ligases serves as scaffolds that facilitate direct transfer of ubiquitin from the E2 to the target protein. HECT E3 ligases engage the E2-ub complex via their HECT domain in the N-lobe forming a thioester bond between ubiquitin and the conserved catalytic cysteine residue in the C-lobe of the HECT E3s. RBR E3 ligases use a combination of the RING and HECT mechanisms, the RING1 engages with the E2-ub complex in a similar manner to the RING E3s, whereas the Rcat acts in a similar fashion to the C-lobe of the HECT E3s by performing a transthiolation reaction to form a thiolester bond between ubiquitin and the catalytic cysteine of the Rcat.

HYPOTHESIS

NSPA regulates NMDA receptors function through posttranslational modifications.

GENERAL OBJECTIVE

To define NSPA function in posttranslational modifications, expression levels and function of NMDA receptors in mice hippocampus.

SPECIFIC OBJECTIVES

1. To analyze NSPA function in NMDAR expression levels and function.

2. To study NSPA function in posttranslational modifications and synaptic abundance of NMDAR.

3. To evaluate NSPA as a possible E3 ubiquitin ligase.

MATERIALS AND METHODS

Antibodies. The following antibodies were used: mouse anti-GluN1 (Cat.#75-272), mouse anti-GluN2A (Cat.#75-288), mouse anti-GluN2B (Cat.#75-101), mouse anti-GluA1 (Cat.#75-327), mouse anti-GluA2 (Cat.#75-002) and mouse anti-PSD95 (Cat.#75-028) from UC Davis/NIH/NeuroMab Facility (UCLA, Davis, CA, USA, USA), rabbit anti-phospho-GluN2B(Tyr1472) (Cat.#4208) (Cell Signaling Technology), rabbit anti-Synapsin I (Cat.#ab8) (Abcam), mouse anti-FYN (Cat.#sc-434) (Santa Cruz Biotechnology), mouse anti-STEP (Cat.#05-730) (Merck Millipore), rabbit anti-PTPN4 (Cat.#10818) (Allele Biotechnology), mouse anti-ubiquitin P4D1 (Cat.#sc-8017) (Santa Cruz Biotechnology), rabbit anti-ZZEF1 (Cat.#ab176594) (Abcam), affinity-purified rabbit anti-APC10, rat anti-BrdU (Abcam), rabbit anti-mcherry (Cat.#ab167253) (Abcam), rabbit anti-Doublecortin (Cat.#460467) (Cell Signaling Technology), mouse anti-Nestin (Cat.#ab11306) (Abcam), rabbit anti-Arc (Cat.#203056) (Abcam) and rabbit anti-Ki67 (Cat.#ab15580) (Abcam), mouse anti-GAPDH (Cat.#CB1001) (Millipore) and mouse anti-β-actin (Cat#ab6276) (Abcam). Primary antibodies for immunoblot were recognized with horseradish peroxidase (HRP)-conjugated antibodies (Rockland). As secondary antibodies for immunofluorescence Alexa (Molecular Probes) and DyLight (Abcam) conjugated antibodies were used.

Lac-Z knockout mice, genotyping and NSPA expression. Mice were maintained under conditions of strict confinement, which included automatic control of temperature (21ºC) and photoperiod (12 h light / 12 h dark). Animals were housed at the Animal Facility of the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile. C57BL/6NTac Zzef1$^{tm2.1(KOMP)vlcg}$ (here called NSPA$^{-/-}$ or NSPA KO) mice were generated from *C57BL/6NTac* mice engineered in Regeneron Pharmaceuticals Inc., New York, using Velocigene technology) (Valenzuela et al 2003) for replacing the entire coding region of the mouse *Zzef1* gene (128 kb) with ZEN-UB1 Cassette containing the LacZ gene that encodes β-galactosidase. Details of the NSPA KO mice and PCR genotyping assay, including the predicted PCR products and the primers, are available at the Velocigene website (http://www.velocigene.com/komp/detail/10007). NSPA mRNA expression was assessed by RT-PCR with the following primers: exons 4-5 (TATAGAAACGTCCTCCAACCC and GCTTCATCTTCAAACGTATCCA), exons 20-22 (GTCAACTGGTCATCTTCCTG and TCACACCTCTCATCAAATTCCA), exons 50-52 (TAGTGACTTTCAGCAGGACC and GATCTCAAACCCTGTCTGGA) for mice mRNA.

Brain β-gal staining. Brains fixed by perfusion in 4% paraformaldehyde (PFA) in PB buffer (0.1M phosphate buffer, pH 7.4) were processed for β-gal staining (Poueymirou et al 2007).

Electron microscopy. Immunogold labeling was performed in tissue fixed with glutaraldehyde 2.5% in 0.1 M cacodylate buffer, pH 7.2 for 6 hours at room temperature and washed with sodium cacodylate buffer 0.1M, pH 7.2 for 18 hours at

4 °C, post-fixed with osmium tetroxide aqueous 1% for 90 min and stained with uranyl acetate aqueous 1% for 60 min. Tissues dehydrated and pre-embedded with epon:acetone 1:1 were finally embedded in epon, sectioned in Sorvall MT-5000 ultramicrotome and stained with uranyl acetate 4% in methanol for 2 min and lead citrate for 5 min.

Subcellular fractionation of mice hippocampi and immunoblotting. Hippocampi of WT or NSPA KO mice were dissected on ice and homogenized in homogenization buffer (0.32M sucrose, 0.5mM EGTA, 5mM Hepes, pH 7.4) supplemented with 4 mg/ml leupeptin, 4 mM PMSF, 4 mg/ml pepstatin, 25mM NaF, 100mM Na_3VO_4 and when specified with 25μM MG-132, using a Potter homogenizer, homogenates were centrifuged twice at 1.000 x g for 10 min at 4ºC (H). Subcellular fractionation was performed following the method of Wyneken et al. 2001 (Wyneken et al 2001). The supernatant (S1) was centrifuged at 12.000 x g for 20 min at 4ºC to obtain the crude synaptosomal membrane fraction (P2). Synaptosomes were collected from the first sucrose gradient at 1/1.2 M interphase and submitted to a hypo-osmotic shock to release intracellular organelles. Synaptic membranes were collected from the second sucrose gradient at the 1/1.2 M interphase and delipidated in 1% Triton to yield PSDs. Protein concentrations were determined using the BCA assay (Pierce, Thermo Fisher Scientific). Samples were resolved by SDS-PAGE, followed by immunoblotting on nitrocellulose membranes. Western blot assays were made as described (Matus et al 2007, Serrano et al 2014).

Immunoprecipitation. For immunoprecipitation of PTPN4, PSD95 and STEP, the crude synaptosomal membrane fraction (P2) from WT or NSPA KO mice hippocampi were solubilized in lysis buffer (50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100), immunoprecipitation was performed with either 1 ml of PTPN4 antibody or 10 ml of STEP antibody prebound to protein G-agarose. Beads were washed three times with lysis buffer, and immunoprecipitated proteins were eluted with SDS sample buffer for subsequent immunoblotting.

Behavioral Tests. Memory flexibility test: A modification of Morris water maze protocol was applied, in which memory retrieval must be selective for a recent episode while long-term memory potentially causes interference (Chen et al 2000, Serrano et al 2014). This water maze protocol requires of up to 15 trials of the rodents to learn to escape onto the hidden platform at one location, and then, the platform is move to a new point. Different areas are used successively during 4 days. As previously described, in such day earlier positions of the platform are encoded in long-term memory, potentially causing interference in the next day. (Chen et al 2000) Up to 15 training trials were performed per day until the criterion of 3 successive trials with an escape latency of <20 s was met. Upon testing completion, the mouse was gently removed from the maze and returned to its cage. Data were collected using a video tracking system coupled to Honestech TVR 2.5 program and analyzed off-line in ANY-MAZE software (Stoelting Co, Wood Dale, IL, USA).

Open field test: WT and NSPA KO mice were placed in a 40×40×40cm box and recorded of their horizontal locomotor activity for 10 min. The center zone line was 10cm apart from the edge. Data were collected using a video tracking system coupled

to Honestech TVR 2.5 program and analyzed off-line in ANY-MAZE software (Stoelting Co, Wood Dale, IL, USA).

Novel object recognition test: WT and NSPA KO mice were habituated to an open-field box for 10 min on day 1, on day 2 they were presented with two identical objects for 10 min, then he mice was removed and four hours later (delay) one of the objects was replaced with a novel one. Mice were allowed to explore freely during 10 min. Object recognition was measured by the amount of time during which the nose of the mouse pointed the object. Data were collected using a video tracking system coupled to Honestech TVR 2.5 program and analyzed off-line in ANY-MAZE software (Stoelting Co, Wood Dale, IL, USA).

BrdU administration and immunofluorescence. 5-Bromo-2′-deoxyuridine (BrdU, Sigma) was injected intraperitoneally (100mg/kg) to mice (8 weeks) for 3 days. Fourthteen days after the last injection, mice were perfused with 4% paraformaldehyde. Immunodetection of BrdU and neuronal markers in tissue sections was carried out as previously described (Abbott et al 2013). BrdU and Ki67 positive cells were counted using a fluorescence microscope (Olympus BX51, Tokyo, Japan) as described (Abbott et al., 2013). Double-labeled sections were analyzed by confocal laser microscopy (Olympus FV 1000). Image analysis and z-projections were made with ImageJ software (NIH, USA).

Electrophysiology. Field excitatory post-synaptic potential (fEPSP). Transverse slices (400 μm) from the dorsal hippocampus of two-month-old mice were prepared, maintained and processed for electrophysiology according with standardized procedures as previously described (Cerpa et al 2010) adding picrotoxin

(PTX; 10 μM) to suppress inhibitory GABA transmission. Recordings were filtered at 2.0–3.0 kHz, sampled at 4.0 kHz using an A/D converter (National Instrument, Austin, TX, USA), and stored with the WinLTP program. The basal excitatory synaptic transmission was measured using an input/output curve protocol, which consisted of eight stimuli ranging from 200 to 900 μA (the interval between stimuli was 10 s). Input-output curves were performed either in ACSF supplemented with 50 μM APV inhibitor of NMDAR or in ACSF lacking magnesium and supplemented with 10 μM NBQX inhibitor of AMPAR to measure the responses of AMPAR and NMDAR respectively. To generate LTP, we used high-frequency stimulation (HFS), which consisted of 3 or 4 trains of 100 pulses at 100 Hz of stimuli with an inter-train interval of 20 s. Data were collected and analyzed offline with pClamp 10 (Molecular Devices, San Jose, CA, USA).

Cell-Based Ubiquitination. HEK293 cells were transfected with NSPA-mcherry and myc-6xHis-ubiquitin, and 24 h after transfection subjected to a His-ubiquitin based assay as described (Tatham et al 2009) with minor modifications. Briefly, cells were harvested in ice-cold PBS, pelleted for 10 minutes at 3,000 rpm, resuspended in buffer A2 (6 M guanidine chloride, 0.1 M Na2HPO4/NaH2PO4, and 10 mM immidazole, pH 8.0) and incubated with 100 ml of Ni-NTA agarose (QIAGEN, Valencia/CA, www.qiagen.com) for 3 hours at room temperature. After binding, Ni-NTA beads were washed twice with buffer A2, twice with buffer A2/TI (1 volume of buffer A2 and 3 volumes of buffer TI) and once with buffer TI (25 mM Tris-HCl, 20 mM imidazole, pH 6.8). Proteins were eluted with buffer ETI (50 mM Tris-HCl, 20 mM imidazole, pH 6.8) and analyzed by SDS-PAGE and Western blotting.

Statistical Analysis. The software GraphPad PRISM Version 6.0c (San Diego, CA) was used for statistical analysis. Data are presented as the mean ± S.E.M values and differences were analyzed with the Student's *t*-test, one-way ANOVA or two-way ANOVA, as indicated. Statistical significances correspond to *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.

RESULTS

1.NSPA function in NMDA receptor expression levels and function.

Previous work showed that NSPA knock-in mice ($NSPA^{tr/tr}$) expressing a truncated form of NSPA lacking the EF-hand and anaphase promoter complex 10 (APC10) domains perform poorly in the Morris water maze and memory flexibility tests and also displayed depressed NMDAR-transmission and impaired LTP (Segovia-Miranda et al 2015). These results suggested that NSPA is some how required for NMDAR function and synaptic plasticity. However, we cannot discard unnoticed effects of the truncated version of NSPA. We now have knockout mice lacking any form of NSPA expression in which we decided to re-evaluate the phenotype regarding NMDAR expression levels and function in hippocampus.

1.1 NSPA knockout mice ($NSPA^{-/-}$) and NSPA expression in the brain.

In the NSPA full knockout mice ($NSPA^{-/-}$; $Zzef1^{tm2.1(KOMP)vlcg}$), the 55 exons of the NSPA-encoding gene *Zzef1* have been replaced with a β-gal cassette. We corroborated the lack of NSPA expression by analyzing different brain regions with reverse transcriptase-PCR (Figure 2B) with three primer pairs for different exons (Figure 2A). NSPA mRNA is expressed in various brain regions of wild type mice, including olfactory bulb, hippocampus, cortex and cerebellum, whereas NSPA mRNA was undetectable in $NSPA^{-/-}$ mice (Figure 2B). Immunoblots using commercial anti-ZZEF1 and our anti-APC10 antibodies, which gave positive reaction in MDCK cells transfected with NSPA-GFP, also demonstrated lack of NSPA expression in $NSPA^{-/-}$ mice (Figure 2C). Both anti-ZZEF1 and anti-APC10 antibodies recognize an epitope

that is absent in the truncated version of NSPA, unfortunately we do not have an antibody that recognizes NSPA truncated thus we could not differentiate between NSPA knockout and NSPA knock-in mice by immunoblot. β-gal staining reflecting the activity of NSPA promoter previously showed high levels of NSPA expression in hippocampus, although restricted to the CA1 and dentate gyrus of the dorsal hippocampus and absent from dorsal CA3 (Segovia-Miranda et al 2015). We corroborated this expression pattern by in the $NSPA^{-/-}$ mice (Figure 2D).

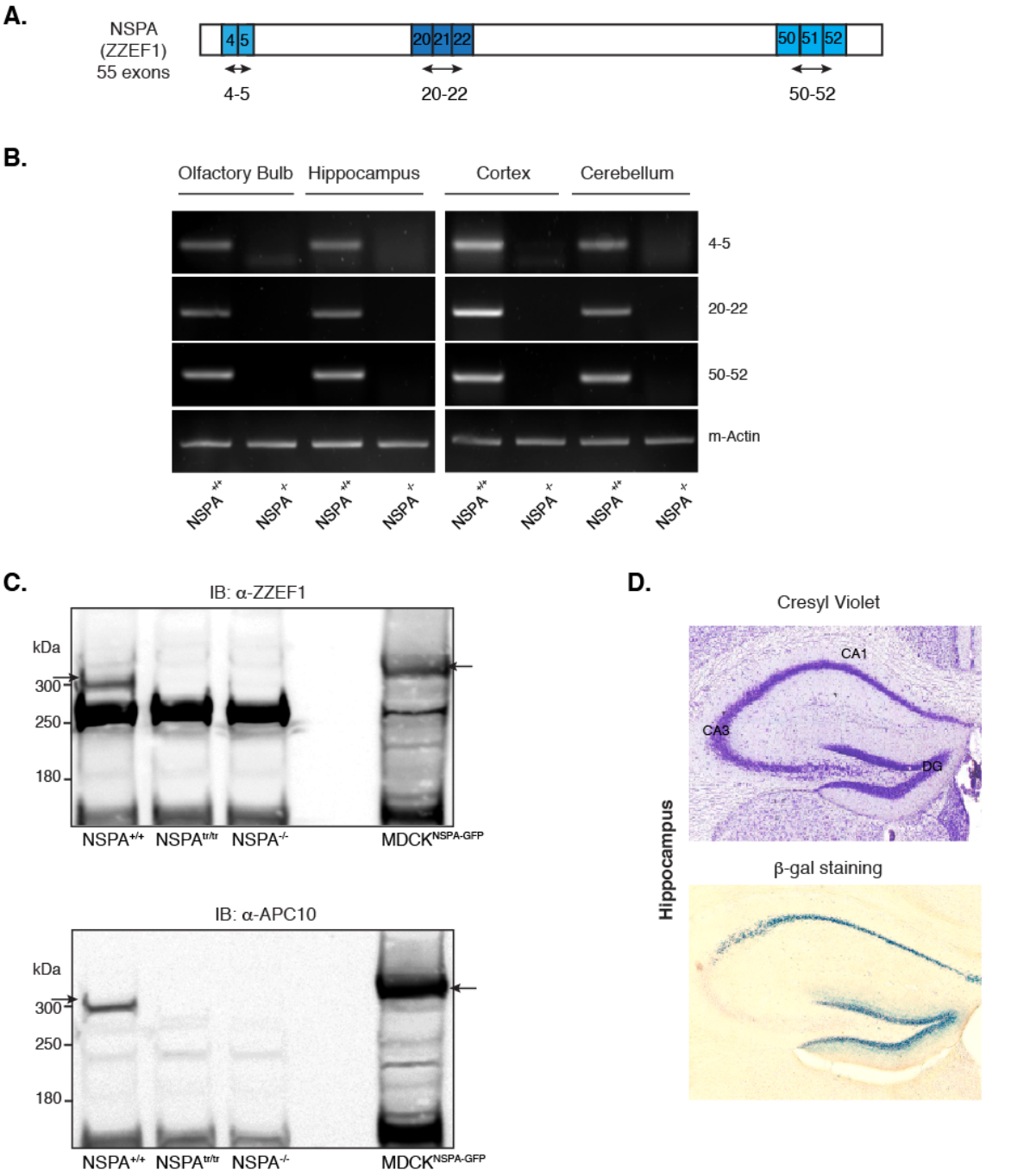


Figure 2. NSPA knockout mice (NSPA$^{-/-}$) and NSPA expression in the brain. (A) Location of the primers for RT-PCR in the mouse ZZEF1 (NSPA) cDNA. (B) RT-PCR of mRNA from NSPA$^{+/+}$ and NSPA$^{-/-}$ mice brain regions. (C) Immunoblot of hippocampal P2 fraction of NSPA$^{+/+}$, NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice with anti-APC10 and anti-ZZEF1 antibodies, extract of MDCK cells transfected with NSPA-GFP was used as a positive control, undetectable expression of NSPA in hippocampus of NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice (**Arrows** indicates NSPA or NSPA-GFP band). (D) Coronal slices of dorsal hippocampus from NSPA$^{+/-}$ mice stained for cresyl violet and β-Gal.

1.2 NSPA transgenic mice display decreased expression of GluN2A and GluN2B NMDAR subunits in hippocampal synaptic region.

Given the alterations in glutamatergic synaptic plasticity and hippocampal-dependent memory in the $NSPA^{tr/tr}$ mice and NSPA possible role as an E3 ubiquitin ligase we evaluated the expression levels of synaptic proteins in $NSPA^{+/+}$, $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice hippocampus. Immunoblot analyses of hippocampal lysates showed no significant differences in synaptic receptors and scaffolding proteins in $NSPA^{+/+}$ and $NSPA^{-/-}$ mice (Figure 3A and C). However, we found significant decreased expression of GluN2A and GluN2B NMDAR subunits in $NSPA^{tr/tr}$ mice whole hippocampal lysates (Figure 3B and C), without changes in the expression of AMPAR and other analyzed synaptic proteins. Because we found a decrease in GluN2 subunits in $NSPA^{tr/tr}$ mice, we analyzed by semiquantitative RT-PCR NMDAR subunits mRNA expression. The levels of GluN2A and GluN2B mRNA were unchanged in the hippocampus of $NSPA^{tr/tr}$ mice (Figure 4). Therefore, GluN2A and GluN2B decrease in $NSPA^{tr/tr}$ mice whole hippocampus seems not be due to a decreased transcription.

To evaluate the expression of synaptic proteins in the synaptic region we isolated synaptosomes from $NSPA^{+/+}$, $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice hippocampus. First we characterized the synaptosomes preparation analyzing synaptic markers like PSD95, synapsin-1 and NMDAR subunits in the crude hippocampal extract (H), supernatant obtained from hippocampal extract centrifugation (S2), crude synaptosomal membranes (P2) and pure synaptosome fraction (PSF). The pure synaptosome fraction showed enrichment in these markers (Figure 5). When we

compared the expression levels of synaptic proteins in hippocampal synaptosomes we found a significant decreased expression of GluN2A and GluN2B subunits of NMDAR in both $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice hippocampal synaptosomes compared to $NSPA^{+/+}$ mice (Figure 6B, C and D). These data indicated that NSPA is required for NMDAR expression levels at the synaptic region. Differences found in GluN2 expression levels in whole hippocampus between $NSPA^{tr/tr}$ and $NSPA^{-/-}$ may be due to the expression of a truncated version of NSPA.

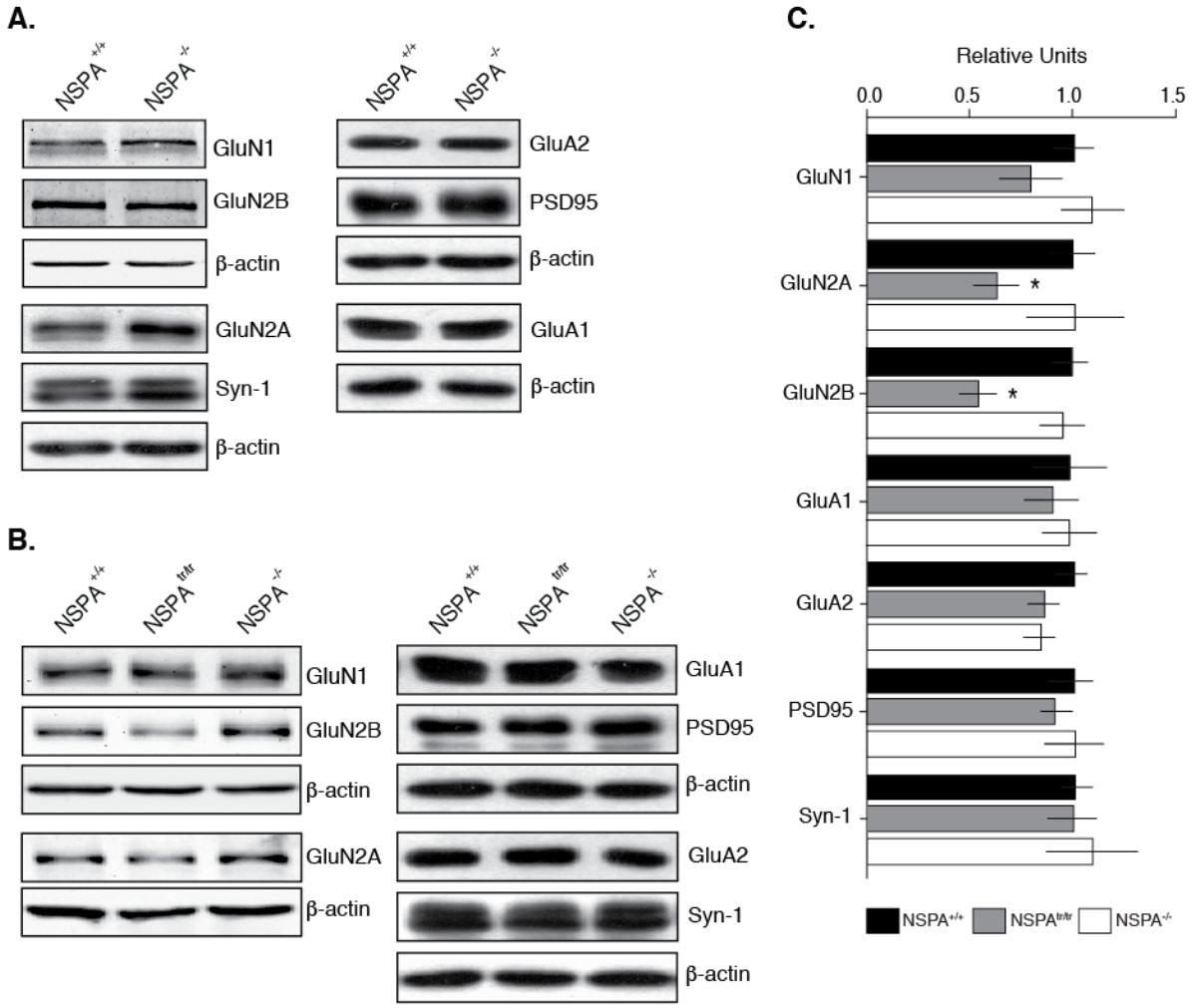


Figure 3. Expression of synaptic proteins in hippocampus of wild type and NSPA transgenic mice. (A) Levels of synaptic proteins were not different between NSPA$^{+/+}$ and NSPA$^{-/-}$ mice in whole hippocampal lysates. (B) NSPA$^{tr/tr}$ mice displayed decreased expression of GluN2A and GluN2B subunits of NMDA receptor in whole hippocampal lysates (C) Densitometric analysis of GluN1, GluN2A, GluN2B, GluA1, GluA2, PSD95 and Synapsin-1(Syn-1) in hippocampal lysates from NSPA$^{+/+}$, NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice. Values are the mean ± SEM (n≥3 per group),*P≤0.05, *t*-test.

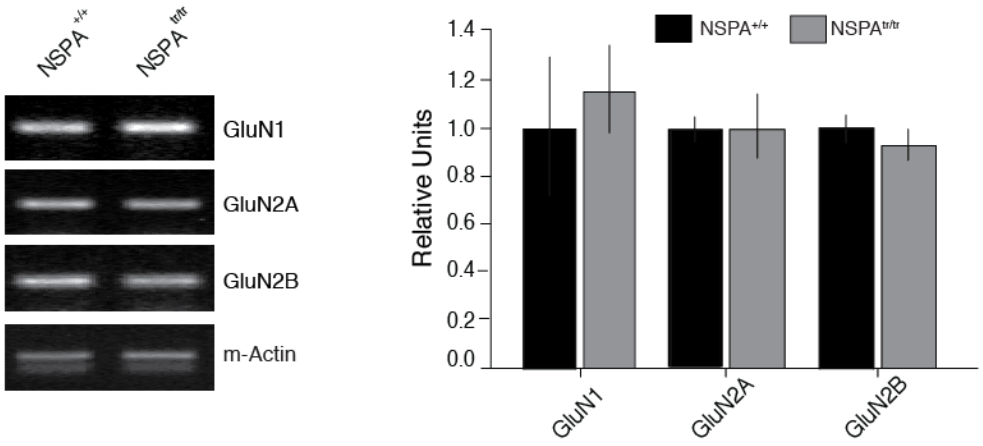


Figure 4. Analysis of mRNA expression of NMDAR subunits in wild type and NSPA knock-in mice. Analysis of GluN1, GluN2A and GluN2B NMDAR subunits by RT-PCR of hippocampal mRNA of NSPA$^{+/+}$ and NSPA$^{tr/tr}$ mice, no differences were found between NSPA$^{+/+}$ and NSPA$^{tr/tr}$ mice. Values are the mean ± SEM (n=3 per group), *t*-test.

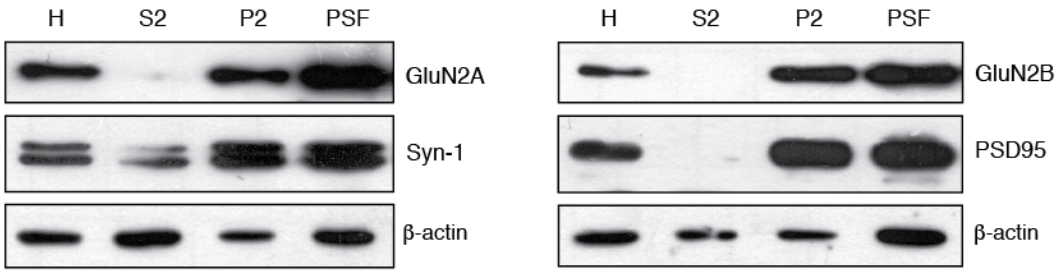


Figure 5. Characterization of hippocampal synaptosomes preparation. Crude hippocampal extract (H), supernatant obtained from hippocampal extract centrifugation (S2), crude synaptosomal membranes (P2) and pure synaptosome fraction (PSF) were analyzed by western blot. Synaptic markers like PSD95, synapsin-1 and NMDAR subunits were enriched in the pure synaptosome fraction.

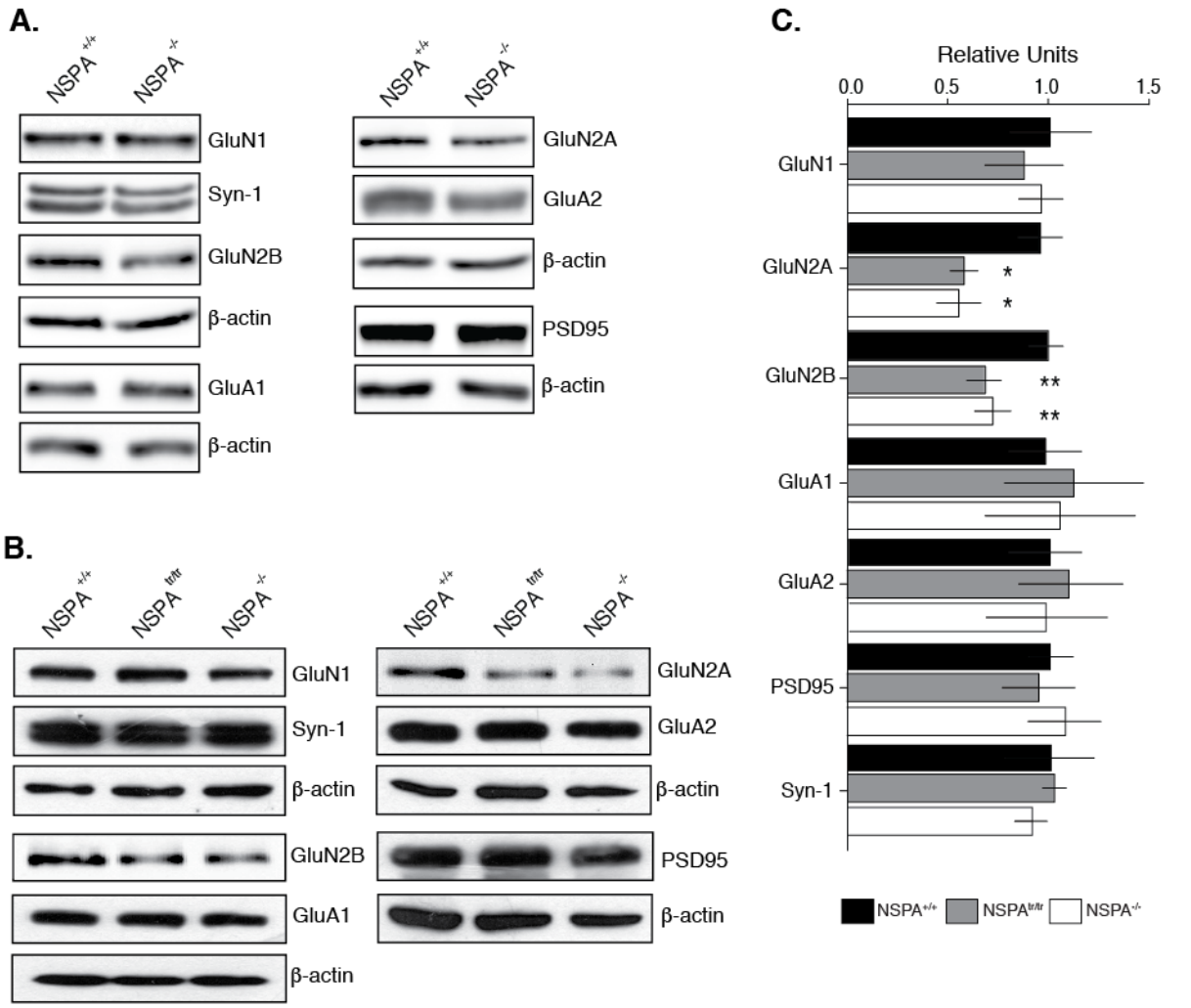


Figure 6. NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice displayed decreased expression of two subunits of NMDA receptor in hippocampal synaptosomes. Expression levels of synaptic proteins were analyzed by immunoblot in hippocampal synaptosomes of (A) NSPA$^{+/+}$ and NSPA$^{-/-}$ mice and of (B) NSPA$^{+/+}$, NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice, decreased expression of GluN2A and GluN2B subunits of NMDAR was found in both NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice compared to NSPA$^{+/+}$. (C) Densitometric analysis of GluN1, GluN2A, GluN2B, GluA1, GluA2, PSD95 and Synapsin-1 (Syn-1) in hippocampal synaptosomes of NSPA$^{+/+}$, NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice. Values are the mean ± SEM (n≥3 per group),*P≤0.05, **P≤0.01, *t*-test.

1.3 NSPA knockout mice have impaired hippocampal-dependent tasks.

Our previous studies showed that NSPA$^{tr/tr}$ mice have impaired NMDAR-mediated neurotransmission accompanied by a decreased synaptic plasticity of SC-CA1 synapses (Segovia-Miranda et al 2015). Collaborative experiments carried out in Dr. Waldo Cerpa's laboratory analyzed electrophysiological input-output analysis for synaptic strength and LTP assays registering fEPSP in the Schaffer collateral-CA1 pathway (SC-CA1 synapses) and the medial perforant pathway synapses on DG granule cells (MPP-DG synapses) in *ex vivo* hippocampal slices of NSPA$^{+/+}$ and NSPA$^{-/-}$ mice. Similar to our previous description in NSPA$^{tr/tr}$ mice (Segovia-Miranda et al 2015), NSPA$^{-/-}$ mice also showed a decreased magnitude of LTP compared with wild-type mice (Annex Figure 1A). The paired pulse facilitation assay showed no alterations in presynaptic activity in SC-CA1 synapses of NSPA$^{-/-}$ mice (Annex Figure 1B). NSPA$^{-/-}$ SC-CA1 synapses also showed normal input-output curve (Annex Figure 1C). NMDAR activity was analyzed in SC-CA1 synapses of NSPA$^{+/+}$ and NSPA$^{-/-}$ mice measuring the sensitivity to AP-V, an NMDAR competitive antagonist (Brady et al 1994). AP-V application had a smaller inhibitory effect in NSPA$^{-/-}$ slices SC-CA1 synapses compared with NSPA$^{+/+}$ slices (Annex Figure 1D). This result suggests an impaired NMDAR-mediated transmission, which correlates with the decreased LTP observed in NSPA$^{-/-}$ SC-CA1 synapses. In MPP-DG synapses of NSPA$^{-/-}$ mice decreased LTP was observed compared with wild-type mice (Annex Figure 2A), with normal paired pulse facilitation (Annex Figure 2B). Increased input-output responses were found in NSPA$^{-/-}$ compared with wild-type mice (Annex Figure 2C), meaning an increase in basal synaptic transmission. This unexpected result does not correlate

with decreased LTP, LTP is NMDAR-dependent while basal synaptic transmission correspond to AMPA/Kainate receptor-mediated synaptic responses, thus this result may be due to an increase in transmission mediated by these receptors.

Theoretical and experimental studies implicate hippocampal LTP with memory and learning (Barnes & McNaughton 1985, Berger 1984, McNaughton et al 1986). Indeed, glutamatergic dysfunctions can affect hippocampal-dependent memory (Morris 1989, Yamada et al 2015). We performed two different behavioral tests that involve hippocampal function and a third test to evaluate locomotion, exploration and anxiety in $NSPA^{+/+}$ and $NSPA^{-/-}$ mice. We tested a modified spatial memory paradigm task to evaluate episodic-like memory (memory flexibility) (Chen et al 2000). $NSPA^{-/-}$ mice performed poorly in this test (Figure 7A). Additionally, we evaluated animals using a recognition memory test (novel object recognition; NOR) (Cohen et al 2013, Cohen & Stackman 2015). $NSPA^{-/-}$ mice exhibited decreased preference for the novel object, contrasting with the significant preference for the novel object shown by $NSPA^{+/+}$ mice (Figure 7B). Then, we applied an open field (OF) test, used to measure locomotor and anxiety-like behaviors (Carola et al 2002), we found that $NSPA^{-/-}$ mice have normal locomotor activity, as measured by the total distance moved (Figure 8A), and spend the same amount of time in the center region of the OF arena that $NSPA^{+/+}$ mice (Figure 8A), thus not displaying anxiety-like behaviors. Vision capability of $NSPA^{-/-}$ mice did not differ from wild-type mice, as determined by Morris water maze visible platform (Figure 8B). Therefore, the $NSPA^{-/-}$ mice have impaired hippocampal-dependent memory without showing anxiety-like behaviors or altered locomotor activity.

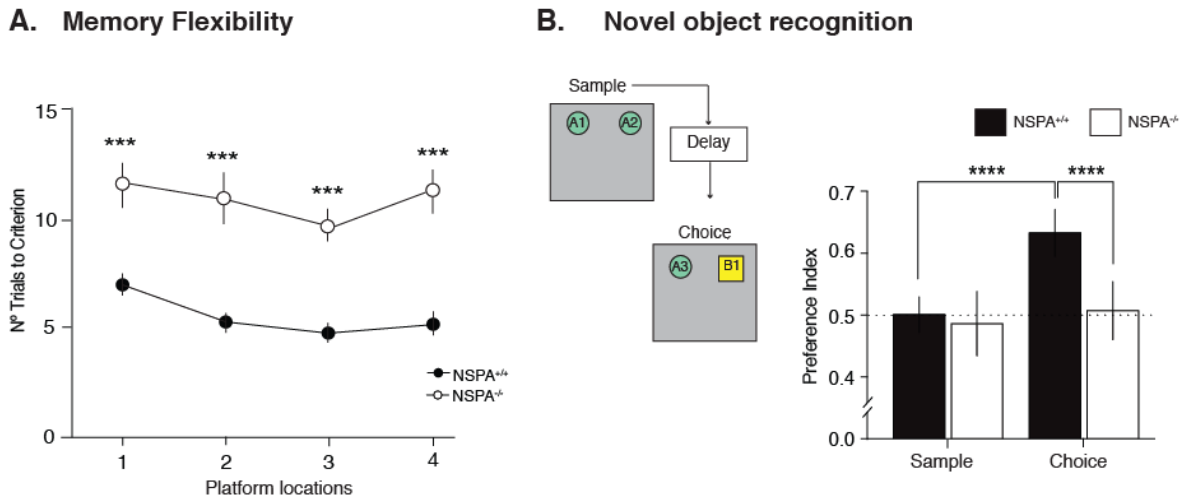


Figure 7. NSPA$^{-/-}$ mice show impairment on hippocampal-dependent tasks. (A) Memory flexibility test average number of trials considering 4 days in the same bar, impaired performance of NSPA$^{-/-}$ mice on memory flexibility test (higher number of trials required to meet the criterion). Values are the mean ± SEM (n=5 mice per group), ***P≤0.001 versus NSPA$^{+/+}$ mice by *t*-test. (B) Schematic of the novel recognition (NOR) task comprising sample (10 min), delay (4 hours) and choise (10 min) phases. A1 and A2 represent identical objects, whereas B1 refears to a novel object, Preference index calculated as time spent with object divided by total exploration time. NSPA$^{+/+}$ mice explored the novel object preferentially, while NSPA$^{-/-}$ mice did not. Values are the mean ± SEM (n=10 mice per group), ****P≤0.0001 versus NSPA$^{+/+}$ mice by one-way ANOVA, followed by Bonferroni post-hoc test.

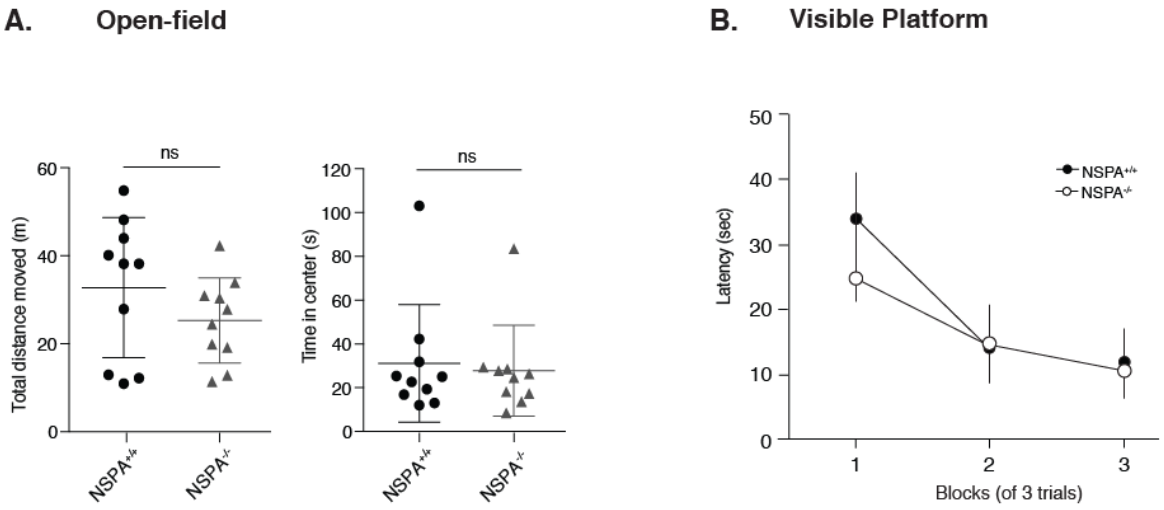


Figure 8. NSPA$^{-/-}$ mice do not display anxiety-like behaviors or alterations in vision capabiliby. (A) NSPA$^{-/-}$ mice show normal locomotor activity in the open field test (novel environment), measured by the total distance moved. The time spent in the center region is also normal, NSPA$^{-/-}$ mice do not display anxiety-like behavior. Values are the mean ± SEM (n=10 mice per group), ns, not significant, *t*-test. (B) Lack of differences in vision and general health between NSPA$^{+/+}$ and NSPA$^{-/-}$ mice, determined by Morris water maze test with visible platform.

1.4 Decreased proliferation of neural progenitors in dentate gyrus of NSPA knockout mice.

Adult neurogenesis has been suggested to be relevant for hippocampal functions such as spatial learning, object recognition and memory (Deng et al 2010, Goncalves et al 2016). In the hippocampus, adult neurogenesis is restricted to the subgranular zone (SGZ) of the dentate gyrus (DG) (Zhao et al 2008), and is known to regulate synaptic plasticity (Drew et al 2013). Thinking that impaired hippocampal function and LTP observed in the DG region of $NSPA^{-/-}$ mice might be associated with alterations in the adult neurogenesis, we compared cell proliferation by immunostaining for the mitotic marker Ki67 (Kee et al 2002) and BrdU, a thymidine analog (Wojtowicz & Kee 2006) in the hippocampus of 2-month-old $NSPA^{+/+}$ and $NSPA^{-/-}$ mice. $NSPA^{-/-}$ mice showed a significant decrease in the total number of Ki67 positive cells (Figure 9A) and in the total number of BrdU positive cells (Figure 9B) in the SGZ compared with wild-type mice. These results indicate that $NSPA^{-/-}$ mice have decreased proliferation of hippocampal neural progenitor cells *in vivo*.

We also evaluated neuronal activity in the SGZ of $NSPA^{+/+}$ and $NSPA^{-/-}$ mice by immunostaining for Arc protein, which is expressed by glutamatergic neurons in response to an increase in synaptic activity (Ramirez-Amaya et al 2005). Arc synthesis is required for LTP and synaptic plasticity is associated with the induction of Arc translation, thus being used as a tool to study synaptic activity (Korb & Finkbeiner 2011, Messaoudi et al 2007). $NSPA^{-/-}$ mice showed a significant decrease in the total number of Arc positive cells (Figure 10A), suggesting less synaptic activity in the SGZ of $NSPA^{-/-}$ mice.

Then, we evaluated differentiation of newborn cells into neurons using immunostaining for BrdU and the immature neuronal marker doublecortin (DCX) (Kempermann et al 2003). The percentage of the $BrdU^{+}$ cells that are also DCX^{+} was not significantly different in $NSPA^{-/-}$ mice (Figure 10B). Therefore, $NSPA^{-/-}$ mice have decreased proliferation of hippocampal neuron progenitors but unaffected differentiation of $BrdU^{+}$ cells into DCX^{+} neuroblast.

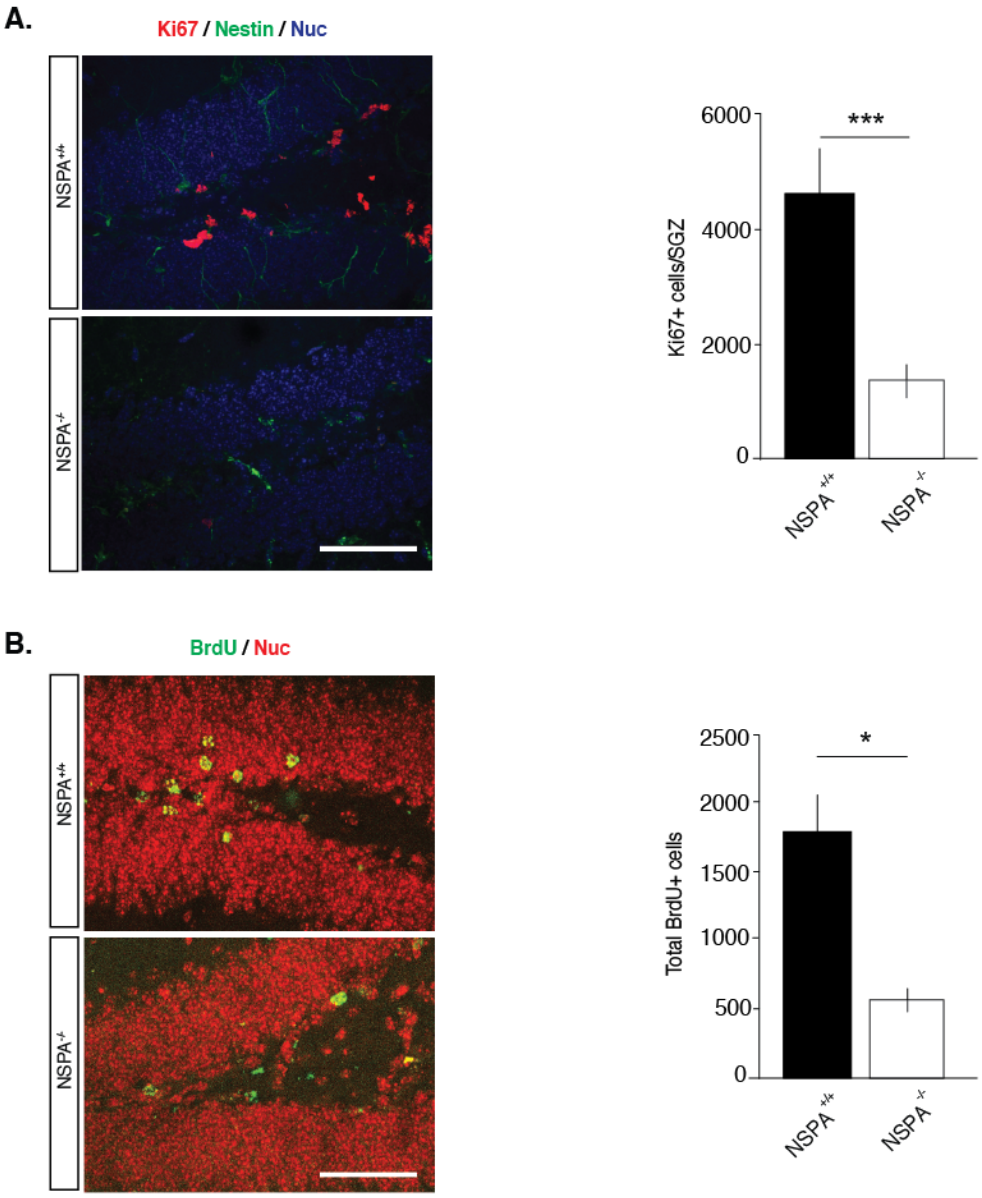


Figure 9. NSPA$^{-/-}$ mice show decreased proliferation of neural progenitors in the adult dentate gyrus (DG). (A) Representative immunofluorescence staining of Ki67 in the DG of NSPA$^{+/+}$ and NSPA$^{-/-}$ mice. NSPA$^{-/-}$ mice show a significant decrease in the total number of Ki67 positive cells (Ki67^{+}). Scale bar: 50 µm. Bars represent mean ± SEM (n=10 mice per experimental group). ***P≤0.001, *t*-test. (B) Representative immunofluorescence staining of BrdU in the DG of NSPA$^{+/+}$ and NSPA$^{-/-}$ mice. NSPA$^{-/-}$ mice show a significant decrease in the total number of proliferating cells (BrdU^{+}). Scale bar: 50 µm. Bars represent mean ± SEM (n=4 mice per experimental group). * P≤0.05, *t*-test.

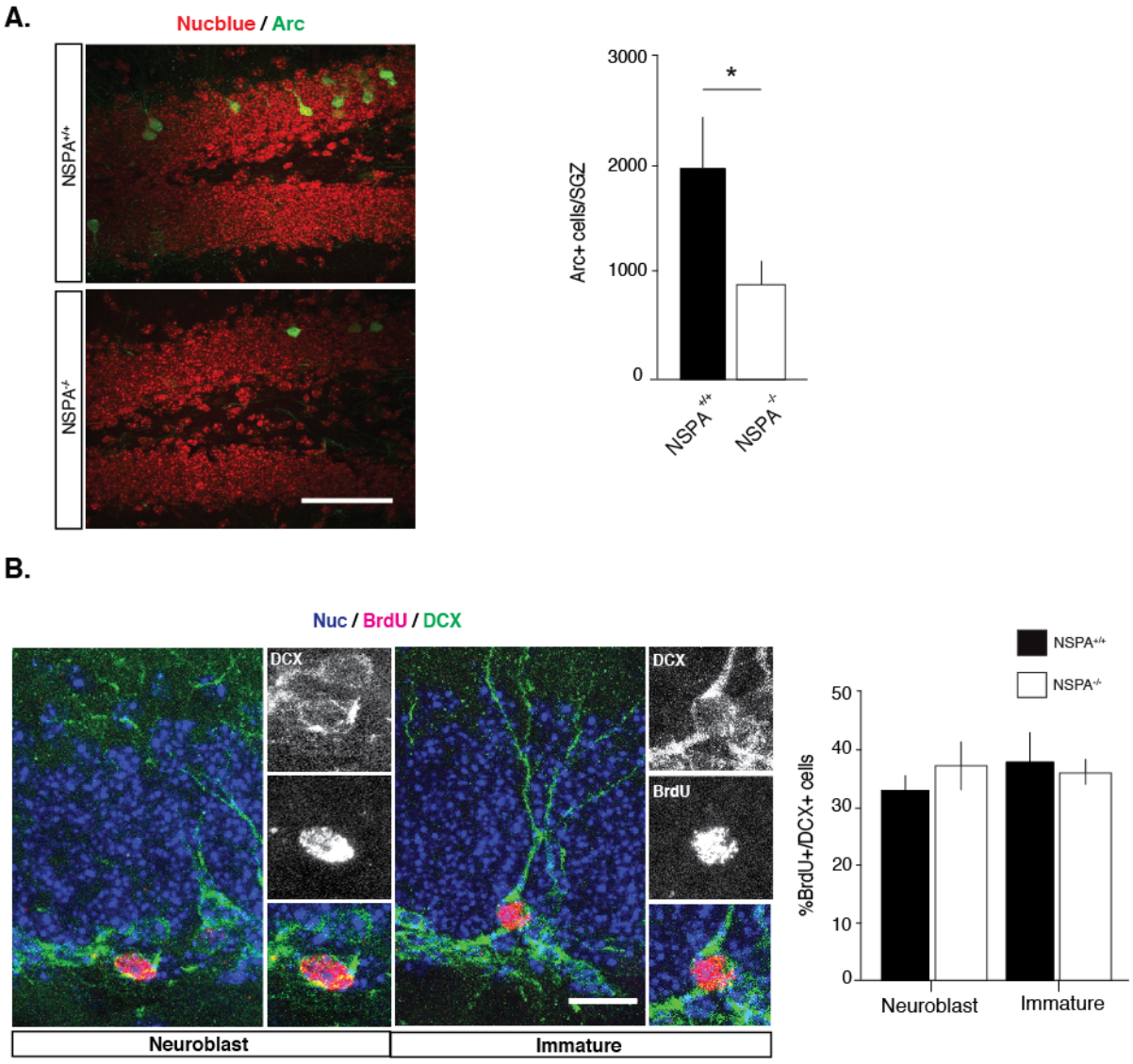


Figure 10. Normal differentiation of newborn cells into neurons in NSPA$^{-/-}$ mice. (A) Representative immunofluorescence staining of Arc in the DG of NSPA$^{+/+}$ and NSPA$^{-/-}$ mice. NSPA$^{-/-}$ mice show a significant decrease in the total number of Arc positive cells (Arc+). Scale bar: 50 µm. Bars represent mean ± SEM (n=3 mice per experimental group). *P≤0.05, *t*-test (B) Representative double labeling of BrdU and the marker for immature neurons doublecortin (DCX) in the DG. The percentage of BrdU^{+} cells that are also positive for DCX (%BrdU^{+}/DCX^{+}) was unchanged in NSPA$^{-/-}$ mice, neuroblast and immature neurons. Scale bar: 10 µm. Bars represent mean ± SEM (n=4 mice per experimental group), *t*-test.

2. NSPA function in posttranslational modifications and postsynaptic abundance of NMDAR.

Posttranslational modifications of NMDAR, including ubiquitination and phosphorylation, alter both the activity and trafficking properties of the receptor (Chen & Roche 2007, Mabb & Ehlers 2010, Mao et al 2011, Qiu et al 2011). Particularly phosphorylation of cytoplasmic C-tails of GluN2A and GluN2B subunits of NMDAR has emerged as a key regulatory mechanism to regulate NMDAR function, trafficking and interactions with cytosolic proteins, modulating NMDARs postsynaptic abundance (Chen & Roche 2007, Lussier et al 2015). Since we found decreased expression of GluN2 NMDAR subunits and impaired LTP in NSPA transgenic mice, we studied the postsynaptic abundance of NMDAR and posttranslational modifications that can regulate NMDARs localization in those mice.

2.1 Reduced postsynaptic abundance of GluN2A and GluN2B NMDAR subunits in NSPA knockout mice.

LTP involves NMDAR-mediated postsynaptic calcium influx leading to biochemical and protein trafficking processes that ultimately result in postsynaptic recruitment of AMPAR (Citri & Malenka 2008, Rao & Finkbeiner 2007). Impaired LTP is most likely due to postsynaptic deficits in synaptic function and/or a reduction in the number of functional synapses (Citri & Malenka 2008). Therefore, we isolated hippocampal postsynaptic densities (PSD) from $NSPA^{+/+}$, $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice to evaluate the abundance of glutamatergic receptors only at the postsynaptic region. We verified the purity of isolated hippocampal PSD by western blot using the presynaptic marker VGlut1 and postsynaptic marker PSD95 (Figure 11), the absence

of presynaptic marker in the PSD fraction showed the purity of the PSD preparation. According to what we observed in synaptosomes, we found decreased expression of GluN2A and GluN2B subunits of NMDAR in the PSD of both NSPA$^{tr/tr}$ (Figure 12A) and NSPA$^{-/-}$ (Figure 12B) mice, without changes in other synaptic proteins. We do not have significance in the experiment with NSPA$^{tr/tr}$ since we only had one preparation of hippocampal PSD from NSPA$^{tr/tr}$ mice, but the effect was the same as in hippocampal PSD from NSPA$^{-/-}$.

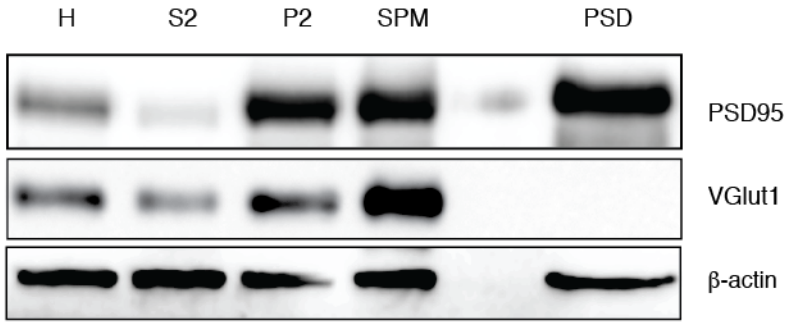


Figure 11. Purity of isolated hippocampal postsynaptic densities (PSD) from NSPA$^{+/+}$ mice verified by western blot with presynaptic marker VGlut1 and postsynaptic marker PSD95. Crude hippocampal extract (H), supernatant obtained from hippocampal extract centrifugation (S2), crude synaptosomal membranes (P2), synaptic membranes (SPM) and postsynaptic densities (PSD).

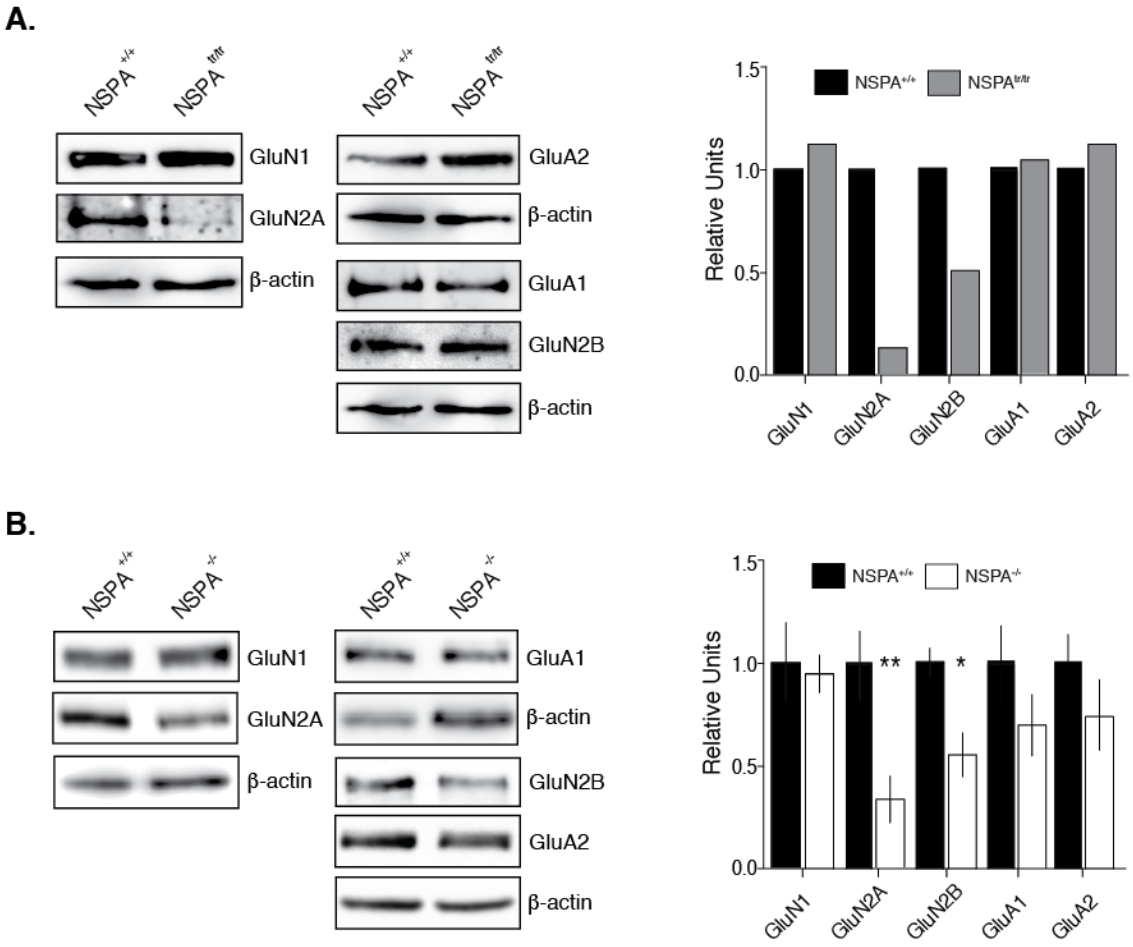
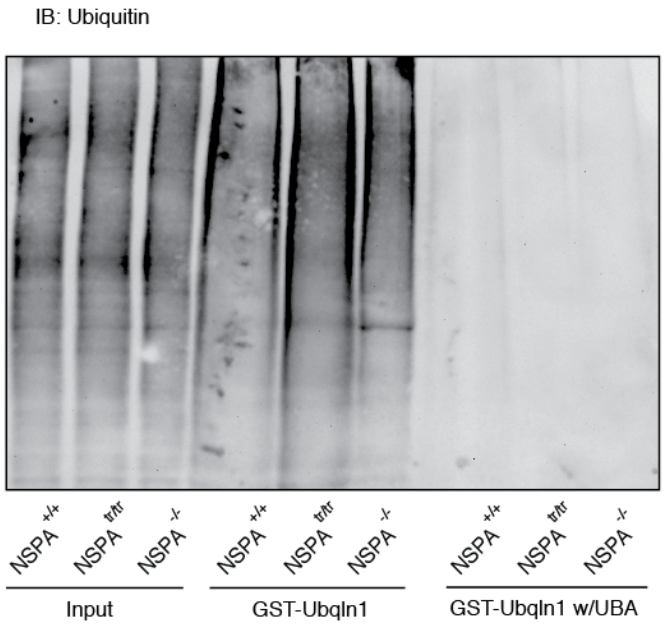


Figure 12. NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice displayed decreased expression of two subunits of NMDA receptor in hippocampal postsynaptic densities (PSD). (A) Hippocampal PSD of NSPA$^{+/+}$ and NSPA$^{tr/tr}$ mice, decreased expression of GluN2A and GluN2B subunits of NMDAR was found in NSPA$^{tr/tr}$ mice compared to NSPA$^{+/+}$, (n=1 per group). (B) Hippocampal PSD of NSPA$^{+/+}$ and NSPA$^{-/-}$ mice, decreased expression of GluN2A and GluN2B subunits of NMDAR was also found in NSPA$^{-/-}$ mice compared to NSPA$^{+/+}$. Values are the mean ± SEM (n=4 per group), *P≤0.05, **P≤0.01, *t*-test.

2.2 Undetectable GluN2B ubiquitination in mice hippocampus.

Although GluN2A and GluN2B subunits can be ubiquitinated (Jurd et al 2008, Nelson et al 2006), only for GluN2A, ubiquitination has been demonstrated in neurons (Yin et al 2011). To evaluate GluN2 ubiquitination levels we used a GST pulldown assay for ubiquitinated proteins. First we characterized the GST pulldown assay, we used GST fusions with Ubiquilin-1 (Ubqln1) or Ubiquilin-1 without UBA domain (Ubqln1 w/UBA). Ubiquilin-1 interacts with ubiquitin chains via its UBA domain (Ko et al 2004). Using hippocampal P2 fraction of $NSPA^{+/+}$, $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice, we determined by immunoblot with anti-Ubiquitin antibody that Ubqln1 but not Ubqln1 w/UBA precipitates ubiquitinates proteins (Figure 13A). Then hippocampal P2 fraction of $NSPA^{+/+}$, $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice was subjected to GST pulldown for ubiquitinated proteins, eluted proteins were probed with GluN2B antibody for the presence of GluN2B, we could not detect ubiquitination in GluN2B subunits in mice hippocampal extracts (Figure 13B).

A.



B.

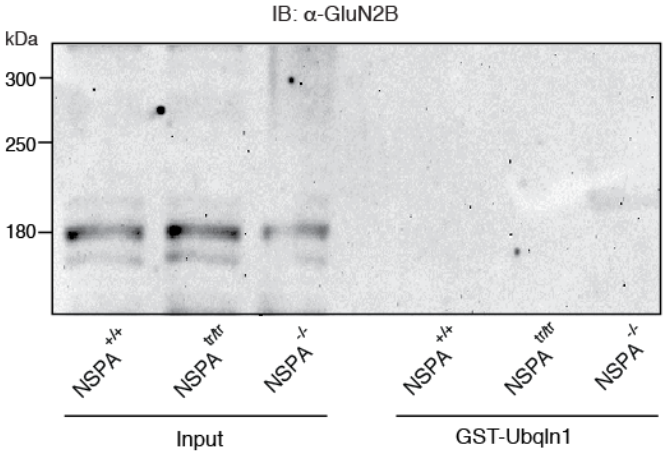


Figure 13. GluN2B ubiquitination was undetectable in NSPA$^{+/+}$, NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice hippocampus. (A) GST pulldown assay for ubiquitinated proteins were performed on hippocampal P2 fraction of NSPA$^{+/+}$, NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice, using either GST fusions with Ubiquilin-1 (Ubqln1) or Ubiquilin-1 without UBA domain (Ubqln1 w/UBA). Ubiquilin-1 interact with ubiquitin chains via its UBA domain. (B) Hippocampal P2 fraction of NSPA$^{+/+}$, NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice was subjected to GST pulldown for ubiquitinated proteins, eluted proteins were probed with GluN2B antibody for the presence of GluN2B.

2.3 Decreased phosphorylation of NMDAR GluN2B subunit at a Tyr residue known to regulate its surface expression.

Then we focused in tyrosine phosphorylation, a posttranslational modification that regulates surface and synaptic expression of NMDARs (Chen & Roche 2007, Lussier et al 2015, Prybylowski et al 2005). GluN2 subunits contain several tyrosine phosphorylation sites that can be phosphorylated (Chen & Roche 2007, Kalia et al 2004, Kohr & Seeburg 1996), for instance phosphorylation of GluN2B subunit at Tyr1472 prevents endocytosis leading to enhanced NMDAR surface expression at the synaptic zone (Prybylowski et al 2005, Xu et al 2006), and phosphorylated Tyr1336 is associated with the enrichment of the receptor in extrasynaptic membranes (Goebel-Goody et al 2009). We tried to evaluate the tyrosine phosphorylation status of GluN2B subunit in hippocampal P2 fraction of $NSPA^{+/+}$, $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice by immunoprecipitation with anti-GluN2B antibody and immunoblot with anti-phosphotyrosine antibody. We detected phosphorylated tyrosine residues of GluN2B that included those proposed that promote enrichment in synaptic (Tyr1472) and extrasynaptic (Tyr1336) membranes among others. Thus, this technique does not differentiate between synaptic and extrasynaptic subpopulations (Figure 14A). The decreased synaptic levels of GluN2B found in both $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice led us to compare GluN2B Tyr1472 phosphorylation levels in the hippocampus of $NSPA^{+/+}$, $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice. Immunoblot analysis of the hippocampal crude synaptosomal membrane fraction (P2) with antibody specific to pTyr1472 revealed a decreased phosphorylation of GluN2B Tyr1472 in $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice (Figure 14B, C and D). These results suggest that deregulation of the

phosphorylation status of GluN2B Tyr1472 might account for its decreased levels at the PSD, as reported in other conditions (Prybylowski et al 2005, Won et al 2016, Xu et al 2006).

Phosphorylation of GluN2B Tyr1472 residue is catalyzed by Fyn kinase (Nakazawa et al 2001), whereas its dephosphorylation involves $STEP_{61}$ phosphatase, which in turn is mainly regulated by ubiquitination (Kurup et al 2010). Therefore, we examined both Fyn and $STEP_{61}$ expression levels in synaptosomal P2 fraction, the last in the absence and presence of the proteasome inhibitor MG-132 (25μM). Immunoblots of Fyn and $STEP_{61}$ showed similar mass levels in $NSPA^{+/+}$ and $NSPA^{-/-}$ synaptosomal P2 fractions (Figure 15A and B).

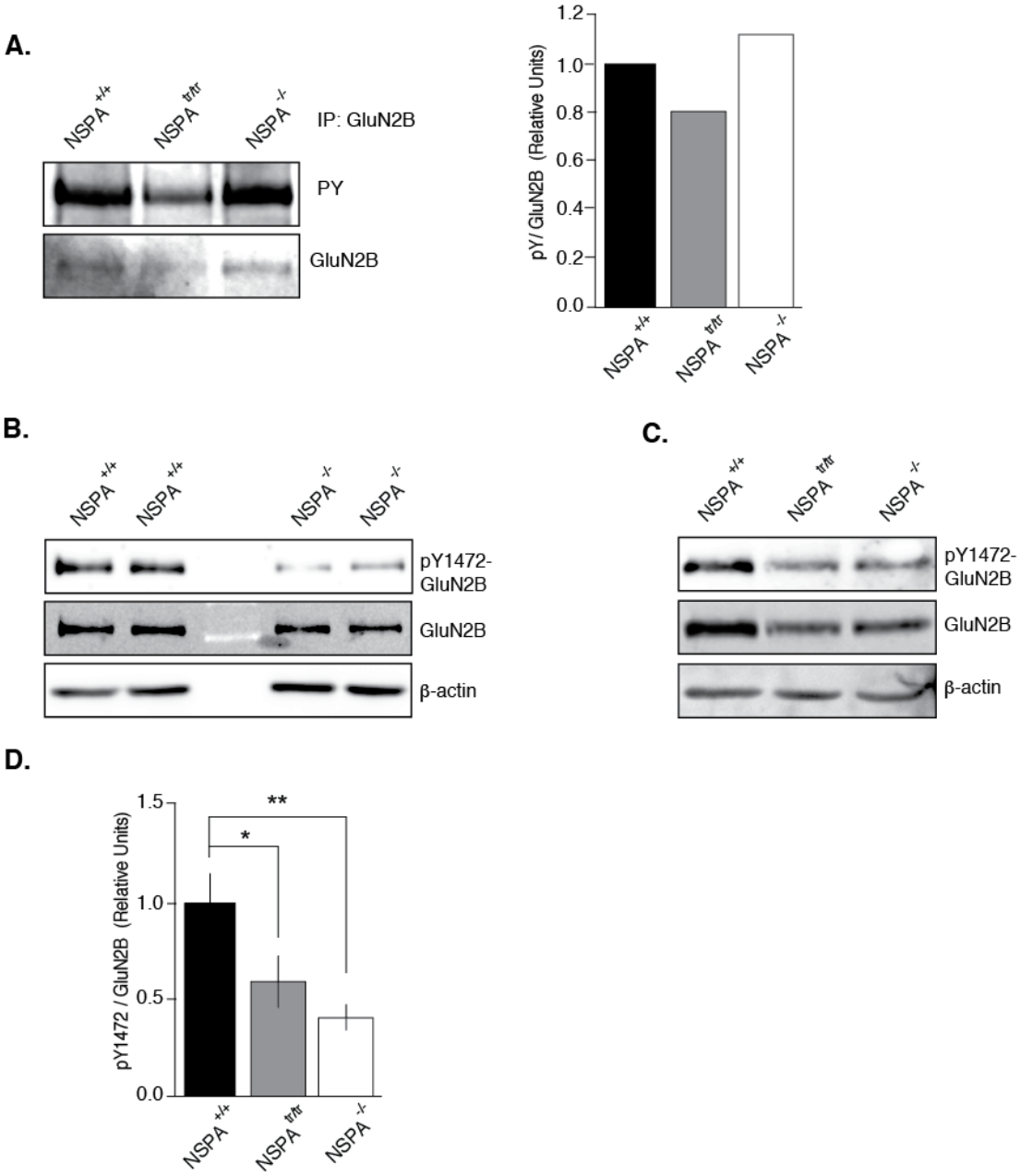


Figure 14. Decreased phosphorylation of GluN2B subunit of NMDAR at Tyr1472 in hippocampus of NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice. (A) GluN2B immunoprecipitates from hippocampal P2 fraction of NSPA$^{+/+}$, NSPA$^{tr/tr}$ and NSPA$^{-/-}$ was subjected to immunoblot with anti-PY antibody. (B) Phosphorylated GluN2B Tyr1472 was analyzed by immunoblot with specific antibody (GluN2B Tyr1472P) in hippocampal P2 fraction of (A) NSPA$^{+/+}$ and NSPA$^{-/-}$ mice and of (C) NSPA$^{+/+}$, NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice. Decreased phosphorylation of GluN2B in Tyr1472 was found in both NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice compared to NSPA$^{+/+}$, (n=5 for NSPA$^{+/+}$ and NSPA$^{-/-}$, n=3 for NSPA$^{tr/tr}$). (D) Densitometric analysis of normalized ratio of phospho-Tyr1472 with GluN2B. Values are the mean ± SEM, *P≤0.05, **P≤ 0.01, one-way ANOVA, Bonferroni's post hoc test or *t*-test.

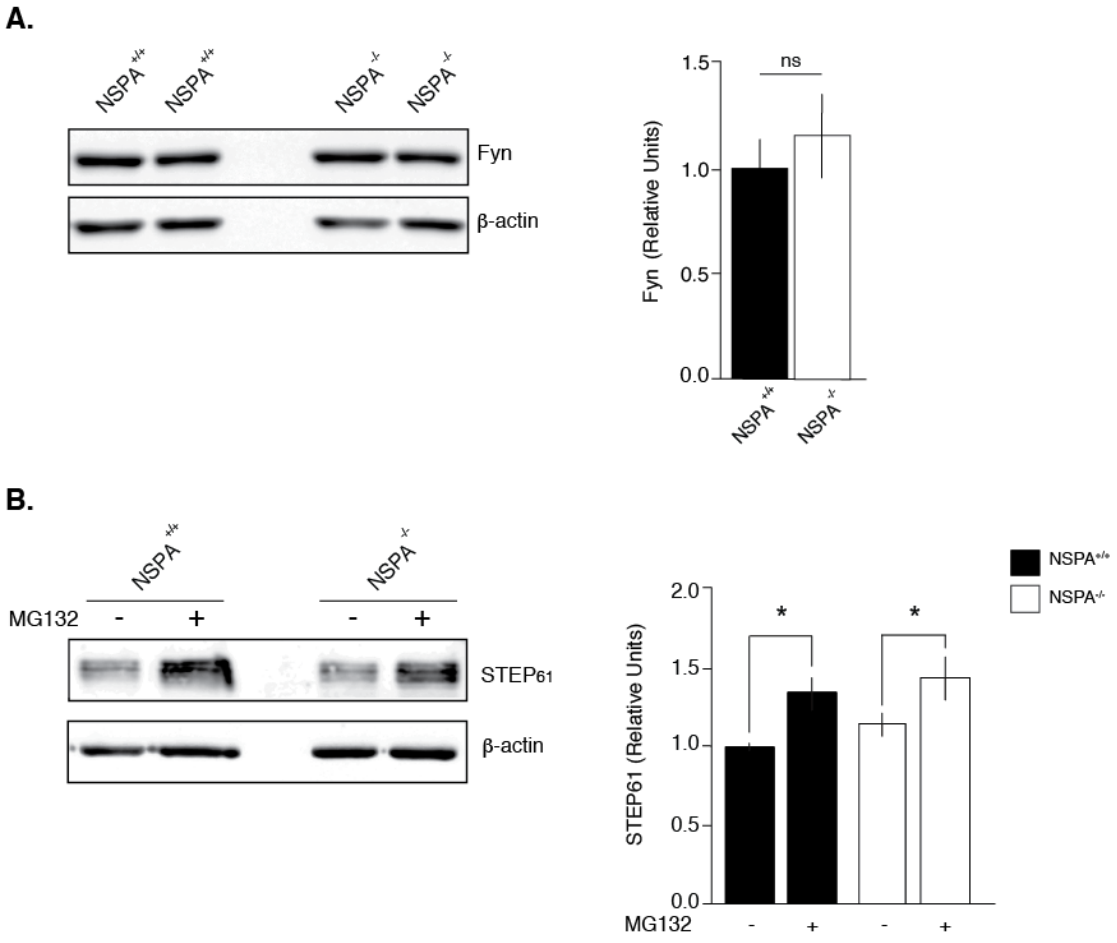


Figure 15. Normal expression levels of Fyn kinase and $STEP_{61}$ phosphatase in $NSPA^{-/-}$ hippocampus. Hippocampal P2 fraction of $NSPA^{+/+}$ and $NSPA^{-/-}$ mice was isolated and immunoblotted for: (A) Fyn kinase with specific antibody, no significant differences in Fyn levels were found comparing $NSPA^{+/+}$ and $NSPA^{-/-}$ mice, (n=4). (B) $STEP_{61}$ phosphatase with specific antibody, no significant differences in $STEP_{61}$ levels were found comparing $NSPA^{+/+}$ and $NSPA^{-/-}$ mice, (n=3). Values are the mean ± SEM, *P≤0.05, one-way ANOVA, Bonferroni's post hoc test or *t*-test.

2.4 PTPN4 tyrosine phosphatase interacts with GluN2 NMDAR subunits and is increased in hippocampus of NSPA knockout mice.

PTPN4 tyrosine phosphatase, also known as PTPMEG, has been shown to interact with NMDAR subunits such as GluN2A (Hironaka et al 2000) and to modulate downstream signaling of glutamate receptors through changes in the phosphorylation of GluD2 and GluN2 subunits of NMDAR (Hironaka et al 2000, Kina et al 2007, Kohda et al 2013). Interestingly, a reported yeast two-hybrid wide-screen (Stelzl et al 2005) analysis of human proteins reported PTPN4 phosphatase as a potential NSPA interacting protein.

PTPN4 is expressed in mouse brain, particularly in thalamus, cerebellum, olfactory bulb, cortex and hippocampus (Hironaka et al 2000) and it has been shown to interact with NMDAR subunits such as GluN2A in mouse telencephalon (Hironaka et al 2000). We examined whether PTPN4 interacts with NMDAR subunits GluN2A and GluN2B in mouse hippocampus. We carried out co-immunoprecipitation experiments with an antibody directed against PTPN4 using crude synaptosomal membrane (P2) fraction from wild type mice hippocampus. We detected GluN2A and GluN2B subunits in the anti-PTPN4 immunoprecipitates (Figure 16A), suggesting that PTPN4 interacts with GluN2A and GluN2B *in vivo*. We found that GluN2B co-immunoprecipitates with PTPN4 only in wild type mice hippocampus, but not in $NSPA^{tr/tr}$ or $NSPA^{-/-}$ hippocampus (Figure 16B). This result may be due to a lower availability of GluN2B to interact with PTPN4 in NSPA transgenic mice.

Then we examined PTPN4 expression levels in hippocampal P2 fraction of $NSPA^{+/+}$ and $NSPA^{-/-}$ mice in absence or presence of the proteasome inhibitor MG-

132 (25μM). Strikingly, we found an increased mass of PTPN4 in NSPA$^{-/-}$ mice compared with wild type mice only in the absence of MG-132 (Figure 16C). The presence of MG-132 shielded the difference. Therefore, in the absence of NSPA, an increased PTPN4 levels very likely accounts for the decreased phosphorylation of GluN2B at Tyr1472. These results also imply that NSPA regulates the levels of PTPN4 through the ubiquitin proteasome system.

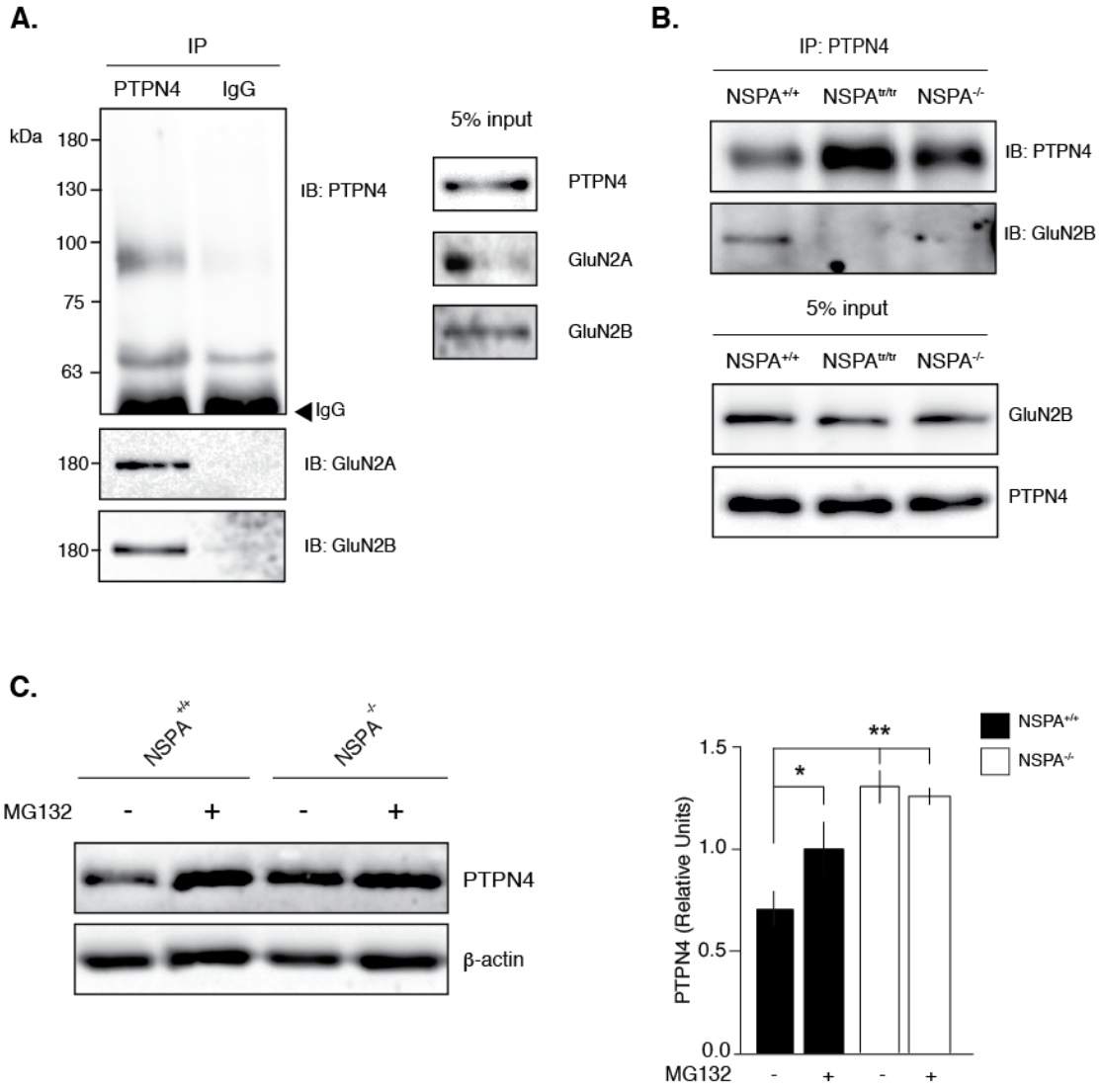


Figure 16. PTPN4 interacts with GluN2 NMDAR subunits and its levels are increased in hippocampal extracts of NSPA$^{-/-}$ mice. (A) Hippocampal P2 fraction of NSPA$^{+/+}$ mice was immunoprecipitated with anti-PTPN4 antibody or IgG as control, and probed with anti-PTPN4, anti-GluN2A and anti-GluN2B antibodies, GluN2A and GluN2B subunits coimmunoprecitated with PTPN4. (B) Hippocampal P2 fraction of NSPA$^{+/+}$, NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice was immunoprecipitated with anti-PTPN4 and probed with anti-PTPN4 and anti-GluN2B antibodies, GluN2B coimmunoprecipitated with PTPN4 in NSPA$^{+/+}$ mice extracts but not in NSPA$^{tr/tr}$ or NSPA$^{-/-}$ mice extracts. (C) Hippocampal P2 fraction of NSPA$^{+/+}$ and NSPA$^{-/-}$ mice was isolated and immunoblotted for PTPN4, increased mass of PTPN4 was found in NSPA$^{-/-}$ mice compared to NSPA$^{+/+}$, in samples prepared with DMSO not with MG-132, (n=3). Values are the mean ± SEM, *P≤0.05, **P≤ 0.01, one-way ANOVA, Bonferroni's post hoc test or *t*-test.

3. Evidence of NSPA as an E3 ubiquitin ligase.

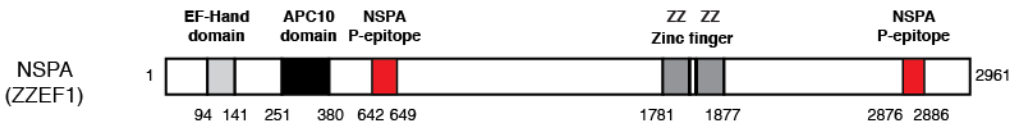
Ubiquitination is an important posttranslational modification involved in the regulation of glutamatergic transmission and synaptic plasticity (Ehlers 2003, Fu et al 2011, Lin & Man 2013, Mabb & Ehlers 2010). In a previous work we presented evidence indicating that NSPA is somehow necessary for a normal glutamatergic transmission and has structural characteristics of E3 ubiquitin ligases (Segovia-Miranda et al 2015). We found alterations in NMDAR-mediated transmission, synaptic plasticity and spatial memory in NSPA knock in mice ($NSPA^{tr/tr}$) which express a truncated form of NSPA lacking the EF-hand and anaphase promoter complex 10 (APC10) domains (Segovia-Miranda et al 2015). E3 ubiquitin ligases are characterized by one of several defining motifs including RING, U-box, F-box, PHD and HECT, among others (Freemont 2000, Metzger et al 2014, Rotin & Kumar 2009). Therefore, an important aim in this work was to obtain experimental evidence supporting a role of NSPA as E3 ubiquitin ligase.

3.1 NSPA functional domains and predicted topology.

We proposed that NSPA might be an ubiquitin ligase based on its anaphase promoter complex 10 (APC10) domain, as this APC10 domain has only been found in proteins that are or are part of E3 ubiquitin ligases (Segovia-Miranda et al 2015). In addition to APC10, NSPA also contains two ZZ-type zinc finger domains (Figure 17A), which belongs to the same Zinc finger family as PHD, RING, and FYVE domains (Legge et al 2004) and are present in some E3 ubiquitin ligases (Araki & Milbrandt 2003, Nishito et al 2006, Smit et al 2012). Delta-Blast analysis against swissprot database of mouse APC10 protein belonging to an E3 ubiquitin ligase,

Anaphase Promoter Complex (APC), showed a list of proteins bearing APC10 domain, only E3 ubiquitin ligases and NSPA showed high homology scores (Figure 17B). NSPA is a transmembrane protein (Matus et al 2007). As it might be expected for a transmembrane E3 ubiquitin ligase, a predicted NSPA topology based in CCTOP prediction server analysis showed that APC10 and both ZZ-type zinc finger domains would be directed towards the intracellular compartment (Figure 17C).

A.



B.

Proteins bearing APC10 domain and homology scores (E-value)

Protein	Function	E-Value
APC10	RING domain E3 ubiquitin ligase APC	2^{e-128}
HECTD3	HECT domain E3 ubiquitin ligase	7^{e-18}
NSPA	Unknown function. E3 ubiquitin ligase?	2^{e-16}
Cul9	RBR domain E3 ubiquitin ligase	3^{e-16}
Herc2	HECT domain E3 ubiquitin ligase	3^{e-14}
Cul7	RING domain E3 ubiquitin ligase	6^{e-13}
MYCBP2	RING domain E3 ubiquitin ligase	4^{e-08}

C.

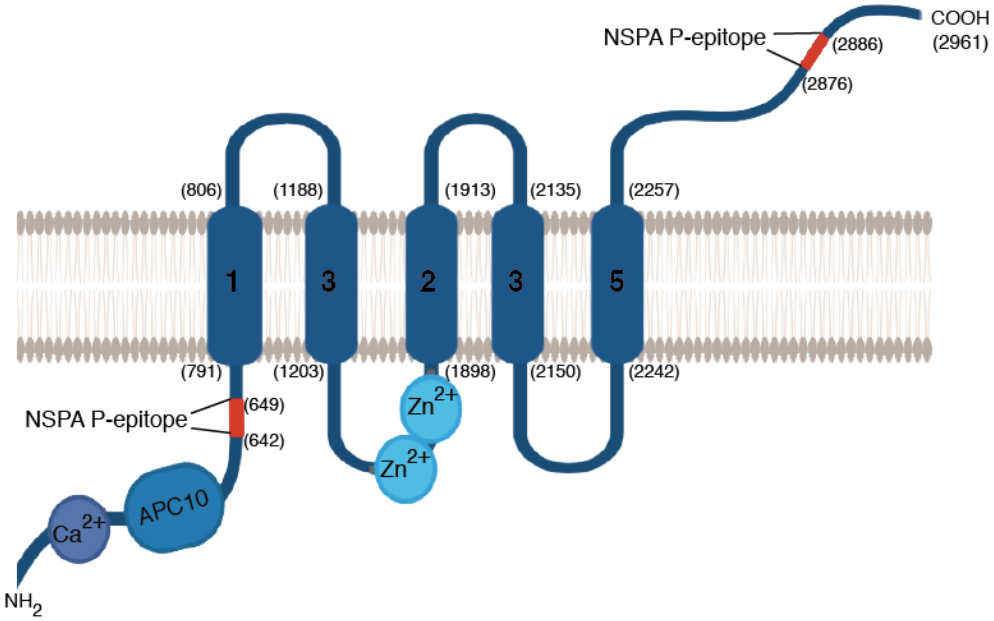


Figure 17. NSPA primary structure, functional domains and predicted topology. (A) Domain structure of human NSPA, EF-Hand (amino acids 94-141), APC10 (251-380) and ZZ domains (1781-1828 and 1830-1877) are shown. (B) Delta-Blast analysis against swissprot database of mouse APC10 protein belonging to an E3 ubiquitin ligase Anaphase Promoter Complex (APC). Only E3 ubiquitin ligases and NSPA show high homology scores. (C) Predicted NSPA topology based in CCTOP prediction server analysis. Numbers in each transmembrane region indicates the number of prediction programs that coincide.

3.2 NSPA is ubiquitinated *in vivo*.

E3 ubiquitin ligases that include both an APC10 and ZZ-type zinc finger domains are exemplified by Cullin9/PARC, an RBR-type E3, and HERC2, a HECT-type E3 (Garcia-Gonzalo & Rosa 2005, Kaustov et al 2007). NSPA is more likely an RBR-type E3 because it does not have a HECT domain (Bekker-Jensen et al 2010) and has two ZZ-type zinc finger domains similar to the RING domains of RBR-type E3 (Spratt et al 2014). Wherever the case, both HECT and RBR E3 types contain an active Cysteine residue to which ubiquitin binds prior to its transfer to the substrate (de Bie & Ciechanover 2011, Smit & Sixma 2014, Wenzel et al 2011). Therefore, to have more direct evidence of NSPA as an E3 ubiquitin ligase we evaluate if it becomes ubiquitinated *in vivo*. We used a cell-based ubiquitination assay in which HEK293 cells were co-transfected with NSPA and myc-6xHis-Ub expression plasmids (Figure 18A). Ni-NTA affinity purification of total ubiquitinated proteins in strong denaturizing conditions clearly demonstrated ubiquitin-NSPA complexes (Figure 18B).

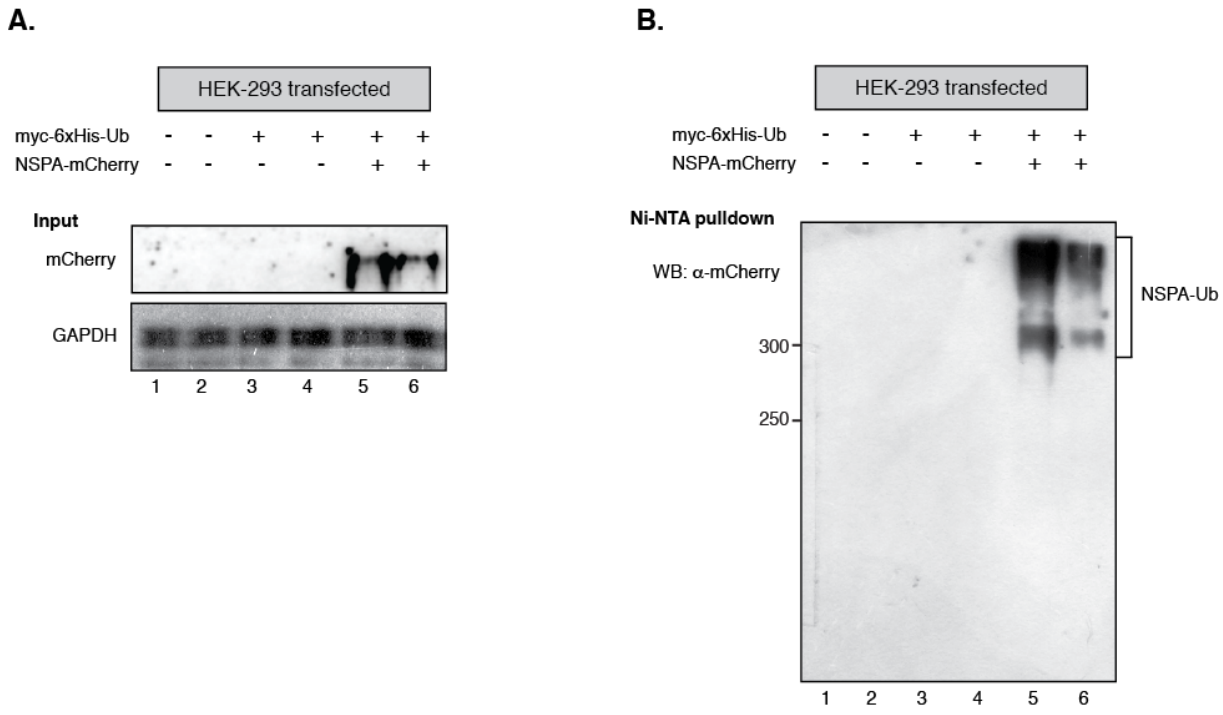


Figure 18. NSPA ubiquitination *in vivo*. (A) HEK-293 cells were transfected with NSPA-mCherry and myc-6xHis-ubiquitin, input of HEK-293 transfected cells to control NSPA-mCherry transfection levels. (B) Denaturing Ni-NTA pulldown, followed by Western blot with mCherry specific antibody, shows ubiquitinated NSPA.

3.3 PTPN4 is found ubiquitinated in wild type but not in NSPA transgenic mice.

Degradation mechanism of PTPN4 is unknown but other members of tyrosine phosphatase non-receptor type proteins are ubiquitinated and degraded by the ubiquitin proteasome system (Jing et al 2007, Won et al 2016, Yamada et al 2013a). Since we found an increase in PTPN4 mass in $NSPA^{-/-}$ mice and also that proteasome inhibition with MG-132 increased PTPN4 expression in wild type mice (Figure 16C), we explored whether PTPN4 is ubiquitinated and if NSPA is regulating its ubiquitination and degradation. We immunoprecipitated PTPN4 from crude hippocampal synaptosome membrane fractions and the immunoblot for ubiquitin revealed a reduction of ubiquitinated PTPN4 in both $NSPA^{tr/tr}$ and $NSPA^{-/-}$ compared with $NSPA^{+/+}$ mice (Figure 19A and B). We also evaluated the ubiquitination status of PSD95 and $STEP_{61}$ known to be ubiquitinated and degraded by the ubiquitin proteasome system (Colledge et al 2003, Kurup et al 2010, Xu et al 2017). We immunoprecipitated PSD95 from P2 fractions of $NSPA^{+/+}$, $NSPA^{tr/tr}$ and $NSPA^{-/-}$ hippocampus, PSD95 ubiquitination status was normal in $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice (Figure 20A). $STEP_{61}$ ubiquitination status also showed no difference between the hippocampus of $NSPA^{+/+}$ and $NSPA^{-/-}$ mice (Figure 20B). These results show that in NSPA transgenic mice there is less ubiquitination of PTPN4, without changes in the ubiquitination status of other proteins like, PSD95 and $STEP_{61}$, thus PTPN4 might be NSPA first identified substrate.

Taken together, these data show that mice lacking NSPA have less ubiquitination of PTPN4, elevated PTPN4 expression, and decreased phosphorylation of GluN2B at Tyr1472 and NMDARs levels at the PSD. These observations likely explain the synaptic and behavioral deficits of NSPA knockout mice. As NSPA it self is very likely an E3 ubiquitin ligase, PTPN4 emerged as its first identified substrate. Therefore, the structural analysis of relevant domains and the decreased ubiquitination of PTPN4 in NSPA silenced mice indicate that NSPA is very likely an E3 or part of an E3 ubiquitin ligase.

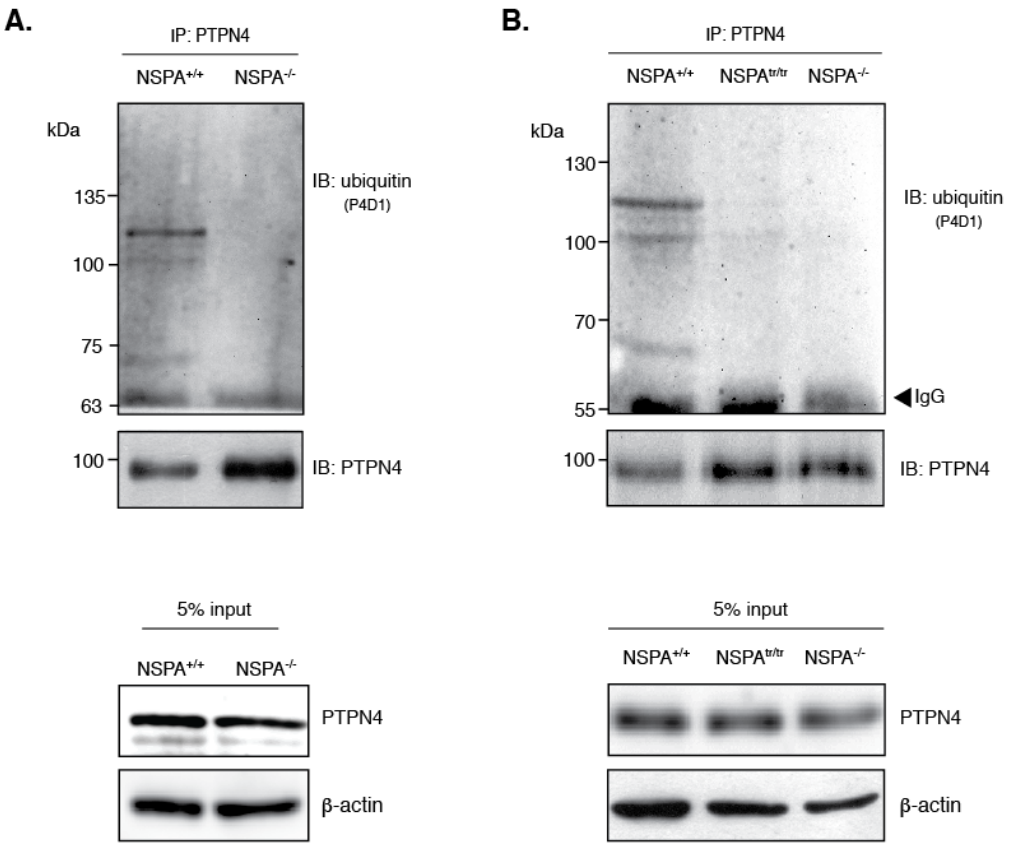


Figure 19. PTPN4 phosphatase is ubiquitinated in NSPA$^{+/+}$ mice hippocampal extracts but not in NSPA$^{tr/tr}$ or NSPA$^{-/-}$ mice. (A) Hippocampal P2 fraction of NSPA$^{+/+}$ and NSPA$^{-/-}$ mice was immunoprecipitated with anti-PTPN4 and probed with anti-PTPN4 and anti-Ubiquitin (P4D1) antibodies. (B) Hippocampal P2 fraction of NSPA$^{+/+}$, NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice was immunoprecipitated with anti-PTPN4 and probed with anti-PTPN4 and anti-Ubiquitin (P4D1) antibodies. PTPN4 was ubiquitinated in NSPA$^{+/+}$ mice extracts but not in NSPA$^{tr/tr}$ or NSPA$^{-/-}$ mice, n=3.

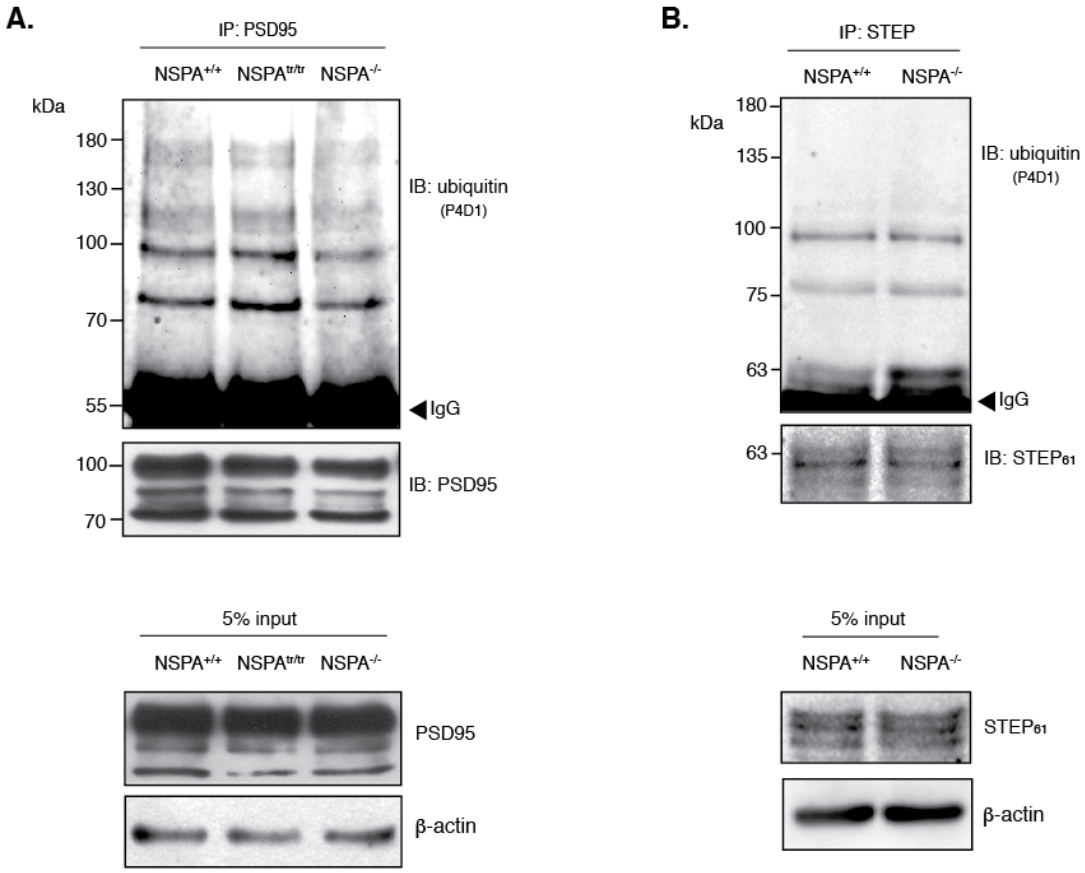
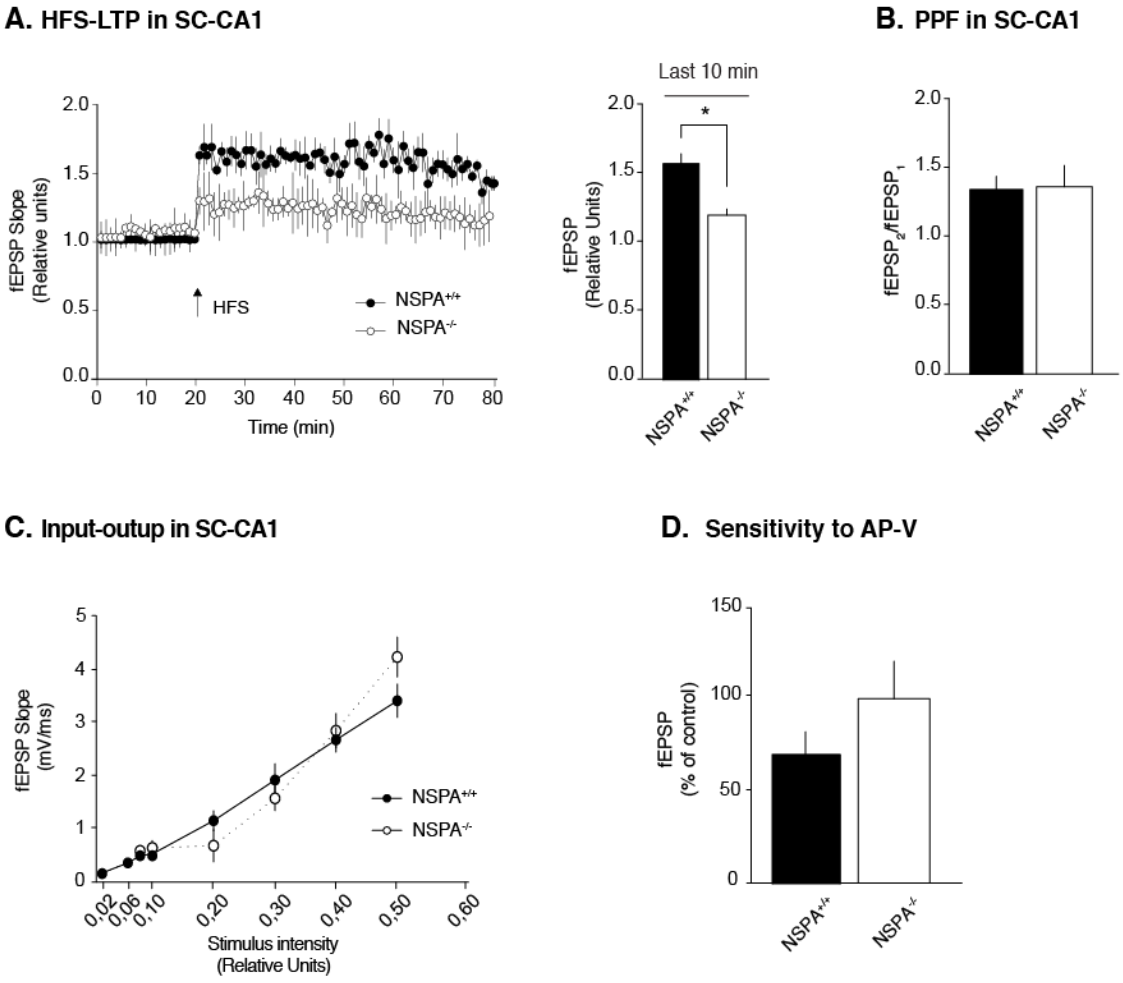
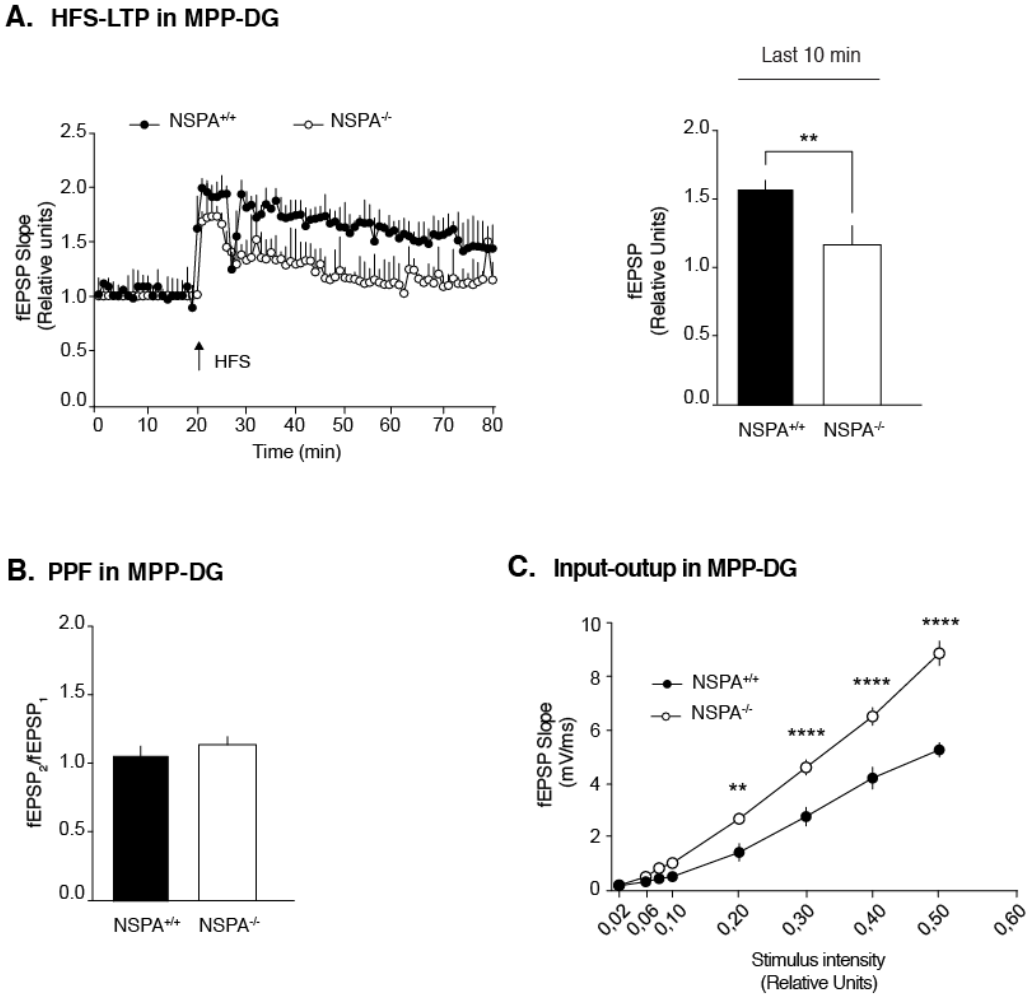


Figure 20. Normal ubiquitination levels of PSD95 and $STEP_{61}$ in the hippocampus of NSPA transgenic mice. (A) Hippocampal P2 fraction of $NSPA^{+/+}$, $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice was immunoprecipitated with anti-PSD95 antibody and probed with anti-PSD95 and anti-Ubiquitin (P4D1) antibodies. (B) Hippocampal P2 fraction of $NSPA^{+/+}$ and $NSPA^{-/-}$ mice was immunoprecipitated with anti-STEP antibody and probed with anti-STEP and anti-Ubiquitin (P4D1) antibodies. No significant changes in PSD95 and $STEP_{61}$ ubiquitination, n=2.



Annex Figure 1. Impaired long-term potentiation (LTP) in CA1 region of NSPA$^{-/-}$ mice. (A) LTP was generated by HFS in the hippocampal CA1 area in slices from NSPA$^{+/+}$ and NSPA$^{-/-}$ mice. Quantification of fEPSP slope 60 min after HFS. The dots and bars represent the mean ± SEM (n=9 slices). Three animals were used per experimental group for electrophysiological experiments. Bars show the last 10 minutes of recording after HFS. NSPA$^{-/-}$ mice showed decreased LTP compared with NSPA$^{+/+}$ mice. *P≤0.05 by *t*-test. (B) Paired pulse facilitation (PPF) of the fEPSP of hippocampal slices from NSPA$^{+/+}$ and NSPA$^{-/-}$ mice showed no alteration in presynaptic activity. The bars represent the mean ± SEM (n=7 slices). (C) fEPSP slope induced by the input-output protocol to record total responses from NSPA$^{+/+}$ and NSPA$^{-/-}$ mice, we did not find significant differences in total currents. Values are the mean ± SEM (n=9 slices). (D) Effects of application of 50 µM APV on fEPSP of hippocampal slices from NSPA$^{+/+}$ and NSPA$^{-/-}$ mice. Percentage of the response with AP-V compared to control response represent AP-V inhibition, decreased sensitivity to APV in NSPA$^{-/-}$ mice. The bars represent the mean ± SEM (n=4 slices for NSPA$^{+/+}$, 3 slices for NSPA$^{-/-}$).



Annex Figure 2. Impaired long-term potentiation (LTP) in dentate gyrus (DG) region of NSPA$^{-/-}$ mice. (A) LTP was generated by HFS in the hippocampal DG area in slices from NSPA$^{+/+}$ and NSPA$^{-/-}$ mice. Quantification of fEPSP slope 60 min after HFS. The dots and bars represent the mean ± SEM (n=9 slices). Three animals were used per experimental group for electrophysiological experiments. Bars show the last 10 minutes of recording after HFS. NSPA$^{-/-}$ mice showed decreased LTP compared with NSPA$^{+/+}$ mice. **P≤0.01 by *t*-test. (B) Paired pulse facilitation (PPF) of the fEPSP of hippocampal slices from NSPA$^{+/+}$ and NSPA$^{-/-}$ mice showed no alteration in presynaptic activity. The bars represent the mean ± SEM (n=7 slices). (C) fEPSP slope induced by the input-output protocol to record total responses from NSPA$^{+/+}$ and NSPA$^{-/-}$ mice, NSPA$^{-/-}$ mice showed a significant increase in total synaptic response. Values are the mean ± SEM (n=9 slices), **P≤0.01, ****P≤0.0001 versus NSPA$^{+/+}$ mice by two-way ANOVA, followed by Bonferroni post-hoc test.

DISCUSSION

This thesis reveals a functional relationship between NSPA and NMDA receptors involving the tyrosine-phosphatase PTPN4 as a potential NSPA ubiquitination substrate in mice hippocampus. Our present study in NSPA knockout mice demonstrates that NSPA is required to ensure adequate NMDAR expression levels at the PSD, synaptic plasticity and memory, PTPN4 ubiquitination and regulation of its expression levels and also is important to maintain normal rates of adult neurogenesis. In addition, biochemical experiments provided evidence supporting a role of NSPA as E3 ubiquitin ligase.

Immunoblots of hippocampus preparations revealed significant reductions in the levels of GluN2A and GluN2B NMDA receptor subunits in $NSPA^{-/-}$ postsynaptic densities. Further analysis of GluN2B posttranslational modifications revealed a significant decrease in phosphorylation of Tyr1472 of GluN2B in $NSPA^{-/-}$ mice, phosphorylation that promotes surface expression of GluN2B-containing NMDARs (Lavezzari et al 2003, Roche et al 2001). The decreased Tyr1472 phosphorylation in GluN2B subunits predicts increased levels of endocytosis of NMDARs that could explain the lower levels of NMDAR found at the PSDs.

PTPN4 tyrosine phosphatase has been associated with regulation of NMDAR phosphorylation (Kina et al 2007) and appeared in a wide screen analysis as a potential NSPA interacting protein (Stelzl et al 2005). We found increased PTPN4 levels in $NSPA^{-/-}$ compared with wild type mice in hippocampus. However, these differences disappeared when the proteasome activity is inhibited with MG-132. This

finding proves that both NSPA and the proteasome are involved in the regulation of PTPN4 levels, suggesting a linking role based on ubiquiination, as the proteasome substrates are ubiquitinated proteins.

We had previously proposed that the presence of an APC10 domain in NSPA implies a possible role as an E3 ubiquitin ligase (Segovia-Miranda et al 2015). All known proteins that bear a APC10 domain are E3 ubiquitin ligases (Boratyn et al 2012, Segovia-Miranda et al 2015). Here we provide some evidence supporting a role of NSPA as E3 ubiquitin ligase, including NSPA ubiquitination *in vivo*. We also show that PTPN4 is less ubiquitinated in hippocampal preparations of NSPA knockout mice. This together with the yeast two-hybrid indication of an interaction of PTPN4 and NSPA (Stelzl et al 2005) makes very likely that PTPN4 is a direct substrate of NSPA acting as E3 ubiquitin ligase. Futhermore, this provides a link between NSPA function regulating PTPN4 ubiquitination and expression of GluN2B-containing NMDARs in hippocampus.

In addition, $NSPA^{-/-}$ mice performed poorly in memory flexibility and object recognition test reflecting an impaired hippocampal-dependent memory, congruent with abnormal synaptic plasticity. $NSPA^{-/-}$ mice displayed decreased synaptic activity and proliferation of neural progenitors without changes in the differentiation of newborn cells in SGZ of DG. Reduced neurogenesis and synaptic activity in the $NSPA^{-/-}$ adult DG might be associated with the decreased LTP and impaired memory in these mice.

NSPA knockout mice (NSPA$^{-/-}$) characterization.

In 2015 our laboratory reported evidence indicating that NSPA participates in NMDA receptor function and in synaptic plasticity, as our NSPA knock-in mice showed impaired memory and decreased NMDAR activity and LTP (Segovia-Miranda et al 2015). However, these NSPA knock-in mice still express a truncated form of NSPA and therefore we decided to further validate the phenotype using knockout mice lacking any form of NSPA expression discarding unnoticed effects of the truncated version of NSPA. We characterized a NSPA full knockout mice (NSPA$^{-/-}$; Zzef1$^{tm2.1(KOMP)vlcg}$) produced by our collaborator Dr. David Valenzuela in Regeneron Pharmaceuticals (Terrytown, New York, USA). RT-PCR for different exons and immunoblots with two antibodies showed that NSPA$^{-/-}$ mice do not express NSPA and neither any truncated version of NSPA (Figure 2B and C).

In NSPA$^{-/-}$ mice, we found decreased synaptic plasticity and impaired NMDAR-mediated transmission in SC-CA1 and MPP-DG synapses of dorsal hippocampus (Annex Figure 1A, 1D and 2A), without alterations in presynaptic activity (Annex Figure 1B and 2B). Therefore, NSPA absence reproduces the phenotype of the truncated version of NSPA in mice at least in electrophysiology experiments.

NSPA modulates NMDAR expression at the postsynaptic region of mice hippocampus.

The results showing that NSPA regulates NMDAR-dependent transmission, prompted us to evaluate the levels of NMDAR and other synaptic proteins comparing NSPA$^{+/+}$, NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice hippocampus. When analyzing hippocampal whole lysates, we did not found significant differences in synaptic receptors and

scaffolding proteins between NSPA$^{+/+}$ and NSPA$^{-/-}$ mice (Figure 3A and C). However, NSPA$^{tr/tr}$ hippocampus showed significant reductions in the levels of GluN2A and GluN2B NMDAR subunits (Figure 3B and C). By RT-PCR we showed that GluN2A and GluN2B mRNA levels were unchanged in the hippocampus of NSPA$^{tr/tr}$ mice (Figure 4). Therefore GluN2A and GluN2B decrease in NSPA$^{tr/tr}$ mice whole hippocampus may not be due to a lower transcription, but most probably to a posttranslational regulation.

In contrast with the experiments in whole hippocampal extracts, we found decreased expression of GluN2A and GluN2B subunits of NMDAR in hippocampal synaptosomes and postsynaptic densities (PSD) from NSPA$^{tr/tr}$ and NSPA$^{-/-}$ mice (Figure 6 and 12), without changes in other synaptic proteins. Further analysis in NSPA$^{-/-}$ mice showed a decrease in GluN2 subunits only in membrane preparations and not in total hippocampal homogenate, suggesting a redistribution of NMDA receptor subunits among subcellular compartments. This decrease at the postsynaptic region could explain the deficits in NMDAR-dependent transmission and synaptic plasticity found in NSPA transgenic mice.

NSPA$^{-/-}$ mice displayed defects in hippocampal-dependent tasks and decreased adult neurogenesis.

Hippocampal NMDARs are crucial elements in the induction of synaptic plasticity and memory acquisition (Gao et al 2010, Quinn et al 2005). Alterations in postsynaptic abundance of NMDAR and glutamatergic transmission can affect hippocampal-dependent memory (Tsien et al 1996), and NSPA$^{tr/tr}$ mice have alterations in spatial memory (Segovia-Miranda et al 2015). Here we found that

NSPA$^{-/-}$ mice also performed poorly in memory flexibility (Figure 7A) and object recognition (Figure 7B) tests, thus reflecting an impaired hippocampal-dependent memory (Stackman et al 2016). These results are congruent with abnormal synaptic plasticity in mice lacking NSPA function.

Adult mice that lack NMDAR-mediated LTP in the CA1 synapses have been shown to exhibit impaired spatial memory (Tsien et al 1996). However, the role of the hippocampus in recognition memory remains controversial. The neural basis of recognition memory implicates several brain regions and some studies reported no effect of hippocampal lesions in object recognition, while others found significant impairments (Barker & Warburton 2011, Cohen et al 2013). Therefore, our findings of NSPA$^{-/-}$ altered object recognition may reflect not only a dysfunction in the hippocampus but also in other brain regions where NSPA is expressed.

The limbic system is a key site for anxiety and includes structures like amygdala, hippocampus, and cingulate cortex among others (Clement & Chapouthier 1998). Since NSPA is expressed in hippocampus as well as in other areas that include cortex and amygdala (Matus et al 2007), we evaluated if NSPA$^{-/-}$ mice presented alterations in anxiety-like behaviors. Measuring the time spend in the center region of an open field arena we did not found differences between the wild type and NSPA$^{-/-}$ group (Figure 8A), thus NSPA knockout mice do not display anxiety-like behaviors.

In addition, we show that NSPA is required for adult neurogenesis in dentate gyrus. Neurogenesis in the hippocampus represents a form of cellular plasticity in the adult brain contributing to hippocampal functions in learning and memory (Deng et al

2010, Goncalves et al 2016, Kheirbek et al 2012, Zhao et al 2008). NSPA$^{-/-}$ mice displayed decreased synaptic activity (Figure 10A) and proliferation of neural progenitors (Figure 9A and B) without changes in the differentiation of newborn cells in SGZ of DG (Figure 10B). Reduced proliferation of neural progenitors and synaptic activity in the NSPA$^{-/-}$ adult DG might be associated with the decreased LTP and impaired memory in these mice. Indeed, reduced adult neurogenesis has been shown to suppress both LTP and LTD, and Arc synthesis, which we found decreased reflecting decreased synaptic activity, is also required for LTP (Massa et al 2011, Messaoudi et al 2007). These results suggest that NSPA role is important in the proliferation but not in the differentiation of neural progenitors. Interestingly, NMDAR-mediated glutamatergic inputs plays an important role in the survival of new neurons, without affecting neuronal maturation (Tashiro et al 2006). Therefore, the decreased NMDAR synaptic levels found in NSPA$^{-/-}$ mice may contribute to reduce proliferation of neural progenitors.

Decreased phosphorylation of GluN2B Tyr1472 in NSPA transgenic mice.

To approach the mechanism by which the GluN2 subunits are decreased in the postsynaptic region of NSPA transgenic mice, we evaluated posttranslational modifications known to regulate NMDARs localization. The cytoplasmic C-tails of NMDAR subunits are substrates for posttranslational modifications that control NMDAR function, trafficking and interactions with cytosolic proteins (Chen & Roche 2007, Goebel-Goody et al 2009, Lin & Man 2013). GluN2A and GluN2B are subjected to differential regulation by several posttranslational mechanisms in their C-tails, including ubiquitination (Jurd et al 2008, Yin et al 2011). Ubiquitination can control

NMDAR localization and degradation and therefore is involved in modulating NMDAR functionality (Jurd et al 2008, Kato et al 2005). GluN2 subunits can be ubiquitinated *in vitro* while *in vivo* ubiquitination has only been described for GluN2A subunit (Yin et al 2011). If NSPA directly ubiquitinates NMDAR subunits it might be expected that increased levels of NMDAR are found in NSPA transgenic mice. But as mentioned we found the opposite, i.e. increased levels of NMDAR subunits in the absence of NSPA. In any case, we tried to evaluate ubiquitination levels of GluN2 subunits and could not detect this modification in hippocampal extracts (Figure 13B). Therefore, we searched for alternative regulation of NMDAR levels through indirect mechanisms involving other proteins.

NMDARs GluN2 subunits undergo active phosphorylation that regulates surface and synaptic expression of NMDARs (Lussier et al 2015). In particular, tyrosine phosphorylation regulates the trafficking of NMDARs from intracellular compartments to the postsynaptic density (PSD) and the stability of NMDARs at the synaptic membrane (Dunah & Standaert 2001, Prybylowski et al 2005). Moreover, GluN2B subunit has been identified as the main tyrosine-phosphorylated protein in the PSD (Moon et al 1994). Its phosphorylation at Tyr1472 prevents endocytosis leading to an enhanced NMDAR surface expression at the synaptic zone (Prybylowski et al 2005, Xu et al 2006). Therefore, we focused on this post-translational modification. Interestingly, immunoblot analysis showed a decrease in phosphorylation of GluN2B Tyr1472 in $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice compared with wild type mice (Figure 14B, C and D). Such a decreased Tyr1472 phosphorylation in

GluN2B subunits predicts increased levels of endocytosis of NMDARs, which indeed may explain the lower levels of NMDAR found at the PSDs.

It remains to evaluate this posttranslational modification in GluN2A subunit, as tyrosine phosphorylation also plays an important role in GluN2A surface stability (Vissel et al 2001).

PTPN4 levels are increased in hippocampus of NSPA knockout mice and is ubiquitinated in wild type but not in NSPA transgenic mice

NMDAR subunits, scaffolding proteins and signaling enzymes compose the NMDAR complex (Husi et al 2000), including Fyn tyrosine kinase and $STEP_{61}$ tyrosine phosphatase (Pelkey et al 2002, Tezuka et al 1999) which are well known to regulate GluN2B Tyr1472 phosphorylation (Kurup et al 2010, Nakazawa et al 2001, Xu et al 2006). However, we found no changes in Fyn and $STEP_{61}$ levels in $NSPA^{-/-}$ mice compared with wild type mice (Figure 15A and B).

The NMDAR complex also contains PTPN4 tyrosine phosphatase (Hironaka et al 2000), which has been involved in learning, spatial memory and cerebellar synaptic plasticity (Kina et al 2007, Kohda et al 2013). PTPN4 modulates signaling downstream of the glutamate receptors through changes in phosphorylation of NMDAR GluD2 and GluN2 subunits (Kina et al 2007) and AMPAR GluA2 subunit (Gallimore et al 2016, Kohda et al 2013). PTPN4 hypofunction has been implicated in clinical cases of neurodevelopmental disorders (Szczaluba et al 2018, Williamson et al 2015). A missense variant in the PTPN4 gene produces a mutated form of PTPN4 that do not reach dendritic spines and therefore cannot interact with and regulate

glutamatergic receptors (Szczaluba et al 2018). Thus alterations in PTPN4 function or levels may alter NMDAR phosphorylation status, localization and function.

PTPN4 has been reported as a potential NSPA interacting protein by a yeast two-hybrid massive screen (Stelzl et al 2005). We show that PTPN4 levels are increased in $NSPA^{-/-}$ compared with wild type mice in hippocampus particularly when the proteasome activity is not inhibited (Figure 16C). If the proteasome is inhibited with MG-132, PTPN4 levels were similar in wild type and $NSPA^{-/-}$ mice (Figure 16C). Also, we showed that PTPN4 is ubiquitinated in mouse hippocampus and is less ubiquitinated in $NSPA^{tr/tr}$ and $NSPA^{-/-}$ mice (Figure 19A and B). These results indicate that PTPN4 is very likely a potential substrate of NSPA regulation that affects NMDAR phosphorylation status. A potential link between NSPA function regulating PTPN4 ubiquitination and levels and surface expression of GluN2B-containing NMDARs in hippocampus is thus revealed. NSPA and the proteasome are involved in the regulation of PTPN4 levels. The increased PTPN4 levels can well account for the decreased phosphorylation of GluN2B at Tyr1472. We also found that PTPN4 interacts with GluN2A and GluN2B subunits of NMDAR in mice hippocampus (Figure 16A), suggesting that it can regulate both NMDAR subunits.

NSPA role as E3 ubiquitin ligase

We had previously proposed that the presence of an APC10 domain in NSPA implies a possible role as an E3 ubiquitin ligase (Segovia-Miranda et al 2015). All known proteins that bear a APC10 domain have been described as E3 ubiquitin ligases (Figure 17B) (Boratyn et al 2012, Segovia-Miranda et al 2015).

E3 ubiquitin ligases are classified in three major classes, RING, HECT and RBR (Zheng & Shabek 2017) (Figure 1). NSPA is more likely an RBR-type E3 because it has two ZZ-type zinc finger domains (Figure 17A) similar to the RING domains of RBR-type E3 (Spratt et al 2014). Those type of E3 ligases contain an active Cysteine residue that is ubiquitinated before the transfer of ubiquitin to the substrate (Wenzel et al 2011). We performed experiments in transfected HEK293 cells that clearly demonstrated ubiquitination of NSPA *in vivo* (Figure 18B), thus accomplishing a crucial characteristic of RBR E3 ubiquitin ligases (Dove et al 2016, Wenzel et al 2011). RBR E3s are defined by a highly conserved catalytic unit of three characteristic domains each of which coordinates two Zn^{2+} ions: RING1, in-between RING (IBR), and RING2 (Wenzel et al 2011) and the active cysteine residue is on RING2 (Smit et al 2012). NSPA does not posses the conserved catalytic domain of RBR E3s, as only two zinc finger domains are present in its sequence. Some studies have shown that IBR-RING2 of Parkin, an RBR E3, can still promote ubiquitination, conserving its catalytic activity in the absence of RING1 (Chew et al 2011, Matsuda et al 2006). NSPA could be a particular RBR type with only two zinc finger domains. Future experiments should explore whether NSPA is ubiquitinated in one of the zinc finger domain, and also its capacity to bind to E2 enzymes.

The results obtained in NSPA transgenic mice points to PTPN4 tyrosine phosphatase as its first possible identified substrate. These findings support the possible role of NSPA as an E3 ligase or as a part of one. Moreover it seems that NSPA regulation over NMDARs phosphorylation status involves specifically PTPN4 phosphatase, and not $STEP_{61}$.

We propose the following model. NSPA normally promotes ubiquitination and proteasome-mediated degradation of PTPN4 and thus regulates its expression levels, which in turn regulates the phosphorylation levels of GluN2B at Y1472 (Figure 21). In NSPA absence, PTPN4 ubiquitination and its proteasomal-mediated degradation becomes reduced and in consequence its increased levels would reduce the phosphorylation of GluN2B at Y1472 leading to a reduced GluN2B-containing NMDARs levels at the PSD (Figure 21). This mechanism can explain the depressed synaptic plasticity, impaired hippocampal-dependent memory and altered adult neurogenesis in NSPA knockout mice.

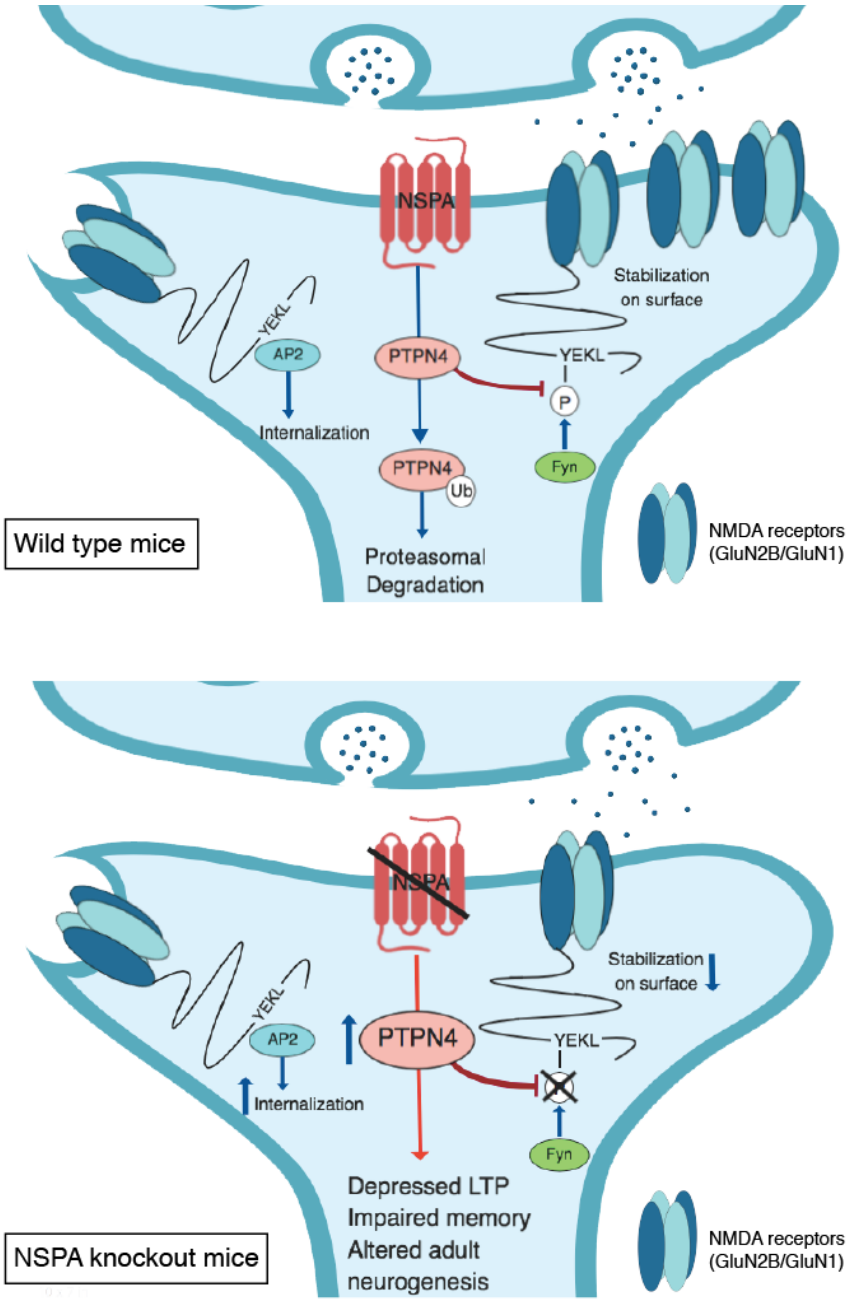


Figure 21. NSPA regulation of GluN2B-containing NMDA receptors. In wild type mice hippocampus, NSPA presence promotes ubiquitination and degradation of PTPN4 through the proteasome, increasing the phosphorylation of GluN2B at Y1472. This phosphorylation enhances the stabilization on surface of GluN2B-containing NMDARs, promoting its synaptic enrichment. In NSPA knockout mice hippocampus, NSPA absence results in reduced ubiquitination and increased levels of PTPN4, which decreases the phosphorylation of GluN2B at Y1472 and GluN2B-containing NMDARs levels at the PSD. Leading to depressed synaptic plasticity (LTP), impaired hippocampal-dependent memory and reduced adult neurogenesis in NSPA knockout mice.

CONCLUDING REMARKS

The present thesis provides evidence that NSPA might be an E3 ubiquitin ligase that regulates PTPN4 phosphatase ubiquitination status and levels, which points PTPN4 as a potential NSPA ubiquitination substrate. On the other hand PTPN4 could be modulating the phosphorylation status of GluN2B-containing NMDARs, and in consequence NMDAR synaptic abundance and function in mouse hippocampus.

Impaired NMDAR-mediated transmission, decreased synaptic plasticity and poor performance in memory tests in NSPA knockout mice correlates with reduced levels of GluN2A and GluN2B NMDAR subunits in the PSD. Decreased GluN2B phosphorylation at Tyr 1472 in NSPA knockout mice may lead to diminished GluN2B levels at the PSD. Interestingly levels of Fyn kinase and $STEP_{61}$ phosphatase, known regulators of GluN2B Tyr 1472 phosphorylation, not dot change in NSPA absence, but there is less ubiquitination of PTPN4, resulting in increased expression. PTPN4 has been shown to interact with and modulate changes in the tyrosine phosphorylation of NMDAR subunits. All these results indicate that NSPA absence leads to a deregulation in PTPN4 ubiquitination and expression levels that result in alterations in phosphorylation of GluN2B at Tyr1472 and NMDARs levels at the PSD, producing synaptic alterations and behavioral deficits in mice.

REFERENCES

Abbott AC, Calderon Toledo C, Aranguiz FC, Inestrosa NC, Varela-Nallar L. 2013. Tetrahydrohyperforin increases adult hippocampal neurogenesis in wild-type and APPswe/PS1DeltaE9 mice. *J Alzheimers Dis* 34: 873-85

Al-Hakim AK, Bashkurov M, Gingras A-C, Durocher D, Pelletier L. 2012. Interaction Proteomics Identify NEURL4 and the HECT E3 Ligase HERC2 as Novel Modulators of Centrosome Architecture. *Molecular & Cellular Proteomics* 11

AlE'ed A, Vega-Fernandez P, Muscal E, Hinze CH, Tucker LB, et al. 2017. Challenges of Diagnosing Cognitive Dysfunction With Neuropsychiatric Systemic Lupus Erythematosus in Childhood. *Arthritis Care Res (Hoboken)* 69: 1449-59

Araki T, Milbrandt J. 2003. ZNRF proteins constitute a family of presynaptic E3 ubiquitin ligases. *J Neurosci* 23: 9385-94

Arinuma Y, Yanagida T, Hirohata S. 2008. Association of cerebrospinal fluid anti-NR2 glutamate receptor antibodies with diffuse neuropsychiatric systemic lupus erythematosus. *Arthritis Rheum* 58: 1130-5

Arvidsson A, Kokaia Z, Lindvall O. 2001. N-methyl-D-aspartate receptor-mediated increase of neurogenesis in adult rat dentate gyrus following stroke. *Eur J Neurosci* 14: 10-18

Barker GR, Warburton EC. 2011. When is the hippocampus involved in recognition memory? *J Neurosci* 31: 10721-31

Barnes CA, McNaughton BL. 1985. An age comparison of the rates of acquisition and forgetting of spatial information in relation to long-term enhancement of hippocampal synapses. *Behav Neurosci* 99: 1040-8

Barria A, Malinow R. 2002. Subunit-specific NMDA receptor trafficking to synapses. *Neuron* 35: 345-53

Barria A, Malinow R. 2005. NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. *Neuron* 48: 289-301

Bekker-Jensen S, Rendtlew Danielsen J, Fugger K, Gromova I, Nerstedt A, et al. 2010. HERC2 coordinates ubiquitin-dependent assembly of DNA repair factors on damaged chromosomes. *Nat Cell Biol* 12: 80-6; sup pp 1-12

Berger TW. 1984. Long-term potentiation of hippocampal synaptic transmission affects rate of behavioral learning. *Science* 224: 627-30

Berndsen CE, Wolberger C. 2014. New insights into ubiquitin E3 ligase mechanism. *Nat Struct Mol Biol* 21: 301-7

Bonfa E, Golombek SJ, Kaufman LD, Skelly S, Weissbach H, et al. 1987. Association between lupus psychosis and anti-ribosomal P protein antibodies. *N Engl J Med* 317: 265-71

Boratyn GM, Schaffer AA, Agarwala R, Altschul SF, Lipman DJ, Madden TL. 2012. Domain enhanced lookup time accelerated BLAST. *Biol Direct* 7: 12

Borden KLB, Boddy MN, Lally J, O'Reilly JO, Martin S, et al. 1995. The solution structure of the RING finger domain from the acute promyelocytic leukaemia proto-oncoprotein PML. *EMBO J* 14: 1532-41

Brady RJ, Gorter JA, Monroe MTM, Swann JW. 1994. Developmental alterations in the sensitivity of hippocampal NMDA receptors to AP5. *Developmental Brain Research* 83: 190-96

Bravo-Zehnder M, Toledo EM, Segovia-Miranda F, Serrano FG, Benito MJ, et al. 2015. Anti-ribosomal P protein autoantibodies from patients with neuropsychiatric lupus impair memory in mice. *Arthritis Rheumatol* 67: 204-14

Buschhorn BA, Petzold G, Galova M, Dube P, Kraft C, et al. 2011. Substrate binding on the APC/C occurs between the coactivator Cdh1 and the processivity factor Doc1. *Nat Struct Mol Biol* 18: 6-13

Cameron HA, McEwen BS, Gould E. 1995. Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus. *Journal of Neuroscience* 15: 4687-92

Carola V, D'Olimpio F, Brunamonti E, Mangia F, Renzi P. 2002. Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. *Behav Brain Res* 134: 49-57

Cercato MC, Colettis N, Snitcofsky M, Aguirre AI, Kornisiuk EE, et al. 2014. Hippocampal NMDA receptors and the previous experience effect on memory. *J Physiol Paris* 108: 263-9

Cerpa W, Farias G, Godoy J, Fuenzalida M, Bonansco C, Inestrosa N. 2010. Wnt-5a occludes Ab oligomer-induced depression of glutamatergic transmission in hippocampal neurons. *Mol Neurodegener* 5: 3

Chen BS, Roche KW. 2007. Regulation of NMDA receptors by phosphorylation. *Neuropharmacology* 53: 362-8

Chen G, Chen KS, Knox J, Inglis J, Bernard A, et al. 2000. A learning deficit related to age and beta-amyloid plaques in a mouse model of Alzheimer's disease. *Nature* 408: 975-9

Chew KC, Matsuda N, Saisho K, Lim GG, Chai C, et al. 2011. Parkin mediates apparent E2-independent monoubiquitination in vitro and contains an intrinsic activity that catalyzes polyubiquitination. *PLoS One* 6: e19720

Chung HJ, Huang YH, Lau LF, Huganir RL. 2004. Regulation of the NMDA receptor complex and trafficking by activity-dependent phosphorylation of the NR2B subunit PDZ ligand. *J Neurosci* 24: 10248-59

Citri A, Malenka RC. 2008. Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology* 33: 18-41

Clement Y, Chapouthier G. 1998. Biological bases of anxiety. *Neurosci Biobehav Rev* 22: 623-33

Cohen SJ, Munchow AH, Rios LM, Zhang G, Asgeirsdottir HN, Stackman RW, Jr. 2013. The rodent hippocampus is essential for nonspatial object memory. *Curr Biol* 23: 1685-90

Cohen SJ, Stackman RW, Jr. 2015. Assessing rodent hippocampal involvement in the novel object recognition task. A review. *Behav Brain Res* 285: 105-17

Colledge M, Snyder EM, Crozier RA, Soderling JA, Jin Y, et al. 2003. Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. *Neuron* 40: 595-607

Collingridge GL, Isaac JT, Wang YT. 2004. Receptor trafficking and synaptic plasticity. *Nat Rev Neurosci* 5: 952-62

Cull-Candy S, Kelly L, Farrant M. 2006. Regulation of Ca2+-permeable AMPA receptors: synaptic plasticity and beyond. *Curr Opin Neurobiol* 16: 288-97

Cull-Candy SG, Leszkiewicz DN. 2004. Role of distinct NMDA receptor subtypes at central synapses. *Sci STKE* 2004: re16

de Bie P, Ciechanover A. 2011. Ubiquitination of E3 ligases: self-regulation of the ubiquitin system via proteolytic and non-proteolytic mechanisms. *Cell Death Differ* 18: 1393-402

DeGiorgio LA, Konstantinov KN, Lee SC, Hardin JA, Volpe BT, Diamond B. 2001. A subset of lupus anti-DNA antibodies cross-reacts with the NR2 glutamate receptor in systemic lupus erythematosus. *Nat Med* 7: 1189-93

Deisseroth K, Singla S, Toda H, Monje M, Palmer TD, Malenka RC. 2004. Excitation-Neurogenesis Coupling in Adult Neural Stem/Progenitor Cells. *Neuron* 42: 535-52

Delgado-Esteban M, Garcia-Higuera I, Maestre C, Moreno S, Almeida A. 2013. APC/C-Cdh1 coordinates neurogenesis and cortical size during development. *Nat Commun* 4: 2879

Deng W, Aimone JB, Gage FH. 2010. New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nat Rev Neurosci* 11: 339-50

Deshaies RJ, Joazeiro CA. 2009. RING domain E3 ubiquitin ligases. *Annu Rev Biochem* 78: 399-434

Diamond B, Honig G, Mader S, Brimberg L, Volpe BT. 2013. Brain-reactive antibodies and disease. *Annu Rev Immunol* 31: 345-85

Diamond B, Huerta PT, Mina-Osorio P, Kowal C, Volpe BT. 2009. Losing your nerves? Maybe it's the antibodies. *Nat Rev Immunol* 9: 449-56

Dove KK, Stieglitz B, Duncan ED, Rittinger K, Klevit RE. 2016. Molecular insights into RBR E3 ligase ubiquitin transfer mechanisms. *EMBO Rep* 17: 1221-35

Drew LJ, Fusi S, Hen R. 2013. Adult neurogenesis in the mammalian hippocampus: why the dentate gyrus? *Learn Mem* 20: 710-29

Dunah AW, Standaert DG. 2001. Dopamine D1 receptor-dependent trafficking of striatal NMDA glutamate receptors to the postsynaptic membrane. *Journal of Neuroscience* 21: 5546-58

Ehlers MD. 2000. Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron* 28: 511-25

Ehlers MD. 2003. Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nat Neurosci* 6: 231-42

Elkon K, Bonfa E, Llovet R, Danho W, Weissbach H, Brot N. 1988. Properties of the ribosomal P2 protein autoantigen are similar to those of foreign protein antigens. *Proc Natl Acad Sci U S A* 85: 5186-89

Elkon K, Skelly S, Parnassa A, Moller W, Danho W, et al. 1986. Identification and chemical synthesis of a ribosomal protein antigenic determinant in systemic lupus erythematosus. *Proc Natl Acad Sci U S A* 87: 7419-23

Faust TW, Chang EH, Kowal C, Berlin R, Gazaryan IG, et al. 2010. Neurotoxic lupus autoantibodies alter brain function through two distinct mechanisms. *Proc Natl Acad Sci U S A* 107: 18569-74

Freemont PS. 2000. RING for destruction? *Curr Biol* 10: R84-R87

Fu AK, Hung KW, Fu WY, Shen C, Chen Y, et al. 2011. APC(Cdh1) mediates EphA4-dependent downregulation of AMPA receptors in homeostatic plasticity. *Nat Neurosci* 14: 181-9

Gallimore AR, Aricescu AR, Yuzaki M, Calinescu R. 2016. A Computational Model for the AMPA Receptor Phosphorylation Master Switch Regulating Cerebellar Long-Term Depression. *PLoS Comput Biol* 12: e1004664

Gamsjaeger R, Liew CK, Loughlin FE, Crossley M, Mackay JP. 2007. Sticky fingers: zinc-fingers as protein-recognition motifs. *Trends Biochem Sci* 32: 63-70

Gao C, Gill MB, Tronson NC, Guedea AL, Guzman YF, et al. 2010. Hippocampal NMDA receptor subunits differentially regulate fear memory formation and neuronal signal propagation. *Hippocampus* 20: 1072-82

Garcia-Gonzalo FR, Rosa JL. 2005. The HERC proteins: functional and evolutionary insights. *Cell Mol Life Sci* 62: 1826-38

Gardoni F, Bellone C, Cattabeni F, Di Luca M. 2001. Protein kinase C activation modulates alpha-calmodulin kinase II binding to NR2A subunit of N-methyl-D-aspartate receptor complex. *J Biol Chem* 276: 7609-13

Glickman MH, Ciechanover A. 2002. The Ubiquitin-Proteasome Proteolytic Pathway: Destruction for the Sake of Construction. *Physiol Rev* 82: 373–428

Goebel-Goody SM, Davies KD, Alvestad Linger RM, Freund RK, Browning MD. 2009. Phospho-regulation of synaptic and extrasynaptic N-methyl-d-aspartate receptors in adult hippocampal slices. *Neuroscience* 158: 1446-59

Goncalves JT, Schafer ST, Gage FH. 2016. Adult Neurogenesis in the Hippocampus: From Stem Cells to Behavior. *Cell* 167: 897-914

Gonzalez A, Massardo L. 2018. Antibodies and the brain: antiribosomal P protein antibody and the clinical effects in patients with systemic lupus erythematosus. *Curr Opin Neurol* 31: 300-05

Haglund K, Dikic I. 2005. Ubiquitylation and cell signaling. *EMBO J* 24: 3353-9

Hanly JG. 2004. ACR classification criteria for systemic lupus erythematosus: limitations and revisions to neuropsychiatric variables. *Lupus* 13: 861-4

Hanly JG. 2014. Diagnosis and management of neuropsychiatric SLE. *Nat Rev Rheumatol* 10: 338-47

Hanly JG, Harrison MJ. 2005. Management of neuropsychiatric lupus. *Best Pract Res Clin Rheumatol* 19: 799-821

Hanly JG, Urowitz MB, Su L, Bae SC, Gordon C, et al. 2011. Autoantibodies as biomarkers for the prediction of neuropsychiatric events in systemic lupus erythematosus. *Ann Rheum Dis* 70: 1726-32

Harboe E, Tjensvoll AB, Maroni S, Goransson LG, Greve OJ, et al. 2009. Neuropsychiatric syndromes in patients with systemic lupus erythematosus and primary Sjogren syndrome: a comparative population-based study. *Ann Rheum Dis* 68: 1541-6

Hegde AN. 2004. Ubiquitin-proteasome-mediated local protein degradation and synaptic plasticity. *Prog Neurobiol* 73: 311-57

Hegde AN, DiAntonio A. 2002. Ubiquitin and the synapse. *Nat Rev Neurosci* 3: 854-61

Hirohata S, Arinuma Y, Takayama M, Yoshio T. 2007. Association of cerebrospinal fluid anti-ribosomal p protein antibodies with diffuse

psychiatric/neuropsychological syndromes in systemic lupus erythematosus. *Arthritis Res Ther* 9: R44

Hironaka K, Umemori H, Tezuka T, Mishina M, Yamamoto T. 2000. The protein-tyrosine phosphatase PTPMEG interacts with glutamate receptor delta 2 and epsilon subunits. *J Biol Chem* 275: 16167-73

Hnia K, Zouiten D, Cantel S, Chazalette D, Hugon G, et al. 2007. ZZ domain of dystrophin and utrophin: topology and mapping of a beta-dystroglycan interaction site. *Biochem J* 401: 667-77

Hu M, Sun YJ, Zhou QG, Chen L, Hu Y, et al. 2008. Negative regulation of neurogenesis and spatial memory by NR2B-containing NMDA receptors. *J Neurochem* 106: 1900-13

Huerta PT, Kowal C, DeGiorgio LA, Volpe BT, Diamond B. 2006. Immunity and behavior: antibodies alter emotion. *Proc Natl Acad Sci U S A* 103: 678-83

Huibregtse JM, Scheffner M, Beaudenon S, Howley PM. 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc Natl Acad Sci U S A* 92: 2563-67

Husi H, Ward MA, Choudhary JS, Blackstock WP, Grant SGN. 2000. Proteomic analysis of NMDA receptor-adhesion protein signalling complexes. *Nat Neurosci* 3: 661-69

Hwang LH, Murray AW. 1997. A novel yeast screen for mitotic arrest mutants identifies DOC1, a new gene involved in cyclin proteolysis. *Mol Biol Cell* 8: 1877-87

Ikura M. 1996. Calcium binding and conformational response in EF-hand proteins. *Trends Biochem Sci* 21: 14-17

Iwamura E, Yamada K, Ichitani Y. 2016. Involvement of hippocampal NMDA receptors in retrieval of spontaneous object recognition memory in rats. *Behav Brain Res* 307: 92-9

Jing M, Bohl J, Brimer N, Kinter M, Vande Pol SB. 2007. Degradation of tyrosine phosphatase PTPN3 (PTPH1) by association with oncogenic human papillomavirus E6 proteins. *J Virol* 81: 2231-9

Jurd R, Thornton C, Wang J, Luong K, Phamluong K, et al. 2008. Mind bomb-2 is an E3 ligase that ubiquitinates the N-methyl-D-aspartate receptor NR2B subunit in a phosphorylation-dependent manner. *J Biol Chem* 283: 301-10

Kalia LV, Gingrich JR, Salter MW. 2004. Src in synaptic transmission and plasticity. *Oncogene* 23: 8007-16

Karpova A, Mikhaylova M, Thomas U, Knopfel T, Behnisch T. 2006. Involvement of protein synthesis and degradation in long-term potentiation of Schaffer collateral CA1 synapses. *J Neurosci* 26: 4949-55

Kato A, Rouach N, Nicoll RA, Bredt DS. 2005. Activity-dependent NMDA receptor degradation mediated by retrotranslocation and ubiquitination. *Proc Natl Acad Sci U S A* 102: 5600-5

Katzav A, Ben-Ziv T, Chapman J, Blank M, Reichlin M, Shoenfeld Y. 2008. Anti-P ribosomal antibodies induce defect in smell capability in a model of CNS -SLE (depression). *J Autoimmun* 31: 393-8

Katzav A, Solodeev I, Brodsky O, Chapman J, Pick CG, et al. 2007. Induction of autoimmune depression in mice by anti-ribosomal P antibodies via the limbic system. *Arthritis Rheum* 56: 938-48

Kaustov L, Lukin J, Lemak A, Duan S, Ho M, et al. 2007. The conserved CPH domains of Cul7 and PARC are protein-protein interaction modules that bind the tetramerization domain of p53. *J Biol Chem* 282: 11300-7

Kee N, Sivalingam S, Boonstra R, Wojtowicz JM. 2002. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. *J Neurosci Methods* 115: 97-105

Kempermann G, Gast D, Kronenberg G, Yamaguchi M, Gage FH. 2003. Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. *Development* 130: 391-99

Kennedy MB. 2000. Signal-Processing Machines at the Postsynaptic Density. *Science* 290: 750-54

Kheirbek MA, Tannenholz L, Hen R. 2012. NR2B-dependent plasticity of adult-born granule cells is necessary for context discrimination. *J Neurosci* 32: 8696-702

Kina S, Tezuka T, Kusakawa S, Kishimoto Y, Kakizawa S, et al. 2007. Involvement of protein-tyrosine phosphatase PTPMEG in motor learning and cerebellar long-term depression. *Eur J Neurosci* 26: 2269-78

Ko HS, Uehara T, Tsuruma K, Nomura Y. 2004. Ubiquilin interacts with ubiquitylated proteins and proteasome through its ubiquitin-associated and ubiquitin-like domains. *FEBS Lett* 566: 110-4

Kohda K, Kakegawa W, Matsuda S, Yamamoto T, Hirano H, Yuzaki M. 2013. The delta2 glutamate receptor gates long-term depression by coordinating interactions between two AMPA receptor phosphorylation sites. *Proc Natl Acad Sci U S A* 110: E948-57

Kohr G, Seeburg PH. 1996. Subtype-specific regulation of recombinant NMDA receptor-channels by protein tyrosine kinases of the src family. *J Physiol* 492 (Pt 2): 445-52

Korb E, Finkbeiner S. 2011. Arc in synaptic plasticity: from gene to behavior. *Trends Neurosci* 34: 591-8

Koren E, Wolfson Reichlin M, Koscec M, Fugate RD, Reichlin M. 1992. Autoantibodies to the Ribosomal P Proteins React with a Plasma Membrane-related Target on Human Cells. *J Clin Invest* 89: 1236-41

Koscec M, Koren E, Wolfson Reichlin M, Fugate RD, Trieu E, et al. 1997. Autoantibodies to Ribosomal P Proteins Penetrate into Live Hepatocytes and Cause Cellular Dysfunction in Culture. *J Immunol* 159: 2033-41

Kowal C, DeGiorgio LA, Nakaoka T, Hetherington H, Huerta PT, et al. 2004. Cognition and immunity; antibody impairs memory. *Immunity* 21: 179-88

Kurup P, Zhang Y, Xu J, Venkitaramani DV, Haroutunian V, et al. 2010. A -Mediated NMDA Receptor Endocytosis in Alzheimer's Disease Involves Ubiquitination of the Tyrosine Phosphatase STEP61. *Journal of Neuroscience* 30: 5948-57

Lapteva L, Nowak M, Yarboro CH, Takada K, Roebuck-Spencer T, et al. 2006. Anti-N-methyl-D-aspartate receptor antibodies, cognitive dysfunction, and depression in systemic lupus erythematosus. *Arthritis Rheum* 54: 2505-14

Laube B, Kuhse J, Betz H. 1998. Evidence for a tetrameric structure of recombinant NMDA receptors. *J Neurosci* 18: 2954-61

Lavezzari G, McCallum J, Lee R, Roche KW. 2003. Differential binding of the AP-2 adaptor complex and PSD-95 to the C-terminus of the NMDA receptor subunit NR2B regulates surface expression. *Neuropharmacology* 45: 729-37

Lee HK. 2006. Synaptic plasticity and phosphorylation. *Pharmacol Ther* 112: 810-32

Legge GB, Martinez-Yamout MA, Hambly DM, Trinh T, Lee BM, et al. 2004. ZZ domain of CBP: an unusual zinc finger fold in a protein interaction module. *J Mol Biol* 343: 1081-93

Li R, Huang FS, Abbas AK, Wigstrom H. 2007. Role of NMDA receptor subtypes in different forms of NMDA-dependent synaptic plasticity. *BMC Neurosci* 8: 55

Li W, Bengtson MH, Ulbrich A, Matsuda A, Reddy VA, et al. 2008. Genome-Wide and Functional Annotation of Human E3 Ubiquitin Ligases Identifies MULAN, a Mitochondrial E3 that Regulates the Organelle's Dynamics and Signaling. *PloS ONE* 3: e1487

Lin AW, Man HY. 2013. Ubiquitination of neurotransmitter receptors and postsynaptic scaffolding proteins. *Neural Plast* 2013: 432057

Lopez-Salon M, Alonso M, Vianna MRM, Viola H, Mello e Souza T, et al. 2001. The ubiquitin-proteasome cascade is required for mammalian long-term memory formation. *Eur J Neurosci* 14: 1820-26

Lussier MP, Sanz-Clemente A, Roche KW. 2015. Dynamic Regulation of N-Methyl-d-aspartate (NMDA) and alpha-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors by Posttranslational Modifications. *J Biol Chem* 290: 28596-603

Mabb AM, Ehlers MD. 2010. Ubiquitination in postsynaptic function and plasticity. *Annu Rev Cell Dev Biol* 26: 179-210

Malenka RC, Bear MF. 2004. LTP and LTD: an embarrassment of riches. *Neuron* 44: 5-21

Mao LM, Guo ML, Jin DZ, Fibuch EE, Choe ES, Wang JQ. 2011. Post-translational modification biology of glutamate receptors and drug addiction. *Front Neuroanat* 5: 19

Mao LM, Wang W, Chu XP, Zhang GC, Liu XY, et al. 2009. Stability of surface NMDA receptors controls synaptic and behavioral adaptations to amphetamine. *Nat Neurosci* 12: 602-10

Marin I, Lucas JI, Gradilla A-C, Ferrus A. 2004. Parkin and relatives: the RBR family of ubiquitin ligases. *Physiol Genomics* 17: 253–63

Massa F, Koehl M, Wiesner T, Grosjean N, Revest J-M, et al. 2011. Correction for Immler et al., Resolving variation in the reproductive tradeoff between sperm size and number. *Proceedings of the National Academy of Sciences* 108: 8065-65

Massardo L, Bravo-Zehnder M, Calderon J, Flores P, Padilla O, et al. 2015. Anti-N-methyl-D-aspartate receptor and anti-ribosomal-P autoantibodies contribute to cognitive dysfunction in systemic lupus erythematosus. *Lupus* 24: 558-68

Matsuda N, Kitami T, Suzuki T, Mizuno Y, Hattori N, Tanaka K. 2006. Diverse effects of pathogenic mutations of Parkin that catalyze multiple monoubiquitylation in vitro. *J Biol Chem* 281: 3204-9

Mattison HA, Hayashi T, Barria A. 2012. Palmitoylation at two cysteine clusters on the C-terminus of GluN2A and GluN2B differentially control synaptic targeting of NMDA receptors. *PLoS One* 7: e49089

Matus S, Burgos PV, Bravo-Zehnder M, Kraft R, Porras OH, et al. 2007. Antiribosomal-P autoantibodies from psychiatric lupus target a novel neuronal surface protein causing calcium influx and apoptosis. *J Exp Med* 204: 3221-34

McNaughton BL, Barnes CA, Rao G, Baldwin J, Rasmussen M. 1986. Long-term enhancement of hippocampal synaptic transmission and the acquisition of spatial information. *J Neurosci* 6: 563-71

Messaoudi E, Kanhema T, Soule J, Tiron A, Dagyte G, et al. 2007. Sustained Arc/Arg3.1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo. *J Neurosci* 27: 10445-55

Metzger MB, Pruneda JN, Klevit RE, Weissman AM. 2014. RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. *Biochim Biophys Acta* 1843: 47-60

Mok CC, Lau CS. 2003. Pathogenesis of systemic lupus erythematosus. *J Clin Pathol* 56: 481-90

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12: 529-40

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, et al. 1992. Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science* 256: 1217-21

Moon IS, Apperson ML, Kennedy MB. 1994. The major tyrosine-phosphorylated protein in the postsynaptic density fraction is N-methyl-D-aspartate receptor subunit 2B. *Proc Natl Acad Sci U S A* 91: 3954-58

Morris RG. 1989. Synaptic plasticity and learning: selective impairment of learning in rats and blockade of long-term potentiation in vivo by the N-methyl-d-aspartate receptor antagonist, AP5. *Journal of Neuroscience* 9: 3040-57

Mu Y, Zhao C, Toni N, Yao J, Gage FH. 2015. Distinct roles of NMDA receptors at different stages of granule cell development in the adult brain. *Elife* 4: e07871

Nacher J, McEwen BS. 2006. The role of N-methyl-D-asparate receptors in neurogenesis. *Hippocampus* 16: 267-70

Nakanishi S. 1992. Molecular diversity of glutamate receptors and implications for brain function. *Science* 258: 597-603

Nakazawa T, Komai S, Tezuka T, Hisatsune C, Umemori H, et al. 2001. Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR epsilon 2 (NR2B) subunit of the N-methyl-D-aspartate receptor. *J Biol Chem* 276: 693-9

Nelson RF, Glenn KA, Miller VM, Wen H, Paulson HL. 2006. A novel route for F-box protein-mediated ubiquitination links CHIP to glycoprotein quality control. *J Biol Chem* 281: 20242-51

Nishito Y, Hasegawa M, Inohara N, Nunez G. 2006. MEX is a testis-specific E3 ubiquitin ligase that promotes death receptor-induced apoptosis. *Biochem J* 396: 411-7

Nourry C, Maksumova L, Pang M, Liu X, Wang T. 2004. Direct interaction between Smad3, APC10, CDH1 and HEF1 in proteasomal degradation of HEF1. *BMC Cell Biol* 5: 20

Paoletti P, Bellone C, Zhou Q. 2013. NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nature Reviews Neuroscience* 14: 383-400

Paoletti P, Neyton J. 2007. NMDA receptor subunits: function and pharmacology. *Curr Opin Pharmacol* 7: 39-47

Passmore LA, McCormack EA, Au SW, Paul A, Willison KR, et al. 2003. Doc1 mediates the activity of the anaphase- promoting complex by contributing to substrate recognition. *EMBO J* 22: 786-96

Patrick GN. 2006. Synapse formation and plasticity: recent insights from the perspective of the ubiquitin proteasome system. *Curr Opin Neurobiol* 16: 90-4

Pelkey KA, Askalan R, Paul S, Kalia LV, Nguyen TH, et al. 2002. Tyrosine phosphatase STEP is a tonic brake on induction of long-term potentiation. *Neuron* 34: 127-38

Perez-Otano I, Ehlers MD. 2004. Learning from NMDA receptor trafficking: clues to the development and maturation of glutamatergic synapses. *Neurosignals* 13: 175-89

Petralia RS, Sans N, Wang YX, Wenthold RJ. 2005. Ontogeny of postsynaptic density proteins at glutamatergic synapses. *Mol Cell Neurosci* 29: 436-52

Pickart C. 2001. Mechanisms underlying ubiquitination *Annu Rev Biochem* 70: 503–33

Ponting CP, Blake DJ, Davies KE, Kendrick-Jones J, Winder SJ. 1996. ZZ and TAZ: new putative zinc fingers in dystrophin and other proteins. *Trends Biochem Sci* 21: 11-13

Poueymirou WT, Auerbach W, Frendewey D, Hickey JF, Escaravage JM, et al. 2007. F0 generation mice fully derived from gene-targeted embryonic stem cells allowing immediate phenotypic analyses. *Nat Biotechnol* 25: 91-9

Prybylowski K, Chang K, Sans N, Kan L, Vicini S, Wenthold RJ. 2005. The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. *Neuron* 47: 845-57

Qiu S, Li XY, Zhuo M. 2011. Post-translational modification of NMDA receptor GluN2B subunit and its roles in chronic pain and memory. *Semin Cell Dev Biol* 22: 521-9

Quinn JJ, Loya F, Ma QD, Fanselow MS. 2005. Dorsal hippocampus NMDA receptors differentially mediate trace and contextual fear conditioning. *Hippocampus* 15: 665-74

Ramirez-Amaya V, Vazdarjanova A, Mikhael D, Rosi S, Worley PF, Barnes CA. 2005. Spatial exploration-induced Arc mRNA and protein expression: evidence for selective, network-specific reactivation. *J Neurosci* 25: 1761-8

Rao VR, Finkbeiner S. 2007. NMDA and AMPA receptors: old channels, new tricks. *Trends Neurosci* 30: 284-91

Reichlin M. 1998. Cellular Dysfunction Induced by Penetration of Autoantibodies into Living Cells: Cellular Damage and Dysfunction Mediated by Antibodies to dsDNA and Ribosomal P Proteins. *J Autoimmun* 11: 557-61

Roche KW, Standley S, McCallum J, Dune Ly C, Ehlers MD, Wenthold RJ. 2001. Molecular determinants of NMDA receptor internalization. *Nat Neurosci* 4: 794-802

Rotin D, Kumar S. 2009. Physiological functions of the HECT family of ubiquitin ligases. *Nat Rev Mol Cell Biol* 10: 398-409

Salter MW, Kalia LV. 2004. Src kinases: a hub for NMDA receptor regulation. *Nat Rev Neurosci* 5: 317-28

Sanz-Clemente A, Nicoll RA, Roche KW. 2013. Diversity in NMDA receptor composition: many regulators, many consequences. *Neuroscientist* 19: 62-75

Schwartz N, Stock AD, Putterman C. 2019. Neuropsychiatric lupus: new mechanistic insights and future treatment directions. *Nat Rev Rheumatol* 15: 137-52

Sciascia S, Bertolaccini ML, Roccatello D, Khamashta MA, Sanna G. 2014. Autoantibodies involved in neuropsychiatric manifestations associated with systemic lupus erythematosus: a systematic review. *J Neurol* 261: 1706-14

Segovia-Miranda F, Serrano F, Dyrda A, Ampuero E, Retamal C, et al. 2015. Pathogenicity of lupus anti-ribosomal P antibodies: role of cross-reacting neuronal surface P antigen in glutamatergic transmission and plasticity in a mouse model. *Arthritis Rheumatol* 67: 1598-610

Serrano FG, Tapia-Rojas C, Carvajal FJ, Hancke J, Cerpa W, Inestrosa NC. 2014. Andrographolide reduces cognitive impairment in young and mature AbetaPPswe/PS-1 mice. *Mol Neurodegener* 9: 61

Sherer Y, Gorstein A, Fritzler MJ, Shoenfeld Y. 2004. Autoantibody explosion in systemic lupus erythematosus: More than 100 different antibodies found in SLE patients. *Seminars in Arthritis and Rheumatism* 34: 501-37

Singer BH, Gamelli AE, Fuller CL, Temme SJ, Parent JM, Murphy GG. 2011. Compensatory network changes in the dentate gyrus restore long-term potentiation following ablation of neurogenesis in young-adult mice. *Proc Natl Acad Sci U S A* 108: 5437-42

Smit JJ, Monteferrario D, Noordermeer SM, van Dijk WJ, van der Reijden BA, Sixma TK. 2012. The E3 ligase HOIP specifies linear ubiquitin chain assembly through its RING-IBR-RING domain and the unique LDD extension. *EMBO J* 31: 3833-44

Smit JJ, Sixma TK. 2014. RBR E3-ligases at work. *EMBO Rep* 15: 142-54

Snyder JS, Kee N, Wojtowicz JM. 2001. Effects of Adult Neurogenesis on Synaptic Plasticity in the Rat Dentate Gyrus. *J Neurophysol* 85: 2423-31

Spratt DE, Walden H, Shaw GS. 2014. RBR E3 ubiquitin ligases: new structures, new insights, new questions. *Biochem J* 458: 421-37

Stackman RW, Jr., Cohen SJ, Lora JC, Rios LM. 2016. Temporary inactivation reveals that the CA1 region of the mouse dorsal hippocampus plays an equivalent role in the retrieval of long-term object memory and spatial memory. *Neurobiol Learn Mem* 133: 118-28

Stafford HA, Chen AE, Anderson CJ, Paul AGA, Wyatt EL, et al. 1997. Anti-ribosomal and "P-peptide" -specific antibodies bind to T lymphocytes. *Clin Exp Immunol* 109: 12-19

Stegmuller J, Konishi Y, Huynh MA, Yuan Z, Dibacco S, Bonni A. 2006. Cell-intrinsic regulation of axonal morphogenesis by the Cdh1-APC target SnoN. *Neuron* 50: 389-400

Stelzl U, Worm U, Lalowski M, Haenig C, Brembeck FH, et al. 2005. A human protein-protein interaction network: a resource for annotating the proteome. *Cell* 122: 957-68

Sun KH, Tang SJ, Lin ML, Wang YS, Sun GH, Liu WT. 2001. Association between lupus psychosis and anti-ribosomal P protein antibodies. *Rheumatology* 40: 750-56

Szczaluba K, Chmielewska JJ, Sokolowska O, Rydzanicz M, Szymanska K, et al. 2018. Neurodevelopmental phenotype caused by a de novo PTPN4 single nucleotide variant disrupting protein localization in neuronal dendritic spines. *Clin Genet* 94: 581-85

Tashiro A, Sandler VM, Toni N, Zhao C, Gage FH. 2006. NMDA-receptor-mediated, cell-specific integration of new neurons in adult dentate gyrus. *Nature* 442: 929-33

Tatham MH, Rodriguez MS, Xirodimas DP, Hay RT. 2009. Detection of protein SUMOylation in vivo. *Nat Protoc* 4: 1363-71

Taylor CJ, He R, Bartlett PF. 2014. The role of the N-methyl-D-aspartate receptor in the proliferation of adult hippocampal neural stem and precursor cells. *Sci China Life Sci* 57: 403-11

Tezuka T, Umemori H, Akiyama T, Nakanishi S, Yamamoto T. 1999. PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A. *Proc Natl Acad Sci U S A* 96: 435-40

Tsien JZ, Huerta PT, Tonegawa S. 1996. The Essential Role of Hippocampal CA1 NMDA Receptor–Dependent Synaptic Plasticity in Spatial Memory. *Cell* 87: 1327-38

Tsokos GC. 2011. Systemic lupus erythematosus. *N Engl J Med* 365: 2110-21

Valenzuela DM, Murphy AJ, Frendewey D, Gale NW, Economides AN, et al. 2003. High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. *Nat Biotechnol* 21: 652-9

Venkitaramani DV, Moura PJ, Picciotto MR, Lombroso PJ. 2011. Striatal-enriched protein tyrosine phosphatase (STEP) knockout mice have enhanced hippocampal memory. *Eur J Neurosci* 33: 2288-98

Vissel B, Krupp JJ, Heinemann SF, Westbrook GL. 2001. A use-dependent tyrosine dephosphorylation of NMDA receptors is independent of ion flux. *Nat Neurosci* 4: 587-96

Wang H, Hu Y, Tsien JZ. 2006. Molecular and systems mechanisms of memory consolidation and storage. *Prog Neurobiol* 79: 123-35

Wenthold RJ, Prybylowski K, Standley S, Sans N, Petralia RS. 2003. Trafficking of NMDA receptors. *Annu Rev Pharmacol Toxicol* 43: 335-58

Wenzel DM, Lissounov A, Brzovic PS, Klevit RE. 2011. UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. *Nature* 474: 105-8

Williamson SL, Ellaway CJ, Peters GB, Pelka GJ, Tam PP, Christodoulou J. 2015. Deletion of protein tyrosine phosphatase, non-receptor type 4 (PTPN4) in twins with a Rett syndrome-like phenotype. *Eur J Hum Genet* 23: 1171-5

Wojtowicz JM, Kee N. 2006. BrdU assay for neurogenesis in rodents. *Nat Protoc* 1: 1399-405

Won S, Incontro S, Nicoll RA, Roche KW. 2016. PSD-95 stabilizes NMDA receptors by inducing the degradation of STEP61. *Proc Natl Acad Sci U S A* 113: E4736-44

Wu W, Sato K, Koike A, Nishikawa H, Koizumi H, et al. 2010. HERC2 is an E3 ligase that targets BRCA1 for degradation. *Cancer Res* 70: 6384-92

Wyneken U, Smalla KH, Marengo JJ, Soto D, de la Cerda A, et al. 2001. Kainate-induced seizures alter protein composition and N-methyl-D-aspartate receptor function of rat forebrain postsynaptic densities. *Neuroscience* 102: 65-74

Xu F, Plummer MR, Len GW, Nakazawa T, Yamamoto T, et al. 2006. Brain-derived neurotrophic factor rapidly increases NMDA receptor channel activity through Fyn-mediated phosphorylation. *Brain Res* 1121: 22-34

Xu J, Kurup P, Nairn AC, Lombroso PJ. 2017. Synaptic NMDA Receptor Activation Induces Ubiquitination and Degradation of STEP61. *Mol Neurobiol* 55: 3096-111

Yamada K, Shimizu M, Kawabe K, Ichitani Y. 2015. Hippocampal AP5 treatment impairs both spatial working and reference memory in radial maze performance in rats. *Eur J Pharmacol* 758: 137-41

Yamada T, Urano-Tashiro Y, Hashi Y, Sakumoto M, Akiyama H, Tashiro F. 2013a. The U-box-type ubiquitin ligase PRP19beta regulates astrocyte differentiation via ubiquitination of PTP1B. *Brain Res* 1524: 12-25

Yamada T, Yang Y, Bonni A. 2013b. Spatial organization of ubiquitin ligase pathways orchestrates neuronal connectivity. *Trends Neurosci* 36: 218-26

Yaniv G, Twig G, Shor DB, Furer A, Sherer Y, et al. 2015. A volcanic explosion of autoantibodies in systemic lupus erythematosus: a diversity of 180 different antibodies found in SLE patients. *Autoimmun Rev* 14: 75-9

Yi JJ, Ehlers MD. 2007. Emerging roles for ubiquitin and protein degradation in neuronal function. *Pharmacol Rev* 59: 14-39

Yin X, Takei Y, Kido MA, Hirokawa N. 2011. Molecular motor KIF17 is fundamental for memory and learning via differential support of synaptic NR2A/2B levels. *Neuron* 70: 310-25

Zhang L, Kang L, Bond W, Zhang N. 2009. Interaction between syntaxin 8 and HECTd3, a HECT domain ligase. *Cell Mol Neurobiol* 29: 115-21

Zhao C, Deng W, Gage FH. 2008. Mechanisms and functional implications of adult neurogenesis. *Cell* 132: 645-60

Zheng N, Shabek N. 2017. Ubiquitin Ligases: Structure, Function, and Regulation. *Annu Rev Biochem* 86: 129-57