Trans-Synaptic Signaling by Activity-Dependent Cleavage of Neuroligin-1

Rui Peixoto

Oeiras, Abril de 2011

Dissertation presented to obtain the Doutoramento (Ph.D.) degree in Neurobiology at the **Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa**.

Com o apoio financeiro da FCT e do FSE no âmbito do Quadro Comunitário de apoio, BD nº SFRH/BD/15217/2004

Aos meus pais, pelo exemplo.

Table of contents

Chapter 2

Signaling pathways Regulating Neuroligin-1 cleavage

Chapter 3

Acute Cleavage of Neuroligin-1 Destabilizes Presynaptic Neurexin-1β and Reduces Neurotransmitter Release

Acknowledgements

This project was undoubtedly the biggest endeavor I ever embarked on and it is a pleasure to thank all those who helped me along the way. First and foremost I want to thank my parents and my sister for their unconditional love and support. It was their examples of courage, dedication and perseverance that gave me the strength to carry through all the frustration and frequent disappointment that lie behind the work presented here. As important to me was my wife Susana. Thank you for your everlasting patience and for being the best friend, partner and colleague one can wish for.

This thesis would have never been possible without the guidance and continued support from my advisor Michael Ehlers whose professionalism and rigor were truly inspiring and will remain a beacon for all my future pursuits. A big thank you goes as well to all the members of the lab, past and present, for their company, friendship, and all the precious tips and tricks that helped me get my experiments working. I am particularly grateful to Ian Davison for going out of his way on many occasions and for often being the voice of reason in more troubled times, and to Cyril Hanus for always speaking his mind and sparking many insightful discussions.

Some of the experiments presented here were done in collaboration with Portia McCoy and Ben Philpot at the University of North Carolina. Ben and Portia are amazing colleagues and it was great to count with their enthusiasm and support. I also want

to thank my committee members at Duke, Anne West, Ryohei Yasuda and Richard Weinberg for all the helpful comments and suggestions.

Regarding my previous advisors, I want to give a special thanks to Sukalyan Chatterjee for the opportunities and trust he gave me and for broadening my horizons at an important time in my life. I am also in debt to my professor Carlos Duarte for always being the most generous colleague and showing me early on that there is more to science than memorizing endless amounts of information.

I would also like to thank everyone involved in the Gulbenkian PhD program and particularly to Miguel Seabra (and Sukalyan) for letting me take part in it. The PGDB was a truly life changing experience and I will be forever grateful for this remarkable opportunity.

At last, I want to thank all my family and friends for their friendship and support. Being abroad is definitely hard sometimes, but it does make one appreciate that some things are even wider the ocean.

Summary

Throughout the brain, patterns of activity in postsynaptic neurons influence the properties of synaptic inputs. Such feedback regulation is central to neural network stability that underlies proper information processing and feature representation in the central nervous system. At the cellular level, tight coupling of presynaptic and postsynaptic function is fundamental to neural computation and synaptic plasticity. The cohort of protein complexes at the pre and postsynaptic membrane allows for tight synapse-specific segregation and integration of diverse molecular and electrical signals. Bridging these scaffolding complexes are trans-synaptic adhesion molecules that organize and stabilize synaptic specializations. Indeed, adhesive contact between pre and post synaptic neurons initiates synapse formation during brain development and provides a natural means of trans-synaptic signaling. Numerous adhesion molecules and their role during synapse development have been described in detail, however, once established, the mechanisms of adhesive disassembly and its function in acute regulation of synaptic transmission remain uncertain.

Among the different classes of synaptic adhesion molecules, Neuroligins (NLGs) have emerged as key regulators of synaptic function. Recent evidence has also implicated Neuroligins as critical proteins involved in the etiology of austistic spectrum disorders (ASDs). Through association with presynaptic Neurexins (NRXs), Neuroligins are able to cluster and organize both pre and postsynaptic molecular scaffolding

complexes involved in synaptic transmission. During development, total NLG levels are correlated with the overall number of synapses generated reflecting the strong synaptogenic potential of this protein family. Furthermore, NLGs regulate synaptic transmission by modulating postsynaptic function and exocytosis of synaptic vesicles at the nerve terminals and have been implicated in synaptic plasticity. Moreover, NLG function is itself dependent of NMDA glutamate receptors (NMDAR) and Ca^{2+}/calmodulin-dependent protein kinase (CaMK) activity indicating that these molecules are themselves regulated by synaptic activity. However, despite the numerous studies addressing NLG function, the basic molecular and cellular mechanisms regulating NLG levels at synapses remain mostly unknown.

Here, I report that neuronal depolarization induces acute loss of NLG1 from mature synapses due to overall protein degradation. This observation provides evidence that synaptic NLG1 is acutely regulated by activity. Using a combination of biochemical and microscopic analysis I show that under these conditions, NLG1 degradation does not occur via the canonical lysosomal pathway but is instead mediated by Matrix Metalloprotease (MMP)-dependent proteolytic cleavage. MMP cleavage of NLG1 is ubiquitous throughout the brain and is regulated over development. Moreover, this process is upregulated by sensory experience in visual cortex during early development, which implicates NLG cleavage in the mechanisms underlying the activity dependent synaptic refinement during critical periods of circuit maturation.

To characterize the molecular mechanisms regulating NLG1 cleavage I developed a novel biochemical method based on surface protein labeling with a biotin conjugate that allows the enrichment and isolation of cleaved protein fragments from the culture media. Using this technique I found that activity dependent NLG1 cleavage requires NMDA receptor activation and Ca^{2+}/calmodulin-dependent protein kinase signaling. Moreover, using pharmacological manipulation of protease activity I determined that NLG1 cleavage occurs in its juxtamembrane extracellular region and is mediated by Matrix Metalloprotease-9 (MMP9). Interestingly, MMP9-dependent cleavage of NLG1 is also upregulated *in vivo*, in the hippocampus, during pilocarpine induced epileptic seizures, unveiling a potential link between NLG function and epileptogenesis.

Due to the redundant and promiscuous nature of MMP activity regulation it has been difficult to address how acute cleavage of adhesion molecules affects synaptic function. To overcome this limitation I developed a new *in vitro* system based on the application of an exogenous protease that allows the specific and temporally controlled cleavage of transmembrane proteins. Combining this method with real-time microscopy imaging analysis, I found that acute NLG1 shedding at the plasma membrane causes rapid destabilization of its presynaptic partner Neurexin-1β (NRX1β). Interestingly, this effect is specific and is not caused by the structural collapse of pre synaptic terminals or due to loss of synaptic vesicles. In turn, electrophysiological measurements of synaptic properties

showed that NLG1 cleavage rapidly depresses synaptic transmission by abruptly reducing presynaptic release probability.

Together, these results indicate that postsynaptic activity influences presynaptic function through NLG1 cleavage. Furthermore, this work describes a new post-translational mechanism of NLG1 regulation that contributes to synapse plasticity, and may provide a general paradigm for trans-synaptic signaling in diverse neural circuits. Moreover, this work implicates NLG function in epilepsy and critical period plasticity. Given the high association of NLG and NRX mutations with ASDs, this work may contribute for the understanding of the pathophysiology underlying ASDs.

Sumário

Os padrões de actividade neuronal influenciam as propriedades funcionais dos aferentes pré-sinápticos ao nível do sistema nervoso central. Este mecanismo regulador de *feedback* é essencial para a estabilização de redes neuronais e para o processamento de informação no cérebro. Ao nível celular, a correcta justaposição das especializações pré- e pós-sinápticas é essencial para a integração e transmissão de informação entre neurónios. Múltiplas famílias de proteínas de adesão trans-sinápticas estão envolvidas na organização e estabilização de sinapses. De facto, a formação de sinapses entre neurónios durante o desenvolvimento é iniciada pela interacção entre moléculas de adesão, o que desencadeia cascatas de sinalização que levam à eventual agregação e estabilização de proteínas sinápticas. Têm sido descrito em detalhe o papel de diversas proteínas de adesão existentes no sistema nervoso, assim como a sua função durante o desenvolvimento. No entanto, existe pouca informação acerca dos mecanismos responsáveis pela regulação destas moléculas no contexto de plasticidade sináptica.

As *Neuroliguinas* (NLGs) são moléculas de adesão com uma função reguladora importante ao nível sináptico. Diversos estudos recentes demonstraram também a associação entre diversas nas NLGs e doenças de espectro austístico, o que evidenciou a importância destas proteínas para o normal funcionamento do sistema nervoso central. Através da interacção com *Neurexinas* (NRXs), que são proteínas de

adesão pré-sinápticas, as NLGs induzem a justaposição e a organização funcional de múltiplas proteínas nas sinapses. Durante o desenvolvimento, os níveis globais de NLG estão correlacionados com o número total de sinapses formadas, o que revela o elevado potencial sinaptogénico destas moléculas. Além disso, estudos recentes indicaram que as NLGs estão envolvidas na regulação de propriedades pós-sinápticas, na modulação de exocitose de vesículas sinápticas e em processos de plasticidade sináptica. Outros estudos demonstraram ainda que a função das NLGs depende da activação de receptores do glutamato do tipo NMDA (NMDAR) e da proteína cinase dependente de cálcio e calmodulina (CaMK), indicando que estas moléculas são elas próprias reguladas pela actividade sináptica. No entanto, apesar do elevado número de estudos centrados na função de NLGs, os mecanismos celulares e moleculares responsáveis pela regulação dos níveis de NLG nas sinapses permanecem desconhecidos.

Neste trabalho, demonstrou-se que a actividade neuronal induz a perda da Neuroligin-1 (NLG1) de sinapses maduras. Esta observação evidencia que os níveis de NLG1 em sinapses podem ser regulados pela actividade neuronal de uma forma aguda. Utilizando métodos bioquímicos e microscópicos mostrou-se que a perda de NLG1 dá-se por clivagem proteolítica mediada por metaloproteinases (MMPs) e não através de degradação em lisosomas. A clivagem de NLG1 ocorre em diversas regiões do cérebro e é regulada durante o desenvolvimento neuronal. Este processo é ainda potenciado no córtex visual em resposta a manipulações da actividade sensorial durante o período crítico de desenvolvimento cortical.

De forma a caracterizar os mecanismos moleculares responsáveis pela regulação da clivagem de NLG1 foi desenvolvida uma nova técnica baseada na marcação de proteínas de superfície por biotina que permite o isolamento de fragmentos proteicos clivados presentes no meio de cultura. Usando este método identificou-se que a clivagem de NLG1 induzida por actividade neuronal depende da activação de receptores NMDA e da proteína cinase CaMK. Além disso, através de manipulações farmacológicas demonstrou-se que a clivagem de NLG1 é mediada pela actividade proteolítica da metaloproteinase 9 (MMP9) e ocorre na região proximal extracelular da proteína. A clivagem de NLG1 através da acção de MMP9 acontece também *in vivo* no hipocampo e é potenciada num modelo animal de epilepsia induzida farmacologicamente, o que revela uma potencial associação entre este mecanismo e processos epileptogénicos.

A elevada redundância e reduzida especificidade da acção das MMPs tem dificultado o estudo dos efeitos específicos mediados pela clivagem de proteínas de adesão sinápticas. De forma a ultrapassar esta limitação, desenvolveu-se uma nova metodologia *in vitro* baseada na aplicação de uma protease exógena que permite a clivagem específica e controlada de proteínas de membrana. Combinando esta técnica com métodos de microscopia em tempo real, demonstrou-se que a proteólise aguda de NLG1 induz a desestabilização rápida da sua parceira de interacção situada ao nível pré-sináptico, *Neurexina-1β* (NRX1β). Este efeito é específico e não resulta do colapso estrutural de terminais pré-sinápticos, ou da redução do número de vesículas pré-sinápticas. Além disso, através da análise de

propriedades electrofisiológicas, demonstrou-se que a clivagem de NLG1 origina uma depressão da transmissão sináptica por redução aguda da probabilidade de exocitose de vesículas ao nível pré-sináptico.

Este estudo identifica assim um novo mecanismo de regulação pós-traducional que regula NLG1, que revela o envolvimento destas moléculas em processos de plasticidade sináptica e evidencia o papel que a clivagem de proteínas de adesão pode ter na mediação de sinalização retrógrada em sinapses maduras. Além disso, este trabalho revela o envolvimento de NLGs em epilepsia e em mecanismos de plasticidade sináptica que ocorrem durante os períodos críticos de maturação de circuitos neuronais. Devido à elevada associação de mutações de NLGs e NRXs em doenças do espectro autístico, este estudo pode assim contribuir para a elucidação de mecanismos moleculares subjacentes à patofisiologia deste tipo de doenças.

Introduction

The vertebrate brain is arguably the most complex multicellular system in all Eukarya. In humans, the average brain contains approximately 100 billion neurons each participating in thousands of precise inter-cellular connections resulting in a network of staggering complexity (Kandel and Schwartz, 1985). Until the early 1900's the brain was conceived as a continuous reticular network of interlacing nerve fibers where electricity could flow freely to and from different regions of the body. This "reticular" theory found a notable opponent in Ramon y Cajal, that by using a novel staining method was able to demonstrate that the brain was instead formed by an ordered array of multiple individual cells (Figure 1)(Cowan and Stevens, 2001).

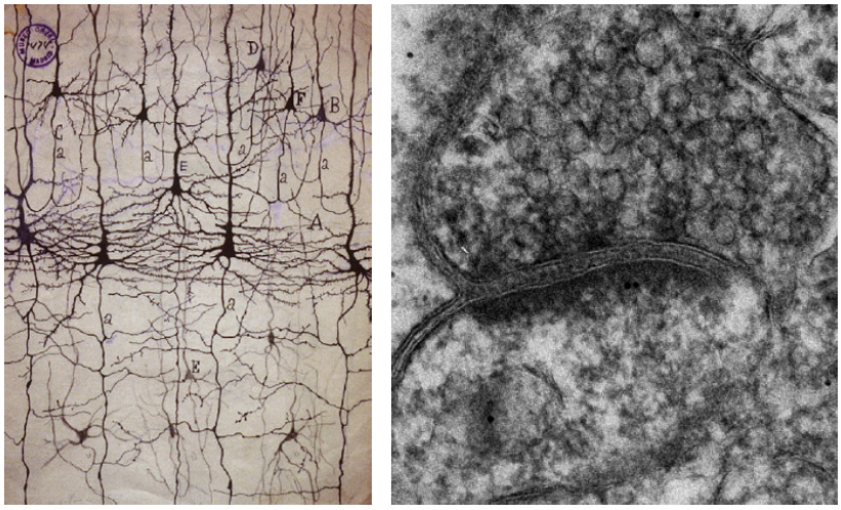

Figure 1. Neuronal synapses are specialized adhesion junctions (Left) Ramon y Cajal drawing based on Golgi staining of neurons in cortical sections of human brain **(Right)** High resolution EM picture of a dendritic spine

highlighting the electron dense material in the synaptic cleft (courtesy of Cam Robinson).

Crucial for the establishment of the new “neuron” theory was the identification of the points of contact between different neurons, sub-micron sized structures named synapses. From a structural perspective neuronal synapses are highly specialized adhesion junctions, whose function is to ensure the proper transfer of information between neurons (Figure 1). Morphologically, synapses resemble tight junctions with closely juxtaposed membranes coated with electron dense material (Stevenson and Keon, 1998). These correspond to the presynaptic active zone of neurotransmitter release where synaptic vesicles fuse and release their cargo and the postsynaptic region where neurotransmitter receptors and signaling molecules are enriched (Garner et al., 2000). In excitatory synapses of the central nervous system (CNS) this last region has a characteristic dense thickening called the postsynaptic density (PSD) (Sheng, 2001). Between the pre and postsynaptic membranes lies the synaptic cleft which performs both mechanical and signaling functions (Garner et al., 2000). In terms of signaling, the synaptic cleft is the region where neurotransmitters and other signaling molecules diffuse and effectively transduce information between the two cells. Mechanically, the cleft orients and maintains the relative apposition between the two synaptic plasma membranes. Interestingly, regardless of their shape or size the tight apposition between pre and postsynaptic elements is observed in all mature synapses of the brain (Lisman and Harris, 1993). This

observation long suggested the presence of strong trans-synaptic adhesive elements that would maintain the juxtaposition of both synaptic sides. Indeed, ultrastructural analysis of neuronal synapses revealed that the synaptic cleft is not empty, but is instead filled with electron dense material (Gray, 1959), which is now recognized to be composed mainly by highly glycosylated membrane proteins and synaptic cell adhesion molecules (CAMs).

Cell adhesion molecules are transmembrane proteins that span across the synaptic cleft and undergo homophilic and heterophilic interactions (Gerrow and El-Husseini, 2006). Importantly, the formation of a mature synapse, or synaptogenesis, is a multi-step process that gets initiated with the transient adhesive contact between two neurons. This initial contact is followed by the establishment of stable sites of cell-cell contact and subsequent recruitment of scaffolding proteins, which in turn contributes for the stabilization of neurotransmitter receptors, voltage-gated ion channels, and various second-messenger signaling molecules (Garner et al., 2002). Hence, CAMs are key structural players in synapses that provide anchoring points to intracellular cytoskeletal and scaffolding elements, and extracellular trans-synaptic adhesive interactions.

Early studies in synaptosomes clearly demonstrated that the adhesive apparatus of excitatory synapses is extremely strong. These structures are pinched-off nerve endings that form spontaneously upon homogenization of brain tissue and retain the postsynaptic density and part of the postsynaptic plasma membrane. Notably, synaptosomes are stable in solution for

days, even in complete absence of ATP (Gray and Whittaker, 1962). Furthermore, the association between pre and postsynaptic sites resists the presence of high salt concentrations and even urea (Cotman and Taylor, 1972). Together, this indicates that the trans-synaptic adhesive complex is extremely strong and thermodynamically stable. Yet, neuronal synapses distinguish themselves from other cellular adhesive specializations for their capacity to undergo extremely fast functional and structural changes in response to brief stimuli (Citri and Malenka, 2008; Trachtenberg et al., 2002). The activity-dependent modification of synaptic properties, or synaptic plasticity, underlies the capacity of neuronal networks to adapt to changes in the environment and effectively process and transmit information. However, this highly dynamic nature of synapse remodeling is in stark contrast with the structural stability of the synaptic adhesive complex observed in synaptosomes. This suggests that the remodeling or disassembly of synaptic structures requires the involvement of active processes. However, the cellular and molecular basis for adhesive disassembly and its potential function in regulating synaptic transmission is still unknown and many questions remain about how nanomolar high-avidity adhesive interactions are uncoupled across the synaptic cleft in contexts of acute synaptic plasticity or elimination.

There are numerous forms of synaptic plasticity that can induce both short-term (ranging from milliseconds to several minutes) (Zucker and Regehr, 2002) to long-term (ranging from hours to years) changes in synaptic properties (Citri and Malenka, 2008). The two most widely studied forms of long-term

plasticity, long-term potentiation (LTP) and long-term depression (LTD), are thought to represent the cellular correlate of learning and memory, and can have different expression mechanisms depending on the neuronal circuits in which they operate (Citri and Malenka, 2008; Malenka and Bear, 2004). Other forms of plasticity act on a much broader regulatory scale. For example, homeostatic synaptic plasticity serves as a negative feedback mechanism in response to global changes in neuronal network activity, resulting in a compensatory and uniform scaling of all synaptic strengths (Pozo and Goda, 2010; Turrigiano, 1999, 2008).

Many synaptic CAMs have important roles in the development and maturation of synapses. Such examples include Neurexins and Neuroligins (Sudhof, 2008), Ephs and ephrins, the immunoglobulin superfamily adhesion molecules, cadherins (Dalva et al., 2007) and integrins (McGeachie et al., 2011). Interestingly, these same classes of proteins are also involved in the regulation of synaptic transmission in mature stages. For instance, blockade of extracellular N-cadherin adhesion with antibodies or peptides impairs LTP at Schaeffer collateral–CA1 synapses without affecting basal synaptic properties (Tang et al., 1998). Moreover, pharmacological induction of LTP upregulates N-Cadherin expression and increases the number of cadherin-positive synaptic puncta (Bozdagi et al., 2000). N-Cadherin localization and dimerization are in turn regulated by NMDAR activation, suggesting that Cadherin based adhesion can be dynamically regulated by activity-induced mechanisms (Tanaka et al., 2000). Interestingly, N-cadherin can also regulate presynaptic function and short-term

plasticity as described in studies using embryonic stem cell-derived neurons from N-cadherin-null mice (Jungling et al., 2006). EphB receptors directly interact with NMDARs extracellularly (Dalva et al., 2000) and regulate receptor function and localization via intracellular enzymatic activity. EphBs also regulate the localization of AMPARs (Kayser et al., 2006) although the consequences of this regulation still require further inquiry. However, several studies have now implicated Eph receptors in several forms of hippocampal synaptic plasticity (Grunwald et al., 2001; Henderson et al., 2001). Multiple lines of evidence also implicate NCAM, a member of the immunoglobulin superfamily of adhesion molecules, in the regulation of synaptic function (Luthl et al., 1994; Venero et al., 2006). Initial studies using blocking antibodies or synthetic peptides to inhibit NCAM-mediated adhesion found normal basal synaptic transmission but reduced LTP in the hippocampus (Luthl et al., 1994; Ronn et al., 1995). In addition, NCAM null mice show hippocampus-dependent long term memory defects (Bukalo et al., 2004; Cremer et al., 1994) and impairment of LTP expression in CA1 area (Muller et al., 2000; Muller et al., 1996). Another class of synaptic adhesion molecules implicated in synaptic plasticity is the integrin protein family. Impairment of integrin function using blocking peptides in acute hippocampal slices compromised the late phase of LTP, which decayed back to baseline levels after 15–30 min of induction, while the baseline transmission was not affected (Staubli et al., 1990). These results provided the first evidence that the integrins have a role in the stabilization of LTP. This was further confirmed by subsequent studies based on genetic ablation of several integrin subunits (Chan et al., 2003). In addition, Neuroligins, postsynaptic adhesion molecules that

were initially thought to play an important role during synaptogenesis (Chih et al., 2005; Graf et al., 2004; Scheiffele et al., 2000), have recently been implicated as critical regulators of synaptic function (Chubykin et al., 2007; Futai et al., 2007; Varoqueaux et al., 2006) and shown to be important for LTP expression in the amygdala (Jung et al., 2010; Kim et al., 2008). Together, these studies clearly demonstrate that cell adhesion molecules play important roles in the regulation of mature synapses. Hence the elucidation of the molecular mechanisms capable of acutely regulating CAMs at synapses is an important step to understand how these structural elements are regulated in the context of rapid synaptic remodeling.

Interestingly, several synaptic CAMs have been shown to undergo regulated ectodomain shedding. For example, NCAM isoforms can undergo proteolytic cleavage via a disintegrin and metalloprotease (ADAM) family of proteases, resulting in soluble extracellular and intracellular domain fragments (Diestel et al., 2005; Hinkle et al., 2006; Hubschmann et al., 2005). Similarly, ephrins are cleaved by ADAM10 in response to Eph receptor binding (Janes et al., 2005), while EphB2 receptors themselves are cleaved by MMP2/MMP9 in response to ephrin B2 ligation (Lin et al., 2008). Moreover, ADAM10 also cleaves N-Cadherin via its metalloproteinase domain and is responsible for initial step of N-cadherin proteolytic processing (Reiss et al., 2005; Uemura et al., 2006). This mechanism also generates extracellular soluble N-terminal fragments (NTFs) and intracellular C-terminal fragments (CTFs). Together these results suggest that ectodomain shedding is an important regulatory mechanism capable of controlling cell

adhesion molecules expressed at the plasma membrane. Moreover, given its acute and irreversible effects, proteolytic cleavage is a plausible mechanism for CAM regulation at synapses during synaptic plasticity. A recent study has employed the application of tissue inhibitor of metalloproteinase-1 (TIMP-1), an ADAM10 inhibitor, and a cell-permeable peptide capable of interfering with ADAM10 synaptic localization and activity to study the effects of N-Cadherin shedding at synapses. ADAM10 inhibition using these methods decreased N-cadherin cleavage, induced a significant increase in size of dendritic spines and potentiated AMPAR currents (Malinverno et al., 2010). However, ADAM10 is capable of targeting several proteins (Janes et al., 2005; Reiss et al., 2005) and TIMP-1 blocks multiple proteases including for example, MMP9 (Ethell and Ethell, 2007). Hence these results, despite showing that ADAM10 activity causes changes in synaptic properties, are inconclusive when it comes to providing information about the specific effects of N-Cadherin shedding at synapses. In fact, this highly redundant nature of ADAM and MMP activity has undermined the study of adhesion molecule shedding and its consequences in synapse maturation and function. Although manipulations of adhesion molecule levels and binding properties can alter synaptic transmission and influence synaptic plasticity, there is virtually no information on the consequences of acute shedding of trans-synaptic adhesion in response to neuronal activation.

This thesis is focused on the regulation of Neuroligins at synapses and describes a previously unknown mechanism of trans-synaptic signaling whereby synaptic activity induces acute proteolytic cleavage of Neuroligin-1 (NLG1), which in turn causes

a direct reduction of synaptic transmission by decreasing presynaptic function. The first chapter describes the initial observation that cleavage of NLG1 occurs in response to neuronal depolarization and is the major form of NLG1 regulation under these conditions. Moreover, I also present data showing that NLG1 cleavage occurs over development and is upregulated in the visual cortex in response to sensory experience during early development. Chapter 2 is centered on the characterization of the signaling mechanisms regulating NLG1 cleavage using a newly developed biochemical method based on surface biotinylation. Using this technique I found that NLG1 cleavage is bi-directionally regulated by activity and is mediated by NMDAR and CaMK signaling. Moreover, I also identified the region where NLG1 is cleaved and that the protease involved in activity dependent NLG1 cleavage *in vitro* and *in vivo* is the Matrix Metalloprotease-9 (MMP9). To finalize, in Chapter 3, I describe the development of a new technique that allows the cleavage of any transmembrane protein in a specific and temporally controlled manner. Using this novel approach in combination with real time microscopy I show that acute cleavage of NLG1 causes rapid destabilization of its presynaptic partner Neurexin-1β (NRX1β), which in turn depresses synaptic transmission by abruptly reducing presynaptic release probability.

Chapter 1

Acute regulation of synaptic Neuroligin-1 by Matrix-Metalloprotease mediated ectodomain shedding

Introduction

In the mammalian brain, neuronal synapses are highly specialized adhesion junctions maintained by a complex network of adhesion molecules that span the synaptic cleft and juxtapose the presynaptic active zone of neurotransmitter release and the postsynaptic density (Dalva et al., 2007; Shapiro et al., 2007; Yamagata et al., 2003). Among these, Neuroligins (NLGs) and Neurexins (NRXs) have emerged as critical regulators of proper circuit development and function (Sudhof, 2008).

Neuroligins are postsynaptic adhesion molecules that interact with presynaptic Neurexins with nanomolar binding affinities (Arac et al., 2007; Chen et al., 2008; Comoletti et al., 2006; Ichtchenko et al., 1995; Song et al., 1999). Structurally, NLGs are type I transmembrane proteins presenting a large extracellular globular domain that undergoes Ca^{2+} dependent dimerization, a highly glycosylated stalk domain and a shorter cytoplasmic tail containing a PDZ binding domain (Ichtchenko et al., 1995). In mammals, there are 4 genes expressing NLGs with NLG3 and NLG4 localized in the X chromosome. NLGs are alternatively spliced at a single canonical site (A) with NLG1 containing an extra splice site (B) (Ichtchenko et al., 1996).

Interestingly, each NLG isoform exhibits a specific pattern of expression and subcellular distribution. In particular, NLG1 and NLG2 are exclusively localized to excitatory and inhibitory synapses, respectively (Graf et al., 2004; Song et al., 1999; Varoqueaux et al., 2004), whereas NLG3 can be present in both (Budreck and Scheiffele, 2007).

Neurexins were identified as receptors for α-latrotoxin (Ushkaryov et al., 1992), a toxin present in the venom of the black widow spider that induces massive release of neurotransmitters (Ushkaryov et al., 2008). The mammalian genome contains 3 NRX genes each encoding a long α-protein and a shorter β-protein from independent promoters (Tabuchi and Sudhof, 2002). Moreover, NRXs are highly polymorphic and through extensive alternative splicing at 5 canonical sites have the potential to generate over 3000 possible isoforms (Ullrich et al., 1995). Interestingly, different NRX splice variants are differentially expressed in specific neuronal types (Ullrich et al., 1995) and have different binding affinities to different NLG isoforms (Chih et al., 2006) (Comoletti et al., 2006) (Ushkaryov and Sudhof, 1993) indicating that NRXs may provide a structural adhesive code at synapses.

NLGs are sufficient to induce functional maturation of presynaptic terminals (Dean et al., 2003; Prange et al., 2004; Scheiffele et al., 2000; Wittenmayer et al., 2009), and transgenic expression of NLG1 results in extended active zones and increased reserve pool size of synaptic vesicles (Dahlhaus et al., 2010). Reciprocally, NRXs trigger the aggregation and clustering

of postsynaptic components (Graf et al., 2004; Heine et al., 2008; Nam and Chen, 2005) and regulate NMDA receptor function trans-synaptically (Kattenstroth et al., 2004). This synaptogenic potential of NLGs and NRXs is due in part to the fact that both these proteins contain intracellular domains that interact with important synaptic scaffold proteins such as PSD95 and CASK (Hata et al., 1996; Irie et al., 1997). Adhesion between NLGs and NRXs thus provides a direct structural bridge between pre- and postsynaptic scaffolding machinery.

The ability to induce trans-aggregation of synaptic components suggested that NLGs and NRXs were critical mediators of synapse formation. This hypothesis was further supported by *in vitro* studies showing that NLG expression levels are correlated with the number of synapses generated during development (Chih et al., 2005; Dean et al., 2003; Graf et al., 2004; Levinson et al., 2005; Prange et al., 2004). However, despite presenting severe deficits in synaptic transmission, NLG1-3 triple knockout (KO) mice exhibit normal synaptogenesis (Varoqueaux et al., 2006). These results demonstrated that *in vivo*, NLGs are not required for the initial stages of synapse formation but are instead critical regulators of synaptic function. In addition to these results, it was later found that NLG1 overexpression in dissociated neuronal cultures increases AMPA and NMDA excitatory postsynaptic currents (EPSCs) (Chubykin et al., 2007). Moreover, recent studies have also shown that NLG1 is required for LTP in the amygdala (Jung et al., 2010; Kim et al., 2008), which reinforces the notion that NLG1 is an important functional component of mature synapses and is able to modulate synaptic transmission in adult stages.

Interestingly, regardless of no apparent change in the number of total synapses generated, NLG1-3 triple KO neurons present reduced evoked excitatory postsynaptic currents (eEPSCs) and decreased miniature EPSCs (mEPSCs) frequency, which is consistent with impaired presynaptic function (Varoqueaux et al., 2006). Moreover, overexpression of NLG1 in hippocampal slices and cultured neurons results in increased release probability through a NRX-dependent mechanism (Futai et al., 2007; Ko et al., 2009b; Stan et al., 2010), suggesting that NLG1 modulates presynaptic function trans-synaptically. Consistent with this hypothesis, neurons lacking αNRX1-3 exhibit deficits in synaptic transmission due to severe impairment of N-type Ca^{2+} channel function (Missler et al., 2003), while disruption of endogenous NLG-NRX interactions with soluble Fc-NRX fragments decreases mEPSC frequency and release probability (Levinson et al., 2005). Taken together, these results suggested a new model by which NLGs and NRXs validate and stabilize synapses during development by modulating synaptic transmission at the presynaptic level.

The importance of the NRX-NLG trans-synaptic complex for normal brain development is emphasized by the strong association of several NLG and NRX mutations with autistic spectrum disorders (ASDs) (Sudhof, 2008). A typical feature of ASDs is that they usually affect the brain during the second or third year of life, which is a period of extensive synapse remodeling and circuit refinement in humans (Lord et al., 2000; Pardo and Eberhart, 2007). The association of NLGs and NRXs with ASDs may then reflect an important role of these molecules during the synaptic activity-dependent maturation processes that

occur during early development (Hensch, 2004, 2005b). As such, the elucidation of the molecular and cellular pathways regulating NLGs may provide new insights regarding the function of these proteins in the etiology of ASDs and in broader terms, the role of adhesion molecules during the functional maturation of neuronal circuits. Despite the numerous studies addressing how NLG affects synaptic properties, little is known about how endogenous NLGs are themselves regulated at synapses. It is also still unclear if NLGs are stable structural elements in synapses or if they can be regulated by changes in neuronal activity.

Here, I address how NLG1 is affected in response to neuronal depolarization. Based on a combination of biochemical methods and immunocytochemistry I show that synaptic NLG1 can be acutely regulated by changes in neuronal activity through Matrix Metalloprotease dependent ectodomain shedding. Moreover, NLG1 cleavage occurs *in vivo*, is regulated over development and is modulated by sensory experience during critical periods of circuit maturation.

Results

To determine the effect of neuronal activity on synaptic NLGs, we treated dissociated hippocampal cultures at 21 days *in vitro* (DIV21) with 30 mM KCl for 2 h, a paradigm that elicits robust depolarization and is widely used as a model for activity-dependent neuronal signaling (Kim et al., 2010; Murase et al., 2002; Sheng et al., 1990).

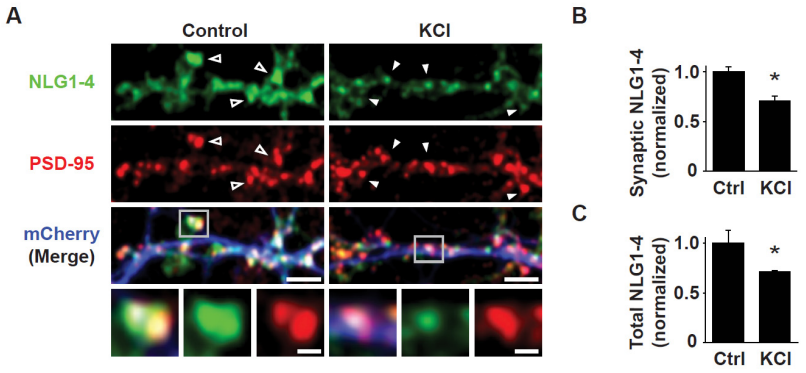

Figure 1. Neuronal activity triggers loss of Neuroligins from synapses. **(A)** Hippocampal neurons (DIV21) were incubated in Neurobasal media (Control) or Neurobasal media supplemented with 30 mM KCl (KCl) for 2 h, fixed, and immunolabeled for endogenous PSD-95 and pan-NLG (NLG1-4). Neurons were transfected with mCherry as a cell fill. Solid arrows show decreased NLG1-4 labeling at PSD-95 positive synapses following KCl incubation. Open arrows depict synaptic NLG1-4 labeling under control conditions. Scale bar, 5 µm. **(B)** Data indicate means ± SEM of NLG1-4 fluorescence intensity in PSD-95 positive dendritic spines (synaptic NLG1-4) or **(C)** over the entire neuron (total NLG1-4) normalized to controls. Control, n = 435 spines from 8 neurons; KCl, n = 546 spines from 9 neurons. * $p < 0.01$.

The level of endogenous NLGs at synapses was assessed by immunocytochemistry using a pan-NLG antibody targeted against the C-terminal domain of NLG1-4 together with immunolabeling for PSD-95 to identify excitatory synapses. Following KCl incubation, pan-NLG labeling at excitatory synapses was reduced by 30 ± 5% (Figures 1A and 1B) indicating that synaptic levels of NLGs are reduced after increased activity. Interestingly, we also observed a similar change in total average fluorescence of pan-NLG signal across neurons (29 ± 1%) indicating that the loss of NLG signal from PSD95 positive sites is not due to a redistribution of NLGs to extra synaptic sites, but due to overall protein degradation (Figure 1C). Among the different NLG isoforms, NLG1 exclusively partitions to and regulates excitatory synapses (Chubykin et al., 2007; Graf et al., 2004; Ko et al., 2009b; Song et al., 1999). Hence, the extensive loss of pan-NLG fluorescent signal from PSD95 positive puncta prompted us to test whether neuronal depolarization depletes NLG1 from the glutamatergic synapses. Due to the lack of NLG1-specific antibodies suitable for immunocytochemistry, we performed the same experiments and measured NLG1 levels in biochemical fractions of PSDs from dissociated cortical cultures treated with KCl and Neurobasal media alone (Ehlers, 2003) using an antibody targeted against the extracellular N-terminal domain (4C12).

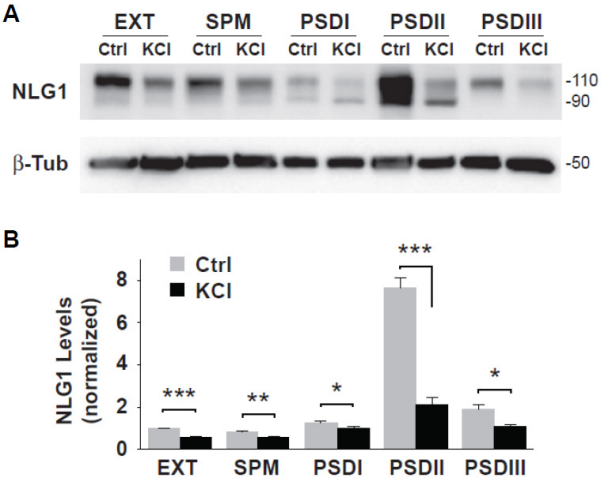

Figure 2. Neuronal activity triggers loss of NLG1 from excitatory synapses. (A) Immunoblot analysis of NLG1 and β-Tubulin in membrane fractions isolated from control or KCl-treated cortical neurons (DIV21). EXT, whole cell extract; SPM, synaptic plasma membrane; PSDI,II, III; sequential postsynaptic density fractions. Note that 4-fold less protein by mass was loaded in PSD fraction lanes. See Experimental Procedures for details. **(B)** Data indicate means ± SEM of NLG1 proteins levels in indicated fractions relative to the total extract control. n = 3, *p < 0.05, **p<0.005, ***p<0.001.

Immunoblot analysis of isolated fractions revealed an extensive enrichment of NLG1 in the PSD (Figure S1). Consistent with the immunocytochemistry data using the pan-NLG antibody, KCl depolarization resulted in a significant loss (48 ± 2%) of NLG1 from total neuronal extracts (Figures 2A and 2B). This reduction in NLG1 was also observed in the synaptic plasma membrane (SPM) and PSD fractions (31 ± 2% decrease in SPM; 24 ± 7% in PSDI; 45 ± 5% in PSDIII) and was particularly pronounced in PSDII fractions (73 ± 5% decrease), where NLG1 is most highly enriched (Figure S1). Thus, together with the immunocytochemistry data using pan-NLG antibody,

these results indicate that the neuronal levels of NLG1 are extensively reduced following 2h of neuronal depolarization.

Several synaptic membrane proteins and receptors are degraded in response to changes in neuronal activity through the lysosomal pathway upon regulated endocytosis (Ehlers, 2000). To address whether the decrease in NLG1 levels is due to increased internalization and lysosomal degradation, we measured internalization rates of NLG1 in dissociated cortical cultures during KCl depolarization using a biochemical strategy based on surface biotinylation of proteins (Ehlers, 2000) (Figure 3A). In these experiments, neurons were pre-incubated with leupeptin for 1 h to inhibit lysosomal proteolysis.

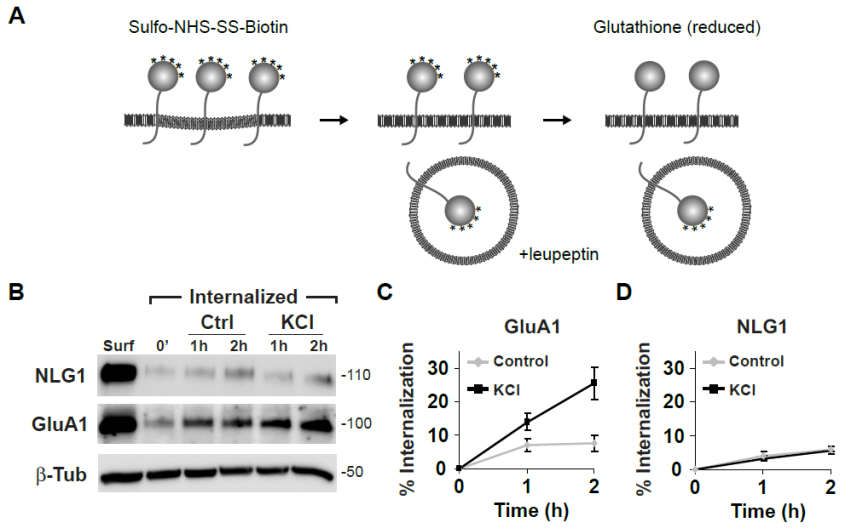

Figure 3. Neuronal depolarization does not increase NLG1 internalization. **(A)** Surface biotinylation based assay of endocytosis performed on cultured cortical neurons. Surface proteins are covalently labeled with 1mg/ml Sulfo-NHS-SS-Biotin for 10min, incubated for 1 h or 2 h at 37°C in neurobasal media

(Ctrl) or in media supplemented with 30 mM KCl (KCl), and subsequently treated with 50mM Glutathione pH8 for 30min to remove surface biotin label **(B)** Immunoblot of GluA1 and NLG1 indicate that GluA1 internalization was increased by KCl while NLG1 was not. **(C)** Quantitative analysis of GluA1 and **(D)** NLG1 internalization over time. Immunoblot values were compared to a calibration standard of total surface protein at time zero (Surf) to quantify percent internalization.

Under basal conditions 5.3 ± 1.2% of surface NLG1 was internalized over 2h (Figures 3B and 3D). This low internalization rate was unaltered by KCl incubation (5.7 ± 0.8% of surface NLG1) indicating that KCl-induced NLG1 loss is not due to increased internalization. The GluA1 AMPA receptor was used as a positive control and exhibited a marked increase in internalization under KCl stimulation (Figure 3B and 3C, 7.4 ± 2.4% of total surface protein internalized in control conditions; 25 ± 4.7% in KCl), similar to previous reports (Ehlers, 2000). These results suggested that KCl induced NLG1 loss is not caused by protein internalization and lysosomal degradation.

Moreover, to further address whether if this effect was sensitive to proteasome or lysosome inhibition we treated DIV21 dissociated cortical cultures with 30mM KCl for 2h in the presence of proteosome inhibitor MG132 and/or lysosomal enzyme inhibitor leupeptin, respectively, and measure how NLG1 degradation was affected.

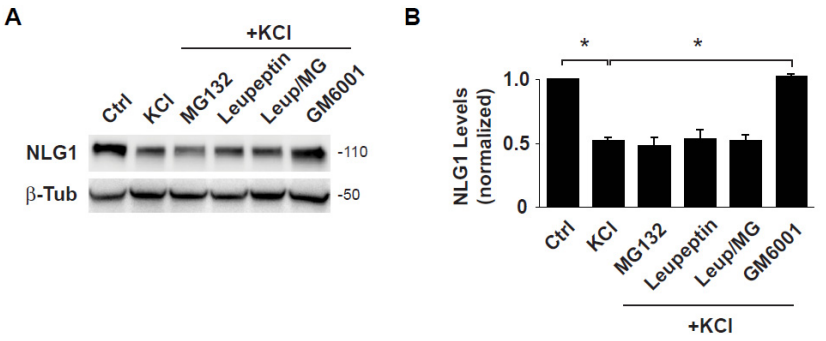

Figure 4. Activity dependent loss of NLG1 is mediated by Metalloproteases. (A) Immunoblot analysis of NLG1 in total lysates from cortical cultures following 2 h incubation in neurobasal medium (Ctrl) or medium supplemented with 30 mM KCl alone (KCl) or with MG132 (10 μM), leupeptin (200 μM), leupeptin plus MG132 (Leup/MG) or GM6001 (10 μM). Note that GM6001 prevents KCl-induced loss of total NLG1. **(B)** Data indicate means ± SEM of total NLG1 levels under the indicated conditions. n = 3, *p < 0.05.

As previously shown (Figure 2) depolarization induced by KCl incubation resulted in a 48 ± 3% reduction in total NLG1 levels. Interestingly, this effect was unaffected by proteasome inhibition (50 μM MG132), blockade of lysosomal degradation (200 μM leupeptin), or both together (46 ± 7% decrease with MG132, 52 ± 6% with leupeptin, 47 ± 4% with both; Figures 4A and 4B). These results indicated that NLG1 degradation was occurring through a different degradation pathway and prompted us to test an alternative hypothesis. Several membrane proteins are targeted and degraded by Matrix Metalloproteases, which are a large family of secreted proteases. Indeed, incubation with the broad-spectrum Matrix Metalloprotease (MMP) inhibitor GM6001 (10 μM) abolished the KCl-induced loss of NLG1 (102.9 ± 1.1% of control; Figures 4A and 4B). This important result indicated that NLG1 can be cleaved by MMPs and that MMP-dependent

proteolysis is the major regulatory mechanism mediating the rapid and extensive degradation of NLG1 in response to KCl depolarization.

To determine if NLG1 is also cleaved *in vivo* we analyzed soluble fractions of cortical, hippocampal, and cerebellar tissue from adult P60 mice (Figure 5A).

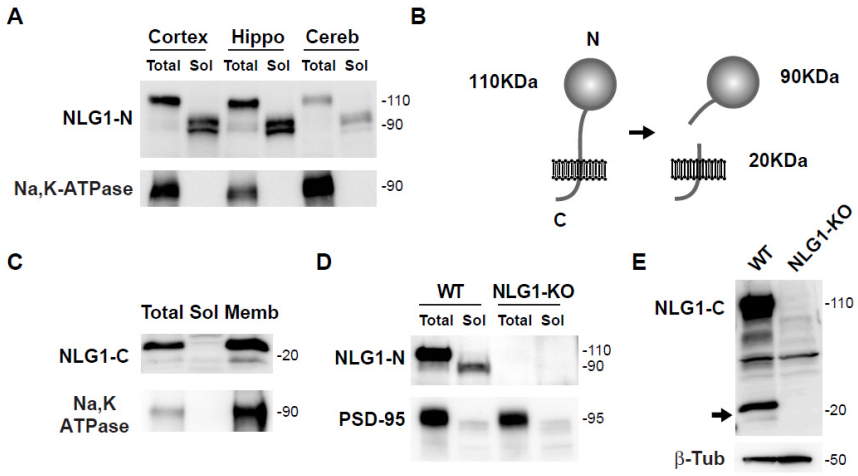

Figure 5. NLG1 cleavage fragments are ubiquitous throughout the brain. **(A)** Immunoblot analysis of NLG1 in whole homogenates (5μg, Total) and soluble (50μg, Sol) fractions obtained from cortical, hippocampal and cerebellar tissue from P60 WT mice reveals soluble ~90 kDa NLG1-NTFs. Na,K-ATPase was used to assess purity of soluble fraction. **(B)** Schematic representation of NLG-1 cleavage fragments **(C)** Immunoblot analysis of NLG1 C-terminal fragments in whole homogenates (Total), soluble (Sol) and membrane enriched (Memb) fractions obtained from cortical tissue from P60 WT mice reveals ~20 kDa NLG1-CTFs. **(D)** Immunoblot analysis of NLG1 in whole brain homogenates (Total) and soluble fractions (Sol) from WT and NLG1-KO mice indicating the absence of NLG1-NTFs in NLG1-KO tissue. **(E)** Immunoblot analysis of NLG1 C-terminal in whole brain homogenates from WT and NLG1-KO mice indicate the absence of NLG1-CTFs in NLG1-KO tissue.

Interestingly, several bands of approximately 90 kDa were recognized by the N-terminal NLG1 antibody in the soluble fractions of extracts of different brain regions, suggesting that multiple NLG1 extracellular cleavage fragments are generated *in vivo*. A logical outcome of the ectodomain shedding of NLG1 is the generation of corresponding intracellular C-terminal fragments of approximately 20kDa (Figure 5B). Analysis of mouse cortical fractions using an antibody targeted against the C-terminal domain of NLG1 (NLG1-C) revealed in fact, multiple membrane bound bands of approximately 20KDa, a size consistent with the predicted mass based on the size of the N-terminal cleavage fragments (Figures 5B and 5C). To further confirm these findings we expressed a dual labeled version of NLG1 with GFP tagged to the N-terminus and HA tagged to the C-terminus (GFP-NLG1-HA) in COS7 cells (Figure S2). Immunoblot analysis of cell extracts using an anti-HA antibody revealed the presence of similar ~20 kDa bands that were absent in extracts of COS7 cells transfected with GFP-NLG1.

To exclude possible nonspecific interactions recognized by the NLG1 antibodies, we tested whether similar bands were detected in extracts of NLG1 null mice (Varoqueaux et al., 2006). Both 110 kDa full form and 90 kDa NLG1 N-terminal fragments (NLG1-NTFs) were absent from NLG1 KO brain extracts and respective soluble fraction (Figure 5D). Similarly, NLG1 C-terminal fragments (NLG1-CTFs) were absent from NLG1 KO brain extracts (Figure 5E). Together, these results indicate that the NTFs and CTFs recognized by the NLG1 antibodies are indeed cleavage fragments of NLG1 and not an artifact due to non-specific antibody binding.

Several 90 kDa NLG1 species have been widely observed throughout the literature and have generally been considered to be immature unglycosylated forms of NLG1 (Ichtchenko et al., 1995; Ko et al., 2009b). This is due to the fact that NLG1 has an amino-acid mass of approximately 90KDa, but due to extensive N- and O-glycosylation, it migrates electrophoretically with an apparent weight of 110kDa. To test whether the 90 kDa NLG polypeptide(s) detected in our western blots correspond to immature or incompletely glycosylated isoforms of NLG1, we enzymatically deglycosylated all N- and O-linked glycans from P5 mouse cortical extracts and respective soluble fraction and measured changes in electrophoretic mobility.

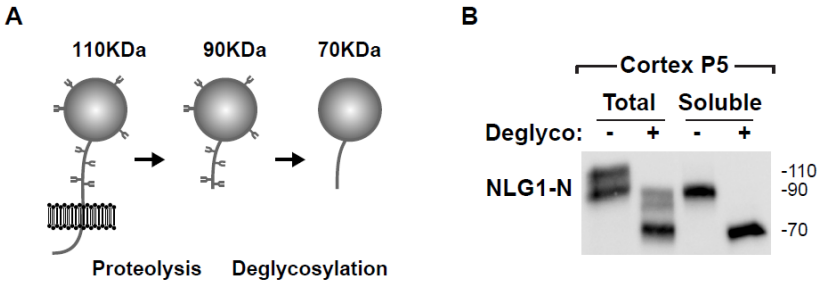

Figure 6. NLG1-NTFs originate from fully glycosylated NLG1. (A) Schematic representation of the electrophoretic mobility shift induced by proteolytic cleavage of the N-terminal domain and subsequent deglycosylation of N- and O-linked glycans of full form NLG1. **(B)** Deglycosylation (Deglyco) of N- and O-linked residues in whole homogenates and soluble fractions of P5 WT mouse cortex results in a 20 kDa apparent mass shift of NLG1-NTFs.

Enzymatic deglycosylation resulted in a significant shift in apparent weight of NLG1-NTFs indicating that they originate from

glycosylated forms of NLG1 (Figures 6A and 6B). Importantly, whereas deglycosylation of total cortical extracts collapsed NLG1 to both a 90 kDa and 70 kDa species corresponding to full-length and N-terminal cleaved NLG1, respectively, deglycosylation of soluble fractions produced only a 70 kDa species (Figures 6B), confirming that all soluble NLG1-NTFs correspond to cleavage fragments of mature, fully glycosylated NLG1.

Together, the data presented here indicates that overall NLG1 levels are modulated by neuronal activity via MMP dependent ectodomain shedding. Interestingly, previous studies have implicated tissue plasminogen activator (tPA) dependent proteolytic mechanisms as critical processes regulating synapse remodeling during early development (Mataga et al., 2004; Mataga et al., 2002). Given the involvement of NLG1 in synapse maturation we also characterized the developmental profile of NLG1 cleavage and addressed if this mechanism is regulated over brain development. Indeed, immunoblot analysis of mouse cortical fractions at various time points from birth to adulthood (P1-P60, Figure 7A) revealed that NLG1-NTFs are present throughout development and are particularly enriched during early development (P1-P7) where they are as abundant as the full form protein (Figures 7A and 7B). This indicated that NLG1 cleavage is not only regulated by neuronal activity changes during mature stages but is also an important mechanism during early development.

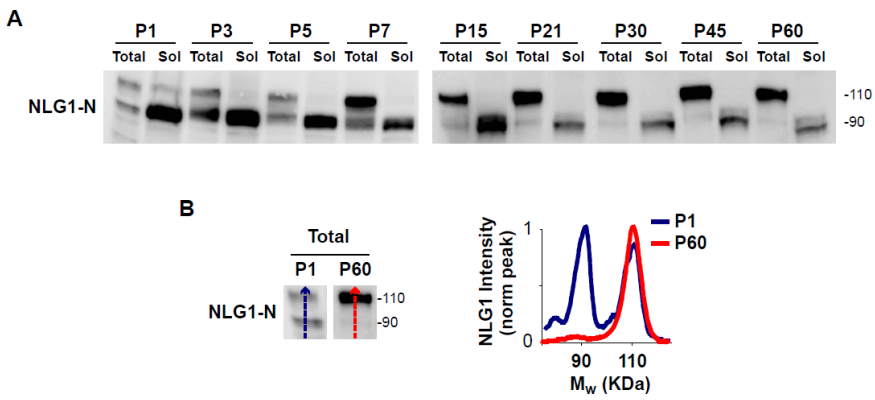

Figure 7. NLG1-NTFs are highly abundant during early development and decrease with age. (A) Developmental profile of NLG1-NTFs from WT mice cortical extracts. Postnatal days (P) are shown at the top. Total, 10 μg whole cortical homogenates; Sol, 50 μg soluble fractions from mouse cortex. **(B)** Analysis of the relative abundance of full form NLG1 and NLG1-NTFs present in whole cortical extracts from P1 and P60 mice. Note that NLG1 cleavage products are more abundant during early developmental stages. The graph plots signal intensity along the lines shown on the blots on the left.

The refinement of synaptic connections by activity dependent processes during early development is crucial for the proper formation of functional neuronal circuits (Hensch, 2004, 2005b). To address if NLG1 cleavage is regulated by sensory experience during critical periods of development we subjected mice to 5 days of dark rearing (DR) and subsequently re-exposed them to light for a brief period of 2 hours (DR+2hL). This protocol induces rapid synaptic remodeling in the primary region of the visual cortex and results in extensive molecular functional and structural synaptic changes (Brakeman et al., 1997; Cotrufo et al., 2003; Philpot et al., 2001; Tropea et al., 2010; Viegi et al., 2002). Dark rearing of mice for 5 days resulted in a significant decrease in the levels of NLG1-NTFs present in V1 cortex

compared with control animals (LR) reared in normal light cycle (DR group - 0.71 ± 0.04 NLG1-NTFs normalized to LR group) (Figures 8B and 8C). Notably, 2 hours of re- exposure to light after dark rearing caused a robust increase in NLG1 cleavage (1.51 ± 0.14 compared with LR group).

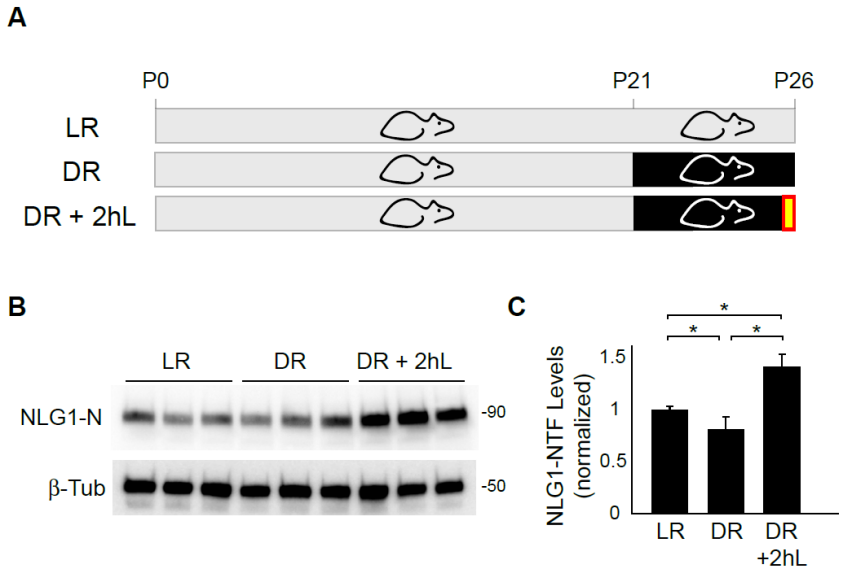

Figure 8. NLG1 cleavage is regulated during the critical period of visual cortex development. (A) Schematic representation of the experimental paradigm. Mice are reared in normal 12h day/night cycle (LR) or submitted to dark rearing for 5 days from P21-P26 (DR). DR+2hL indicates experimental group submitted to a brief 2h light exposure following light deprivation. **(B)** immunoblot of P26 mouse primary visual cortex of animals reared under different conditions. Note that 2h of light exposure after dark rearing induces increase in NLG1-NTF levels. **(C)** Data indicate means ± SEM of NLG1-NTFs levels under the indicated conditions normalized to LR animals. n = 12 animals per condition, *p < 0.005.

These results indicate that NLG1 cleavage is acutely regulated by changes in sensory experience *in vivo* and suggest

that this mechanism may be involved in the shaping of neuronal circuits during development. Taken together, the results presented here unveil a new activity dependent form of post-translational regulation of NLG1 that is ubiquitous in the brain and present throughout all developmental stages.

Discussion

Although highly studied for its role in synapse maturation and stabilization, it has been unclear whether the NLG-NRX trans-synaptic complex undergoes dynamic regulation and dissociation. Here we have shown that acute depolarization leads to a robust decrease in synaptic NLG1 levels. MMP inhibitors but not lysosome or proteasome inhibitors block this effect, indicating that under these conditions NLG1 is predominantly regulated by proteolytic cleavage. The extent of NLG1 loss observed using biochemical methods (Figures 2 and 4) is somewhat greater than that observed by immunostaining (Figure 1A). This disparity may be attributed to the broad specificity of the pan-NLG antibody used for immunolabeling or to the fact that it targets the C-terminal domain of NLGs, an epitope that may be differently regulated after ectodomain shedding. In addition, the presence of a residual C-terminal fragment (CTF) of cleaved NLG-1 (Figure 5C) raises the possibility that further intracellular processing of NLG1 may trigger downstream signaling cascades similar to what has been described for other membrane proteins such as Notch and E-Cadherin (Marambaud et al., 2002; Mumm et al., 2000). Moreover, these results suggest that caution is warranted in interpreting changes in Neuroligin synaptic abundance measured exclusively by an N-terminal or C-terminal antibody.

The N-terminal AChE homolog domain of Neuroligins binds the extracellular domain of Neurexins and this interaction has, to date, been thought to occur exclusively through membrane-associated Neuroligin (Arac et al., 2007; Chen et al., 2008; Comoletti et al., 2006). Here we have shown that the

Neurexin-binding domain of NLG-1 is released as a soluble N-terminal fragment (NTF) and is abundant in brain. Glutamatergic synapses are densely packed in brain tissue *in vivo* and, much like other soluble factors such as brain-derived neurotrophic factor (BDNF) (Desai et al., 1999; Rutherford et al., 1998), NLG1-NTFs could potentially mediate heterosynaptic effects by diffusing to neighboring synapses. Intriguingly, hetereosynaptic depression of neurotransmitter release has been reported at several CNS synapses, although the molecular basis for such depression has not always been clear (Chistiakova and Volgushev, 2009; Grover and Teyler, 1993; Huang et al., 2008). Indeed, cleavage of the Neural Cell Adhesion Molecule (NCAM) originates soluble extracellular fragments (NCAM-EC) that induce several structural and functional defects in cortical neurons (Brennaman and Maness, 2008; Pillai-Nair et al., 2005). Moreover, cleavage of E-cadherin by MMP3 and MMP7 results in disrupted aggregation and cell migration in neighboring cells due to diffusion of soluble cleavage products (Lochter et al., 1997; Noe et al., 2001). We further note that several studies have employed exogenous application of recombinant soluble NRX extracellular fragments as a means to disrupt Neuroligin interactions (Levinson et al., 2005; Scheiffele et al., 2000) raising the possibility that NLG-NTFs present in soluble brain fractions may exert similar functions. Alternatively, release of soluble NLG-NTFs could act to locally suppress pro-synaptogenic NLG-NRX interactions during brain development, ensuring proper synapse patterning and providing a potentially novel mechanism for synapse competition.

Neuroligins have been strongly implicated in the maturation of synapses during development. The regulation of NLG1-NTF levels by sensory experience during the critical period of primary visual cortex maturation indicates that NLG1 cleavage is a physiological process regulated by activity *in vivo* and suggests that this mechanism may be involved in the shaping of neuronal circuits during early development. There is wide evidence that neuronal circuits are shaped and refined by sensory experience during critical periods of heightened plasticity (Hensch, 2004, 2005b). During this process, single neurons tune their functional properties in order to ultimately create stable and functional neuronal networks (Hensch, 2004, 2005a). A key requirement for this process is the establishment of the proper balance between excitation and inhibition (Bavelier et al., 2010; Hensch and Fagiolini, 2005; Hensch et al., 1998). Notably, the relative levels of NLG1 and NLG2 determines the overall balance between excitation and inhibition (Levinson et al., 2005; Prange et al., 2004) suggesting that differential cleavage of different NLG isoforms may contribute for the establishment of proper E/I ratio. Moreover, manipulation of NLG levels during development determines the overall number of synapses generated (Chih et al., 2005; Dean et al., 2003; Graf et al., 2004; Levinson et al., 2005; Prange et al., 2004) suggesting that NLGs may directly regulate the stability of synapses. Our results indicate that NLG cleavage is highly upregulated during the first postnatal week and declines over time reaching a minimum at around 2 weeks of age. Interestingly, this is the developmental period where more dendritic spines are observed in neurons indicating that the decrease in NLG1-NTFs is correlated with the stabilization synapses during development. Moreover, previous studies have

shown that tissue plasminogen activator (tPA), a potent activator of proteolytic cascades, is involved in the stabilization and pruning of synapses during early development (Mataga et al., 2004; Mataga et al., 2002). In addition, visual experience during the critical period affects dendritic spine morphology in visual cortex and leads to an increase in the fraction of thin spines and filopodia (Tropea et al., 2010). Together, this raises the interesting possibility that NLG cleavage may contribute for the maturation of synapses during cortical development.

Moreover, manipulation of sensory experience during critical period alters the ratio of NR2A/NR2B-only NMDARs layer 2/3 neurons of visual cortex (Philpot et al., 2001). The regulation of this balance alters the metaplastic state of excitatory synapses with NR2A increasing and NR2B decreasing the threshold for LTP induction (Philpot et al., 2007). Interestingly, NLG1 overexpression increases the ratio of NMDAR/AMPAR currents in cultured neurons (Chubykin et al., 2007). In addition, NLG1 KO animals have lower NMDAR/AMPAR ratio and exhibit decreased NR1 expression and NMDAR-mediated synaptic transmission (Chubykin et al., 2007; Jung et al., 2010; Varoqueaux et al., 2006), suggesting that NLG1 is important for recruitment of NMDAR to postsynaptic sites. A recent study has also suggested that NLG1 may induce clustering of NMDARs directly without the presence of PSD95 (Barrow et al., 2009). Hence it is possible that NLG cleavage may induce a shift in NMDAR subunits in synapses, either by preferentially binding a particular type of receptor or by enabling a structural remodeling of the postsynaptic density that allows for a shift in synaptic receptors, which subsequently could lead to synaptic plasticity.

Together the findings presented here describe a novel post-translational mechanism that acutely regulates NLG1 in synapses and that implicates this class of molecules in the molecular mechanisms underlying critical period plasticity. Given the strong association between several NLG and NRX mutations with ASDs (Sudhof, 2008) the acute proteolytic regulation of Neuroligins may provide novel insight into the pathophysiological mechanisms and therapeutic strategies for synaptic dysfunction in ASDs. More broadly, such proteolytic mechanism may provide a general paradigm for regulation of cell-cell trans-synaptic adhesion in the context of synaptic plasticity.

Methods

Reagents and Antibodies

Dissociated primary neuronal cultures were prepared from hippocampi or cortex of embryonic day 18 or 19 Wistar rats. Tissue was dissociated by enzymatic papain digestion followed by brief mechanical trituration. Cultures were grown in Neurobasal media supplemented with B27 and Glutamax. For biochemical experiments 700k cortical cells were plated onto 60mm petri dishes. For immunocytochemistry, 100k hippocampal cells were plated onto 12 well plates containing Poly-L-Lysine coated 18mm glass coverslips. Plasmid transfection was done using lipid mediated gene transfer using Lipofectamine 2000 (Invitrogen). Briefly, for each coverslip, 1μg total DNA was mixed with 1μl Lipofectamine in 100 μl of Neurobasal media for 15min. Following incubation time 500 μl of conditioned media was mixed to the reaction and added to cell cultures for 30min. After this period, lipofectamine containing media was removed and replaced with a 1:1 mixture of conditioned media + fresh growth media. COS7 cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum. Plasmid tranfections were performed at approximately 70% confluency using 3 μg of DNA and 3 μl of Lipofectamine per 60mm petri dish and let express for 2 days.

Commercial antibodies used include N-terminal NLG1 antibody clone 4C12 (mouse, Synaptic Systems), C-terminal NLG1 polyclonal antibody (rabbit, Synaptic Systems), PSD-95

(mouse, Chemicon), Na^+,K^+-ATPase (rabbit, Cell Signaling), GFP (mouse, Millipore), HA.11 (mouse, Covance). Rabbit anti-panNLG antibody used for immunocytochemistry was a gift from Peter Scheiffele (Biozentrum).

Imaging

Confocal images of fixed samples were obtained using a Perkin Elmer Ultraview spinning disc confocal microscope with either a 40x 1.3 N.A. objective or a 60x 1.4 N.A. objective. Images were analyzed using Metamorph software (Molecular devices, Universal Imaging Corporation). Maximum projections of z series (0.5 mm steps) were used for quantification. For immunocytochemistry, DIV21 hippocampal neurons were fixed in 4% paraformaldehyde/4% sucrose in PBS for 20 min, permeabilized with -20ºC methanol for 10 min, and incubated with indicated antibodies at 1:250 (panNLG) or 1:1000 (PSD-95) dilution overnight at 4ºC. Quantification of integrated fluorescence intensity at individual puncta was performed using fixed region sizes. For PSD95 positive puncta quantification only regions containing 3x background level of PSD95 immunofluorescence were considered for analyzes. For each neuron the average fluorescence intensity for all puncta was determined. For determination of total pan-NLG fluorescence cell was traced based on mCherry cell fill and average intensity of pan-NLG fluorescence was determined. Results depicted correspond to the average of all neurons per condition.

Biochemical Analysis of Brain and PSD Fractions

Mouse brains or brain regions were homogenized in homogenization buffer (4 mM HEPES, 0.32 M sucrose pH 7.4) with protease and phosphatase inhibitors (Roche). Crude membrane fractionation was performed by centrifugation at 150,000 x g for 30 min. High density cortical cultures were incubated in Neurobasal media or media supplemented with 30 mM KCl for 2 h. Whole cell, synaptic plasma membrane, and PSD fractions from cultured cortical neurons were prepared as described previously (Ehlers, 2003) and immunoblotted for proteins of interest. For PSD fractions, 5 μg of protein was loaded per lane, while for remaining fractions 20 μg protein was loaded. Deglycosylation was performed using an enzymatic deglycosylation kit according to the manufacturer's instructions (Calbiochem). All protein concentrations were measured with Dc protein assay (Bio-Rad).

Biotinylation-Based Internalization Assay

High-density cortical neuron cultures were incubated with 100 μg/ml of the lysosomal protease inhibitor leupeptin beginning 1 hr prior to biotinylation with 1 mg/ml sulfo- NHS-SS-biotin (Ehlers, 2000). Leupeptin was present throughout all steps and incubations except the 4ºC biotinylation reaction. Neurons were then incubated at either 4ºC to block membrane trafficking or 37ºC for various times to allow endocytosis to occur. The remaining surface biotin was cleaved by reducing its disulfide

linkage with glutathione cleavage buffer (50 mM glutathione in 75 mM NaCl and 10 mM EDTA containing 1% BSA and 0.075 N NaOH) two times for 15 min each at 4ºC. Whole cell extracts were prepared, and biotinylated proteins were precipitated essentially as described (Ehlers, 2000). Biotinylated receptors were detected by immunoblot (ECL Plus, Amersham), and quantification was performed on an LAS-3000 gel reader (Fujifilm), using Multigauge 3.0 software. The percent receptor internalized was determined by measuring the band intensity after 37ºC incubation, subtracting the nonspecific band intensity obtained after 4ºC incubation (always <5%), and comparing to the total surface receptor calibration curve.

Dark rearing experiments

Male and female mice were reared in a normal light dark cycle (12 h light/12 h light) from birth until P26 (LR group) or were transferred to a dark room in complete darkness at P21 (DR group). Handling of animals in the dark room was done with the aid of night vision goggles and infra-red light. Mice were all sacrificed at P26 at the peak of the critical period of visual cortex development (Gordon and Stryker, 1996). One experimental group (DR+2hL) was re-exposed to light for 2 hours before being euthanized. Animals were sacrificed after 2 hours of light exposure and primary visual cortex was extracted, homogenized in homogenization buffer (4 mM HEPES, 0.32 M sucrose pH 7.4) containing protease and phosphatase inhibitors (Roche). Soluble fraction was obtained by centrifugation at 150,000 x g for 30 min. Protein concentration was determined using the Bradford reagent

(Bio-Rad) and 50μg of protein were loaded on western blot and probed for NLG1-NTFs using a NLG1 specific antibody targeted against the N-terminal domain (clone 4C12, Synaptic Systems).

Supplementary Figures

Figure S1

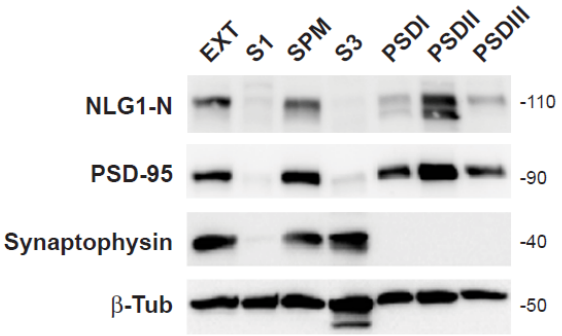

Figure S1. Biochemical Fractionation of Cultured Cortical Neurons.
Shown are immunoblots of biochemical fractions from cortical neurons cultures. EXT, whole cell extract; S1, supernatant; SPM, synaptic plasma membranes; S3, synaptic vesicle fraction; PSDI, Triton-washed SPM pellet; PSDII, Triton-washed PSDI pellet; PSDIII, Sarcosyl-washed PSDI pellet. Note that 4-fold less protein by mass was loaded in PSD fraction lanes. See Chapter1 Experimental Procedures for details.

Figure S2

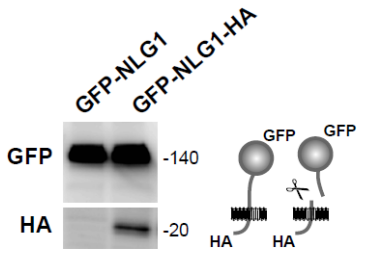

Figure S2. Generation of NLG1-CTFs in COS7 cells.
Lysates from COS7 cells expressing GFP-NLG1 or GFP-NLG1-HA were analysed by immunoblot using anti-GFP and anti-HA antibodies. Anti-HA immunoblot revealed a fragment of approximately 20 kDa corresponding to a C-terminal fragment.

Acknowledgements

For the realization of this work, Irina Lebedeva and Marguerita Klein prepared the neuronal cultures from dissociated hippocampus and cortex. The dark rearing experiments were done at the laboratory of Ben Philpot at the University of North Carolina. In these experiments Portia McCoy was responsible for breeding and housing the animals in the dark room and helping with tissue extraction.

Chapter 2

Signaling Pathways Regulating Neuroligin-1 Cleavage

Introduction

Neuroligins and Neurexins undergo high affinity molecular interactions and subsequently activate multiple trans-synaptic signaling mechanisms resulting in stabilization and maturation of synapses during development (Dean et al., 2003; Graf et al., 2004; Heine et al., 2008; Ichtchenko et al., 1995; Nam and Chen, 2005; Prange et al., 2004; Scheiffele et al., 2000; Song et al., 1999; Wittenmayer et al., 2009). Furthermore, several studies indicate that manipulation of NLG levels *in vitro* and *in vivo* alters synaptic function (Chubykin et al., 2007; Futai et al., 2007; Varoqueaux et al., 2006). However, the detailed molecular and cellular mechanisms regulating endogenous NLGs in neuronal synapses remain mostly unknown.

In the previous chapter I demonstrated that Matrix Metalloprotease (MMP) dependent ectodomain shedding is an important mechanism regulating NLG1 in response to neuronal depolarization and sensory experience during critical periods of cortical development. MMPs are a large family of Zn^{2+}-dependent proteolytic enzymes that were first identified for their capacity to cleave and remodel the extracellular matrix. However, growing evidence indicates that MMPs can also target multiple pericellular proteins including proteinases, growth factors, cytokines, cell

surface receptors and cell adhesion molecules (Hubschmann et al., 2005; Maretzky et al., 2005; Monea et al., 2006; Sternlicht and Werb, 2001). Hence, MMPs can regulate a large array of biological processes in diverse tissues from cell migration and tissue morphogenesis to wound repair and inflammation (Ethell and Ethell, 2007; Sternlicht and Werb, 2001). Moreover, the expression profile of different MMPs can be tuned in response to specific stimuli or different cellular contexts. Given the potential deleterious effects caused by unrestrained proteolytic activity, MMPs are tightly regulated at the level of transcription, compartmentalization and enzymatic activity. Importantly, MMPs are secreted as inactive zymogens containing an auto-inhibitory N-terminal prodomain with a cysteine residue that blocks Zn^{2+} in the active catalytic site. Removal of this prodomain via proteolysis or S-nitrosylation exposes the catalytic site and activates the protein (Gu et al., 2002; Morgunova et al., 1999; Van Wart and Birkedal-Hansen, 1990).

There are 24 identified MMPs in the mammalian genome with distinct yet overlapping target specificities (Ethell and Ethell, 2007). MMP2, MMP3 and MMP9 are the most abundant MMPs in the brain and have been implicated in synapse formation, multiple forms of synaptic plasticity and numerous neuropathological conditions (Yong, 2005). In neurons, several studies have reported an increased expression of MMP3 and MMP9 in response to chemically induced seizures in the dentate gyrus (Jourquin et al., 2003; Szklarczyk et al., 2002). Moreover, MMP9 is located in the synaptic cleft of glutamatergic synapses and in putative secretory vesicles in dendritic spines and dendrites suggesting that it can be rapidly activated and secreted

in response to activity (Sbai et al., 2010; Wilczynski et al., 2008). This is further supported by recent work based on high resolution in situ zymography showing that gelatinase activity is increased in glutamatergic synapses of hippocampal neurons 2h after kainite (KA) induced seizures (Gawlak et al., 2009). Epileptogenesis, the process that leads to recurrent seizures after the initial epileptic episode, involves extensive circuit remodeling and fiber sprouting in the hippocampus (Pitkanen and Sutula, 2002). Thus, the robust upregulation of MMP9 levels and activity after seizures long suggested a potential involvement of this protease in synaptic remodeling. This hypothesis has been recently confirmed in a study showing that MMP9-KO mice do not exhibit synapse remodeling following seizures (Wilczynski et al., 2008). This significant result indicates that cleavage of MMP9 targets constitutes an important regulatory step mediating synapse stability in the hippocampus after *status epilepticus*.

Besides being involved in the molecular signaling cascades induced by epileptogenic activity, MMP9 is also upregulated in CA1 after LTP induction in acute hippocampal slices (Nagy et al., 2006) and *in vivo* (Bozdagi et al., 2007). Moreover, MMP-9 blockade impairs LTP under these conditions whereas recombinant MMP9 is sufficient to induce LTP (Wang et al., 2008). The substrates of MMP-9 associated with hippocampal LTP remain however, unknown. Interestingly, MMP9 cleaves the NRX binding partner β-dystroglycan (Sugita et al., 2001) *in vitro* in response to glutamate or bicuculline incubation and *in vivo* following KA induced seizures (Michaluk et al., 2007). Moreover, MMP-9 can activate TrkB signaling in cultured cortical neurons, whereas MMP inhibitors reduce zinc-

mediated TrkB activation (Hwang et al., 2005). In fact, zinc has been shown to be released from presynaptic sites during high-frequency stimulation, suggesting that this may be an additional regulatory mechanism of MMP activity at synapses (Vogt et al., 2000). In addition, NMDAR antagonists also block the MMP9 effect in LTP suggesting that MMP9 dependent proteolysis can be regulated by synaptic activity (Nagy et al., 2006).

Interestingly, the synaptogenic potential of NLG1 is dependent on NMDAR signaling, as incubation with NMDAR receptor antagonist APV abrogates the increase in synapse number caused by NLG1 overexpression in cultured neurons (Chubykin et al., 2007). Moreover, APV and CaMK inhibitor KN93 also abrogate the increase in EPSCs mediated by NLG1 overexpression. These results indicate that NLG function at synapses is regulated by neuronal activity and is dependent of NMDAR and CaMK signaling. In addition, high frequency field stimulation induces fast morphological changes of NLG1 puncta further supporting that NLG1 can be acutely regulated by activity (Gutierrez et al., 2009). However the mechanisms mediating this effect remain unknown. Recent work has also shown that induction of LTD using (*S*)-3,5-Dihydroxyphenylglycine (DHPG) in both hippocampal slices and dissociated cultures, acutely reduces the surface levels of NLG1/3 through a putative dynein dependent process (Schapitz et al., 2010). Conversely, chemical LTP induction using Forskolin/Rolipram increases surface NLG1/3 levels. These results suggest that the trafficking of NLGs can also be acutely regulated by activity. Moreover, brief stimulation with 50mM KCl increased NLG1 and NRX1 surface expression in hippocampal neurons by decreasing internalization

(Thyagarajan and Ting, 2010). Interestingly, this effect is also blocked by NMDAR antagonist APV suggesting that NMDAR can regulate NLG1 trafficking. Hence, several lines of evidence suggest that NLG levels at synapses can be modulated in response to changes in neuronal activity via NMDAR dependent processes.

The previous results described in Chapter 1 indicate that NLG1 cleavage is modulated by neuronal depolarization in mature neurons and is differentially regulated throughout development. However, the molecular mechanisms and specific proteases regulating NLG1 cleavage remain unknown. Here, using biochemical labeling of surface proteins and pharmacological manipulation of enzymatic activity, I demonstrate that activity dependent NLG1 cleavage is bi-directionally regulated by neuronal activity, requires NMDA receptor activation and Ca^{2+}/calmodulin-dependent protein kinase signaling. Moreover, NLG1 cleavage occurs near its juxtamembrane extracellular region and is mediated by Matrix Metalloprotease-9 (MMP9). Interestingly, MMP9 dependent cleavage of NLG1 is also upregulated *in vivo*, in the hippocampus, during pilocarpine induced epileptic seizures furthering reinforcing the notion that NLG1 cleavage is an important mechanism regulating neuronal function *in vivo*.

Results

To dissect the signaling mechanisms regulating NLG1 cleavage, we developed a novel *in vitro* assay based on surface biotinylation that allows the purification of protein fragments cleaved and released to the culture media (Figure 1A).

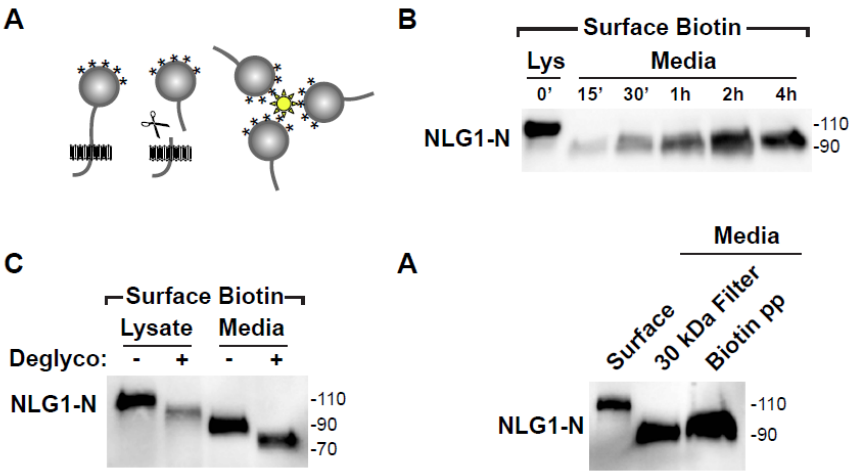

Figure 1. Surface biotinylation based assay reveals NLG1-NTFs in neuronal cultures. (A) Schematic diagram indicating the biotinylation-based cleavage assay. Media from cultured neurons previously subjected to surface biotinylation is collected and soluble biotin conjugates isolated by streptavidin precipitation. **(B)** Precipitation of soluble biotinylated proteins released to the media followed by immunoblot detection reveals time-dependent release of soluble ~90 kDa NLG1 N-terminal fragment (NLG-NTF). The ~110 kDa full form NLG1 in a total lysate (Lys, 10% of precipitate) is shown. Molecular mass markers in kDa are shown on the right. **(C)** Deglycosylation (Deglyco) of N- and O-linked glycans induces an equivalent mass shift of both full-length NLG1 (lysate) **(D)** Collection and 30 kDa mass cutoff filtration of media collected from cultured cortical neurons (DIV21) reveals the presence of ~90 kDa NLG1-NTFs similar to those isolated by surface biotinylation and strepavidin pull-down

(Biotin pp). The slight increase in molecular mass of the streptavidin-isolated NLG1-NTF relative to filter-isolated NLG1-NTF is likely due to biotinylation.

Briefly, neuronal cultures are covalently labeled with cell impermeable biotin (Sulfo-LC-Biotin-NHS, 1 mg/ml) for 10 min to exclusively label surface proteins. Following incubation at 37ºC, culture media was collected, centrifuged, and incubated overnight with streptavidin beads to isolate released soluble biotin-conjugates prior to immunoblot analysis. Compared with traditional filtration and protein precipitation techniques this method specifically isolates protein fragments that originate from proteins expressed at the plasma membrane at the time of labeling and excludes peptides released via the secretory pathway. Moreover, the labeling of surface proteins allows the use of conditioned media during experiments given that previous accumulation of NTFs in the media does not confound the results.

Using this assay we detected a set of ~90 kDa polypeptide(s) recognized by N-terminal NLG1 antibody that accumulated in a time-dependent manner (Figure 1B). Deglycosylation of the precipitated fragments resulted in a migration shift for both full-length NLG1 and the soluble 90 kDa NLG1-NTF species (Figure 1C), identifying the latter as a cleaved N-terminal fragment rather than an immature under-glycosylated species (See Chapter 1, Figure 6). To control for possible artifacts generated by surface biotinylation, we also filtered conditioned media from neuronal culture media using a

30 kDa cutoff filter. Similar ~90 kDa bands were detected in concentrated filtered fractions (Figure 1D), indicating that NLG1 N-terminal fragments (NLG1-NTFs) are not a by-product of biotin labeling.

Neuroligin-based signaling is regulated by activity (Chubykin et al., 2007) although the underlying mechanisms remain obscure. Using our biotinylation based assay we investigated whether activity regulates NLG1 cleavage *in vitro*. For this, we first bidirectionally manipulated network activity in cortical neuron cultures using a pharmacological approach.

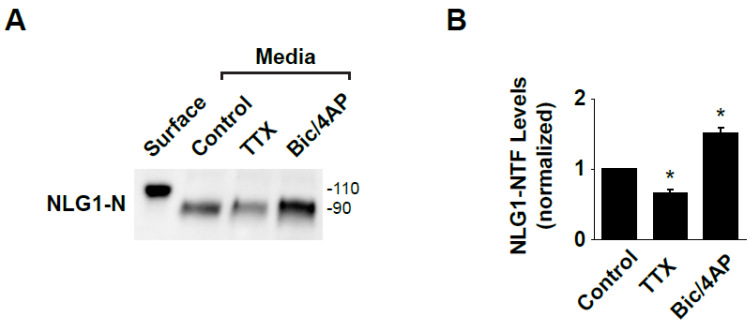

Figure 2. NLG1 cleavage is bidirectionally regulated by activity. (A) Biotinylation-based isolation and detection of NLG1-NTFs from cortical neurons incubated under control conditions or in the presence of TTX (2 μM) or bicuculline (50 μM) plus 4AP (25 μM, Bic/4AP) to decrease or increase network activity, respectively. Total surface-labeled NLG1 corresponding to 10% of precipitated protein is shown in the left lane (Surface). Molecular mass markers in kDa are shown on the right. **(B)** Data represent means ± SEMs of NLG1-NTFs produced under the indicated conditions normalized to control. n = 3, *p < 0.05 relative to control.

Interestingly, whereas blocking action potentials across the entire neuronal network using tetrodotoxin (TTX, 2 μM)

resulted in decreased generation of NLG1-NTFs (0.65 ± 0.09 of control), increasing network activity by simultaneously blocking inhibitory synaptic transmission with the $GABA_A$ receptor antagonist bicuculline (50 μM) and K^+ channels with 4-aminopyridine (4AP, 25 μM) significantly increased NLG1 cleavage (Bic/4AP, 1.5 ± 0.1 of control) (Figures 2A and 2B). Besides confirming our previous data showing that NLG1 cleavage is increased by neuronal depolarization these results also show that this process is bidirectionally regulated and can be reduced in response to decreased neuronal activity.

To mimic conditions that lead to robust loss of synaptic NLG1 (See Chapter 1, Figures 1 and 2), we depolarized cortical neurons by incubation with KCl (30 mM, 2 h).

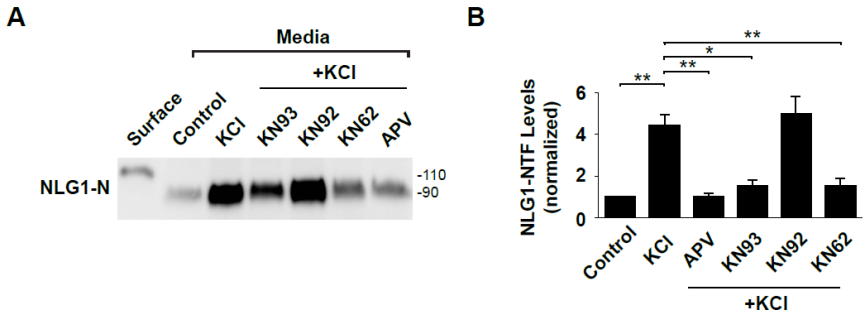

Figure 3. Activity Induces NLG1 Cleavage Through NMDARs and CaMK. **(A)** Biotinylation-based isolation and detection of NLG1-NTFs from cortical neurons incubated under control conditions or in the presence of KCl (30 mM, 2 h) with or without indicated pharmacological agents. Note the robust increase in NLG1-NTFs induced by KCl depolarization that is blocked by the NMDA receptor antagonist APV and CaMK inhibitors KN93 and KN62. **(B)** Data represent means ± SEMs of NLG1-NTFs produced under the indicated conditions normalized to control. n = 4, * p< 0.05, ** p < 0.01.

As previously described (Chapter 1, Figures 1 and 2) KCl mediated depolarization led to a dramatic increase in NLG1-NTFs (4.4 ± 0.5 fold) compared to control conditions (Figures 3A and 3B). Interestingly, this depolarization-induced cleavage of NLG1 was abolished by the NMDA receptor antagonist APV (50 μM). Activation of NMDA receptors elicits Ca^{2+} influx and activation of Ca^{2+}/calmodulin-dependent kinases (CaMK) including CaMKII (Fink and Meyer, 2002). Accordingly, inhibition of CaMK using either KN93 (5 μM) or KN62 (10 μM), but not the inactive isomer KN92 (5 μM), significantly reduced activity-induced cleavage of NLG1 (KN93, 1.5 ± 0.6; KN62, 1.5 ± 0.5; KN92, 5.0 ± 1.1 fold increase in NLG1-NTFs relative to control). These results show that activity-induced cleavage of NLG1 is triggered by NMDA receptor activation and requires Ca^{2+}/calmodulin-dependent kinase activity.

What enzyme is responsible for NLG1 cleavage? Among the diverse cellular proteolytic enzymes, MMPs comprise a large family of secreted endoproteases that mediate cell migration, signaling, morphogenesis, and development in diverse tissues (Ethell and Ethell, 2007; Sternlicht and Werb, 2001). Our previous results described in Chapter 1 demonstrated that the KCl-induced loss of full-length NLG1 in cortical neuron lysates was abrogated by the broad spectrum MMP inhibitor GM6001 (See Chapter 1, Figure 4). This result was confirmed using our biotinylation-based isolation method where incubation with GM6001 (10 μM) reduced basal NLG1 cleavage and prevented KCl-induced cleavage of NLG1 (fold increase relative to control: KCl, 2.62 ± 0.20; KCl + GM6001, 0.53 ± 0.05; Figures 4A and

4B). In order to determine the specific Matrix Metalloprotease involved in NLG1 ectodomain shedding we followed a candidate approach based on pharmacological inhibition of MMP activity. MMP-2, -3 and -9 are the most abundant MMPs in the brain and have been implicated in synaptic maturation and multiple forms of synaptic plasticity (Ethell and Ethell, 2007; Yong, 2005). This prompted us to address how these specific proteases affected KCl-induced cleavage of NLG1.

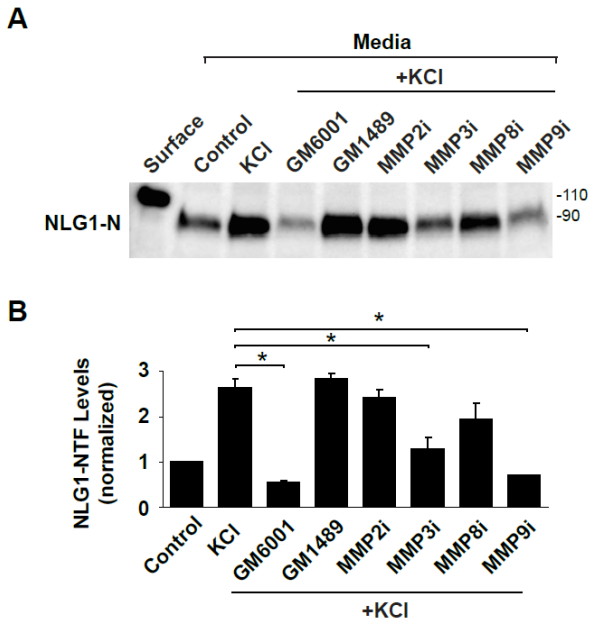

Figure 4. MMP9 Mediates Activity-Dependent Cleavage of NLG1. (A) Isolation and detection of ~90 kDa NLG1-NTFs following biotinylation of cortical neurons incubated under control conditions or in the presence of KCl with or without MMP inhibitors. Molecular mass markers in kDa are shown on the right. **(B)** Data represent means ± SEMs of NLG1-NTFs produced under the indicated conditions normalized to control. n = 4, *p < 0.01.

Incubation with the selective MMP9 inhibitor I (0.5 μM) or MMP3 inhibitor III (50 μM) significantly reduced KCl-induced cleavage (0.68 ± 0.02 and 1.27 ± 0.27 fold increase in NLG1-NTFs relatively to control, respectively), with MMP9 inhibitor I eliciting a more robust reduction. Conversely, the MMP1 inhibitor GM1489 (5 nM), MMP2 inhibitor III (50 μM) and MMP8 inhibitor I (10 μM) had no significant effect on activity-induced NLG1 cleavage (2.82 ± 0.14, 2.40 ± 0.19 and 1.92 ± 0.37 relatively to control, respectively).

MMPs are secreted as inactive zymogens and require further processing to become enzymatically active (Ethell and Ethell, 2007; Van Wart and Birkedal-Hansen, 1990). MMP3 is an upstream activator of MMP9 capable of cleaving its inhibitory prodomain (Ogata et al., 1992). This raised the possibility that the partial blockade of KCl-induced NLG1 cleavage by MMP3 inhibition could be due to compromised MMP9 activation. To test this hypothesis and determine which MMP is the terminal protease cleaving NLG1 we treated neurons with 4-aminophenylmercuric acetate (APMA), a mercury-based compound that indiscriminately activates all MMPs by cleaving their prodomain (Van Wart and Birkedal-Hansen, 1990), and tested the effect of specific MMP inhibitors on APMA mediated cleavage. Since APMA activates all MMPs, only inhibition of the final effector protease will impair NLG1 cleavage.

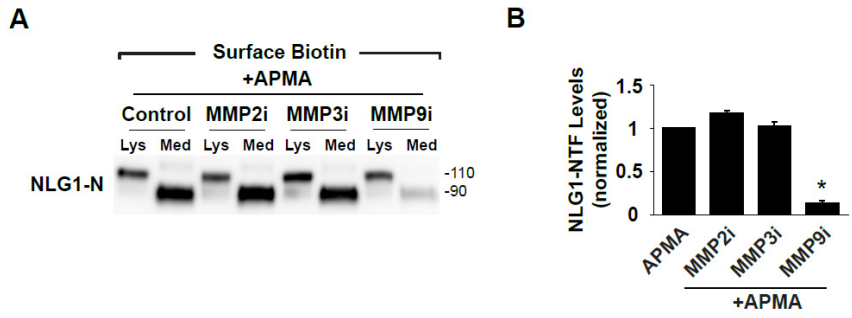

Figure 5. MMP9 Mediates Activity-Dependent Cleavage of NLG1. (A) Following surface biotinylation, cortical neurons were treated with the nonselective MMP activator APMA (0.5 mM, 15 min) with or without MMP inhibitors. Lysates (Lys) and soluble media (Med) were subjected to streptavidin precipitation prior to immunoblot analysis for NLG1 using an N-terminal antibody (4C12). Note that MMP9 inhibitors block APMA-induced cleavage. **(B)** Data represent means ± SEMs of NLG1-NTFs produced under the indicated conditions normalized to control. n = 3, *p < 0.05 relative to APMA alone.

Indeed, brief incubation of cortical neurons with 0.5 mM APMA for 15 min induced robust generation of NLG1-NTFs (Figures 5A and 5B). Co-incubation with MMP9 inhibitor I almost completely blocked APMA-induced cleavage while inhibition of MMP3 or MMP2 had no effect. Based on these results we conclude that MMP9 is most likely the terminal effector protease responsible for cleavage of NLG1 while MMP3 might be an upstream component of the signaling cascade activating MMP9.

Deglycosylation of endogenous NLG1-NTFs produces a ~70 kDa species (Chapter 1, Figure 6) which, based on amino acid mass, indicates that cleavage occurs in the extracellular juxtamembrane domain of NLG1. To determine the specific region of NLG1 targeted for cleavage, we generated a series of

mutants with sequential deletions and amino acid replacements in its juxtamembrane domain (Figure 6A).

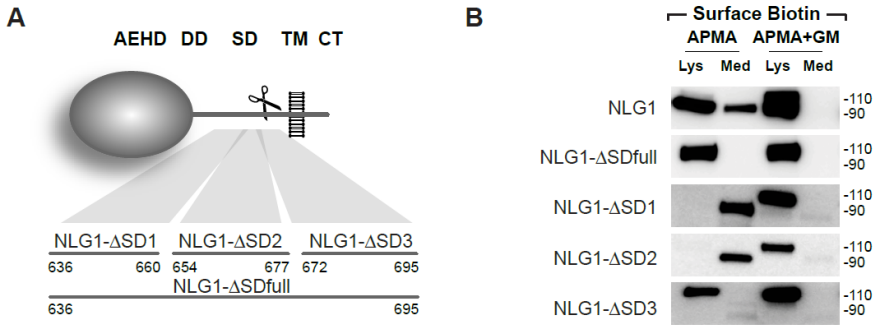

Figure 6. Extracellular Cleavage of NLG1 Occurs Near Its Transmembrane Domain. (A) Model illustrating the location of the NLG1-ΔSD substitution mutants in relation to NLG1 domains: AEHD, acetylcholinesterase homology domain; DD, dimerization domain; SD, stalk domain; TM, transmembrane domain; CT, C-terminal domain. Numbers indicate amino acids. **(B)** Biotinylation-based cleavage assay of COS7 cells expressing the indicated NLG1 mutants. Total lysates (Lys) or media (Med) were collected and subjected to immunoblot analysis following treatment with the MMP activator APMA with or without the broad spectrum MMP inhibitor GM6001 (GM). Both NLG1-ΔSDfull and NLG-ΔSD3 were resistant to APMA-induced cleavage indicating that MMP cleavage sites lie between residues 672 and 695. Molecular mass markers are shown at the right.

These newly generated NLG1 mutants were tested for their resistance to APMA-induced cleavage using biotinylation-based labeling and isolation of NLG1-NTFs in COS7 cells (Figure 6). The presence of surface biotinylation labeling indicates that surface trafficking and expression of all mutants is not compromised. Brief incubation with APMA results in extensive ectodomain shedding of wild-type GFP-NLG1 (Figure 6B). This effect was fully blocked by GM6001 confirming that NLG1

cleavage is MMP-dependent under these conditions (Figure 6B). Substituting 60 amino acids (aa) of the NLG1 stalk domain (aa 636-695) with the polylinker GAAAAA resulted in a mutant (NLG1-ΔSDfull) that is resistant to APMA-induced cleavage (Figures 6A and 6B). Within this 60 residue stretch, deletion of amino acids 672-695 (NLG1-ΔSD3) and replacement with the polylinker GAAAAA likewise abolished APMA-induced cleavage whereas mutation of more membrane-distal sequences did not (aa 636-660, NLG-ΔSD1; aa 654-677, NLG-ΔSD2). This indicates that NLG1 cleavage occurs in it juxtramembrane region within the 24 aminoacid sequence upstream of the transmembrane domain. We attempted to further resolve the precise cleavage site, but shorter deletions or different single site substitution mutants were all cleaved upon APMA treatment (data not shown), suggesting the presence of multiple MMP target sequences within this 24 aa domain. However, these results indicate that cleavage of NLG1 occurs in the region corresponding to amino acids 672-695 of NLG1.

Different NLG isoforms partition to and regulate different subsets of synapses (Budreck and Scheiffele, 2007; Graf et al., 2004; Song et al., 1999; Varoqueaux et al., 2004). Hence, the differential regulation of NLG isoforms may have important implications for neuronal function (Dahlhaus et al., 2010; Hines et al., 2008; Prange et al., 2004). To determine if all NLGs are processed similarly in response to activity we measure total NLG levels following KCl depolarization (Figure 7A).

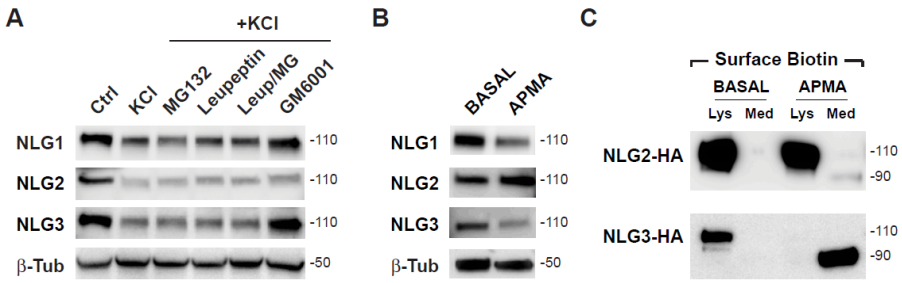

Figure 7. Differential regulation of NLG2 cleavage. (A) Immunoblot analysis of NLG1, NLG2 and NLG3 in total lysates from cortical cultures following 2 h incubation in neurobasal medium (Ctrl) or medium supplemented with 30 mM KCl alone (KCl) or with MG132 (10 μM), leupeptin (200 μM), leupeptin plus MG132 (Leup/MG) or GM6001 (10 μM). Note that GM6001 prevents KCl-induced loss of total NLG1 and NLG3 but not NLG2. **(B)** Immunoblot analysis of NLG1, NLG2 and NLG3 in total lysates from cortical cultures following 15min incubation in neurobasal medium (Ctrl) or medium supplemented with 0.5 mM APMA (APMA). Note that NLG2 levels are not reduced by APMA **(C)** Biotinylation-based cleavage assay of COS7 cells expressing the indicated NLG isoform HA tagged mutants. Total lysates (Lys) or media (Med) were collected and subjected to immunoblot analysis following treatment with the MMP activator APMA. Note the presence of residual soluble NLG2-HA bands in the media.

All NLGs exhibit a robust decrease in overall levels following KCl treatment that is unaffected by lysosome and proteasome inhibition. This suggests that all NLG isoforms are processed by similar mechanisms upon KCl depolarization. However, only degradation of NLG1 and NLG3, but not NLG2, could be completely rescued by application of the broad spectrum MMP inhibitor GM6001 (Figure 7A). This indicates that NLG1 and NLG3 are cleaved my MMPs under these conditions and suggests that NLG2 is regulated by a different mechanism. Moreover, incubation of cortical cultures with 0.5mM APMA for

15 min resulted in a dramatic reduction of NLG1 and NLG3 levels, but not NLG2 (Figure 7B). Again, this is consistent with a differential regulation of NLG2 compared with NLG1 and NLG3. Unfortunately, due to the lack of NLG2 specific antibodies that target the N-terminal domain of the protein we could not address NLG2 cleavage based on surface biotinylation. However, to address if NLG2 also undergoes ectodomain shedding we expressed HA-tagged forms of NLG2 and NLG3 in COS7 cells and measured the release of NLG-NTFs after APMA treatment using surface biotinylation (Figure 7C). While surface NLG3-HA was completely cleaved and released to the extracellular media in response to APMA stimulation, NLG2-HA remained mostly associated with the membrane fraction. This result is consistent with lack of NLG2 degradation following APMA incubation in neuronal cultures (Figure 7B). However, residual amounts of soluble NLG2-HA fragments could still be detected in the media of APMA treated cultures (Figure 7C, top panel) indicating that NLG2 can also undergo ectodomain shedding. Nevertheless, despite being cleaved this data indicates that NLG2 is differentially regulated when compared with other NLG isoforms, potentially by a different set of proteases that are not blocked by GM6001 and not activated by APMA.

The upregulation of MMP9 in response to seizures (Szklarczyk et al., 2002) suggested that NLG1 cleavage could also be regulated by activity during adult stages. To address this hypothesis we measured NLG1-NTFs generated during pilocarpine induced *status epilepticus* (PSE) in mice. This paradigm is characterized by recurrent activation of hippocampal circuits (Curia et al., 2008).

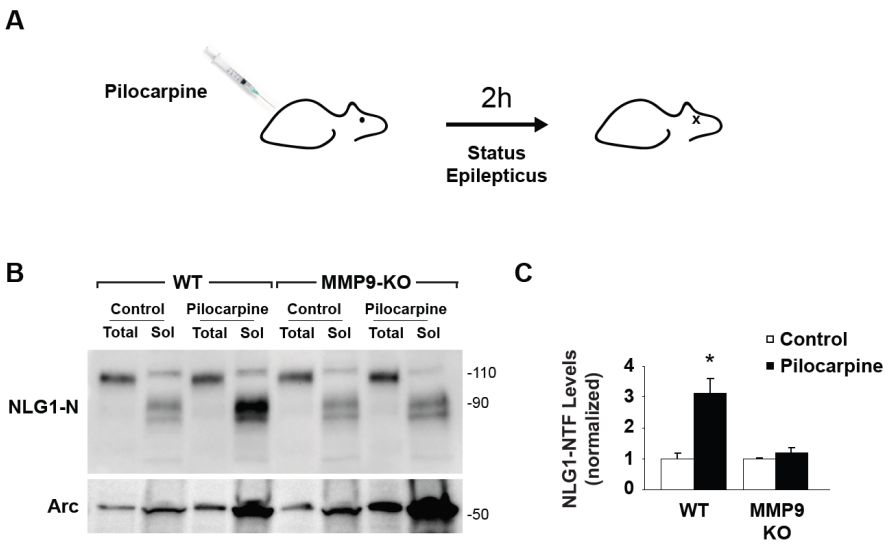

Figure 8. NLG1 is cleaved in response to epileptic seizures in the hippocampus by MMP-9. (A) Schematic of the experimental conditions **(B)** WT and MMP9 KO mice were injected with pilocarpine (315 mg/kg) to induce status epilepticus (Pilocarpine) or saline (Control). After 2 h, hippocampi were isolated and homogenized and either total extracts (5 μg) or soluble fractions (Sol, 50 μg) were immunoblotted for NLG1. Note the increased NLG1-NTFs after pilocarpine-induced seizures in WT and the abrogation of this response in MMP9-KO animals. Pilocarpine upregulates Arc in both wildtype and MMP9 KO animals. **(C)** Data represent means ± SEMs of NLG1-NTFs produced under the indicated conditions normalized to control. n = 9, *$p < 0.05$.

Intraperitoneal administration of pilocarpine in P60 mice induced robust epileptic seizures and resulted in a 3.12 ± 0.47 fold increase of soluble NLG1-NTFs in the hippocampus after 2h of PSE induction (Figures 8B and 8C). These findings indicate that increased neuronal activity also upregulates NLG1 cleavage *in vivo.* To address if MMP9 also mediates NLG1 cleavage under pilocarpine induced *status epilepticus*, we repeated these experiments in MMP9 null mice. Interestingly, soluble fractions of

MMP9-KO hippocampus under basal conditions still exhibit normal levels of NLG1-NTFs indicating that NLG1 cleavage still occurs in the absence of MMP9. However, 2h of PSE characterized by robust seizures failed to elevate soluble NLG1-NTFs in hippocampus when compared to saline controls (Figures 8B and 8C). As a control for seizure activity we measured levels of the activity-regulated cytoskeleton-associated protein (Arc) and both wild-type and MMP9 KO mice exhibited significant Arc upregulation after pilocarpine administration (Figure 8B). These findings indicate that activity triggers NLG1 cleavage *in vivo* through MMP9 activation. Moreover, these results suggest a potential link between NLG1 cleavage and circuit remodeling during epileptogenesis.

Discussion

In this section, we have characterized the molecular and signaling pathways regulating activity-dependent NLG1 cleavage. Using a newly developed assay based on biotinylation of surface proteins we found that cleavage of NLG1 at the plasma membrane is bidirectionally regulated by neuronal activity, requires NMDA receptor signaling, Ca^{2+}/calmodulin-dependent kinase activity and is mediated by the activity-regulated and brain-expressed Matrix Metalloprotease-9.

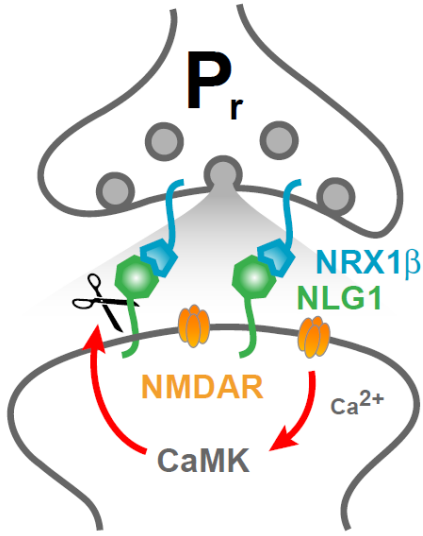

Figure 9. Schematic model for activity-induced MMP9 cleavage of NLG1. Increased neuronal activity activates a proteolytic cascade of MMPs via NMDAR and CaMK signaling. Ultimately, MMP9 activation cleaves NLG1 releasing NLG1-NTFs to the media. NMDAR, N-methyl-D-aspartate receptor; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; Scissors represent MMP9.

Moreover, these results define a novel link between NLG-NRX adhesion and a NMDAR-CaMK-MMP9 signaling cascade. In addition, we found that cleavage of NLG1 is highly upregulated in the hippocampus during epileptic seizures. Previous data described in Chapter 1 already has shown that NLG1 cleavage can be regulated by changes in neuronal activity *in vivo*. Besides providing additional evidence that NLG1 shedding is dynamically regulated by activity *in vivo,* the increase of NLG1-NTFs levels during epileptic seizures indicates that this process can also occur in adult brains outside developmental periods of circuit maturation. Indeed, recent work has implicated NLG1 in the expression of LTP in the amygdala suggesting that NLG1 is not only involved in the maturation and stabilization of synapses but is also required for synaptic function in later stages of development (Jung et al., 2010; Kim et al., 2008). Moreover, the increase in NLG1-NTFs following pilocarpine injection was absent in MMP9 KO mice indicating that MMP9 is required for seizure-induced cleavage of NLG1 in the hippocampus. This result is consistent with our findings in dissociated cultures where MMP9 inhibition abrogated the increase in NLG1-NTFs induced by KCl stimulation. Thus, MMP9 is likely an important protease regulating NLG1 cleavage in contexts of increased neuronal activity.

Epileptogenic patterns of activity in the hippocampus lead to extensive fiber sprouting and circuit remodeling which are thought to underlie the generation of recurrent seizures in later stages of the disease (Isokawa, 1998, 2000). Interestingly, not only is MMP9 upregulated in response to epileptic seizures but it is also required for the synaptic pruning observed in the days

following seizure induction (Szklarczyk et al., 2002; Wilczynski et al., 2008). This important finding suggests that the molecular targets of MMP9 in hippocampal neurons may be critical regulators of synapse maintenance and stability during epileptogenesis. Indeed, MMP7, an upstream activator of MMP-9, induces morphological changes in dendritic spines converting them to elongated filopodia-like structures (Bilousova et al., 2006). Conversely, MMP9 inhibition accelerates spine maturation in neuronal cultures and increases the total fraction of mushroom spines (Bilousova et al., 2009). Together these results suggest that MMP9 activity may indeed be an important regulator of synapse maintenance.

Besides interacting with NLGs, presynaptic NRXs also bind to other postsynaptic molecules such as β-dystroglycan (Sugita et al., 1999; Sugita et al., 2001). Interestingly, β-dystroglycan is also cleaved by MMP9 in response to kainate induced seizures (Michaluk et al., 2007), suggesting that MMP9 may be an important regulator of NRX function by targeting multiple of its postsynaptic binding partners. Given the well-established role of NLGs and NRXs in synaptic maturation and maintenance (Chubykin et al., 2007; Varoqueaux et al., 2006), MMP9 could then potentially regulate synapse stability by controlling NLG-NRX function. Moreover, the developmental profile of NLG1-NTFs shown in Chapter1 (Figure 8) indicates that NLG1 cleavage is highly upregulated during early developmental stages and declines during the first 2 postnatal weeks, which is the period of synapse formation in the neocortex. This hypothesis is further supported by upregulation of NLG1-NTFs observed in

response to visual experience during critical period, a paradigm that also elicits subsequent synapse remodeling. Hence, cleavage of NLG1 emerges as a potential general mechanism associated with synaptic remodeling during development and disease.

Alternatively, cleavage of NLG1 induced by strong activity could also provide a protective mechanism to ensure proper balance between excitation and inhibition in complex circuits. In fact, we found that NLG1 cleavage is bidirectionally regulated by activity both *in vitro,* in response to Bic/4AP and TTX treatment (Figure 2) and *in vivo* in response to dark rearing and brief light exposure after dark rearing (Chapter 1, Figure 8). Given that NLG1 increases overall excitatory drive by preferentially stabilizing (Chubykin et al., 2007) and augmenting the release probability of excitatory synapses (Futai et al., 2007), the bidirectional control of NLG1 levels by proteolytic regulation could mediate homeostatic compensatory changes in synaptic transmission to balance overall excitability. Interestingly, our data indicates that NLG2 cleavage exhibits a different and specific pattern of regulation compared with NLG1 and NLG3. Since NLG2 and NLG1/3 exhibit a preferential enrichment in inhibitory and excitatory synapses, respectively (Budreck and Scheiffele, 2007; Graf et al., 2004; Song et al., 1999; Varoqueaux et al., 2004), the activation of specific proteolytic pathways targeting NLG2 could also in principle, affect the balance between excitation and inhibition. In fact, deletion of NLG2 decreases IPSC amplitudes in acute cortical slices (Chubykin et al., 2007) and increases the excitatory-to-inhibitory ratio in the hippocampus (Poulopoulos et al., 2009) and cortex (Gibson et

al., 2009), whereas transgenic overexpression of NLG2 leads to increased mIPSC frequency in prefrontal cortical regions (Hines et al., 2008). This interesting hypothesis will certainly be the focus of future studies.

Excitotoxicity driven by unrestrained glutamate release contributes to neuronal degeneration in many acute CNS diseases, including ischemia, trauma, and epilepsy (Arundine and Tymianski, 2003). In particular, excessive NMDAR dependent Ca2+ influx has been shown to be prone to induce deleterious effects in neurons (Arundine and Tymianski, 2004). Interestingly, NLG1 levels in dissociated neurons are directly correlated with NMDAR EPSC amplitudes (Chubykin et al., 2007). Moreover, αNRX deletion induces loss of NMDAR function in cortical slices (Kattenstroth et al., 2004). Hence, an acute loss of NLG1 in response to increased activity could mediate decreased NMDAR dependent Ca^{2+} influx, protecting neurons from glutamate excitotoxicity. Consistent with this hypothesis, it has been shown that MMP9 activity increases synaptic NMDAR mobility which potentially decreases NMDAR synaptic currents (Michaluk et al., 2009). This effect has been attributed to Integrin-β1 signaling however integrin function was assessed by overnight antibody cross linking which may cause indirect secondary effects. Thus, other mechanisms may be involved in the destabilization of synaptic NMDAR by MMP9.

It is important to note that basal levels of NLG1-NTFs are still present in MMP9 KO brain extracts. This indicates that even in the absence of MMP9 NLG1 cleavage still occurs which

indicates the existence of parallel proteolytic mechanisms targeting NLG1 under basal conditions. Consistent with this idea, none of the pharmacological MMP inhibitors tested in our *in vitro* assays was able to fully block NLG1 cleavage. Moreover, multiple soluble NLG1-NTF species can be detected throughout the brain (Chapter 1, Figure 5), indicating that NLG1 probably contains multiple cleavage sites. This observation provides a plausible explanation for why single amino acid point mutations in the stalk region of NLG1 failed to generate mutants that would resist APMA induced MMP-dependent cleavage, which instead required substitution of a longer 24 amino acid segment (Figures 6A and 6B). Hence, these results suggest that the regulation of NLG1 cleavage is probably under control of multiple proteolytic mechanisms that may be differently regulated depending of cellular context. It will be important for future studies to define in detail the activity-independent and MMP9-independent mechanisms responsible for NLG1 cleavage and their potential role in synapse development and plasticity.

Finally, our results indicate that the activity-induced cleavage of NLG1 requires NMDAR and CaMK signaling. Previous studies have shown that the synaptogenic effect and functional increase in EPSCs caused by NLG1 overexpression in cultured neurons are also abrogated by APV and CaMK inhibitor KN93 (Chubykin et al., 2007), however these experiments were based on chronic application of APV for 4 days. Nevertheless, our data suggest that NMDAR signaling controls NLG1 levels at synapses through MMP activity. Consistent with this hypothesis, APV blocks MMP9 upregulation in response to LTP suggesting that NMDAR controls MMP9 function in other activity-dependent

contexts (Nagy et al., 2006). Recent work has further shown that in response to brief depolarization, NLG1 internalization is halted via NMDAR signaling (Thyagarajan and Ting, 2010). Taken together, this data suggests that NLG1 is tightly regulated by NMDAR and CaMK activity at multiple levels and that the regulation of proteolysis and trafficking of NLGs may occur in tandem though similar signaling mechanisms.

Taken together, the results discussed here indicate that cleavage of NLGs is a highly regulated process that is involved in multiple activity-dependent cellular contexts and has the potential to generate different functional outcomes due to specific regulation of each NLG isoform. Moreover, our data implicates NLG processing during the early stages of epileptogenesis which may provide new insights regarding the pathophysiology of epileptic disorders.

Methods

Reagents and Antibodies

Dissociated primary neuronal cultures were prepared from cortex of embryonic day 18 or 19 Wistar rats. Tissue was dissociated by enzymatic papain digestion followed by brief mechanical trituration. Cultures were grown in Neurobasal media supplemented with B27 and Glutamax. For surface biotinylation experiments 700k cortical cells were plated onto 60mm petri dishes. Commercial antibodies used include N-terminal NLG1 antibody clone 4C12 (mouse, Synaptic Systems), C-terminal NLG2 polyclonal antibody (rabbit, Synaptic Systems), C-terminal NLG3 polyclonal antibody (rabbit, Synaptic Systems), Arc (C-7, rabbit, Santa Cruz) and HA.11 (mouse, Covance). Pharmacological inhibitors used include KN93 (Calbiochem), KN62 (Calbiochem), KN92 (Calbiochem), APV (Tocris), TTX (Tocris), GM6001 (Calbiochem), MMP9 inhibitor I (Calbiochem), MMP3 inhibitor III (Calbiochem), GM1489 (Calbiochem), MMP2 inhibitor III (Calbiochem), and MMP8 inhibitor I (Calbiochem), MG132 (SIGMA), Leupeptin (SIGMA) and APMA (SIGMA).

NLG1-ΔSDfull mutant was obtained by substitution of endogenous 636-695 residues with GAAAAA linker using primers 5'-ggcgccgcagcagcagcactaagtgtcacaatcgcagtg-3' and 5'-tgctgctgctgcggcgccattatgcagatgaggtacc-3'; NLG1-ΔSD1 (636-660) 5'-ggcgccgcagcagcagcaaggaaaaattccactccag-3' and 5'-tgctgctgctgcggcgccattatgcagatgaggtacc-3; NLG1-ΔSD2 (654-677) 5'-ggcgccgcagcagcagcacccaaacaacaaccaagtc-3' and 5'-

tgctgctgctgcggcgccgtctgttgatggcactttag-3'; NLG1-ΔSD3 (672-695) 5'-ggcgccgcagcagcagcactaagtgtcacaatcgcagtg-3'and 5'-tgctgctgctgcggcgccgggaaaggctgatgtgactgg-3'. For GFP-NLG1-HA the HA peptide YPYDVPDYA was inserted at the end of GFP-NLG1(ΔA) using primer 5'- gcatctcgagctaagcgtaatctggaaca tcgtatgggtataccctggttgttgaatgtg-3'. In all reactions the two self-annealing PCR products were templates in a reaction with primer 5'-gttggagctggtacctcatc-3' containing a KpnI site and 5'-gtcactcgagctataccctggttgttgaatg-3'. The respective product was subcloned into pcDNA3-GFP-NLG1 using KpnI and XhoI.

Surface Biotinylation and Isolation of Soluble Cleavage Products

For surface protein labeling, cortical cultures (700,000 cells/6cm plate) were incubated in PBS/Ca^{2+} supplemented with 1 mg/ml Sulfo-LC-Biotin-NHS (Pierce) for 10 min at 37ºC. Cultures were washed in PBS/Ca^{2+} containing 10 mM Tris pH7.4 and rinsed twice in PBS/Ca^{2+} and incubated in conditioned media at 37ºC for the indicated experimental time period. Pharmacological inhibitors were added 5 minutes prior to manipulations of neuronal activity. Following incubation at 37ºC, culture media was collected, supplemented with protease inhibitor cocktail (Roche) and centrifuged at 16,000 x g for 20 min. For surface-labeled controls, cultures were washed twice with PBS/Ca^{2+}, immediately lysed with precipitation buffer (PBS with 1% Triton-X100, 5 mM EDTA, 10 mM L-lysine pH7.4), sonicated and centrifuged at 16,000 x g for 20 min. Supernantants were incubated overnight with 50 µl NeutrAvidin Plus UltraLink Resin

(Pierce) at 4ºC and precipitated biotin conjugates subsequently washed twice and liberated by boiling in 2x sample buffer. Precipitated proteins were then resolved by SDS-PAGE prior to immunoblot analysis with the indicated antibodies

Pilocarpine-Induced Status Epilepticus

Two month female FVB or FVB.Cg-*Mmp9*^{tm1Tvu}/J (MMP9 KO) mice (~18–25 g) were injected intraperitoneally (i.p.) with 1 mg/kg scopolamine methyl-nitrate (Sigma) 30 min prior to the experiment. Status epilepticus was induced by i.p. injection of 315 mg/kg pilocarpine hydrochloride (Sigma) while controls were injected with vehicle alone (sterile 0.9% NaCl). The experiment was terminated two hours after pilocarpine injection and hippocampal tissue was collected and flash frozen. In the pilocarpine group, only animals that exhibited class IV or higher seizures during the course of the experiment were used for analysis.

Acknowledgements

For the realization of this work, Irina Lebedeva and Marguerita Klein prepared the neuronal cultures from dissociated mouse hippocampus and cortex. Susana da Silva kindly provided assistance in the pilocarpine induced seizure experiments.

Chapter 3

Acute Cleavage of Neuroligin-1 Destabilizes Presynaptic Neurexin-1β and Reduces Neurotransmitter Release

Introduction

The close association between pre and postsynaptic elements has long suggested that changes on either side of the synapse could be transmitted trans-synaptically (Lisman and Harris, 1993). In fact, whether the expression of Hebbian-forms of long term synaptic plasticity involve retrograde modulation of presynaptic function has been a source of heated debate for more than 20 years and remains an open question in the field (Bear and Malenka, 1994; Enoki et al., 2009; Malenka and Bear, 2004). However, a less controversial form of trans-synaptic signaling occurs during the homeostatic adaptation of neurotransmitter release to chronic changes in neuronal activity.

In mature hippocampal neurons, suppression of excitability by expression of an inward rectifier potassium channel (Kir2.1) increases mEPSC frequency (Burrone et al., 2002). Moreover, chronic silencing of network activity using tetrodotoxin (TTX) increases the size of presynaptic terminals and their respective pool of synaptic vesicles, with a concomitant increase in release probability (Murthy et al., 2001). Interestingly, a similar response is observed in response to chronic inhibition of AMPAR currents using the antagonist 3-dihydroxy-6-nitro-7-sulfamoyl-

benzo[f]quinoxaline-2,3-dione (NBQX) suggesting that retrograde signals may be involved in the generation of this response (Thiagarajan et al., 2005). Conversely, increased KCl concentration during development results in lower release probability and loss of excitatory inputs (Moulder et al., 2004), whereas 2h of 2Hz field stimulation of dissociated cultures causes an acute decrease in release probability (Branco et al., 2008). Importantly, this latter effect is blocked by NMDAR and AMPAR antagonists suggesting that this homeostatic response is induced postsynaptically and subsequently transduced by retrograde messengers. A similar adaptive response has been described in the *Drosophila* neuromuscular junction where blockade of postsynaptic glutamate receptors (Frank et al., 2006), or reduced cellular depolarization due to Kir2.1 expression (Paradis et al., 2001) increase presynaptic release of neurotransmitter without altering postsynaptic properties. Hence, several lines of evidence indicate that chronic changes in postsynaptic activity result in a homeostatic compensation of afferent neurotransmitter release.

Retrograde signaling can be mediated by many types of molecular mechanisms. Diffusible retrograde messengers including nitric oxide and endocannabinoids (eCB) are two well characterized systems capable of modulating neurotransmitter release in response to changes in postsynaptic activity (Regehr et al., 2009). However, given their confined localization at synapses and restricted number of interactions, adhesion molecules offer the potential for tight local control over presynaptic function. For instance, postsynaptic ablation of N-Cadherin induces defects in synaptic vesicle release under

conditions of high-release activity (Jungling et al., 2006), whereas acute activation of presynaptic ephrin-B2 by soluble forms of EphB2 induces a rapid increase in release probability in optic tectum neurons (Lim et al., 2008).

Recently, NLGs have emerged as important trans-synaptic signaling mediators. NLGs are capable of inducing functional maturation of presynaptic terminals (Dean et al., 2003; Prange et al., 2004; Scheiffele et al., 2000; Wittenmayer et al., 2009), while transgenic expression of NLG1 results in extended active zones and increased reserve pool size (Dahlhaus et al., 2010). Interestingly, overexpression of NLG1 in hippocampal slices and cultured neurons results in increased release probability through a Neurexin (NRX) dependent mechanism (Futai et al., 2007; Ko et al., 2009b; Stan et al., 2010), suggesting that NLG1 can modulate presynaptic function trans-synaptically. Indeed, neurons lacking NLG1-3 present decreased miniature EPSCs (mEPSCs) frequency consistent with impaired presynaptic function (Varoqueaux et al., 2006). Moreover, neurons lacking αNRX1-3 exhibit deficits in synaptic transmission due to impaired N-type Ca^{2+} channel function despite normal synaptic vesicle readily releasable pool size (Missler et al., 2003). In addition, chronic disruption of endogenous NLG-NRX interactions with long term incubation of soluble Fc-NRX fragments decreases mEPSC frequency and release probability (Levinson et al., 2005). Taken together, these results suggest that NLGs and NRXs are important regulators of presynaptic function and that NLGs can signal retrogradely through NRXs. However, a limitation of these studies is that they all rely on long term manipulations of NLG and NRX function, which may

generate indirect compensatory mechanisms. Hence, whether trans-synaptic NLG-NRX adhesion can acutely regulate synapse transmission remains unclear.

The work described in the previous chapters, demonstrates that NLGs can be rapidly degraded in response to increased neuronal activity via MMP dependent proteolysis. Considering the strong association between NLGs and NRXs, acute proteolytic cleavage of NLGs could be an effective way to disassociate the NLG-NRX interaction and mediate fast synaptic signaling. In fact, acute proteolysis has been shown to elicit fast changes in synaptic transmission. For instance, acute application of recombinant MMP9 is able to induce LTP in hippocampal slices (Nagy et al., 2006) and increase NMDAR mobility in matter of minutes (Michaluk et al., 2009). Moreover, acute inhibition or displacement of the metalloprotease ADAM10 from synapses induces a rapid expansion of dendritic spine size and increase AMPA EPSCs (Malinverno et al., 2010). However, the promiscuous nature of MMP activity, where each MMP can cleave multiple targets and activate parallel proteolytic cascades (Ethell and Ethell, 2007), has been a limitation to address the specific functional consequences of cell adhesion molecule cleavage.

To overcome this limitation and address the functional consequence of NLG1 cleavage we developed a novel strategy using an exogenous protease that allows the cleavage of specific membrane proteins in a temporally controlled manner. Combining this strategy with real time confocal microscopy and electrophysiological measurements of neuronal activity we

demonstrate that acute cleavage of NLG1 results in destabilization and loss of its presynaptic partner Neurexin-1β. This effect is specific and not an indirect consequence of pre synaptic terminal collapse or loss of synaptic vesicle pools. In turn, NLG1 cleavage rapidly depresses synaptic transmission by abruptly reducing presynaptic release probability. Together, these results indicate that postsynaptic activity influences presynaptic function through NLG1 cleavage unveiling a novel form of trans-synaptic signaling that may be involved in multiple forms of synaptic plasticity.

Results

To address the functional effect of acute cleavage of NLG1 we developed a system to acutely and selectively cleave NLG1 (Figure 1A).

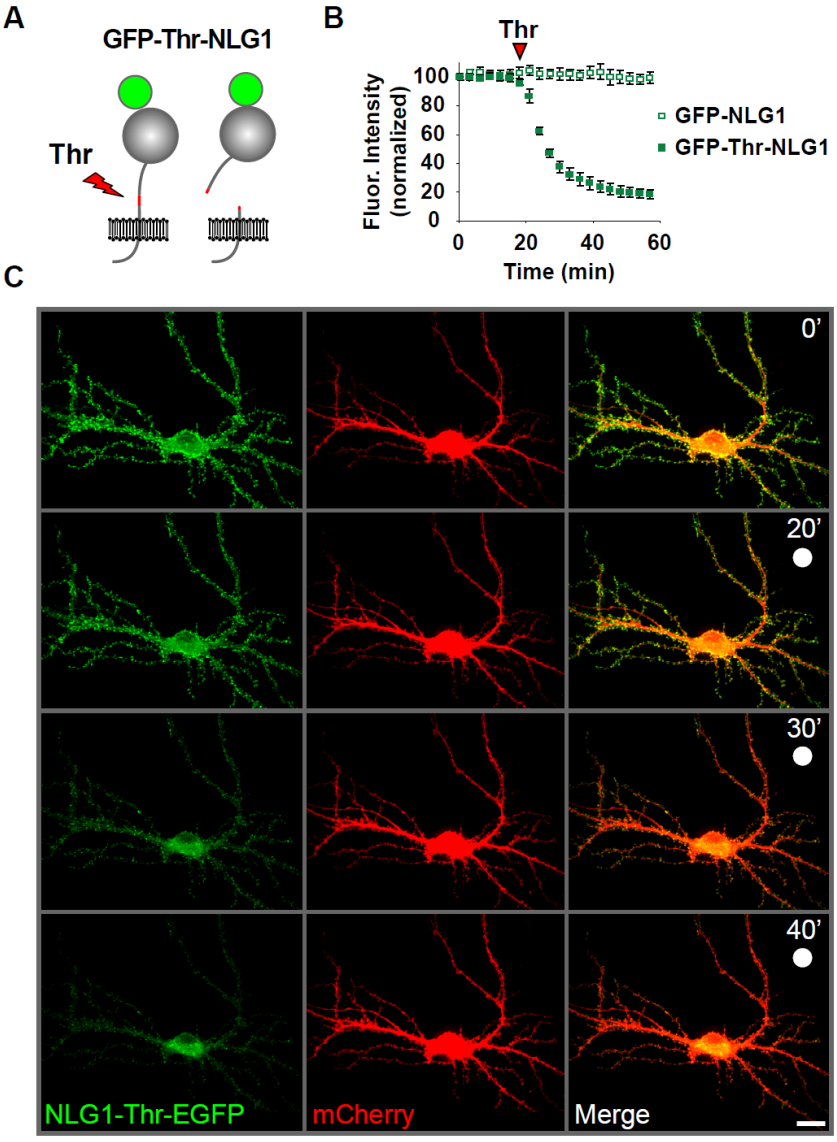

Figure 1. Acute cleavage of NLG1 using Thrombin. (A) Schematic of thrombin-cleavable NLG1 construct. Red region represents insertion of thrombin recognition sequence LVPRGS in the stalk domain of NLG1. **(B)** Quantification of time lapse experiment measuring fluorescence intensity before and after thrombin application of neurons expressing GFP-Thr-NLG1. **(C)** Hippocampal neuron (DIV21) expressing GFP-Thr-NLG1 and mCherry imaged live by confocal microscopy before and during thrombin incubation (5U/ml, 30 min). Thrombin application is indicated by the white circle. Note the robust loss of GFP fluorescence due to GFP-Thr-NLG1 cleavage. Time is indicated in minutes. Scale bar, 20 μm.

To achieve this, we inserted the thrombin recognition sequence LVPRGS at the stalk domain of NLG1 replacing the endogenous sequence TTTKVP, downstream of the dimerization domain. In these experiments, the NLG1ΔA splice variant lacking splice site A was chosen due to its stringent partition into excitatory synapses (Chih et al., 2006). The thrombin-cleavable mutant GFP-Thr-NLG1 targeted to synapses in a manner indistinguishable from wild-type GFP-NLG1 (Figure 1C). Incubation with 5U/ml thrombin for 30 min results in a marked (80.4 ± 3.4%) reduction in GFP-Thr-NLG1 fluorescence (Figures 1B and 1C). Control neurons transfected with GFP-NLG1 lacking a thrombin recognition sequence exhibited no change in GFP fluorescence after thrombin treatment (Figure 1B, 99.9 ± 1.5% of initial intensity), indicating that the reduced GFP fluorescence was not due to photobleaching. This can also be inferred by the fact that the internal pools of NLG1-GFP are not affected by thrombin incubation (see lower panel in Figure 1C - GFP signal is present in the soma after thrombin application potentially corresponding to NLG1-GFP pool present in the secretory

pathway). Nevertheless, the extensive and rapid loss of GFP fluorescence after thrombin incubation indicates that a large fraction of NLG1 extracellular fragment is released from synaptic sites after cleavage.

To test the functional effect of acute NLG1 cleavage, we used whole-cell electrophysiological recordings to measure synaptic transmission on neurons expressing GFP-Thr-NLG1. This system allowed us to monitor synaptic transmission in the same neuron before, during, and after the selective thrombin-induced cleavage of NLG1 overcoming the inherent variability of synaptic properties in cultured neurons (Figure 2A). Electrophysiological measurements from neurons expressing wild-type GFP-NLG1 lacking the thrombin cleavage site were used to control for possible off-target effects of thrombin application. We first examined the effect of NLG1 cleavage on miniature excitatory postsynaptic currents (mEPSCs). Thrombin application reduced mEPSC frequency (44 ± 10% of baseline, $n = 8$, $p<0.01$; Figures 2B and 2C) with no significant effect on mEPSC amplitude (91 ± 8% of baseline, $n = 8$, $p>0.05$; Figures 2B and 2D) indicating that NLG1 cleavage does not affect postsynaptic AMPA receptor number or function, but rather reduces neurotransmitter release or decreases the number of functional excitatory synapses. Thrombin had no effect on mEPSCs in neurons expressing GFP-NLG1 (mEPSC Frequency, 100 ± 3% of baseline, $n = 5$, $p>0.05$; amplitude, 102 ± 4% of baseline, $n = 5$, $p>0.05$; Figures 2C and 2D), indicating that the effects of thrombin on mEPSC frequency are specifically generated by NLG1 cleavage.

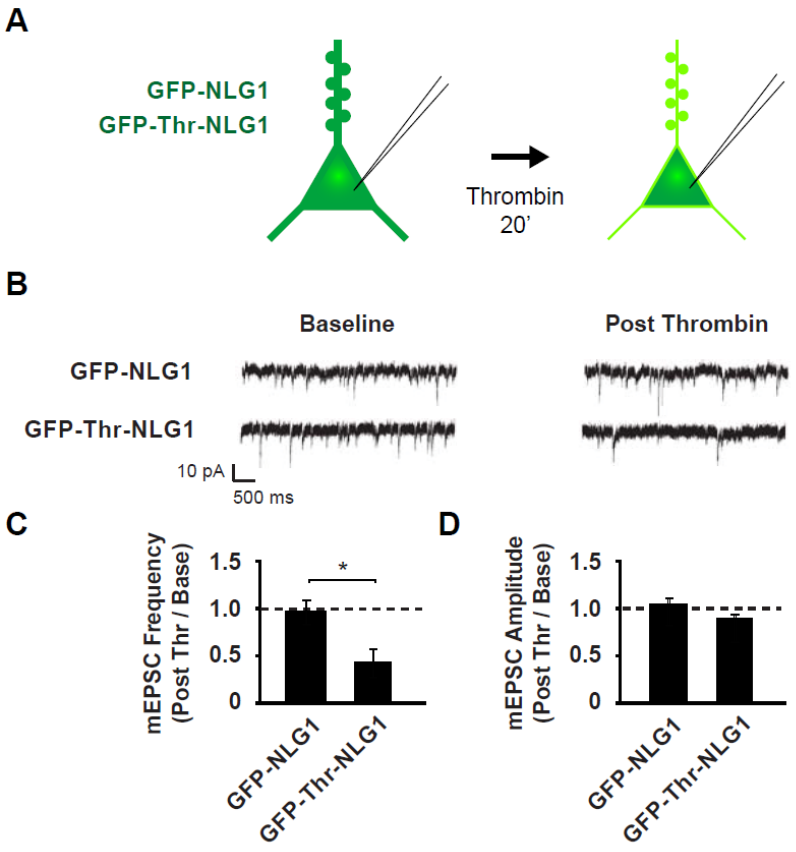

Figure 2. NLG1 cleavage acutely dampens excitatory synaptic transmission. (A) Schematic representation of the experimental design. Electrophysiological recordings of hippocampal neurons (DIV18) expressing either thrombin-cleavable GFP-Thr-NLG1 or non-cleavable GFP-NLG1 control were performed before and after thrombin incubation. **(B)** Representative miniature EPSCs (mEPSCs) recorded before (Baseline) and after (Post) thrombin. **(C)** Quantification showing that thrombin-induced cleavage of NLG1 reduces mEPSC frequencies without affecting **(D)** amplitudes. Thrombin has no effect on mEPSC frequency or amplitude in neurons expressing NLG1-EGFP lacking a thrombin cleavage site. n = 5, 8 for GFP-Thr-NLG1 and GFP-NLG, respectively. *p < 0.01, unpaired t-test.

Interestingly, NLG1 cleavage failed to alter mIPSC frequency or amplitude (mIPSC Frequency, 103 ± 3%; mIPSC Amplitude, 98 ± 8% of baseline, n = 6, p>0.05; Figure 3), indicating that NLG1 cleavage does not alter neurotransmitter release at inhibitory synapses. These results are consistent with previous studies demonstrating that NLG1 predominantly localizes to and regulates glutamatergic synapses (Song et al., 1999) (Chubykin et al., 2007; Graf et al., 2004).

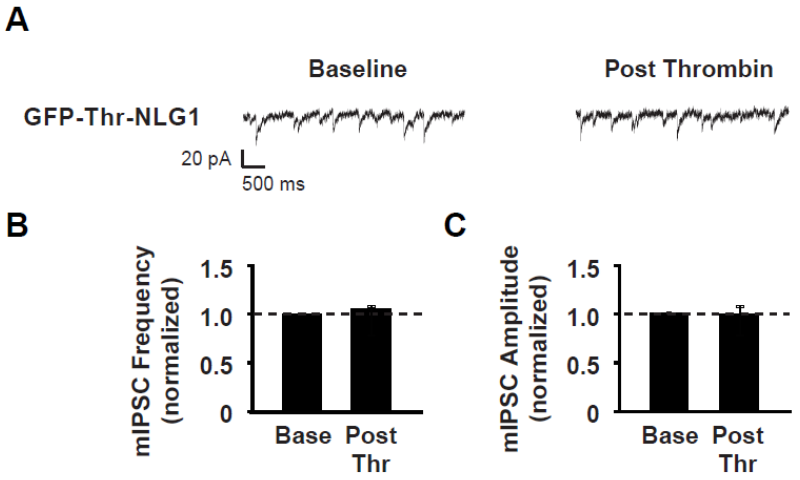

Figure 3. NLG1 cleavage has no effect in inhibitory synaptic transmission. **(A)** Representative mIPSCs before (Baseline) or after (Post) thrombin treatment in neurons expressing GFP-Thr-NLG1. **(B)** Quantification showing that NLG1 cleavage does not affect mIPSC frequency or **(C)** amplitude (right). Data in bar graphs represent mean ± SEM. n = 6.

The reduction observed in mEPSC frequency (Figures 2B and 2C) without any visible loss of dendritic spines (Figures 1C and 5A-C) suggested that NLG1 cleavage could be affecting presynaptic function.

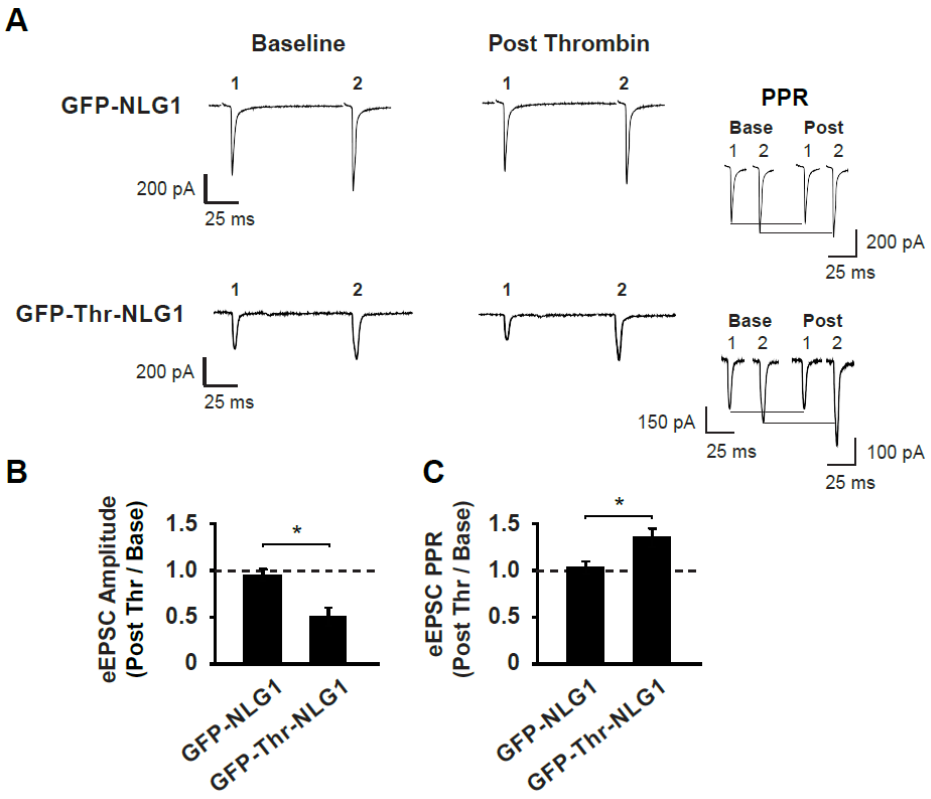

Figure 4. **Cleavage of NLG1 reduces release probability at excitatory synapses. (A)** Representative paired-pulse evoked EPSCs (eEPSCs) recorded before (Baseline) or after (Post) thrombin treatment in hippocampal neurons (DIV18) expressing either thrombin-cleavable GFP-Thr-NLG1 or GFL-NLG1 lacking a thrombin cleavage site. Insets depict traces before and after thrombin treatment, normalized to the amplitude of the first EPSP to indicate the increase in paired pulse ratio (PPR) after thrombin cleavage in cells expressing GFP-Thr-NLG1. **(B)** NLG1 cleavage reduces eEPSC amplitude. Data represent means ± SEM of eEPSC amplitudes after thrombin treatment normalized to amplitudes prior to thrombin treatment. n's = 6; *p < 0.05, unpaired t-test. **(C)** NLG1 cleavage increases paired pulse ratio (PPR). Data indicate means ± SEM of paired-pulse ratios after (Post) and before (Baseline) thrombin normalized to the GFP-NLG1 control. n's = 6; *p < 0.05, unpaired t-test. Note that an increase in PPR is consistent with a decrease in release probability.

To test this hypothesis further, we examined the effects of thrombin-induced NLG1 cleavage on evoked excitatory synaptic currents (eEPSCs) and their paired-pulse responses (Dobrunz and Stevens, 1997). Evoked responses were elicited by stimulating nearby cells with an interstimulus interval of 100 msec (Figure 4A). Recordings were made on the same cell before and after thrombin application. Thrombin treatment of neurons expressing GFP-Thr-NLG1 reduced the amplitude of the first response and increased the paired-pulse ratio (PPR) (eEPSC amplitude, 50 ± 18% of baseline, $n = 6$, $p < 0.05$; PPR, 139 ± 8% of baseline, $n = 6$, $p < 0.05$, Figure 4), strongly suggesting that NLG1 cleavage decreases the probability of neurotransmitter release (Dobrunz and Stevens, 1997). This effect was not attributable to thrombin treatment per se, as there was no change in amplitude or PPR in neurons expressing wild type NLG1-EGFP lacking a thrombin cleavage sequence (eEPSC amplitude, 95 ± 8% of baseline, $n = 6$, $p > 0.05$; PPR, 106 ± 6% of baseline, $n = 6$, $p > 0.05$; Figure 4). Together with the observed decrease in mEPSC frequency (Figure 2C), these findings demonstrate that juxtamembrane cleavage of postsynaptic NLG1 acutely dampens synaptic transmission due to a reduction in presynaptic neurotransmitter release. Thus, activity-dependent cleavage of Neuroligin defines a retrograde signal for rapid trans-synaptic tuning of synaptic strength.

To determine the molecular mechanism underlying this retrograde modulation of neurotransmitter we performed a series of studies based on microscopic analysis of different synaptic proteins during acute NLG1 cleavage. We initially addressed whether postsynaptic morphology was affected by thrombin

incubation. To achieve this we measured fluorescence variations after 30 min of thrombin incubation in neurons co-transfected with GFP-Thr-NLG1, and mCherry or PSD95-mCh.

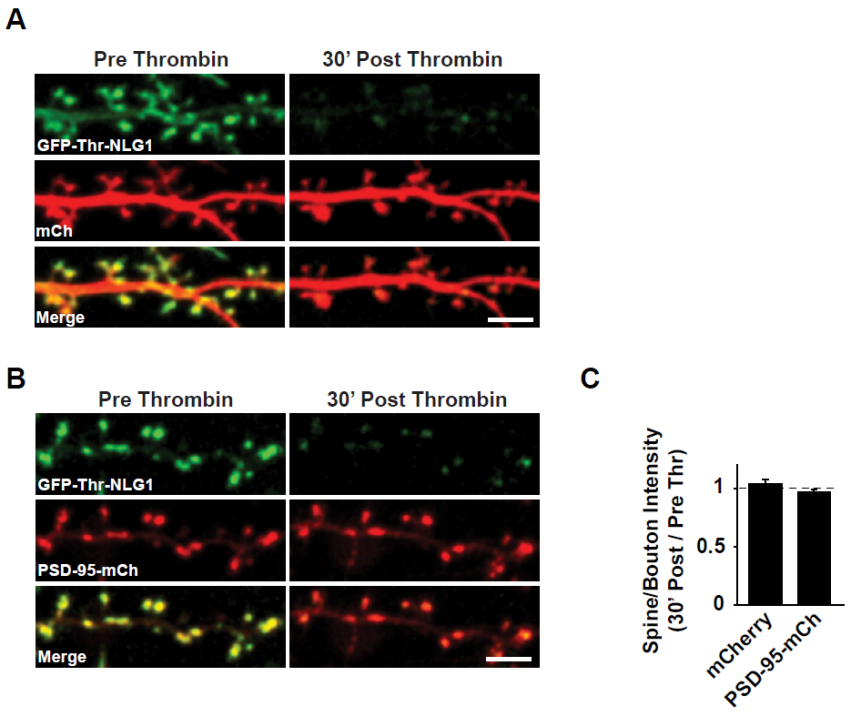

Figure 5. Spine volume and PSD95 levels unaffected by acute cleavage of NLG1. (A) Hippocampal neurons expressing GFP-Thr-NLG1 and mCherry were imaged before (Pre) and 30 min after (Post) thrombin incubation. Note the loss of GFP fluorescence upon thrombin cleavage. Scale bar, 5 μm. **(B)** Same as (A) but hippocampal neurons were transfected with GFP-Thr-NLG1 and PSD-95-mCh. Scale bar, 5 μm. **(C)** Ratio of fluorescence intensities 30 min after thrombin (Thr) cleavage relative to before thrombin addition at synapses containing GFP-Thr-NLG1. Data represent means ± SEM. mCherry, n = 307 spines; PSD-95-mCh, n = 403 spines.

Interestingly, no significant changes in fluorescence intensity were detected after thrombin treatment in mCherry cell fill ($\Delta F/F$; 1.04 ± 0.03; n=307 spines; Figures 5A and 5C) and PSD95-mCh puncta ($\Delta F/F$; 0.97 ± 0 .02; n=403 spines; Figures 5B and 5C). This indicates that acute NLG1 cleavage has no gross impact in PSD structure or dendritic spine volume. These results are consistent with the invariance observed in mEPSC amplitude after thrombin incubation and reinforce the hypothesis that NLG1 cleavage predominantly affects presynaptic function.

NLG1 forms a high affinity trans-synaptic complex with the presynaptic membrane protein Neurexin-1β (NRX1β), thereby regulating presynaptic assembly and function (Arac et al., 2007; Chen et al., 2008; Comoletti et al., 2007; Dean et al., 2003; Fabrichny et al., 2007; Futai et al., 2007; Ichtchenko et al., 1995; Prange et al., 2004; Scheiffele et al., 2000; Varoqueaux et al., 2006; Wittenmayer et al., 2009). To test whether acute cleavage of NLG1 alters the stability of the Neuroligin-Neurexin trans-synaptic adhesion complex we sequentially transfected cultures with GFP-Thr-NLG1 and NRX1β–mCh lacking splice site 4 in separate days. This method results in the transfection of two distinct neuronal populations with each construct, resulting in the labeling of pre and postsynaptic pairs with NRX1β-mCh and GFP-Thr-NLG, respectively (Figure 6A). At dually-labeled synapses, acute thrombin incubation caused a rapid and pronounced decrease in NRX1β-mCh fluorescence (Figures 6B and 6C; 0.51 ± 0.04 of initial fluorescence after 30 min of thrombin, n=87 synaptic pairs, $p<0.05$).

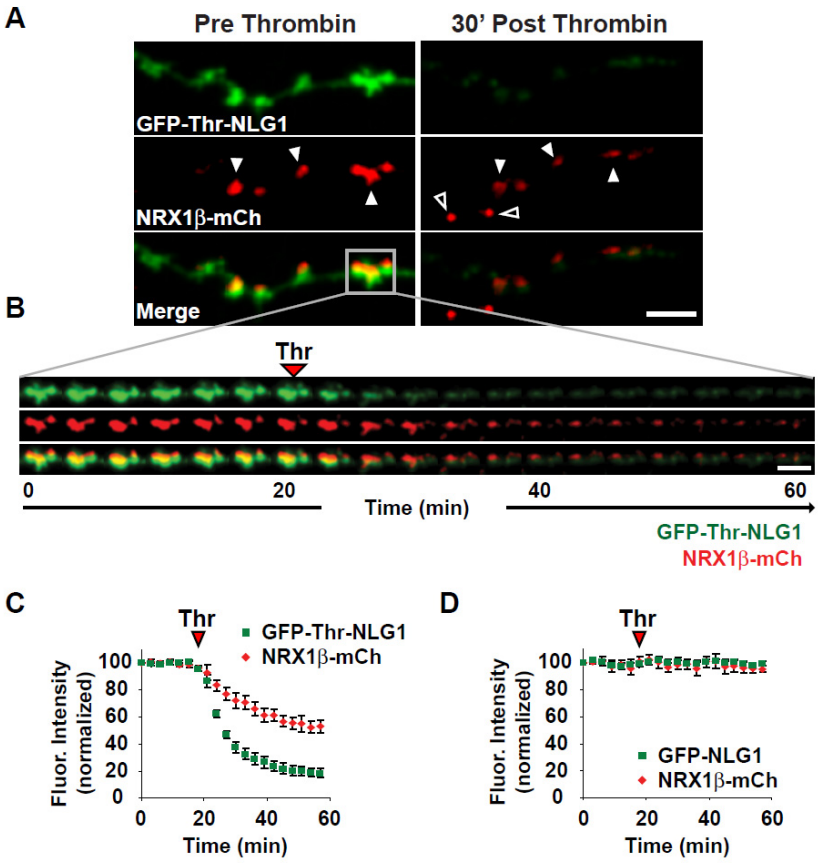

Figure 6. Acute NLG1 cleavage destabilizes presynaptic NRX1β. (A) Hippocampal cultures were sequentially transfected with GFP-Thr-NLG1 and NRX1β-mCh to label distinct postsynaptic and presynaptic neurons, respectively. Neurons were incubated with thrombin and imaged live by confocal microscopy. Solid arrowheads indicate presynaptic NRX1β-mCh apposed to postsynaptic GFP-Thr-NLG1 puncta. Note the thrombin-induced loss of NRX1β-mCh. Open arrows show newly generated mobile NRX1β-mCh puncta along the axon. Scale bar, 5 μm. **(B)** Timelapse of the highlighted region depicted in (A). The red arrowhead shows the time of thrombin (Thr) application. Note that thrombin-induced cleavage of GFP-Thr-NLG1 is followed by rapid loss of NRX1β-mCh. Scale bar, 5 μm. **(C)** Quantification of time lapse experiments as in (B). Plotted is fluorescence intensity before and after thrombin application at synapses containing presynaptic NRX1β-mCh along

with postsynaptic GFP-Thr-NLG1. The red arrowhead shows the time of thrombin (Thr) application. n = 87 synaptic pairs. **(D)** Same as (C) except neurons expressed GFP-NLG1 lacking a thrombin cleavage site. n = 76 synaptic pairs.

These results demonstrate that NLG1 cleavage destabilizes presynaptic NRX1β-mCh and suggest that the decrease in release probability observed after NLG1 shedding is due, at least in part, to loss of presynaptic Neurexins. Importantly, this destabilization is not an artifact caused by thrombin off-target effects as NRX1β-mCh puncta opposed to NLG1-GFP expressing synapses were unaffected by thrombin incubation (Figures 6A and 6D).

Interestingly, we noted the appearance of several mobile puncta containing high levels of NRX1β-mCh after thrombin incubation which raised the possibility that NLG1 cleavage could result in overall presynaptic terminal collapse or loss of synaptic vesicles from synaptic sites. To address this possibility we repeated the same experiments using Synaptophysin-mCh instead of NRX1β-mCh. We detected no changes in presynaptic Synaptophysin-mCh signal intensity (0.99 ± 0.08, n=43 synaptic pairs, Figures 7A and 7B) indicating that destabilization of NRX1β by NLG1 cleavage is specific and not due to indirect structural changes in presynaptic terminals.

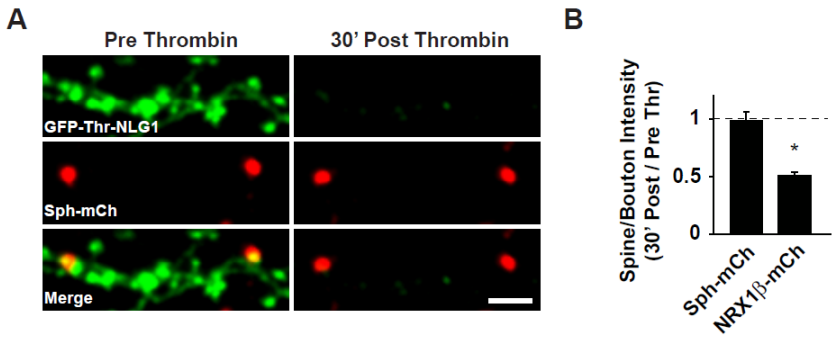

Figure 7. Acute NLG1 cleavage does not affect presynaptic Synaptophysin-mCh. (A) Hippocampal cultures were sequentially transfected with GFP-Thr-NLG1 and Synaptophysin-mCh (Sph-mCh). Neurons were incubated with thrombin and imaged live by confocal microscopy. Note that cleavage of postsynaptic NLG1 does not elicit loss of presynaptic Sph-mCh . Scale bar, 5 μm. **(B)** Ratio of fluorescence intensities 30 min after thrombin (Thr) cleavage relative to before thrombin at synapses containing GFP-Thr-NLG1. Data represent means ± SEM. Sph-mCh, n = 43 synaptic pairs; NRX1β-mCh, n = 87 synaptic pairs, *p <0.05.

These results were further confirmed by measuring endogenous synaptic vesicle pool size by immunolabeling of vesicular glutamate transporter VGLUT1 in neurons expressing GFP-NLG1 or GFP-Thr-NLG1 before and after 30 minutes of thrombin incubation. Consistent with the stability of Synaptophysin-mCh puncta observed after NLG1 cleavage, we detected no change in VGLUT1 puncta number or intensity in both neuronal populations after thrombin treatment (Figure 8), indicating that NLG1 cleavage affects presynaptic Neurexin levels specifically.

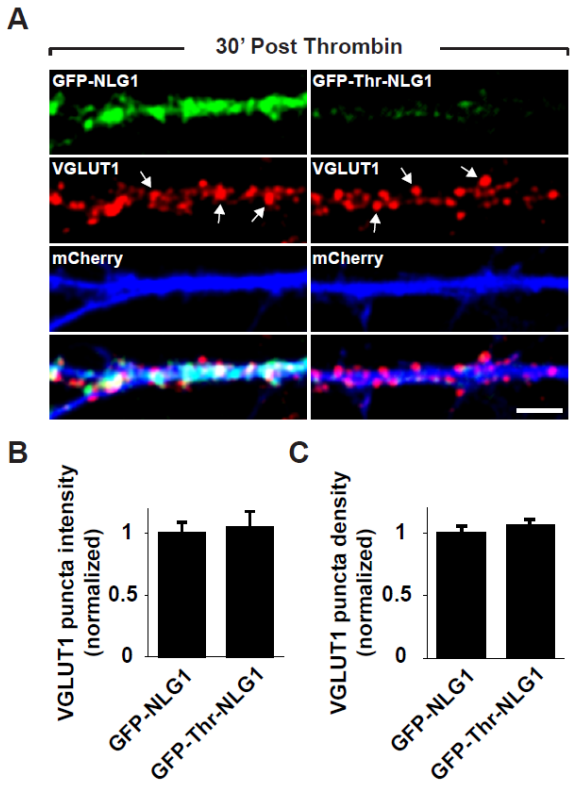

Figure 8. Neuroligin-1 cleavage does not alter presynaptic VGLUT1. (A) Hippocampal neurons (DIV21) expressing mCherry and either GFP-NLG1 or GFP-Thr-NLG1 were incubated in 5U/ml thrombin for 30 min, fixed, and immunolabeled for endogenous VGLUT1. Arrows indicate VGLUT1-positive presynaptic terminals. Note that VGLUT1 staining is unaffected by the thrombin-induced loss of GFP-Thr-NLG1. Scale bar, 5 µm. **(B)** Data represent means ± SEM of VGLUT1 puncta intensity apposing GFP-Thr-NLG1 or GFP-NLG1 after thrombin treatment normalized to the GFP-NLG1 group. GFP-NLG1, n = 2316 puncta from 9 neurons; GFP-Thr-NLG1, n = 2408 puncta from 8 neurons. **(C)** Data represent means ± SEM of VGLUT1 puncta density after thrombin normalized to GFP-NLG1 group. n values as in (B).

Together, the results presented here define a novel trans-synaptic signaling mechanism induced by NLG1 cleavage. NLG1

shedding results in destabilization and loss of presynaptic NRX1β with concomitant reduction of release probability. Given the involvement of NLG1 cleavage during early development and synaptic plasticity described in previous chapters these findings indicate that acute regulation of NLG1 at synapses has direct consequences over synaptic transmission and provide new insight about how NLGs regulate mature synapses.

Discussion

Neurexins and Neuroligins are highly synaptogenic proteins capable of aggregating pre and postsynaptic scaffolding complexes (Dean et al., 2003; Graf et al., 2004; Heine et al., 2008; Nam and Chen, 2005; Prange et al., 2004; Scheiffele et al., 2000; Wittenmayer et al., 2009). Moreover, recent work has also shown that NLGs can regulate synaptic transmission (Chubykin et al., 2007; Futai et al., 2007; Varoqueaux et al., 2006) and are required for Hebbian forms of synaptic plasticity (Jung et al., 2010; Kim et al., 2008). However, these studies were based on chronic long-term manipulations of NLG and NRX levels, which are susceptible to indirect compensatory mechanisms. Hence, it remained unclear whether acute modulation of the NLG-NRX trans-synaptic interaction can directly affect synaptic transmission. The experiments described in previous chapters unveiled a new form of NLG1 regulation based on ectodomain shedding by MMP9. Due to the acute and irreversible nature of MMP proteolysis, this mechanism has the potential to elicit rapid and long lasting changes in synaptic function. However, the functional consequences induced by shedding of specific adhesion molecules have been difficult to address due to the highly redundant and promiscuous nature of MMP activity. Here, we have developed a novel strategy capable of specifically and acutely cleaving surface NLG1. Using this method we demonstrate that acute cleavage of NLG1 causes rapid loss of presynaptic NRX1β and reduces synaptic transmission by decreasing presynaptic release probability (Figure 9).

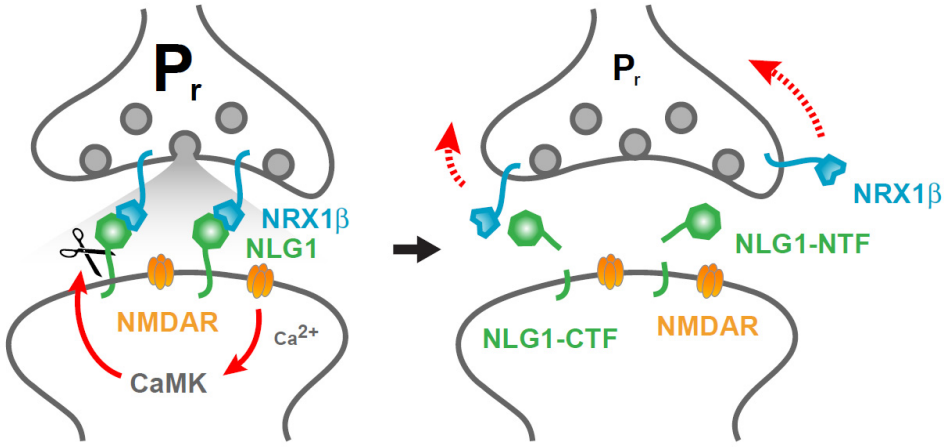

Figure 9. Schematic model for synaptic effects induced by cleavage of NLG1. NLG1 cleavage decreases NRX1β stability and reduces transmitter release probability (P_r) without inducing changes in overall presynaptic structure or synaptic vesicle pool size. NMDAR, N-methyl-D-asparate receptor; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; NTFs, N-terminal fragments; NLG1-CTFs, NLG1 C-terminal fragments. Scissors represent MMP9.

These results provide first evidence that NLGs can acutely regulate synaptic transmission and suggest that cleavage of other adhesion molecules may elicit similar effects. Indeed, acute activation of presynaptic ephrin-B2 by soluble forms of EphB2 induces a rapid increase in release probability in optic tectum neurons (Lim et al., 2008). This suggests that EphB2-ephrin interaction has the potential to mediate acute retrograde modulation of presynaptic function. Interestingly, MMP9/MMP2 have been shown to target and cleave EphB2 (Lin et al., 2008) suggesting that MMP9 activation can potentially activate parallel signaling cascades that converge in the silencing of presynaptic vesicle release. In addition to Eph cleavage by MMPs, ephrins can also be cleaved by ADAM10 in response to Eph binding

(Janes et al., 2005). This indicates that the same trans-synaptic adhesive complex may be under control of different proteolytic pathways. Another cell adhesion molecule that has been shown to regulate presynaptic function is N-Cadherin (NCAD). NCAD deficient neurons have defects in synaptic vesicle release from incoming afferents under conditions of high neuronal activity (Jungling et al., 2006). Notably, ADAM10 can also cleave N-cadherin, although the potential effect of NCAD cleavage in presynaptic function has not been addressed. Together, these results demonstrate that shedding of adhesion molecules has the potential to induce retrograde modulation of presynaptic release probability, but also illustrate the highly complex regulatory network that these processes will likely entail. Nevertheless, the strategies presented here may provide significant aid in addressing how cleavage of specific adhesion molecules affects synaptic function.

Previous studies have shown that deletion of αNRXs in mice reduces action-potential-evoked neurotransmitter release due to impaired presynaptic Ca^{2+} channel function (Missler et al., 2003). However, due to the role of NRXs in synapse maturation (Dean et al., 2003; Prange et al., 2004; Scheiffele et al., 2000; Wittenmayer et al., 2009) it has been unclear whether this effect is due to indirect developmental effects or reflects an ongoing role for NRXs in neurotransmitter release. Using the highly selective thrombin-induced cleavage of NLG1, we have demonstrated an overall reduction in excitatory transmission and release probability concurrent with NRX1β loss. This finding is consistent with a role of NRXs as regulators of neurotransmitter release (Futai et al., 2007; Missler et al., 2003; Zhang et al.,

2005) and further suggests that NRX-dependent regulation of presynaptic function can occur in time scales of minutes. Interestingly, the destabilization of NRX1β observed after acute NLG1 cleavage is specific, since presynaptic protein Synaptophysin was unaffected under similar conditions. This also indicates that acute ectodomain shedding of NLG1 is not accompanied by gross structural changes of presynaptic terminals or by loss of synaptic vesicles. Besides interacting with NLGs, NRXs bind to several other postsynaptic transmembrane proteins such as members of the Leucine Rich Repeat Transmembrane Molecule (LRRTM) family (de Wit et al., 2009; Ko et al., 2009a), Cerebellin precursor proteins (Joo et al., 2011; Matsuda and Yuzaki, 2011), Neurexophilins (Petrenko et al., 1996), β-Dystroglycan (Sugita et al., 2001) and also to GABA(A) receptors (Zhang et al., 2010). The function of NRX interaction with each of these specific postsynaptic partners and its potential contribution for synaptic regulation remains unclear. However, in contrast to NLGs no functional effect of binding of Neurexophilin or β-Dystroglycan to NRXs has been observed so far (Sudhof, 2008). Nevertheless, our results suggest that postsynaptic NLG1 is a major stabilizing element controlling the recruitment and abundance of NRX1β at synapses. Moreover, the increase in PPR observed after NLG1 cleavage indicates that the NLG-NRX interaction directly contributes for the regulation of release probability.

Neurons undergo compensatory, homeostatic synaptic changes in order to maintain their overall firing rate (Davis, 2006; Turrigiano, 2008). In mature hippocampal and cortical cultures

postsynaptic receptor blockade causes an increase in mEPSC frequency (Burrone et al., 2002; Thiagarajan et al., 2005; Wierenga et al., 2006) and augments presynaptic terminal size and release probability (Murthy et al., 2001; Thiagarajan et al., 2005). In contrast, KCl-induced depolarization results in lower release probability and loss of excitatory inputs (Moulder et al., 2004) indicating that homeostatic regulation of presynaptic function is a bidirectional process. Elegant experiments performed in dissociated cultures further demonstrated that local dendritic activity regulates release probability of presynaptic terminals (Branco et al., 2008) implying the existence of trans-synaptic signals capable of transducing postsynaptic messages. The identity of the molecular signals involved in this response remains however, unknown. At the *Drosophila* neuromuscular junction, compromised postsynaptic activity results in a compensatory increase of presynaptic neurotransmitter release (Davis, 2006) with two distinct phases: an initial protein synthesis-independent phase operating within minutes, and a slower protein synthesis-dependent phase that is required for the long term maintenance of the presynaptic adaptation (Frank et al., 2006). Interestingly, the latter component involves modulation of Cav2.1 calcium channels by Eph receptor activation (Frank et al., 2009), resembling the effect of αNRX1-3 ablation on N-type Ca^{2+} channel function (Missler et al., 2003). Although quite different in molecular mechanism, these observations together with the findings presented here suggest an evolutionarily conserved module whereby postsynaptic activity signals retrogradely by adhesion molecule coupling to presynaptic voltage-gated Ca^{2+} channels. Hence, we propose that bidirectional regulation of NLG1 cleavage by neuronal activity

may provide a trans-synaptic signal for homeostatic plasticity that dampens or augments presynaptic function in response to increased or decreased postsynaptic activity, respectively.

Several other retrograde signaling mechanisms can directly affect presynaptic function. Notable examples are mediated by nitric oxide (NO) and the endocannabinoid signaling system (eCB). Both these signaling mechanisms are activated by elevated postsynaptic Ca^{2+} levels and ultimately result in changes in presynaptic function. Production of eCBs is triggered by intracellular calcium and G_q-coupled receptor activation, which in turn drive the conversion of Phosphatidylinositol 4,5-bisphosphate (PIP2) into Diacylglycerol (DAG) by phospholipase-C beta (PLCβ), and is subsequently converted into the endocannabinoid 2-AG by DAG lipase (Regehr et al., 2009). In turn, 2-AG is released from the postsynaptic cell and activates Cannabinoid Receptors type 1 (CB1Rs) located in presynaptic terminals. Similarly, NMDAR signaling activates NO production by Nitric Oxide Synthetase (iNOS) in postsynaptic cells (Garthwaite, 2008) that is able to freely diffuse across membranes and activate multiple targets in presynaptic terminals such as soluble guanylyl cyclase (Garthwaite et al., 1989). Activation of eCB and NO signaling pathways have direct implications for presynaptic function. For example, CBR1 agonists suppress synaptic transmission by decreasing release probability through Ca^{2+} channel regulation (Brown et al., 2003; Brown et al., 2004), and eCBs have been shown to suppress seizures in the hippocampus (Monory et al., 2006). Conversely, NO activity has been implicated in several forms of presynaptic LTP (Nugent et al., 2007). Hence, there are multiple signaling

mechanisms capable of transducing retrograde messages across the synapse. However, in comparison with signaling mechanisms based on small diffusible messengers, shedding of adhesion molecules has the potential for increased temporal and spatial control over trans-synaptic signaling. Indeed, eCB and NO have been shown to induce heterosynaptic effects (Lancaster, 1994; Wilson and Nicoll, 2001). Moreover, long-term signaling requires continuous production of intermediate messengers which may be unfavorable from an energetic perspective. Signaling through adhesion molecule cleavage may then offer the possibility to induce permanent structural changes in synapses which may then be remodeled by new incoming molecules. Future work will be required to characterize in detail how different retrograde signaling mechanisms are regulated and how they specifically affect synaptic function. Interestingly, NO can regulate enzymatic function in many target proteins through S-nitrosylation including MMP9 proteolytic activity (Gu et al., 2002). It is tempting to speculate that some of the reported effects of NO may occur in tandem with, or even be caused by, MMP9 activation.

Consistent with results demonstrating that NLG1 predominantly regulates excitatory synaptic function (Chubykin et al., 2007; Ko et al., 2009b), we found no effect of NLG1 cleavage on mIPSCs (Figure 3). Moreover, mEPSC amplitudes were unaffected by acute NLG1 cleavage, indicating that postsynaptic function was largely intact. This is consistent with live-cell imaging data showing that dendritic spines and PSD95-mCh puncta are unaffected by acute NLG1 cleavage. Our results described in Chapter 2 indicate that NLG2 can also be cleaved, but through a different proteolytic pathway. It will be interesting to

address if cleavage of NLG2 may be involved in the regulation of inhibitory synapse function. Notably, long term potentiation of GABAergic synapses on dopaminergic neurons in the ventral tegmental area (VTA) is expressed presynaptically, as evidenced by a change in paired-pulse ratio, yet it can be impaired by blocking postsynaptic calcium elevations with BAPTA, suggesting a requirement for a retrograde messenger (Nugent et al., 2007). Hence, the future elucidation of the mechanisms regulating cleavage of different NLG isoforms may have important implications for understanding retrograde signaling at different synapses.

Together, the results discussed here indicate that postsynaptic activity influences presynaptic function by NLG1 cleavage. Furthermore, these findings reveal a new post-translational mechanism of NLG1 regulation that contributes to synapse plasticity and may provide a general paradigm for acute retrograde signaling in diverse neural circuits.

Methods

Reagents and Antibodies

Dissociated primary neuronal cultures were prepared from hippocampi of embryonic day 18 or 19 Wistar rats. The hippocampi were dissociated by enzymatic papain digestion followed by brief mechanical trituration. Cultures were grown in Neurobasal media supplemented with B27 and Glutamax. For immunocytochemistry, 100k hippocampal cells were plated onto 12 well plates containing Poly-L-Lysine coated 18mm glass coverslips. Plasmid transfection was done using lipid mediated gene transfer using Lipofectamine 2000 (Invitrogen). Briefly, for each coverslip, 1μg total DNA was mixed with 1μl Lipofectamine in 100 μl of Neurobasal media for 15min. Following incubation time 500 μl of conditioned media was mixed to the reaction and added to cell cultures for 30min. After this period, lipofectamine containing media was removed and replaced with a 1:1 mixture of conditioned media + fresh growth media. Commercial antibodies used include VGLUT1 (mouse, Synaptic Systems).

DNA Constructs and Antibodies

PSD95-mCh was a gift from Thomas Blanpied (University of Maryland). mCherry-N1 vector was a gift from Roger Tsien (University of California, San Diego). Synaptophysin-mCh was a gift from Matthew Kennedy (Duke University). Rat GFP-NLG1 in pcDNA3 (ΔA splice variant) was kindly provided by Anne Marie

Craig (University of British Columbia), and NRX1β-mCh was a gift from Thomas Sudhof (Stanford University). Thrombin inducible GFP-Thr-NLG1 was generated by replacing the endogenous sequence TTTKVP from GFP-NLG1(ΔA) with the thrombin recognition sequence LVPRGS. This was achieved by sequential PCR using primers 5'-ctagtacctagaggatcctcaacagaca tcactctcag-3' and 5'-ggatcctctaggtactagcgaggtatactgagaaatgtca-3', and primers 5'-ggatcctctaggtactagctgagaaatgtcattgag-3' and 5'-gttggagctggtacctcatc-3'. The two self-annealing PCR products were templates in a reaction with primer 5'-gttggagctggtacctcatc-3' containing KpnI site and 5'-gtcactcgagctataccctggttgttgaatg-3'. The respective product was subcloned into pcDNA3-GFP-NLG1 using KpnI and XhoI.

Imaging

Confocal images of live cells were obtained using a Perkin Elmer Ultraview spinning disc confocal microscope with a 60x 1.4 N.A. objective. Images were analyzed using Metamorph software (Molecular Devices, Universal Imaging Corporation). For immunocytochemistry, DIV21 hippocampal neurons were fixed in 4% paraformaldehyde/4% sucrose in PBS for 20 min, permeabilized with 0.2% Triton X-100 for 15 min, and incubated with 1:1000 VGLUT1 antibody in 3% goat serum/ 3% BSA overnight at 4ºC. For live imaging, cells were imaged in E4 media (10 mM HEPES, 120 mM NaCl, 3 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$) at 37ºC. Images were taken every 3 min and 5U/ml thrombin was added at t = 20 min.

Quantification of fluorescence intensity at individual puncta was performed before and 30 min after thrombin application. For each neuron the average fluorescence intensity ratios for all puncta were determined. Results depicted correspond to the average of all neurons per condition.

Electrophysiological Recordings

For EPSC recordings, patch pipettes (6-8 MΩ tip resistance) were filled with internal solution (30 mM $CsSO_4$, 70 mM K_2SO_4, 25 mM HEPES, 25 mM N-methly-D-glucamine, 0.1 mM $CaCl_2$, 1 mM EGTA, 2 mM (Na)ATP, and 0.1 mM leupeptin, pH7.2, ~300 mOsm). Cells were bathed in external solution (150 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 30 mM D-glucose, 2 mM $CaCl_2$, and 30 μM bicuculline). Only cells with series resistance < 30 MΩ and having <20% change in input resistance, series resistance, and holding current were included for analyses. EPSCs were evoked from a concentric bipolar stimulating electrode. Paired-pulse responses, with an inter-pulse interval of 100 ms were evoked every 30 sec by extracellular stimulation (200 μs duration) of nearby cells. Currents were recorded in neurons voltage-clamped at -70 mV. Similar conditions were used to record mEPSCs, except the external solution additionally contained 1 μM TTX (to block action potentials mediated by voltage-gated sodium channels). For mIPSC recordings, patch pipettes were filled with internal solution (150 mM KCl, 3 mM $MgCl_2$, 15 mM HEPES, 0.1 mM $CaCl_2$, 1 mM EGTA, 2 mM Na_2ATP, and 0.1 mM leupeptin, pH7.2, ~300 mOsm). Cells were

bathed in external solution containing (150 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 30 mM D-glucose, 2 mM $CaCl_2$, 1 μM TTX (to block voltage-gated sodium channels), 50 μM D-APV (to block NMDA receptors), and 10 μM CNQX (to block AMPA/kainate receptors). Only cells with series resistance < 30 MΩ and having <20% change in input resistance, series resistance, and holding current were included for analyses. Currents were recorded in neurons voltage-clamped at -70 mV.

Acknowledgements

For the realization of this work, Portia McCoy performed all the electrophysiological recordings and analyzed the data from those experiments. Irina Lebedeva and Marguerita Klein prepared the neuronal cultures from dissociated mouse hippocampus. I also thank Matt Kennedy for generously providing the Synaptophysin-mCh construct.

Final Discussion

Although highly studied for their role during synapse maturation (Chih et al., 2005; Graf et al., 2004; Levinson et al., 2005; Scheiffele et al., 2000; Varoqueaux et al., 2006; Wittenmayer et al., 2009), it has remained unclear whether Neuroligins acutely regulate synaptic function and whether the Neuroligin-Neurexin trans-synaptic complex undergoes dynamic dissociation. In this study, we have shown that neuronal activity triggers cleavage and loss of NLG1 at glutamatergic synapses. Cleavage of NLG1 requires NMDA receptor activation and Ca^{2+}/calmodulin-dependent kinase activity, and is mediated by the activity-regulated brain-expressed protease MMP9. Cleavage of NLG1 occurs within its juxtamembrane ectodomain, releasing soluble N-terminal cleavage fragments that are present in brain and are mostly abundant during early postnatal development. Furthermore, acute cleavage of NLG1 causes rapid loss of presynaptic NRX1β, reduces synaptic strength, and decreases presynaptic release probability. These results define a novel trans-synaptic signaling mechanism coupling postsynaptic activity to presynaptic function, and link Neuroligin-Neurexin synaptic adhesion to a NMDAR-CaMK-MMP9 signaling cascade. Moreover, these findings suggest that acute cleavage of synaptic adhesion molecules by activity-regulated proteolysis may provide a general mechanism linking synaptic activity to structural remodeling of mature and developing neuronal circuits.

Taken together, the data presented here suggests that NLG1 cleavage is under complex regulation and may have

multiple functional outcomes depending on cellular context. Moreover, it is likely that NLG1 cleavage may be regulated by other proteases besides MMP9. In mature synapses, NLG1 cleavage is upregulated in response to increased neuronal activity *in vitro* and *in vivo* resulting in acute downregulation of synaptic transmission due to changes in presynaptic release probability. As discussed in Chapters 2 and 3, this effect may underlie a homeostatic mechanism of negative feedback responsible for maintaining proper levels of excitation in neurons. However, our results also demonstrate that NLG1 cleavage is particularly prominent during the first weeks of life and can be bi-directionally regulated by sensory experience during critical periods of cortical development. This suggests that besides mediating acute trans-synaptic modulation of presynaptic activity, NLG cleavage may also regulate synapse formation and maturation at a different scale. Moreover, it is worth noting that although the effects of NLG1 cleavage described here are exclusively presynaptic, it remains to be determined whether prolonged loss or further processing of NLG1 can impact postsynaptic function.

Despite initial studies suggesting that NLG could be involved in the early steps of synaptogenesis (Chih et al., 2005; Graf et al., 2004; Levinson et al., 2005; Scheiffele et al., 2000; Varoqueaux et al., 2006; Wittenmayer et al., 2009), recent data suggests different model whereby NLGs regulate synapse maintenance and pruning during later stages of development through activity dependent processes (Chubykin et al., 2007; Varoqueaux et al., 2006). Interestingly, the synaptogenic effect of NLG1 overexpression is blocked by NMDAR antagonist and

CaMK inhibitors, indicating that the same signaling pathways regulating NLG1 cleavage also control NLG-induced synaptogenesis. Hence, it is possible that MMP-dependent cleavage of NLG is involved in synapse maturation.

Thrombospondins (TSPs) are secreted glycoproteins that promote synapse formation in CNS neurons (Christopherson et al., 2005). A recent study has shown that TSP1 can bind to NLG1 and that TSP1 induced synapse formation is abrogated in the absence of NLG1 (Xu et al., 2010). This suggests that TSP1 acts through NLG1 to promote synapse stabilization, although the detailed molecular mechanisms mediating this response remain unknown. Interestingly, TSP1 blocks MMP9 activity (Bein and Simons, 2000) raising the interesting possibility that TSP1 synaptogenic properties may be due, at least in part, to inhibition of MMP9-dependent NLG1 cleavage. Moreover, as previously discussed in Chapter 2, MMP9 inhibition accelerates spine maturation in neuron cultures and increases the total fraction of mushroom spines (Bilousova et al., 2009), whereas MMP7, an upstream activator of MMP9, induces morphological changes in dendritic spines reverting them to elongated filopodia-like structures (Bilousova et al., 2006). Thus, although our work was centered on the function of NLG1 cleavage in mature synapses, the characterization of the specific molecular mechanisms and proteases regulating NLG cleavage during development may provide important insight regarding the molecular mechanisms controlling synapse maturation and neuronal circuit formation during development.

It is also important to note that NLG1 cleavage is upregulated during epileptic seizures in the hippocampus and also in the visual cortex in response to light stimulation after dark rearing. Interestingly, both these experimental paradigms result in synapse remodeling and circuit rearrangements that last for days after stimuli induction (Suzuki et al., 1997; Tropea et al., 2010). Moreover, activity induced processes leading to synaptic depression have been associated with synaptic pruning and loss (Bastrikova et al., 2008; Becker et al., 2008; Mysore et al., 2007). It is then tempting to speculate that persistent NLG1 downregulation, perhaps coupled with other signals, could lead to synapse disassembly and elimination. In this putative model (Figure 1), specific patterns of neuronal activity would decrease overall surface levels of NLGs through MMP-dependent cleavage. In turn, loss of synaptic NLGs would result in silencing of synapses due to reduced release probability. The decreased levels of surface NLGs would then trigger synaptic competition for the remaining pools of the protein. It is still unclear whether MMP-dependent NLG cleavage operates at a local or global scale. However, synaptic NLG1 molecules exhibit a relatively fast exchange rate (τ=100s) and a significant mobile fraction at excitatory synapses (40% of total) (data not shown), indicating that NLG molecules remaining at the plasma membrane can diffuse and be redistributed among neighboring synapses. In this scenario, synapses able to sequester and recruit remaining NLGs would restore normal levels of activity and be maintained, while others unable to do so, would be eliminated.

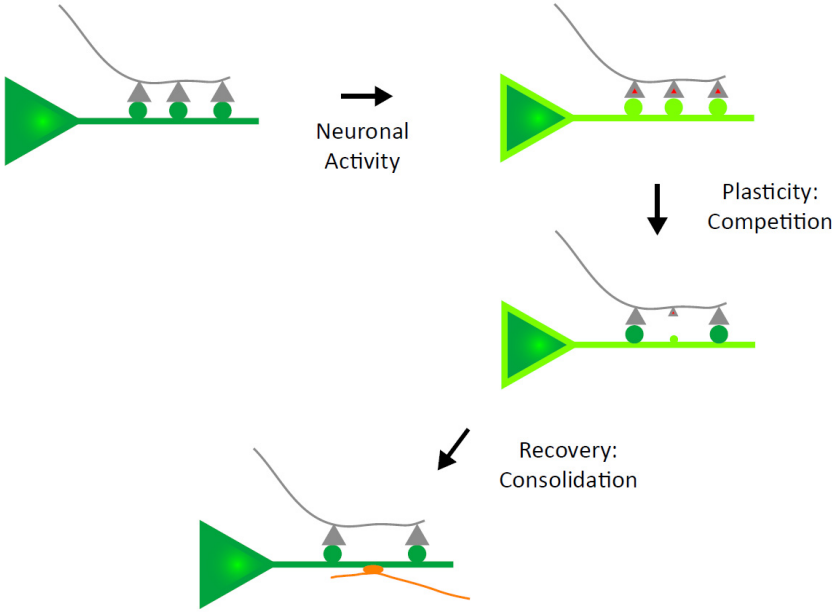

Figure 1. Putative model for circuit remodeling after NLG cleavage. Increased neuronal activity drives NLG cleavage reducing overall surface levels (dark to light green). In turn, decreased surface induces silencing of presynaptic function (depicted as red presynaptic terminals). This leads to synaptic competition for remaining pools of NLG. Synapses that are able to sequester and recruit NLG restore normal levels of activity and are maintained, while others are eliminated. Transcription and translation of newly synthesized NLG molecules may replenish total protein levels but alter the relative pools of different NLG isoforms and splice variants. In turn, this affects new synapse formation resulting in circuit remodeling and changes in E/I ratio (Orange terminal depicts GABAergic synapse).

Another important topic of future research will be to address how neurons respond to NLG cleavage and how the pools of degraded NLGs are replenished. An interesting possibility is that NLG C-terminal fragments originated by NLG shedding may have intracellular signaling functions and affect

downstream transcription similar to other biologically active CTFs (Mumm et al., 2000; Schettini et al., 2010). Moreover, different NLG isoforms partition to and regulate specific synaptic subsets differently (Chubykin et al., 2007; Gibson et al., 2009) and in addition, variations in alternative splicing can have a high impact in the binding for affinity between different NLG and NRX isoforms (Boucard et al., 2005; Chih et al., 2006; Ullrich et al., 1995). Hence, a shift in the relative levels of different NLG isoforms or splice variants caused by changes in transcriptional regulation could have profound implications in the types of new synapses generated, which could in turn lead to circuit rearrangements or changes in overall E/I ratio.

Finally, several NLG and NRX mutations have been linked with autism spectrum disorders (ASDs) (Sudhof, 2008). A typical feature of ASDs is that they develop before two or three years of age which corresponds to the period of heightened synaptic maturation in humans (Lord et al., 2000; Pardo and Eberhart, 2007). Moreover, 30% of autistic patients have epileptic seizures and many more may suffer from subclinical epileptoform conditions (Lewine et al., 1999). In addition, neuroimaging and postmortem studies of patients with autism suggest that changes in the ratio of excitation and inhibition in neural circuits may underlie the etiology of ASDs (Rubenstein and Merzenich, 2003). Together, these results suggest that at least a subset of ASDs may arise from deficient synaptic maturation during critical periods of development that in turn destabilize circuit function by altering the E/I ratio. Interestingly, NLG levels during development control overall excitability and regulate the ratio of excitation and inhibition in neurons (Chubykin et al., 2007;

Gibson et al., 2009; Poulopoulos et al., 2009; Prange et al., 2004). Our results indicate that NLG cleavage is regulated by sensory experience during critical periods of circuit maturation and in response to epileptic seizures in the hippocampus. Although the characterizations of the detailed molecular mechanisms regulating NLG in these different contexts will require further inquiry, this data suggests a potential link between NLG function, critical period plasticity and epileptogenesis. Hence, the proteolytic regulation of NLGs reported here may provide novel insight into the pathophysiological mechanisms and therapeutic strategies for synaptic dysfunction in ASDs.

References

Arac, D., Boucard, A.A., Ozkan, E., Strop, P., Newell, E., Sudhof, T.C., and Brunger, A.T. (2007). Structures of neuroligin-1 and the neuroligin-1/neurexin-1 beta complex reveal specific protein-protein and protein-Ca2+ interactions. Neuron *56*, 992-1003.

Arundine, M., and Tymianski, M. (2003). Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell Calcium *34*, 325-337.

Arundine, M., and Tymianski, M. (2004). Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. Cell Mol Life Sci *61*, 657-668.

Barrow, S.L., Constable, J.R., Clark, E., El-Sabeawy, F., McAllister, A.K., and Washbourne, P. (2009). Neuroligin1: a cell adhesion molecule that recruits PSD-95 and NMDA receptors by distinct mechanisms during synaptogenesis. Neural Dev *4*, 17.

Bastrikova, N., Gardner, G.A., Reece, J.M., Jeromin, A., and Dudek, S.M. (2008). Synapse elimination accompanies functional plasticity in hippocampal neurons. Proc Natl Acad Sci U S A *105*, 3123-3127.

Bavelier, D., Levi, D.M., Li, R.W., Dan, Y., and Hensch, T.K. (2010). Removing brakes on adult brain plasticity: from molecular to behavioral interventions. J Neurosci *30*, 14964-14971.

Bear, M.F., and Malenka, R.C. (1994). Synaptic plasticity: LTP and LTD. Curr Opin Neurobiol *4*, 389-399.

Becker, N., Wierenga, C.J., Fonseca, R., Bonhoeffer, T., and Nagerl, U.V. (2008). LTD induction causes morphological changes of presynaptic boutons and reduces their contacts with spines. Neuron *60*, 590-597.

Bein, K., and Simons, M. (2000). Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. J Biol Chem *275*, 32167-32173.

Bilousova, T.V., Dansie, L., Ngo, M., Aye, J., Charles, J.R., Ethell, D.W., and Ethell, I.M. (2009). Minocycline promotes dendritic spine maturation and improves behavioural performance in the fragile X mouse model. J Med Genet *46*, 94-102.

Bilousova, T.V., Rusakov, D.A., Ethell, D.W., and Ethell, I.M. (2006). Matrix metalloproteinase-7 disrupts dendritic spines in hippocampal neurons through NMDA receptor activation. J Neurochem *97*, 44-56.

Boucard, A.A., Chubykin, A.A., Comoletti, D., Taylor, P., and Sudhof, T.C. (2005). A splice code for trans-synaptic cell adhesion mediated by binding of neuroligin 1 to alpha- and beta-neurexins. Neuron *48*, 229-236.

Bozdagi, O., Nagy, V., Kwei, K.T., and Huntley, G.W. (2007). In vivo roles for matrix metalloproteinase-9 in mature hippocampal synaptic physiology and plasticity. J Neurophysiol *98*, 334-344.

Bozdagi, O., Shan, W., Tanaka, H., Benson, D.L., and Huntley, G.W. (2000). Increasing numbers of synaptic puncta during late-phase LTP: N-cadherin is synthesized, recruited to synaptic sites, and required for potentiation. Neuron *28*, 245-259.

Brakeman, P.R., Lanahan, A.A., O'Brien, R., Roche, K., Barnes, C.A., Huganir, R.L., and Worley, P.F. (1997). Homer: a protein that selectively binds metabotropic glutamate receptors. Nature *386*, 284-288.

Branco, T., Staras, K., Darcy, K.J., and Goda, Y. (2008). Local dendritic activity sets release probability at hippocampal synapses. Neuron *59*, 475-485.

Brennaman, L.H., and Maness, P.F. (2008). Developmental regulation of GABAergic interneuron branching and synaptic development in the prefrontal cortex by soluble neural cell adhesion molecule. Mol Cell Neurosci *37*, 781-793.

Brown, S.P., Brenowitz, S.D., and Regehr, W.G. (2003). Brief presynaptic bursts evoke synapse-specific retrograde inhibition mediated by endogenous cannabinoids. Nat Neurosci *6*, 1048-1057.

Brown, S.P., Safo, P.K., and Regehr, W.G. (2004). Endocannabinoids inhibit transmission at granule cell to Purkinje cell synapses by modulating three types of presynaptic calcium channels. J Neurosci *24*, 5623-5631.

Budreck, E.C., and Scheiffele, P. (2007). Neuroligin-3 is a neuronal adhesion protein at GABAergic and glutamatergic synapses. Eur J Neurosci *26*, 1738-1748.

Bukalo, O., Fentrop, N., Lee, A.Y., Salmen, B., Law, J.W., Wotjak, C.T., Schweizer, M., Dityatev, A., and Schachner, M. (2004). Conditional ablation of the neural cell adhesion molecule reduces precision of spatial learning, long-term potentiation, and depression in the CA1 subfield of mouse hippocampus. J Neurosci *24*, 1565-1577.

Burrone, J., O'Byrne, M., and Murthy, V.N. (2002). Multiple forms of synaptic plasticity triggered by selective suppression of activity in individual neurons. Nature *420*, 414-418.

Chan, C.S., Weeber, E.J., Kurup, S., Sweatt, J.D., and Davis, R.L. (2003). Integrin requirement for hippocampal synaptic plasticity and spatial memory. J Neurosci *23*, 7107-7116.

Chen, X., Liu, H., Shim, A.H., Focia, P.J., and He, X. (2008). Structural basis for synaptic adhesion mediated by neuroligin-neurexin interactions. Nat Struct Mol Biol *15*, 50-56.

Chih, B., Engelman, H., and Scheiffele, P. (2005). Control of excitatory and inhibitory synapse formation by neuroligins. Science *307*, 1324-1328.

Chih, B., Gollan, L., and Scheiffele, P. (2006). Alternative splicing controls selective trans-synaptic interactions of the neuroligin-neurexin complex. Neuron *51*, 171-178.

Chistiakova, M., and Volgushev, M. (2009). Heterosynaptic plasticity in the neocortex. Exp Brain Res.

Christopherson, K.S., Ullian, E.M., Stokes, C.C., Mullowney, C.E., Hell, J.W., Agah, A., Lawler, J., Mosher, D.F., Bornstein, P., and Barres, B.A. (2005). Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell *120*, 421-433.

Chubykin, A.A., Atasoy, D., Etherton, M.R., Brose, N., Kavalali, E.T., Gibson, J.R., and Sudhof, T.C. (2007). Activity-dependent validation of excitatory versus inhibitory synapses by neuroligin-1 versus neuroligin-2. Neuron *54*, 919-931.

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

Comoletti, D., Flynn, R.E., Boucard, A.A., Demeler, B., Schirf, V., Shi, J., Jennings, L.L., Newlin, H.R., Sudhof, T.C., and Taylor, P. (2006). Gene selection, alternative splicing, and post-translational processing regulate neuroligin selectivity for beta-neurexins. Biochemistry *45*, 12816-12827.

Comoletti, D., Grishaev, A., Whitten, A.E., Tsigelny, I., Taylor, P., and Trewhella, J. (2007). Synaptic arrangement of the neuroligin/beta-neurexin complex revealed by X-ray and neutron scattering. Structure *15*, 693-705.

Cotman, C.W., and Taylor, D. (1972). Isolation and structural studies on synaptic complexes from rat brain. J Cell Biol *55*, 696-711.

Cotrufo, T., Viegi, A., Berardi, N., Bozzi, Y., Mascia, L., and Maffei, L. (2003). Effects of neurotrophins on synaptic protein expression in the visual cortex of dark-reared rats. J Neurosci *23*, 3566-3571.

Cowan, W.M., Sudhof, T.C., Stevens, F.S. (2001). Synapses. The Johns Hopkins University Press.

Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P., Scheff, S.*, et al.* (1994). Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature *367*, 455-459.

Curia, G., Longo, D., Biagini, G., Jones, R.S., and Avoli, M. (2008). The pilocarpine model of temporal lobe epilepsy. J Neurosci Methods *172*, 143-157.

Dahlhaus, R., Hines, R.M., Eadie, B.D., Kannangara, T.S., Hines, D.J., Brown, C.E., Christie, B.R., and El-Husseini, A. (2010). Overexpression of the cell adhesion protein neuroligin-1 induces learning deficits and impairs synaptic plasticity by altering the ratio of excitation to inhibition in the hippocampus. Hippocampus *20*, 305-322.

Dalva, M.B., McClelland, A.C., and Kayser, M.S. (2007). Cell adhesion molecules: signalling functions at the synapse. Nat Rev Neurosci *8*, 206-220.

Dalva, M.B., Takasu, M.A., Lin, M.Z., Shamah, S.M., Hu, L., Gale, N.W., and Greenberg, M.E. (2000). EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. Cell *103*, 945-956.

Davis, G.W. (2006). Homeostatic control of neural activity: from phenomenology to molecular design. Annu Rev Neurosci *29*, 307-323.

de Wit, J., Sylwestrak, E., O'Sullivan, M.L., Otto, S., Tiglio, K., Savas, J.N., Yates, J.R., 3rd, Comoletti, D., Taylor, P., and Ghosh, A. (2009). LRRTM2 interacts with Neurexin1 and regulates excitatory synapse formation. Neuron *64*, 799-806.

Dean, C., Scholl, F.G., Choih, J., DeMaria, S., Berger, J., Isacoff, E., and Scheiffele, P. (2003). Neurexin mediates the assembly of presynaptic terminals. Nat Neurosci *6*, 708-716.

Desai, N.S., Rutherford, L.C., and Turrigiano, G.G. (1999). BDNF regulates the intrinsic excitability of cortical neurons. Learn Mem *6*, 284-291.

Diestel, S., Hinkle, C.L., Schmitz, B., and Maness, P.F. (2005). NCAM140 stimulates integrin-dependent cell migration by ectodomain shedding. J Neurochem *95*, 1777-1784.

Dobrunz, L.E., and Stevens, C.F. (1997). Heterogeneity of release probability, facilitation, and depletion at central synapses. Neuron *18*, 995-1008.

Ehlers, M.D. (2000). Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. Neuron *28*, 511-525.

Ehlers, M.D. (2003). Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. Nat Neurosci *6*, 231-242.

Enoki, R., Hu, Y.L., Hamilton, D., and Fine, A. (2009). Expression of long-term plasticity at individual synapses in hippocampus is graded, bidirectional, and mainly presynaptic: optical quantal analysis. Neuron *62*, 242-253.

Ethell, I.M., and Ethell, D.W. (2007). Matrix metalloproteinases in brain development and remodeling: synaptic functions and targets. J Neurosci Res *85*, 2813-2823.

Fabrichny, I.P., Leone, P., Sulzenbacher, G., Comoletti, D., Miller, M.T., Taylor, P., Bourne, Y., and Marchot, P. (2007). Structural analysis of the synaptic

protein neuroligin and its beta-neurexin complex: determinants for folding and cell adhesion. Neuron *56*, 979-991.

Fink, C.C., and Meyer, T. (2002). Molecular mechanisms of CaMKII activation in neuronal plasticity. Curr Opin Neurobiol *12*, 293-299.

Frank, C.A., Kennedy, M.J., Goold, C.P., Marek, K.W., and Davis, G.W. (2006). Mechanisms underlying the rapid induction and sustained expression of synaptic homeostasis. Neuron *52*, 663-677.

Frank, C.A., Pielage, J., and Davis, G.W. (2009). A presynaptic homeostatic signaling system composed of the Eph receptor, ephexin, Cdc42, and CaV2.1 calcium channels. Neuron *61*, 556-569.

Futai, K., Kim, M.J., Hashikawa, T., Scheiffele, P., Sheng, M., and Hayashi, Y. (2007). Retrograde modulation of presynaptic release probability through signaling mediated by PSD-95-neuroligin. Nat Neurosci *10*, 186-195.

Garner, C.C., Kindler, S., and Gundelfinger, E.D. (2000). Molecular determinants of presynaptic active zones. Curr Opin Neurobiol *10*, 321-327.

Garner, C.C., Zhai, R.G., Gundelfinger, E.D., and Ziv, N.E. (2002). Molecular mechanisms of CNS synaptogenesis. Trends Neurosci *25*, 243-251.

Garthwaite, J. (2008). Concepts of neural nitric oxide-mediated transmission. Eur J Neurosci *27*, 2783-2802.

Garthwaite, J., Garthwaite, G., Palmer, R.M., and Moncada, S. (1989). NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. Eur J Pharmacol *172*, 413-416.

Gawlak, M., Gorkiewicz, T., Gorlewicz, A., Konopacki, F.A., Kaczmarek, L., and Wilczynski, G.M. (2009). High resolution in situ zymography reveals matrix metalloproteinase activity at glutamatergic synapses. Neuroscience *158*, 167-176.

Gerrow, K., and El-Husseini, A. (2006). Cell adhesion molecules at the synapse. Front Biosci *11*, 2400-2419.

Gibson, J.R., Huber, K.M., and Sudhof, T.C. (2009). Neuroligin-2 deletion selectively decreases inhibitory synaptic transmission originating from fast-spiking but not from somatostatin-positive interneurons. J Neurosci *29*, 13883-13897.

Gordon, J.A., and Stryker, M.P. (1996). Experience-dependent plasticity of binocular responses in the primary visual cortex of the mouse. J Neurosci *16*, 3274-3286.

Graf, E.R., Zhang, X., Jin, S.X., Linhoff, M.W., and Craig, A.M. (2004). Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. Cell *119*, 1013-1026.

Gray, E.G. (1959). Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. J Anat *93*, 420-433.

Gray, E.G., and Whittaker, V.P. (1962). The isolation of nerve endings from brain: an electron-microscopic study of cell fragments derived by homogenization and centrifugation. J Anat *96*, 79-88.

Grover, L.M., and Teyler, T.J. (1993). Presynaptic mechanism for heterosynaptic, posttetanic depression in area CA1 of rat hippocampus. Synapse *15*, 149-157.

Grunwald, I.C., Korte, M., Wolfer, D., Wilkinson, G.A., Unsicker, K., Lipp, H.P., Bonhoeffer, T., and Klein, R. (2001). Kinase-independent requirement of EphB2 receptors in hippocampal synaptic plasticity. Neuron *32*, 1027-1040.

Gu, Z., Kaul, M., Yan, B., Kridel, S.J., Cui, J., Strongin, A., Smith, J.W., Liddington, R.C., and Lipton, S.A. (2002). S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. Science *297*, 1186-1190.

Gutierrez, R.C., Flynn, R., Hung, J., Kertesz, A.C., Sullivan, A., Zamponi, G.W., El-Husseini, A., and Colicos, M.A. (2009). Activity-driven mobilization of post-synaptic proteins. Eur J Neurosci *30*, 2042-2052.

Hata, Y., Butz, S., and Sudhof, T.C. (1996). CASK: a novel dlg/PSD95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins. J Neurosci *16*, 2488-2494.

Heine, M., Thoumine, O., Mondin, M., Tessier, B., Giannone, G., and Choquet, D. (2008). Activity-independent and subunit-specific recruitment of functional AMPA receptors at neurexin/neuroligin contacts. Proc Natl Acad Sci U S A *105*, 20947-20952.

Henderson, J.T., Georgiou, J., Jia, Z., Robertson, J., Elowe, S., Roder, J.C., and Pawson, T. (2001). The receptor tyrosine kinase EphB2 regulates NMDA-dependent synaptic function. Neuron *32*, 1041-1056.

Hensch, T.K. (2004). Critical period regulation. Annu Rev Neurosci *27*, 549-579.

Hensch, T.K. (2005a). Critical period mechanisms in developing visual cortex. Curr Top Dev Biol *69*, 215-237.

Hensch, T.K. (2005b). Critical period plasticity in local cortical circuits. Nat Rev Neurosci *6*, 877-888.

Hensch, T.K., and Fagiolini, M. (2005). Excitatory-inhibitory balance and critical period plasticity in developing visual cortex. Prog Brain Res *147*, 115-124.

Hensch, T.K., Fagiolini, M., Mataga, N., Stryker, M.P., Baekkeskov, S., and Kash, S.F. (1998). Local GABA circuit control of experience-dependent plasticity in developing visual cortex. Science *282*, 1504-1508.

Hines, R.M., Wu, L., Hines, D.J., Steenland, H., Mansour, S., Dahlhaus, R., Singaraja, R.R., Cao, X., Sammler, E., Hormuzdi, S.G*., et al.* (2008). Synaptic imbalance, stereotypies, and impaired social interactions in mice with altered neuroligin 2 expression. J Neurosci *28*, 6055-6067.

Hinkle, C.L., Diestel, S., Lieberman, J., and Maness, P.F. (2006). Metalloprotease-induced ectodomain shedding of neural cell adhesion molecule (NCAM). J Neurobiol *66*, 1378-1395.

Huang, Y., Yasuda, H., Sarihi, A., and Tsumoto, T. (2008). Roles of endocannabinoids in heterosynaptic long-term depression of excitatory synaptic transmission in visual cortex of young mice. J Neurosci *28*, 7074-7083.

Hubschmann, M.V., Skladchikova, G., Bock, E., and Berezin, V. (2005). Neural cell adhesion molecule function is regulated by metalloproteinase-mediated ectodomain release. J Neurosci Res *80*, 826-837.

Hwang, J.J., Park, M.H., Choi, S.Y., and Koh, J.Y. (2005). Activation of the Trk signaling pathway by extracellular zinc. Role of metalloproteinases. J Biol Chem *280*, 11995-12001.

Ichtchenko, K., Hata, Y., Nguyen, T., Ullrich, B., Missler, M., Moomaw, C., and Sudhof, T.C. (1995). Neuroligin 1: a splice site-specific ligand for beta-neurexins. Cell *81*, 435-443.

Ichtchenko, K., Nguyen, T., and Sudhof, T.C. (1996). Structures, alternative splicing, and neurexin binding of multiple neuroligins. J Biol Chem *271*, 2676-2682.

Irie, M., Hata, Y., Takeuchi, M., Ichtchenko, K., Toyoda, A., Hirao, K., Takai, Y., Rosahl, T.W., and Sudhof, T.C. (1997). Binding of neuroligins to PSD-95. Science *277*, 1511-1515.

Isokawa, M. (1998). Remodeling dendritic spines in the rat pilocarpine model of temporal lobe epilepsy. Neurosci Lett *258*, 73-76.

Isokawa, M. (2000). Remodeling dendritic spines of dentate granule cells in temporal lobe epilepsy patients and the rat pilocarpine model. Epilepsia *41 Suppl 6*, S14-17.

Janes, P.W., Saha, N., Barton, W.A., Kolev, M.V., Wimmer-Kleikamp, S.H., Nievergall, E., Blobel, C.P., Himanen, J.P., Lackmann, M., and Nikolov, D.B. (2005). Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. Cell *123*, 291-304.

Joo, J.Y., Lee, S.J., Uemura, T., Yoshida, T., Yasumura, M., Watanabe, M., and Mishina, M. (2011). Differential interactions of cerebellin precursor protein (Cbln) subtypes and neurexin variants for synapse formation of cortical neurons. Biochem Biophys Res Commun.

Jourquin, J., Tremblay, E., Decanis, N., Charton, G., Hanessian, S., Chollet, A.M., Le Diguardher, T., Khrestchatisky, M., and Rivera, S. (2003). Neuronal

activity-dependent increase of net matrix metalloproteinase activity is associated with MMP-9 neurotoxicity after kainate. Eur J Neurosci *18*, 1507-1517.

Jung, S.Y., Kim, J., Kwon, O.B., Jung, J.H., An, K., Jeong, A.Y., Lee, C.J., Choi, Y.B., Bailey, C.H., Kandel, E.R.*, et al.* (2010). Input-specific synaptic plasticity in the amygdala is regulated by neuroligin-1 via postsynaptic NMDA receptors. Proc Natl Acad Sci U S A *107*, 4710-4715.

Jungling, K., Eulenburg, V., Moore, R., Kemler, R., Lessmann, V., and Gottmann, K. (2006). N-cadherin transsynaptically regulates short-term plasticity at glutamatergic synapses in embryonic stem cell-derived neurons. J Neurosci *26*, 6968-6978.

Kandel ER, Schwartz JH (1985) Principles of Neural Science, 2nd Edition: Elsevier.

Kattenstroth, G., Tantalaki, E., Sudhof, T.C., Gottmann, K., and Missler, M. (2004). Postsynaptic N-methyl-D-aspartate receptor function requires alpha-neurexins. Proc Natl Acad Sci U S A *101*, 2607-2612.

Kayser, M.S., McClelland, A.C., Hughes, E.G., and Dalva, M.B. (2006). Intracellular and trans-synaptic regulation of glutamatergic synaptogenesis by EphB receptors. J Neurosci *26*, 12152-12164.

Kim, J., Jung, S.Y., Lee, Y.K., Park, S., Choi, J.S., Lee, C.J., Kim, H.S., Choi, Y.B., Scheiffele, P., Bailey, C.H.*, et al.* (2008). Neuroligin-1 is required for normal expression of LTP and associative fear memory in the amygdala of adult animals. Proc Natl Acad Sci U S A *105*, 9087-9092.

Kim, T.K., Hemberg, M., Gray, J.M., Costa, A.M., Bear, D.M., Wu, J., Harmin, D.A., Laptewicz, M., Barbara-Haley, K., Kuersten, S.*, et al.* (2010). Widespread transcription at neuronal activity-regulated enhancers. Nature *465*, 182-187.

Ko, J., Fuccillo, M.V., Malenka, R.C., and Sudhof, T.C. (2009a). LRRTM2 functions as a neurexin ligand in promoting excitatory synapse formation. Neuron *64*, 791-798.

Ko, J., Zhang, C., Arac, D., Boucard, A.A., Brunger, A.T., and Sudhof, T.C. (2009b). Neuroligin-1 performs neurexin-dependent and neurexin-independent functions in synapse validation. EMBO J *28*, 3244-3255.

Lancaster, J.R., Jr. (1994). Simulation of the diffusion and reaction of endogenously produced nitric oxide. Proc Natl Acad Sci U S A *91*, 8137-8141.

Levinson, J.N., Chery, N., Huang, K., Wong, T.P., Gerrow, K., Kang, R., Prange, O., Wang, Y.T., and El-Husseini, A. (2005). Neuroligins mediate excitatory and inhibitory synapse formation: involvement of PSD-95 and neurexin-1beta in neuroligin-induced synaptic specificity. J Biol Chem *280*, 17312-17319.

Lewine, J.D., Andrews, R., Chez, M., Patil, A.A., Devinsky, O., Smith, M., Kanner, A., Davis, J.T., Funke, M., Jones, G., *et al.* (1999). Magnetoencephalographic patterns of epileptiform activity in children with regressive autism spectrum disorders. Pediatrics *104*, 405-418.

Lim, B.K., Matsuda, N., and Poo, M.M. (2008). Ephrin-B reverse signaling promotes structural and functional synaptic maturation in vivo. Nat Neurosci *11*, 160-169.

Lin, K.T., Sloniowski, S., Ethell, D.W., and Ethell, I.M. (2008). Ephrin-B2-induced cleavage of EphB2 receptor is mediated by matrix metalloproteinases to trigger cell repulsion. J Biol Chem *283*, 28969-28979.

Lisman, J.E., and Harris, K.M. (1993). Quantal analysis and synaptic anatomy--integrating two views of hippocampal plasticity. Trends Neurosci *16*, 141-147.

Lochter, A., Galosy, S., Muschler, J., Freedman, N., Werb, Z., and Bissell, M.J. (1997). Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. J Cell Biol *139*, 1861-1872.

Lord, C., Cook, E.H., Leventhal, B.L., and Amaral, D.G. (2000). Autism spectrum disorders. Neuron *28*, 355-363.

Luthl, A., Laurent, J.P., Figurov, A., Muller, D., and Schachner, M. (1994). Hippocampal long-term potentiation and neural cell adhesion molecules L1 and NCAM. Nature *372*, 777-779.

Malenka, R.C., and Bear, M.F. (2004). LTP and LTD: an embarrassment of riches. Neuron *44*, 5-21.

Malinverno, M., Carta, M., Epis, R., Marcello, E., Verpelli, C., Cattabeni, F., Sala, C., Mulle, C., Di Luca, M., and Gardoni, F. (2010). Synaptic localization and activity of ADAM10 regulate excitatory synapses through N-cadherin cleavage. J Neurosci *30*, 16343-16355.

Marambaud, P., Shioi, J., Serban, G., Georgakopoulos, A., Sarner, S., Nagy, V., Baki, L., Wen, P., Efthimiopoulos, S., Shao, Z., *et al.* (2002). A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. EMBO J *21*, 1948-1956.

Maretzky, T., Schulte, M., Ludwig, A., Rose-John, S., Blobel, C., Hartmann, D., Altevogt, P., Saftig, P., and Reiss, K. (2005). L1 is sequentially processed by two differently activated metalloproteases and presenilin/gamma-secretase and regulates neural cell adhesion, cell migration, and neurite outgrowth. Mol Cell Biol *25*, 9040-9053.

Mataga, N., Mizuguchi, Y., and Hensch, T.K. (2004). Experience-dependent pruning of dendritic spines in visual cortex by tissue plasminogen activator. Neuron *44*, 1031-1041.

Mataga, N., Nagai, N., and Hensch, T.K. (2002). Permissive proteolytic activity for visual cortical plasticity. Proc Natl Acad Sci U S A *99*, 7717-7721.

Matsuda, K., and Yuzaki, M. (2011). Cbln family proteins promote synapse formation by regulating distinct neurexin signaling pathways in various brain regions. Eur J Neurosci.

McGeachie, A.B., Cingolani, L.A., and Goda, Y. (2011). A stabilising influence: Integrins in regulation of synaptic plasticity. Neurosci Res.

Michaluk, P., Kolodziej, L., Mioduszewska, B., Wilczynski, G.M., Dzwonek, J., Jaworski, J., Gorecki, D.C., Ottersen, O.P., and Kaczmarek, L. (2007). Beta-dystroglycan as a target for MMP-9, in response to enhanced neuronal activity. J Biol Chem *282*, 16036-16041.

Michaluk, P., Mikasova, L., Groc, L., Frischknecht, R., Choquet, D., and Kaczmarek, L. (2009). Matrix metalloproteinase-9 controls NMDA receptor surface diffusion through integrin beta1 signaling. J Neurosci *29*, 6007-6012.

Missler, M., Zhang, W., Rohlmann, A., Kattenstroth, G., Hammer, R.E., Gottmann, K., and Sudhof, T.C. (2003). Alpha-neurexins couple Ca2+ channels to synaptic vesicle exocytosis. Nature *423*, 939-948.

Monea, S., Jordan, B.A., Srivastava, S., DeSouza, S., and Ziff, E.B. (2006). Membrane localization of membrane type 5 matrix metalloproteinase by AMPA receptor binding protein and cleavage of cadherins. J Neurosci *26*, 2300-2312.

Monory, K., Massa, F., Egertova, M., Eder, M., Blaudzun, H., Westenbroek, R., Kelsch, W., Jacob, W., Marsch, R., Ekker, M.*, et al.* (2006). The endocannabinoid system controls key epileptogenic circuits in the hippocampus. Neuron *51*, 455-466.

Morgunova, E., Tuuttila, A., Bergmann, U., Isupov, M., Lindqvist, Y., Schneider, G., and Tryggvason, K. (1999). Structure of human pro-matrix metalloproteinase-2: activation mechanism revealed. Science *284*, 1667-1670.

Moulder, K.L., Meeks, J.P., Shute, A.A., Hamilton, C.K., de Erausquin, G., and Mennerick, S. (2004). Plastic elimination of functional glutamate release sites by depolarization. Neuron *42*, 423-435.

Muller, D., Djebbara-Hannas, Z., Jourdain, P., Vutskits, L., Durbec, P., Rougon, G., and Kiss, J.Z. (2000). Brain-derived neurotrophic factor restores long-term potentiation in polysialic acid-neural cell adhesion molecule-deficient hippocampus. Proc Natl Acad Sci U S A *97*, 4315-4320.

Muller, D., Wang, C., Skibo, G., Toni, N., Cremer, H., Calaora, V., Rougon, G., and Kiss, J.Z. (1996). PSA-NCAM is required for activity-induced synaptic plasticity. Neuron *17*, 413-422.

Mumm, J.S., Schroeter, E.H., Saxena, M.T., Griesemer, A., Tian, X., Pan, D.J., Ray, W.J., and Kopan, R. (2000). A ligand-induced extracellular cleavage

regulates gamma-secretase-like proteolytic activation of Notch1. Mol Cell *5*, 197-206.

Murase, S., Mosser, E., and Schuman, E.M. (2002). Depolarization drives beta-Catenin into neuronal spines promoting changes in synaptic structure and function. Neuron *35*, 91-105.

Murthy, V.N., Schikorski, T., Stevens, C.F., and Zhu, Y. (2001). Inactivity produces increases in neurotransmitter release and synapse size. Neuron *32*, 673-682.

Mysore, S.P., Tai, C.Y., and Schuman, E.M. (2007). Effects of N-cadherin disruption on spine morphological dynamics. Front Cell Neurosci *1*, 1.

Nagy, V., Bozdagi, O., Matynia, A., Balcerzyk, M., Okulski, P., Dzwonek, J., Costa, R.M., Silva, A.J., Kaczmarek, L., and Huntley, G.W. (2006). Matrix metalloproteinase-9 is required for hippocampal late-phase long-term potentiation and memory. J Neurosci *26*, 1923-1934.

Nam, C.I., and Chen, L. (2005). Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter. Proc Natl Acad Sci U S A *102*, 6137-6142.

Noe, V., Fingleton, B., Jacobs, K., Crawford, H.C., Vermeulen, S., Steelant, W., Bruyneel, E., Matrisian, L.M., and Mareel, M. (2001). Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. J Cell Sci *114*, 111-118.

Nugent, F.S., Penick, E.C., and Kauer, J.A. (2007). Opioids block long-term potentiation of inhibitory synapses. Nature *446*, 1086-1090.

Ogata, Y., Enghild, J.J., and Nagase, H. (1992). Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. J Biol Chem *267*, 3581-3584.

Paradis, S., Sweeney, S.T., and Davis, G.W. (2001). Homeostatic control of presynaptic release is triggered by postsynaptic membrane depolarization. Neuron *30*, 737-749.

Pardo, C.A., and Eberhart, C.G. (2007). The neurobiology of autism. Brain Pathol *17*, 434-447.

Petrenko, A.G., Ullrich, B., Missler, M., Krasnoperov, V., Rosahl, T.W., and Sudhof, T.C. (1996). Structure and evolution of neurexophilin. J Neurosci *16*, 4360-4369.

Philpot, B.D., Cho, K.K., and Bear, M.F. (2007). Obligatory role of NR2A for metaplasticity in visual cortex. Neuron *53*, 495-502.

Philpot, B.D., Sekhar, A.K., Shouval, H.Z., and Bear, M.F. (2001). Visual experience and deprivation bidirectionally modify the composition and function of NMDA receptors in visual cortex. Neuron *29*, 157-169.

Pillai-Nair, N., Panicker, A.K., Rodriguiz, R.M., Gilmore, K.L., Demyanenko, G.P., Huang, J.Z., Wetsel, W.C., and Maness, P.F. (2005). Neural cell adhesion molecule-secreting transgenic mice display abnormalities in GABAergic interneurons and alterations in behavior. J Neurosci *25*, 4659-4671.

Pitkanen, A., and Sutula, T.P. (2002). Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. Lancet Neurol *1*, 173-181.

Poulopoulos, A., Aramuni, G., Meyer, G., Soykan, T., Hoon, M., Papadopoulos, T., Zhang, M., Paarmann, I., Fuchs, C., Harvey, K.*, et al.* (2009). Neuroligin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. Neuron *63*, 628-642.

Pozo, K., and Goda, Y. (2010). Unraveling mechanisms of homeostatic synaptic plasticity. Neuron *66*, 337-351.

Prange, O., Wong, T.P., Gerrow, K., Wang, Y.T., and El-Husseini, A. (2004). A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. Proc Natl Acad Sci U S A *101*, 13915-13920.

Regehr, W.G., Carey, M.R., and Best, A.R. (2009). Activity-dependent regulation of synapses by retrograde messengers. Neuron *63*, 154-170.

Reiss, K., Maretzky, T., Ludwig, A., Tousseyn, T., de Strooper, B., Hartmann, D., and Saftig, P. (2005). ADAM10 cleavage of N-cadherin and regulation of cell-cell adhesion and beta-catenin nuclear signalling. EMBO J *24*, 742-752.

Ronn, L.C., Bock, E., Linnemann, D., and Jahnsen, H. (1995). NCAM-antibodies modulate induction of long-term potentiation in rat hippocampal CA1. Brain Res *677*, 145-151.

Rubenstein, J.L., and Merzenich, M.M. (2003). Model of autism: increased ratio of excitation/inhibition in key neural systems. Genes Brain Behav *2*, 255-267.

Rutherford, L.C., Nelson, S.B., and Turrigiano, G.G. (1998). BDNF has opposite effects on the quantal amplitude of pyramidal neuron and interneuron excitatory synapses. Neuron *21*, 521-530.

Sbai, O., Ould-Yahoui, A., Ferhat, L., Gueye, Y., Bernard, A., Charrat, E., Mehanna, A., Risso, J.J., Chauvin, J.P., Fenouillet, E.*, et al.* (2010). Differential vesicular distribution and trafficking of MMP-2, MMP-9, and their inhibitors in astrocytes. Glia *58*, 344-366.

Schapitz, I.U., Behrend, B., Pechmann, Y., Lappe-Siefke, C., Kneussel, S.J., Wallace, K.E., Stempel, A.V., Buck, F., Grant, S.G., Schweizer, M.*, et al.* (2010). Neuroligin 1 is dynamically exchanged at postsynaptic sites. J Neurosci *30*, 12733-12744.

Scheiffele, P., Fan, J., Choih, J., Fetter, R., and Serafini, T. (2000). Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. Cell *101*, 657-669.

Schettini, G., Govoni, S., Racchi, M., and Rodriguez, G. (2010). Phosphorylation of APP-CTF-AICD domains and interaction with adaptor proteins: signal transduction and/or transcriptional role--relevance for Alzheimer pathology. J Neurochem *115*, 1299-1308.

Shapiro, L., Love, J., and Colman, D.R. (2007). Adhesion molecules in the nervous system: structural insights into function and diversity. Annu Rev Neurosci *30*, 451-474.

Sheng, M. (2001). Molecular organization of the postsynaptic specialization. Proc Natl Acad Sci U S A *98*, 7058-7061.

Sheng, M., McFadden, G., and Greenberg, M.E. (1990). Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron *4*, 571-582.

Song, J.Y., Ichtchenko, K., Sudhof, T.C., and Brose, N. (1999). Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. Proc Natl Acad Sci U S A *96*, 1100-1105.

Stan, A., Pielarski, K.N., Brigadski, T., Wittenmayer, N., Fedorchenko, O., Gohla, A., Lessmann, V., Dresbach, T., and Gottmann, K. (2010). Essential cooperation of N-cadherin and neuroligin-1 in the transsynaptic control of vesicle accumulation. Proc Natl Acad Sci U S A *107*, 11116-11121.

Staubli, U., Vanderklish, P., and Lynch, G. (1990). An inhibitor of integrin receptors blocks long-term potentiation. Behav Neural Biol *53*, 1-5.

Sternlicht, M.D., and Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol *17*, 463-516.

Stevenson, B.R., and Keon, B.H. (1998). The tight junction: morphology to molecules. Annu Rev Cell Dev Biol *14*, 89-109.

Sudhof, T.C. (2008). Neuroligins and neurexins link synaptic function to cognitive disease. Nature *455*, 903-911.

Sugita, S., Khvochtev, M., and Sudhof, T.C. (1999). Neurexins are functional alpha-latrotoxin receptors. Neuron *22*, 489-496.

Sugita, S., Saito, F., Tang, J., Satz, J., Campbell, K., and Sudhof, T.C. (2001). A stoichiometric complex of neurexins and dystroglycan in brain. J Cell Biol *154*, 435-445.

Suzuki, F., Makiura, Y., Guilhem, D., Sorensen, J.C., and Onteniente, B. (1997). Correlated axonal sprouting and dendritic spine formation during kainate-induced neuronal morphogenesis in the dentate gyrus of adult mice. Exp Neurol *145*, 203-213.

Szklarczyk, A., Lapinska, J., Rylski, M., McKay, R.D., and Kaczmarek, L. (2002). Matrix metalloproteinase-9 undergoes expression and activation during dendritic remodeling in adult hippocampus. J Neurosci *22*, 920-930.

Tabuchi, K., and Sudhof, T.C. (2002). Structure and evolution of neurexin genes: insight into the mechanism of alternative splicing. Genomics *79*, 849-859.

Tanaka, H., Shan, W., Phillips, G.R., Arndt, K., Bozdagi, O., Shapiro, L., Huntley, G.W., Benson, D.L., and Colman, D.R. (2000). Molecular modification of N-cadherin in response to synaptic activity. Neuron *25*, 93-107.

Tang, L., Hung, C.P., and Schuman, E.M. (1998). A role for the cadherin family of cell adhesion molecules in hippocampal long-term potentiation. Neuron *20*, 1165-1175.

Thiagarajan, T.C., Lindskog, M., and Tsien, R.W. (2005). Adaptation to synaptic inactivity in hippocampal neurons. Neuron *47*, 725-737.

Thyagarajan, A., and Ting, A.Y. (2010). Imaging activity-dependent regulation of neurexin-neuroligin interactions using trans-synaptic enzymatic biotinylation. Cell *143*, 456-469.

Trachtenberg, J.T., Chen, B.E., Knott, G.W., Feng, G., Sanes, J.R., Welker, E., and Svoboda, K. (2002). Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature *420*, 788-794.

Tropea, D., Majewska, A.K., Garcia, R., and Sur, M. (2010). Structural dynamics of synapses in vivo correlate with functional changes during experience-dependent plasticity in visual cortex. J Neurosci *30*, 11086-11095.

Turrigiano, G.G. (1999). Homeostatic plasticity in neuronal networks: the more things change, the more they stay the same. Trends Neurosci *22*, 221-227.

Turrigiano, G.G. (2008). The self-tuning neuron: synaptic scaling of excitatory synapses. Cell *135*, 422-435.

Uemura, K., Kihara, T., Kuzuya, A., Okawa, K., Nishimoto, T., Ninomiya, H., Sugimoto, H., Kinoshita, A., and Shimohama, S. (2006). Characterization of sequential N-cadherin cleavage by ADAM10 and PS1. Neurosci Lett *402*, 278-283.

Ullrich, B., Ushkaryov, Y.A., and Sudhof, T.C. (1995). Cartography of neurexins: more than 1000 isoforms generated by alternative splicing and expressed in distinct subsets of neurons. Neuron *14*, 497-507.

Ushkaryov, Y.A., Petrenko, A.G., Geppert, M., and Sudhof, T.C. (1992). Neurexins: synaptic cell surface proteins related to the alpha-latrotoxin receptor and laminin. Science *257*, 50-56.

Ushkaryov, Y.A., Rohou, A., and Sugita, S. (2008). alpha-Latrotoxin and its receptors. Handb Exp Pharmacol, 171-206.

Ushkaryov, Y.A., and Sudhof, T.C. (1993). Neurexin III alpha: extensive alternative splicing generates membrane-bound and soluble forms. Proc Natl Acad Sci U S A *90*, 6410-6414.

Van Wart, H.E., and Birkedal-Hansen, H. (1990). The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci U S A *87*, 5578-5582.

Varoqueaux, F., Aramuni, G., Rawson, R.L., Mohrmann, R., Missler, M., Gottmann, K., Zhang, W., Sudhof, T.C., and Brose, N. (2006). Neuroligins determine synapse maturation and function. Neuron *51*, 741-754.

Varoqueaux, F., Jamain, S., and Brose, N. (2004). Neuroligin 2 is exclusively localized to inhibitory synapses. Eur J Cell Biol *83*, 449-456.

Venero, C., Herrero, A.I., Touyarot, K., Cambon, K., Lopez-Fernandez, M.A., Berezin, V., Bock, E., and Sandi, C. (2006). Hippocampal up-regulation of NCAM expression and polysialylation plays a key role on spatial memory. Eur J Neurosci *23*, 1585-1595.

Viegi, A., Cotrufo, T., Berardi, N., Mascia, L., and Maffei, L. (2002). Effects of dark rearing on phosphorylation of neurotrophin Trk receptors. Eur J Neurosci *16*, 1925-1930.

Vogt, K., Mellor, J., Tong, G., and Nicoll, R. (2000). The actions of synaptically released zinc at hippocampal mossy fiber synapses. Neuron *26*, 187-196.

Wang, X.B., Bozdagi, O., Nikitczuk, J.S., Zhai, Z.W., Zhou, Q., and Huntley, G.W. (2008). Extracellular proteolysis by matrix metalloproteinase-9 drives dendritic spine enlargement and long-term potentiation coordinately. Proc Natl Acad Sci U S A *105*, 19520-19525.

Wierenga, C.J., Walsh, M.F., and Turrigiano, G.G. (2006). Temporal regulation of the expression locus of homeostatic plasticity. J Neurophysiol *96*, 2127-2133.

Wilczynski, G.M., Konopacki, F.A., Wilczek, E., Lasiecka, Z., Gorlewicz, A., Michaluk, P., Wawrzyniak, M., Malinowska, M., Okulski, P., Kolodziej, L.R.*, et al.* (2008). Important role of matrix metalloproteinase 9 in epileptogenesis. J Cell Biol *180*, 1021-1035.

Wilson, R.I., and Nicoll, R.A. (2001). Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. Nature *410*, 588-592.

Wittenmayer, N., Korber, C., Liu, H., Kremer, T., Varoqueaux, F., Chapman, E.R., Brose, N., Kuner, T., and Dresbach, T. (2009). Postsynaptic Neuroligin1 regulates presynaptic maturation. Proc Natl Acad Sci U S A *106*, 13564-13569.

Xu, J., Xiao, N., and Xia, J. (2010). Thrombospondin 1 accelerates synaptogenesis in hippocampal neurons through neuroligin 1. Nat Neurosci *13*, 22-24.

Yamagata, M., Sanes, J.R., and Weiner, J.A. (2003). Synaptic adhesion molecules. Curr Opin Cell Biol *15*, 621-632.

Yong, V.W. (2005). Metalloproteinases: mediators of pathology and regeneration in the CNS. Nat Rev Neurosci *6*, 931-944.

Zhang, C., Atasoy, D., Arac, D., Yang, X., Fucillo, M.V., Robison, A.J., Ko, J., Brunger, A.T., and Sudhof, T.C. (2010). Neurexins physically and functionally interact with GABA(A) receptors. Neuron *66*, 403-416.

Zhang, W., Rohlmann, A., Sargsyan, V., Aramuni, G., Hammer, R.E., Sudhof, T.C., and Missler, M. (2005). Extracellular domains of alpha-neurexins participate in regulating synaptic transmission by selectively affecting N- and P/Q-type Ca2+ channels. J Neurosci *25*, 4330-4342.

Zucker, R.S., and Regehr, W.G. (2002). Short-term synaptic plasticity. Annu Rev Physiol *64*, 355-405.