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UNDERSTANDING THE INTERACTIONS BETWEEN $GABA_AR$ AND SYNAPTIC ADHESION PROTEINS NEUROLIGIN1 AND NEUROLIGIN2

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ABSTRACT

Gamma aminobutyric acid type A receptors ($GABA_A$Rs) are the principal inhibitory receptors in the brain. Disruption of their function was shown to be a factor in familiar epilepsy and is further implicated in a number of other neurological and neuropsychiatric disorders. Of particular interest is the mechanism of trafficking and stabilization of $GABA_A$Rs in the postsynaptic cell membrane. Preliminary findings in our lab had demonstrated that $GABA_A$R subunits display differential interaction with different members of a family of synaptic adhesion molecules known as neuroligins (NLs). In this study, I went on to explore these interactions further, through analysis of modified NL constructs and the testing of additional $GABA_A$R subunits. I observed efficientimmuno-copurification of $GABA_A$R subunits and NLs from transfected human embryo kidney cells. Specifically, I found that the γ2 and β2 subunits interact stronger with NL2 than with NL1, while the α1 and α2 subunits show no preference for interaction with NL2 vs. NL1. Additionally, I observed some evidence of proteolytic degradation of NL2following coexpression specifically of the β3 subunit - no such effect was evident in the case of α1, 2 and the γ2 subunits. These results support the hypothesis that $GABA_A$Rs and neuroligins corroborate in their postsynaptic targeting. It is possible that the interaction between γ2 and NL2 serves to stabilize specific subtypes of $GABA_A$Rs at synaptic sites. However, the role of β3 in NL2 degradation has to be explored further and controlled for in future experiments.

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Acknowledgements

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Chapter 1
Introduction

GABA-A receptors

The neurotransmitter primarily responsible for regulating inhibitory neurotransmission in the brain is γ-aminobutyric acid (GABA). The GABA type A receptors ($GABA_AR$s) responds to GABA signaling by changing membrane permeability to chloride ions. These are heteropentameric receptors that are part of the Cys-loop ligand-gated ion channel superfamily (Barnard et al., 1998). A total of 19 genes encoding $GABA_AR$ subunits have been identified: α(1–6), β(1–3), γ(1–3), δ, ε, θ, π, and 3 ρ subunits (Olsen and Sieghart, 2009). Alternative splicing of the γ2 (Whiting et al., 1990) and the β2 (McKinley et al., 1995) further enhance receptor diversity. This variety in $GABA_AR$ subunits implies that a large number of variable $GABA_AR$ subtypes exist. In fact, 11 distinct receptor subtypes have been conclusively identified in the brain (Luscher et al., 2011).

$GABA_AR$s containing the γ2 subunit in association with α1, α2 or α3 and unspecified β subunits predominantly mediate phasic inhibition, while receptors that contain the γ2 or δ subunit in combination with α4, α5 or α6 subunits are either predominantly or exclusively extrasynaptic and mediate tonic inhibition (Farrant and Nusser, 2005).

Of particular interest is the β3 $GABA_AR$ subunit. Homomeric β3 ion channels have been described in transfected HEK cells and *Xenopus* oocytes and shown to mediate a GABA-independent resting membrane conductance (Wooltorton et al., 1997). The resulting ion channels could be modulated by the $GABA_AR$ antagonists picrotoxin and zinc, but they were insensitive to GABA itself (Wooltorton et al., 1997). In contrast, there is no evidence of other $GABA_AR$

subunits forming homomeric ion channels, with the exception of the β1 subunit expressed in oocytes (Krishek et al., 1996).

Neuroligin 1 and 2

Synaptic adhesion complexes play an important role in the structural and functional maturation of synapses. Four different neuroligin (NL) genes have been identified, each with specific localization (Xu et al., 2012). They interact with pre-synaptic proteins know as neurexins (Ichtchenko et al., 1995, Ichtchenko et al., 1996), and with intracellular scaffolding proteins such as postsynaptic density protein 95 (Sudhof, 2008). At the same time, neurexins interact with the presynaptic calcium/calmodulin-dependent serine protein kinase scaffolding protein (Sudhof, 2008). Mutations in NL/neurexin complexes together account for a significant proportion of autism patients (Sudhof, 2008). In terms of localization, NL1 is typically found at excitatory glutamatergic synapses (Song et al., 1999). Additionally, NL1 has been found to be an important component for functional nicotinic synapses (Conroy et al., 2007). In contrast, NL2 is localized at inhibitory GABAergic synapses (Varoqueaux et al., 2004). Further, ectopically expressed NL1 can preferentially induce glutamatergic synapse formation, while overexpression of NL2 induces the formation of GABAergic synapses (Graf et al., 2004, Chih et al., 2005). Glycinergic synapses, a different type of inhibitory synapses, contain NL4 (Hoon et al., 2009). Neuroligin 3 is thought to be present at both GABAergic and glutamatergic synapses (Budreck and Scheiffele, 2007).

In the present study, the aim was to determine the presence and the nature of interactions between NL2 and $GABA_AR$ subunits. We originally intended to use NL1 as a negative control for interactions with $GABA_AR$ subunits as it is not normally concentrated at inhibitory GABAergic synapses. Numerous research findings suggest either direct or indirect links between

$GABA_A$Rs and NL2. Deletion of NL2 reduces $GABA_A$R clustering, and perturbs GABAergic synaptic transmission (Hoon et al., 2009, Poulopoulos et al., 2009), while mislocalized expression of NL2 dispersed synaptic proteins, among them $GABA_A$Rs (Graf et al., 2004).NL2 and $GABA_A$Rs can even reconstitute functional GABAergic postsynaptic complexes in cotransfected HEK cells (Dong et al., 2007, Wu et al., 2012). Additionally, our lab has seen that NL2 disappears from synapses in γ2 KO mice (Masuda S, 2007). Moreover, reduced cell surface expression of α and β subunits of $GABA_A$Rs correlated with reduced expression of NL2 at the neural cell surface (Masuda S, 2007). Therefore, we hypothesize that $GABA_A$Rs and NL2 are part of one and the same complex and may interact directly with each other, or though indirect interaction through the scaffolding protein gephyrin must also be considered as gephyrin interacts both with $GABA_A$R subunits α1 (Mukherjee et al., 2011), α2 (Tretter et al., 2008), α3 (Tretter et al., 2011) and NL2 (Poulopoulos et al., 2009, Tretter et al., 2012).

Recent publications indicate that in order to fully understand the interactions between $GABA_A$Rs and NLs, these interactions must be considered in context of cleavage of NLs by matrixmetalloproteinases (MMPs), α-, and γ-secretases (Peixoto et al., 2012, Suzuki et al., 2012).The level of NL1 is a determining factor for both synapse number and activity-dependent synaptogenesis (Kwon et al., 2012). NL1 undergoes ectodomain shedding at the juxtamembrane stalk region to generate a secreted soluble form of NL1 and a membrane bound, C-terminal portion. The C-terminal membrane bound region is subsequently cleaved by presenilin/γ-secretase (Suzuki et al., 2012). The same research group found that NL2 is processed in a manner similar to NL1, though the specific proteases responsible have not yet been identified (Suzuki et al., 2012). A parallel finding is that, synaptic activity triggers MMP-9 dependent proteolysis of NL1, an effect reversed by MMP inhibitors (Peixoto et al., 2012). Further,

increases in MMP-9 mRNA levels have been detected in the hippocampus two hours post-seizure onset induced by administration of the $GABA_A$ receptor antagonist pentylenetetrazole (Huntley, 2012). It is likely that the mechanism of NL processing parallel and redundant, with the specific protease responsible for NL1 processing depends on the exact circumstances. Both studies indicate that ectodomain shedding of NL1 is regulated by synaptic activity, pointing to a novel activity-dependent mechanism that regulates synapse formation and function (Peixoto et al., 2012, Suzuki et al., 2012).

In this study, I assessed the interactions between $GABA_AR$ subunits and NLs. In order to do so, the co-immunoprecipitation (Co-IP) technique was used. $GABA_AR$ subunits and NLs were co-expressed in HEK-293T cells. Next, agarose beads charged with either myc-tagged $GABA_AR$ subunits or HA-NLs were used to precipitate either HA-NLs or $GABA_AR$ subunits respectively. The resulting complexes were analyzed via SDS-PAGE/western blots. Western blot analysis of IP complexes revealed different affinities of $GABA_AR$ subunits to NLs. These differences in affinity could explain subunit-composition-dependent $GABA_AR$ clustering at inhibitory synapses. However, evidence of proteolytic processing means that these results have to be confirmed using more stringent conditions.

Chapter 2
Methods

Plasmids

Myc-tagged mouse GABAR α1 cDNA in pRK5 vector was obtained from Mike Lumb, Moss Lab (p502). Myc-tagged rat GABAR α2 mature cDNA in pEGFP vector (Clontech), produced by Lüscher lab (p655).The myc-tagged mouse GABA α1 and β2 cDNAs in pRK5 vector wereobtained from Steve Moss (Tufts University, Boston, MA) (p503).The plasmid encoding the myc-tagged mouse $GABA_AR$ γ2 cDNA (p636) waspreviously described(Alldred et al., 2005). The myc-tagged GABAR γ2 mutant cDNA (3C-SA) in pGBKT7 vector was generously provided by Xu Yuan (Luscher lab, p644). It is identical with P636 except for the three Cys residues that were mutated to Ser or Ala in the cytoplasmic loop of the g2 subunit. The untagged mouse $GABA_AR$ α1, β3 and γ2 cDNAs in pRK5 were obtained from Steven Moss(p690, p693, and p694, resp.). HA-tagged mouse neuroligin-1 (NL1, p779) and neuroligin-2 (NL2,p780) cDNAsin pNICE vector were obtained from Scheiffele lab (Scheiffele et al., 2000).C-terminally truncated HA-NL1 (HA-NL1ΔC) and HA-NL2ΔC (p790) in pNICE (Clontech) were produced by Zhen Ren, Luscher Lab.

Cell Culture and Transfection

Human Embryonic Kidney (HEK) 293T cells were used for all co-immunoprecipitation (CoIP) experiments. Cells were cultured in 10cm plates with 10ml High-Fructose Dulbecco's Modified Eagle Medium (DMEM) (cat. No 11965, Gibco, Life Technologies Co, Grand Island, NY), 10% fetal bovine serum (FBS), penicillin/streptomycin (cat. No 081M0845, SIGMA, St. Louis, MO). Upon reaching near confluency the cells were split 1/20 into new 6 cm plates. They were transfected approx. 48-72 h. after plating. Lipid transfection was used in all experiments. First, plasmid DNA (1-4 µg) was thoroughly mixed in Opti-MEM (LifeTechnologies, Carlsbad,

CA) (200µl/plate). Secondly, an appropriate amount of polyethyleneimine (PEI) (Life Technologies) was added to the mix with the amount determined as 4 x µg of DNA. The solution was thoroughly mixed, left alone for five min., and subsequently distributed into plates evenly. The medium was changed between four and five h. after transfection and the cells were grown to confluency.

Protein Extraction and Preparation

To collect cells, first all culture media was aspirated. Cells were washed twice with 3ml ice-cold Phosphate-Buffered Saline (PBS), subsequently suspended in 1ml cold PBS, and transferred to a cold 1.5ml Eppendorf tube. Cells were centrifuged at 1000 x g for two min. at 4° C, and the supernatant (SN) discarded. The cell pellet was gently re-suspended and washed twice with 1ml cold PBS, SN discarded.

For 6cm plates with cells confluent or almost confluent, 300µl DOC protein extraction buffer was used (10 mMTris-HCl pH 8.0, 150 mM NaCl, 0.5% deoxycholate (cat. No D5760, SIGMA), 0.05% phosphatidyl choline, 1mM benzamidine, 100 µg/ml bacitracin (cat. No 11805, Amersham, USB, Cleveland, OH). Prior to use, fresh protease inhibitors were added to a final concentration of 1 pg/ml leupeptin, 1 pg/ml pepstatinA, 1 pg/mlaprotinin, 1pg/ml antipain as a 1000x stock in methanol. Additionally, fresh phenylmethanesulfonylfluoride (PMSF) was added to a final concentration of 1mM as a 100x stock in isopropanol. After addition of the DOC protein extraction buffer, cells were gently resuspended using a cut-off 1000 µl pipette tip. Cells were sonicated for 10 sec. on setting of 2.5 of continuous pulse (Sonifier 450, 3 mm Double-Step microtip, Branson Ultrasonics, Danbury, CT), and immediately returned to ice. After incubation on ice for 30 min., cells were centrifuged at 10,000g for 5 min. to remove insoluble material, and the SN was transferred while carefully and strictly avoiding any of the pellet.

In order to account for the differing affinity of agarose types to different antibody (Ab) source animals, different agarose types were used. Protein-A-Agarose (cat. No P7786, SIGMA) was used for rabbit-sourced antibodies, and Protein-G-Agarose (cat. No P7700, SIGMA) was used for rat-sourced antibodies. Agarose beads were prepared in the following manner. Agarose beads were either dissolved from powder or used as bought in cold PBS. To the agarose bead suspension, 1ml cold PBS and 10µl of 10mg/ml BSA (New England Biolabs, Ipswich, MA) was added. The solution was mixed, and spun at 2,500 x g for 2 min. at 4°C. The SN was discarded, and the beads were washed twice with 1ml PBS. After the second wash, 30-40 µl of 10 mg/ml BSA was added to the resin, mixed, and 950 µl of cold PBS was added. The resin was spun as above, and the SN discarded.

Co-Immunoprecipitation

In order to eliminate the possibility of unwanted interactions between proteins of interest and the agarose beads, the protein solution was pre-cleared as follows: 5 µl of freshly prepared agarose beads were added to the protein solution and the solution was rotated at 4° C for 1hr. The agarose beads were then centrifuged out at maximum speed (18,000 x g) for five min., and the SN was transferred to a new tube.

For co-immunoprecipitation (co-IP), the following antibodies were used. Rabbit anti-myc tag IgG from (MBL International Corporation, Woburn, MA). Rat anti-HA tag monoclonal IgG (Roche Pharmaceuticals, Nutley, New Jersey). HRP-conjugated donkey anti-rabbit secondary antibody (Amersham, Pittsburgh, PA) and HRP-conjugated anti-rat secondary antibody (GE Healthcare, Piscataway, NJ) were used for western blot detection.

To the IP protein solution, 3-5 µg appropriate antibody was added. The sample was incubated with end-over-end mixing O/N at 4° C. In order to remove unwanted protein

precipitates that formed, the solution was spun at 18,000 g for 10 min., and the SN removed into Sigma-coated (SIGMA) Eppendorf tubes. Affinity resin amount equal to the amount of antibodies was added into the Eppendorf tube using a cut-off, Sigma-coated pipette tips. The solution was incubated end-over-end for three to four h. at 4° C.

To wash away proteins that interact un-specifically, IP wash buffer was used (50mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% deoxycholate, 1% Triton X-100, 0.02% NaN_3). Prior to use of the wash buffer, protease inhibitors and PMSF were added as described above. Samples were spun at 2,500 x g for 2 min. at 4° C. The SN was saved for new experiments, but discarded for repeat experiments. The resin was washed three times for 20-30 min. using 1ml IP wash buffer for each wash. Between washes, samples were spun at 1,000 x g for 2 min. at 4° C, taking care not to disturb the cell pellet. Next, the resin was washed with 1ml cold PBS for 15 min. at 4° C to remove traces of Triton X-100 which could interfere with SDS-Page gel Resolution. After the final wash step, the samples were spun at 1,000 x g for two min. at 4° C, and the SN carefully removed to leave 5-10 µl PBS covering the resin. An equal amount of 2x SDS sample buffer (20 mM Tris-HCl pH 6.8, 200 mM DTT added fresh, 4 M urea, 2% SDS, 20% glycerol, 0.2% bromophenol blue) was added to the resin, and the samples were stored at -80° C, or processed further on an SDS-Page blot.

Western Blot

Separating gels contained either 10 or 12% acrylamide (cat. No E347, AMPRESCO, Solon, OH) depending on desired resolution. Ten ml of 10% separating gel (0.375 M Tris-HCl, pH 7.5, 0.001 % SDS, 10 % acrylamide/bis, 0.0005 % ammonium persulfate, and 5 µl tetramethylethylenediamine) was poured into a 1.5 mm thick gel mold, and overlaid with isoporopanol until polymerization. After rinsing the separating gel with dH_2O and wicking away

remaining water with a paper towel, a 4 % stacking gel was poured, and a 15-well comb placed on top. Prior to loading, samples (10 µl/well) were heated at 95°C for 5-10 min. The electrophoresis gel was ran at 175 V, 0.13 A for 1-2 h. depending on the desired separation. Consequently, a semi-dry transfer using an OWL Hep-1 (Thermo Scientific) was performed onto 0.2µm Westran S Polyvinylidine Fluoride (PVDF) membrane (GE Healthcare) by applying a constant voltage of 15V for 1h.

The membrane was subsequently blocked O/N at 4° C in 5 % dry milk (Walmart, Bentonville, AZ) in Tris-buffered saline with 0.05 % Tween 20 (TBST). Afterwards, primary antibodies at a dilution of 1:1000 in 3ml 5% (w/v) dried milk in TBST were applied to the membrane during continuous rotation at 4°C O/N. The membrane was then washed once in RIPEA (20 mM Tris-HCl pH 7.5, 60 mM NaCl, 2 mM EDTA pH 8.0, 0.4 % SDS, 0.4 % Triton X-100) buffer, and three times in TBST buffer for 20min./wash, blocked for 30min-1h in 5% milk TBST, and an HRP-conjugated secondary antibody applied at a dilution of 1:5000 for either 2h. at RT, or O/N at 4°C with shaking.

Prior to development, the membrane was rinsed in RIPEA and TBST buffers as described above and developed using the ECL Prime Western Blotting Detection Reagent (GE Healthcare) as per manufacturer instructions. If needed, membranes were stripped using 0.2M NaOH, and re-probed.

Chapter 3
Results

Preliminary Data

Experiments previously conducted by Zhen Ren in our lab suggested that myc-tagged $GABA_AR$ subunits display differential interaction with HA-NL2 (Fig. 1). It was therefore important to explore these interactions further. My objective was to clarify the subunit and neuroligin subtype specificity of these interactions.

In Zhen's experiment, human embryonic kidney (HEK) 293T cells were co-transfected with either myc-tagged $GABA_AR$ subunits (α1, β2, γ2) or untagged subunits (α1, β2, δ) as putative negative controls with HA-tagged NL2. All plasmids were transfected at 2 μg each. NL-$GABA_AR$ complexes were immunopurified using protein-A-agarose resin charged with rabbit anti-myc and analyzed by 10% acrylamide SDS-PAGE/western blots probed with rat anti-HA as described above. Aliquots of total cell extracts were analyzed on parallel gels and probed analogously with rat anti-HA to control for altered expression of transfected proteins as a loading control.

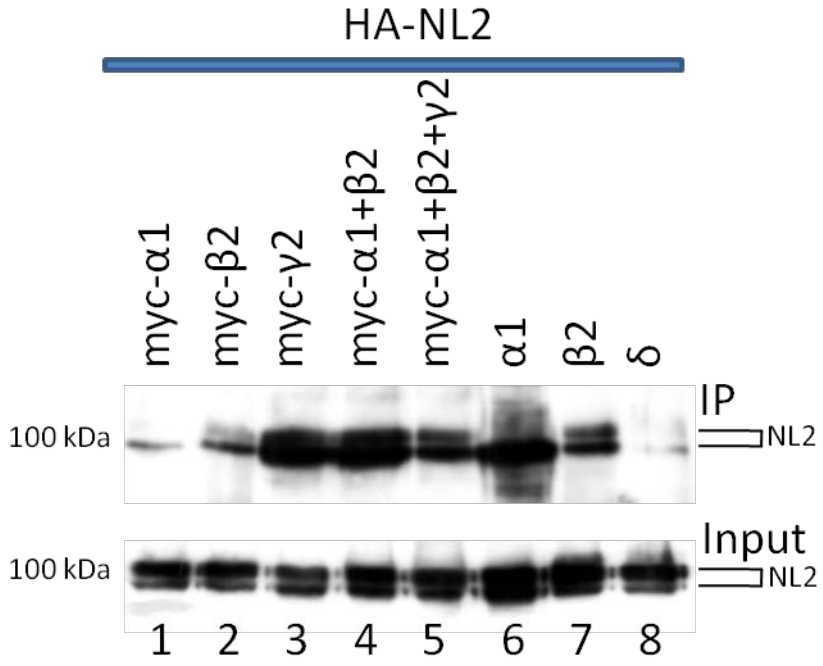

Figure 1: Immunoblot analysis of co-immunoprecipitation of HA-NL2 with $GABA_AR$ subunits. HEK 293T cells were co-transfected with either myc-tagged $GABA_AR$ subunits (α1, β2, γ2) or untagged subunits (α1, β2, δ) as putative negative controls, and HA-tagged NL2. (All constructs were transfected at 2 µg each.) The pull-down was performed with rabbit α-myc Ab, and HA-NL2 was detected with rat α-HA Ab (1:1000) and HRP anti-Rat 2' Ab (1:5000). Complexes were analyzed by PAGE(10% acrylamide)Note that different subunits have differing ability to precipitate HA-NL2 (lanes 1-5), and that untagged subunits intended as negative controls precipitate HA-NL2 nonetheless (lanes 6,7). Contributed by Zhen Ren.

It was observed that different subunits immunopurified NL2 with differing efficiency. The presence of myc-α1 or myc-β2 resulted in weak immunopurification (Fig. 1, lanes 1,2). The presence of myc-γ2 resulted in efficient immunopurification HA-NL2 (Fig. 1, lane 3). The combination of α1/β2 subunits, as well as a triple combination of α1/β2/γ2 also resulted in efficient immunopurification HA-NL2 (Fig. 1, lanes 3,4,5). Untagged $GABA_AR$ subunits α1, β2, and δ (lanes 6,7,8) were intended as negative controls, however immunopurification of HA-NL2occurred regardless (Fig. 1, lanes 6,7). Given that the negative controls did not in fact show a lack of interaction (with the exception of the δ subunit), the experiment showed that $GABA_AR$ subunits are inherently insoluble, and a pre-clearing step was needed in future experiments to get

rid of unspecific precipitates. Additionally, a spin down step after Ab incubation, but before resin addition would be introduced for future experiments to solve the same problem (see materials and methods). These steps would have eliminated interactions between HA-NL2 and the resin, and insoluble HA-NL2-containing complexes respectively.

GABAAR subunit γ2 interacts strongest with NL1ΔC andNL2ΔC

Towards examining the NL domains that mediate the interaction of NL1/2 with $GABA_A$Rs, plasmids encoding C-terminally truncated versions of HA-NL1 and HA-NL2 were created (HA-NL1ΔC and HA-NL2ΔC respectively), that lack the terminal cytoplasmic domain. This was accomplished by introducing a stop codon at position 703 for NL2. Co-immunoprecipitations were then performed with these constructs to address whether extracellular and transmembrane domains are sufficient for interaction with $GABA_A$R subunits.

HEK 293T cells were co-transfected with myc-tagged γ2 $GABA_A$R subunits and HA-tagged NL1/NL1ΔC/NL2/NL2ΔC. NLs were transfected at 2 µg DNA/plate with the exception of NL2 (1 µg DNA/plate), and all myc-γ2 constructs were transfected at 2 µg DNA/plate. Additionally, to test whether palmitoylation of the γ2 subunit plays a role in the interactions, I performed parallel co-IPs with a myc-γ2 constructs in which the cytoplasmic Cys residues required for palmitoylation of the γ2 subunit were mutated to Ala or Ser (Fang et al., 2006).

NL-γ2 complexes of duplicate transfections were immunopurified using protein-A-agarose resin charged with rabbit anti-myc and analyzed by SDS-PAGE/western blots (10% acrylamide) probed with rat anti-HA antiserum as described above. Aliquots of the total cell extracts were analyzed on parallel gels and probed analogously with rabbit anti-HA to control for altered expression of transfected proteins and as a loading control.

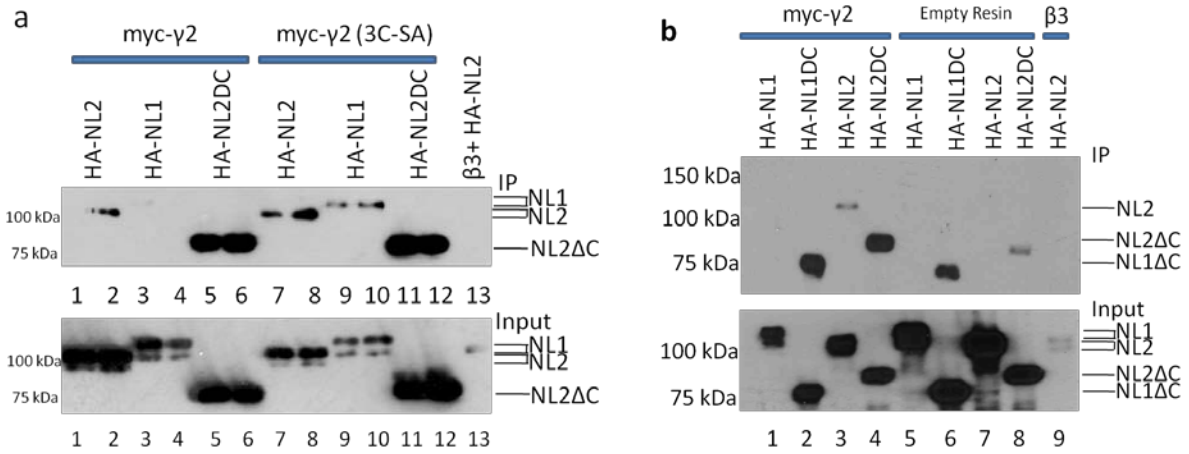

Figure 2: Analysis of interactions between HA-tagged neuroligin constructs and myc-tagged $GABA_AR\gamma2$ subunit constructs in extracts of transfected HEK cells. (a) HA-tagged Neuroligin adhesion proteins were pulled down with myc-γ2 constructs, with experiments performed in duplicate. (b) HA-tagged Neuroligin adhesion proteins were pulled down with myc-γ2 constructs to confirm immunoprecipitations seen in a. Blots were probed with Rat αHA 1' Ab (1:1000) and HRP αRat 2'Ab (1:5000) (All plasmids were transfected at 2 µg/plate with the exception of HA-NL2 at 1 µg/plate). Note the effective immunopurification of NL1ΔC and NL2ΔC with myc-γ2 beads (Fig. 2a, lanes 5,6,11,12; Fig. 2b, lanes 2,4). Also note the changes in expression level of NLs across the input blot (Fig. 2a, input). Double bands seen for NLs in input lanes likely show the presence of post-translational modifications.

The results show that myc-γ2 interacts more strongly with HA-NL2ΔC than with HA-NL2 or HA-NL1 (Fig. 2a, lanes1-6). Moreover, stronger bands for HA-NL1, HA-NL2, and HA-NL2ΔC appear following immunoprecipitation with a myc-γ2 variant lacking palmitoylation sites, even though input bands for NL1 and NL2 are weaker (Fig. 2a, lanes 7-10). Given that this effect was seen with both full length NL constructs and constructs that lack a cytoplasmic domain (the latter being unable to interact with cytoplasmic γ2 subunit domains), the effect of substituting cytoplasmic palmitoylation sites of the γ2 subunit was most likely due to variable expression efficiency of the mutant γ2 plasmids (Fig. 2a, Input).A further experiment confirmed the interaction between myc-γ2 and HA-NL2ΔC, and interaction with HA-NL1ΔC was also shown (Fig. 2b, lanes 2,4). Though there are bands corresponding to NL1ΔC and NL2ΔC in lanes intended as negative controls, those bands are weaker than those showing interaction (Fig.

2b, lanes 6,8 vs. 2,4), despite the input lanes showing a greater amount of NL present (Fig. 2b, input lanes 5-8).

Some interesting results were observed. First, levels of HA-NL2 in the input lane in the presence of the untagged β3 subunit are reproducibly low (Fig. 2a, Input lane 13; Fig. 2b, Input lane 9). This may relate to the ability of the β3 subunit to make homomeric ion channels in HEK cells (Wooltorton et al., 1997). Secondly, the truncated versions of the HA-tagged NLs appear to interact stronger with myc-γ2, indicating not only that the cytoplasmic loop of NLs are dispensable for interaction but also consistent with evidence that these NL fragments accumulate in intracellular compartments (B Luscher, personal communication) and hence, unlike full-length constructs, are not subject to proteolytic processing.

GABRAR subunits α1, β2, and γ2 interact differentially with truncated versions of Neuroligins

During the course of these experiments it was discovered by Pexioto et al 2012 and Suzuki et al. 2012 that NLs are subject to ectodomain shedding. This finding suggests that supposedly negative interactions between NL1/2 and β subunits could be due to excessive proteolysis of NL constructs, depending on the presence of β subunits, which in HEK cells are able to reach the cell surface independently of α and γ subunits (Krishek et al., 1996, Wooltorton et al., 1997).

Further experiments were carried out in order to compare the interaction of myc-γ2 and NLs to that of other subunits. HA-tagged NL1ΔC and NL2ΔC constructs were coexpressed with either myc-tagged $GABA_AR$ subunits (α1, β2, γ2) or untagged $GABA_AR$ subunits (α1, β3, γ2) as controls. HA-tagged NL1ΔC and NL2ΔC –myc-tagged $GABA_AR$ complexes were immunopurified using protein-A-agarose in charged with rabbit anti-myc IgG, and analyzed by

SDS-PAGE/western blots (10% acrylamide) with rat anti-HA IgG and HRP-conjugated anti-rat Ab (Fig. 3).

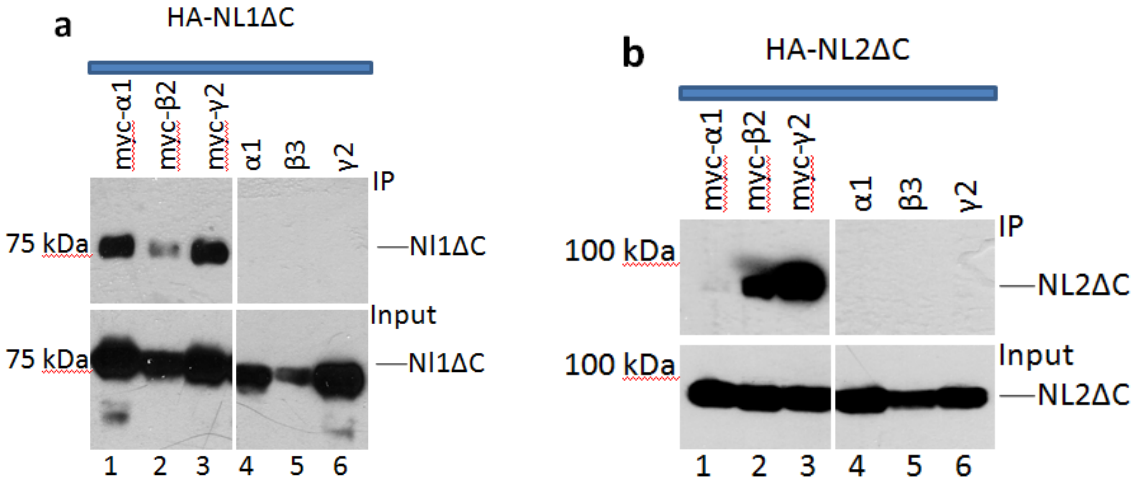

Figure 3: GABA$_A$R subunits α1 and β2 display different affinities for NL1ΔC and NL2ΔC. (a) Myc-α1 and myc-γ2 efficiently immunoprecipitate HA-NL1ΔC. (b) In contrast, myc-β2 and myc-γ2 efficiently immunoprecipitate HA-NL2ΔC.All plasmids were transfected at 2 µg/plate. Gels were 10% acrylamide.

Fig. 3 shows that while myc-γ2 interacts with both HA-NL1ΔC and HA-NL2ΔC, myc-α1 has a preference for HA-NL1ΔC, while myc-β2 has a preference for HA-NL2ΔC. Also, Note that the level of NL1ΔC fluctuates with the presence of different GABA$_A$R subunits (Fig. 3a, lanes 1-6). Specifically, the level of NL1ΔC is lowest when co-transfected with the β3 subunit (Fig. 3a, Input lane 5). This is an unexpected and currently unexplained effect as these NL fragments accumulate in intracellular compartments (B Luscher, personal communication) and hence, unlike full-length constructs, should not be subject to proteolytic processing by matrix metallo-proteinases.

$GABA_AR$ subunits α1 and α2 interact with NL constructs in a similar fashion.

Further we wondered whether α1 and α2 $GABA_AR$ subunits display differential interaction with NL constructs that could explain the different postsynaptic clustering properties of α1-containing $GABA_ARs$ vs. α2-containing $GABA_ARs$. In contrast to previous experiments where myc-tagged $GABA_AR$ subunits were used as bait, in this case, HA-tagged NLs were used as bait, and myc-tagged $GABA_AR$ subunits were detected.

Myc-tagged $GABA_AR$ subunits were coexpressed with HA-tagged full length and truncated NL1/NL2 proteins in HEK 293T cells. For negative controls, no HA-tagged NLs were transfected. All plasmids were transfected at 2 µg per plate with the exception of HA-NL2 (1µg) $GABA_AR$-NL complexes were immunopurified using protein-G-agarose resin charged with rat anti-HA IgG and analyzed by SDS-PAGE/western blots probed with rabbit anti-myc IgG. For better resolution of the 49 kDa bands of $GABA_AR$ subunits α1/α2, 12% acrylamide gels were used for SDS-PAGE.

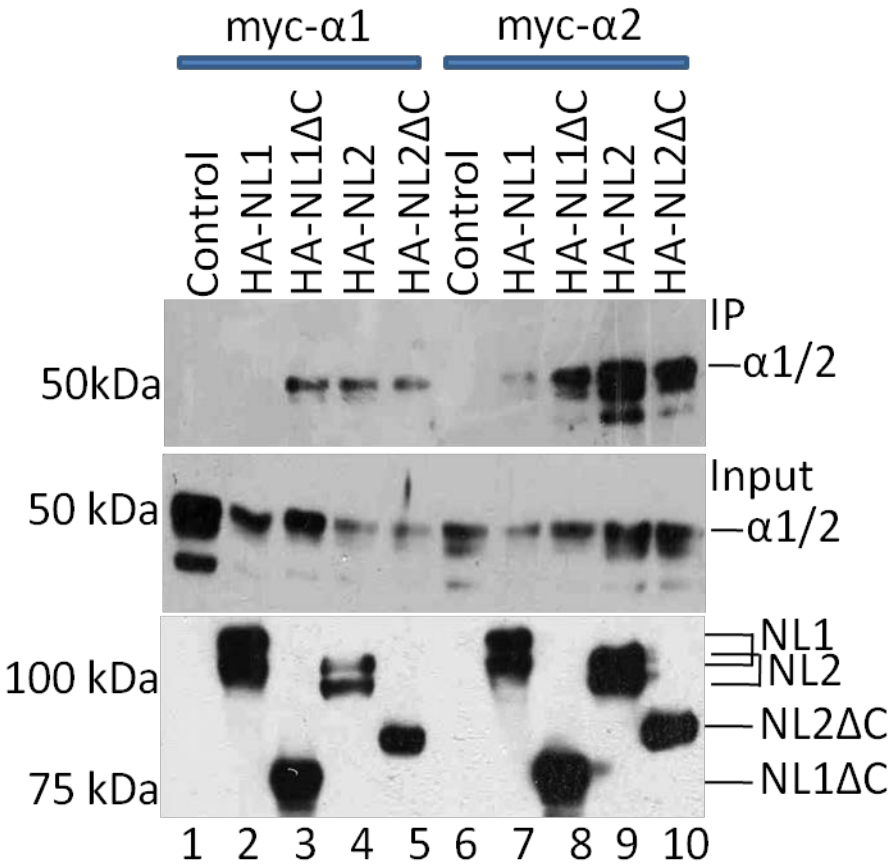

Figure 4: Analysis of interactions between myc-tagged α1/α2 $GABA_AR$ subunits and HA-tagged NL constructs. myc-α1/α2 – NL complexes were immunopurified using protein-G-agarose resin charged with rat anti-HA IgG, and analyzed using SDS-Page/western blot using rabbit anti-myc IgG. All plasmids were transfected at 2 µg/plate with the exception of NL2 (1 µg/plate). Gels were 12% acrylamide. . Note that the α2 subunits co-IPs more effectively than the α1 subunit with NLΔC, NL2, and NL2ΔC. Note the similar immunoprecipitation efficiency observed between myc-α1 and myc-α2 $GABA_AR$ subunits and HA-tagged NLs.

The myc-α2 subunit immunopurified most effectively in the presence of HA-NL2, and slightly less so in the presence of HA-NL1ΔC and NL2ΔC (Fig 4., lanes 8,9,10).Similarly, the myc-α1 subunit immunopurified most effectively in the presence of HA-NL1ΔC, HA-NL2, and HA-NL2ΔC (Fig 4., lanes 3,4,5).

Chapter 4
Discussion

I found that the gamma-2 subunit immunopurified NL2 more effectively than with NL1 (Fig. 2a). This could indicate that NL2 serves to stabilize gamma-2 containing $GABA_AR$s at synaptic sites (Fig. 2). Myc-tagged α1 and α2 subunits display similar preference for interaction with NL1ΔC, NL2, and NL2ΔC, while α2 appears to have stronger interaction with NL1ΔC, NL2, and NL2ΔC (Fig. 4). The β2 subunit co-immunopurified more efficiently with NL2ΔC rather than NL1ΔC (Fig. 3). This is in accordance with the finding that β2-containing $GABA_AR$s are synaptic are enriched at synapses (Farrant and Nusser, 2005). Therefore, interaction between β2 and NL2 could contribute to this observed enrichment.

Among these experiments, two approaches were used. Both approaches test for interaction, but the bait and predator proteins positions were switched. In the first approach, protein-A-agarose charged with rabbit anti-myc IgG was used to immunopurify HA-tagged NLs using $GABA_AR$ subunits as bait (Fig. 1,2,3). The results of the first approach are summarized in Table 1 below. The second approach was an inverse of the first: protein-G-agarose charged with rat anti-HA IgG was used to immunopurify myc-tagged $GABA_AR$ subunits, using HA-tagged NLs as bait (Fig. 4). The results of the second approach are summarized in Table 2 below.

Bait	**Prey Proteins**			
Protein	HA-NL1	HA-NL1ΔC	HA-NL2	HA-NL2ΔC
Myc-α1	n/a	+++	n/a	+
Myc-β2	n/a	+	n/a	++
Myc-γ2	-	+++	+	+++
Myc-γ2 (3C-SA)	+	n/a	++	+++

Table 1: Summary of observations using myc-tagged $GABA_AR$ subunits as bait proteins. Strength of interaction is indicated by -, +, ++, +++ (from lack of interaction to strongest interaction respectively). Interactions not tested for are marked as "n/a". The results from Fig. 1 are not included in the table due to a lack of definitive negative controls.

Bait Protein	Prey Proteins	
	Myc-α1	Myc-α2
HA-NL1	-	-
HA-NL1ΔC	+	++
HA-NL2	+	+++
HA-NL2ΔC	+	++

Table 2: Summary of observations using HA-tagged NLs as bait proteins. Strength of interaction is indicated by -, +, ++, +++ (from lack of interaction to strongest interaction respectively). The results from Fig. 1 are not included in the table due to a lack of definitive negative controls.

One interaction confirmed in both directions was the interaction between HA-NL1ΔC and myc-α1 $GABA_AR$ subunit (Fig. 3a, lane 1; Fig. 4, lane 3). No contradicting results were observed, though several experiments have yet to be completed to confirm interactions in both directions (myc-α2 as bait protein, and myc-α2 as bait protein against full length HA-NL1/NL2). In the future, inverse experiments such as these should be used as a robust method for verifying interactions; though detecting immunopurified myc-tagged $GABA_AR$ subunits is problematic due to the presence of an IgG band at 50kD (data not shown).

Overall, the finding that $GABA_AR$ subunits interact with not only NL2, but also NL1 is unexpected (Table 1, Table 2). Our expectation was to see a specificity of $GABA_AR$ subunit interaction with NL2 only, as NL2 is closely associated with GABAergic synapses (Varoqueaux et al., 2004). Instead, only tendencies to interact stronger with either NL1 or NL2 versions were observed. The β2 subunit for example interacted stronger with NL2ΔC than with NL1ΔC. Similarly, although both the α1 and α2 subunits were shown to interact with NL1ΔC and NL2ΔC (Fig. 3,4), the α2 subunit shows stronger interaction with NLs (Table 2). The γ2 subunit was found to interact equally well with both NL1 and NL2 (Fig. 2, 3). One future experiment should include NL constructs lacking the extracellular domain in order to specify the specific domain responsible for interaction.

These unexpected interactions with NL1 could have several explanations. First, the interactions could be happening exclusively in the HEK 293T cell environment, and not in actual

neurons. To resolve this problem, co-IP experiments using either transfected cultured neurons or brain extracts could be performed. Secondly, even though interactions with NL1 are observed, there are differences in the apparent strengths of these interactions. For example, the β2 and α2 subunits appear to interact more strongly with NL2 than with NL1 (Fig. 3, 4). Perhaps, interaction with only strictly one NL isoform would make the synapses overly stable, whereas interaction with both NL1 and NL2 could contribute to dynamic movement of receptors.

Another question that has to be addressed in future experiments is the localization of $GABA_AR$ subunits and isoforms of NLs in HEK cells. With the exception of β subunits, $GABA_AR$ subunits such as γ2, α1/2 do not localize to the surface of HEK cells, and a co-transfection of α/β/γ subunits is needed for receptor assembly. (G Chen, personal communication). C-terminally truncated NL constructs also do not seem to localize to the surface of HEK cells, and are instead accumulated intracellularly (B Luscher, personal communication), but their exact localization has not been characterized. In future experiments, immunostaining for C-terminally truncated NL constructs should explain the observation that truncated NLs interact stronger with $GABA_AR$ subunits than do full length NL isoforms.

Throughout these experiments, various negative controls were used. One example of a negative control is the absence of a bait protein (Fig 4., lanes 1,6). This type of negative control shows that the interaction depends on the presence of a bait protein. However, amounts of transfected DNA have to be compensated with empty vector in order to compensate for toxicity of the PEI transfection reagent. This was not done in the above experiments, and the high levels of myc-tagged subunits in Fig. 4 in the absence of NL are examples of the observed problem. Another example of a negative control is the substitution of a tagged bait protein for one that is untagged (Fig. 1,2,3). This negative control confirms that the tagged bait protein is indeed

responsible for immunopurifying the predator protein.

However, these theoretically simple negative controls can still yield unexpected and interesting results. It was observed that with when co-expressed with the β3 subunit, levels of HA-tagged NL2 were low. This effect was reproducibly observed in two independent experiments (Fig. 2 a,b). The β3 subunit has been shown to be able to form homomeric $GABA_A$Rs in HEK cells (Wooltorton et al., 1997). Additionally, increases in MMP9 mRNA levels have been detected in the hippocampus 2 hours post-seizure onset following administration of the $GABA_A$ receptor antagonist pentylenetetrazole (Rylski et al., 2008, Huntley, 2012). While MMP-9 has not been shown to cleave NL2, MMP-9 dependent proteolysis of NL1 has been observed, an effect reversed by MMP inhibitors (Peixoto et al., 2012). It is therefore possible to presume that a similar process is at play with NL2. There are several potential scenarios for this that should be explored. It is possible that NL2 brings subunits to the surface of neurons, and is subsequently degraded. Alternatively, it is possible that β3 receptors form a type of excitatory synapses, causing NL2 to be degraded by MMPs. This resting conductance of homomeric channels may contribute to degradation of coexpressed neuroligins in HEK cells. In future experiments, immunostaining should either confirm or disprove this theory.

Additionally, I would like to address the considerations involved in using co-immunoprecipitation for studying interactions between $GABA_A$Rs and NLs in HEK 293T cells, instead of neurons. In these experiments, HEK cells were used to simplify the interpretation of experiments – they express only the $GABA_A$R subunits that are transfected, and lack other $GABA_A$R interacting proteins that would complicate interpretation. Additionally, HEK cells are easy to transfect and maintain in cell culture. They provide a robust system for protein expression, are easy to transfect, culture, and maintain. However, cultured neurons would

provide a better platform for the same studies because they model native conditions more closely. Neurons, on the other hand are in limited supply, have to be isolated from embryonic brain and are harder to transfect. When analysis is done in HEK cells, factors such as altered $GABA_AR$ trafficking and protein localization must be considered. Specifically, the unique ability of the β3 $GABA_AR$ subunit to form homomeric constitutively active channels, and the downstream implications of this formation are important. There remain many unknown factors of receptor trafficking, and degradation.

In order to enhance the reproducibility of future experiments, conditions of cell culture, protein isolation, and co-immunoprecipitation have to be better controlled. Specifically, protease inhibitors should be included in culture media to prevent cleavage of full-length NL constructs. Surface NL1has recently been shown to be cleaved by proteases such as MMP-9 or ADAM10, and NL2 is cleaved as well, though the specific proteases involved are unknown (Peixoto et al., 2012, Suzuki et al., 2012). In order to isolate the interactions between $GABA_AR$ subunits and NLs in HEK cells, potential proteolytic cleavage by proteases must be controlled.

Additionally, DNA amount equalization using empty plasmid vectors is needed to ensure that any differences observed in complex formation between different plasmid combinations are not due to differences in the amount of DNA transfected or variation of the amount of PEI used for transfection. PEI's toxicity to cells was regularly seen as the level of input proteins correlated with the amount of DNA transfected, and the amount of PEI used (Fig. 2b, Fig 4). Alternatively, equal amounts of PEI reagent can be used in all transfections, with the amount determined by the highest amounts of DNA needed for any particular sample. In addition, probing western blots for the housekeeping protein actin will reveal the true level of protein in each lane.

Another important factor to consider is the meaning of a strong or weak band observed on a

western blot. Multiple factors can be responsible for the strength of the band, and as many as possible have to be controlled for. The amount of protein in a cell will depend primarily on transfection amount, expression rate, and degradation rate. The amount of protein detected on a western blot pre-immunoprecipitation will depend on the amount of cells. The amount of protein detected a western blot post-immunoprecipitation will ideally depend on the affinity of the protein to the pull-down protein. Finally, reproducible laboratory techniques are essential.

In conclusion, I have demonstrated that differential co-immunopurification efficiency between myc-tagged $GABA_AR$ subunits and HA-tagged NL constructs. These results have implications for $GABA_AR$ localization at inhibitory synapses. Additionally, an unexpected effect of β3 $GABA_AR$ subunit expression on detected levels of NL2 gives clues about NL proteolysis, and should be both controlled for in future experiments, and explored further utilizing different NL isoforms. Deficits in $GABA_AR$-mediated GABAergic transmission are implicated in the etiology of neurological disorders such as epilepsy (Fritschy, 2008), and mental disorders such as schizophrenia (Charych et al., 2009) and anxiety disorders (Lydiard, 2003). These deficits could be caused by altered $GABA_AR$ trafficking, which in turn is affected by affinity of $GABA_AR$ subunits to other multiple membrane proteins such as NLs. The strength of GABAergic synapses changes in proportion with the number of postsynaptic $GABA_ARs$ (Luscher et al., 2011).Therefore, it is important to understand and explore all of the numerous factors that can contribute to the changing number and location of $GABA_ARs$ on the post-synaptic neuron.

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