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AMPA Receptor Trafficking in Schizophrenia

John Hammond
University of Alabama at Birmingham

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by

John C Hammond

Robert McCullumsmith, M.D., Ph.D., CHAIR
James Meador-Woodruff, M.D.
Lynn Dobrunz, Ph.D.
Robin Lester, Ph.D.
Robin Lorenz, M.D., Ph.D.

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John C Hammond

DEPARTMENT OF NEUROBIOLOGY

ABSTRACT

The glutamate hypothesis of schizophrenia is based primarily on NMDA receptor dysfunction. Recent studies have led to an expansion of this hypothesis to include AMPA receptors which are essential for neurotransmission through NMDA receptors. Examination of total AMPA receptor protein expression in schizophrenia has been inconsistent and led to examination of AMPA receptor interacting proteins and trafficking and subcellular localization of the receptors. AMPA receptors are highly trafficked from the endoplasmic reticulum to the synapse and in a complex system of endosomes. Alterations in the subcellular localization of these receptors may be a part of the underlying pathophysiology of schizophrenia. I measured expression of multiple proteins that interact with AMPA receptors in postmortem dorsolateral prefrontal cortex tissue from patients with schizophrenia. I developed immunoisolation techniques to isolate two endosomal compartments and used a modified centrifugation protocol to isolate the endoplasmic reticulum from these samples as well. Following western blot and electron microscopic verification of subcellular fraction isolation and enrichment, I measured the protein expression of the AMPA receptor subunits. I found increased expression of two proteins involved in forward trafficking of AMPA receptors, SAP97 and GRIP1, in schizophrenia. In the isolated early endosomes, I found increased expression of the AMPA receptor subunit GluR1. Taken together, these findings implicate altered forward trafficking of AMPA receptors in schizophrenia. However, the

lack of significant changes in multiple other proteins, as well as lack of changes in protein expression in any of the AMPA receptor subunits in the late endosomes or endoplasmic reticulum diminishes the likelihood that altered AMPA receptor trafficking and subcellular localization is a prominent feature in the underlying pathophysiology. We must consider that with the exception of a change in one AMPA receptor subunit in one subcellular fraction, AMPA receptor trafficking may be largely intact in schizophrenia.

Keywords: Schizophrenia, AMPA receptors, endosomes, endoplasmic reticulum, subcellular fractionation, postmortem

DEDICATION

To my parents, John and Mary, for always believing in me and keeping me moving forward by asking, “How much longer will you be in school?”

To my wonderful wife, Katherine, for standing by my side and helping me to keep perspective when things seemed to be turning upside-down.

To Adam, who has made this lab journey with me since the beginning and helped me to remember that it is all in my head.

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CHAPTER 1

INTRODUCTION

1.1 Schizophrenia

Schizophrenia is a debilitating mental illness that affects nearly 1% of the adult population worldwide (1, 2). Development of schizophrenia at a relatively young age, late teens to early twenties for men and twenties to early thirties for women, creates significant burdens for afflicted persons, their families, and society (3, 4). Patients with schizophrenia typically endure multiple hospitalizations, medication side effects, and psychotic symptoms that hinder their ability to live independently and cost society billions of dollars annually (5, 6).

Schizophrenia is characterized by a myriad of clinical findings, including positive, negative and cognitive symptoms (6, 7). Positive symptoms include delusions, hallucinations, or agitation (7). Oftentimes, patients report auditory hallucinations in the form of a running commentary of the patient's thoughts and actions (4, 7). Negative symptoms, including lack of drive, social withdrawal, decreased eye contact, apathy, and diminished spontaneous movement, may be more debilitating to patients and are often not addressed by pharmacotherapy (7-9). In addition to positive and negative symptoms, some patients have cognitive deficits such as disorganized thinking and deficits in executive functioning (7, 10).

While there are well-established criteria in place for making the diagnosis of schizophrenia, the cause is still unknown. Most recent evidence supports a combination of genetic and environmental factors contributing to the development of the disorder (11). To date, no one gene has been consistently linked to the illness and it is likely that multiple genes create a predisposition to developing schizophrenia (11). There have been several proposed hypotheses regarding the underlying pathophysiology of schizophrenia, ranging from neurodevelopmental abnormalities to physical abnormalities in the brain to imbalance of neurotransmission (12-17).

One of the first neurotransmitters implicated in schizophrenia was dopamine. Early studies revealed that large doses or prolonged use of amphetamines, which act as dopamine agonists, may cause psychoses, including hallucinations and paranoia similar to that seen in schizophrenia (9, 18, 19). The discovery of chlorpromazine as a potent antipsychotic medication was critical in the further development of the dopamine hypothesis of schizophrenia. Chlorpromazine is a typical antipsychotic that exerts its antipsychotic effects through dopamine D_2 receptor blockade (20-22). Together, these data suggest excess dopaminergic activity contributes to the positive symptoms of schizophrenia. However, typical antipsychotics that affect the dopaminergic system are not efficacious in managing the negative and cognitive symptoms of schizophrenia (9, 23, 24). Consequently, further research has implicated other neurotransmitter system involvement in the pathophysiology of schizophrenia.

1.2 Glutamate Hypothesis of Schizophrenia

Glutamate facilitates excitatory neurotransmission via activation of ionotropic (NMDA, AMPA, KA) and metabotropic ($mGluR_1$ - $mGluR_8$) glutamate receptors. Converging lines of evidence implicate dysfunction of glutamate receptors in schizophrenia. Administration of phencyclidine (PCP), ketamine, or other NMDA receptor antagonists induces a schizophrenia-like psychosis in control subjects (25-27). Moreover, administration of ketamine to patients with schizophrenia leads to an exacerbation of symptoms (28). Interestingly, administration of compounds that block NMDA receptors can induce or exacerbate positive, negative, and cognitive symptoms (14, 29).

Under normal resting conditions, activation of NMDA-type glutamate receptors should lead to opening of cation channels followed by influx of calcium and sodium into and efflux of potassium from the cell (30, 31). However, prior to activation of NMDA receptors, a magnesium blockade must be removed via partial depolarization of the cell (32). Typically, it is the activation of AMPA-type glutamate receptors that provides the depolarization necessary to remove the magnesium blockade (32). Therefore, though the glutamate hypothesis posits NMDA receptor hypofunction, the hypothesis can be expanded to include other glutamate receptor subtypes including AMPA receptors.

1.3 AMPA receptors

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors are a subset of ligand-gated ionotropic glutamate receptors (33). AMPA receptors are essential for fast excitatory synaptic transmission in the central nervous system (34, 35). Function

of AMPA receptors is dependent upon structure, subunit assembly, interacting proteins, trafficking, and localization.

1.3.1 AMPA receptor function and structure

There are four AMPA receptor subunits, GluR1-4, that are typically assembled as dimers into a tetrameric complex in the endoplasmic reticulum (36-38). The structure of each GluR subunit contains four domains. An extracellular N-terminus consisting of approximately 370 amino acids is homologous to LIVBP, a bacterial amino acid binding protein (39-41). There are three-transmembrane-spanning domains in each receptor subunit (42, 43). Each receptor subunit contains one re-entrant domain which does not span across the membrane from the intracellular space to the extracellular space but turns back into the intracellular space (43, 44). Along with the three transmembrane domains, the re-entrant domain is part of the pore forming unit of the receptor (44). Finally, each receptor subunit contains an intracellular C-terminus that is the site of interaction for various molecules (43, 45).

Assembled AMPA receptors serve as cation channels in the cell membrane (33, 34). Each subunit contains a ligand binding domain proposed to be located between the extracellular N-terminus and the extracellular loop between two transmembrane domains (42, 46). When at least two of the binding sites are occupied, a shift occurs in the subunits and a pore is opened allowing for the flux of cations (47, 48). Most assembled AMPA receptors contain GluR2 and one of the other subunits, GluR1, GluR3 or GluR4 (49). The presence of GluR2 in the receptor confers gating of calcium through the pore whereas receptors lacking GluR2 subunits are permeable to calcium, sodium, and potassium

influx (49-51). Unlike NMDA receptors, AMPA receptors generally open and close quickly initiating fast excitatory synaptic transmission (35).

1.3.2 AMPA receptor assembly and trafficking

AMPA receptor subunits, like other proteins, are synthesized in the endoplasmic reticulum. Each of the four AMPA receptor subunits, GluR1-4, is encoded by a gene, GRIA1-4, which can be modified via RNA editing and post-transcriptional modifications (52, 53). For example, the gating ability of GluR2 arises from a glutamine (Q) to arginine (R) switch within the channel pore (54, 55). When the uncharged amino acid glutamine is replaced by the positively charged arginine, it becomes energetically unfavorable for the positively charged calcium ions to move through the pore (56, 57). Further modification of AMPA receptor subunits takes place in the transmembrane domain just prior to the C-terminus in a 38 amino acid sequence commonly referred to as the flip/flop sequence (43, 58). Studies of this sequence have shown that different isoforms can alter the desensitization and resensitization kinetics of the channel. The expression of flip and flop variants is developmentally regulated (59). Furthermore, the flip isoform of the receptor is more likely to be correctly processed for exit from the endoplasmic reticulum (60).

1.3.3 Posttranslational Modifications of AMPA Receptors

Posttranslational modifications of AMPA receptors are important for forward trafficking of the AMPA receptors. Processing of AMPA receptors in the endoplasmic reticulum involves the addition and removal of a complex series of sugar moieties in a process known as glycosylation (45, 61). Glycosylation of AMPA receptor subunits at as many as 4-6 sites on each subunit can protect proteins from proteolytic degradation (45).

When high-mannose sugars are attached to receptors, the receptors are retained in the ER (45). This process of adding high-mannose sugars may maintain a pool of GluR2 receptor subunits in the ER (62). Proper glycosylation is required for dimerization of the receptors which begins in the endoplasmic reticulum but is not required for ion channel function (63).

Phosphorylation is also an important posttranslational modification of receptors. Phosphorylation of specific residues on AMPA receptors subunits regulates channel properties as well as trafficking to the synapse (64). Serine 818 of GluR1 is phosphorylated by PKC during LTP, leading to insertion of GluR1-containing AMPA receptors in the synapse (65). Phosphorylation of serine 831 of GluR1 also regulates trafficking of AMPA receptors during LTP (66, 67). This phosphorylation of GluR1 at S818 and S831 is important for stabilization of the receptors at the post-synaptic density and if lost may lead to receptor internalization. Phosphorylation of the GluR2 AMPA subunit at serine 880 causes internalization of the AMPA receptor which colocalizes with internalized PICK1 in the dendrite (68, 69). Further studies of S880 phosphorylation reveal that when phosphorylation is blocked, the GluR2 subunit is rapidly recycled back to the synapse (70, 71).

1.3.4 Subunit Composition of AMPA Receptors

In addition to modifications of the receptors, trafficking of AMPA receptors in the ER and Golgi is facilitated by AMPA receptor subunit composition and AMPA receptor interacting proteins. Although AMPA receptors are usually a dimer of dimers, it is often the case that one subunit exerts dominant effects over the other in heteromers (72, 73).

For example, in GluR1/GluR2 heteromers, GluR1 dominates the trafficking of the receptor (72). Synapse-associated protein 97 (SAP97) is a member of the MAGUK (membrane-associated guanylate kinase) family of proteins and contains three PDZ (postsynaptic density 95/Discs large/zona occludens-1) domains (74). One of the PDZ domains of SAP97 interacts with the PDZ domain on the C-terminus of GluR1 in GluR1/GluR2 hetero-oligomers (75, 76). This early interaction of SAP97 and GluR1 in the ER allows for fast trafficking of the receptors from the ER to the Golgi (45, 77). Another interacting protein, Protein interacting with C Kinase 1 (PICK1) binds to the C-terminal domain of GluR2 (78, 79). Interaction of PICK1 and GluR2 in the ER leads to slower exit of GluR2/GluR3 containing receptors from the ER (45).

1.4 AMPA Receptor Interacting Proteins

Upon leaving the ER, AMPA receptors must reach the synapse in order to facilitate neurotransmission. Transport of assembled AMPA receptors from the ER to the dendritic spine occurs along the cytoskeletal spine of the neuron in a microtubule and actin-filament based system (45) (Figure 1.1). SAP97, bound to GluR1/GluR2 receptors also interacts with MyosinVI, a motor protein (80). This interaction is important for the forward trafficking of the receptors and if it is interrupted, AMPA receptors may not be trafficked to the synapse (81). Glutamate receptor interacting protein 1 (GRIP1) is a multi-PDZ-containing neuronal scaffolding protein (82). One of the PDZ domains of GRIP1 directly interacts with the GluR2 C-terminus PDZ domain (83, 84). Additionally, GRIP1 associates with the protein Liprin α, a ubiquitous member of the LAR protein-tyrosine phosphatase-interacting protein family (85, 86). In turn, Liprin α binds to the motor protein KIF5 for trafficking of GluR2-containing receptors to the dendritic spine

(45, 85, 87). NSF (*N*-ethylmaleimide-sensitive fusion) protein also binds to GluR2 and directs the insertion of GluR2 containing AMPA receptors at the synapse (88). The 4.1 family of proteins is associated with the cytoskeletal membrane in cells (89). The protein 4.1N binds to GluR1 and GluR4 once the AMPA receptors are within the shaft, and assists in the transport of these receptors along the shaft to the dendritic spine (45, 89).

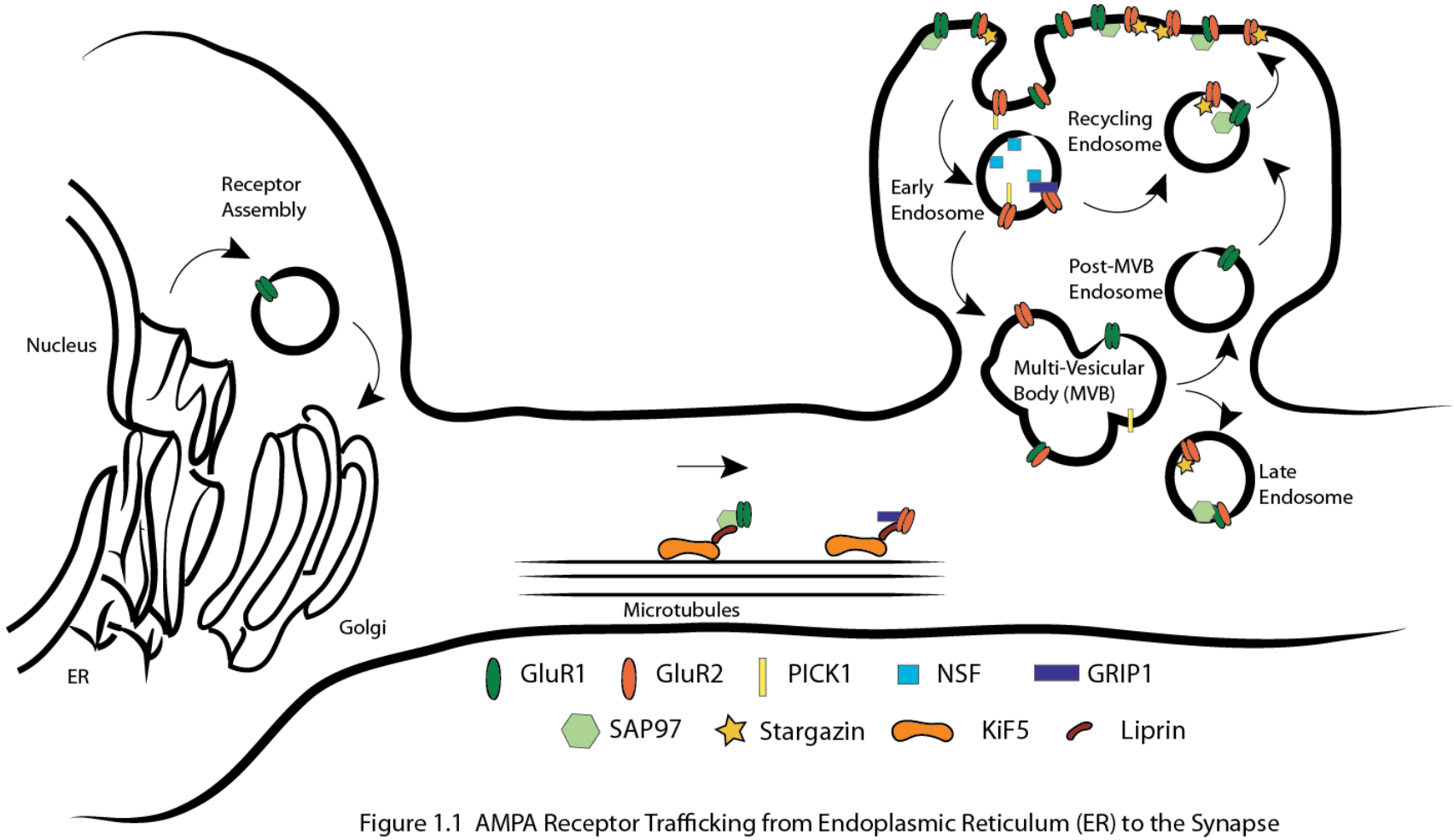

Figure 1.1 AMPA Receptor Trafficking from Endoplasmic Reticulum (ER) to the Synapse

1.5 AMPA Receptor Exocytosis

Much of what is known about AMPA receptor trafficking and turnover comes from studies of long term potentiation (LTP) and long term depression (LTD). After AMPA receptors have moved into the dendritic spine, they are localized to the plasma membrane in exocytic vesicles (45, 90). Although initially the process of AMPA receptor localization to the synapse was unclear, recent studies have described that AMPA receptor insertion can occur either directly to the synapse or to an extra-synaptic area (72, 73, 91). GluR1/GluR2 receptors are inserted into the extra-synaptic membrane and move laterally to the synapse (92, 93). The lateral movement and insertion of GluR1/GluR2 follows induction of LTP (36, 91). This movement of GluR1/GluR2 receptors following LTP induction is referred to as the regulated receptor pool (94). GluR2/GluR3-containing AMPA receptors are inserted directly into the synapse in an activity-independent manner (72, 73). Following insertion at the synapse, GluR2/GluR3 containing receptors are cycled between the synapse and an intracellular domain (95, 96). This cycling of GluR2/GluR3 containing receptors is referred to as the constitutive cycling pathway (97).

AMPA receptor PDZ domains and are unable to directly interact with PSD95 (98). Stabilization of AMPA receptors at the synapse is directed by interactions between the AMPA receptor subunits and AMPA receptor interacting proteins (90, 99). SAP97, bound to GluR1/GluR2 hetero-oligomers, interacts with the post-synaptic density (100). This interaction is important for the localization and stabilization of GluR1/GluR2 AMPA receptors to the synaptic area (101, 102). PICK1 links to the PDZ-binding domain of GluR2 and plays a role in the clustering of the subunit and tethering of the receptor to

the post-synaptic density and plasma membrane (103-105). Stargazin also plays a role in the trafficking and localization of AMPA receptors at the cell surface, participating in the lateralization of AMPA receptors from extrasynaptic membrane to the synapse (106-108). Through interactions with the PDZ domain of GluR2, GRIP1 plays a role in maintaining GluR2 containing receptors at the synapse (82, 109). Point mutations in the sequence of residues that bind GluR4 and the 4.1 protein interfere with the binding and prevent the surface expression of GluR4 (110).

1.6 AMPA Receptor trafficking in endosomes

AMPA receptor expression at the synapse is a tightly controlled process that allows for turnover of receptors and recruitment of new receptors for proper synaptic functioning (111) (Figure 1.2). Once localized to the synapse, AMPA receptors can be brought back into the cell in vesicles called endosomes (112). Endosomes are membrane-bound organelles comprised of lipid bilayers (113, 114). These compartments are often derived from the plasma membrane and may contain receptors that were located on the cell surface. Early endosomes are formed directly from the plasma membrane in an area referred to as the endocytic zone (115). In a clathrin-dependent process, a small pocket forms in the membrane, followed by invagination of the membrane and a closing off of the newly formed endosome via dynamin (113). These newly formed endosomes express early endosome antigen-1 (EEA1) on their surface. EEA1 is used as a tethering molecule providing inward directionality for endosomes (116). Activation of NMDA receptors and long-term depression can lead to removal of AMPA receptors from the synapse into the early endosomes (95, 112, 117). Interestingly, pharmacological blockade of early endosome formation prevents the internalization of AMPA receptors (113, 118).

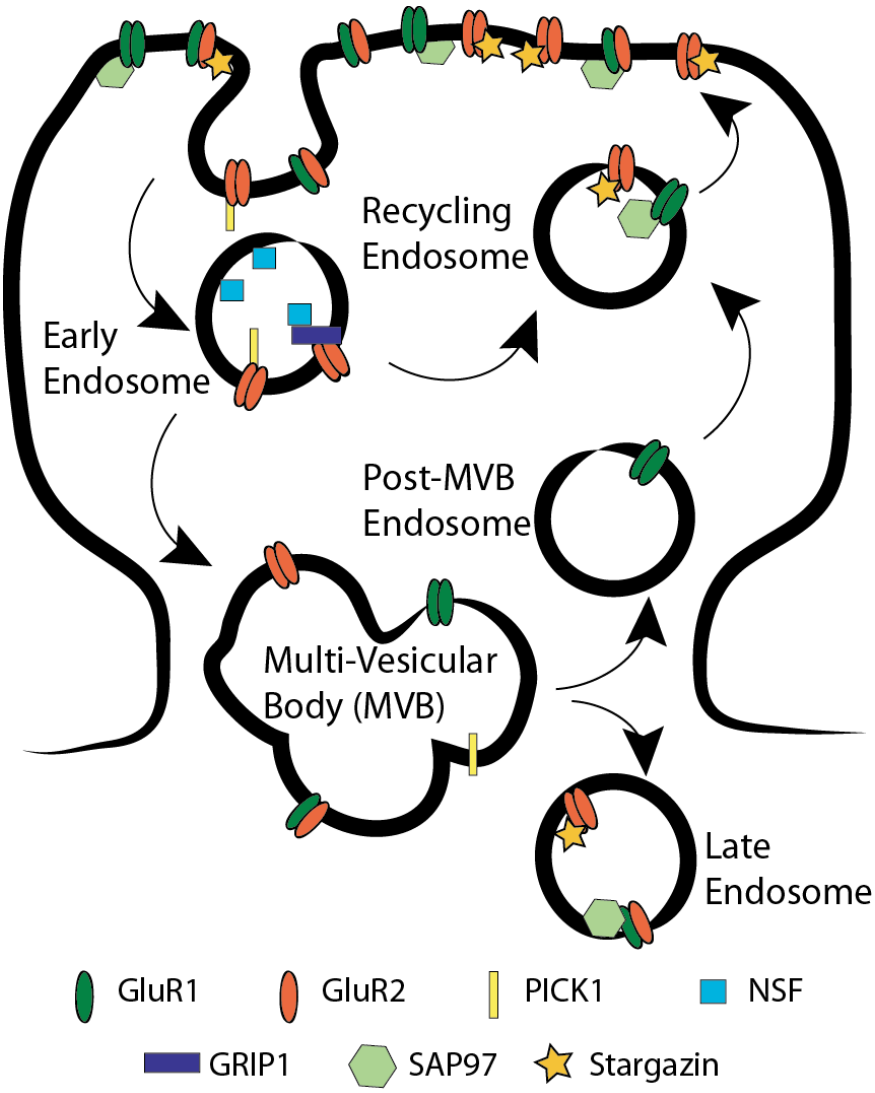

Figure 1.2 AMPA Receptor Trafficking in Endosomes

From the early endosome, AMPA receptors are sorted to either the late endosome or recycling endosome (119). Late endosomes contain the marker GTPase Rab7, have a lower pH, and carry contents to the lysosome for degradation (120, 121). Following endocytosis without NMDA activation, AMPA receptors are targeted for degradation (119). AMPA receptors containing the GluR3 subunit are also targeted to late endosomes (122). Alternatively, AMPA receptors from early endosomes can be brought to the

recycling endosomes (119). Recycling endosomes, which contain the GTPase Rab11, bring receptors that have been endocytosed back to the cell surface (123, 124). Interestingly, the spine morphology changes that are seen following LTP are likely due to the insertion of AMPA receptors into the membrane from Rab11 positive recycling endosomes (124-126).

As with insertion and stabilization of AMPA receptors at the synapse, AMPA receptor subunit composition and interacting proteins are important in the endosomal trafficking of the receptors. The endocytosis of receptors occurs in an area referred to as the endocytic zone which is located near the post-synaptic density (127). Interactions between Dynamin3 and other scaffolding proteins maintain the endocytic zone (128). Arc/ARG3.1 is an immediate early gene product whose function was poorly understood until recently (129). Arc/ARG3.1 interacts with dynamin in the endocytic zone to induce rapid internalization of AMPA receptors (129, 130). The loss of Arc/ARG3.1 leads to a decrease in the internalization of AMPA receptors, overexpression of AMPA receptors at the synapse and loss of homeostatic signaling (131).

Activity at the synapse and receptor subunit composition also direct the trafficking of AMPA receptors in endosomes. Following NMDA receptor activation, AMPA receptor subunits may be unclustered from PICK1 (117). Unclustering of AMPA receptors from PICK1 can lead to recycling of the receptor back to the synapse, likely via recycling endosomes (117, 132). Homomeric GluR2 receptors and receptors with GluR1 subunits are also preferentially recycled (122).

The complicated sorting of AMPA receptors to the recycling endosomes requires additional interacting proteins. Dysregulated trafficking and localization of AMPA receptors may alter excitatory neurotransmission mediated by these receptors. NEEP21 (neuronal endosomal protein of 21 kDa) is a small protein that interacts with the GluR2/GluR3 containing AMPA receptors and GRIP1 (133, 134). Expression of a NEEP21 fragment containing the GRIP1 binding site causes a reduction of GluR2 surface expression and an accumulation of these receptors in early endosomes (133). GRASP1 (GRIP-associated protein-1) expression is a protein that interacts with NEEP21, early, and recycling endosomes (135). Expression of GRASP1 is necessary for the coupling of EEA1-containing early endosomes with Rab11-containing recycling endosomes and thus sorting of AMPA receptors between these compartments (136). NSF may also disassemble GRIP1/GluR2 complexes (137, 138). Following disassociation with GRIP1, GluR2-containing AMPA receptors are then sorted from early endosomes to the recycling endosomes and re-expressed on the cell surface (139, 140). There is evidence that with inhibition of NSF, AMPA receptors will remain in the early endosomes and not be trafficked back to the surface (138).

Once localized to recycling endosomes, AMPA receptors still must be returned to the cell surface to facilitate neurotransmission. Again, multiple proteins assist in the trafficking and targeting of the receptors to the surface. In addition to sorting receptors to the recycling endosomes, NEEP21 also assists in the targeting of AMPA receptors back to the synapse (141). Liprin α, which trafficked AMPA receptors along the shaft with KIF5, and associated protein family members also function as scaffolding proteins (142). Liprin α helps to mature the synapse and provides targeting for AMPA receptor

reinsertion through interactions with GIT1 and GRIP1 (86, 143). Syntaxin13, a member of the syntaxin family, is present in recycling endosomes (144). Syntaxin13 interacts with the plasma membrane and is essential in the fusion of recycling endosomes with the membrane and the return of AMPA receptors to the synapse (145).

Alterations in the expression or trafficking of AMPA receptors can drastically affect proper neurotransmission and cellular signaling. Inability of proper AMPA receptor localization and receptor firing for a variety of reasons can prevent depolarization of the cell. Improper depolarization of the cell may lead to failed release of the magnesium block in NMDA receptors and a state physiologically similar to NMDA-receptor hypofunction, as proposed by the glutamate hypothesis of schizophrenia.

1.7 AMPA receptors and interacting proteins in schizophrenia

To date, many studies have examined the expression of AMPA receptors and AMPA receptor interacting proteins in schizophrenia. When studying AMPA receptor expression in postmortem tissue, there are inherent complications arriving from the complexity and overlapping nature of the AMPA receptor subunits (4). There are also multiple levels of gene expression that can be examined with various techniques including receptor binding sites, mRNA expression, and protein expression. Data from these studies (summarized in TABLE 1.1), have yielded inconsistent results. Studies of AMPA receptor subunit binding site, mRNA and protein expression have found increases (146-148), decreases (149-155), and no changes (156-161) in various areas in the brain.

Table 1.1 AMPA receptor binding and expression in schizophrenia

Level of Gene Expression	Technique	Probe(s)	Finding	Brain Region	Reference
Receptor Binding Sites	Autoradiography	CNQX	↑	PFC	(148)
	Autoradiography	AMPA	↓	HC	(153)
	Homogenate Binding	AMPA	No change	PFC, Striatum	(157)
	Homogenate Binding	AMPA	No change	FC	(158)
	Autoradiography	AMPA	No change	DLPFC	(161)
Subunit mRNA Expression	qPCR	GluR1 GluR2-4	↑ No change	DLPFC DLPFC	(146)
	qPCR	GluR1, GluR4 GluR2, GluR3	↑ No change	DLPFC DLPFC	(147)
	ISH	GluR1, GluR2	↓	HC	(149)
	qPCR	GluR2	↓	HC	(150)
	qPCR	GluR1	↓	FC	(154)
	ISH	GluR1, GluR3	↓	Thalamus	(155)
	qPCR	GluR1-4	No change	DLPFC	(156)
	ISH	GluR1-4	No change	PFC, Striatum	(157)
Subunit Protein Expression	Western blot	GluR2, GluR3	No change	Cingulate	(159)

Abbreviations: *in situ* hybridization (ISH), quantitative-polymerase chain reaction (qPCR),prefrontal cortex (PFC), hippocampus (HC), frontal cortex (FC), dorsolateral frontal cortex (DLPFC).

Glycosylation of AMPA receptors is essential for the proper trafficking of AMPA receptors (45, 61). Unpublished observations from our lab implicate abnormal glycosylation of GluR2 protein. Since protein glycosylation is crucial for forward trafficking of proteins from the ER to the Golgi, alterations in this posttranslational modification would lead to alterations in the trafficking of the proteins involved.

These inconsistencies and the complex biology of the AMPA receptors have led to the examination of the molecules involved in trafficking of AMPA receptors in

schizophrenia (summarized in TABLE 1.2). In the dorsolateral prefrontal cortex (DLPFC), PICK1 mRNA was decreased, while stargazin mRNA was increased, suggesting a problem with the clustering of AMPA receptors at the synapse (162). Transcripts for SAP97, which anchors GluR1 in the synapse, were decreased in the prefrontal cortex, suggesting abnormal synaptic localization in schizophrenia (163). NSF mRNA was decreased in the cortex, suggesting that AMPA recycling may be impaired (138, 164).

Table 1.2 AMPA Receptor Interacting Proteins in DLPFC in Schizophrenia

Interacting Protein	Level of gene expression	Technique	Finding	Reference
PICK1	mRNA	qPCR	No change	(147)
	mRNA	ISH	No change	(162)
Stargazin	mRNA	ISH	↓	(165)
SAP97	mRNA	qPCR	No change	(147)
	protein	western	↓	(163)
NSF	mRNA	ISH, microarray	↓	(164)
	mRNA	ISH	No change	(162)
	mRNA protein	qPCR western	No change No change	(166)
	protein	western	No change	(167)
GRIP1	mRNA	qPCR	↑	(147)
	protein	western	No change	(163)

Abbreviations: Protein interacting with C Kinase 1 (PICK1), Synapse-associated protein 97 (SAP97), Glutamate receptor interacting protein 1 (GRIP1), *in situ* hybridization (ISH), quantitative-polymerase chain reaction (qPCR).

1.8 Refinement of the glutamate hypothesis of schizophrenia

Discovery of these alterations in the AMPA receptor subunits and interacting proteins further support the involvement of AMPA receptors in addition to NMDA receptors in the underlying pathophysiology of schizophrenia. However, the

inconsistencies of the AMPA receptor subunit expression data are enigmatic when examined in isolation. Most studies that examine AMPA receptor subunit expression, whether through binding site, mRNA, or protein expression, utilize whole slices or homogenates of tissue. While these studies are valuable for looking at regional differences in AMPA receptor expression, AMPA receptors are highly mobile. Expression of mRNA or protein as measured through RT-PCR or western blotting is a measure of all AMPA receptor expression in the cell, from the ER to the synapse and in any member of the complex endosomal trafficking system. Taken together with the inconsistencies in AMPA receptor subunit expression, alterations of AMPA receptor interacting proteins support the hypothesis that AMPA receptor dysfunction in schizophrenia is a problem of trafficking, rather than one of simply too much or too little receptor expression. These data and theoretical considerations highlight the importance of examining AMPA receptors in subcellular fractions.

1.9 Measuring AMPA receptor expression in postmortem human tissue

Psychiatric illness, including schizophrenia, can be studied in a number of ways, including animal models, clinical studies with living patients, and tissue studies from the deceased (168). While beneficial for understanding some facets of the illness, no current animal model can completely mirror the complex phenotype of schizophrenia (169). Studies using living patients have the advantage measuring any changes in a system representing the complexities of the illness, but are limited by the importance of safety to the patient (170, 171). Use of postmortem tissue presents with its own unique set of challenges, but provides the opportunity to perform in-depth cellular and molecular analyses (168).

1.9.1 Dorsolateral Prefrontal Cortex

Schizophrenia is a complex mental illness with a broad range of symptoms. This broad range of symptoms suggests that multiple regions of the brain may be involved in the illness. While much emphasis and focus is placed on the positive symptoms, cognitive impairment may be a core feature of schizophrenia (172, 173). Although positive and negative symptoms progress over time, cognitive impairments are relatively stable (174, 175). Long-term functional outcome of patients with schizophrenia may also be correlated with level of cognitive impairment (176). In the case of patients with schizophrenia, cognitive deficits seem to arise from problems with central executive function and working memory for auditory and visual information (172, 177-179). The dorsolateral prefrontal cortex (DLPFC) is one brain region where this higher order executive functioning takes place. Alterations in DLPFC activation are present in schizophrenia, but not other mood disorders such as major depression (180).

The DLPFC, or Brodmann area 46 and part of Brodmann area 9, is a grey matter region in the frontal cortex of the brain. A region with granular cytoarchitecture, the DLPFC has 6 discernible layers (181, 182). Within these layers are pyramidal cell neurons, so named for their triangular shaped cell body, with excitatory projections to various other brain regions. Typically, pyramidal neurons in layers 2 and 3 project to other cortical structures, while those in layer 5 project to the striatum and those in layer 6 project to the thalamus (181). The deep layer 3 and layer 4 receive input from the thalamus (182). Pyramidal neurons comprise nearly 75% of the cortical neurons while the other 25% are interneurons (172).

Together, the function and composition, as well as findings of previous studies, make the DLPFC an ideal brain region to study with regards to alterations in trafficking of glutamate receptors in schizophrenia.

1.9.2 Subcellular fractionation

Previous reports of alterations in AMPA receptors and AMPA receptor interacting proteins in schizophrenia have examined expression on a regional level. Current evidence supports the hypothesis of alterations of trafficking and subcellular localization of AMPA receptors in schizophrenia. In order to examine whether or not such alterations exist in this illness, it is important to measure AMPA receptor expression as the receptors are trafficked in the cell. However, a limitation of using postmortem tissue is that measuring active trafficking of receptors is not a viable option. It is possible to measure AMPA receptors in various subcellular regions within the cell as a proxy for trafficking. Specifically, since their role in assembly and turnover of AMPA receptors is integral to receptor trafficking, the endoplasmic reticulum and endosomes are excellent targets for measuring AMPA receptor subunit expression. In addition to their central roles in AMPA receptor trafficking, examination of the ER and the endosomes provides a proximal and distal view of AMPA receptor subcellular localization.

Traditional isolation of subcellular compartments is through multiple centrifugations and sucrose density gradients (183, 184). While these protocols are suitable for fresh tissue and cell culture samples, blurring of some subcellular fractions in postmortem tissue is possible. It is possible to enrich various subcellular fractions using

this technique, but only if the fraction is relatively unique. Furthermore, though various endosome subtypes have distinct protein markers and morphology, they are similar in size and weight making traditional centrifugation ill-suited for subcellular fractionation. In cases such as this, alternative techniques must be utilized. One such technique is immunoisolation using an antibody specific for the fraction of interest. Once a fraction is isolated, it is possible to measure proteins associated with that fraction. In this way, measurement of proteins in a particular fraction may be used to approximate AMPA receptor subcellular localization.

1.10 Goals of dissertation research

While previous studies have measured AMPA receptor subunits and AMPA receptor interacting proteins at a regional level, the results are inconsistent. The glutamate hypothesis of schizophrenia suggests hypofunction of NMDA receptors, yet AMPA receptors are essential in cellular depolarization and functioning of NMDA receptors. AMPA receptor trafficking and subcellular localization are crucial for proper functioning of AMPA receptors and improper trafficking and subcellular localization of AMPA receptors may lead to failed NMDA receptor functioning. Trafficking and subcellular localization of AMPA receptors has yet to be studied in the prefrontal cortex in schizophrenia. The goals of this dissertation research are to test the following hypotheses:

1) Expression of AMPA receptor subunits, GluR1-4, and AMPA interacting proteins in early endosomes are altered in schizophrenia.
2) Expression of AMPA receptor subunits, GluR1-4, in late endosomes and proteins involved in the endosome pathway are altered in schizophrenia.
3) Expression of AMPA receptor subunits, GluR1-4, in the endoplasmic reticulum are altered in schizophrenia.

Evidence for Abnormal Forward Trafficking of AMPA Receptors in Frontal Cortex of Elderly Patients with Schizophrenia

by

John C. Hammond, Robert E. McCullumsmith, Adam J. Funk, Vahram Haroutunian and James H. Meador-Woodruff

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2.1 Abstract

Several lines of evidence point to alterations of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor trafficking in schizophrenia. Multiple proteins, including Synapse Associated Protein 97 (SAP97), Glutamate Receptor Interacting Protein 1 (GRIP1), and N-ethylmaleimide Sensitive Factor (NSF), facilitate the forward trafficking of AMPA receptors toward the synapse. Once localized to the synapse, AMPA receptors are trafficked in a complex endosomal system. We hypothesized that alterations in the expression of these proteins and alterations in the subcellular localization of AMPA receptors in endosomes may contribute to the pathophysiology of schizophrenia. Accordingly, we measured protein expression of SAP97, GRIP1, and NSF in the dorsolateral prefrontal cortex and found an increase in the expression of SAP97 and GRIP1 in schizophrenia. To determine the subcellular localization of AMPA receptor subunits, we developed a technique to isolate early endosomes from postmortem tissue. We found increased GluR1 receptor subunit protein in early endosomes in subjects with schizophrenia. Together, these data suggest that there is an alteration of forward trafficking of AMPA receptors as well as changes in the subcellular localization of an AMPA receptor subunit in schizophrenia.

2.2 Introduction

There is a growing body of evidence that schizophrenia may be linked to abnormalities of glutamate transmission. While early evidence implicated NMDA receptor hypofunction (Allen and Young, 1978; Barbon *et al*, 2007; Coyle *et al*, 2003; Ellison, 1995), preclinical literature has also implicated involvement of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors in the pathophysiology of this illness (Dracheva *et al*, 2005; Meador-Woodruff *et al*, 2001; O'Connor *et al*, 2007). Several studies have examined AMPA receptor expression in multiple brain regions with inconsistent results (Breese *et al*, 1995; Eastwood *et al*, 1995; Freed *et al*, 1993; Healy *et al*, 1998; Noga *et al*, 2001; Scarr *et al*, 2005). Measuring total AMPA receptor expression levels may be less informative, however, since AMPA receptors are highly regulated via trafficking between subcellular organelles from the endoplasmic reticulum to localization at the synapse(Greger and Esteban, 2007; Jiang *et al*, 2006; Zhu, 2003). Measurement of total cellular AMPA receptor expression cannot distinguish localization in these subcellular compartments, yet abnormalities in subcellular localization may have significant functional implications.

The lack of consistent findings and the complexity of AMPA receptor trafficking have led to the examination of proteins that interact with AMPA receptors. Several interacting proteins, including Synapse Associated Protein 97 (SAP97), Glutamate Receptor Interacting Protein 1 (GRIP1), and N-ethylmaleimide Sensitive Factor (NSF) have a significant role in the trafficking and localization of AMPA receptors. SAP97 interacts with the AMPA receptor subunit GluR1 and the myosin motor protein

responsible for transport of the receptor along the dendritic shaft (Wu *et al*, 2002). SAP97 has also been described in the stabilization of GluR1-containing AMPA receptors at the synapse (Nash *et al*, 2010). GRIP1 interacts with the AMPA receptor subunit GluR2 and assists in the transport of the AMPA receptor along the dendritic shaft and stabilization at the synapse (Guo and Wang, 2007). NSF helps regulate surface expression of GluR2-containing AMPA receptors (Noel *et al*, 1999). Because of the complexity of AMPA receptor trafficking, these molecules are potential candidates to study in schizophrenia.

Studies have found alterations in proteins associated with forward trafficking of AMPA receptors in schizophrenia (Beneyto and Meador-Woodruff, 2006; Mirnics *et al*, 2000; Toyooka *et al*, 2002; Whiteheart and Matveeva, 2004). Transcripts and protein for SAP97 were decreased in the prefrontal cortex, but not the hippocampus or occipital cortex, suggesting a region specific deficit in AMPA receptor trafficking (Toyooka *et al*, 2002). Two other studies found decreases in NSF mRNA levels in the prefrontal cortex (Mirnics *et al*, 2000; Whiteheart *et al*, 2004) possibly indicating a problem with AMPA receptor recycling at the synapse, which is facilitated by endosomes. These abnormalities in the proteins specifically involved in the forward trafficking of AMPA receptors suggest that endosomal trafficking of this receptor may be abnormal in schizophrenia.

Endosomal trafficking of neurotransmitter receptors facilitates changes in synaptic strength via surface expression and localization of receptors to the synapse, as well as degradation of receptors. The endocytic pathway and the turnover of (AMPA) receptors have been extensively studied in the context of long-term potentiation (LTP) and long-term depression (LTD), correlates of learning and memory that may be affected in psychiatric illness. Endosomes are small (about 1μm) spherical structures with a

phospholipid bilayer that facilitate sorting of AMPA receptors between intracellular compartments and the cell surface (Beattie *et al*, 2000; Boehm *et al*, 2006; Lüscher *et al*, 1999). Subclasses of endosomes are identified by the presence or absence of specific marker proteins, such as early endosomes antigen-1 (EEA1), Rab7, and Rab11 (Carroll *et al*, 1999; Ehlers, 2000; Gerges *et al*, 2004). Each subclass of endosome has a particular morphology and role in the trafficking of proteins (Ehlers, 2000; Lee *et al*, 2004; Park *et al*, 2004; Tjelle *et al*, 1996). Early endosomes, containing EEA1, are the primary subclass that receives proteins endocytosed from the cell surface. From the early endosomes, proteins are sorted to recycling endosomes, containing Rab11, or late endosomes, containing Rab7, for degradation.

We hypothesized that forward trafficking and endosomal handling of AMPA receptors may be associated with the pathophysiology of schizophrenia. To test this, we measured expression of proteins associated with forward trafficking of AMPA receptors in brains from subjects with schizophrenia and a comparison group. In addition, we developed a technique to isolate intact early endosomes from postmortem brain tissue, characterized the isolated early endosomes, and measured the expression of AMPA receptor subunits and trafficking molecules in endosomes from these same subjects.

2.3 Methods

2.3.1 Subjects and tissue preparation

Subjects from the Mount Sinai Medical Center brain bank were recruited prospectively and underwent extensive antemortem diagnostic and clinical assessment (Table 1). Exclusion criteria included a history of alcoholism, substance abuse, death by

suicide, or coma for more than 6 hours prior to death. Consent was obtained from next of kin for each subject. Brains were collected and cut coronally in 10 mm slabs. The dorsolateral prefrontal cortex was dissected from the coronal slabs, snap frozen and stored at -80^0 C. This tissue was pulverized, adding small amounts of liquid nitrogen as necessary, and stored at -80^0 C until used.

Tissue was prepared for western blots as previously described (Funk *et al*, 2009). Tissue was reconstituted in 5 mM Tris-HCl pH 7.4, 0.32 M sucrose, and a protease inhibitor tablet (Complete Mini, Roche Diagnostics, Mannheim, Germany). Tissue was homogenized using a Power Gen 125 homogenizer (Thermo Fisher Scientific, Rockford, Illinois, USA) at speed 5 for 60 s. Homogenates were assayed for protein concentration using a BCA protein assay kit (Thermo Scientific, Rockford, Illinois, USA), and stored at -80^0 C.

2.3.2 Western blot analysis

Commercially available antibodies were used for the western blot analyses with antisera dilutions determined empirically (Table 2). Samples for western blots were placed in reducing buffer containing β-mercaptoethanol and heated at 70^0 C for 10 minutes. Samples for each subject were then run in duplicate by SDS-polyacrylamide gel electrophoresis on Invitrogen (Carlsbad, California, USA) 4-12% gradient gels, and transferred to polyvinylidene fluoride membrane using Bio-Rad semi-dry transblotter (Hercules, California, USA). The membranes were blocked in LiCor blocking buffer for 1 hour at room temperature, and probed with primary antibody in 0.1% Tween LiCor blocking buffer at the dilutions and for the durations indicated in table 2. Membranes

were then washed four times for 5 minutes each with 0.01% Tween phosphate buffered saline. Membranes were probed with IR-dye labeled secondary antibody in 0.1% Tween, 0.01% SDS LiCor Blocking buffer for 1 hour at room temperature in the dark. Membranes were washed again with 0.01% Tween phosphate buffered saline four times for 5 minutes each and then briefly rinsed 3 times in distilled water. The blots were stored in distilled water at 4^0 C until scanned using the LI-COR Odyssey® laser-based image detection method (Bond *et al*, 2008). We tested each antibody using varying concentrations of total protein from homogenized human cortical tissue to confirm we were in the linear range of the assay.

2.3.3 Immunoisolation of early endosomes

A subset of subjects (Table 1) was used for early endosome isolation due to the large amounts of tissue required for this technique. For each subject, isolation was performed in duplicate. Eighty (80) μL [6.7×10^8 beads/mL] of sheep anti-rabbit Dynabead M280 magnetic beads (Invitrogen, Carlsbad, California, USA) were washed three times with ice-cold phosphate buffered saline. All washes consisted of 5 minutes rotating at 4^0 C and 2 minutes on the magnet [Dynal MPC-S, Invitrogen Carlsbad, California, USA]). Beads were then resuspended in 70 μL of phosphate buffered saline and 7.5 μg of rabbit anti-EEA1 antibody (Abcam Inc., Cambridge, Massachusetts, USA). The bead-antibody solution was incubated while rotating at 4^0 C for 16-18 hours to form a bead-antibody complex. Seventy (70) μL of fresh beads were chilled on ice and washed three times with ice cold phosphate buffered saline. We added 130 μg of homogenized tissue in 5mM Tris HCl (final volume 200 μL) to the freshly washed beads, and

precleared the tissue for 1 hour while rotating at 4^0 C. The bead-antibody complex was washed three times with ice cold phosphate buffered saline. After the 1 hour incubation, the precleared tissue homogenate was collected and incubated with the bead-antibody complex for 3 hours while rotating at 4^0 C to isolate early endosomes. The supernatant of the bead-antibody-endosome complex was collected and saved, and the bead-antibody-endosome complex was washed 3 times with ice cold phosphate buffered saline. This complex was reconstituted in 20 μL of distilled Milli-Q water and samples were prepared for western blot analysis or electron microscopy. Samples for western blot analysis were heated in reducing buffer containing β-mercaptoethanol at 70^0 C for 10 minutes. Samples were placed in the Dynal magnet for 2 minutes prior to loading on the gel.

2.3.4 Electron Microscopy

Immediately after immunoisolation and reconstitution in Milli-Q water, bead-antibody-endosome complexes were embedded in agarose and then fixed with 4% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) overnight at room temperature. The complexes were then washed and treated with 1% osmium tetroxide for 1 hour, mordanted with 0.25% uranyl acetate in acetate buffer for 30 minutes to overnight, washed and dehydrated with a graded series of ethanol washes and propylene oxide. Finally, the samples were embedded in epoxy resin, thin sectioned and counterstained with uranyl acetate and lead citrate. Images were captured using an FEI Tecnai Spirit 20-120kv Transmission Electron Microscope.

2.3.5 Data analysis

Near-infrared fluorescent signals obtained from the LiCor Odyssey scanner were expressed as raw integrated intensity with top/bottom median intralane background subtraction using Odyssey 3.0 analytical software (LiCor, Lincoln, Nebraska, USA) (Bond *et al*, 2008). For homogenate protein studies, duplicate lanes of protein expression from each subject were normalized to β-tubulin as an in-lane loading control. β-tubulin was chosen because no changes have previously been detected in subjects with schizophrenia compared to control subjects (Bauer *et al*, 2009). For immunoisolation studies, duplicate lanes of protein expression from each subject were normalized to EEA1 as an in-lane loading control.

To confirm the immunocapture of endosomes and to assess capture efficiency, 1650X direct magnification electron micrograph images of preclear, negative control, and immunoisolation samples, were printed, coded, and randomly sorted. Counts were made by an observer blind to condition. Beads or endosomes on the borders of each image were not included in the counts.

Data were analyzed using Statistica (Statsoft, Tulsa, Oklahoma, USA). Correlation analyses were done to identify any associations between the dependent variables and pH, age, and post-mortem interval. One-way analysis of covariance was performed if significant correlations were found. If no correlations were present, data were analyzed with one-way analysis of variance. Secondary analyses were performed using sex and medication status as the independent measure.

2.4 Results

2.4.1 Protein expression in tissue homogenates

We examined the expression of the AMPA receptor interacting proteins GRIP1, NSF, and SAP97 in schizophrenia and a comparison group using β-tubulin as a loading control (Figure 1). As previously reported(Bauer *et al*, 2009), we found no changes in β-tubulin (non-normalized) in schizophrenia. Despite a significant difference in PMI between the schizophrenia and comparison group [$F(1,63) = 6.59$, $p = 0.01$], we found no significant correlation between PMI and protein expression for GRIP1 [$F(1,55) = 1.32$, $p = 0.26$], SAP97 [$F(1,54) = 1.76$, $p = 0.19$], or NSF [$F(1,53) = 0.008$, $p = 0.92$]. We also found no significant correlations between protein expression and age or pH in our samples. Additionally, we found no effect of either sex or medication status in these homogenate studies. We found significant increases in GRIP1 [$F(1,62) = 18.659$, $p < 0.01$] and SAP97 [$F(1,62) = 7.719$, $p < 0.01$], but not NSF [$F(1,62) = 2.616$, $p = 0.11$] in subjects with schizophrenia. We also found no significant difference in the expression of EEA1 [$F(1,52) = 0.135$, $p = 0.71$], the marker we used to isolate early endosomes. We also examined total expression of GluR1-3 and found no change in schizophrenia (data not shown). We found a significant correlation between GluR1 and SAP97 expression in schizophrenia [$F(1,29) = 5.29$, $p = 0.03$] , but not in our comparison group. We found no correlation between GluR2 and GRIP1 in either diagnostic group.

2.4.2 Early Endosome enrichment

To analyze alterations in early endosome content in schizophrenia, we used magnetic beads bound to an early endosome-specific antibody to obtain an enriched early endosome fraction from postmortem tissue (Figure 2). When starting with tissue homogenate, we detected non-specific binding of PSD95 to our magnetic beads (Figure 2, non-precleared, IM, Pellet lane). We incubated tissue with fresh beads to preclear the homogenate (Figure 2, preclear bead lane). Using this precleared tissue, we performed an immunoisolation (Figure 2, IM) with beads complexed to the EEA1 antibody. The negative control (Figure 2, - ctrl) consists of precleared homogenate with beads alone. When starting with the precleared samples, we found markedly diminished nonspecific expression of PSD95 in our endosome preparation, while EEA1 protein levels were preserved (Figure 2, Precleared, IM, Pellet lane). Substituting preimmune IgG for the EEA1 capture antibody gave identical results as beads alone (data not shown).

Using electron microscopy, we measured the number of endosomes per bead in preclear, negative control, and immunoisolation samples. We found a 6.15-fold increase in the endosome to bead ratio in our immunoisolation samples relative to our preclear beads samples (Figure 3, Table 3).

To verify the specificity of our immunoisolation, we used western blot analysis to measure expression of proteins not expected to be expressed in early endosomes, including those found in the post-synaptic density (PSD95), endoplasmic reticulum (GRP78/BiP), astrocytes (Glutamine Synthetase) and late endosomes (Rab7). As anticipated, we did not find any of these markers in our early endosome preparation (Figure 4).

2.4.3 Protein expression in early endosomes

We examined the expression of the AMPA receptor subunits, GluR1 – 3, in early endosomes samples (Figure 5). All protein expression was measured relative to EEA1 expression in the same lane. We found a significant increase in GluR1 [$F(1,37) = 4.189$, $p = 0.048$], but not GluR2 [$F(1,41) = 0.030$, $p = 0.864$] or GluR3 [$F(1,41) = 0.067$, $p = 0.797$] in the enriched endosome fraction. We found no significant correlations between protein expression and age, pH, or PMI in our isolated endosome samples. Additionally, we found no influence of sex in our isolated endosome studies.

Further analyses were done to determine if medication status had an effect on the expression of AMPA receptor subunits in early endosome isolation samples. We found a significant increase in GluR1 [$F(2,36) = 6.65$, $p = 0.004$], but not GluR2 [$F(2,40) = 1.92$, $p = 0.159$] or GluR3 [$F(2,40) = 0.13$, $p = 0.876$] in the enriched endosome fraction for patients off medication for 6 weeks or more prior to death compared to the comparison group ($p = 0.003$) or patients on medication 6 weeks or less prior to death ($p = 0.018$).

We also examined the enriched fraction to determine if there were alterations in the expression of the AMPA receptor interacting proteins, NSF or SAP97. We found no significant change in the expression of NSF [$F(1,35) = 0.895$, $p = 0.351$] or SAP97 [$F(1,38) = 0.403$, $p = 0.529$] in our early endosome enriched fraction (Figure 7). We found no significant associations with NSF/EEA1 or SAP97/EEA1 and medication status or sex.

2.5 Discussion

Previous studies have attempted to link alterations in AMPA receptor trafficking with the underlying pathophyisology of schizophrenia. These studies have examined expression of polymorphisms, transcripts, and proteins associated with AMPA receptor forward trafficking (Beneyto *et al*, 2006; Scarr *et al*, 2005). Transcripts for the GluR2 interacting protein, GRIP1, were decreased in one study, but increased in another (Choi *et al*, 2002; Dracheva *et al*, 2005). A different study found no changes in GRIP1 protein expression in the frontal cortex (Toyooka *et al*, 2002). Binding site and protein expression of another AMPA trafficking molecule, NSF, was unchanged in the DLPFC (Beneyto *et al*, 2006; Gray *et al*, 2006; Imai *et al*, 2001), while another study found decreased SAP97 protein expression in the DLPFC, but not in the hippocampus or occipital cortex (Toyooka *et al*, 2002). In contrast to these studies, we found an increase in expression of two proteins associated with AMPA receptor trafficking, GRIP1 and SAP97 in the DLPFC. There are several possible explanations for these divergent results, including differences in western blot protocol, level of gene expression, subject age, or tissue source. For example, in one previous report (Toyooka *et al*, 2002), SAP97 was normalized to NSE, a neuronal cytoplasmic marker, while we used β-tubulin for normalization, a structural microtubule protein that is unchanged in schizophrenia (Bauer *et al*, 2009). In addition, the cohort in the current study is elderly, while the subjects from other studies are generally younger. Regardless of the direction of change in expression, several studies have reported alterations in molecules associated with the trafficking of AMPA receptors in schizophrenia (McCullumsmith *et al*, 2004). Such alterations in

trafficking proteins suggests abnormal trafficking of AMPA receptors along the dendrite and to the cell surface at the synapse.

To further understand AMPA receptor trafficking and its potential role in the pathophysiology of schizophrenia, we examined one subcellular fraction that is essential in the turnover of AMPA receptors at the cell surface, early endosomes. Following endocytosis, endocytic vesicles fuse with early endosomes as the initial step in the sorting of receptors to recycling or late endosomes (Gruenberg, 2001; Hirling, 2008). The isolation protocol we developed provided intact early endosomes with no evidence of cross-contamination from other subcellular organelles. Isolated endosomes (Figure 3) were similar in size (~ 1 micron) and shape (spheroid) to descriptions of early endosomes in preclinical literature (Gruenberg, 2001; Tjelle *et al*, 1996). Further examination revealed that the AMPA receptor subunits GluR1, GluR2, and GluR3, as well as the AMPA receptor interacting proteins, SAP97 and NSF, are present in the early endosomes in postmortem brain tissue. Thus, we used this preparation to measure the contents of early endosomes in schizophrenia.

We found no change in total EEA1 protein expression, a marker of early endosomes, between the subjects with schizophrenia and our comparison group, suggesting that the total number of early endosomes is unchanged between the groups. We also found no change in expression of GluR1-3 in total homogenate, suggesting that there is not a problem with too much or too little AMPA receptor expression, but a problem with receptor trafficking. Western blot analysis of the isolated endosomes revealed an increase in GluR1 relative to EEA1 expression in subjects with schizophrenia. The increase in GluR1 protein expression in endosomes, combined with

the increase in SAP97 and GRIP1 in homogenate, is consistent with increased forward trafficking of the subunit to the cell surface and an increase in endocytosis. Supporting these findings, we detected a significant positive correlation between SAP97 and GluR1 expression in tissue homogenates from subjects with schizophrenia, but not control subjects. We speculate that increased SAP97 might be a compensation for increased levels of GluR1 on the cell surface, with increased internalization of excess receptor complexes, in a manner similar to NMDA-induced long-term depression (Biou *et al*, 2008; Brown *et al*, 2005). Recent developments in the glutamate hypothesis of schizophrenia suggest that increased synaptic glutamate might contribute to pathophysiology in schizophrenia (Krystal, 2008). In preclinical studies, elevated synaptic glutamate levels led to a selective increase in the internalization of GluR1-containing AMPA receptors (Lissin *et al*, 1999). Such a mechanism is consistent with our findings of altered trafficking molecules and GluR1 in early endosomes and the hypothesis of increased synaptic glutamate in schizophrenia.

Alternatively, there may be dysfunction in the stabilization of GluR1 containing AMPA receptors at the synapse. The consequences of diminished levels of GluR1-containing AMPA receptors in the synapse has been examined in rodent models. One study using a GluR1 knockout mouse model found an increase in behavioral endophenotypes associated with schizophrenia (Wiedholz *et al*, 2008). Other studies have found removal of GluR1-containing AMPA receptors from the synapse leads to a decrease in long-term potentiation and cognitive dysfunction in rodents (Johnson *et al*, 2005; Mead and Stephens, 2003; Rumpel *et al*, 2005). If the GluR1-containing receptors are not stabilized at the synapse, they may become trapped in the early endosomes.

Regardless of the cause, our finding of increased GluR1 in early endosomes suggests abnormal intracellular localization of AMPA receptors in schizophrenia.

While GluR1 exists as part of a heteromeric AMPA receptor complex, we did not find an increase in other AMPA receptor subunits in the early endosomes. It may be that trafficking of GluR2- and GluR3-containing AMPA receptors through early endosomes is preserved in schizophrenia. To gain a clear understanding of where GluR2 and GluR3 may be localized, other subcellular fractions, including the ER and PSD, must be examined.

Many patients with schizophrenia have a long history of taking antipsychotic medication. In order to partially control for this effect, we examined a subset of subjects who were off medication for 6 weeks or more at the time of death. We found no medication related changes in GluR2 or GluR3 in early endosomes, however patients off medication had increased GluR1 protein expression in early endosomes relative to both the comparison group and the subjects on medication at the time of death (Figure 6). Thus, antipsychotic treatment may mask changes in AMPA receptor trafficking and localization, by decreasing the amount of GluR1-containing AMPA receptors that are present in early endosomes, without changing the overall protein expression level. Additionally, there may be a neuroleptic effect on other trafficking molecules. However, this effect may be confined to the endosomes as we found no change in SAP97 or GRIP1 in early endosomes, despite a non-drug related increase in these proteins in total homogenate. In order to further understand how drug related changes affect trafficking, additional studies would be beneficial.

While previous studies have examined surface binding of AMPA receptor subunits (Beneyto *et al*, 2006; Dracheva *et al*, 2005; Scarr *et al*, 2005), we have examined a subcellular compartment that directly contributes to the trafficking and surface expression of AMPA receptors. In order to isolate endosomes, we developed a modified immunoisolation protocol rather than using a standard subcellular fractionation technique involving high-speed centrifugation that may lead to cross-contamination of the fractions, due to the processing typically associated with postmortem tissue collection (Aniento and Gruenberg, 2003; German and Howe, 2009). In addition, we found that when using magnetic or sepharose beads, there was non-specific binding of some proteins to the beads, including PSD95. In order to remove this non-specific binding, we found that a preclear step was required, highlighting the importance of appropriate control studies when utilizing bead capture techniques in postmortem tissue.

In summary, we found an increase in proteins involved in forward trafficking of AMPA receptors, SAP97 and GRIP1. This increase may lead to increased forward trafficking of the AMPA receptors and more AMPA receptors in the endosomal compartment. We also found an increase in an AMPA receptor subunit in early endosomes in the dorsolateral prefrontal cortex in schizophrenia, supporting the hypothesis that forward trafficking of AMPA receptors is altered in schizophrenia and suggesting that alterations in endosome contents may be associated with the underlying pathophyisology of the illness.

2.6 Conflict of Interest

Dr. James Meador-Woodruff receives an honorarium as editor of N*europsychopharmacology*. The other authors have nothing to disclose.

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Table 1: Subject Demographics

	Homogenate studies		Endosome studies	
	comparison	schizophrenia	comparison	schizophrenia
N	31	35	21	20
Sex	12m/19f	23m/12f	8m/13f	15m/5f
Tissue pH	6.4 ± 0.2	6.4 ± 0.3	6.4 ± 0.3	6.4 ± 0.3
PMI (hours)	8.1 ± 6.9	12.5 ± 6.6	8.5 ± 7.6	12.8 ± 6.4
Age (years)	78 ± 14	74 ± 12	79 ± 12	73 ± 12
Medication (on/off)	0/31	24/11	0/21	12/8

Values presented as mean ± standard deviation. Male (m), female (f), postmortem interval (PMI)

Table 2: Antibodies used for Western blot studies

Antibody	Species	Concentration	Incubation	Company
GluR1	mouse	1 : 100	16 hours	Santa Cruz Biotechnology Inc, Santa Cruz, CA
GluR2	mouse	1 : 1000	2 hours	US Biological, Swampscott, MA
GluR3	mouse	1 : 500	16 hours	US Biological, Swampscott, MA
GRIP1	rabbit	1 : 1000	16 hours	Upstate, Lake Placid, NY
NSF	mouse	1 : 4000	16 hours	Abcam Inc., Cambridge, MA
SAP97	mouse	1 : 1000	16 hours	Abcam Inc., Cambridge, MA
EEA1	mouse	1 : 1000	2 hours	BD Transduction, San Jose, CA
PSD95	mouse	1 : 1000	1 hour	Millipore, Bellarica, MA
GRP78/BiP	mouse	1 : 250	1 hour	BD Transduction, San Jose, CA
GS	mouse	1 : 5000	1 hour	BD Transduction, San Jose, CA
Rab5	mouse	1 : 2000	1 hour	Abcam Inc., Cambridge, MA
β-Tubulin	mouse	1 : 10,000	1 hour	Upstate, Lake Placid, NY

Abbreviations: Glutamate Receptor (GluR), Glutamate Receptor Interacting Protein 1 (GRIP1), N-ethylmalemide Sensitive Factor (NSF), Synapse Associated Protein 97 (SAP97), Early Endosome Antigen 1 (EEA1), Post-synaptic Density 95 (PSD95), Glucose Regulated Protein 78/Binding Protein (GRP78/BiP), Glutamine Synthetase (GS).

Table 3: Endosome Counts from Electron Micrograph Studies

Condition	Antibody	Beads Counted	Endosomes/Bead
Preclear (non-specific binding)	None	890	0.047
Negative Control	Rabbit IgG	175	0.000
Immunoisolation (specific binding)	Rabbit α EEA1	560	0.276

Abbreviations: Early Endosome Antigen 1 (EEA1)

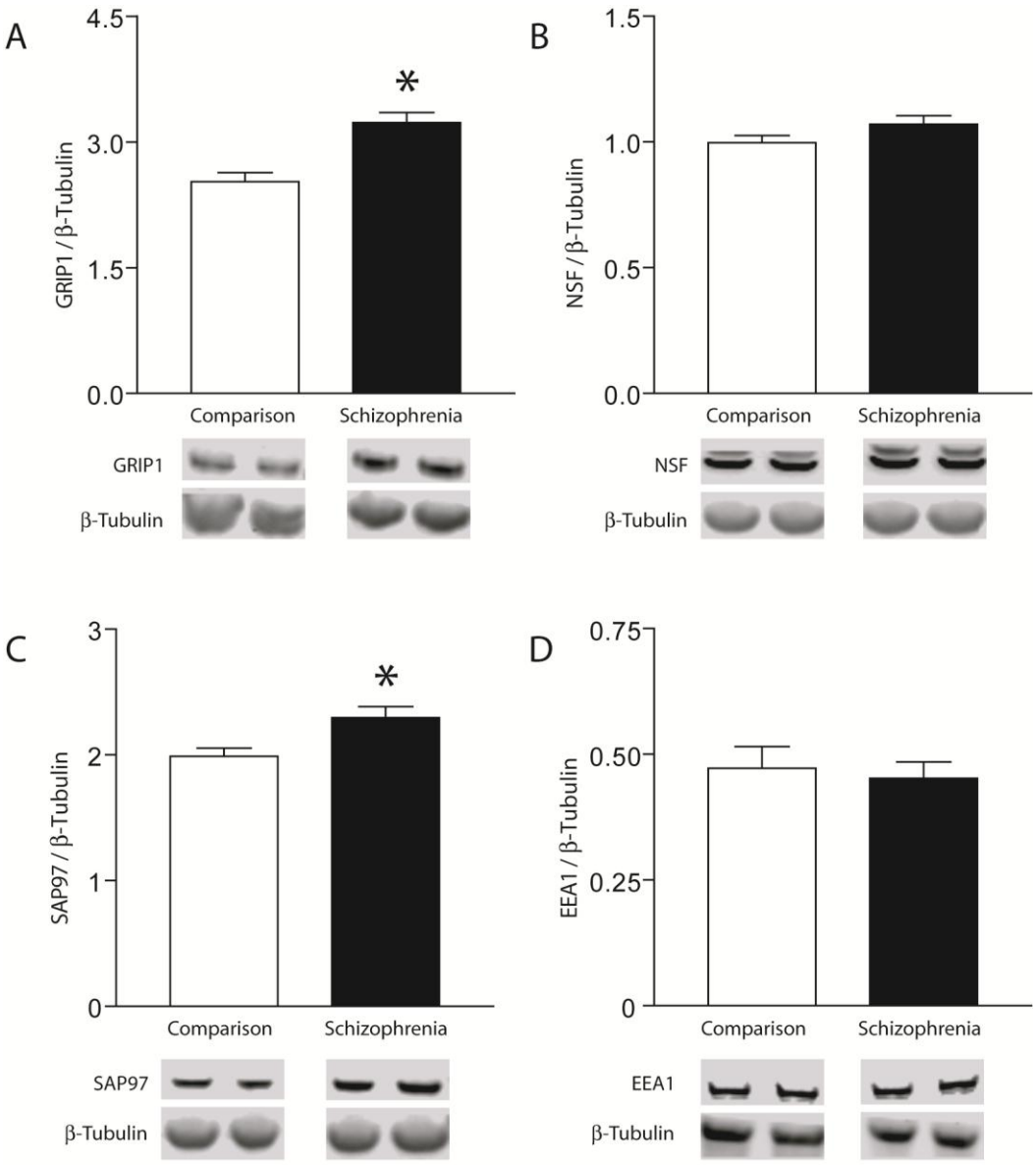

Figure 1. Western blot analysis of AMPA interacting proteins (A – Glutamate Receptor Interacting Protein 1 (GRIP1), B – N-ethylmaleimide Sensitive Factor (NSF), C – Synapse Associated Protein 97 (SAP97) and D – Early Endosome Antigen 1 (EEA1)) in total brain homogenate normalized to β-tubulin. * $p < 0.05$

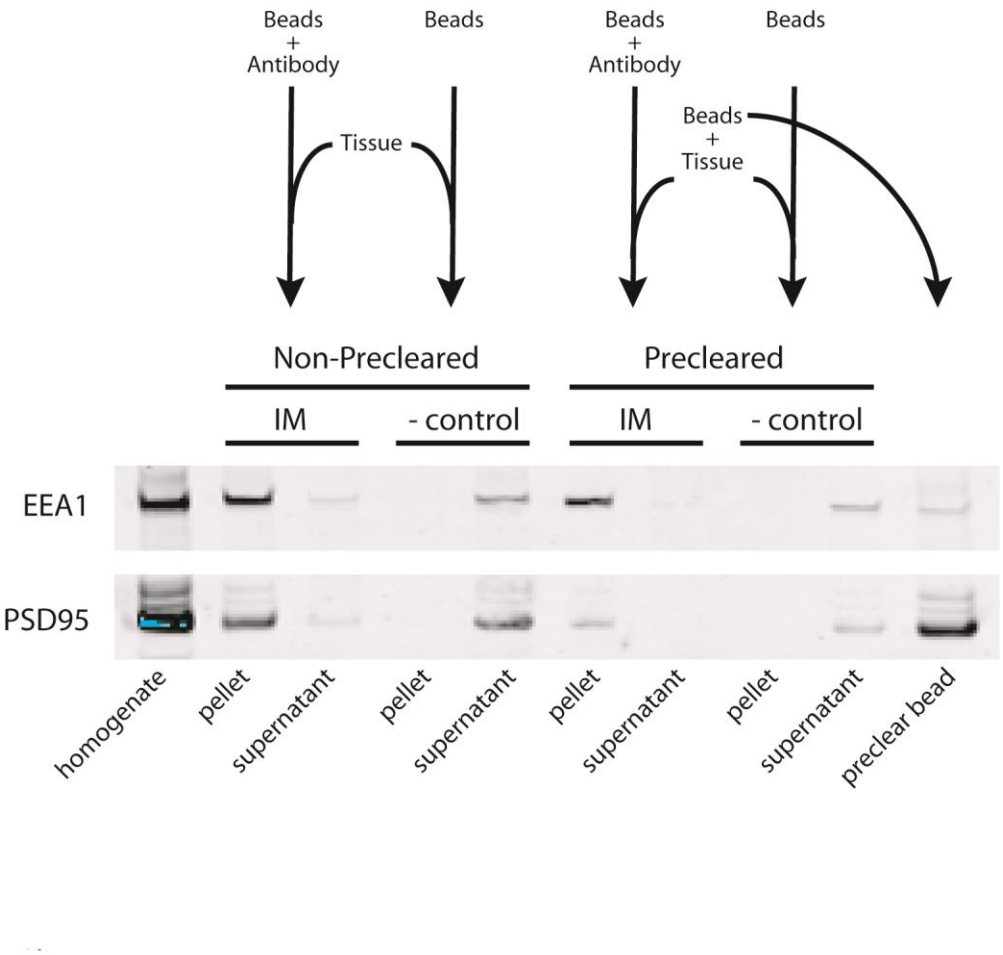

Figure 2. Flow chart of EEA1 immunoisolation protocol with non-precleared and precleared tissue. Western blotting was used to determine protein expression of EEA1 and PSD95. Non-specific binding of PSD95 in EEA1 immunoisolation is present with non-precleared tissue (Non-precleared, IM, Pellet lane). EEA1 immunoisolation with precleared tissue has markedly reduced expression of PSD95 but maintains EEA1 expression (Precleared, IM, Pellet lane). PSD95 sticks non-specifically to beads (preclear bead lane). Immunoisolation (IM). Negative control (- control).

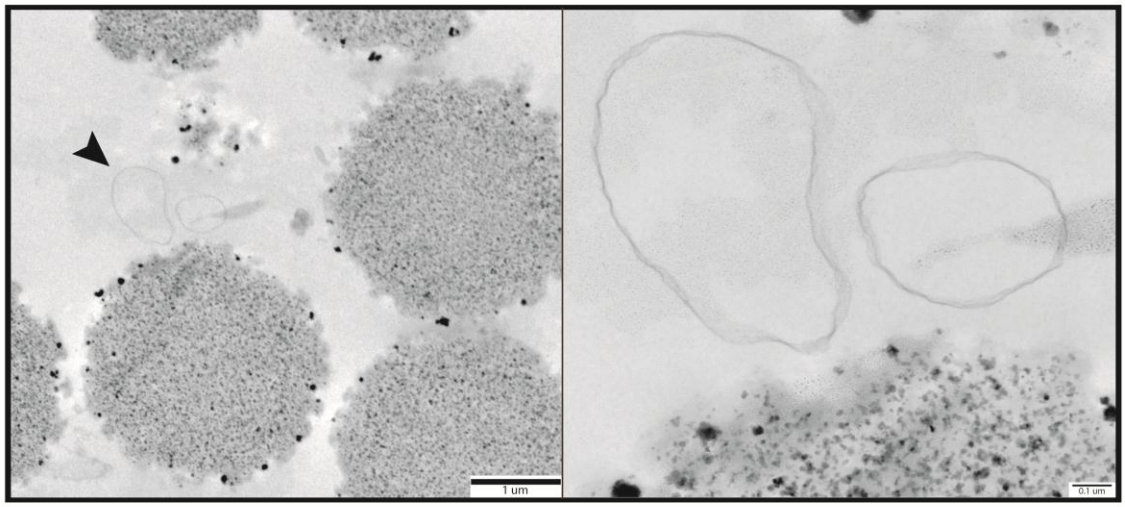

Figure 3. Electron micrograph of early endosome immunoisolation. Early endosomes were isolated using magnetic beads and EEA1 capture antibody and imaged using electron microscopy. The panel on the right is an enlarged view of the area indicated by the arrow on the left.

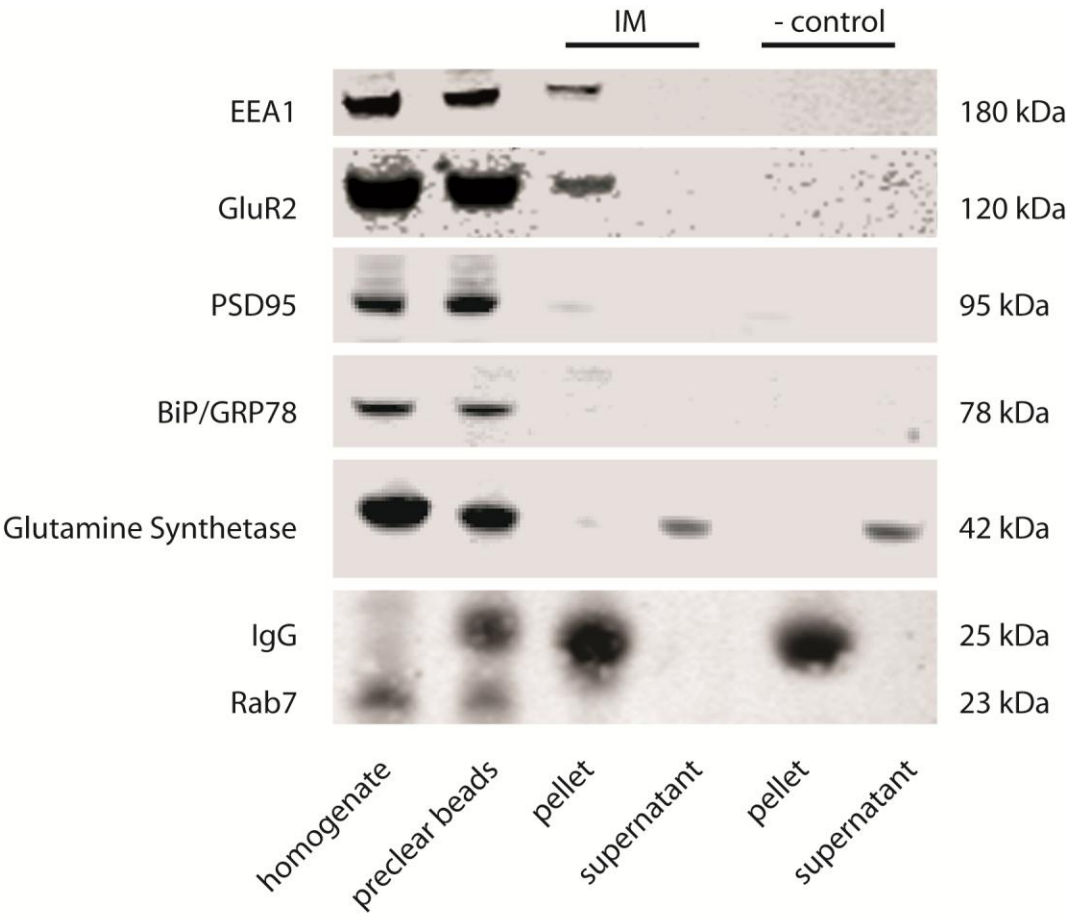

Figure 4. Characterization of early endosome isolation by western blot analysis. Proteins not expressed in early endosomes (IM – Pellet) include those found in the post-synaptic density (PSD95), endoplasmic reticulum (GRP78/BiP), astrocytes (glutamine synthetase), and late endosomes (Rab7). Expression of the AMPA receptor subunit (GluR2) is present in early endosomes (IM – Pellet). Immunoisolation (IM). Negative control (-ctrl). Early Endosome Antigen 1 (EEA1). Post-synaptic density 95 (PSD95). Glucose Regulated Protein 78/Binding immunoglobulin protein (GRP78/BiP). Ionotropic Glutamate Receptor 2 (GluR2).

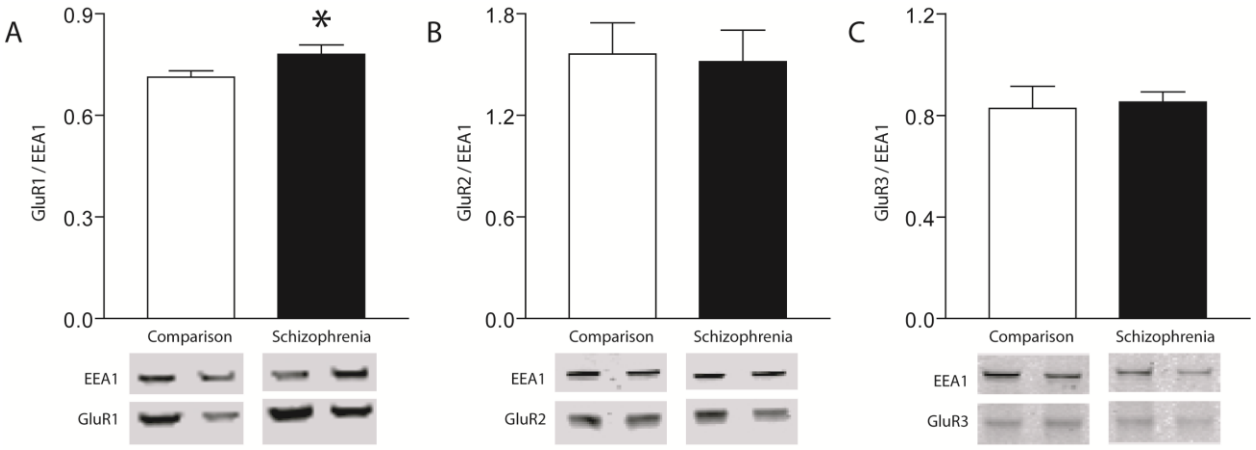

Figure 5. Western blot analysis of AMPA receptor subunits GluR1 (A), GluR2 (B), and GluR3 (C) in early endosomes normalized to EEA1 levels. Data are expressed as the ratio of subunit relative to isolated endosome expression. * $p < 0.05$

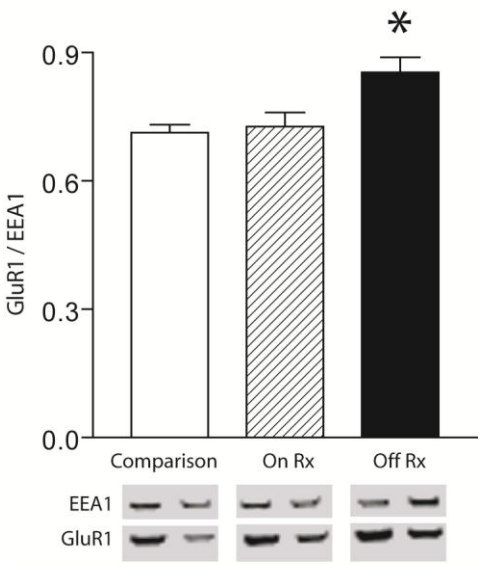

Figure 6. Analysis of antipsychotic effects on GluR1 expression relative to expression of isolated early endosomes. Post-hoc analysis of expression of GluR1 relative to expression of isolated early endosomes. Patients on medication at time of death (On Rx). Patients off medication for ≥ 6 weeks at time of death (Off Rx). * $p < 0.05$

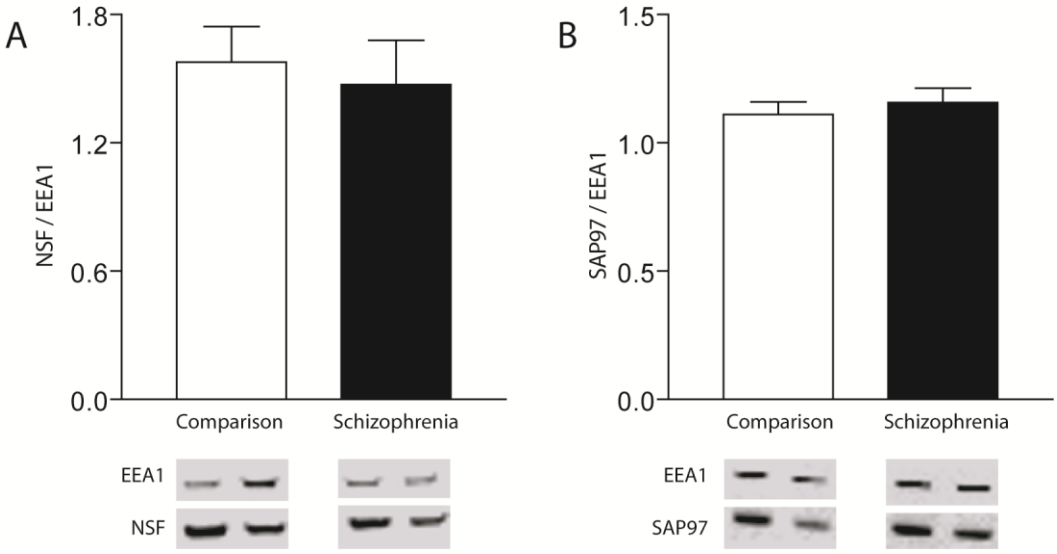

Figure 7. AMPA receptor interacting protein expression in early endosomes. The expression of the AMPA receptor interacting proteins, NSF (A) and SAP97 (B), relative to expression of EEA1 was detected by western blot following enrichment of early endosomes.

Endosomal Trafficking of AMPA Receptors in Frontal Cortex of Elderly Patients with Schizophrenia

by

John C. Hammond, Robert E. McCullumsmith, Vahram Haroutunian, James H. Meador-Woodruff

Schizophrenia Research

Format adapted for dissertation

3.1 Abstract

Several lines of evidence indicate altered trafficking of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors in schizophrenia. Previous reports have implicated alterations in the endosomal trafficking of AMPA receptors in this illness. We hypothesized that late endosome content of AMPA receptor subunits is altered in schizophrenia. Accordingly, we developed a technique to isolate and measure contents of late endosomes from postmortem human tissue. We found no changes in the expression of the AMPA subunits, GluR1-4, in late endosomes from the dorsolateral prefrontal cortex in schizophrenia. We also hypothesized that proteins involved in the sorting and trafficking of AMPA receptors between endosomal compartments would be altered in schizophrenia. We found no changes in expression of multiple proteins associated with these processes (dynamin3, Arc/ARG3.1, NEEP21, GRASP1, liprin α, and syntaxin13). Together, these data suggest that endosomal trafficking of AMPA receptors in the prefrontal cortex may be largely intact in schizophrenia.

3.2 Introduction

Recent studies suggest a link between the pathophysiology of schizophrenia and abnormalities of glutamate receptor expression and neurotransmission (Beneyto et al., 2007; Dracheva et al., 2005; McCullumsmith et al., 2004). The glutamate hypothesis of schizophrenia posits decreased signaling through the *N*-methyl *D*-aspartate (NMDA), and possibly α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) subtypes of glutamate receptors (Coyle, 1996; Coyle et al., 2003). Some recent evidence has suggested abnormalities of trafficking of the AMPA subtype of glutamate receptor in this illness (Beneyto and Meador-Woodruff, 2006; Hammond et al., 2010; Toyooka et al., 2002).

AMPA receptor trafficking is tightly regulated from the endoplasmic reticulum (ER) to expression at the synapse (Greger and Esteban, 2007; Zhu, 2003). Mature tetrameric AMPA receptors exit the ER and Golgi and are trafficked along the shaft of the cell toward the synapse with assistance from AMPA receptor interacting proteins such as SAP97 and GRIP1 (Goldstein and Yang, 2000; Jiang et al., 2006; Sans et al., 2001; Setou et al., 2002). Upon reaching the synapse, AMPA receptor insertion into the cell membrane is activity-dependent (Greger and Esteban, 2007; Jin et al., 2006; Zhu, 2003). AMPA receptors expressed at the synapse can be inactivated through removal from the cell membrane via internalization into endosomes. Endosomes are small, spherical compartments that traffic AMPA receptors between intracellular compartments and the neuronal surface (Hirling, 2008). From early endosomes, AMPA receptors can be sorted to recycling endosomes or to late endosomes for degradation (Ehlers, 2000).

AMPA receptors may also be pooled in the endosomes as a reserve (Lüscher and Frerking, 2001; Park et al., 2004). There is evidence of an increased pool of AMPA receptors in early endosomes in schizophrenia (Hammond et al., 2010).

Specific markers identify different endosomal subclasses: Rab5 is expressed in early endosomes, Rab7 in late endosomes, and Rab11 in recycling endosomes (Hanley, 2010; Ng and Tang, 2008; Wang et al., 2011). In addition to the rabs, other molecules are involved in the sorting and trafficking of AMPA receptors between these endosomal subclasses. Dynamin3 and Arc/ARG3.1 facilitate endocytosis of AMPA receptors into early endosomes, while NEEP21 and GRASP1 sort AMPA receptors from early endosomes to recycling endosomes (Bramham et al., 2008; Chowdhury et al., 2006; Hoogenraad et al., 2010; Hoogenraad and van der Sluijs, 2010; Lu et al., 2007; Steiner et al., 2005). Liprin α and syntaxin13 facilitate postsynaptic targeting and reinsertion of AMPA receptors in the plasma membrane (Ko et al., 2003; Park et al., 2006; Spangler and Hoogenraad, 2007). Dysregulated trafficking and localization of AMPA receptors may alter excitatory neurotransmission mediated by these receptors.

We postulate that altered endosomal trafficking of AMPA receptors may be associated with the pathophysiology of schizophrenia. Consistent with this hypothesis, we have previously reported increased GluR1 in an early endosome compartment isolated from postmortem cortex samples in schizophrenia (Hammond et al., 2010). To further test this hypothesis, in the present study we isolated and characterized intact late endosomes from postmortem brain tissue and measured the expression of AMPA receptor subunits and trafficking molecules in these endosomes from subjects with schizophrenia and a comparison group. To assess the fidelity of the endosomal system,

we also measured expression of proteins associated with endosomal handling of AMPA receptors in brains from these same subjects.

3.3 Materials and Methods

3.3.1 Subjects and Tissue Preparation

Subjects from the Mount Sinai Medical Center brain bank were recruited prospectively and underwent extensive antemortem diagnostic and clinical assessment (Table 1). Patients were considered to be "off" medication if they had not taken antipsychotics for 6 weeks or more at the time of death. Exclusion criteria included a history of alcoholism, substance abuse, death by suicide, or coma for >6 h before death. Consent was obtained from next of kin for each subject. Brains were collected and cut coronally in 10 mm slabs. The dorsolateral prefrontal cortex was dissected from the coronal slabs, snap frozen, and stored at −80 °C. This tissue was pulverized, adding small amounts of liquid nitrogen as necessary, and stored at −80 °C until used.

Tissue was prepared for western blots as previously described (Funk et al., 2009). Tissue was reconstituted in 5 mM Tris-HCl pH 7.4, 0.32 M sucrose, and a protease inhibitor tablet (Complete Mini, Roche Diagnostics, Mannheim, Germany). Tissue was homogenized using a Power Gen 125 homogenizer (Thermo Fisher Scientific, Rockford, Illinois) at speed 5 for 60 s. Homogenates were assayed for protein concentration using a BCA protein assay kit (Thermo Scientific, Rockford, Illinois), and stored at −80 °C.

3.3.2 Western Blot Analysis

Commercially available antibodies were used for the western blot analyses with antisera dilutions determined empirically (Table 2). Samples for western blots were placed in reducing buffer-containing β-mercaptoethanol and heated at 70 °C for 10 min.

Samples for each subject were then run in duplicate by SDS-polyacrylamide gel electrophoresis on Invitrogen (Carlsbad, California) 4–12% gradient gels, and transferred to polyvinylidene fluoride membrane using Bio-Rad semi-dry transblotter (Hercules, California). The membranes were blocked in LiCor (Lincoln, Nebraska) blocking buffer or 1% BSA for 1 h at room temperature, and probed with primary antibody in 0.1% Tween LiCor blocking buffer or 0.1% Tween in 1% BSA at the dilutions and for the durations indicated in Table 2. Membranes were then washed four times for 5 min each with 0.01% Tween phosphate-buffered saline. Membranes were probed with IR-dye labeled secondary antibody in 0.1% Tween, 0.01% SDS LiCor blocking buffer or 0.1% Tween, 0.01% SDS 1% BSA for 1 h at room temperature in the dark. Membranes were washed again with 0.01% Tween phosphate-buffered saline four times for 5 min each and then briefly rinsed three times in distilled water. The blots were stored in distilled water at 4 °C until scanned using the LI-COR Odyssey laser-based image detection method (Bond et al., 2008). We tested each antibody using varying concentrations of total protein from homogenized human cortical tissue to confirm we were in the linear range of the assay.

3.3.3 Immunoisolation of Late Endosomes

A subset of subjects (Table 1) was used for late endosome isolation because of the large amounts of tissue required for this technique. For each subject, isolation was performed in duplicate. In total, 80 µl (6.7×10^8 beads/ml) of sheep anti-mouse Dynabead M280 magnetic beads (Invitrogen) were washed three times with ice-cold phosphate-buffered saline. All washes consisted of 5 min rotating at 4 °C and 2 min on the magnet (Dynal MPC-S, Invitrogen). Beads were then resuspended in 70 µl of

phosphate-buffered saline and 20 μg of mouse anti-Rab7 antibody (Abcam, Cambridge, Massachusetts). The bead-antibody solution was incubated while rotating at 4 °C for 16–18 h to form a bead-antibody complex. Subsequently, 80 μl of fresh beads were chilled on ice and washed three times with ice-cold phosphate-buffered saline. We added 260 μg of homogenized tissue in 5 mM Tris-HCl (final volume 200 μl) to the freshly washed beads, and precleared the tissue for 1 h while rotating at 4 °C. The bead-antibody complex was washed three times with ice-cold phosphate-buffered saline. After the 1-h incubation, the precleared tissue homogenate was collected and incubated with the bead-antibody complex for 3 h while rotating at 4 °C to isolate late endosomes. The supernatant of the bead-antibody-endosome complex was collected and saved, and the bead-antibody-endosome complex was washed three times with ice-cold phosphate-buffered saline. This complex was reconstituted in 20 μl of distilled Milli-Q water and samples were prepared for western blot analysis or electron microscopy. Samples for western blot analysis were heated in reducing buffer-containing β-mercaptoethanol at 70 °C for 10 min. Samples were placed in the Dynal magnet for 2 min before loading on the gel.

3.3.4 Electron Microscopy

Immediately after immunoisolation and reconstitution in Milli-Q water, bead-antibody-endosome complexes were embedded in agarose and then fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at room temperature. The complexes were then washed and treated with 1% osmium tetroxide for 1 h, mordanted with 0.25% uranyl acetate in acetate buffer for 30 min to overnight, washed and dehydrated with a graded series of ethanol washes and propylene oxide. Finally, the samples were embedded in epoxy resin, thin sectioned and counterstained with uranyl

acetate and lead citrate. Images were captured using an FEI Tecnai Spirit 20–120kv Transmission Electron Microscope.

3.3.5 Data Analysis

Near-infrared fluorescent signals obtained from the LiCor Odyssey scanner were expressed as raw integrated intensity with top–bottom median intralane background subtraction using Odyssey 3.0 analytical software (LiCor) (Bond et al, 2008). For homogenate protein studies, duplicate lanes of protein expression from each subject were normalized to valosin-containing protein (VCP) as an in-lane loading control. VCP was chosen because no changes have previously been detected in subjects with schizophrenia compared with control subjects (Bauer et al., 2009; Hammond et al., 2010). For immunoisolation studies, duplicate lanes of protein expression from each subject were normalized to Rab7 as an in-lane loading control.

To confirm the immunocapture of endosomes and to assess capture efficiency, 1650X direct magnification electron micrograph images of preclear, negative control, and immunoisolation samples, were printed, coded, and randomly sorted. Counts were made by an observer blind to condition. Beads or endosomes on the borders of each image were not included in the counts.

Data were analyzed using Statistica (Statsoft, Tulsa, Oklahoma). Correlation analyses were carried out to identify any associations between the dependent variables and pH, age, and postmortem interval. One-way analysis of covariance was performed if significant correlations were found. If no correlations were present, data were analyzed with one-way analysis of variance. Secondary analyses were performed using sex and

medication status (receiving antipsychotics at the time of death vs. off medication for six weeks or more at the time of death) as the independent measure.

3.4 Results

3.4.1 Late Endosome Enrichment

To analyze alterations in late endosome content in schizophrenia, we used magnetic beads bound to a late endosome-specific antibody to obtain an enriched late endosome fraction from postmortem tissue (Figure 1). To verify the enrichment of late endosomes in our immunoisolation, we used western blot analysis to measure expression of proteins not expected to be expressed in late endosomes, including those found in early endosomes (EEA1), endoplasmic reticulum (GRP78/BiP), and astrocytes (glutamine synthetase). As anticipated, we did not find any of these markers in our late endosome preparation (Figure 1 – IM, pellet). We do have evidence of minor non-specific PSD95 remnants in our late endosome enrichment that also occurs in our negative control preparation (Figure 1A – IM, pellet; - control, pellet). Additionally, as expected we have evidence of an AMPA receptor subunit (GluR1) expressed in our late endosome enriched fraction (Figure 1A – IM, pellet).

Using electron microscopy, we confirmed the isolation of intact late endosomes in our immunoisolation. Further, we measured the number of endosomes per bead in preclear, negative control, and immunoisolation samples. We found a 1.7-fold increase in the endosome to bead ratio in our immunoisolation samples relative to our preclear beads samples and a 17.3-fold increase in the endosome to bead ratio in our immunoisolation samples relative to our negative control samples.

3.4.2 Protein Expression in Late Endosomes

We examined the expression of the AMPA receptor subunits, GluR1-4, in Rab7 positive late endosomes samples. All protein expression was measured relative to Rab7 expression in the same lane. We found no significant differences in GluR1, GluR2, GluR3, or GluR4 in the enriched endosome fraction (Figure 2 and Table 3). We found no significant correlations between protein expression and age, pH, or PMI in our isolated endosome samples. In addition, we found no influence of sex or medication status in our isolated endosome studies.

We also examined the enriched fraction to determine if there were alterations in the expression of the AMPA receptor-interacting proteins, GRIP1, NSF, or SAP97. We found no significant change in the expression of GRIP1, NSF or SAP97 in our late endosome-enriched fraction (Table 3). We found no significant associations with GRIP1/Rab7, NSF/Rab7, or SAP97/Rab7 and medication status or sex.

3.4.3 Protein Expression in Tissue Homogenates

We have examined the expression of proteins involved in the sorting of AMPA receptors in endosomes as well as those involved in the fusion of endosome subtypes using VCP as a loading control (Figure 3). As previously reported (Bauer et al., 2009; Hammond et al., 2010), we found no changes in VCP (non-normalized) in schizophrenia. We found no significant correlations between protein expression and PMI, pH, or age in our samples. In addition, we found no significant effect of either sex or medication status in these samples. We found no significant difference in the expression of Arc/ARG3.1, NEEP21, Liprin α, dynamin3, GRASP1 or syntaxin13 (Figure 2 and Table 4).

Additionally, we found no significant difference in the expression of Rab7, the marker we used to isolate late endosomes (Figure 2 and Table 4).

3.5 Discussion

We have previously reported increased expression of GluR1 in isolated early endosomes in frontal cortex of schizophrenia (Hammond et al., 2010). Our interpretation of these results was that the increase in GluR1 in early endosomes reflected an increase in the forward trafficking of AMPA receptors. From the early endosome, AMPA receptors may be sorted to late endosomes. An increase in AMPA receptors in early endosomes may indicate an increase in AMPA receptors in other endosomal compartments. To extend this hypothesis, we isolated late endosomes and examined expression of the AMPA receptor subunits, GluR1-4, and AMPA receptor interacting proteins, GRIP1, NSF, and SAP97, in this fraction. We predicted that we would find an increase in AMPA receptor subunits in the late endosomes, consistent with our previous finding in early endosomes, further implicating altered endosomal handling and trafficking of AMPA receptors in schizophrenia.

Based on western blot protein characterization, our late endosome isolation is free from contaminants of other subcellular fractions with the exception of the postsynaptic density (Figure 1A – IM, pellet lane). The postsynaptic density expressed in our isolation is no greater than the expression in our negative control (Figure 1A – negative (-) control, pellet lane) and intact postsynaptic densities were not visualized by electron microscopy. While the early endosome (EEA1) expression is not as prominent as late endosome (Rab7) expression in the homogenate lane (Figure 1A – homogenate), we did not detect cross-contamination with early endosomes (EEA1) by western blot (Figure 1A – IM,

pellet lane) or morphological profile in electron microscopy. Furthermore, our isolated late endosomes have similar morphologic features to previously published reports on late endosomes in the preclinical literature (Figure 1B) (Kamsteeg et al., 2006; Kobayashi et al., 1998). Using this enriched fraction of late endosomes, we found no significant changes in the expression of any of the AMPA receptor subunits, GluR1-4, or the three AMPA receptor interacting proteins in schizophrenia. These results suggest that AMPA receptor trafficking in late endosomes is not altered in schizophrenia. Taken together with our earlier data, in two separate endosomal compartments, early and late, we have only identified increased GluR1 expression in early endosomes (Hammond et al., 2010).

We also tested the hypothesis that there are abnormalities of endosomal machinery in schizophrenia. Specifically, we focused on proteins that interact with AMPA receptors, such as dynamin3, NEEP21, and liprin α, as well as proteins that are more directly involved in endosome machinery, Arc/ARG3.1, GRASP1, and syntaxin13. Disruption of any of these proteins may alter the endosomal trafficking of AMPA receptors. Together, the necessity of these proteins in the trafficking of AMPA receptors near the synapse and the hypothesis that endosomal trafficking may be altered in schizophrenia made these six proteins ideal targets to measure. We found no significant changes in any of these endosomal trafficking proteins, suggesting that AMPA receptor-associated trafficking machinery is intact in schizophrenia.

It is conceivable that the magnitude of changes in these proteins in schizophrenia is too small to detect with our current sample size. We feel that this is unlikely given that in our total homogenate studies, we analyzed protein expression from 63 subjects, and in our endosome isolation studies we analyzed protein expression from 40 subjects. There is

also the possibility that our in-lane normalizing proteins, VCP in total homogenate and Rab7 in endosome studies, masked any differences that may be present. However, our data were also analyzed without normalization and no significant changes were found.

While there are reports detailing changes in AMPA receptor interacting protein expression in schizophrenia, there are no previous reports that have examined multiple proteins involved in a localized but complex trafficking system as presented in this report. Our lack of significant findings suggests that with the exception of our earlier GluR1 finding in early endosomes, the endosomal trafficking system of AMPA receptors is largely intact in schizophrenia. Though we did not find distal changes in AMPA receptor trafficking, there may be abnormalities in AMPA receptor trafficking proximally in the endoplasmic reticulum or Golgi apparatus, an area we are currently pursuing.

3.6 Acknowledgements

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Table 1: Subject Demographics

	Homogenate studies		Endosome studies	
	comparison	schizophrenia	comparison	schizophrenia
N	28	35	20	20
Sex	11m/17f	23m/12f	8m/12f	15m/5f
Tissue pH	6.4 ± 0.2	6.4 ± 0.3	6.4 ± 0.3	6.4 ± 0.3
PMI (hours)	7.8 ± 7.0	12.5 ± 6.6	8.5 ± 7.8	13.3 ± 6.3
Age (years)	78 ± 14	74 ± 12	78 ± 12	73 ± 11
Medication (on/off)	0/28	24/11	0/21	12/8

Values presented as mean ± standard deviation. Male (m), female (f), postmortem interval (PMI). “Off” medication indicates no antipsychotics for six weeks or more prior to death.

Table 2: Antibodies used for Western blot studies

Antibody	Species	Concentration	Blocking buffer	Incubation	Company
Dynamin3	rabbit	1:1000	Licor	16 hours	Abcam Inc., Cambridge, MA
NEEP21	mouse	1:100	Licor	16 hours	Santa Cruz Biotechnology, Santa Cruz, CA
GRASP1	rabbit	1:1000	Licor	16 hours	Abcam Inc., Cambridge, MA
Arc/Arg3.1	rabbit	1:1000	1% BSA	16 hours	Abcam Inc., Cambridge, MA
Syntaxin13	mouse	1:1000	1% BSA	16 hours	Abcam Inc., Cambridge, MA
Liprin α	rabbit	1:1000	Licor	16 hours	Abcam Inc., Cambridge, MA
GluR1	rabbit	1:1000	Licor	16 hours	Millipore, Bellarica, MA
GluR2	rabbit	1:250	1% BSA	16 hours	Abcam Inc., Cambridge, MA
GluR3	rabbit	1:1000	Licor	16 hours	Cell Signaling, Danvers, MA
GluR4	rabbit	1:500	Licor	16 hours	Millipore, Bellarica, MA
GRIP1	rabbit	1:500	Licor	16 hours	Abcam Inc., Cambridge, MA
NSF	rabbit	1:2000	Licor	16 hours	Abcam Inc., Cambridge, MA
SAP97	rabbit	1:1000	Licor	16 hours	Abcam Inc., Cambridge, MA
EEA1	rabbit	1:1000	Licor	2 hours	Abcam Inc., Cambridge, MA
PSD95	mouse	1:1000	Licor	1 hour	Millipore, Bellarica, MA
GRP78/BiP	mouse	1:250	Licor	1 hour	BD Transduction, San Jose, CA
GS	mouse	1:5000	Licor	1 hour	BD Transduction, San Jose, CA
VCP	mouse	1:5000	Licor	2 hours	Abcam Inc., Cambridge, MA

Abbreviations: Neuron Enriched Endosomal Protein 21 (NEEP21), GRIP Associated Protein1 (GRASP1), Glutamate Receptor (GluR), Glutamate Receptor Interacting Protein 1 (GRIP1), N-ethylmalemide Sensitive Factor (NSF), Synapse Associated Protein 97 (SAP97), Early Endosome Antigen 1 (EEA1), Post-synaptic Density 95 (PSD95), Glucose Regulated Protein 78/Binding Protein (GRP78/BiP), Glutamine Synthetase (GS), Valosin-containing protein (VCP).

Table 3: Statistical Analysis of Dependent Measures

Late Endosomes Isolation Studies			
Dependent Measure	F statistic	df	p value
GluR1	0.186	1, 36	0.67
GluR2	0.020	1, 38	0.89
GluR3	0.00019	1, 38	0.99
GluR4	0.508	1, 38	0.48
GRIP1	0.00010	1, 38	0.99
NSF	0.238	1, 38	0.63
SAP97	0.00011	1, 38	0.99

Abbreviations: degrees of freedom (df). Statistical values presented from ANOVA calculation.

Table 4: Statistical Analysis of Dependent Measures

Total Homogenate Studies			
Dependent Measure	F statistic	df	p value
Arc/ARG3.1	0.063	1, 61	0.80
NEEP21	0.142	1, 54	0.71
Liprin α	0.009	1, 57	0.92
dynamin3	0.210	1, 54	0.65
GRASP1	0.019	1, 61	0.89
syntaxin13	0.115	1, 52	0.74
Rab7	0.00035	1, 54	0.99

Abbreviations: degrees of freedom (df). Statistical values presented from ANOVA calculation.

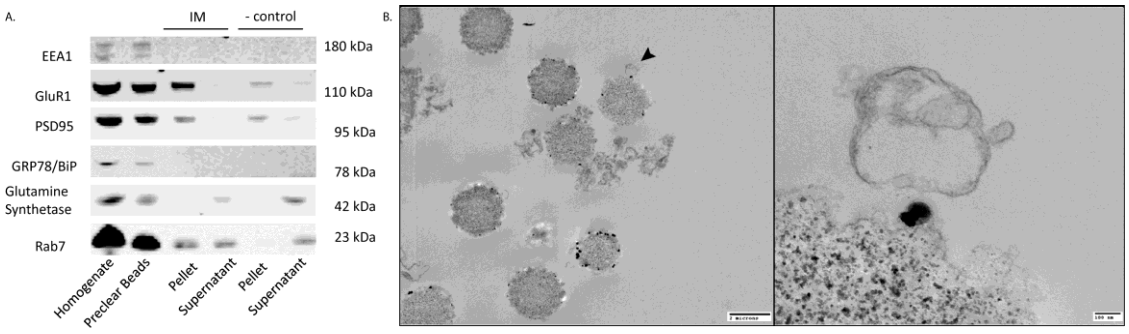

Figure 1. Characterization and visualization of late endosomes. A. Proteins not expressed in late endosomes (IM – Pellet) include those found in early endosomes (EEA1), endoplasmic reticulum (GRP78/BiP), and astrocytes (glutamine synthetase). Expression of the AMPA receptor subunit (GluR1) is present in early endosomes (IM – Pellet). Immunoisolation (IM). Negative control (-ctrl). Early Endosome Antigen 1 (EEA1). Post-synaptic density 95 (PSD95). Glucose Regulated Protein 78/Binding immunoglobulin protein (GRP78/BiP). Ionotropic Glutamate Receptor 2 (GluR2). B. Electron micrograph of late endosome immunoisolation. Late endosomes were isolated using magnetic beads and Rab7 capture antibody and imaged using electron microscopy. The panel on the right is an enlarged view of the area indicated by the arrow on the left.

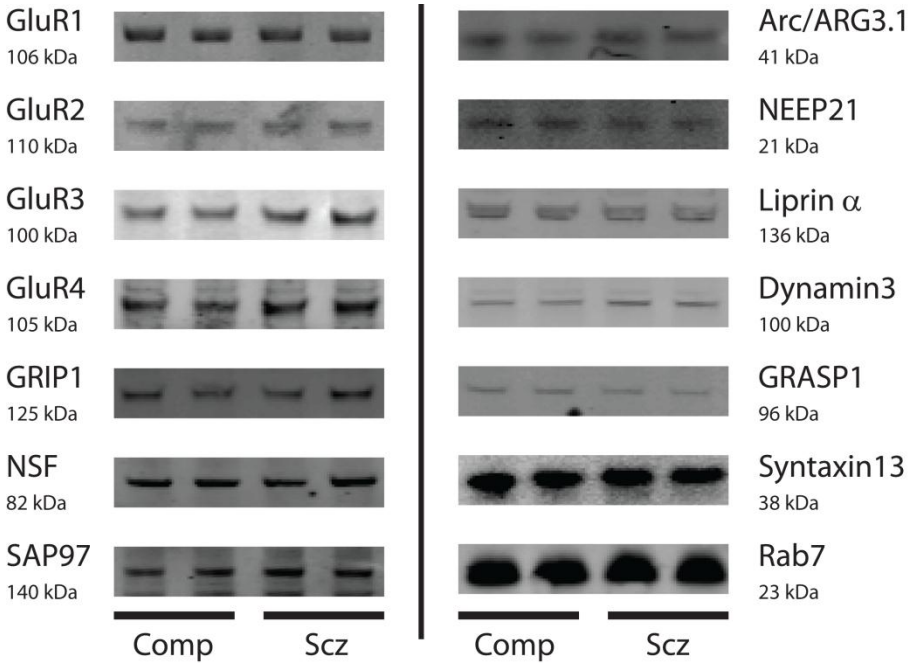

Figure 2. Representative western blots of proteins in late endosomes (left panel) and total homogenate (right panel). Expression of markers for quantified proteins is shown in duplicate. Comparison (comp). Schizophrenia (scz). Ionotropic Glutamate Receptor 1 (GluR1). Ionotropic Glutamate Receptor 2 (GluR2). Ionotropic Glutamate Receptor 3 (GluR3). Ionotropic Glutamate Receptor 4 (GluR4). N-ethylmaleimide Sensitive Factor (NSF). Synapse Associated Protein 97 (SAP97). Neuron-Enriched Endosomal Protein 21 (NEEP21). GRIP-Associate Protein 1 (GRASP1).

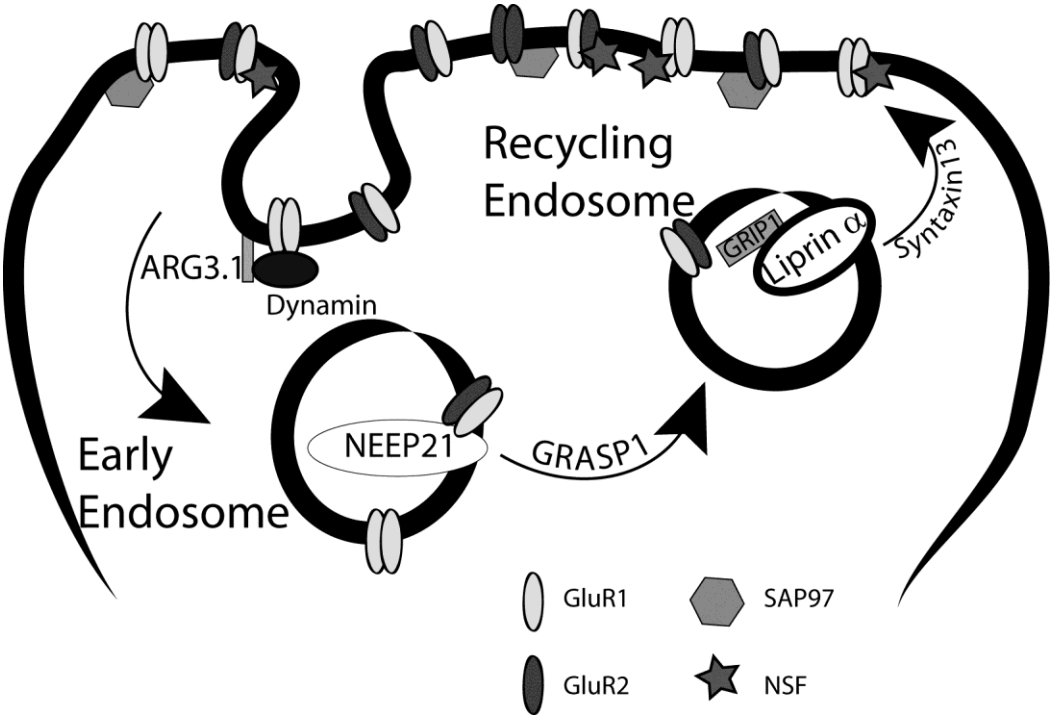

Figure 3. Representation of multiple proteins involved in the endosomal trafficking of AMPA receptors. Arc/ARG3.1 and dynamin assist in the endocytosis of AMPA receptors from the cell membrane. NEEP21 associates with AMPA receptors in early endosomes. GRASP1 is involved in the sorting of AMPA receptors from the early endosomes to recycling endosomes. Liprin α and syntaxin13 assist in the postsynaptic localization and reinsertion of AMPA receptors into the cell membrane. Ionotropic Glutamate Receptor 1 (GluR1). Ionotropic Glutamate Receptor 2 (GluR2). Neuron-Enriched Endosomal Protein 21 (NEEP21). GRIP-Associate Protein 1 (GRASP1). Glutamate Receptor Interacting Protein 1 (GRIP1). Synapse Associated Protein 97 (SAP97). N-ethylmaleimide Sensitive Factor (NSF).

AMPA Receptor Subunit Expression in the Endoplasmic Reticulum in Frontal Cortex of Elderly Patients with Schizophrenia

by

John C. Hammond, James H. Meador-Woodruff, Vahram Haroutunian, Robert E. McCullumsmith

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4.1 Abstract

Several lines of evidence indicate altered trafficking of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors in schizophrenia. Previous reports have shown potential changes in the trafficking of AMPA receptors based on subunit expression of endosomes, subcellular organelles located near the synapse. We hypothesized that alterations in AMPA receptor trafficking through the endoplasmic reticulum (ER) may also be altered in schizophrenia. Accordingly, we developed a technique to isolate and measure content of the ER from postmortem brain tissue. We used western blot and electron microscopy imaging to confirm the enrichment of the ER in our isolation. We found no changes in the expression of the AMPA receptor subunits, GluR1-4, in the ER from the dorsolateral prefrontal cortex in schizophrenia. These data suggest that AMPA receptor subcellular localization in and trafficking through the ER is largely intact in schizophrenia.

4.2 Introduction

Schizophrenia, a severe mental illness, may be linked to abnormalities of glutamate receptor neurotransmission and expression (1, 2). Recent findings support the hypothesis that alterations in glutamate neurotransmission may be part of the underlying pathophysiology of schizophrenia (3). More recent evidence has implicated abnormalities in trafficking of AMPA-type glutamate receptors in this illness (4-6). Alterations of AMPA receptor interacting proteins, as well as altered subcellular localization of AMPA receptor subunits, have been described in this illness (4, 5).

The AMPA receptor consists of four subunits, GluR1-4, that are typically assembled as dimers into a tetrameric complex in the endoplasmic reticulum (ER) (7, 8). The AMPA receptor subunit composition influences the rate of transit of AMPA receptors through the ER, with AMPA receptors containing GluR1/GluR2 subunits trafficking faster than GluR2/GluR3 containing receptors (9). Additionally, AMPA receptor interacting proteins alter the rate of trafficking of AMPA receptors through the ER and to the synapse. Synapse-associated protein 97 (SAP97) binds to the PDZ (postsynaptic density 95/Discs large/zona occludens-1) domain of GluR1 in GluR1/GluR2 hetero-oligomers for fast trafficking from the ER to the Golgi (10, 11). Protein interacting with C Kinase 1 (PICK1) binds to the C-terminal domain of GluR2 in GluR2/GluR3 containing receptors, leading to slower exit from the ER (9). Trafficking of assembled AMPA receptors from the ER to the distal dendrites occurs along the cytoskeletal spine. Glutamate Receptor Interacting Protein 1 (GRIP1) interacts with GluR2 containing receptors and kinesin motor proteins to bring assembled AMPA

receptors along the dendrites to the synapse (12-14). AMPA receptors in the distal dendrites can be surface expressed at the synapse in an activity-dependent manner (15, 16). Surface expressed AMPA receptors can be removed from and returned to the synapse in a tightly regulated endosomal system, facilitating changes in synaptic strength (17).

Previous studies have measured the expression of AMPA receptor protein and mRNA at a regional level and found no changes in schizophrenia (18-20). Recent studies have examined expression of AMPA interacting proteins and subcellular localization of AMPA receptors in schizophrenia (4, 6, 21). In postmortem human dorsolateral prefrontal cortex samples, we have previously found an increase in the AMPA receptor GluR1 subunit in early endosomes (4, 21). Additionally, SAP97 and GRIP1 were increased in schizophrenia, suggesting an increase in the rate of forward trafficking of AMPA receptors from the ER to the synapse (4).

Based on these findings, we hypothesized that accelerated proximal forward trafficking and decreased ER retention of AMPA receptor subunits may be associated with the pathophysiology of schizophrenia. To further test this hypothesis, we developed a technique to isolate ER from postmortem human brain tissue, characterized this ER fraction, and measured the expression of AMPA receptor subunits in this fraction from subjects with schizophrenia and a comparison group.

4.3 Results

4.3.1 Endoplasmic Reticulum Isolation and Characterization

We used differential sucrose gradient centrifugation to obtain an ER enriched fraction from postmortem brain tissue (Figure 1). In addition to an enriched ER fraction,

we obtained 3 other fractions. To verify and characterize the identity of each fraction, we used a wide screen of antibodies specific for various subcellular compartments (Figure 2). As expected, bands for each antibody were present in total brain homogenate. The putative ER fraction (pellet 4) contained calnexin, an ER marker, as well as GluR2, which is expected to be in the ER (22-24). In supernatant 3, we detected Grp75 (mitochondria), and EEA1 (early endosomes). In pellet 2, we detected calnexin, Grp75, PSD95 (postsynaptic density), α 1,2-mannosidase (Golgi), and GluR2. In pellet 1, we detected calnexin, histone3 (nuclei), Grp75, PSD95, α 1,2-mannosidase, and GluR2.

Enriched subcellular fractions were further characterized using electron microscopy (Figure 3). As expected, pellet 4 largely contained structures resembling ER and did not contain intact mitochondria, nuclei, or postsynaptic density-like structures. Supernatant 3 was void of mitochondria, nuclei, or postsynaptic density-like structures, but did contain endosome-like structures. Pellet 2 contained an enrichment of mitochondria and postsynaptic density-like structures. Pellet 1 contained an enrichment of nuclei as well as postsynaptic density-like structures.

4.3.2 AMPA Subunit Protein Expression in Total Homogenate

We measured the expression by western blot analysis of the AMPA receptor subunits, GluR1-4, in total homogenate samples. All protein expression was measured relative to the amount of protein loaded. We found no significant changes in GluR1, GluR2, GluR3, or GluR4 in the total homogenate. We found no significant correlations between protein expression and age, pH, or PMI. In addition, we found no influence of sex on the expression of these subunits.

4.3.3 AMPA Subunit Protein Expression in Enriched Endoplasmic Reticulum

We examined the expression of the AMPA receptor subunits, GluR1-4, in ER samples (Figure 4). All protein expression was measured relative to the corresponding protein in the total homogenate lane. We found no significant changes in GluR1, GluR2, GluR3, or GluR4 in the enriched ER fraction. We found no significant correlations between protein expression and age, pH, or PMI in our enriched ER samples. In addition, we found no influence of sex on these protein levels.

4.4 Discussion

We have previously reported increased expression in DLPFC in schizophrenia of SAP97 and GRIP1, two proteins involved in the forward trafficking of AMPA receptors from the ER to the synapse (4). We also found no changes in total AMPA receptor subunit expression but an increase in GluR1 in one distal endosome compartment (4). Taken together, these previous findings suggest alterations in the subcellular localization of AMPA receptors in schizophrenia. To extend this hypothesis, we measured the expression of the AMPA receptor subunits, GluR1-4, in an ER enriched brain fraction from human brain.

Based on western blot protein characterization and electron microscopy imaging, we have identified contents of the four fractions obtained using our differential sucrose gradient centrifugation protocol. As predicted, in our ER fraction we find proteins expected to be in the ER (calnexin and GluR2) and no markers from other comparments (histone3, GRP75, EEA1, PSD95, and α 1,2-mannosidase). The western blot and electron microscopy profile of supernatant 3 implicates that this fraction contains light membranes

and cytosol. The presence of Grp75, a stress protein typically found in the mitochondrial matrix, in this fraction may indicate damage to the mitochondria either during the postmortem interval or processing of the samples. Using western blot and electron microscopy imaging of pellet 2, we have characterized this fraction as containing heavy membranes and intact mitochondria. We have characterized pellet 1 as containing heavy membranes and intact nuclei based on western blot analysis and electron microscopy. There is evidence of contamination in these fractions with calnexin and α 1,2-mannosidase. Importantly, our fraction of interest, the ER fraction, is generally free from contamination by other subcellular organelles, including mitochondria, nuclei, and postsynaptic densities. Thus, we used this preparation to measure the contents of the ER in schizophrenia.

We found no change in the total GluR1-4 AMPA receptor subunit expression in schizophrenia, suggesting that the total expression of AMPA receptor subunits is unchanged between the groups. Using the expression of AMPA receptor subunits in total homogenate as a control, we measured the AMPA receptor subunits in the ER fraction relative to the amount in the homogenate. We found no change in the expression of GluR1-4 in the ER in schizophrenia. These results suggest that although AMPA receptor interacting proteins responsible for trafficking of AMPA receptors from the ER to the synapse are increased in schizophrenia, the expression of the AMPA receptor subunits themselves is not altered in the ER in this illness.

Similar to other studies using postmortem tissue, there are technical limitations associated with this work. It is conceivable that small changes below our level of detection may be present in schizophrenia and our negative findings are the result of a

Type II error. Further, while there was no correlation with expression of any of the subunits with age, all of the subjects included in this study were from an older population, and these findings may not be generalizable to younger patients. The effect of antipsychotic treatment was not assessed in this study because of the small number of patients off of antipsychotic medications at the time of death. Thus, we cannot exclude that this lack of an effect might be due to normalization of these subunits by antipsychotic treatment.

In summary, we found no significant changes in the expression of AMPA receptor subunits in the ER in DLPFC in schizophrenia. In our previous studies, we examined distal AMPA receptor trafficking in schizophrenia, while in the current study we examined proximal localization of AMPA receptors. With the exception of our earlier GluR1 finding in early endosomes, our findings suggest that proximal and distal subcellular localization of AMPA receptors is largely unchanged in schizophrenia. However, AMPA receptor interacting proteins responsible for the trafficking of the receptors are abnormal in schizophrenia, suggesting that AMPA receptor trafficking may be associated with the illness. Importantly, the subcellular expression of the AMPA receptors in isolated recycling endosomes and the postsynaptic density has not been examined in schizophrenia, areas that we are actively pursuing.

4.5 Materials and Methods

4.5.1 Subjects

Subjects from the Mount Sinai Medical Center brain bank were recruited prospectively and underwent extensive antemortem diagnostic and clinical assessment (Table 1). Exclusion criteria included a history of alcoholism, substance abuse, death by

suicide, or coma for >6 h before death. Consent was obtained from next of kin for each subject. Brains were collected and cut coronally into 10 mm slabs. The dorsolateral prefrontal cortex was dissected from the coronal slabs, snap frozen, and stored at −80 °C. This tissue was pulverized, adding small amounts of liquid nitrogen as necessary, and stored at −80 °C until used. Each of the eighteen (18) subjects with schizophrenia was matched for sex, and as closely as possible, for age and tissue pH with one comparison subject. Subjects groups did not significantly differ in mean age or tissue pH (Table 2). Subject groups did significantly differ in length of postmortem interval; however PMI was not correlated with expression of any of these subunits.

4.5.2 ER Isolation

Isolation of an ER enriched fraction was performed in parallel for each pair of subjects, one control and one schizophrenia subject, using nitrogen cavitation and differential sucrose gradient centrifugation (modified from (25)) (Figure 1). A starting amount of about 65 mg of pulverized tissue was used for each subject. All steps were carried out on ice or at 4 ˚C for the centrifugation steps. Pulverized tissue was reconstituted in 1.2 mL of 1X ER extraction buffer (Sigma Aldrich) and subjected to nitrogen cavitation at 450 psi for 8 minutes. A 60 μL aliquot of this was saved as total homogenate. A low speed centrifugation (700 x g) for 10 minutes was used to remove intact nuclei and large cellular debris (pellet 1). A subsequent 15000 x g centrifugation for 10 minutes of the supernatant (supernatant 1) was done to pellet intact mitochondria and other debris (pellet 2). The resulting supernatant (supernatant 2) was loaded onto a three-layered sucrose gradient (2.0 M sucrose, 1.5 M sucrose, and 1.3 M sucrose) and centrifuged at 126000 x g for 70 minutes on a Beckman L8-M ultracentrifuge in a

SW60Ti rotor (Beckman). Following this centrifugation, the upper 200 μL of supernatant was collected (supernatant 3) and 100 to 300 μL of a dense white band between the top layer and the 1.3 M sucrose layer was collected (pellet 3). Pellet 3 was gently mixed by inversion with ice cold MTE buffer (premixed with 200 mM PMSF buffer). This mixture was centrifuged at 126000 x g for 45 minutes resulting in a large, translucent pellet (pellet 4).

For each subject, pellets 1 and 2 were each reconstituted in 500 μL of PBS and pellet 4 was reconstituted in 50 μL of PBS. All samples were stored at -20 C until ready for use.

4.5.3 Electron Microscopy

To visualize the content and confirm morphology of each fraction, samples were prepared for electron microscopy as previously described (4). Briefly, fractions were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at room temperature. Samples were then washed and treated with 1% osmium tetroxide for 1 h, mordanted with 0.25% uranyl acetate in acetate buffer for 30 min to overnight, washed and dehydrated with a graded series of ethanol washes and propylene oxide. Finally, the samples were embedded in epoxy resin, thin sectioned and counterstained with uranyl acetate and lead citrate. Images were captured using an FEI Tecnai Spirit 20-120kv Transmission Electron Microscope.

4.5.4 Western Blot Analysis

Prior to Western blot analysis, the amount of protein in each fraction was measured using a BCA protein assay kit (Thermo scientific). For each pair of subjects,

samples for western blot were prepared in duplicate with 5-10 μg of protein for each fraction placed in reducing buffer containing β-mercaptoethanol and heated at 70 ˚C for 10 minutes. Protein loaded was consistent for each fraction within subject pairs, but not necessarily between subject pairs. Samples were then run by SDS-PAGE on Invitrogen (Carlsbad, California) 4-12% gradient gels, and transferred to PVDF membrane using Bio-Rad semi-dry transblotter (Hercules, California). Membranes were blocked in LiCor (Lincoln, Nebraska) blocking buffer for 1 h at room temperature before overnight incubation at 4 ˚C with commercially available antibodies in 0.1% Tween LiCor blocking buffer with antisera dilutions determined empirically (Table 3). Following incubation, membranes were washed four times for 5 minutes each with PBS. Membranes were probed with IR-dye labeled secondary antibody in 0.1% Tween, 0.01% SDS LiCor blocking buffer for 1 h at room temperature in the dark. Membranes were washed again with PBS four times for 5 minutes each and then briefly rinsed three times in distilled water. The blots were stored in distilled water at 4 ˚C until scanned using the LiCor Odyssey laser-based image detection method (26, 27).

4.5.5 Data Analysis

Image analysis from ER isolation Western blot studies was performed as previously described (4, 27). Briefly, near-infrared fluorescent signals obtained from the LiCor Odyssey scanner were expressed as raw integrated intensity with top-bottom median intralane background subtraction using Odyssey 3.0 analytical software (LiCor). Protein band intensity for each protein in the ER isolation was normalized to the protein band intensity for the same protein in the total homogenate lane. All statistical analyses were performed using Statistica 7.1 (Statsoft, Tulsa, Oklahoma). Correlation analyses

were carried out to identify any associations between the dependent variables and pH, age, and postmortem interval. One-way analysis of covariance (ANCOVA) was performed if significant correlations were found. If no correlations were present, data were analyzed with one-way analysis of variance (ANOVA). An additional secondary analysis was performed using sex as the independent measure.

4.6 Acknowledgements

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Table 1: Paired Subject Demographics

Pair	Subject	Sex/Age, y	pH	PMI, h
1	Comparison	F/63	6.20	20.2
	Schizophrenia	F/62	6.74	23.7
2	Comparison	F/73	6.98	3
	Schizophrenia	F/71	6.60	5.5
3	Comparison	F/74	6.32	4.7
	Schizophrenia	F/75	6.49	21.5
4	Comparison	F/76	6.46	4.2
	Schizophrenia	F/77	6.01	9.7
5	Comparison	F/79	6.38	10.1
	Schizophrenia	F/79	6.80	9.9
6	Comparison	F/81	6.37	19.4
	Schizophrenia	F/81	6.67	15.1
7	Comparison	F/85	6.30	4.3
	Schizophrenia	F/84	6.64	
8	Comparison	F/96	6.30	4.5
	Schizophrenia	F/89	6.20	9.6
9	Comparison	M/59	6.67	20.4
	Schizophrenia	M/57	6.40	20.6
10	Comparison	M/65	6.82	3.8
	Schizophrenia	M/66	6.50	12.1
11	Comparison	M/69	6.67	7.4
	Schizophrenia	M/70	6.35	7.1
12	Comparison	M/73	6.17	14.9
	Schizophrenia	M/73	7.30	11.7
13	Comparison	M/76	6.32	2.9
	Schizophrenia	M/76	6.70	16.5
14	Comparison	M/84	6.83	11.4
	Schizophrenia	M/84	6.71	17.7
15	Comparison	M/93	6.28	4.1
	Schizophrenia	M/92	6.67	17.7
16	Comparison	M/68	6.55	2.7
	Schizophrenia	M/70	6.36	17.3
17	Comparison	M/75	6.43	5.0
	Schizophrenia	M/78	6.64	26.1
18	Comparison	M/95	6.53	4.1
	Schizophrenia	M/97	6.50	9.2

Abbreviations: Years (y), Postmortem Interval (PMI). Hours (h). Female (F). Male (M).

Table 2: Pooled Subject Demographics

Parameter	Comparison Subjects	Subjects with Schizophrenia
Sex, M/F	10/8	10/8
Age, years	76.8 ± 10.6	76.7 ± 10.3
pH	6.52 ± 0.23	6.51 ± 0.21
PMI, hours	8.2 ± 6.3	14.8 ± 6.8

Abbreviations: Male (M). Female (F). Postmortem Interval (PMI). Data are presented as mean ± SD.

Table 3: Antibodies used for western blots

Antibody	Species	Concentration	Company
Calnexin	Rabbit	1:5000	CalBiochem, Gibbstown, NJ
Histone3	Rabbit	1:1000	Cell Signaling, Danvers, MA
Grp75	Rabbit	1:5000	Abcam Inc., Cambridge, MA
EEA1	Mouse	1:500	BD Bioscience, San Jose, CA
PSD95	Mouse	1:1000	Millipore, Bellarica, MA
α 1,2-Mannosidase	Rabbit	1:1000	Abcam Inc., Cambridge, MA
GluR1	Rabbit	1:200	US Biological, Swampscott, MA
GluR2	Mouse	1:1000	US Biological, Swampscott, MA
GluR3	Rabbit	1:200	US Biological, Swampscott, MA
GluR4	Goat	1:100	Santa Cruz Biotechnology, Santa Cruz, CA

Abbreviations: Glucose Regulated 75 kDa Protein (GRP75). Early Endosome Antigen 1 (EEA1). Postsynaptic Density 95 (PSD95). Ionotropic Glutamate Receptor 1 (GluR1). Ionotropic Glutamate Receptor 2 (GluR2). Ionotropic Glutamate Receptor 3 (GluR3). Ionotropic Glutamate Receptor 4 (GluR4).

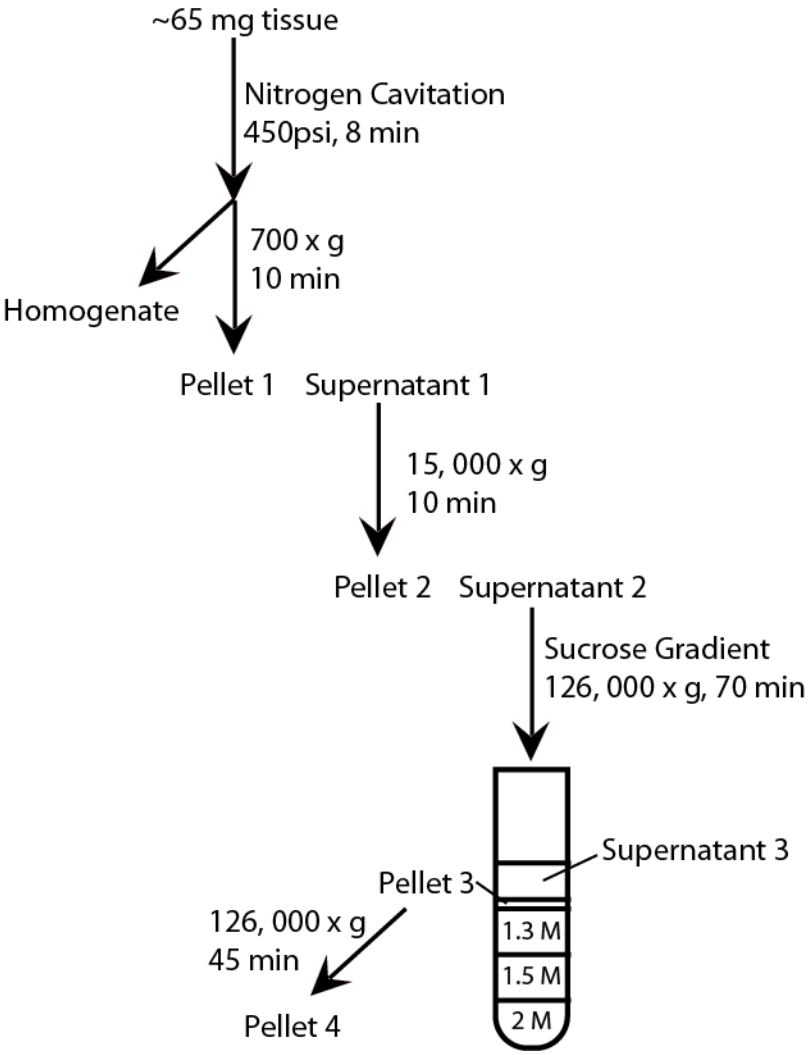

Figure 1. Flow chart of endoplasmic reticulum isolation. Nitrogen cavitation of tissue was followed by a series of centrifugation steps and sucrose density gradient centrifugation.

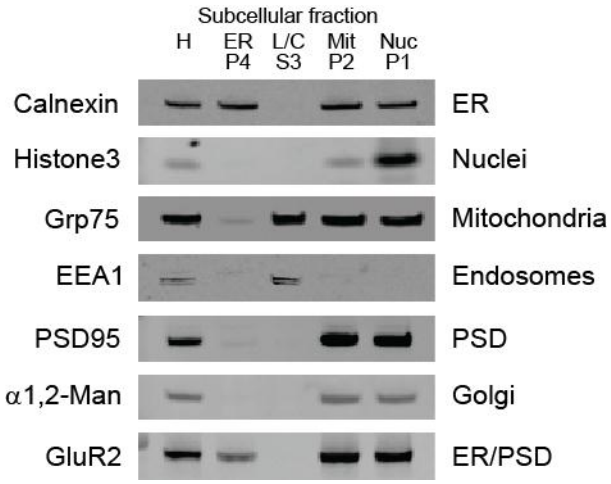

Figure 2. Neurochemical characterization of subcellular fractions isolated from postmortem human frontal cortex. Fractions enriched for endoplasmic reticulum (P4), cytosol and 'light' membranes (S3), mitochondria (P2), and nuclei (P1) were assayed by Western blot for compartment-specific markers: Calnexin (ER), histone3 (Nuc), glucose regulated 75 kDa protein (Grp75, Mito), early endosome antigen 1 (EEA1, endosomes), postsynaptic density 95 (PSD95, PSD), and □1,2 mannosidase (□1,2-Man, Golgi). The PSD segregated with the other 'heavy' membranes in the Mit and Nuc fractions. As expected, AMPA receptor subunit 2 (GluR2) was detected in the ER and fractions containing the PSD.

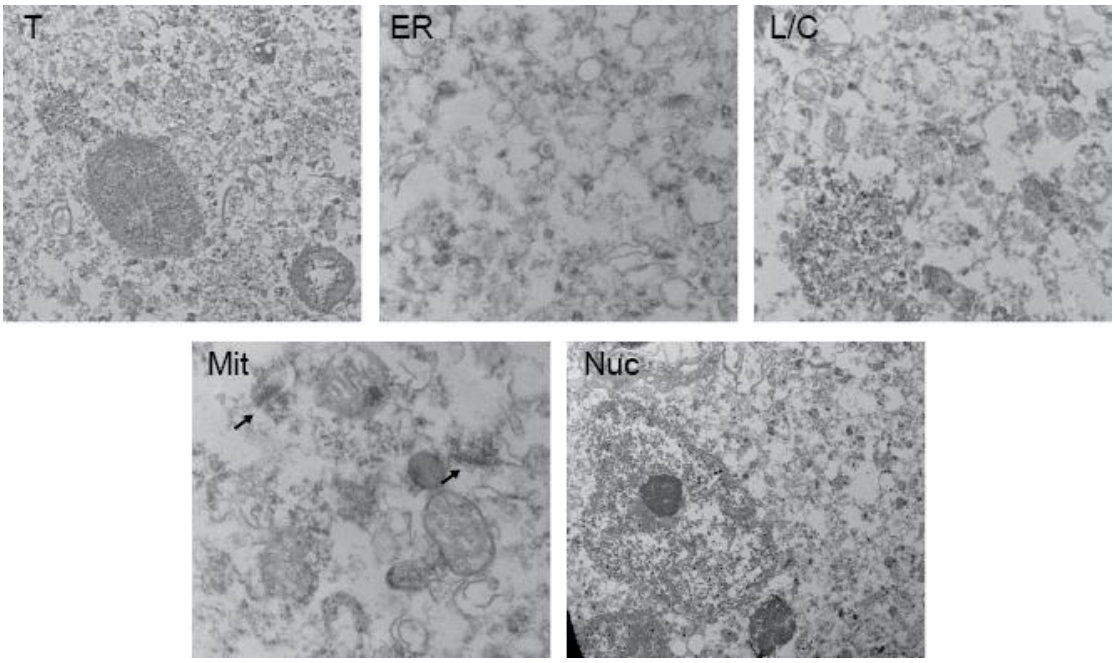

Figure 3. Assessment of enriched subcellular fractions isolated from postmortem human frontal cortex by electron microscopy. 100 mg of total unfractionated sample (T, 1650x magnification), endoplasmic reticulum (P4, 11,000x), light membranes and cytosol (S3, 4,400x), mitochondria (P2, 11,000x), and nuclei (P1, 1650x) were processed for electron microscopy. The ER and L/C fractions do not have intact Mit, Nuc, or postsynaptic density (PSD)-like structures. The Mit and Nuc fractions have PSD-like structures (black arrows).

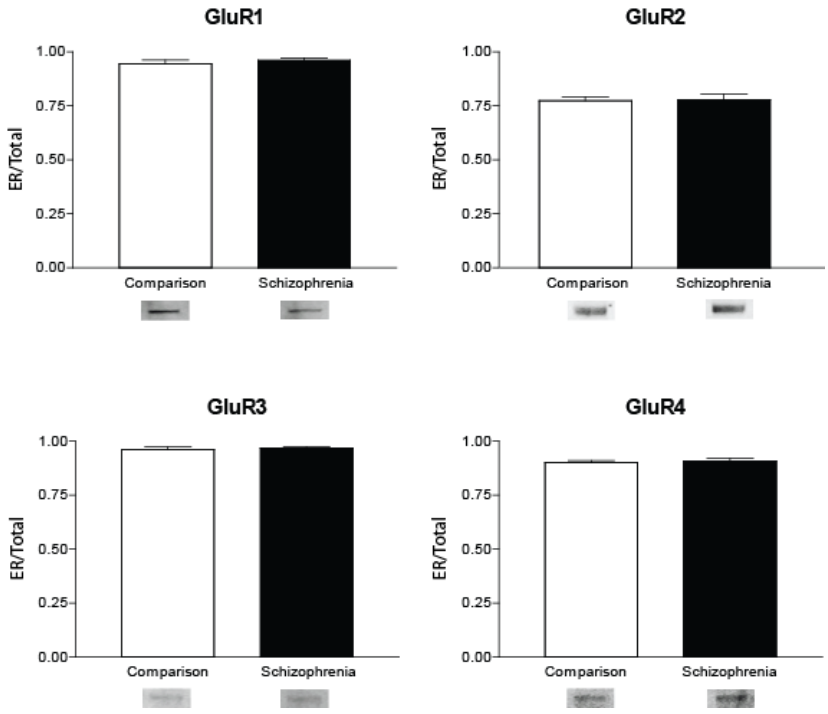

Figure 4. Western blot analysis of AMPA Receptor subunit expression in endoplasmic reticulum (A – GluR1, B – GluR2, C – GluR3, D – GluR4) normalized to relative expression in total homogenate. Representative blots of ER fraction. Error bars represent standard deviation.

CHAPTER FIVE

DISCUSSION

5.1 Summary of Findings

The objective of this dissertation was to examine the trafficking and subcellular localization of AMPA-type glutamate receptors in schizophrenia. AMPA receptors are assembled in the endoplasmic reticulum, trafficked along the dendritic shaft, expressed in the plasma membrane, and endocytosed, recycled or degraded in a complex series of endosomes. We attempted to determine if there were alterations in the subcellular localization and trafficking of these AMPA receptor subunits in schizophrenia. First, we measured total protein expression of AMPA receptor interacting proteins in the dorsolateral prefrontal cortex (DLPFC). We found increased expression of two proteins associated with the forward trafficking and synaptic localization of AMPA receptors, GRIP1 and SAP97, but no change in NSF expression. We also isolated and verified isolation of intact early endosomes from postmortem tissue using a modified immunoisolation protocol. When normalized to EEA1, the protein used to isolate the early endosomes, we detected no change in GluR2 or GluR3 protein expression, but increased expression of the AMPA receptor subunit GluR1 in early endosomes. Further analysis of these data revealed a significant increase in GluR1 protein expression in early

endosomes in patients with schizophrenia off medication for 6 weeks or more prior to the time of death. Next, we isolated intact late endosomes using Rab7 as our capture antibody. Following verification of late endosome isolation, we found no change in the expression of GluR1-4, GRIP1, NSF, or SAP97 proteins in our isolated late endosomes. We measured total protein expression using tissue homogenate for several proteins involved in the sorting and handling of AMPA receptors in endosomes. We found no significant changes in the protein expression of Arc/ARG3.1, NEEP21, Liprin α, Dynamin3, GRASP1, or Syntaxin13. Additionally, we found no significant differences in proteins specific to early (EEA1), late (Rab7), or recycling (Rab11) endosomes. Finally, we used a modified ultracentrifugation and sucrose density gradient protocol to isolate endoplasmic reticulum (ER) from postmortem human tissue. After verifying the specificity of our isolated ER, we measured protein expression of GluR1-4. When normalized to the respective expression of GluR1-4 protein in total homogenate, we found no significant changes in GluR1-4 protein expression in our isolated ER fraction.

5.2 Schizophrenia as a Disorder of AMPA Receptor Trafficking

Initially, the glutamate hypothesis of schizophrenia suggested hypofunction of NMDA-type glutamate receptors in schizophrenia (14). Based on preclinical and glutamate receptor literature, the hypothesis has been expanded to include the AMPA-type glutamate receptors. We measured proteins involved in the trafficking and stabilization of AMPA receptors in schizophrenia. Additionally, we isolated subcellular fractions from postmortem tissue and measured AMPA receptor subunit protein expression in the fractions as a proxy for trafficking of the receptors. We found an increase in SAP97 and PICK1 protein expression, two proteins involved in the forward

trafficking and stabilization of AMPA receptors. In our isolated early endosome fraction, we found an increase in GluR1 protein expression. We did not find any significant differences in total protein expression of GluR1. Taken together, these data indicate an alteration of forward trafficking and subcellular localization of the GluR1 subunit of AMPA receptors in schizophrenia.

We found a positive correlation between SAP97 and GluR1 protein expression in schizophrenia. We speculate that the increase in SAP97 may be in compensation for increased levels of GluR1 on the cell surface as SAP97 is important in anchoring GluR1 at the synapse (101, 102). Recent evidence suggests that increased synaptic glutamate might contribute to the pathophysiology of schizophrenia (185). We suspect the increase in GluR1 in early endosomes may be the result of an increased internalization of excess receptor complexes, in a manner similar to NMDA-induced long-term depression (186). Preclinical studies have reported that elevated synaptic glutamate levels may lead to a selective increase in the internalization of GluR1-containing AMPA receptors (187). This explanation of altered trafficking molecules and increased GluR1 protein expression in early endosomes is consistent with the hypothesis of increased synaptic glutamate in schizophrenia (188, 189).

It is well established that blockade of NMDA receptors using certain compounds, such as PCP or ketamine, may lead to a schizophrenia-like phenotype or an exacerbation of symptoms (25, 26, 28). In more recent studies, the effect of these NMDA receptor blockers on the presynaptic release of glutamate has been examined (188). Models of schizophrenia that use NMDA blockade may be blocking NMDA receptors leading to an increase in glutamate in the synapse in a feedback dependent mechanism. An increase in

glutamate at the synapse may cause an internalization of AMPA-type glutamate receptors when the cell attempts to maintain homeostasis.

An alternative explanation of increased GluR1 in early endosomes may be that there is a dysfunction in the stabilization of GluR1-containing AMPA receptors at the synapse. Diminished expression of GluR1 containing AMPA receptors at the synapse in preclinical models leads to a decrease in cognitive function and an increase in behavioral phenotypes associated with schizophrenia (190-192). If GluR1 containing AMPA receptors are not stabilized at the synapse, they may become trapped in early endosomes. Regardless of the cause, we found an increase in GluR1 protein expression in isolated early endosomes suggesting abnormal subcellular localization of these receptors in schizophrenia.

However, when these results are taken in context with our other results, the support for altered AMPA receptor trafficking and subcellular localization in schizophrenia as a prominent feature of the underlying pathophysiology of the illness is diminished. GluR1 exists as part of a heteromeric AMPA receptor complex, yet we did not find an increase in other AMPA receptor subunits in early endosomes in schizophrenia. Isolation of intact late endosomes and subsequent measurement of AMPA receptor subunit protein in the late endosomes revealed no alterations in schizophrenia. We found no significant difference in AMPA receptor protein expression following isolation of the endoplasmic reticulum. With the exception of increased SAP97 and GRIP1 protein expression in total homogenate, we found no significant changes in the expression of 10 other AMPA receptor interacting proteins or endosomal related proteins in schizophrenia.

Attempts have been made to pharmacologically augment signaling through the AMPA-type glutamate receptors using AMPAkines (193). These compounds have been used in patients with schizophrenia and large scale clinical trials (194-196). However, Cortex pharmaceutical compound CX516 was used in a clinical trial and was found to be no more effective than placebo in treating cognitive symptoms of patients with schizophrenia (197). While AMPAkines and AMPA receptor signal augmentation were promising targets for treatment in schizophrenia, the lack of improvement in symptoms suggest that alterations in schizophrenia may not be due to changes in AMPA receptor trafficking.

We hypothesized that we would find alterations in the subcellular localization of AMPA receptors in schizophrenia. AMPA receptors and NMDA receptors are closely linked in glutamatergic signaling and alterations in trafficking of one glutamate receptor subtype may implicate changes in other glutamate receptor subtypes. Previous studies in our lab have examined NMDA receptor trafficking and found alterations in schizophrenia. It has been proposed that increased clearance of NMDA receptors from the endoplasmic reticulum may be a compensatory mechanism for compromised forward trafficking (198). An alternatively spliced variant of NR1, NR1C2', which confers accelerated trafficking of the NMDA receptor through the ER was increased in the ACC in schizophrenia (199). NR2B protein was found to be decreased in an isolated ER fraction in schizophrenia, further implicating altered NMDA receptor trafficking in the underlying pathophysiology of schizophrenia (200). Additionally, interacting proteins involved with the forward trafficking of NMDA receptors are altered in the ACC and DLPFC in schizophrenia (199).

Alterations in AMPA receptor trafficking have also been studied in schizophrenia. A previous study has suggested that GluR2 may be alternatively edited in schizophrenia. The ratio of GluR2 flip/flop variants revealed an increase in the flip variant in schizophrenia (150). The flip variant does not contain an ER retention sequence found in the flop variant, indicating the GluR2 subunit may be processed too quickly in the ER in schizophrenia. Additionally, in situ hybridization studies revealed a decrease in GluR2 and GluR4 subunit expression but protein binding studies using the same cohort found no changes in AMPA receptor subunit expression (162). Taken together, these studies implicate a problem with intracellular processing and trafficking of AMPA-type and NMDA-type glutamate receptors in schizophrenia.

However, when examined alongside the mixed findings of previous studies of AMPA-type glutamate receptors in schizophrenia, it is not surprising that many of our results are negative. Previous studies have measured AMPA receptor subunit expression via receptor binding, in situ hybridization, qPCR, microarray analysis, and western blot in the DLPFC (146, 148, 154, 156, 157, 162, 165). While some studies reported no change in AMPA receptor subunit expression in schizophrenia (157), others reported decreased subunit expression (201) while others still reported an increase in subunit expression (147).

5.3 Limitations of These Studies

As with all scientific studies, certain limitations must be considered when interpreting any findings, negative or otherwise. These limitations range from tissue

condition to methodological concerns to data collection and analysis. While performing our studies, we attempted to anticipate and control for these limitations.

5.3.1 Limitations of Subject Demographics

The subjects used for these studies were obtained from the Mt. Sinai brain bank. Extensive antemortem diagnostic and clinical data were collected for each subject including age, sex, postmortem interval, and tissue pH. Subjects used in these studies represent an elderly population relative to cohorts in other studies. Schizophrenia is a lifelong illness with positive and negative symptoms that may become more pronounced as a patient ages (9). Changes found in the population used in these studies may not reflect changes that might be found in studies with a cohort of younger patents (156).

In some respects, it is advantageous that we studied an elderly population. Patients with schizophrenia have high comorbidities with drug use and suicide (202). Survival of patients with schizophrenia to an older age may mean that these patients were well cared for, did not abuse alcohol or drugs, and likely died of natural causes and thus do not have the same confounds as a younger cohort (6).

5.3.2 Limitations of Postmortem Tissue

In these studies, we used postmortem tissue to measure AMPA receptor interacting proteins and AMPA receptor protein trafficking and subcellular localization. The use of postmortem tissue to study psychiatric illness carries advantages and disadvantages over other methods (168). While cell culture and animal models are helpful to study particular aspects of psychiatric illness, postmortem human tissue does not rely on assumed mechanisms and pathophysiology. For this reason, postmortem

tissue is extremely valuable in terms of selective use for experiments. However, use of postmortem tissue does have disadvantages. For instance, the postmortem interval (PMI), the length of time between patient death and collection of the sample, must be considered as a factor that may influence the data. During the PMI, changes may be occurring that also affect the expression and interactions of various proteins. In order to control for any changes, we checked for correlations between our proteins of interest and the PMI and found no correlations between PMI and out dependent variables.

5.3.3 Limitations of Subcellular Fractionation

Since postmortem human tissue represents a single time point, we are unable to directly assess trafficking in this tissue. In these studies, we used protein expression in various subcellular fractions as a proxy for measuring trafficking of these proteins. For a more robust design, we chose to analyze one proximal (ER) and two distal (early and late endosomes) fractions. In order to isolate these fractions, we used homogenized postmortem tissue. While the homogenization process is relatively mild and done without detergents that may disrupt the plasma membrane, it may be the case that some subcellular integrity was lost. This loss of integrity is evidenced by the presence of certain markers, such as calnexin, in nearly all of our various fractions. In these studies, based on western blot analyses and electron microscopy, we assumed that endosome and ER integrity remained intact during the PMI. The may still be some cross-contamination between subcellular compartments.

5.3.4 Limitations of the Endosome Immunoisolation

Endosomes are dynamic vesicles that are involved in the endocytosis of AMPA receptors from the synapse and trafficking of the receptors back to the synapse or to late endosomes for degradation. Each endosome subtype carries a distinct protein marker, early (EEA1), late (rab7), and recycling (Rab11). However, the endosome subtypes represent distinct overlapping compartments in order to traffic proteins between the endosome subtypes (91). Following isolation of early and late endosomes, we confirmed the specificity of our isolation using western blot and electron microscopy. Our captured endosomes contained markers specific for the target endosome, but no other endosomes. With the dynamic nature of endosomes, it is expected that there would be some overlap. It may be that our immunisolation protocol was only effective at capturing healthy, intact endosomes of the particular targeted isolation. Based on electron microscopy images, it is unlikely we captured partial endosomes in our preparation. Based on the specificity of the western blot profiles, it is unlikely that we captured endosomes in a transition period when endosome subtypes may be joined for protein trafficking. Thus, it is possible that we may be missing changes in AMPA receptor subcellular localization by examining only healthy, intact endosomes.

Another technical limitation of our endosome immunoisolation involves cross-reactivity of antibodies for western blot studies. Since our technique requires the use of an antibody specific for the isolation of endosomes, we must contend with immunoglobulin from the capture antibody present in our isolation. In many cases, we were able to avoid cross-reactivity by using different species of capture and western blot antibodies, but it is an important limitation that must be considered in these studies.

5.3.5 Limitations of the Endoplasmic Reticulum Enrichment

The modification of an established ER isolation protocol was integral in our ability to obtain isolated ER from postmortem tissue (203). As with the endosome fraction isolations, we confirmed the specificity of the ER isolation using western blot and electron microscopy. There is little to no evidence of PSD, mitochondria, or nuclear protein contamination in our ER fraction indicating that we are measuring AMPA receptor subunit expression in a pure ER fraction. However, we failed to see any enrichment of ER related proteins, such as calnexin, in our ER fraction relative to other fractions indicating that we may be losing some of the ER protein in other fractions. It may be that any changes in AMPA receptor protein expression are lost in the ER in the other fractions.

5.3.6 Limitations of Protein Quantification

In these studies, we used the Licor Odyssey image based gel analysis system that uses near-infrared technology to quantify our protein bands of interest. While the use of the Licor Odyssey for western blot protein studies has been well-established, traditional methods of protein quantification use ECL and film transfer (204-206). We have found that the Licor method allows for greater detection of low level protein expression. It may be that use of the Licor is allowing us to measure proteins that we would fail to quantify using a more traditional method. If this were the case, our lack of changes may be related to the sensitivity of the western blot quantification method.

5.4 Antipsychotic Effects

The population used in these studies was from an elderly cohort of patients that were institutionalized. These patients were likely well cared for and received antipsychotic medications in order to help manage their symptoms. Considering the young age of onset of schizophrenia and the age at time of death for these patients, some of these patients may have been receiving medication for several decades. It must be considered that any changes or lack of changes found in postmortem tissue from this population may be due in part or whole to the effects of antipsychotic medication.

In the isolated early endosomes, we found an increase in GluR1 protein expression. Further analysis of this finding, taking medication status into account, revealed that this increase in GluR1 expression was significant in patients who were off medication for 6 weeks or more prior to death. GluR1 expression in early endosomes in patients receiving medication at time of death was not significantly different than expression in the comparison group. It may be the case that antipsychotic medications were altering the subcellular localization of GluR1 receptors and masking a primary increase of GluR1 protein expression in early endosomes in some manner. Alternatively, the increase in GluR1 in early endosomes in patients off antipsychotic medication may have been a compensatory effect for some other facet of the underlying pathophysiology of the illness.

In these studies, we considered patients to be off medication if they were not taking antipsychotics for 6 weeks or more prior to death. We did not have any medication naïve patients in these studies. As stated above, these patients may have received

medication for decades before stopping treatment. It is unknown whether the stoppage of medication for 6 weeks would reverse decades of neuroplastic changes brought on by the medication.

Interpretation of our findings must be done with the understanding that antipsychotics may alter our dependent measures. In most of our studies, we examined the effect of medication status on our dependent measures. However, in our ER isolation studies, our limited number of subjects off medication did not provide the statistical power to make definitive statements regarding medication effects. We do know, however, that chronic administration of antipsychotic medication can affect mRNA expression of the AMPA receptor subunits in the DLPFC of rhesus monkeys (156).

5.5 Future Directions

Current work presented here has provided many testable hypotheses. These future directions are an attempt to address questions that have arisen during this work. First, we propose expansion of our current studies. Then, we propose more subcellular fractionation studies to further describe the subcellular localization of the AMPA receptors. Next, we describe experiments examining posttranslational modifications of the AMPA receptors. Fourth, we propose an alternative immunoisolation study that may further elucidate our findings here. And finally, we describe animal studies that may be useful for understanding the underlying pathophysiology of schizophrenia.

5.5.1 Expansion of Current Studies

The studies we have done so far have focused on the protein expression of AMPA-type glutamate receptors in the ER and early and late endosomes in the DLPFC.

The glutamate hypothesis of schizophrenia has been expanded to include other glutamate receptors including AMPA, kainite, and metabotropic glutamate receptors, but not to the exclusion of NMDA receptors. There is evidence of altered expression of NMDA receptor subunits in an isolated ER fraction in the same cohort of patients used in our studies (200). We propose that we could measure other glutamate receptors in our subcellular fractions. Subcellular fractionation of postmortem tissue can be used as a proxy for trafficking of receptors which is important since these receptors are dynamic rather than static in the living system.

We found increased expression of SAP97 and GRIP1 proteins in total homogenate, but no changes in expression in early or late endosomes. These proteins are also essential for the forward trafficking of AMPA receptors from the ER to the Golgi. It would be informative to measure expression of these and other AMPA interacting proteins in our ER fraction.

Schizophrenia is a complicated illness that affects multiple brain regions. In our current studies we focused on the DLPFC. The anterior cingulate cortex (ACC) is involved in selective attention and working memory, both of which may be altered in schizophrenia (16, 173, 207, 208). Structural abnormalities of the ACC in schizophrenia have been described as well as alterations in blood flow following ketamine administration (28, 208). Other studies have examined changes of glutamate receptor mRNA and protein expression in the ACC (199, 204). In our unpublished observations, we did not find significant changes in AMPA receptor subunit expression in early endosomes in the ACC. However, findings in the DLPFC are not always consistent with findings in the ACC (199, 204, 209). We propose that our current studies could be

expanded to include the ACC as well as the DLPFC to examine possible brain region specific changes in schizophrenia.

5.5.2 Isolation of Recycling Endosomes

In our current studies, we isolated early and late endosomes. Proper AMPA receptor trafficking in endosomes is critical for glutamatergic neurotransmission (91). AMPA receptors are sorted from early endosomes to late or recycling endosomes. Though our current findings suggest AMPA receptor trafficking in endosomes and the endosomal trafficking system in general is intact in schizophrenia, isolation of recycling endosomes and subsequent measurement of AMPA receptor protein expression would provide a crucial piece in understanding the trafficking of the receptors.

It is possible that with the correct antibody, immunocapture and isolation of intact recycling endosomes from postmortem tissue should be possible. Rab11 is a protein specifically expressed in recycling endosomes that deliver AMPA receptors to the synapse (111). It would be necessary to verify the immunoisolation of intact recycling endosomes using western blot and electron microscopy in a manner similar to the verification of early and late endosome immunoisolations. Using the isolated recycling endosomes, we would be able to measure expression of AMPA receptor subunits in this fraction.

5.5.3 Isolation of Postsynaptic Density

Glutamatergic signaling may be altered in schizophrenia (14). Neurotransmission through glutamate receptors requires synaptic localization and expression of glutamate receptors, including AMPA and NMDA receptors. While we have measured AMPA

receptor subcellular localization in a proximal (ER) and two distal (early and late endosomes) fractions, we have not measured expression of AMPA receptors in the postsynaptic density. We have chosen in our current experiments to perform subcellular fractionation because regional measurement of AMPA receptor proteins cannot differentiate between AMPA receptors that are actively participating in neurotransmission, those at the synapse, and AMPA receptors that are being trafficked in the cell anywhere from the ER to the synapse. Isolation of the PSD would allow for the measurement of functional AMPA receptors expressed in an area of active signaling.

While we have used immunoisolation and centrifugation to isolate subcellular fractions in these studies, isolation of the PSD has proven difficult. In our initial early endosome isolation experiment, we noted that in order to obtain a clean fraction of early endosomes, a step to preclear nonspecific binding of the tissue to the beads was necessary. Using the protocol as we describe it for isolating early endosomes leads to most of the PSD in the homogenate, as evidenced by PSD95 western blot, binding nonspecifically to the beads. There is also nonspecific binding of other subcellular compartments so this preclear step is not a viable method to obtain PSD free from other fractions.

Similarly, we obtain a fraction that has a large amount of PSD using our centrifugation to isolate the ER. Again, by western blot and electron microscopy, this is not a clean fraction of PSD but has contamination of other subcellular organelles. In this case, this is to be expected as the protocol was optimized for obtaining the ER and not the PSD. Work to develop a protocol to obtain a PSD fraction free from contamination would allow for the measurement of AMPA receptors at the synapse and provide more data in

support for or against the hypothesis of alterations in AMPA receptor subcellular localization in schizophrenia.

5.5.4 Measurement of Posttranslational Modifications

5.5.4a Glycosylation Studies Posttranslational modifications of AMPA receptors are a critical part of AMPA receptor trafficking (45). Glycosylation is the process of adding and modifying sugar moieties to proteins as they are processed in the ER and Golgi. Complex glycosylation of AMPA receptor subunits has been associated with trafficking of AMPA receptors to the cell surface (210). Importantly, there are glyosylation sites in the ligand-binding domain of AMPA receptors (44). Studies have demonstrated that N-linked glycosylation of ionotropic glutamate receptors plays a role in the affinity of the AMPA binding site of the receptors (211). EndoH is an enzyme that can be utilized to cleave sugar residues from proteins. Following this cleave, a shift in the band size for a particular protein can be observed via western blot (45, 209). Sugar residues become insensitive to the EndoH enzyme once they have left the ER and are being processed in the Golgi.

Unpublished observations in our lab have found an increase in the ratio of EndoH insensitive to EndoH sensitive GluR2 protein in the DLPFC in schizophrenia. These data suggest that GluR2 is trafficked faster through the ER in schizophrenia. We did not find a decrease in GluR2 protein expression in our ER isolation studies. Though there was no evidence of α 1,2-mannosidase in our isolation, we cannot rule out the possibility that our fraction contained some Golgi as well. With some modification to our ER isolation protocol, we should be able to use an EndoH enzyme to deglycosylate the AMPA

receptor subunits in our fraction. If AMPA receptor forward trafficking is altered in schizophrenia, we would expect to see a decrease in the amount of EndoH sensitive AMPA receptor proteins in our ER fraction.

5.5.4b Phosphorylation Studies Protein phosphorylation is critical for protein interaction and regulation. In particular, phosphorylation of AMPA receptors and interacting protein is essential for AMPA receptor localization and function (212, 213). In our unpublished observations above, we discussed a lack of change in the expression of phosphorylated AMPA receptors at various sites (pGluR1S818, pGluR1S845, pGluR2S880). These sites were chosen because of their importance in AMPA receptor trafficking and stabilization as well as availability of antibodies. We should be able to develop phosphorylation site specific antibodies for multiple AMPA receptor sites including, pGluR1S831 and pGluR4S842. Phosphorylation of pGluR1S831 by protein kinase A (PKA) or calcium CAMKII is thought to be important in regulation of LTP and LTD (66, 67). Transgenic mice with mutations at pGluR1S831 have reduced LTP and therefore may have alterations in AMPA receptor trafficking (214). GluR4 at serine 842 is phosphorylated by PKA and is both necessary and sufficient for AMPA receptor delivery to synapses, especially during early postnatal development (215). Any alterations in expression of these phosphorylated AMPA receptors, in total homogenate or in a subcellular fraction, may implicate abnormal localization of AMPA receptors in schizophrenia. However, it must also be considered that decreased expression of the phosphorylated AMPA receptor may be a problem with activity or expression of the kinases involved.

5.5.5 Alternatives to Subcellular Fractionation

In our studies of total homogenate protein expression, we found a significant increase in expression of two proteins involved in forward trafficking and stabilization of AMPA receptors, SAP97 and GRIP1. SAP97 contains 3 PDZ domains and interacts with GluR1 and myosin motor proteins (74, 75, 80). GRIP1, through multiple PDZ domains, interacts with GluR2 and a Liprin α which in turn binds to a motor protein (82, 83, 86). Our interpretation of this increased expression of these proteins was related to an increased forward trafficking of the AMPA receptors from the ER to the plasma membrane. While these proteins are involved in the forward trafficking of the AMPA receptors, it may be that SAP97 and GRIP1 are increased but not all of the increased proteins are interacting with AMPA receptors.

If some of the increased SAP97 and GRIP1 proteins are not interacting with the AMPA receptors, it may be that AMPA receptor trafficking via these proteins is normal in schizophrenia. We propose that an immunoprecipitation of these and other AMPA interacting proteins and subsequent measurement of AMPA receptor coimmunoprecipitation would better elucidate the effect of increased interacting proteins in schizophrenia. While this immunoprecipitation would not provide information regarding the subcellular localization of AMPA receptors, it would provide vital information regarding the trafficking of these receptors in this illness.

5.5.6 Altered AMPA Receptor Trafficking in Animal Models

Attempts have been made to model some features of schizophrenia in animals (169, 216). These studies are limited by the complex nature and unknown

pathophysiology of the illness. One model is the chronic administration of NMDA-receptor antagonists such as PCP or ketamine (217, 218). Chronic administration of these compounds in rodents or primates is necessary to model the lifelong effects of schizophrenia, but has limitations including being an incomplete model of the illness and enormous cost of these types of studies (219).

While AMPA receptor protein and mRNA expression has been measured in studies involving chronic treatment of animals with NMDA antagonists, these studies suffer the same limitations as some postmortem studies – regional measurement of expression will not reveal deficits in trafficking and subcellular localization (217, 220). We propose that subcellular fractionation and measurement of AMPA receptor localization and trafficking following chronic NMDA receptor antagonism would further reveal the involvement or lack thereof of altered AMPA receptor trafficking in this model of schizophrenia. These findings may then be applied to postmortem studies.

Alternatively, we may be able to take what we have learned in postmortem studies and produce an animal model. It may be possible to mimic increased expression of AMPA interacting proteins, including SAP97 and GRIP1, using a viral expression vector. We could then examine the behavior of the animals on tasks implicated in schizophrenia such as working memory and prepulse inhibition. Further, we would be able to perform electrophysiology on the brains of these animals to determine if altering AMPA receptor subunit expression has an effect of neurotransmission.

5.6 Conclusions

In this dissertation work, I used immunoisolation and centrifugation to obtain subcellular fractions from postmortem tissue in the dorsolateral prefrontal cortex in schizophrenia. I confirmed the specificity of the subcellular fractions using western blot and electron microscopy. Subsequently, I measured the expression of the AMPA receptor subunits in these fractions as well as the expression of proteins involved in the trafficking of AMPA receptors in total homogenate tissue. I found increased protein expression of SAP97 and GRIP1 in total homogenate and increased expression of GluR1 in early endosomes. While these data suggest increased forward trafficking of AMPA-type glutamate receptors in schizophrenia, a lack of significant changes in AMPA receptor protein expression in late endosomes or the endoplasmic reticulum, as well as a lack of changes in 10 other proteins involved in the trafficking of AMPA receptors diminishes support for altered AMPA receptor trafficking as a prominent feature in the underlying pathophysiology of schizophrenia. Our data provide substantial evidence toward the acceptance of the null hypothesis, suggesting that AMPA receptor trafficking, previously a promising target in the glutamate hypothesis of schizophrenia, may have no physiological role in the pathophysiology of schizophrenia.

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APPENDIX A

UNPUBLISHED FINDINGS

In addition to the numerous proteins that we measured and have presented in the previous chapters, we measured expression of three phosphorylated AMPA receptor sites involved in subcellular localization and trafficking as well as interacting proteins and isolated early endosome content in a second brain region, the anterior cingulate cortex, ACC. Also, we measured expression of some NMDA receptor subunits in isolated early endosomes in the ACC and DLPFC.

Phosphorylated AMPA Receptor Protein Expression

Using tissue homogenate from the DLPFC and ACC we measured protein expression of three phosphorylated AMPA receptor sites involved in AMPA receptor trafficking. We found no significant changes in phosphoGluR1 at serine 818, phosphoGluR1 at serine 845, and phosphoGluR2 at serine 880 in schizophrenia in the ACC or DLPFC.

AMPA Interacting Proteins in Anterior Cingulate Cortex

The anterior cingulate cortex, discussed further below, is a brain region involved in higher cognitive functioning including working memory and selective attention. From the same cohort of subjects as the DLPFC subjects with 3 additional subjects, we measured protein expression of NSF, GRIP1, and SAP97 in the ACC. We found no significant changes in these proteins in schizophrenia.

Early Endosomes in Anterior Cingulate Cortex

Using the same immunoisolation protocol, we isolated intact early endosomes from the ACC in patients with schizophrenia and a control group. We found no significant difference in protein expression of GluR1, GluR2 or GluR3 in isolated early endosomes in schizophrenia. Additionally, we found no significant changes in NSF or SAP97 protein expression in isolated early endosomes.

NMDA Receptor Expression in Early Endosomes

In our isolated early endosomes, we measured expression of some NMDA receptor subunits. We found no significant change in NR1 or NR2B protein expression in early endosomes in schizophrenia in the DLPFC. We found no significant difference in NR2A or NR2B protein expression in schizophrenia in the ACC.

Conclusion

Taken together with our published findings, these data further suggest a lack of significant changes in AMPA receptor trafficking and subcellular localization in schizophrenia. While these studies were not as comprehensive as our published studies, they implicate that a similar lack of significant differences in AMPA trafficking may be seen in the ACC as we found in the DLPFC. Our unpublished findings regarding NMDA receptors in the early endosomes are difficult to interpret as we did not measure all of the receptor subunits.

APPENDIX B

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: July 8, 2009

TO: Meador-Woodruff, James H.
SC-560C 0017
996-6171

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Subcellular Glutamate Receptor Defects in Schizophrenia
Sponsor: NIH
Animal Project Number: 090707844

On July 8, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Rats	A	30

Animal use is scheduled for review one year from July 2009. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 090707844 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
1670 University Boulevard
205.934.7692
FAX 205.934.1188

Mailing Address:
VH B10
1530 3RD AVE S
BIRMINGHAM AL 35294-0019