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EVIDENCE OF ALTERED AMPA RECEPTOR LOCALIZATION AND REGULATION IN SCHIZOHPRENIA

by

JANA BENESH DRUMMOND

JAMES H. MEADOR-WOODRUFF, CHAIR
SARAH CLINTON
RITA COWELL
ANNE THEIBERT
SCOTT WILSON

A DISSERTATION

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EVIDENCE OF ALTERED AMPA RECEPTOR LOCALIZATION AND REGULATION IN SCHIZOHPRENIA

JANA BENESH DRUMMOND

NEUROSCIENCE

ABSTRACT

Although the glutamate hypothesis of schizophrenia posits altered glutamatergic transmission is occurring in this illness, the precise mechanisms behind these proposed changes in schizophrenia brain remain elusive. Recent evidence from our laboratory supports a model of altered forward trafficking of glutamate receptors to synaptic membranes in schizophrenia, which could contribute to changes in neurotransmission. The AMPA subtype of ionotropic glutamate receptor (AMPAR), is the main facilitator of fast, excitatory neurotransmission in the brain, and changes in AMPAR number at synapses may control synaptic strength and plasticity. One mechanism that could alter AMPAR trafficking to synapses is abnormal expression of AMPAR auxiliary proteins, such as the transmembrane AMPAR regulatory proteins (TARPs) or Cornichon homologues (CNIHs). These proteins coassemble with AMPARs in endoplasmic reticulum (ER), and traffic AMPARs from the ER to synapses. We measured TARP and CNIH protein expression in brain homogenates from anterior cingulate cortex (ACC) and found increased protein expression for TARP γ-3 and γ-5, and decreased expression for γ-2, γ-4, γ-7, γ-8, and CNIH-2 in schizophrenia, consistent with other reports of diminished AMPAR trafficking. To test if fewer AMPARs were being trafficked to synapses in schizophrenia, we developed a strategy to isolate and enrich synapses from ACC and measured AMPAR, TARP, and CNIH expression in this fraction. We found decreased expression of the AMPAR subunit GluA1 at synapses in schizophrenia, which

corresponded to fewer GluA1 subunits in a fraction enriched for ER, and decreased total protein levels in homogenates. We found no changes in subcellular protein expression for TARP or CNIH proteins, but ratios of GluA1 to AMPAR, TARP, and CNIH proteins were decreased in schizophrenia, suggesting that early processing of these complexes may be disrupted at ER, and may be hindering the trafficking of GluA1-containing receptors to the synapse. We also evaluated NMDAR subunit expression and found no intracellular changes. Taken together, these studies demonstrate a reliable method for the isolation of synapses in postmortem tissue, and provide evidence that altered regulation of receptor trafficking and early processing may be an underlying mechanism contributing to glutamate dysregulation in schizophrenia.

Keywords: postmortem, anterior cingulate cortex, postsynaptic density, ER, TARP, Cornichon

DEDICATION

To Mom, with love and appreciation for your support and encouragement, and for always believing in me. Thank you for instilling in me the value of higher education.

And to William, may you always seek the unknown, for it is there you will find yourself. Keep reaching.

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The Clinical Presentation of Schizophrenia

Schizophrenia is a devastating and severe mental illness that affects approximately 1% of the world's population, irrespective of race, ethnicity, or socioeconomic status. Patients often receive a diagnosis of schizophrenia after their first psychotic break, usually during their late-adolescence or early-adulthood years, and the timing of onset is a consistent feature of the illness, although cases of earlier and later onsets have been documented (Hui *et al*, 2014). This onset importantly corresponds with the final stages of cortical brain maturation, which does not occur until adulthood (Lebel and Beaulieu, 2011). Thus, the etiology of schizophrenia is largely proposed to be developmental, although the precise mechanistic details remain elusive.

Schizophrenia onset is characterized by three main categories of symptoms. Positive symptoms are comprised of hallucinations, delusions, and disordered thought and movement, and have varying levels of severity. These symptoms are the most easily observed by clinicians and others close to the patient, and have been traditional hallmarks of the illness. Second are negative symptoms, which comprise behaviors that are considered lacking from normal behaviors and emotions. These include depression, a flat affect, and lack of motivation. Third, are cognitive deficits, and involve problems with executive functions such as attention and working memory. Generally these symptoms are subjective, as no biomarker or diagnostic tool aside from clinical observation and assessment

currently exists to derive or predict a schizophrenia diagnosis. However, physical changes to brain structure have been reported among schizophrenia patients, including changes in dendrite morphology, white matter tracks, decreased brain volume, and enlarged ventricles (Antonova *et al*, 2004; Glausier and Lewis, 2013). These studies highlight a seemingly paradoxical finding of decreased brain size and dendritic spine morphology, but no corresponding loss of cell number, which together indicate that schizophrenia may contain components of altered spine development and connectivity in its pathophysiology although is not a neurodegenerative illness.

Few treatments currently exist for patients suffering from schizophrenia. Antipsychotic classes of drugs were serendipitously discovered during the 1960s to help manage positive symptoms, and were later determined to block the D2 type of dopamine receptor in brain (Javitt *et al*, 2012). More recent attempts to improve the efficacy of antipsychotic treatments have failed, and these early treatments remain the primary course of treatment for patients today. However, their mechanisms of action remain poorly understood. The discovery of antipsychotic antagonism for D2 receptors spurred the dopamine hypothesis of schizophrenia, which proposed that dopamine was present at excessive levels in patients and contributed to the presentation of positive symptoms. Models of dopamine antagonism in animals, as well as further studies of dopamine modulation in schizophrenia and comparison subjects, suggest that negative and cognitive symptoms occur independently from dopamine scaling, and spurred the field to find an alternative hypothesis to potentially explain the full spectrum of symptoms.

The Glutamate Hypothesis of Schizophrenia

During the early 1960s, another important observation was made: unaffected individuals who ingested phencyclidine (PCP, or angel dust), were clinically indistinguishable from patients suffering from schizophrenia. In fact, effects of this drug closely resembled many aspects of schizophrenia, and included positive, negative, and cognitive symptoms. Conversely, when PCP was administered to patients with schizophrenia, symptoms were exacerbated, which was an intriguing finding that strongly indicated parallel affected molecular pathways between PCP and schizophrenia. PCP was later discovered during the 1980s to also be an antagonist for a neurotransmitter (Javitt, 2012), but this time the target was specific for the NMDA subtype of glutamate receptors (NMDARs). This discovery was the beginning of the glutamate hypothesis of schizophrenia, which posited that these receptors may be hypofunctional since their blockade in unaffected individuals mimicked schizophrenia-like symptoms. Rapidly expanding evidence that has converged in support of this hypothesis over the last few decades has expanded the focus of the glutamate hypothesis from NMDARs to other glutamatergic molecules including other classes of ionotropic and metabotropic glutamate receptors, transporters, enzymes, and regulatory proteins. The developmental origin of these deficits, and the precise mechanistic pathways contributing to these changes, remain poorly understood and a priority of current and future investigations.

In conclusion, schizophrenia is a complex biological illness characterized by a constellation of behavioral and molecular disturbances involving many potential etiologies, yet affects a consistent proportion of the population worldwide. Although the pathophysiology of schizophrenia remains elusive, it is clear that there are specific, quantifia-

ble anatomical and circuity changes in brains of patients with this illness, and that these changes may be driven by a complex interplay of disordered neurotransmitter systems beginning in early development. Effective treatments that address the full spectrum of associated symptoms, along with predictive and diagnostic biomarkers, are also lacking, and are especially challenging to advance due to the inherent challenges in modeling schizophrenia in other systems. The clinical observations that lead to both the dopamine and glutamate hypotheses, and the subsequent ante- and postmortem studies that further support them, are compelling; however, more direct evidence of how these receptors are regulated in schizophrenia, and future studies examining how their potential dysfunction may arise, will be critical for further validation of these hypotheses, and our understanding of the underlying pathophysiology of this illness. Since the most recent evidence has converged on glutamate dysfunction in schizophrenia, this system will be the focus of the remainder of this document, as glutamate is associated with the core of the illness: the negative and cognitive symptoms.

The Regulation of Glutamate Neurotransmission

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system. Glutamate levels are dynamically regulated to maintain appropriate homeostatic levels high enough to support executive functioning, but low enough to prevent excitotoxicity and neuronal cell death. Glutamate cycles through the glutamate-glutamine pathway (see Anderson and Swanson, 2000 for a review), which begins with its initial synthesis from glutamine via glutaminase in the presynaptic neuron (Figure 1). Once synthesized, glutamate is packaged into vesicles, and trafficked by glutamate transporters

(vGLUTs) to the active zone of presynaptic membranes, where the vesicle fuses and releases its contents into the synaptic cleft. Glutamate binds and activates pre- and postsynaptic glutamate receptors, and receptors located on astrocytes. Together these cells form a tripartite glutamatergic synapse (Figure 1). Excess extrasynaptic glutamate is rapidly cleared from the cleft by astrocytic excitatory amino acid transporters (EAATs) before either entering the tricarboxylic acid cycle (TCA) as α-ketoglutarate, or converting back to glutamine by glutamine synthetase for transport back to the presynaptic cell. Recent reports from our laboratory have demonstrated altered cortical expression of EAATs and associated interacting proteins (Bauer *et al*, 2008), and abnormal glycosylation of EAATs (Bauer *et al*, 2010) in schizophrenia, both of which suggest that glutamate reuptake from the synaptic cleft may be abnormal in this illness. Consistent with this, recent reports have indicated abnormal levels of glutamate in brain (Kraguljac *et al*, 2013), which are in agreement with the hypothesis of altered glutamatergic neurotransmission in schizophrenia.

Mechanisms of Glutamate Receptor Regulation and Trafficking

The regulation of glutamate receptors at synapses is important for maintaining appropriate levels of glutamate in neurons, and is also necessary for normal excitatory neurotransmission and executive functions such as learning and memory. Ionotropic glutamate receptors are heterotetrameric ligand-gated cation channels that mediate the majority of fast, excitatory neurotransmission in the brain. The main subtypes of these receptors consist of either N-methyl-d-aspartate (NMDA) receptors or α-amino-3-hydroxy-5-methylisoazole-4-proprionic acid (AMPA) receptors, comprised of GluN1-3 or GluA1-

4 subunits, respectively (Table 1). A third subtype, kainate receptors (subunits GluK1-5), are structurally similar to AMPARs, but unlike AMPARs, they predominantly localize to presynaptic membranes (reviewed in Rubio *et al*, 2012). Both NMDARs and AMPARs are largely regarded as postsynaptic receptors, although some reports suggest localization and function can also occur at presynaptic sites on GABAergic cell membranes (Biou *et al*, 2008). The postsynaptic density (PSD) is an electron-dense organelle at the postsynaptic membrane (Figure 1), and is composed of glutamate receptors and the PSD95 family of MAGUK scaffolding proteins (PSD95, PSD93, SAP97, and SAP102. MAGUK proteins contain PDZ domains that can directly bind to NMDARs, and indirectly to AMPARs through other PDZ domain-containing accessory proteins (Sheng and Sala, 2001).

Glutamate receptors are rapidly targeted and trafficked towards and away from the synapse via exocytic and endocytic pathways, or trafficked within the postsynaptic density (PSD) by lateral diffusion (see Groc and Choquet, 2006 for a review). The dynamic regulation of receptor number at synapses is driven by both synaptic activity and complex interactions with scaffolding and auxiliary proteins to mediate rates of trafficking. Scaling the number of receptors at synapses directly impacts synaptic strength, and the trafficking of AMPARs in particular to synapses is considered a key mechanism underlying some forms of learning and memory processes (Malenka and Nicoll, 1999).

Glutamate Receptor Biosynthesis and Regulation at Endoplasmic Reticulum (ER)

Genes encoding the glutamate receptors are under developmental and cell-type specific control, and hence are not expressed equally throughout development. The majority of synapses spanning from early to prepubescent brain development contain mostly

NMDARs and gain AMPARs over time, a change that effectively unsilences these synapses (Atwood and Wojtowicz, 1999; Hanse *et al*, 2013; Pickard *et al*, 2000). GluA4 is primarily expressed during early development, and tapers off during adulthood (Wenthold *et al*, 1996; Zhu *et al*, 2000). The abundance of GluA2 increases as development progresses compared to GluA1 (Pickard *et al*, 2000), and layer 5 pyramidal neurons in rat neocortex have been shown to undergo a switch from GluA1- and GluA4-containing AMPARs to those containing GluA2 by postnatal day 16 (Kumar *et al*, 2002). Similarly, the NMDAR subunit GluN2B predominates during early development, but GluN2A is upregulated in mature neurons compared to GluN2B (Barria and Malinow, 2002). This activity-dependent switch from GluN2B to GluN2A is associated with developmental long-term potentiation (LTP), but not LTP in mature brain (Bellone and Nicoll, 2007; Sanz-Clemente *et al*, 2013). NMDAR expression can also switch from GluN2B to GluN2C in cerebellar granule cells around 2 weeks postnatal (Farrant *et al*, 1994; Monyer *et al*, 1994; Watanabe *et al*, 1994). GluN3A has also been shown to be highly expressed during early postnatal development and to decrease towards puberty (Henson *et al*, 2012; Roberts *et al*, 2009). Many studies have extrapolated how expression of these genes are transcriptionally controlled in neurons, and have found similar mechanisms including multiple transcriptional start sites, and predominantly neuronal-localized mRNAs that often give rise to multiple splice variants (see Bredt and Nicoll, 2003; Myers *et al*, 1999 for reviews).

Glutamate receptor synthesis and early trafficking through the secretory pathway involves initial assembly and processing at the rough endoplasmic reticulum (ER), and subsequent modifications in the Golgi network before translocation to the synaptic mem-

brane. Briefly, receptor mRNAs are directed to attach to the ER membrane via an N-terminal signal sequence, and the mRNA is translated on clusters of ribosomes attached to surface ER, or polysomes. This synthesis of receptor subunits occurs at ER in the somatic compartment, but recent experiments in neuronal cultures has demonstrated AMPAR synthesis also occurs at ER membranes proximal to dendritic spines (Ju *et al*, 2004). Both NMDAR and AMPAR complexes are formed as homodimers or heterodimers that then assemble by dimerization of dimers into the final heteromer form. Receptors are typically assembled in ER as either GluN1/GluN2 for NMDARs (Schorge and Colquhoun, 2003) or GluA1/A2 and GluA2/A3 for AMPARs (Greger *et al*, 2002; Shi *et al*, 2001; Wenthold *et al*, 1996) in brain. NMDARs may also exist as triheteromeric GluN1/GluN2A/GluN2B receptors, however, the abundance of this complex is unclear and has been estimated to be anywhere from 0 and 60% (Al-Hallaq *et al*, 2007; Blahos and Wenthold, 1996; Chazot and Stephenson, 1997; Luo *et al*, 1997; Sheng *et al*, 1994). Proteins are translocated across ER membranes to undergo extensive posttranslational modifications, such as phosphorylation and glycosylation, before being packaged into transport vesicles that bud off and travel to the *cis* side of the Golgi apparatus. Here, the vesicles fuse with the membrane of the Golgi cisternae, and undergo a series of vesicular transport steps where they are further modified through enzymatic reactions and additional posttranslational modifications. Proteins leave the *trans*-Golgi network by budding off in vesicles that are trafficked to the cell membrane for fusion via exocytosis.

Classically, ER exit has been regarded as a tightly regulated check-point that ensures that protein complexes are appropriately assembled before trafficking to the synaptic membrane (Kennedy and Ehlers, 2006). However, recent studies have shown that

changes in synaptic activity can also mediate the synthesis, retention, or exit of glutamate receptors in ER, and that this directly affects receptor number at the synapse. Prolonged blockade of activity excludes exon 22 of GluN1 and causes its synthesis to include the C2 splice cassette, which acts as an ER retention signal (Mu *et al*, 2003). Conversely, increased activity includes exon 22, and leads newly synthesized GluN1 subunits to contain a C2' domain which increases the exit of NMDARs from the ER, and increases trafficking to synapses (Mu *et al*, 2003). This accelerated exit may require interaction of the PDZ-interacting domain of C2' (Scott *et al*, 2001; Standley *et al*, 2000; Xia *et al*, 2001), however, it is unclear if this interaction involves another PDZ-domain-containing protein. SAP102 (Lau *et al*, 1996; Müller *et al*, 1996) and Sec8 (Sans *et al*, 2003a) are proteins previously shown to interact with NMDARs in the ER to promote forward trafficking of NMDARs to synapses. Additionally, Protein Kinase A (PKA) and Protein Kinase C (PKC) directly phosphorylate GluN1 subunits that are retained in ER, facilitating their exit (Scott *et al*, 2003).

For AMPARs, activity blockade induces strengthening of synapses, and increases synthesis and trafficking of these receptors to synapses (Ju *et al*, 2004; Sutton *et al*, 2006; Turrigiano *et al*, 1998). AMPAR exit from ER may also require interactions between the PDZ motif located on cytosolic C-terminal domains, and a PDZ domain-containing accessory protein. PICK1 may be required for GluA2 ER exit (Greger 2002), whereas SAP97 associates with GluA1 in ER (Sans *et al*, 2001). Transmembrane AMPAR regulatory proteins (TARPs) have also been shown to mediate AMPAR trafficking early in the biosynthetic pathway. The *stargazer* mouse lacking TARP γ-2 in cerebellar granule cells also lacks functional AMPARs in these cells, and GluA2 subunits specifically show im-

mature ER-specific glycosylation (Tomita *et al*, 2001). In another study where γ-2 was deleted, the ER unfolded-protein response (UPR) was induced in cerebellar granule cells (Vandenberghe *et al*, 2005a). Together these reports suggest that TARPs play an important role in AMPAR maturation. AMPARs are also associated with ER chaperone proteins BIP and calnexin in ER, but their precise roles in AMPAR exit or retention in the ER are unclear (Rubio and Wenthold, 1999; Sommer *et al*, 1991). Cornichon proteins have also been recently discovered to mediate AMPAR trafficking (Schwenk *et al*, 2009), and may have distinct roles in regulating ER exit of AMPARs due to their evolutionary conserved roles as ER chaperones in other systems (Castro *et al*, 2007a; Shi *et al*, 2010).

Receptor exit from the ER also appears to be subunit specific. A majority of NMDARs and AMPARs contain GluN1 or GluA2, respectively, and these subunits are both retained in ER potentially for rapid accessibility for incorporation into functional receptors (Greger *et al*, 2002; Wenthold *et al*, 2003). The idea that GluN1 remains in ER until assembly with GluN2 before export to synapses is supported by the findings that GluN1 subunits are synthesized in excess (approximately 10 fold) compared to GluN2 subunits (Huh and Wenthold, 1999), and that overexpression of GluN1 splice variants do not change NMDAR currents, but overexpression of GluN2A or GluN2B subunits increases the number of functional NMDARs (Prybylowski *et al*, 2002). These data suggest that increased pools of GluN1 do not result in increased insertion of functional NMDARs at synaptic membranes, and that retained or unused receptor subunits are degraded.

GluA2 subunits that remain in ER possess immature glycosylation, are associated with GluA3 rather than GluA1, and contain a charged arginine residue (R607) present in the pore-forming segment at a position typically occupied by glutamine (Q) in other

AMPAR subunits (Greger *et al*, 2002; Mansuy and Suter, 2000). The presence of GluA2 confers calcium impermeability to the AMPAR complex via the Q/R editing site, suggesting specific roles for this subunit in governing synaptic excitability. GluA1/A2 AMPARs traffic more quickly from the ER than GluA2/A3, possibly due to a faster maturation rate of GluA1 compared to GluA2 (Greger *et al*, 2002). Interestingly, these differences in kinetics reverse for synaptic insertion, and GluA1/A2 progresses more slowly at this stage of the secretory pathway compared to GluA2/A3 (Greger *et al*, 2002; Passafaro *et al*, 2001). These findings demonstrate subunit-specific regulation at ER, and may be indicative of distinct pathways governing receptor trafficking to synapses.

Trafficking from ER to Synaptic Membranes

Changes in activity, interactions between PDZ domains and accessory proteins, targeting to extrasynaptic sites and subsequent lateral diffusion to the postsynaptic density (PSD), or direct delivery to the synapse, are all potential trafficking pathways of glutamate receptors destined for synapse insertion. Once these proteins exit from ER, they are further processed and modified in the Golgi apparatus, and trafficked to the *trans* Golgi network (TGN) and endosomes. It is unclear what percentage of functional NMDARs or AMPARs that are targeted to synapses were processed in the cell body versus ER and Golgi networks proximal to dendritic spines. Local regulation would be advantageous, as it would allow for rapid synaptic response compared to slower, distal processing that would entail transport from the cell body to the synapse. Interestingly, local synthesis and regulation has recently been demonstrated in cell culture to occur for GluA1 and GluA2 subunits (Ju *et al*, 2004).

While processing and early secretory trafficking of NMDAR and AMPARs appears to be similar, many studies are in agreement that once these receptors are targeted to the synapse, NMDARs are more stable and less dynamic when compared to AMPARs (Allison *et al*, 1998). Although both receptor types have been shown to rapidly traffic between synaptic and extrasynatpic sites, presumably due to lateral diffusion (Tovar and Westbrook, 2002), AMPARs are continually diffusing between these locations, and trafficking through the endocytic pathway. Several studies examining the basal rate of endocytosis of synaptic AMPARs in cell culture have found it nearly threefold that of NMDARs (Ehlers, 2000; Huh and Wenthold, 1999). Hence, this may explain the link between changes in AMPAR number and localization, and the regulation of synaptic plasticity. When either receptor type is removed from the synapse by endocytosis, it is trafficked by clathrin-coated vesicles away from the synaptic membrane to endosomes for either recycling or degradation. Vesicles containing these internalized receptors fuse with intracellular lysosomal vacuoles, or early endosomes, where receptors to be recycled back to the cell surface are sorted from those tagged for degradation. Proteins destined for degradation are further trafficked to late endosome-lysosome complexes where they are destroyed.

Similarly to ER exit, AMPAR trafficking towards and within the PSD is dependent on activity level, subunit type, and interactions with accessory proteins. The *constitutive pathway* is mediated by the short C termini of GluA2 and GluA3 subunits and is activity-independent, continual, and important for homeostatic balance, whereas the *activity-driven pathway* involves the long carboxyl termini specific to GluA1 and GluA4 subunits (Greger *et al*, 2002) and is required for trafficking of AMPARs in response to chang-

es in synaptic activity. These pathways likely require other AMPAR-interacting proteins like GRIP1 (Steiner *et al*, 2005), PICK1 (Anggono *et al*, 2011; Citri *et al*, 2010; Clem *et al*, 2010), and NSF (Song *et al*, 1998) to regulate trafficking in or away from the PSD.

AMPAR auxiliary proteins. A third and novel pathway is the *auxiliary protein-mediated pathway*, which involves binding of AMPAR auxiliary proteins to the AMPAR complex initially in the ER, and subsequent regulation of receptor trafficking to, and function at, synapses. The existence of bona fide AMPAR auxiliary proteins was recently discovered through characterizations of the naturally occurring mutant *stargazer* mouse (Chen *et al*, 1999, 2000; Letts *et al*, 2003). The *stargazer* mouse possesses an intriguing behavioral phenotype consisting of severe ataxia, head-tossing, and spike-wave discharges typical of absence epilepsy in human patients. It was discovered that this phenotype was due to a single recessive mutation on mouse chromosome 15 that mapped to a previously unidentified and brain-specific protein that was homologous to the voltage-gated calcium channel subunit γ-1 (Letts *et al*, 1997, 1998; Noebels *et al*, 1990). Thus, this protein was aptly named stargazin, and is alternatively referred to as transmembrane AMPAR regulatory protein (TARP) γ-2. AMPAR-mediated currents were found to be mostly absent from cerebellar granule neurons in the *stargazer* mouse, however, NMDAR-mediated responses were normal suggesting normal synapse development and function (Chen *et al*, 1999; Hashimoto *et al*, 1999). Transfection of full-length stargazin protein into cerebellar granule neurons fully restored AMPAR currents, which provided the first real evidence that TARPs were involved with trafficking and functioning of AMPARs at synapses (Chen *et al*, 2000).

It is now known that the TARP family is comprised of 8 distinct γ subunits (1-8) that are expressed throughout the brain (Burgess *et al*, 1999; Klugbauer *et al*, 2000; Moss *et al*, 2002), and that these subunits specifically bind and modulate each of the four AMPAR subunits. Since TARPs have been shown to associate exclusively with tetrameric AMPARs (Shanks *et al*, 2010; Vandenberghe *et al*, 2005b), it is likely that TARPs bind AMPARs before the complex leaves the ER. A current model proposes that TARPs mediate the forward trafficking of AMPARs via a two-step process, where first they traffic to extrasynaptic membranes, and second, laterally translocate the receptor complex into the PSD. Once at the PSD, the PDZ domain of TARPs bind to PDZ domains of scaffolding proteins such as PSD95, and this interaction forms the physical anchor that docks AMPARs within the synaptic membrane. In addition to forward trafficking and synaptic targeting and docking, TARPs also mediate biophysical properties of synaptic AMPARs (see Jackson and Nicoll, 2011 for a review), and can increase channel conductance and affinity for glutamate binding. It is unclear if the original TARP subunits that bind to the AMPAR complex at the ER remain part of the same complex at the PSD, and if TARPs play a role in endocytic trafficking.

Other AMPAR auxiliary proteins have recently been discovered in addition to TARPs. The Cornichon homologs (CNIH-1-4) can also bind as part of the AMPAR complex at ER and mediate the forward trafficking and biophysical properties of AMPARs (Schwenk *et al*, 2009). Additionally, CNIH proteins also have evolutionary conserved roles as ER chaperones in other systems (Castro *et al*, 2007b; Shi *et al*, 2010), suggesting they may contribute to mechanisms controlling AMPAR exit from ER. The proportion of AMPAR complexes containing these proteins, and how many TARP subunits or CNIH

proteins are bound to AMPARs is currently being debated (Menuz *et al*, 2007; Schwenk *et al*, 2009; Tomita *et al*, 2003), but a recent report estimates as many as 4 TARP subunits and 0-4 CNIHs may be bound to a single AMPAR (Herring *et al*, 2013).

True auxiliary proteins that modulate trafficking and functional properties of NMDARs at synapses have not yet been discovered. However, NMDARs have been shown to interact with other modulatory proteins that can contribute to their forward trafficking. For instance, cargo vesicles containing NMDAR complexes may associate with mLin-2, mLin7, mLin-10 and KIF17 to assist vesicle transport down dendritic microtubules towards synapses (Setou *et al*, 2000). Other reports suggest a role for NETO1 in the regulation and targeting of GluN2A to the synapse (Ng *et al*, 2009), and that NF-L interacts with GluN1 to block ubiquitination and degradation of this subunit (Ehlers *et al*, 1998; Ratnam and Teichberg, 2005).

Glutamate Receptor Studies

The glutamate hypothesis of schizophrenia suggests that NMDARs are hypofunctional, and are contributing to altered excitatory neurotransmission in this illness. However, it is unclear what effect this has on AMPARs in schizophrenia, or if AMPARs are potentially contributing to NMDAR hypofunction. Also unclear is if the altered function of NMDARs may reflect too little or too much synaptic glutamate due to altered presynaptic release or postsynaptic clearance. Regardless, alterations in the expression of both NMDARs and AMPARs have been reported in schizophrenia, and experiments evaluating genetic and pharmacological manipulations on glutamate receptors have determined

their increasing relevance in synaptic events like maturation and plasticity, and to the development and persistence of schizophrenia-related symptoms.

Genetic manipulations of NMDARs and AMPARs have yielded striking behavioral phenotypes spanning from lethality to changes in learning and memory capabilities (Table 2). These conditions also alter the expression, localization, and function of these receptors. Many studies investigating the effects of receptor dysfunction report that the subsequent synaptic reorganization that results can occur independently of protein synthesis (Crump *et al*, 2001; Follesa and Ticku, 1996; Rao and Craig, 1997), suggesting alterations in glutamate receptor subunit trafficking.

NMDA Receptors

Blockade of NMDAR activity can cause paradoxical increases in synaptic strength, and increases in both NMDAR and AMPAR subunit expression (Allison *et al*, 1998; Follesa and Ticku, 1996; Sutton *et al*, 2006). For NMDARs, this has been shown to involve increased colocalization with PSD95 (a marker of excitatory synapses) but no change in the number of synapses (Rao and Craig, 1997). GluN2 subunit expression has also been shown to increase, and GluN1 localization at synapses increases (Crump *et al*, 2001; Follesa and Ticku, 1996; Rao and Craig, 1997). Similarly, blockade of synaptic activity or treatment with NMDAR antagonists such as MK-801 have been shown to increase AMPAR responses, and increase localization of AMPARs to excitatory synapses (Hanse *et al*, 2013; Sutton *et al*, 2006).

Genetic deletion of the GluN1 subunit is lethal, but when expression is reduced to 5-10% of endogenous levels, mice exhibit some behavioral traits similar to schizophrenia

that are reversed by treatment with the antipsychotic drugs haloperidol or clozapine (Forrest *et al*, 1994; Li *et al*, 1994; Mohn *et al*, 1999). These findings further establish GluN1 as an essential NMDAR subunit in brain. Mice with reduced GluN2B fail to thrive (Kutsuwada *et al*, 1996), but when GluN2B is overexpressed, mice display enhancements of learning and memory (Tang *et al*, 1999) and increased number of functional receptors (Prybylowski *et al*, 2002). Genetic manipulations of other NMDAR subunits produce mice that are viable, with varying effects on learning, memory, and NMDAR responses (Table 2).

In studies of schizophrenia, NMDAR transcripts have been studied more extensively than protein expression, and variable results have been reported (Table 3). At the protein level, GluN1 has been found to be increased in homogenates from cortex (Kristiansen *et al*, 2006), and GluN2B decreased in a fraction enriched for ER (Kristiansen *et al*, 2010b) in schizophrenia. NMDAR accessory proteins that regulate trafficking have also been evaluated in schizophrenia, and many are downregulated in this illness (Table 4), suggesting abnormal trafficking of NMDARs may be occurring.

In summary, NMDARs are necessary for survival and normal learning and memory processes. Their blockade results in counterintuitive increases in synaptic strength, which is thought to arise from increased trafficking of AMPARs containing the GluA1 subunit. These findings, when taken together with the observations of PCP effects in humans, strongly suggest that altered NMDARs and resulting changes in synaptic activity are a compelling model for schizophrenia, and that AMPARs may also be dysfunctional in this illness.

AMPA Receptors

Similarly to NMDARs, genetically manipulated AMPAR subunits have been extensively studied in mice. Interestingly, the only mutation that causes a failure to thrive involves the Q/R editing site of GluA2 that confers calcium impermeability to the receptor (Table 2). These mice have decreased editing at this site and show high calcium permeability, develop seizures, and die within the first 3 weeks of age (Brusa *et al*, 1995). Disruptions of the other subunits cause behavioral changes in learning and memory tasks, altered long-term potentiation or long-term depression, and expression and localization changes in other AMPAR subunits (Table 2). Also, recent studies have demonstrated that GABAergic inhibitory neurons express high levels of GluA2-lacking AMPARs (Biou *et al*, 2008), and knockout of AMPAR subunits in GABAergic interneurons show reduced excitation, demonstrating the capability of altered AMPARs to modulate inhibitory activity (Fuchs *et al*, 2007).

These subunits have also been well characterized in cortical regions in schizophrenia. At the protein level, GluA1, GluA2, and GluA4 have been demonstrated to be decreased, although this was not always corroborated in subsequent analyses (Table 3). Recently, GluA1 was found to be increased in a fraction enriched for early endosomes in schizophrenia (Hammond *et al*, 2010).

AMPAR Auxiliary Proteins

The generation of TARP mutant mice has demonstrated that TARPs specifically mediate AMPAR expression, localization, and activity at synapses. In the *stargazer* mouse which contains a spontaneous mutation of the γ-2 subunit, severe loss of synaptic

and extrasynaptic AMPARs have been reported in cerebellar granule neurons (Chen *et al*, 1999, 2000; Letts *et al*, 1998; Noebels *et al*, 1990). Similar alterations of AMPAR trafficking have been shown in knockout mice for other members of the TARP family including γ-4 (Letts, 2005; Milstein *et al*, 2007), γ-7 (Yamazaki *et al*, 2010), and γ-8 (Rouach *et al*, 2005). Further, studies of double- and triple knockout mice often show that these manipulations result in failure to thrive or death (reviewed in Jackson and Nicoll, 2011), suggesting that TARP subunits are able to compensate for loss of other subunits, and that they are necessary modulators of AMPAR trafficking and excitatory transmission.

Increasing evidence also supports a role for Cornichon homologs (CNIHs) in the regulation of AMPAR trafficking and targeting to synapses. Recent studies have demonstrated these proteins can directly bind to AMPARs, and mediate their expression and function at synapses (Schwenk *et al*, 2009). Another report shows that CNIH-2/-3 knockout mice have marked loss of GluA1 at synapses, and results in diminished AMPAR-mediated synaptic transmission (Herring *et al*, 2013). This study also demonstrated that CNIH-2/-3 selectively binds GluA1, increasing delivery of GluA1/A2 AMPAR complexes to the surface, and that the presence of TARP γ-8 blocks CNIH interaction with other, non GluA1-containing AMPARs.

In schizophrenia, transcript levels of γ-2 were increased in dorsolateral prefrontal cortex (Beneyto and Meador-Woodruff, 2006), but expression of other TARPs have not yet been characterized in this illness. Altered levels of other AMPAR-interacting proteins have been reported in schizophrenia, although mostly at transcript levels (Table 4). Recent reports have found conflicting protein expression levels of SAP97 in schizophrenia

cortex (Hammond *et al*, 2010; Toyooka *et al*, 2002), which has been shown to bind directly to GluA1 and is involved with forward trafficking of AMPARs to synapses (Hayashi *et al*, 2000; Nakagawa *et al*, 2004; Rumbaugh *et al*, 2003). Further, SAP97 may play a specific role in trafficking immature GluA1 from ER to the Golgi network, but dissociates from GluA1 once at the membrane (Sans *et al*, 2001).

These studies demonstrate key roles for AMPARs in the control of synaptic plasticity, learning, and memory, and therefore in schizophrenia pathophysiology. Considering the reports of altered AMPAR expression in schizophrenia, their coexpression with NMDARs, and that they are dynamically regulated, AMPARs are intriguing targets. Further, these studies demonstrate the relevance of NMDAR antagonism as a model of schizophrenia, and that this directly affects AMPAR number due to potential changes in trafficking. Changes in trafficking may be due, in part, to AMPAR interactions with TARPs and CNIHs, which have been shown to directly regulate trafficking and function of AMPARs in a subunit-specific manner. Hence, AMPAR dysregulation may be an important element underlying negative and cognitive symptoms of schizophrenia.

Conclusions and Research Aims

These studies highlight a potential role for altered AMPAR trafficking in schizophrenia brain consistent with the glutamate hypothesis, since these receptors are important for normal NMDAR function and executive functions like attention, learning, and memory. Changes in AMPAR localization has many implications for synaptic function, and has been proposed as a key mechanism behind learning and memory processes. Further, since the lifecycle of AMPARs is highly dynamic, this may suggest a greater margin

for disruption of AMPAR trafficking and regulation in neuropsychiatric illness than exists for NMDARs. Recent evidence from our laboratory suggests that glutamate receptors may exhibit abnormal forward trafficking in schizophrenia, and that the AMPAR auxiliary protein TARP γ-2 which forms as part of the AMPAR complex and functions to mediate receptor trafficking and function at synapses, is also altered, and may be affecting localization of AMPARs to excitatory synapses. Therefore, we hypothesize that expression of AMPAR auxiliary proteins in schizophrenia is abnormal, and results in altered forward trafficking of AMPARs from ER, as well as significantly reduced AMPAR localization and insertion at the synapse. We tested this hypothesis by the following aims.

Specific Aim 1: Determine if AMPAR Trafficking Proteins Are Abnormally Expressed in Anterior Cingulate Cortex (ACC) in Schizophrenia.

Proteins such as TARPs have been shown to regulate trafficking and localization of AMPA receptors in neurons, but their protein expression has not been evaluated in schizophrenia. We previously found altered transcript expression of γ-2 in dorsolateral prefrontal cortex in schizophrenia, as well as changes in other AMPAR-associated proteins in cortex. Therefore, we hypothesize that TARP protein expression is decreased in schizophrenia. Additional proteins important for AMPAR trafficking and localization, such as the Cornichon homologues (CNIHs), will also be measured by Western blot analysis in order to determine the extent of AMPAR auxiliary protein abnormalities in the ACC in this illness.

Specific Aim 2: Determine if Abnormal Expression of AMPARs and Their Auxiliary Trafficking Proteins Occurs in ER in Schizophrenia.

Abnormal expression of AMPARs may be present within subcellular compartments, affecting the regulation pathways of AMPARs. To test our hypothesis of altered forward trafficking, we will examine fractions containing isolated ER from ACC for altered expression of AMPARs and proteins associated with AMPAR trafficking using a subcellular fractionation protocol our laboratory has recently developed to enrich the ER from postmortem brain. AMPARs form complexes with auxiliary trafficking proteins during their assembly in the ER before they are trafficked to, and inserted at, the PSD. Therefore, the ER is the most likely proximal location of AMPAR trafficking dysregulation. Consistent with disrupted AMPAR trafficking, we hypothesize that AMPAR protein expression is increased and AMPAR trafficking protein expression is decreased within the ER in schizophrenia. Measuring AMPARs, TARPs and CNIHs specifically in the ER will provide important information on potential AMPAR dysregulation and altered forward trafficking in schizophrenia.

Specific Aim 3: Determine if Abnormal Expression of AMPARs and Their Auxiliary Trafficking Proteins Occurs at Synapses in Schizophrenia.

Alterations of proteins that mediate distal AMPAR trafficking in the dendritic spine may cause abnormal AMPAR internalization and localization at synapses. We hypothesize that there is decreased protein expression of AMPARs and AMPAR auxiliary protein expression at synapses in schizophrenia, potentially contributing to abnormal AMPAR subcellular localization. Protocols utilizing fresh animal tissue are well established for synapse isolation and show specific enrichment of this structure based on protein anal-

yses. We will use these protocols to aid our development of a technique to isolate synapses from postmortem brain. Evaluating AMPAR expression in the PSD is a novel approach to study these receptors and their regulators in schizophrenia.

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Figure 1: The glutamatergic synapse. Glutamate is packaged into presynaptic vesicles and released to the synaptic cleft where it acts upon ionotropic and metabotropic receptors and is rapidly cleared by EAATs. In the astrocyte, glutamate either enters the TCA cycle or is converted to glutamine by the enzyme glutamine synthetase. Glutamine is then released to the presynaptic neuron where it is converted to glutamate and packaged into vesicles for further release.

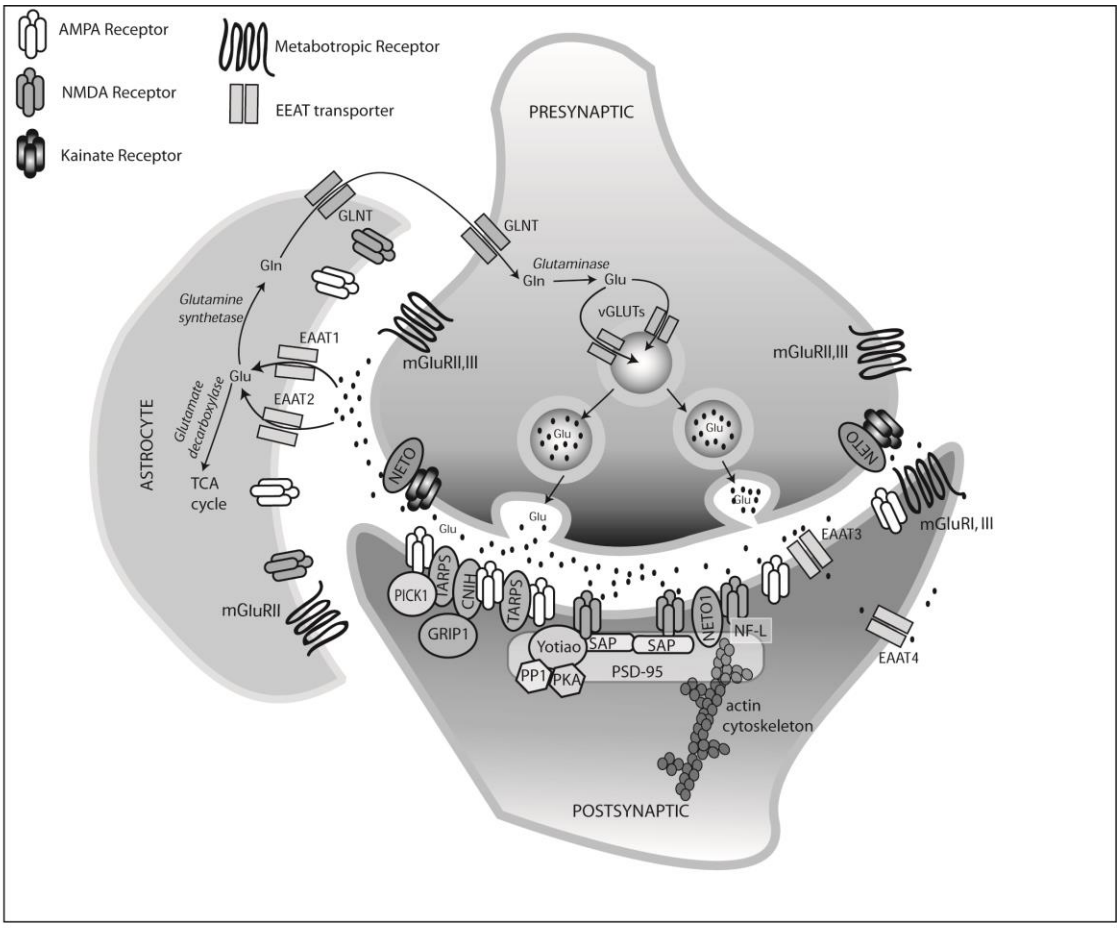

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Table 1. Classification and features of ionotropic glutamate receptors.

FAMILY	SUBUNIT	CHARACTERISTICS	ALTERNATIVE NOMENCLATURE
NMDA	GluN1	-Glycine binding -Obligatory subunit	GLU_{N1}, NMDA-R1, NR1, GluRξ1
	GluN2A	-Glutamate binding -Primarily synaptic -Ubiquitous expression in adult brain -High channel conductance -High sensitivity to Mg^{2+}	GLU_{N2A}, NMDA-R2A, NR2A, GluRε1
	GluN2B	-Glutamate and polyamine binding -Primarily extrasynaptic -Highly expressed in early development -Primarily expressed in adult forebrain -High channel conductance -High sensitivity to Mg^{2+}	GLU_{N2B}, NMDA-R2B, NR2B, hNR3, GluRε2
	GluN2C	-Glutamate binding -Primarily expressed in adult cerebellum -Low channel conductance -Low sensitivity to Mg^{2+}	GLU_{N2C}, NMDA-R2C, NR2C, GluRε3
	GluN2D	-Glutamate binding -Primarily extrasynaptic -Highly expressed in early development -Low channel conductance -Low sensitivity to Mg^{2+}	GLU_{N2D}, NMDA-R2D, NR2D, GluRε4
	GluN3A	-Glycine binding -Primarily extrasynaptic -Highly expressed in early development -Low channel conductance -Low sensitivity to Mg^{2+}	GLU_{N3A}, NMDA-R3A, NMDAR-L, chi-1
	GluN3B	-Glycine binding -Primarily extrasynaptic -Highly expressed in the spinal cord, pons, midbrain and medulla. -Low channel conductance -Low sensitivity to Mg^{2+}	GLU_{N3B}, NMDA-R3B
AMPA	GluA1	-Ca^{2+} permeable -Impermeable when coupled to edited GluA2 -Higher conductance	Glu_{A1},GluR1, GluRA, GluR-A, GluR-K1, HBGR1
	GluA2	-Q/R edited: Linear current–voltage relationship Impermeable to Ca^{2+} Low single-channel conductance -Q/R unedited: Inwardly rectifying when blocked by endogenous intracellular polyamines Ca^{2+} permeable Higher conductance	Glu_{A2},GluR2, GluRB, GluR-B, GluR-K2, HBGR2
	GluA3	-Ca^{2+} permeable -Impermeable when coupled to edited GluA2 -Higher conductance	GLU_{A3}, GluR3, GluRC, GluR-C, GluR-K3
	GluA4	-Ca^{2+} permeable -Impermeable when coupled to edited GluA2 -Higher conductance	GLU_{A4}, GluR4, GluRD, GluR-D

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Table 2. Summary of behavioral and molecular changes observed in glutamate receptor mutant mice

GENE	METHOD	MOLECULAR AND BEHAVIOR EFFECTS IN MUTANT MICE
NMDARS		
GLUN1	Knockout	Lethal; complete loss of GluN1 protein[1]
	Knockdown	Live to adulthood; resulted in 90-95% reduction of NR1 and behavioral phenotype closely resembling schizophrenia; increased locomotor activity; reversal of impairements by treating with antipsychotic drugs haloperidol or clozapine[1]
	Overexpression of splice variants	No change in NMDAR currents/number of functional NMDARs in CGNs[2]
GLUN2A	Knockout	Mild impairments in spatial and contextual learning, NMDAR currents, and LTP at hippocampal CA1 synapses[3]
		Reduced anxiety and immobility; normal locomotor activity and neurological and other behaviors (see Table 1)[4]
		Mild hyperactivity; deficits in learning[5]
		Deficits in eyeblink conditioning[6]
	Overexpression	Increased number of functional NMDARs and surface cluster density of GluN1 in CGNs[2]
GLUN2B	Homologous recombination	Pups display abnormal suckling and premature death; lack of NMDAR responses and LTD[7]
	Overexpression	Increased activation of NMDARs and enhanced learning and memory[8]
		Increased number of functional NMDARs and surface cluster density of GluN1 in CGNs[2]
GLUN2C	Knockout	No observed behavioral effects; larger EPSCs and single-channel conductance of NMDARs in granule cells[9]
GLUN2D	Knockout	Reduced spontaneous activity[10]
	Overexpression	Smaller NMDAR-mediated currents and slower kinetics; reduced LTD at 3 weeks, and LTP at 2 months in CA1[11]
GLUN3A	Knockout	No observed behavioral effects; no change in GluN1, GluN2A or GluN2B protein expression; increased dendritic spines and altered spine morphology; increased NMDA responses[12]
GLUN3B	Knockout	Impaired motor learning/coordination; altered social behaviors[13]
AMPARS		
GLUA1	Knockout	Show AMPA-mediated neurotransmission but reduced number of functional AMPARs and extrasynaptic AMPARs; altered localization of GluA2 at cell body; absence of associative LTP in Schaffer collateral pathway in adults; normal spatial learning via Morris water maze[14]

		Normal LTP in young mice (P14-P28), but diminished by P42; altered expression and distribution of GluA2, and increased number of synapses containing GluA2[15]
		Increased learned helplessness; reduced serotonin and norepinephrine levels; increased glutamate levels; increased GluN1 protein expression in HC in a fraction enriched for synapses[16]
		Impaired LTP at active and silent CA3 synapses; decreased AMPAR responses between pairs of CA3 neurons; normal LTD[17]
GLUA2	Knockout	Enhaced LTP in CA1; normal excitation and paired-pulse facilitation; increased calcium permeability[18]
		Diminished exploration, spatial and non-spatial learning; altered motor coordination[19]
		Deficient in LTD[20]
		Increased formation of GluA1/A3 hetermomers, and GluA1 and GluA3 homomers[21]
	Knockdown	Increased GluA1 endocytosis[22]
	Gene replacement (decreased Q/R editing)	Lethal within first 3 weeks; increased calcium permeability of AMPARs; seizures[23]
		Lethal within first 3 weeks; seizures; altered dendritic morphology; increased calcium permeability that lead to NMDAR-independent LTP in HC pyramidal cells; observed no calcium-mediated neuronal cell death[24]
	Gene replacement (increased Q/R editing)	No observed effects[25]
GLUA3	Knockout	Altered regulation of sleep and breathing; seizures[26]
		Normal LTD in cortex[20]
GLUA4	Knockout	Normal LTP in HC; normal locomotor activity; impaired spatial reference memory bur normal consolidation/retention; modest improvements in spatial working memory; impaired PPI[27]
GLUA2/A3	Double-knockout	Severe impairement of basal synaptic transmission in CA1 region of HC; GluA1 sufficient for LTP, LTD, depotentiation and dedepression[28]
		Normal synapse and spine formation; no change in NMDAR-induced endocytosis and recycling of AMPARs; normal GluA1 levels at synapses[29]

Abbreviations: CGNs, cerebellar granule neurons; LTP, long-term potentiation; LTD, long-term depression; HC, hippocampus; PPI, paired-pulse inhibition.

[1]Forrest *et al*, 1994; Li *et al*, 1994; Mohn *et al*, 1999
[2]Prybylowski *et al*, 2002
[3]Kiyama *et al*, 1998; Sakimura *et al*, 1995
[4]Boyce-Rustay and Holmes, 2006
[5]Miyamoto *et al*, 2001
[6]Kishimoto *et al*, 2001
[7]Kutsuwada *et al*, 1996
[8]Tang *et al*, 1999
[9]Ebralidze *et al*, 1996
[10]Ikeda *et al*, 1995
[11]Okabe *et al*, 1998
[12]Das *et al*, 1998
[13]Niemann *et al*, 2007
[14]Zamanillo, 1999
[15]Jensen *et al*, 2003
[16]Chourbaji *et al*, 2008
[17]Selcher *et al*, 2012
[18]Jia *et al*, 1996
[19]Gerlai *et al*, 1998
[20]Toyoda *et al*, 2007
[21]Sans *et al*, 2003b
[22]Lee *et al*, 2004
[23]Brusa *et al*, 1995
[24]Feldmeyer *et al*, 1999
[25]Kask *et al*, 1998
[26]Steenland *et al*, 2008
[27]Sagata *et al*, 2010
[28]Meng *et al*, 2003
[29]Biou *et al*, 2008

Table 3. Cortical glutamate receptor abnormalities in schizophrenia versus comparison subjects.

FAMILY	SUBUNIT	TRANSCRIPT	PROTEIN
NMDA	GluN1	↓[1-3]/↑[4, 5]/↔[6]	↑[7]↔[8]
	GluN2A	↓[3]/↑[4]/↔[6]	
	GluN2B	↔[3, 6, 9]	↓[8*]
	GluN2C	↓[3]/↔[6, 9]	
	GluN2D	↑[9]/↔[3, 6]	
	GluN3A	↑[10]	
AMPA	GluA1	↓[1]/↑[11]/↔[3, 6]	↓[12]/↑[13*]/↔[14*]
	GluA2	↓[15]/↑[9]/↔[6, 11]	↓[12, 16]/↔[14*, 17]
	GluA3	↔[6, 11, 15]	↔[14*, 17]
	GluA4	↓[15]/↑[11]/↔[6]	↓[16]/↔[14*]

Abbreviations; ↓, decreased; ↑, increased; ↔, unchanged; * indicates change in subcellular fractions.

[1]Sokolov, 1998
[2]Humphries *et al*, 1996
[3]Beneyto and Meador-Woodruff, 2008
[4]Dracheva *et al*, 2001
[5]Le Corre *et al*, 2000
[6]Beneyto *et al*, 2007
[7]Kristiansen *et al*, 2006
[8]Kristiansen *et al*, 2010*
[9]Akbarian *et al*, 1996
[10]Mueller and Meador-Woodruff, 2004
[11]Dracheva *et al*, 2005
[12]Corti *et al*, 2011
[13]Hammond *et al*, 2010
[14]Hammond *et al*, 2012*
[15]Beneyto and Meador-Woodruff, 2006
[16]Tucholski *et al*, 2013
[17]Breese *et al*, 1995

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Table 4. Cortical glutamate receptor accessory protein abnormalities in schizophrenia versus comparison subjects.

FAMILY	SUBUNIT	TRANSCRIPT	PROTEIN
NMDAR ACCESSORY PROTEINS	PSD-93	↑[1]	↓[1]
	PSD-95	↑[1, 2]/↓[3]/↔[4]	↓[1, 5, 6]
	SAP102	↔[4]	
	NF-L	↓[4]/↑[1]	↓[1]
	SynGAP		↓[5]
	APBA1	↑[8]	↔[8]
	mLin2/CASK	↑[8]	↓[8]
	mLin7A/Veli-1	↑[8]	↔[8]
	mLin7C/Veli-3	↑[8]	↓[8]
	Kif17	↔[8]	↔[8]
AMPAR ACCESSORY PROTEINS	GRIP1	↑[9]	↑[10]/↔[12]
	TARP2	↑[11]	
	PICK1	↓[11]	
	SAP97	↓[7]	↓[12]/↑[10]
	NSF	↓[12, 13]/↔[11]	↔[11]
	Syntenin	↔[11]	

Abbreviations: ↓, decreased; ↑, increased; ↔, unchanged.

[1]Kristiansen *et al*, 2006
[2]Dracheva *et al*, 2001
[3]Ohnuma *et al*, 2000
[4]Beneyto and Meador-Woodruff, 2008
[5]Funk *et al*, 2009
[6]Kristiansen *et al*, 2010b
[7]Toyooka *et al*, 2002
[8]Kristiansen *et al*, 2010a
[9]Dracheva *et al*, 2005
[10]Hammond *et al*, 2010
[11]Beneyto and Meador-Woodruff, 2006
[12]Mirnics *et al*, 2000
[13]Whiteheart and Matveeva, 2004

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TRANSMEMBRANE AMPA RECEPTOR REGULATORY PROTEIN (TARP) DYSREGULATION IN ANTERIOR CINGULATE CORTEX IN SCHIZOPHRENIA

by

JANA B. DRUMMOND, JANUSZ TUCHOLSKI, VAHRAM HAROUTUNIAN, JAMES H. MEADOR-WOODRUFF

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Abstract

The glutamate hypothesis of schizophrenia proposes that abnormal glutamatergic neurotransmission occurs in this illness, and a major contribution may involve dysregulation of the AMPA subtype of ionotropic glutamate receptor (AMPAR). Transmembrane AMPAR regulatory proteins (TARPs) form direct associations with AMPARs to modulate the trafficking and biophysical functions of these receptors, and their dysregulation may alter the localization and activity of AMPARs, thus having a potential role in the pathophysiology of schizophrenia. We performed comparative quantitative real-time PCR and Western blot analysis to measure transcript (schizophrenia, N = 25; comparison subjects, N = 25) and protein (schizophrenia, N = 36; comparison subjects, N = 33) expression of TARPs (γ subunits 1-8) in the anterior cingulate cortex (ACC) in schizophrenia and a comparison group. TARP expression was also measured in frontal cortex of rats chronically treated with haloperidol decanoate (28.5 mg/kg every three weeks for nine months) to determine the effect of antipsychotic treatment on the expression of these molecules. We found decreased transcript expression of TARP γ-8 in schizophrenia. At the protein level, γ-3 and γ-5 were increased, while γ-4, γ-7 and γ-8 were decreased in schizophrenia. No changes in any of the molecules were noted in the frontal cortex of haloperidol-treated rats. TARPs are abnormally expressed at transcript and protein levels in ACC in schizophrenia, and these changes are likely due to the illness and not antipsychotic treatment. Alterations in the expression of TARPs may contribute to the pathophysiology of schizophrenia, and represent a potential mechanism of glutamatergic dysregulation in this illness.

Key Words: AMPA receptor auxiliary protein, glutamate receptor, trafficking, postmortem, human

1. Introduction

The glutamate hypothesis of schizophrenia originally proposed that NMDA receptor (NMDAR) hypofunction is associated with the pathophysiology of this illness, and was based in part on the observation that NMDAR antagonists, such as phencyclidine (PCP) and ketamine can induce schizophrenia-like symptoms in normal individuals and exacerbate symptoms in patients with schizophrenia (Allen and Young, 1978; Barbon et al., 2007; Coyle, 1996; Coyle et al., 2003; Ellison, 1995; Lahti et al., 1995; Meador-Woodruff and Healy, 2000). A potential mechanism to explain such hypofunction is abnormal expression and localization of the AMPA subtype of glutamate receptor, whose activation and colocalization with NMDARs at the postsynaptic density (PSD) is required for NMDAR activation and long-term potentiation, and thus critical for glutamatergic neurotransmission (Coyle et al., 2003; Meador-Woodruff and Healy, 2000). Changes in the activity of AMPARs at the PSD, due to either dysregulation of receptor expression or altered insertion stemming from abnormal trafficking, could decrease NMDAR activity and thus contribute to the manifestation of psychotic symptoms. AMPAR modulators, such as ampakines, have been reported to improve cognitive function in schizophrenia (Coyle, 1996), and increased AMPAR binding has been reported in the cortex in this illness (Noga et al., 2001; Zavitsanou et al., 2002). These findings suggest that AMPARs may be abnormally expressed in schizophrenia. Direct examination of AMPAR expression in postmortem brain in schizophrenia, however, has yielded inconsistent results (Breese et al., 1995; Dracheva et al., 2005; Eastwood et al., 1995; Freed et al., 1993; Hammond et al., 2010; Healy et al., 1998; Meador-Woodruff and Healy, 2000; Scarr et al., 2005).

A potential mechanism underlying AMPAR disturbances in schizophrenia is abnormal expression of AMPAR auxiliary proteins that regulate AMPAR function, localization and trafficking (Beneyto and Meador-Woodruff, 2006; Dracheva et al., 2005; Hammond et al., 2010; Malinow and Malenka, 2002; Mirnics et al., 2000; Song and Huganir, 2002; Toyooka et al., 2002; Whiteheart and Matveeva, 2004). Transmembrane AMPAR regulatory protein gamma subunit 2 (TARP γ-2, or stargazin), was the first protein found to interact with AMPARs (Chen et al., 2000; Díaz, 2010; Nakagawa and Sheng, 2000; Tomita et al., 2001; Vandenberghe et al., 2005). γ-2 was initially identified in the naturally occurring mutant stargazer mouse that lacks functional AMPARs at cerebellar granule cell synapses (Chen et al., 1999; Chen et al., 2000; Hashimoto et al., 1999; Letts et al., 1998; Noebels et al., 1990). The gene encoding γ-2 is known as voltage-dependent calcium channel gamma subunit 2 (CACNG2) due to its homology to the skeletal muscle calcium channel subunit γ-1 (Chen et al., 2000; Letts et al., 1998). Currently, eight TARP subunits have been identified, each having varying roles in AMPAR trafficking to the PSD (Chen et al., 2003; Chen et al., 2007; Coombs and Cull-Candy, 2009; Díaz, 2010; Jackson and Nicoll, 2011a; Kato et al., 2007; Klugbauer et al., 2000; Tomita et al., 2003). The TARPs also have biophysical effects on AMPARs, including controlling channel gating, receptor kinetics, glutamate binding affinity, activation and desensitization rates, and receptor stability (Coombs and Cull-Candy, 2009; Kato et al., 2010b; Malinow and Malenka, 2002; Osten and Stern-Bach, 2006; Sager et al., 2009; Tomita, 2010; Ziff, 2007).

In a previous study, we reported increased TARP γ-2 transcript expression in the dorsolateral prefrontal cortex (DLPFC) in schizophrenia (Beneyto and Meador-

Woodruff, 2006), but beyond this, little is known about the expression of this family of genes in schizophrenia. In this study, we hypothesized that TARPs are abnormally expressed in cortex in schizophrenia, which in turn may be associated with AMPAR abnormalities in this illness. We determined both transcript and protein expression of the TARP family using quantitative real-time PCR (qPCR) and Western blot analysis in the anterior cingulate cortex (ACC) from subjects with schizophrenia and a comparison group.

2. Methods and Materials

2.1 Tissue acquisition and preparation

Samples from the full thickness of grey matter from ACC were obtained from the Mount Sinai Medical Center Schizophrenia Brain Collection. Tissue was obtained in compliance with the Mount Sinai School of Medicine Institutional Review Board protocol for postmortem tissue, and was obtained and prepared as previously described (Funk et al., 2012; Hammond et al., 2010). Briefly, patients were diagnosed with schizophrenia using DSM-III-R criteria, and had a documented history of psychiatric symptoms before the age of 40, as well as 10 or more years of hospitalization with a diagnosis of schizophrenia as determined by 2 clinicians. Patients were prospectively recruited and underwent ante mortem clinical assessments, and those with histories of alcoholism, substance abuse, death by suicide, or coma for more than 6 h before death were excluded from study. Neuropathological examinations found no neurodegenerative diseases, including Alzheimer's disease, in any patient. Next of kin consent was obtained for each patient.

Prospective comparison subjects were selected using a formal blinded medical chart review instrument. Subjects were limited to those with no history of alcohol abuse, drug abuse, psychiatric illness, or neurological disease. Assessments included the CERAD battery, the Clinical Dementia Rating Scale and the Positive and Negative Syndrome Scale (Powchik et al., 1998). Additionally, comparison subjects with a diagnosis of dementia or neurodegenerative disease were excluded from study.

Two different sample sets were used for transcript (schizophrenia, N = 25; comparison, N = 25) and protein (schizophrenia, N = 36; comparison, N = 33) studies (Table 1). From these two tissue sets, 11 schizophrenia and 11 comparison samples overlap, and, in several cases, not every subject was available for study in each experiment. A detailed list of subject characteristics is shown in Supplementary Table 1.

2.2 RNA isolation for transcript studies

RNA was isolated from homogenized tissue samples with an AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA), and RNA concentration was determined by UV spectrophotometry. Approximately 1 μg of RNA from each subject was immediately treated following isolation with DNase I (Promega, Madison, WI, USA) for 30 min at 37° C. DNase I was subsequently deactivated by incubating for 15 min at 65° C before reverse transcribing using a High-Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA, USA) containing random primers, dNTPs and transcriptase.

2.3 Comparative quantitative real-time polymerase chain reaction (qPCR)

Taqman® assays (Applied Biosystems, USA) were obtained for all known TARPs, as well as for three housekeeping genes, peptidyl-prolyl isomerase A/cyclophilin A (PPIA), beta-2 microglobulin (B2M) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Supplementary Table 2). qPCR was performed using a Stratagene Mx 3000P qPCR system (Agilent Technologies, Santa Clara, CA, USA) and a FAM-490 detection procedure. Each qPCR reaction contained 0.5 µl Taqman® assay at 1x concentration, 5 µl Jumpstart^{TM} Taq ReadyMix^{TM} (Sigma-Aldrich, St. Louis, MO, USA), 2 µl RNase/DNase-free water (Molecular Devices, Sunnyvale, CA, USA) and 2.5 µl of cDNA diluted 1:3 for a total volume of 10 µl. Each subject sample was loaded in duplicate into 96-well optical reaction plates (Stratagene, La Jolla, CA, USA), with one gene per plate. In addition, each plate included negative control wells lacking cDNA. To generate a standard curve for the quantification of each gene, a pooled calibrator cDNA sample and a set of serial dilutions ranging from 1:5 to 1:40 were made from aliquots of a pool derived from all subject samples and loaded in triplicate into each plate. Cycling conditions consisted of a 2 min hold at 50º C, followed by one denaturing cycle of 95º C for 10 min, and 50 subsequent denature-anneal cycles of 95º C for 15 s and 60º C for 1 min.

2.4 Quantification and statistical analysis of transcript studies

A standard curve method was used to quantify transcript expression (Larionov et al., 2005). Briefly, cycle thresholds (C_t) were taken during the linear range of the standard curve, averaged, and normalized for each subject to the geometric mean of three

housekeeping genes. Multiple regression analyses were performed to determine any significant correlations of dependent measures with sex, age at time of death, tissue pH or postmortem interval (PMI). Differences in gene expression were tested by performing analysis of variance (ANOVA), or covariance (ANCOVA) if a potential covariate was found to be correlated with dependent measures, using Statistica software (StatSoft, Inc., Tulsa, OK, USA). Outliers more than 4 standard deviations from the mean were excluded from statistical analysis. For all tests, $\alpha = 0.05$.

2.5 Western blot analysis

Tissue was prepared for protein analysis as previously described (Funk et al., 2009). Briefly, previously snap-frozen tissue was reconstituted in 5 mM Tris-HCl, pH 7.4, 0.32M sucrose, homogenized using a Power Gen 125 homogenizer (Thermo Fisher Scientific, Rockford, IL, USA) at speed 5 for 60 s, assayed for protein concentration with a BCA protein assay kit, (Thermo Fisher Scientific, USA) and stored at -80° C. Homogenized samples containing 20 µg protein/well were reduced in buffer containing β-mercaptoethanol, and denatured at 70° C for 10 min. Samples were subsequently run in duplicate via electrophoresis on 4-12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) and transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen, USA) using a semi-dry transblotter (Bio-Rad, Hercules, CA, USA).

Commercially available primary antibodies were used for each of the TARP subunits and valosin-containing protein (VCP), and conditions for each antibody were individually optimized (Table 2). Briefly, blots were blocked with LI-COR blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) or 5% (w/v) bovine serum albumin

(BSA) and tris-buffered saline (TBS) for 1 hr at room temperature before incubating with primary antibody in the same buffer overnight at 4° C. Membranes were then washed 2 x 5 min with 0.05% Tween/TBS and subsequently incubated with the corresponding IR-dye labeled rabbit or mouse secondary antibody (LI-COR Biosciences, USA) for 1-4 hrs in the dark at room temperature. Blots were washed 2 x 5 min with 0.05% Tween/TBS and scanned with the LI-COR Odyssey laser-based image detection system (LI-COR Biosciences, USA).

Individual bands from all subjects were measured using Odyssey 3.0 analytical software (LI-COR Biosciences, USA), averaged, and normalized to VCP as a loading control. Changes in protein expression were determined by performing analysis of variance, (ANOVA) or covariance (ANCOVA) if a potential covariate was found to be correlated with dependent measures, using Statistica software (StatSoft, Inc., USA). Outliers more than 4 standard deviations from the mean were excluded from statistical analysis. For all tests, $\alpha = 0.05$.

2.6 Haloperidol-treated rats

Haloperidol decanoate (28.5 mg/kg) or vehicle (sesame oil) injections were injected intramuscularly to house-paired male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) once every three weeks for nine months for a total of 12 injections. This dose was chosen based on previous reports (Harte et al., 2005; Kashihara et al., 1986; Mithani et al., 1987). The animals were sacrificed in compliance with the University of Alabama at Birmingham's IACUC regulations, and the brains immediately harvested, dissected, and stored at -80° C.

Tissue from 10 haloperidol and 10 vehicle treated animals were used for these experiments. For the transcript studies, 1 µg of total rat frontal cortex was stabilized with RNAlater®-ICE (Life Technologies, Carlsbad, CA, USA) and RNA isolated using an RNeasy® Mini RNA isolation kit (Qiagen, USA). Isolated RNA was reverse transcribed using a High-Capacity cDNA RT Kit (Applied Biosystems, USA) containing random primers, dNTPs and transcriptase. Rat-specific Taqman® assays (Applied Biosystems, USA) were obtained for the TARPs, as well as for three housekeeping genes, peptidyl-prolyl isomerase A/cyclophilin A (PPIA), beta-2 microglobulin (B2M) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Supplementary Table 3). Data were analyzed as described above for the human studies.

For protein studies, rat frontal cortex was prepared in homogenization buffer containing 2.5 mM HEPES, pH 7.7, 2 mM EDTA, 2% SDS and a protease inhibitor tablet (Complete Mini, Roche Diagnostics, Mannheim, Germany) using a Model 100 Sonic Dismembrator (Thermo Fisher Scientific, USA) at speed 6 for 3 pulses of 5 s each. The homogenates were assayed for protein concentration with a BCA protein assay kit (Thermo Fisher Scientific, USA) and stored at -80° C. Western blotting procedures and data analyses were performed as described above for the human studies. The same antisera were used in the human and rat studies due to cross-reactivity and antigen homology across both species.

3. Results

3.1 TARP transcript expression in schizophrenia

mRNA levels were measured by qPCR in schizophrenia and comparison subjects (Figure 1). The three housekeeping genes PPIA, B2M and GAPDH were unchanged between diagnostic groups, (average housekeeping C_t = 24.6) and all TARPs except γ-1 and γ-6 were identified and quantifiable (average TARP C_t = 30.1). TARP γ-8 was significantly correlated with PMI (r = 0.32, p = 0.03); ANCOVA showed a main effect for diagnosis, (N, scz = 24, c = 23; F (1, 44) = 4.4, p = 0.042) and γ-8 was significantly decreased in schizophrenia. None of the other TARP transcripts were significantly changed in this illness.

3.2 TARP protein expression in schizophrenia

TARP protein expression was determined by Western blot analysis in schizophrenia and comparison subjects (Figure 2). The loading control protein VCP was unchanged between groups. γ-4, (N, scz = 29, c = 27; F (1, 54) = 8.17, p = 0.006); γ-7, (N, scz = 31, c = 31; F (1, 60) = 9.69, p = 0.003); and γ-8, (N, scz = 30, c = 26; F (1, 54) = 5.03, p = 0.029) were all decreased in schizophrenia. None of these proteins were significantly associated or correlated with sex, age, pH or PMI. TARP γ-3 protein expression was significantly correlated with pH, (r = 0.37, p = 0.003) and ANCOVA showed a main effect for diagnosis, (N, scz = 34, c = 29; F (1, 60) = 10.9, p = 0.002) and γ-3 was significantly increased in schizophrenia. γ-5 (N, scz = 31, c = 28; F (1, 57) = 5.35, p = 0.024) was also increased in schizophrenia, and not significantly correlated with any covariant.

3.3 Effects of antipsychotic treatment on TARP expression

To determine whether the changes found for TARP mRNA and protein expression in schizophrenia might be due to the effects of chronic antipsychotic treatment, we conducted parallel transcript and protein studies in the frontal cortex of rats chronically treated with haloperidol. TARP expression in these animals was not significantly different for either transcript (Figure 3) or protein expression (Figure 4).

4. Discussion

In this study, we measured TARP transcript and protein expression in the ACC and found decreased γ-8 mRNA (Figure 1), but unchanged levels of other transcripts. We also found decreased γ-4, γ-7 and γ-8, and increased γ-3 and γ-5 protein levels in schizophrenia (Figure 2). Further, we measured TARP transcript and protein expression in frontal cortex of rats treated chronically with haloperidol, and determined that all TARPs were unchanged (Figures 3 & 4). These data are consistent with AMPAR abnormalities in schizophrenia, and suggest diminished AMPAR trafficking and function in this illness.

A major finding in the current study is that TARP γ-4, γ-7 and γ-8 are all decreased in parallel in schizophrenia. These three subunits have two different yet dynamic functions: first, they specifically increase trafficking of AMPARs to the synapse following AMPAR-TARP coassembly in the endoplasmic reticulum (ER), and second, they modulate biophysical activity of AMPARs by decreasing desensitization and deactivation rates, and increasing resensitization rates, channel conductance and affinity for glutamate, thus effectively increasing the activity of the channel (Díaz, 2010; Jackson

and Nicoll, 2011a; Sager et al., 2009; Tomita, 2010). Decreased expression of γ-4, γ-7 and γ-8 suggests fewer TARP-AMPAR complexes within the postsynaptic density, as well as reduced activity of AMPA channels, consistent with the hypothesis of decreased AMPAR activity in schizophrenia.

In contrast, TARP γ-3 and γ-5 were found to be increased. The effect of γ-5 on AMPAR trafficking appears to be minimal; however, this subunit has been shown to increase desensitization and deactivation rates of AMPARs and decrease glutamate affinity, while the other TARP subunits have the opposite effects (Jackson and Nicoll, 2011a; Kato et al., 2008). Further, γ-5 appears to be the only subunit capable of affecting AMPA channel peak open probability (Jackson and Nicoll, 2011a; Soto et al., 2009). Accordingly, in concert with decreased γ-4, γ-7 and γ-8, increased γ-5 in schizophrenia may also be consistent with decreased activity of AMPA channels by increasing desensitization rates, and decreasing open probability and glutamate affinity.

The increased TARP γ-3 protein expression we found is likely to have opposing effects from the convergence of other changes we found on AMPAR regulation in schizophrenia, by increasing the number of AMPARs delivered to the synapse and increasing AMPA channel activity. We suggest that this change in γ-3 is a partial compensatory response to decreased expression of γ-4, γ-7 and γ-8, and increased expression of γ-5. In support, studies performed in TARP knockout mice suggest the ability of these proteins to compensate for loss of function (Jackson and Nicoll, 2011a; Menuz et al., 2008), and increased γ-3 may be a reflection of this mechanism.

Although NMDAR hypofunction has been proposed as a component of the pathophysiology of schizophrenia, this may be associated instead with AMPAR

dysregulation. NMDARs and AMPARs are colocalized at the excitatory synaptic membrane, and their sequential coactivation is required for normal glutamatergic neurotransmission. Thus, any abnormality of AMPARs due to alterations of receptor expression, localization or function that resulted in diminished AMPAR function could result in an apparent alteration of NMDAR activity. These considerations have led to hypotheses of potential AMPAR dysfunction in schizophrenia.

Once AMPARs are coassembled with TARPs in the ER they are trafficked to the extrasynaptic membrane before being laterally translocated to the synaptic cleft for insertion (Coombs and Cull-Candy, 2009; Opazo and Choquet, 2011; Tomita et al., 2003). AMPAR complexes are tethered to the postsynaptic membrane by TARP-PSD95 interaction via PDZ binding domains, but they do not become static: AMPARs remain in constant flux, entering in and out of recycling pathways as needed to regulate synaptic strength and plasticity (Bats et al., 2007; Hanley, 2008; Malinow and Malenka, 2002; Opazo and Choquet, 2011; Sager et al., 2009; Schnell et al., 2002). TARPs are direct modulators of this trafficking pathway, and their dysregulation could contribute to altered AMPAR localization and function at the excitatory synapse. Given the altered expression of TARPs in the ACC, we propose a working model of altered forward trafficking of AMPARs in which abnormal expression of AMPAR auxiliary proteins, such as TARPs, contribute to dysregulated AMPAR trafficking and tethering within intracellular compartments that in turn result in a reduction of functional AMPARs at the synapse. In support of this model are previous reports of alterations in schizophrenia of other AMPAR-associated proteins with known roles in receptor assembly, trafficking, and synaptic localization, including PICK1 (Beneyto and Meador-Woodruff, 2006; Dev et al.,

1999; Lu and Ziff, 2005), GRIP1 (Dracheva et al., 2005; Hammond et al., 2010), SAP97 (Hammond et al., 2010; Toyooka et al., 2002), and NSF (Mirnics et al., 2000).

The precise mechanisms underlying TARP-mediated AMPAR trafficking are not yet well understood. TARP mutant mice demonstrate cell-specific and TARP-specific involvement in AMPAR trafficking to synaptic and extrasynaptic membranes (Chen et al., 1999; Chen et al., 2000; Hashimoto et al., 1999; Jackson and Nicoll, 2011a, b; Letts et al., 2005; Menuz et al., 2009; Menuz and Nicoll, 2008; Rouach et al., 2005; Yamazaki et al., 2010). The functions of the TARP subunits are known to significantly overlap, serving to not only increase trafficking and targeting of AMPARs to the synapse, but also to affect biophysical properties of the ion channel (Jackson and Nicoll, 2011a; Sager et al., 2009). TARPs may exhibit binding preferences for specific AMPAR subtypes, (Kato et al., 2008; Kott et al., 2007; Soto et al., 2007; Soto et al., 2009; Suzuki et al., 2008; Zonouzi et al., 2011) or bind in combination with other AMPAR accessory proteins such as the recently described cornichons (Jackson and Nicoll, 2011a; Kato et al., 2010a; Schwenk et al., 2012; Schwenk et al., 2009). AMPARs are not likely to remain bound to the same TARP(s) throughout their lifecycle (Morimoto-Tomita et al., 2009; Tomita et al., 2004), further increasing the complexity of TARP involvement in AMPAR regulation.

A limitation of this study is the potential impact of chronic antipsychotic treatment on transcript and protein expression. To address potential medication effects in this study, we performed parallel transcript and protein studies in frontal cortex from rats chronically treated with haloperidol, and found no significant changes in TARP transcript or protein expression between haloperidol and vehicle-treated control animals. To

attempt to address this issue directly in patients in addition to these rodent studies, we performed *post hoc* analyses for each dependent measure grouped by antipsychotic treatment status within the schizophrenia group. Patients were grouped by treatment status, and were either receiving antipsychotic treatment at the time of death, or not treated if they were receiving no antipsychotics for 6 weeks or more prior to death. These analyses revealed no differences in TARP transcript or protein expression in subjects with schizophrenia on or off of these medications. Taken together, these data suggest that the changes in TARP expression we found in schizophrenia may not be due to chronic antipsychotic treatment but rather the illness itself.

A second limitation of this study is that all of the subjects were elderly and generally in late stages in the progression of this illness with primarily negative and cognitive symptoms. Accordingly, generalization of these findings to younger patients, or those with predominantly positive symptoms, should be made with caution.

In summary, multiple members of the TARP family of AMPAR accessory proteins are abnormally expressed in the ACC in schizophrenia, consistent with our model of abnormal AMPAR trafficking in this illness. Decreased TARP γ-4, γ-7 and γ-8, and increased γ-5 are consistent with abnormal AMPAR localization and decreased function at the synapse in schizophrenia. TARP subunits may work alone or synergistically with other AMPAR auxiliary proteins to modulate the lifecycle and function of AMPARs, potentially affecting normal glutamatergic neurotransmission and contributing to the pathophysiology of schizophrenia.

5. Author Disclosure

All authors have no disclosures to report.

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Figure Legends

Figure 1.

TARP transcript expression is altered in the ACC in schizophrenia. TARPs were measured by quantitative comparative real-time PCR (qPCR) in schizophrenia (N = 25) and comparison (N = 25) subjects. Data are presented as ratio of gene of interest to the geometric mean of three housekeeping genes. TARP γ-1 and γ-6 mRNAs were not detected. γ-8 transcript expression is significantly decreased in schizophrenia. Data are expressed as means ± SEM. *p < 0.05. Comp, comparison subjects; Scz, schizophrenia.

Figure 2.

TARP proteins are abnormally expressed in schizophrenia. TARPs were assayed by Western blot analysis using commercially available antibodies. Samples from schizophrenia (N = 36) and comparison (N = 33) subjects were run in duplicate and normalized to valosin-containing protein (VCP) as a within-lane loading control. Representative blots of each TARP and corresponding VCP blot are shown for both groups. Data are expressed as means ± SEM. *p < 0.05, **p < 0.01. Comp, comparison subjects; Scz, schizophrenia.

Figure 3.

TARP transcript expression in rats treated for 9 months with haloperidol. TARP transcript expression was assayed by qPCR from frontal cortex of haloperidol (N = 10) and vehicle treated (N = 10) rats. Chronic treatment with haloperidol did not affect TARP transcript expression. Data are expressed as means ± SEM.

Figure 4.

TARP protein expression in rats treated for 9 months with haloperidol. TARP protein expression was assayed by Western blot analysis from frontal cortex of haloperidol (N = 10) and vehicle treated (N = 10) rats using commercially available antibodies. Chronic treatment with haloperidol did not affect TARP protein expression. Representative blots of each TARP and corresponding valosin-containing protein (VCP) are shown below for both animal groups. Data are expressed as means ± SEM.

Table 1. Subject characteristics.

	Transcript Studies		Protein Studies	
	Schizophrenia	Comparison	Schizophrenia	Comparison
N	25	25	36	33
Sex	9 F, 16 M	13 F, 12 M	11 F, 25 M	19 F, 14 M
Age	75.2 ± 12.9	75.8 ± 11.4	74.3 ± 11.7	77.8 ± 14.0
Tissue pH	6.5 ± 0.2	6.6 ± 0.3	6.4 ± 0.3	6.4 ± 0.2
PMI	17.1 ± 10.1	9.6 ± 7.0	13.6 ± 8.2	8.3 ± 6.8
Rx (on/off)	17/8	0/25	25/11	0/33

Data are means ± SD. Abbreviation: PMI, postmortem interval (hours); Rx, treatment with antipsychotic medication (on, receiving these drugs at time of death; off, no antipsychotic treatment for 6 weeks or more prior to death).

Table 2. Antisera and conditions used for Western blot analyses.

Protein	Company	Species/ Molecular weight (kDa)	1°/Buffer [a]	2° Species	2°/Time [b]
TARP γ-2	Cell Signaling	Human/40	1:1000/BSA	Rabbit	1:5000/4hrs
TARP γ-3	Lifespan Biosciences	Human/36	1:100/BSA	Rabbit	1:5000/2hrs
TARP γ-4	Lifespan Biosciences	Human/37	1:1000/LI-COR	Rabbit	1:5000/4hrs
TARP γ-5	Lifespan Biosciences	Human/31	1:500/LI-COR	Rabbit	1:5000/2hrs
TARP γ-7	Lifespan Biosciences	Human/31	1:500/LI-COR	Rabbit	1:5000/3hrs
TARP γ-8	Lifespan Biosciences	Human/43	1:500/LI-COR	Rabbit	1:5000/2hrs
VCP	Abcam	Human/90	1:10000/LI-COR	Mouse	1:10000/1hr

[a] Dilution of primary antisera and corresponding blocking buffer. Bovine serum albumin (BSA) was used at 5% (w/v) in tris-buffered saline (TBS), and all blots were incubated overnight at 4° C. [b] Dilution of secondary antisera incubated in same buffer as primary antisera, and incubation time in the dark at room temperature.

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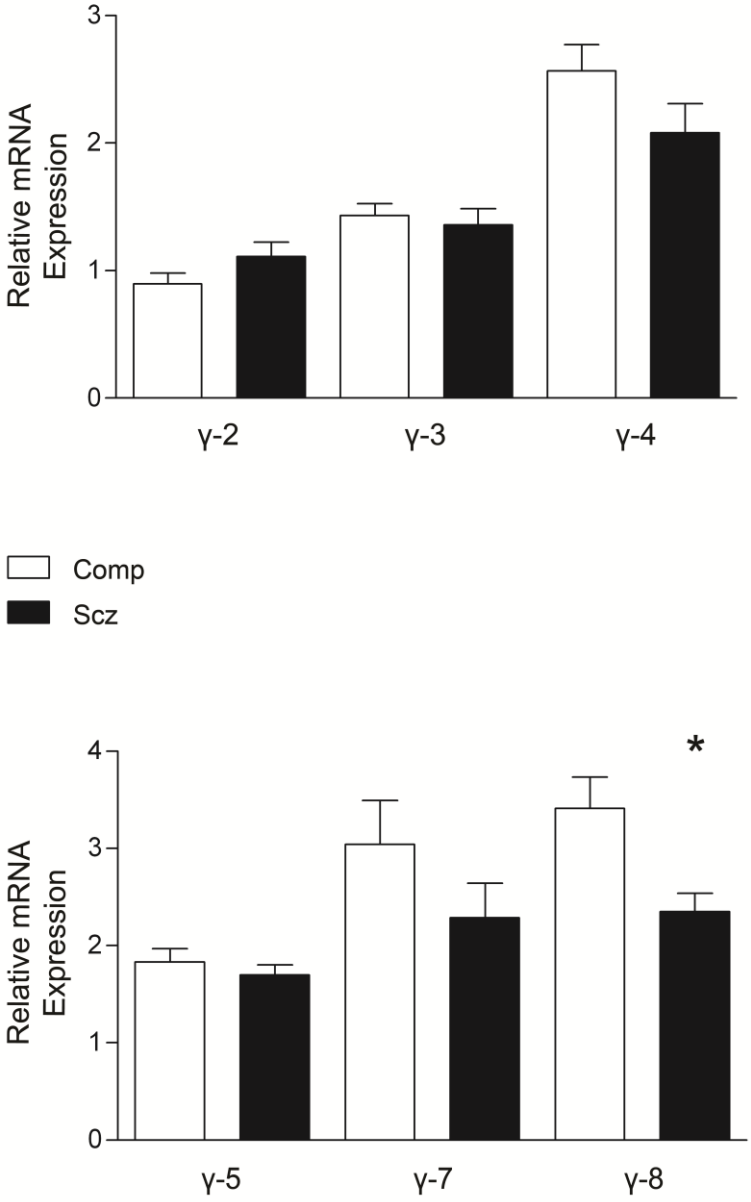
Contributors

JBD, JT and JHMW designed the study. JBD performed the experiments and statistical analyses, and wrote the first draft of the manuscript. VH provided the human tissue. All authors contributed to and have approved the final manuscript.

Conflict of Interest

All authors declare that they have no conflicts of interest.

Figure 1.


Relative mRNA Expression
3
2
1
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γ-2
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γ-4
Comp
Scz
Relative mRNA Expression
4
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γ-5
γ-7
γ-8

Figure 2.

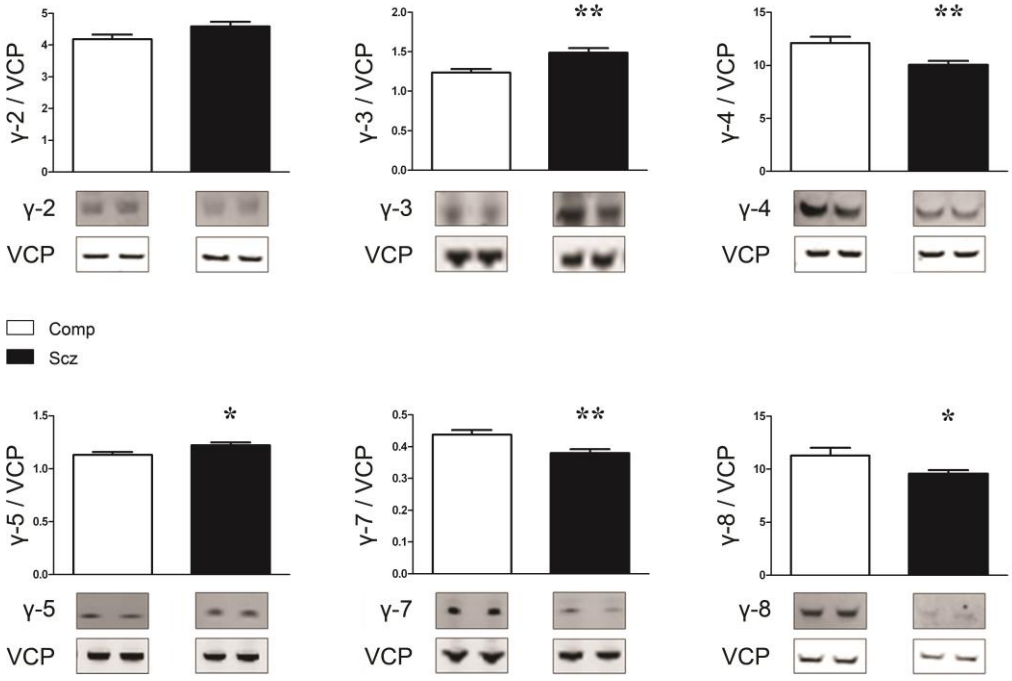
γ-2 / VCP
γ-3 / VCP
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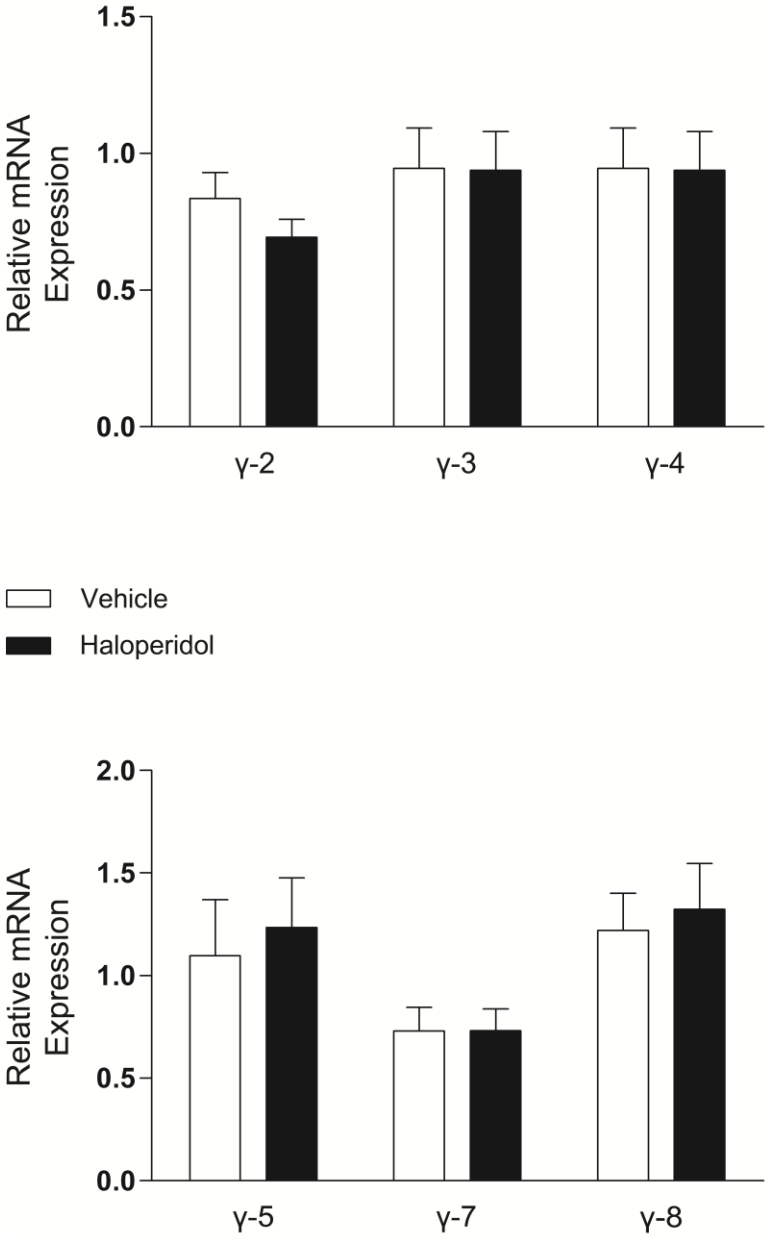
Figure 3.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Vehicle
Haloperidol
Relative mRNA Expression
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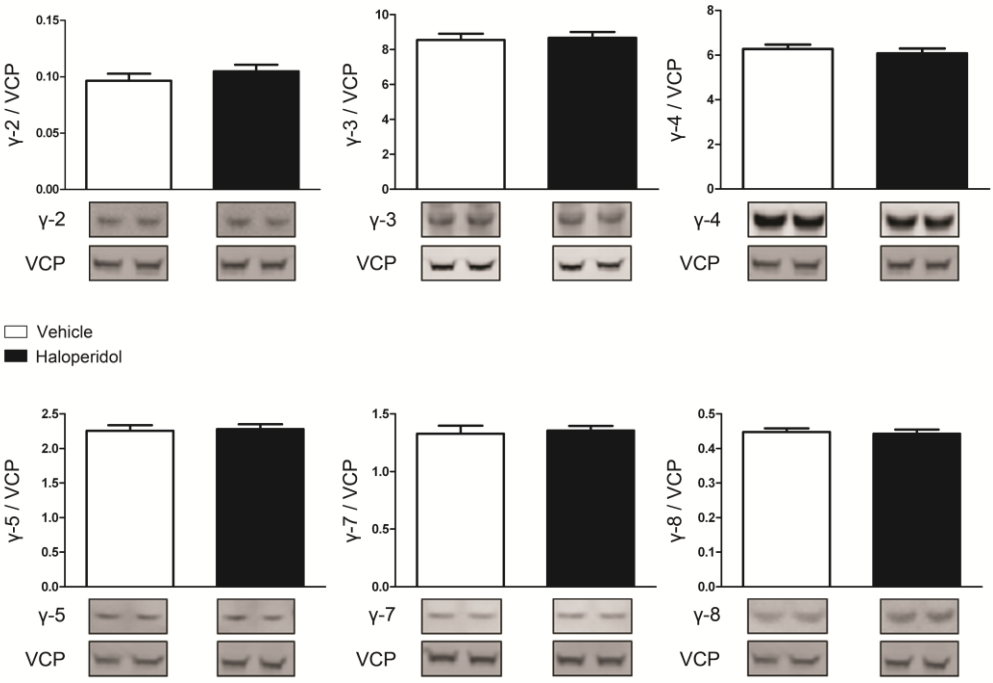
γ-5
γ-7
γ-8

Figure 4.


γ-2 / VCP
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γ-7 / VCP
γ-8 / VCP
γ-5
γ-7
γ-8
VCP

Supplementary Table 1. Individual subject characteristics.

Diagnosis	Sex	Age	pH	PMI	Diagnosis	Sex	Age	pH	PMI
Schizophrenia *	F	79	6.8	9.9	Comparison *	F	85	6.3	4.3
Schizophrenia *	F	89	6.2	9.6	Comparison *	F	96	6.3	4.5
Schizophrenia *	F	77	6.0	9.7	Comparison *	F	89	6.7	2.3
Schizophrenia	F	86	5.8	6.9	Comparison *	F	79	6.4	10.1
Schizophrenia	F	76	6.1	8.5	Comparison *	F	74	6.3	4.8
Schizophrenia	F	71	6.6	5.5	Comparison	F	86	6.5	4.7
Schizophrenia	F	69	6.2	13.7	Comparison	F	96	6.7	3.3
Schizophrenia	F	76	6.1	21.2	Comparison	F	74	6.0	3.0
Schizophrenia	F	82	6.6	18.8	Comparison	F	98	6.6	1.4
Schizophrenia	F	74	6.3	7.0	Comparison	F	82	6.1	5.7
Schizophrenia	F	81	5.9	12.5	Comparison	F	92	6.2	3.5
Schizophrenia	F	90	6.0	7.8	Comparison	F	80	6.2	4.8
Schizophrenia	F	84	6.6	52.3	Comparison	F	102	6.5	7.1
Schizophrenia	F	81	6.5	15.1	Comparison	F	73	6.3	3.4
Schizophrenia	F	75	6.5	21.5	Comparison	F	84	6.2	18.5
Schizophrenia	F	62	6.7	23.7	Comparison	F	83	6.8	6.2
Schizophrenia	F	70	6.4	12.0	Comparison	F	75	6.0	6.5
Schizophrenia *	M	57	6.4	20.7	Comparison	F	40	6.5	3.7
Schizophrenia *	M	73	6.5	7.9	Comparison	F	81	6.4	19.4
Schizophrenia *	M	66	6.5	12.1	Comparison	F	66	6.9	22.6
Schizophrenia *	M	97	6.5	9.3	Comparison	F	63	6.2	20.2
Schizophrenia *	M	92	6.7	26.0	Comparison	F	76	6.5	4.3
Schizophrenia *	M	76	6.7	16.6	Comparison	F	73	7.0	3.0
Schizophrenia *	M	82	6.7	11.4	Comparison	F	85	7.3	8.0
Schizophrenia *	M	70	6.4	7.2	Comparison	F	60	6.8	14.9
Schizophrenia	M	58	6.2	6.7	Comparison	F	88	6.6	9.0
Schizophrenia	M	52	5.9	29.5	Comparison	F	66	6.5	16.0
Schizophrenia	M	84	6.5	6.2	Comparison *	M	95	6.5	4.1
Schizophrenia	M	58	6.9	13.3	Comparison *	M	65	6.8	3.8
Schizophrenia	M	57	6.1	30.3	Comparison *	M	76	6.3	2.9
Schizophrenia	M	63	6.3	6.2	Comparison *	M	93	6.3	4.2
Schizophrenia	M	87	6.5	11.2	Comparison *	M	59	6.7	20.4
Schizophrenia	M	68	6.8	5.6	Comparison *	M	73	6.2	14.9
Schizophrenia	M	85	6.3	5.3	Comparison	M	69	6.3	4.3
Schizophrenia	M	73	6.3	11.7	Comparison	M	66	6.6	7.6
Schizophrenia	M	86	6.7	14.1	Comparison	M	69	6.7	7.4
Schizophrenia	M	66	6.7	8.4	Comparison	M	75	6.4	5.0
Schizophrenia	M	93	6.6	17.7	Comparison	M	60	6.6	28.8
Schizophrenia	M	68	6.6	17.3	Comparison	M	64	6.4	4.2
Schizophrenia	M	69	6.7	40.2	Comparison	M	75	6.3	16.0
Schizophrenia	M	84	6.7	17.7	Comparison	M	92	6.4	20.0
Schizophrenia	M	73	6.2	8.8	Comparison	M	58	6.7	12.3
Schizophrenia	M	77	6.4	24.0	Comparison	M	60	6.6	11.6
Schizophrenia	M	56	6.5	13.5	Comparison	M	64	6.7	23.8
Schizophrenia	M	68	6.3	8.9	Comparison	M	68	6.6	2.8
Schizophrenia	M	86	6.5	15.4	Comparison	M	84	6.8	11.4
Schizophrenia	M	32	6.7	17.0	Comparison	M	70	6.9	18.0
Schizophrenia	M	70	6.4	17.3					
Schizophrenia	M	78	6.6	26.1					
Schizophrenia	M	71	6.5	9.5					

Abbreviations: PMI, postmortem interval (hours). * indicates subjects included in both transcript and protein studies.

Supplementary Table 2. Taqman® assays for human qPCR studies.

Gene Symbol	Assay ID	Amplicon length
PPIA	Hs99999904_m1	98
B2M	Hs99999907_m1	75
GAPDH	Hs99999905_m1	122
CACNG2	Hs00196045_m1	78
CACNG3	Hs00197985_m1	84
CACNG4	Hs01061935_m1	73
CACNG5	Hs00945126_m1	106
CACNG7	Hs00259061_m1	54
CACNG8	Hs01100182_m1	73

CACNG: Calcium channel, voltage-dependent, gamma subunit.

Supplementary Table 3. Taqman® assays for rat qPCR studies.

Gene Symbol	Assay ID	Amplicon Length
PPIA	Rn00690933_m1	149
B2M	Rn00560865_m1	58
GAPDH	Rn01775763_g1	175
CACNG2	Rn00584355_m1	109
CACNG3	Rn00589900_m1	95
CACNG4	Rn00589903_m1	84
CACNG5	Rn00589905_m1	74
CACNG7	Rn00679230_m1	62
CACNG8	Rn00589915_m1	61

CACNG: Calcium channel, voltage-dependent, gamma subunit.

UPREGULATION OF CORNICHON TRANSCRIPTS IN DORSOLATERAL PREFRONTAL CORTEX IN SCHIZOPHRENIA

by

JANA B. DRUMMOND, MICAH SIMMONS, VAHRAM HAROUTUNIAN, JAMES H. MEADOR-WOODRUFF

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Abstract:

Schizophrenia has been proposed to be associated with abnormal glutamatergic neurotransmission. The AMPA subtype of glutamate receptors (AMPARs) mediate fast excitatory synaptic transmission in the brain, and their trafficking and function is regulated in part by AMPAR auxiliary proteins including the cornichons and transmembrane AMPAR regulatory proteins (TARPs). Abnormal regulation of AMPARs via altered expression of these auxiliary proteins could result in changes in glutamatergic neurotransmission, and thus the pathophysiology of schizophrenia. In this study, transcript expression of cornichon homologs 1-4 was measured in dorsolateral prefrontal cortex from schizophrenia (N = 25) and comparison (N = 25) subjects by comparative quantitative real-time PCR. Significant upregulation of CNIH-1, CNIH-2 and CNIH-3 mRNA expression were found in schizophrenia, with no change in CNIH-4 expression. To evaluate the effect of antipsychotic treatment on the expression of these genes, cornichon mRNA expression was assayed in frontal cortex of rats treated chronically with haloperidol decanoate and no changes in any of the cornichon transcripts were found. Abnormal expression of the CNIH family of genes is consistent with cornichon-mediated AMPAR trafficking abnormalities in schizophrenia, and suggests a new mechanism contributing to the pathophysiology of this illness.

Key Words: CNIH, glutamate, AMPA receptor, trafficking, auxiliary, postmortem, human brain

Introduction

Schizophrenia is a chronic psychiatric illness associated with incompletely understood genetic and environmental factors that contribute to the development of cognitive and other symptoms of schizophrenia. Multiple neurotransmitter systems have been proposed to underlie the pathophysiology of this illness, and recent attention has focused on glutamatergic abnormalities. The AMPA subtype of ionotropic glutamate receptor (AMPAR), which mediates most of the fast excitatory neurotransmission in the brain, has been found in some studies to have altered binding and expression levels in schizophrenia [1].

Recent discovery of AMPAR auxiliary protein families such as the cornichons and transmembrane AMPAR regulatory proteins (TARPs) have led to studies characterizing their roles in AMPAR regulation, and have found similar yet distinct roles for these families in mediating AMPAR trafficking, localization and biophysical properties at the synapse [2-5]. TARP-mediated AMPAR trafficking and regulation is more well understood than the role of cornichons [2]. Converging evidence suggests a role for TARPs in schizophrenia. Homozygosity mapping of a consanguineous family chosen for high incidence of schizophrenia, epilepsy and/or hearing deficiency found a link between psychosis and an area on chromosome 22 that contains CACNG2, the TARP gene encoding the γ-2 (stargazin) protein [6]. In another study, evaluation of genetic markers on chromosome 22 in families with high occurrences of schizophrenia determined an association between the illness and CACNG2 [7]. In postmortem brain, TARP γ-2 transcript expression is increased in the dorsolateral prefrontal cortex (DLPFC) in schizophrenia [8].

The cornichons have not yet been studied in the brain in schizophrenia. Given earlier reports suggesting TARP abnormalities in schizophrenia, we hypothesized that the cornichon genes may also be associated with this illness. In this study, we measured transcript expression levels of each cornichon homolog (CNIH-1-4) in DLPFC in schizophrenia and comparison groups, as well as in frontal cortex of rats treated chronically with haloperidol or vehicle.

Methods

Tissue acquisition and preparation

Samples from the full thickness of grey matter from dorsolateral prefrontal cortex (DLPFC) were obtained from the Mount Sinai Medical Center Schizophrenia Brain Collection (Table 1). Tissue was obtained in compliance with the Mount Sinai School of Medicine Institutional Review Board protocol for postmortem tissue and prepared as previously described [9,10]. Patients were diagnosed with schizophrenia using DSM-III-R criteria, and had a documented history of psychiatric symptoms before the age of 40, as well as 10 or more years of hospitalization with a diagnosis of schizophrenia as determined by 2 clinicians. Exclusion criteria for both schizophrenia and comparison groups included histories of alcoholism, substance abuse, death by suicide, or neurodegenerative disease.

RNA isolation

RNA was isolated from homogenized tissue samples with an AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA), and RNA concentration was determined by UV

spectrophotometry. Approximately 1 µg of RNA from each subject was immediately treated following isolation with DNase I (Promega, Madison, WI, USA) for 30 min at 37° C. DNase I was subsequently deactivated by incubating for 15 min at 65° C before reverse transcribing using a High-Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA, USA) containing random primers, dNTPs and transcriptase. RNA integrity was assessed by assaying for RIN, which averaged 7.0 for the schizophrenia group and 6.7 for the comparison subjects.

Comparative quantitative real-time polymerase chain reaction (qPCR)

Commercially available Taqman® assays (Applied Biosystems, USA) were obtained for three housekeeping genes, peptidyl-prolyl isomerase/cyclophilin (PPIA), beta-2 microglobulin (B2M) and glyceraldehydes-3-phosphate dehydrogenase, (GAPDH) and the four cornichons (CNIH-1, Hs00916484_m1; CNIH-2, Hs00704421_s1; CNIH-3, Hs00379269_m1; CNIH-4, Hs00825651_m1). Primers for each cornichon assay are located on the 3' end for CNIH-1-3, and the 5' end for CNIH-4. The assays do not target the 5' untranslated region (UTR) of each gene of interest, and were chosen to detect all known splice variants of each. qPCR was performed using a Stratagene Mx 3000P qPCR system (Agilent Technologies, Santa Clara, CA, USA) and a FAM-490 detection procedure. Each qPCR reaction contained 0.5 µl Taqman® assay at 1x concentration, 5 µl Jumpstart^{TM} Taq ReadyMix^{TM} (Sigma-Aldrich, St. Louis, MO, USA), 2 µl RNase/DNase-free water (Molecular Devices, Sunnyvale, CA, USA) and 2.5 µl of cDNA diluted 1:3 for a total volume of 10 µl. Each subject sample was loaded in duplicate into 96-well optical reaction plates (Stratagene, La Jolla, CA, USA), with one gene per plate.

In addition, each plate included negative control wells lacking cDNA. Cycling conditions consisted of a 2 min hold at 50º C, followed by one denaturing cycle of 95º C for 10 min, and 50 subsequent denature-anneal cycles of 95º C for 15 s and 60º C for 1 min. A standard curve method was used to quantify transcript expression [11]. Briefly, a pooled calibrator cDNA sample and a set of serial dilutions ranging from 1:5 to 1:40 were made from aliquots of a pool derived from all subject samples and loaded in triplicate into each plate. Cycle thresholds (C_t) were subsequently taken during the linear range of the standard curve, averaged, and normalized for each subject to the geometric mean of three housekeeping genes. This value, obtained from duplicates of each subject, was used for statistical analysis.

Statistical analysis

Multiple regression analyses were performed to determine if any dependent measures correlated with age at time of death, tissue pH or postmortem interval (PMI); additionally, secondary analyses were performed to determine sex differences between males and females in the schizophrenia group. Differences in gene expression were tested by performing analysis of variance, (ANOVA) or covariance (ANCOVA) if a potential covariate was found to be correlated with a dependent variable, using Statistica software (StatSoft, Inc., Tulsa, OK, USA). Outliers more than 4 standard deviations from the mean were excluded from statistical analysis. For all tests, $\alpha = 0.05$.

Haloperidol-treated rats

Haloperidol decanoate (28.5 mg/kg) or vehicle (sesame oil) injections were injected intramuscularly in house-paired male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) once every three weeks for nine months for a total of 12 injections. This dose was chosen based on previous reports [12-14]. The animals were sacrificed in compliance with the University of Alabama at Birmingham's IACUC regulations, and the brains immediately harvested, dissected, and stored at -80° C.

Tissue from 10 haloperidol and 10 vehicle treated animals were used for these experiments. 1 µg of RNA from rat frontal cortex was stabilized with RNAlater®-ICE (Life Technologies, Carlsbad, CA, USA) and RNA isolated using an RNeasy® Mini RNA isolation kit (Qiagen, USA). Isolated RNA was reverse transcribed using a High-Capacity cDNA RT Kit (Applied Biosystems, USA) containing random primers, dNTPs and transcriptase. Rat-specific Taqman® assays (Applied Biosystems, USA) were obtained for the cornichons, as well as for the three housekeeping genes used in the human experiments. Data were analyzed as described above.

Results

Cornichon transcript expression is upregulated in DLPFC in schizophrenia

Transcripts encoding cornichon homologs 1-4 were measured by qPCR in DLPFC from schizophrenia subjects and comparison subjects; each cornichon mRNA was detected consistent with other reports of cornichon expression in human brain (http://biogps.gnf.org). CNIH-1 mRNA expression was significantly correlated with age ($r = 0.35$, $p = 0.02$); ANCOVA revealed a main effect for diagnosis, ($F(1, 43) = 8.3$, $p =$

0.006) with CNIH-1 mRNA significantly increased in schizophrenia (Figure 1A). CNIH-2 ($F(1, 45) = 5.7$, $p = 0.02$) and CNIH-3 ($F(1, 45) = 4.6$, $p = 0.04$) transcripts were also increased in schizophrenia (Figure 1A). CNIH-4 mRNA expression was correlated with PMI, ($r = 0.33$; $p = 0.02$) but ANCOVA revealed no differences in expression between the two subject groups. No significant correlations with age, tissue pH or PMI were found for CNIH-2 and -3. Secondary analyses found no sex-specific differences in the schizophrenia group for any of the CNIH mRNAs.

Effects of antipsychotic treatment on Cornichon expression

To determine whether the changes found for cornichon mRNA expression in schizophrenia might be due to the effects of chronic antipsychotic treatment rather than the illness itself, we conducted a parallel transcript study in the frontal cortex of rats chronically treated with haloperidol. Cornichon transcript expression was not significantly altered by this treatment (Figure 1B).

Discussion

Changes occurring at the molecular level that may alter the regulation of glutamate and its receptors are potentially important for our understanding of the pathophysiology of schizophrenia. We previously reported increased TARP γ-2 (stargazin) transcript expression in DLPFC in this illness, [8] and predicted increased CNIH expression given the similarities between these families of AMPAR auxiliary proteins [4,5]. In this study, we found significantly increased transcript expression for CNIH-1, -2 and -3, but not CNIH-4. These data suggest excitatory synaptic defects

associated with AMPARs as one potential mechanism underlying glutamatergic dysfunction in this illness. It is likely that abnormal expression of these genes negatively affects trafficking, function, and stability of AMPA receptors within intracellular compartments and the synapse.

Cornichons are a multi-gene family originally described in *Drosophila* and yeast as endoplasmic reticulum (ER) chaperones that mediate forward trafficking of epidermal growth factor receptor (EGFR) ligands from the ER to the Golgi Apparatus [15-18]. Recent studies have identified the mammalian cornichon homologs as AMPAR auxiliary subunits based on their direct physical contact with AMPARs and their ability to modulate AMPAR surface expression and gating [3,5,19]. Similar to TARPs, cornichons are likely coassembled with AMPARs within the ER, but their role in AMPAR trafficking is not as clear. One study performed in rat brain found approximately 70% of AMPARs were associated with CNIH-2/3 and that these associations increased surface expression and gating of AMPARs [3]. In neurons derived from stargazer mice, however, CNIH involvement in AMPAR trafficking is minimal, suggestive of an ER chaperone role [4]. Since cornichon function appears to be closely conserved across species, [2,18] and immunostaining of CNIH-2 expressed in stargazer neurons shows colocalization with the *cis*-Golgi marker GM130, [4] CNIH may also function as an ER chaperone and mediate transit of proteins into the Golgi in human neurons.

Increased cornichon transcript expression in schizophrenia may be suggestive of abnormal intracellular localization of immature AMPARs in this illness. This could occur via accelerated AMPAR exit from the ER to the Golgi or increased retrograde trafficking from the Golgi to ER, which would not only result in retention of immature AMPARs

within these compartments, but also a subsequent decrease in AMPAR processing, function and surface expression at the postsynaptic density (PSD). In support of this model are studies examining the biosynthesis of AMPARs, demonstrating that glutamate-induced conformation changes can function as ER trafficking checkpoints to prevent immature AMPARs from being transported to the synapse [20-22]. Taken together, increased CNIH expression parallels earlier findings of altered AMPARs and AMPAR auxiliary proteins in schizophrenia brain, [1,8,9] and may be associated with abnormal glutamatergic neurotransmission in this illness.

The subjects with schizophrenia had all been treated with antipsychotic medications. To address whether these genes may be transcriptionally modified by antipsychotic treatment, we measured CNIH-1-4 mRNA expression in frontal cortex from rats treated chronically with haloperidol. We found no significant changes in CNIH gene expression in these animals, suggesting that altered cornichon gene expression in schizophrenia is illness-specific rather than an effect of antipsychotic treatment. Like CACNG2, the human TARP γ-2/stargazin gene, cornichon genes may be novel schizophrenia susceptibility markers.

Conclusion

CNIH-1, -2 and -3 are transcriptionally upregulated in DLPFC in schizophrenia. These data suggest a link between CNIH expression, AMPAR dysregulation and the pathophysiology of schizophrenia, and may reflect a new avenue for pharmacological discovery.

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Table 1. Subject characteristics.

	Schizophrenia	*Comparison*
N	25	25
Sex	9 F, 16 M	13 F, 12 M
Age	75.2 ± 12.9	75.8 ± 11.4
Tissue pH	6.5 ± 0.2	6.6 ± 0.3
PMI (hr)	17.1 ± 10.1	9.6 ± 7.0
Medication (on/off)	14/11	0/25

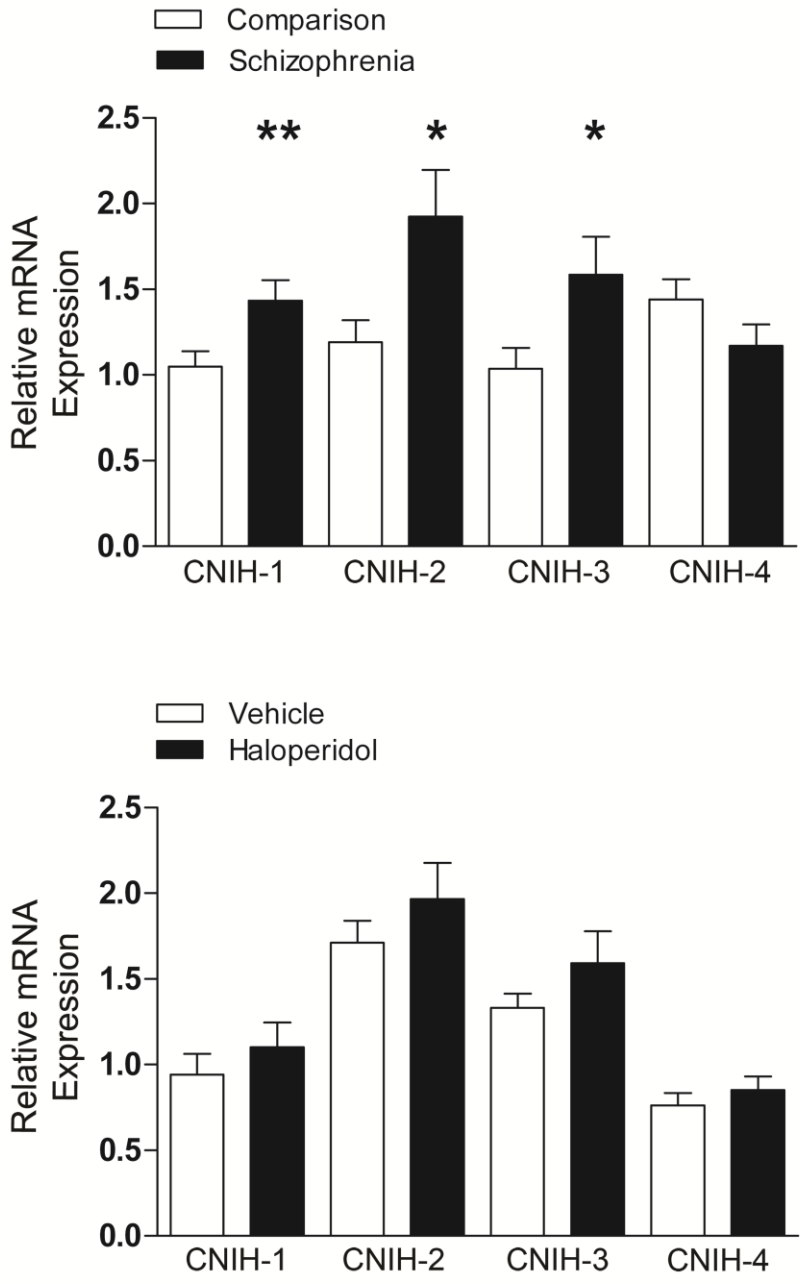
Data are means ± SD. Medication: on or off antipsychotic treatment for > 6 weeks prior to death. The cause of death for all schizophrenia and nearly all of the comparison subjects was cardiopulmonary failure/arrest.

Figure Legend:

Figure 1.

Cornichon (CNIH) transcript expression is upregulated in schizophrenia but not in haloperidol-treated rats. (A) CNIH-1-4 were assayed by quantitative comparative real-time PCR (qPCR) in dorsolateral prefrontal cortex (DLPFC) in schizophrenia (N = 25) and comparison (N = 25) subjects. Data are presented as ratio of gene of interest to the geometric mean of three housekeeping genes: peptidyl-prolyl isomerase/cyclophilin (PPIA), beta-2 microglobulin (B2M) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). CNIH-1, CNIH-2 and CNIH-3 mRNA expression are significantly increased in schizophrenia. (B) Parallel qPCR analysis of CNIH transcript expression in frontal cortex of rats treated for 9 months with haloperidol (N = 10) or vehicle (N = 10). Chronic treatment with haloperidol did not affect CNIH transcript expression in rats. Bars represent mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$.

Figure 1.


Comparison
Schizophrenia
Relative mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
**
*
*
CNIH-1
CNIH-2
CNIH-3
CNIH-4
Vehicle
Haloperidol
Relative mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
CNIH-1
CNIH-2
CNIH-3
CNIH-4

ALTERED SUBCELLULAR AMPA RECEPTOR SUBUNIT LOCALIZATION IN SCHIZOPHRENIA BRAIN

by

JANA B. DRUMMOND, VAHRAM HAROUTUNIAN, JAMES H. MEADOR-WOODRUFF

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Abstract

The glutamate hypothesis of schizophrenia suggests that altered glutamatergic transmission occurs in this illness, although precise mechanisms remain elusive. AMPA receptors (AMPARs), a subtype of ionotropic glutamate receptor, are the main facilitators of fast, excitatory neurotransmission in the brain, and changes in AMPAR number at synapses can regulate synaptic strength and plasticity. Recent evidence from our laboratory suggests that AMPAR auxiliary proteins that modulate receptor trafficking and function are abnormally expressed in schizophrenia, which we propose could lead to less AMPARs localized to excitatory synapses. To test this hypothesis, we isolated a subcellular fraction enriched for synapses from anterior cingulate cortex from 18 matched schizophrenia and comparison subject pairs, and measured by Western blot analysis AMPARs and their auxiliary modulators, transmembrane AMPAR regulatory proteins (TARPs), and cornichons (CNIHs) in this synaptic fraction. We found decreased expression of the AMPAR subunit GluA1 in the synaptic fraction in schizophrenia, which corresponded to fewer GluA1 subunits in a fraction enriched for endoplasmic reticulum (ER). We found no changes in either subcellular compartment of protein expression for any TARP or CNIH proteins, although TARP γ-2 and CNIH-2 protein expression were decreased in total tissue homogenates in schizophrenia. Ratios of the expression of GluA1 to AMPAR, TARP, and CNIH proteins in these subcellular compartments were also decreased in schizophrenia, suggesting that assembly of these subunits into complexes may be disrupted in ER, and subsequently affecting forward trafficking of GluA1-containing AMPARs to the synapse. We also evaluated NMDAR protein expression in these same subjects and found no changes in NMDAR subunit

expression in these subcellular compartments schizophrenia, but total levels of the GluN2A subunit were decreased, suggesting a shift in synaptic NMDAR subunit composition in schizophrenia. Taken together, these data provide evidence that altered regulation of AMPAR trafficking and early receptor processing may result in abnormal AMPAR localization and stoichiometry at the synapse, and may underlie glutamate dysregulation in schizophrenia.

Keywords: postmortem, anterior cingulate cortex, postsynaptic density, ER, TARP, cornichon, NMDA receptor

Introduction

Many studies in schizophrenia have converged in support of the glutamate hypothesis, which have focused on dysregulation of the NMDA subtype of glutamate receptor (Javitt, 2007). The AMPA subtypes of glutamate receptor (AMPAR) are also relevant to the pathophysiology of schizophrenia, given their central role in synaptic events including plasticity, neuronal maturation, memory formation, and synaptogenesis (Hanse *et al*, 2013; Kumar *et al*, 2002; Song and Huganir, 2002). More recently, roles of auxiliary proteins have been identified in the dynamic regulation of AMPARs following the identification of stargazin, the prototypical member of the family of transmembrane AMPAR regulatory proteins (TARPs) (Chen *et al*, 1999, 2000; Hashimoto *et al*, 1999; Tomita *et al*, 2003). TARPs directly bind to AMPAR complexes during receptor assembly in the endoplasmic reticulum (ER), and traffic the receptor complex through the secretory pathway before forming a physical anchor that docks AMPARs at synapses (Jackson and Nicoll, 2011). Genetic and pharmacological manipulations of TARPs, as well as cornichons (CNIHs), a second family of AMPAR auxiliary proteins (Brockie *et al*, 2013; Schwenk *et al*, 2009), have shown that these proteins directly control AMPAR number and activity by modulating their intracellular trafficking and biophysical channel properties at synapses (Herring *et al*, 2013; Sumioka, 2013).

We have previously reported altered protein and transcript expression of TARPs and CNIHs in anterior cingulate cortex (ACC) in schizophrenia (Beneyto and Meador-Woodruff, 2006; Drummond *et al*, 2012, 2013), and hypothesized that this may be associated with abnormal numbers of AMPARs reaching postsynaptic membranes due to altered stoichiometry of auxiliary proteins during receptor complex assembly in the ER.

To address the possibility that there are altered numbers of synaptic AMPARs in schizophrenia in the face of abnormal auxiliary protein expression, we isolated subcellular fractions from ACC to measure AMPAR, TARP, and CNIH expression in fractions enriched for a set of intracellular compartments in matched pairs of schizophrenia and comparison subjects. We found that AMPARs are abnormally localized in subcellular compartments in schizophrenia, and exhibit abnormal subunit composition at the synapse. We also found different patterns in the relationships of AMPARs and auxiliary proteins in schizophrenia that may represent a mechanistic explanation for differential AMPAR localization in schizophrenia. Finally, we found that subunit stoichiometry changes for both AMPA and NMDA receptors are present in schizophrenia that are consistent with altered intracellular trafficking of these receptors to synapses. Taken together, these results suggest an important role of altered glutamate receptor trafficking in the pathophysiology of schizophrenia.

Materials and Methods

Tissue acquisition and preparation

Tissue from the full thickness of gray matter from ACC was obtained from the Mount Sinai Medical Center Schizophrenia Brain Collection, and in compliance with the Mount Sinai School of Medicine Institutional Review Board protocol for acquisition of postmortem tissue. Criteria for patient inclusion, and details of tissue preparation have been previously described (Funk *et al*, 2012). Briefly, patients were diagnosed with schizophrenia using DSM-III-R criteria, and both schizophrenia and comparison subjects underwent antemortem clinical assessments. Following brain removal, neuropathological

assessment was completed and subjects and were excluded from study if there was evidence of neurodegenerative disease, or a history of substance abuse, death by suicide, or coma for more than 6 hours before death. Brain tissue was dissected, snap frozen in liquid nitrogen, and stored at -80° C prior to use.

Schizophrenia and comparison subjects (n=18 pairs) were matched for sex, age at time of death, and tissue pH (Table 1). Tissue blocks from each subject were homogenized and prepared simultaneously in pairs. An aliquot of each fraction was saved from each fractionation step, and protein concentration of each sample was measured using a BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA). Extraction and homogenization buffers used during fractionation contained protease inhibitor tablets (Roche Applied Science, Mannheim, Germany), and each step was performed on ice or at 4° C. All fractions were immediately frozen at -20° C before final storage at -80° C.

Subcellular fractionation for the isolation of synaptic membranes

Protocols that utilize Triton X-100 to isolate synaptic membranes from brain have been previously reported (Billa *et al*, 2010; Goebel-Goody *et al*, 2009; Hahn *et al*, 2009; Morón *et al*, 2007), as well as studies demonstrating the effectiveness of nitrogen cavitation in preserving intracellular organelle structure (Hammond *et al*, 2012; Simpson, 2010). We developed a synapse isolation technique based on these reports (Fig 1). 1.2 ml of 1X isotonic extraction buffer (Sigma-Aldrich, St. Louis, MO, USA) was added to 50 mg of ACC from each subject and dounce-homogenized for 10 passes in a glass tissue grinder with a Teflon pestle, followed by nitrogen cavitation at 450 psi for 8 min to obtain total homogenate fractions (T). These fractions were centrifuged at 700 x g for 10 min, followed by separation of the supernatant (S1) for subsequent centrifugation at 15

000 x g for 10 min. The resulting supernatant (S2) was stored at -80° C for further processing of ER membranes, and the pellet (P2) was combined with P1. This P1 + P2 fraction was re-suspended in 100 µl homogenization buffer (320 mM sucrose, 10 mM Tris (pH 7.4), 1 mM Na_3VO_4, 5 mM NaF, 1 mM EDTA, 1 mM EGTA), mixed, and treated with 8 vol 0.5% (v/v) Triton X-100 homogenization buffer for 20 min with gentle rotation before centrifugation at 32 000 x g for 20 min. The resulting supernatant (S4) was removed for processing of soluble membranes, and the remaining pellet containing insoluble, synaptic membranes (P4, or Syn), was rinsed 2x with homogenization buffer, reconstituted in 60 µl homogenization buffer with gentle mixing, and stored at -80° C for Western blot analysis. To precipitate protein in S4, 8 vol of 100% acetone (-20° C) was added and incubated overnight at -20° C. The following day, the supernatant was removed, and the remaining sample centrifuged 2x at 3 000 x g for 5 min to remove any residual acetone (S5). The final pellet (P5, or ExSyn), was air dried approximately 5 min before rinsing 2x with homogenization buffer, and reconstituted in 60 µl homogenization buffer for storage at -80° C.

Subcellular fractionation for the isolation of endoplasmic reticulum (ER)

Processing of S2 to generate ER membranes has previously been described (Hammond *et al*, 2012). Briefly, S2 was layered onto a discontinuous gradient consisting of layered 1.3 M, 1.5 M, and 2.0 M sucrose buffers (also containing 10 mM Tris, 0.1 mM EDTA, pH 7.6), and centrifuged at 126 000 x g for 70 min (Fig 1). ER membranes segregated to the top interface of the 1.3M sucrose layer, and were extracted from the gradient following removal of the top layer (S3). 3 ml of 1X MTE/PMSF buffer (270 mM

D-mannitol, 10 mM Tris, 0.1 mM EDTA, 200 mM PMSF, pH 7.4) was added to ER membranes and mixed by inversion. The samples were then centrifuged at 126 000 x g for 45 min to obtain pelleted ER (P3). These pellets were rinsed 2x with homogenization buffer before being reconstituted in 50 µl phosphate-buffered saline (PBS) buffer (pH 7.4) containing 0.5% Triton X-100, and stored at -80° C.

Electron Microscopy (EM) and Western blot analysis (WB)

For validation of isolated PSDs, Syn and ExSyn fraction samples were thin sectioned and post-stained with uranyl acetate and lead citrate for EM imaging. To quantitate protein expression, samples containing 10 µg protein and 6X β-mercaptoethanol buffer were denatured at 70° C for 10 min, and allowed to cool before loading onto 4-12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA, USA), 1 subject pair/gel, for electrophoresis. Protein was transferred to either polyvinylidene fluoride (PVDF) or nitrocellulose membranes (Invitrogen, USA) by semi-dry transfer (Bio-Rad, Hercules, CA, USA).

Blots were blocked 1 hr in either Li-COR blocking buffer (Li-COR Biosciences, Lincoln, NE, USA), or buffer containing 2% (w/v) bovine serum albumin (BSA) and phosphate-buffered saline (PBS; pH 7.4) at room temperature (Table S1). Commercially available primary antisera targeting PSD95, JM4, synaptophysin (Syp), β-tubulin, VDAC, H3, AMPA receptor subunits, NMDA receptor subunits, TARPs and CNIHs were individually optimized for use in these studies (SI Table 1), visualized using IR-dye labeled rabbit or mouse secondary antibodies, and scanned with a Li-COR Odyssey laser-based image detection system using Odyssey 3.0 analytical software (Li-COR

Biosciences, USA). Blots were washed in 0.05% Tween-20/PBS, 2x 5 min each, following each primary and secondary antibody incubation, and rinsed with distilled water prior to imaging.

Statistical analyses

For all dependent measures, protein levels are presented as signal intensity normalized to the signal intensity of a fraction-specific marker: β-tubulin for total homogenates, PSD95 for synaptic membranes, and JM4 for ER. Each fraction-specific marker was analyzed both as original data or normalized to β-tubulin, and no changes were found between schizophrenia and comparison subjects for any of these normalization markers.

All statistical analyses were performed using GraphPad Prism 6 (La Jolla, CA, USA). Each data set was assessed for normal distribution using the D'Agostino-Pearson omnibus test, and differences between subject pairs were evaluated using paired t tests (t). If data were not normally distributed, values were logarithmically transformed, reassessed for normal distribution, and analyzed using either paired t tests on the transformed data (t*) if normally distributed, or Wilcoxon matched-pairs signed rank tests (W) on the original data if logarithmic transformation failed to result in normally distributed data. All p values reported from Wilcoxon analyses are exact values rather than approximations. For all statistical tests, alpha = 0.05.

Post hoc analyses of schizophrenia subjects were performed to examine potential effects of antipsychotic treatment on dependent variables significantly different between schizophrenia and comparison subjects. Assessment of treatment status was defined as

'on,' for patients with schizophrenia receiving antipsychotic medications at time of death, and 'off,' for those not taking antipsychotic medications 6 weeks or more prior to death. For these tests, treatment status of on (n = 11) or off (n = 7) was the independent variable. No significant differences between treatment groups for any dependent measures were found.

Results

Isolation of synapses from postmortem brain

To determine if AMPARs are abnormally localized in synapses in schizophrenia, we adapted a method that capitalizes on synaptic membrane insolubility in Triton X-100 (Goebel-Goody *et al*, 2009), to enrich synapses from brain (Fig 1). Consistent with previous reports (Carlin *et al*, 1980; Cho *et al*, 1992; Ferrario *et al*, 2011; Goebel *et al*, 2005; Goebel-Goody *et al*, 2009), synaptic (Syn) and corresponding extrasynaptic membrane-containing (ExSyn) fractions showed specific expression of the postsynaptic density marker PSD95, and presynaptic marker synaptophysin (Syp), respectively (Fig 2A). EM analyses found no detectible synapses in ExSyn (Fig 2B), and enrichment of synaptic membranes in Syn (Fig 2C-H). No evidence of other intact organelles was found by EM in either fraction.

Subcellular localization of AMPAR subunits in schizophrenia

We measured AMPAR subunit protein expression in the synaptic fraction, and predicted decreased levels in schizophrenia given our previous findings of decreased protein levels of several TARPs in ACC (Drummond *et al*, 2013). GluA1 protein levels

in Syn were decreased in schizophrenia (t* (17) = 2.3, p = 0.031) (Fig 3A and Table 2). Other AMPAR subunits were not changed in this fraction (Table 2).

A potential explanation for decreased synaptic levels of GluA1 in schizophrenia is reduced forward trafficking of GluA1-containing AMPAR complexes from ER to postsynaptic membranes, resulting in accumulation in ER. We thus measured GluA1 protein expression in ER, and found it decreased in schizophrenia (t* (17) = 2.9, p = 0.0097) (Fig 3B and Table 2). No other changes in AMPAR subunits were found (Table 2). Since we detected less ER GluA1 in schizophrenia, this suggests that GluA1 subunits are not being retained in ER, but rather are being more rapidly removed from this compartment.

As previous studies from our laboratory have found minimal changes in AMPAR transcript and protein levels from multiple cortical regions in schizophrenia (Beneyto and Meador-Woodruff, 2006; Beneyto *et al*, 2007; Healy *et al*, 1998; Mueller *et al*, 2004; Tucholski *et al*, 2013), we predicted that the subcellular changes we observed for GluA1 expression would not simply be due to decreased total GluA1 protein in tissue homogenates. Surprisingly, we found that total GluA1 protein expression was decreased in schizophrenia (t (17) = 2.2, p = 0.044) (Fig 3C and Table 2). We next calculated the ratio of GluA1/A2 and found decreases in homogenates (W, p = 0.002) and in Syn (t (17) = 3.1, p = 0.006), but no change in ER in schizophrenia (Table 3). We also found that the ratio of GluA1/A3 was unchanged in homogenates and Syn, but decreased in ER (t* (15) = 2.9, p = 0.011) in schizophrenia (Table 3). These data suggest abnormal trafficking of GluA1 to synapses with a corresponding shift in synaptic AMPAR subunit stoichiometry.

Subcellular localization of AMPAR auxiliary proteins in schizophrenia

AMPAR auxiliary proteins mediate targeting and forward trafficking of AMPARs to synapses (Jackson and Nicoll, 2011), and TARPs additionally serve a stabilizing role by binding directly to the postsynaptic density scaffolding protein PSD95 (Chen *et al*, 2000). We next asked if reduced ER and Syn expression of GluA1 might be associated with altered availability of TARP or CNIH proteins to properly regulate trafficking as well as synaptic targeting and localization. We found no changes in subcellular expression for any of the TARP or CNIH proteins (Table 4). Expression of PSD95 in these fractions was also unchanged. We also measured expression levels of TARP and CNIH proteins in total tissue homogenates, and consistent with our previous results (Drummond *et al*, 2012, 2013), found decreased TARP γ-2 ($t(17) = 2.6$, $p = 0.019$) and CNIH-2 (W, $p = 0.044$) in schizophrenia (Table 4). We next addressed the stoichiometry of these proteins and GluA1. In ER, we found decreased ratios of GluA1 to γ-2, γ-4, CNIH-1, CNIH-2, and CNIH-4 in schizophrenia (Table 5). Ratios of Syn expression of GluA1 to γ-3, γ-4, γ-8, and CNIH-4 were also decreased in schizophrenia, and GluA1 to γ-7 was increased (Table 5).

Subcellular localization of NMDA receptor (NMDAR) subunits

Relative loss of synaptic GluA1-containing AMPARs can result in decreased synaptic neurotransmission (Herring *et al*, 2013; Hoffman *et al*, 2002; Schmitt *et al*, 2005; Zamanillo, 1999). Thus, we asked if NMDARs were also abnormally localized in these subjects. We measured GluN1, GluN2A, and GluN2B protein expression and found no changes in subcellular expression, although total protein expression of GluN2A was

decreased (t (17) = 3.4, p = 0.004) in schizophrenia (Table 6). We also found a decrease of the ratio GluN1/2B in Syn in schizophrenia (t (17) = 2.2, p = 0.038), but GluN1/2A was unchanged in this fraction (Table 3). The ratios of GluN1/2A and GluN1/2B were not changed in homogenates (Table 3), suggesting a shift in synaptic subunit composition similar to what we found for AMPARs.

Discussion

In this study, we found that GluA1 is decreased in schizophrenia in subcellular fractions enriched for postsynaptic densities, in fractions enriched for ER, and in total tissue homogenates. These findings support a role for GluA1 in the synaptic pathophysiology of schizophrenia, and expand on previous studies of abnormalities of cortical AMPAR protein expression in this illness (Breese *et al*, 1995; Corti *et al*, 2011; Hammond *et al*, 2010, 2012; Tucholski *et al*, 2013), and studies of transgenic GluA1 mice that exhibit phenotypic features similar to some aspects of schizophrenia (Inta *et al*, 2010; Wiedholz *et al*, 2008; Zamanillo, 1999).

Since AMPAR complexes are first assembled and modified in ER before being trafficked to the synapse (Bredt and Nicoll, 2003), the ER may be the initial site of synaptic AMPAR dysregulation in schizophrenia. Consistent with this, we have reported abnormal N-linked glycosylation of GluA2 in schizophrenia which we speculated was consistent with accelerated ER exit of this subunit (Tucholski *et al*, 2013). While we found in the current study that GluA2 subcellular localization was unchanged in schizophrenia, the ratio of GluA1/A2 is consistent with this model, reflecting a shift to more GluA2-containing receptor complexes at synapses in schizophrenia. We did not

observe changes in ER in schizophrenia of either GluA2 expression or the ratio of GluA1/A2, which may reflect differential subunit trafficking mechanisms that are activity-specific. GluA2 primarily associates with GluA3 in ER, while synaptic GluA2 is present in complexes containing GluA1 and to a lesser extent GluA3 (Greger *et al*, 2002; Lu *et al*, 2009; Wenthold *et al*, 1996). Unlike GluA1, insertion of GluA2 at the synapse is not activity-dependent, but rather is driven by constitutive and continual trafficking pathways thought to underlie homeostatic regulation (Greger *et al*, 2002; Hayashi *et al*, 2000; Passafaro *et al*, 2001; Shi *et al*, 2001).

Recent studies have highlighted the importance of AMPAR auxiliary proteins in AMPAR regulation and trafficking (Bedoukian *et al*, 2006; Jackson and Nicoll, 2011; Vandenberghe *et al*, 2005; Ziff, 2007), and their abnormal expression in schizophrenia (Drummond *et al*, 2012, 2013). In this study, although we did not find abnormal subcellular expression of TARP and CNIH proteins in schizophrenia, the stoichiometry of GluA1 to TARP and CNIH was altered in schizophrenia in both ER and synaptic fractions, suggesting they may contribute to GluA1 trafficking abnormalities through differential assembly with GluA1-containing receptor complexes. Decreased ratios in ER of GluA1 to CNIHs in schizophrenia may specifically reflect dysregulation of the role of these accessory proteins as ER chaperones (Castro *et al*, 2007; Shi *et al*, 2010). In light of recent evidence that suggests auxiliary protein-mediated trafficking of AMPARs may occur independently from their other modulatory effects on receptor targeting and biophysical properties (Bedoukian *et al*, 2008), the effects of altered proportions of GluA1 to auxiliary proteins in ER may be two-fold. First, this may initially restrict GluA1 ER retention thereby promoting receptor misassembly and accelerated ER exit. As

a secondary consequence, auxiliary proteins may then contribute to abnormal forward trafficking of AMPARs and in turn alterations at the synapse.

Although we did not find altered subcellular expression of NMDARs in schizophrenia, total levels of GluN2A were decreased and subunit stoichiometry altered, both consistent with dysfunction and altered trafficking of NMDARs in these subjects. We have previously reported data that we suggested are consistent with accelerated ER exit and forward trafficking of NMDARs in schizophrenia. We have previously found that NMDAR trafficking proteins including CASK and Veli-3 were decreased in ACC in schizophrenia (Kristiansen *et al*, 2010). We also found that the GluN1-C2' splice variant was increased in ACC in schizophrenia, while the GluN1-C2 variant was not changed (Kristiansen *et al*, 2006). This is intriguing given that the C2' variant has been shown to accelerate forward trafficking of NMDARs from the ER in an activity-dependent manner (Mu *et al*, 2003). Whether the GluA1 changes we observed in subcellular fractions in schizophrenia are consistent with auxiliary protein-dependent mechanisms, or are due to a more generalized ER dysfunction, these findings are consistent with accelerated ER exit and subsequent abnormal forward trafficking of glutamate receptors in schizophrenia.

An alternative possibility is that subcellular changes in GluA1 expression may be due to decreased protein synthesis in ER rather than reflecting trafficking abnormalities, since NMDAR antagonists have been shown to alter the rate of dendritic GluA1 synthesis (Ju *et al*, 2004; Sutton *et al*, 2006). However, minimal changes in AMPAR transcript levels have been previously reported in schizophrenia (reviewed in Rubio *et al*, 2012), suggesting that decreased GluA1 is not due to decreased gene expression. Since we found decreased GluA1 protein expression and a decreased ratio of GluA1/A2 in tissue

homogenates, we also measured the ratio GluA1/A2 in the subcellular fractions and found it decreased in the synaptic fraction but not changed in ER. These findings are consistent with decreased expression of GluA1 in ER being more likely due to accelerated forward trafficking rather than to altered synthesis. Future studies examining whether abnormalities in GluA1 localization might be due to changes in local dendritic synthesis or other distal dysregulation would be intriguing given the importance of rapid GluA1 availability at proximal sites of exocytosis for cellular response to synaptic stimuli.

Our current findings are consistent with altered glutamatergic synaptic activity in schizophrenia. The glutamate hypothesis of schizophrenia posits decreased NMDAR-mediated neurotransmission in this illness, an area of much recent focus. Since activation of AMPARs is required for removal of the magnesium block and subsequent activation of NMDARs (Song and Huganir, 2002), NMDAR hypofunction could be secondary to dysregulated AMPARs in schizophrenia. On the other hand, animal studies that have examined reduced NMDAR function have shown that this can cause paradoxical strengthening of synapses, as well as increased numbers of functional synapses containing AMPARs (Hanse *et al*, 2013; Herring *et al*, 2013; Myers *et al*, 1999). AMPAR activation in the face of NMDAR inactivity can promote internalization of AMPARs, and specifically target them to endosomes for lysosomal degradation (Ehlers, 2000). Our finding of decreased GluA1 in the synaptic fraction in schizophrenia could reflect increased synaptic strength and subsequent endocytosis of GluA1, leaving fewer GluA1-containing receptors in this synaptic compartment, but more GluA1 in endosomes and targeted for degradation. Consistent with this model is our previous report of

increased GluA1 protein expression in an early endosome fraction isolated from dorsolateral prefrontal cortex in schizophrenia (Hammond *et al*, 2010), and a recent proteomic study on isolated synapses from ACC finding alterations in schizophrenia of proteins involved in the regulation of endocytosis, NMDARs, calcium signaling, and long-term potentiation (Föcking *et al*, 2014).

There are limitations to this work that are common to all studies in postmortem brain in psychiatric illnesses. A challenge in studies of schizophrenia is that nearly all subjects have been treated with chronic antipsychotic medications. To begin to address this confound, we performed post hoc analyses for schizophrenia subjects on or off antipsychotic treatment at the time of death, and found no differences in any dependent measures for which we found changes in the entire schizophrenia group. Although this analysis is relatively underpowered (on, n=11; off, n=7), it suggests that changes we observed may not be due to antipsychotic treatment, but rather associated with the illness. Previous studies have reported no changes in GluA1, TARP, or CNIH expression in animals treated chronically with haloperidol (Drummond *et al*, 2012, 2013; Eastwood *et al*, 1996; O'Connor *et al*, 2007). To address the possibility that changes we observed in GluA1 expression were due to differential protein loss from the fractionation protocol, we calculated percentage recovery of GluA1 from all fractions, and found that we could account for on average 84% of starting GluA1 protein for all subjects, suggesting that the vast majority of protein is recovered after all fractionation steps.

In summary, these data suggest accelerated forward trafficking of GluA1 in schizophrenia, and support a model of both AMPAR and NMDAR dysfunction in this illness that may be due, in part, to abnormal receptor assembly in the ER. Since our

findings were specific to the GluA1 subtype of AMPAR, this may be an indication that cellular machinery associated with the regulation of this subunit during activity-driven events is altered in this illness. Whether decreased synaptic GluA1 is due to accelerated ER exit and abnormal subunit assembly, or is a secondary consequence to altered synaptic activity, a key finding is that regardless of the underlying mechanism, there are less GluA1 subunits of the AMPAR in the synapse in schizophrenia.

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Figure legends

Figure 1.

Subcellular fractionation technique used for the isolation of subcellular compartments in postmortem brain. 50 mg of anterior cingulate cortex (ACC) was homogenized and subsequently fractionated with a series of centrifugation, sucrose gradient ultracentrifugation, and Triton X-100 separation steps to obtain final fractions enriched for endoplasmic reticulum (ER), synapses (Syn), and extrasynaptic membranes (ExSyn). P, pellet; S, supernatant.

Figure 2.

Validation of isolated synaptic membranes. (A.) Western blot analysis of the synaptic, Triton X-100-insoluble fraction (Syn) showed specific and enriched expression of the postsynaptic density marker PSD95, no expression of the presynaptic marker synaptophysin (Syp), and minimal expression of markers for endoplasmic reticulum (ER) (JM4), mitochondria (VDAC), and nuclei (H3) when compared to Total (T) or preliminary P1 and P2 crude fractions. In contrast, the Triton X-100-soluble fraction containing extrasynaptic membranes (ExSyn) exhibited no detectible PSD95 expression, yet showed AMPAR localization (GluA2 subunit) and enrichment of synaptophysin. ER fractions exhibited enrichment of JM4. (B-D.) Electron microscopy (EM) analyses revealed that no intact synapses were visualized in ExSyn (B, 1 650x magnification), but enrichment of synapses was seen in Syn (C, 2 700x magnification, arrowheads indicate synaptic membranes; D, 3 200x magnification of top inset). (E-H.) Representative images of isolated synapses from Syn at 11 000x magnification. No intact structures of other intracellular organelles were identified by EM in either the Syn or ExSyn fractions.

Figure 3.

Subcellular expression of the GluA1 AMPA receptor subunit in schizophrenia. AMPAR subunit expression was measured by Western blot analysis in subcellular compartments using commercially available antibodies in matched schizophrenia and comparison subjects. Data are expressed as the signal intensity of GluA1 normalized to the signal intensity of a corresponding fraction-specific marker. GluA1 expression was significantly decreased in synaptic membranes (A), and ER (B) in schizophrenia. GluA1 was also decreased in total tissue homogenates in schizophrenia (C). *$p < 0.05$; **$p < 0.01$; Scz, schizophrenia; Comp, comparison.

Table 1. Subject pairs

Pair	Diagnosis	Sex	Age	pH	PMI	Rx	Pair	Diagnosis	Sex	Age	pH	PMI	Rx
1	Comparison	M	64	6.7	15	---	10	Comparison	M	93	6.28	4	---
	Schizophrenia	M	73	6.5	8	on		Schizophrenia	M	92	6.67	18	on
2	Comparison	F	74	6.3	5	---	11	Comparison	F	79	6.38	10	---
	Schizophrenia	F	70	6.4	12	on		Schizophrenia	F	79	6.8	10	on
3	Comparison	F	73	7	3	---	12	Comparison	F	81	6.37	19	---
	Schizophrenia	F	71	6.6	6	on		Schizophrenia	F	81	6.67	15	off
4	Comparison	M	73	6.2	15	---	13	Comparison	M	84	6.83	11	---
	Schizophrenia	M	73	6.3	12	on		Schizophrenia	M	84	6.71	18	on
5	Comparison	M	69	6.7	7	---	14	Comparison	F	96	6.3	5	---
	Schizophrenia	M	70	6.4	7	off		Schizophrenia	F	89	6.2	10	off
6	Comparison	M	59	6.7	20	---	15	Comparison	M	95	6.53	4	---
	Schizophrenia	M	57	6.4	21	on		Schizophrenia	M	97	6.5	9	on
7	Comparison	F	60	6.8	15	---	16	Comparison	F	66	6.85	23	---
	Schizophrenia	F	62	6.7	24	on		Schizophrenia	F	77	6.01	10	off
8	Comparison	M	76	6.3	3	---	17	Comparison	M	68	6.55	3	---
	Schizophrenia	M	76	6.7	17	off		Schizophrenia	M	70	6.36	17	off
9	Comparison	F	76	6.5	4	---	18	Comparison	M	75	6.43	5	---
	Schizophrenia	F	75	6.5	22	off		Schizophrenia	M	71	6.51	10	on

Abbreviations: pH, tissue pH; PMI, postmortem interval (hrs), Rx, treatment with antipsychotic medication: on, receiving treatment at time of death; off, no treatment for 6 weeks or more prior to death.

Table 2. AMPAR protein expression in subcellular fractions

Protein	Schizophrenia means ± SEM or [95% CI]	Comparison means ± SEM or [95% CI]	Pairs (n)	Test statistic	P
GluA1					
Syn GluA1	0.22 [0.13, 0.36]	0.35 [0.27, 0.45]	18	t* = 2.3	0.031
ER GluA1	0.01 [0.001, 0.02]	0.05 [0.02, 0.12]	18	t* = 2.9	0.0097
Total GluA1	1.03 ± 0.14	1.71 ± 0.29	18	t = 2.2	0.044
GluA2					
Syn GluA2	0.17 [0.10, 0.30]	0.17 [0.12, 0.23]	18	t* = 0.1	ns
ER GluA2	0.06 [0.05, 0.08]	0.10 [0.06, 0.16]	18	t* = 1.7	ns
Total GluA2	1.18 ± 0.12	1.05 ± 0.11	18	t = 0.8	ns
GluA3					
Syn GluA3	1.26 ± 0.17	1.18 ± 0.17	18	t = 0.3	ns
ER GluA3	0.41, [0.27, 0.61]	0.27 [0.17, 0.42]	15	t* = 1.4	ns
Total GluA3	0.27 ± 0.02	0.24 ± 0.03	16	t = 1.1	ns
GluA4					
Syn GluA4	0.04 [0.03, 0.06]	0.03 [0.02, 0.05]	18	t* = 1.1	ns
ER GluA4	0.11 [0.07, 0.18]	0.10 [0.07, 0.15]	16	t* = 0.4	ns
Total GluA4	0.70 [0.56, 0.87]	0.64 [0.51, 0.80]	16	t* = 0.9	ns

Abbreviations: Syn, synaptic enriched subcellular fraction; ER, endoplasmic reticulum enriched subcellular fraction; Total, tissue homogenates. Statistics on lognormal data are designated by t*, and statistics for these sets are presented as the geometric mean with 95% confidence intervals. Data are presented as the signal intensity of each dependent measure normalized to the signal intensity of a fraction-specific marker: PSD95 for Syn, JM4 for ER, and β-tubulin for total. For all tests, alpha = 0.05.

Table 3. Glutamate receptor subunit stoichiometry in homogenates and subcellular fractions

Ratio	Schizophrenia means ± SEM or [95% CI]	Comparison means ± SEM or [95% CI]	Pair (n)	Test statistic	P
AMPAR					
GluA1 Syn / GluA2 Syn	1.44 ± 0.16	2.25 ± 0.21	18	t = 3.1	0.006
GluA1 ER / GluA2 ER	0.58 ± 0.14	23.01 ± 22.18	18	W	ns
GluA1 Total / GluA2 Total	0.94 ± 0.12	1.71 ± 0.26	18	W	0.002
GluA1 Syn / GluA3 Syn	0.33 ± 0.09	0.50 ± 0.11	18	t = 1.7	ns
GluA1 ER / GluA3 ER	0.02 [0.01, 0.10]	0.13 [0.04, 0.39]	16	t* = 2.9	0.011
GluA1 Total / GluA3 Total	3.17 [2.00, 5.05]	5.63 [3.24, 9.78]	16	t* = 1.8	ns
NMDAR					
GluN1 Syn / GluN2A Syn	5.37 ± 1.21	4.47 ± 0.53	18	W	ns
GluN1 Total / GluN2A Total	0.78 ± 0.08	0.62 ± 0.15	16	t = 1.0	ns
GluN1 Syn / GluN2B Syn	16.25 ± 2.02	20.60 ± 2.30	18	t = 2.2	0.038
GluN1 Total / GluN2B Total	3.97 ± 1.09	2.81 ± 0.64	15	t = 0.2	ns

Abbreviations: Syn, synaptic enriched subcellular fraction; ER, endoplasmic reticulum enriched subcellular fraction; Total, tissue homogenates; W, Wilcoxon matched-pairs signed rank test. Statistics on lognormal data are designated by t*, and statistics for these sets are presented as the geometric mean with 95% confidence intervals. Data are presented as the signal intensity of each dependent measure normalized to the signal intensity of a fraction-specific marker: PSD95 for Syn, JM4 for ER, and β-tubulin for total. For all tests, alpha = 0.05.

Table 4. TARP and CNIH protein expression in subcellular fractions

Protein	Schizophrenia means ± SEM or [95% CI]	Comparison means ± SEM or [95% CI]	Pairs (n)	Test statistic	P
TARP γ-2					
Syn γ-2	0.09 ± 0.05	0.05 ± 0.01	18	W	ns
ER γ-2	0.01 ± 0.002	0.12 ± 0.08	18	W	ns
Total γ-2	0.12 ± 0.03	0.22 ± 0.03	18	t = 2.6	0.019
TARP γ-3					
Syn γ-3	0.37 ± 0.08	0.29 ± 0.05	18	W	ns
ER γ-3	0.28 ± 0.01	0.30 ± 0.02	16	t = 0.4	ns
Total γ-3	0.71 ± 0.08	0.80 ± 0.12	16	t = 0.7	ns
TARP γ-4					
Syn γ-4	30.02 ± 28.83	0.54 ± 0.18	18	W	ns
ER γ-4	27.74 [20.91, 36.81]	25.73 [18.08, 36.60]	16	t* = 0.5	ns
Total γ-4	1.93 ± 0.06	2.06 ± 0.17	16	W	ns
TARP γ-5					
Syn γ-5	0.43 ± 0.23	0.10 ± 0.02	18	W	ns
Total γ-5	0.06 ± 0.01	0.12 ± 0.05	9	t = 0.3	ns
TARP γ-7					
Syn γ-7	0.74 [0.36, 1.51]	0.51 [0.30, 0.88]	18	t* = 0.9	ns
Total γ-7	0.01 ± 0.002	0.01 ± 0.003	9	t = 0.02	ns
TARP γ-8					
Syn γ-8	0.19 ± 0.06	0.10 ± 0.02	18	W	ns
ER γ-8	0.18 ± 0.06	0.23 ± 0.08	16	t = 1.0	ns
Total γ-8	1.11 ± 0.18	1.21 ± 0.14	16	t = 0.6	ns
CNIH-1					
Syn CNIH-1	0.80 ± 0.49	0.64 ± 0.29	18	W	ns
ER CNIH-1	0.03 ± 0.005	0.14 ± 0.07	18	W	ns
Total CNIH-1	0.14 ± 0.04	0.12 ± 0.03	9	W	ns
CNIH-2					
Syn CNIH-2	0.11 ± 0.05	0.08 ± 0.01	18	W	ns
ER CNIH-2	0.09 ± 0.01	0.10 ± 0.02	15	t = 0.6	ns
Total CNIH-2	0.09 ± 0.01	0.14 ± 0.02	16	W	0.044
CNIH-3					
Syn CNIH-3	0.11 [0.06, 0.19]	0.12 [0.06, 0.24]	18	t* = 0.2	ns
Total CNIH-3	0.02 [0.01, 0.03]	0.02 [0.009, 0.03]	9	t* = 1.7	ns
CNIH-4					
Syn CNIH-4	0.18 ± 0.12	0.06 ± 0.01	18	W	ns
ER CNIH-4	0.01 ± 0.002	0.13 ± 0.08	18	W	ns
Total CNIH-4	0.09 [0.07, 0.13]	0.06 [0.03, 0.15]	18	t* = 1.0	ns

Abbreviations: Syn, synaptic enriched subcellular fraction; ER, endoplasmic reticulum enriched subcellular fraction; Total, tissue homogenates; W, Wilcoxon matched-pairs

signed rank test. Statistics on lognormal data are designated by t*, and statistics for these sets are presented as the geometric mean with 95% confidence intervals. Data are presented as the signal intensity normalized to the signal intensity of a fraction-specific marker: PSD95 for Syn, JM4 for ER, and β-tubulin for total. γ-5, γ-7, and CNIH-3 were not quantifiable in ER. For all tests, alpha = 0.05.

Table 5. GluA1-associated auxiliary protein stoichiometry in subcellular fractions

Ratio	Schizophrenia means ± SEM or [95% CI]	Comparison means ± SEM or [95% CI]	Pairs (n)	Test statistic	P
Syn : Syn					
GluA1 / TARP γ-2	5.63 [3.78, 8.37]	9.23 [6.87, 12.39]	18	t* = 2.0	ns
GluA1 / TARP γ-3	1.60 ± 0.46	2.40 ± 0.56	18	W	0.014
GluA1 / TARP γ-4	1.00 ± 0.31	4.34 ± 1.74	18	W	0.030
GluA1 / TARP γ-5	206.80 ± 204.40	247.10 ± 239.5	18	W	ns
GluA1 / TARP γ-7	1.20 ± 0.88	1.00 ± 0.19	18	W	0.010
GluA1 / TARP γ-8	1.58 [1.08, 2.30]	4.20 [2.66, 6.64]	18	t* = 4.7	0.0002
GluA1 / CNIH-1	117.50 ± 116.00	2.36 ± 0.53	18	W	ns
GluA1 / CNIH-2	8.21 ± 2.25	157.90 ± 150.80	18	W	ns
GluA1 / CNIH-3	2.01 [1.34, 3.02]	2.96 [1.44, 6.10]	18	t* = 0.9	ns
GluA1 / CNIH-4	4.29 [3.01, 6.11]	8.02 [5.14, 12.49]	17	t* = 2.3	0.037
ER : ER					
GluA1 / TARP γ-2	0.65 [0.13, 3.21]	4.62 [1.74, 12.23]	17	t* = 2.3	0.038
GluA1 / TARP γ-3	0.15 ± 0.04	0.21 ± 0.04	16	t = 1.2	ns
GluA1 / TARP γ-4	0.0002 [0.00004, 0.001]	0.001 [0.0005, 0.004]	16	t* = 2.4	0.027
GluA1 / TARP γ-8	0.28 [0.04, 2.08]	0.39 [0.17, 0.95]	16	t* = 0.4	ns
GluA1 / CNIH-1	0.28 [0.06, 1.26]	3.14 [1.38, 7.13]	18	t* = 2.8	0.013
GluA1 / CNIH-2	0.07 [0.01, 0.38]	0.63 [0.23, 1.76]	16	t* = 2.2	0.042
GluA1 / CNIH-4	0.51 [0.10, 2.60]	3.17 [1.23, 8.15]	16	t* = 2.3	0.039

Abbreviations: Syn, synaptic enriched subcellular fraction; ER, endoplasmic reticulum enriched subcellular fraction; W, Wilcoxon matched-pairs signed rank test. Statistics on lognormal data are designated by t*, and statistics for these sets are presented as the geometric mean with 95% confidence intervals. Data are presented as the signal intensity of each dependent measure normalized to the signal intensity of a fraction-specific marker: PSD95 for Syn and JM4 for ER. γ-5, γ-7, and CNIH-3 were not quantifiable in ER. For all tests, alpha = 0.05.

Table 6. NMDAR protein expression in subcellular fractions

Protein	Schizophrenia means ± SEM or [95% CI]	Comparison means ± SEM or [95% CI]	Pairs (n)	Test statistic	P
GluN1					
Syn GluN1	1.20 ± 0.70	0.64 ± 0.07	18	W	ns
ER GluN1	0.04 ± 0.006	0.04 ± 0.007	16	t = 0.3	ns
Total GluN1	0.15 ± 0.02	0.13 ± 0.02	16	t = 1.3	ns
GluN2A					
Syn GluN2A	0.21 ± 0.07	0.15 ± 0.01	18	W	ns
ER GluN2A	0.01 [0.005, 0.02]	0.02 [0.01, 0.05]	18	t* = 1.8	ns
Total GluN2A	0.22 ± 0.02	0.35 ± 0.05	18	t = 3.4	0.004
GluN2B					
Syn GluN2B	0.04 [0.02, 0.08]	0.03 [0.02, 0.04]	18	t* = 0.9	ns
ER GluN2B	0.009 [0.005, 0.01]	0.005 [0.003, 0.009]	18	t* = 1.9	ns
Total GluN2B	0.07 ± 0.02	0.08 ± 0.01	18	t = 0.5	ns

Abbreviations: Syn, synaptic enriched subcellular fraction; ER, endoplasmic reticulum enriched subcellular fraction; Total, tissue homogenates; W, Wilcoxon matched-pairs signed rank test. Statistics on lognormal data are designated by t*, and statistics for these sets are presented as the geometric mean with 95% confidence intervals. Data are presented as the signal intensity of each dependent measure normalized to the signal intensity of a fraction-specific marker: PSD95 for Syn, JM4 for ER, and β-tubulin for total. For all tests, alpha = 0.05.

Figure 1.

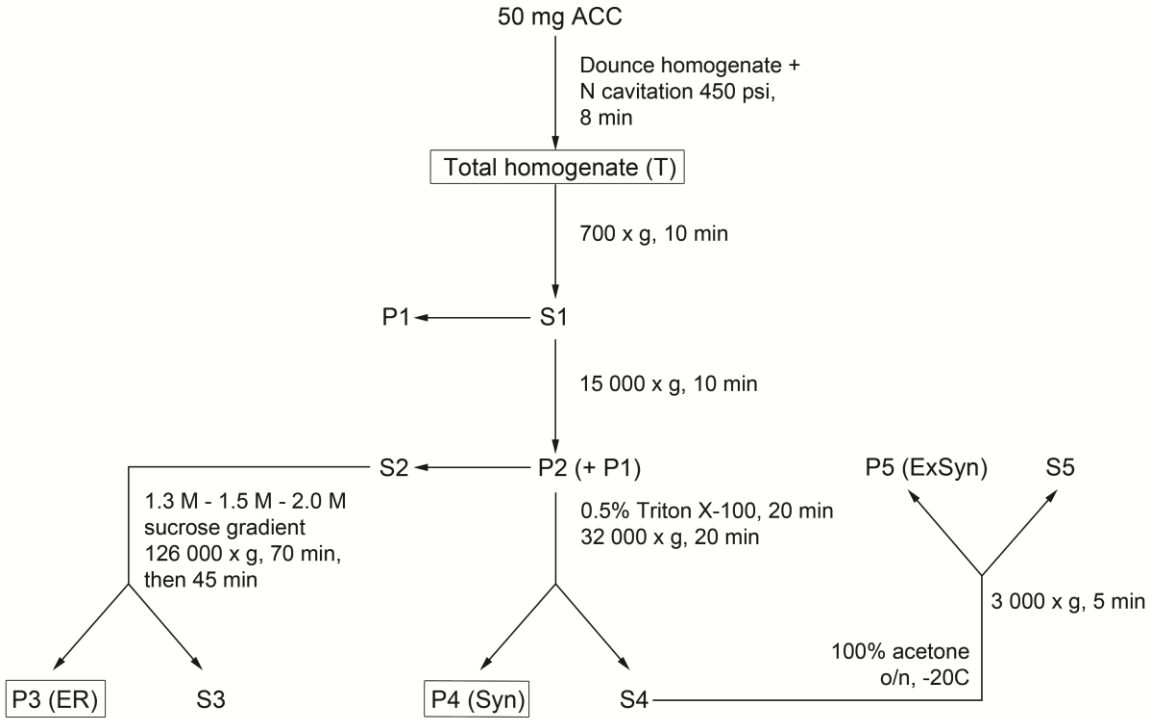

50 mg ACC
Dounce homogenate +
N cavitation 450 psi,
8 min
Total homogenate (T)
700 x g, 10 min
P1
S1
15 000 x g, 10 min
S2
P2 (+ P1)
P5 (ExSyn)
S5
1.3 M - 1.5 M - 2.0 M
sucrose gradient
126 000 x g, 70 min,
then 45 min
0.5% Triton X-100, 20 min
32 000 x g, 20 min
3 000 x g, 5 min
100% acetone
o/n, -20C
P3 (ER)
S3
P4 (Syn)
S4

Figure 2.

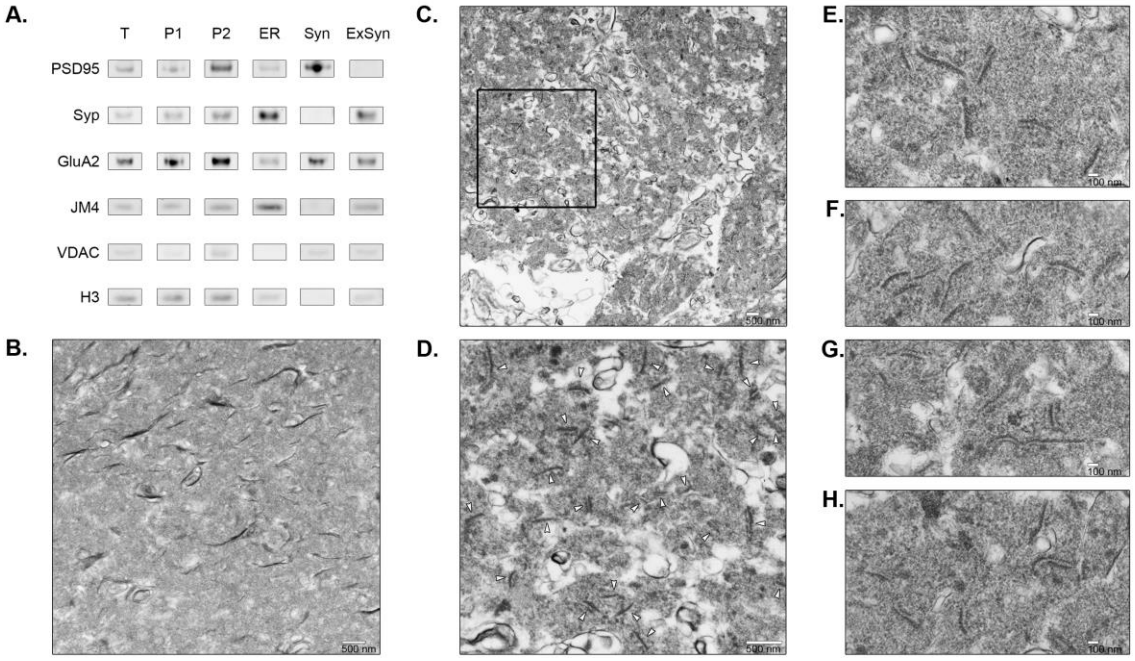
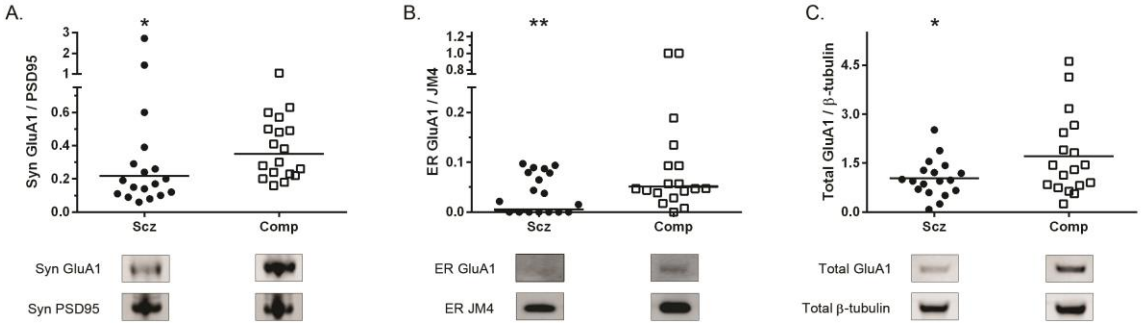

A.
T
P1
P2
ER
Syn
ExSyn
PSD95
Syp
GluA2
JM4
VDAC
H3
B.
C.
D.
E.
F.
G.
H.
500 nm
100 nm

Figure 3.


A.
*
Syn GluA1 / PSD95
3
2
1
0.6
0.4
0.2
0.0
Scz
Comp
Syn GluA1
Syn PSD95
B.
**
ER GluA1 / JM4
1.2
1.0
0.8
0.2
0.1
0.0
Scz
Comp
ER GluA1
ER JM4
C.
*
Total GluA1 / β-tubulin
4.5
3.0
1.5
0.0
Scz
Comp
Total GluA1
Total β-tubulin

Supplemental Table 1. Antisera and assay conditions for Western blot analysis

Antibody	Manufacturer/stock #	1° conditions[1]	2° conditions[2]
AMPARs			
GluA1	Millipore/AB1504	1:500/Li-COR	1:10 000, 2 hr
GluA2	US Biological/G3500-13G	1:1 000/Li-COR	1:10 000, 2 hr
GluA3	Millipore/MAB5416	1:1 000/Li-COR	1:10 000, 2 hr
GluA4	Cell Signaling/8070	1:1 000/Li-COR	1:10 000, 2 hr
NMDARs			
GluN1	Novus Biologicals/NB300-118	1:1 000/Li-COR	1:10 000, 2 hr
GluN2A	Novus Biologicals/NB300-105	1:1 000/Li-COR	1:10 000, 2 hr
GluN2B	Life Technologies/32-0700	1:1 000/Li-COR	1:10 000, 2 hr
GluN3A	Millipore/07-356	1:1 000/Li-COR	1:10 000, 2 hr
TARPs			
γ-2	Cell Signaling/2503	1:1 000/BSA	1:5 000, 4 hr
γ-3	LifeSpan BioSciences/LS-C94053	1:100/BSA	1:5 000, 2 hr
γ-4	Acris Antibodies/AP50706PU-N	1:1000/Li-COR	1:5 000, 3 hr
γ-5	LifeSpan BioSciences/LS-C94070	1:500/Li-COR	1:5 000, 2 hr
γ-7	LifeSpan BioSciences/LS-C94075	1:500/Li-COR	1:5 000, 3 hr
γ-8	LifeSpan BioSciences/LS-C94077	1:500/Li-COR	1:5 000, 2 hr
CNIHs			
CNIH-1	Acris Antibodies/AP09950PU-N	1:1 000/Li-COR	1:5 000, 2 hr
CNIH-2	Acris Antibodies/AP50986PU-N	1:1 000/BSA	1:5 000, 2 hr
CNIH-3	Abnova/H00149111-B01P	1:500/BSA	1:5 000, 2 hr
CNIH-4	Abnova/H00029097-M01	1:500/BSA	1:5 000, 2 hr
Markers			
PSD95	Millipore/MAB1596	1:5 000/Li-COR, 2 hr	1:10 000, 1 hr
Syp	Millipore/MAB368	1:5 000/Li-COR, 2 hr	1:10 000, 1 hr
JM4	abcam/ab53113	1:5 000/Li-COR	1:10 000, 1 hr
VDAC	Santa Cruz Biotechnology/sc-32064	1:5 000/Li-COR	1:10 000, 1 hr
H3	Cell Signaling/9715	1:5 000/Li-COR	1:10 000, 1 hr
β-tubulin	Millipore/05-661	1:10 000/Li-COR, 1 hr	1:10 000, 1 hr

Abbreviations: Li-COR, Li-COR blocking buffer; BSA, 2% (w/v) bovine serum albumin in phosphate-buffered saline (PBS) with 0.05% Tween-20. [1]: Primary antisera concentration and corresponding blocking buffer were incubated overnight at 4° C with gentle rocking, unless otherwise noted. [2]: Dilution of secondary

antisera was made in same buffer as primary antisera, and incubated for noted time in the dark at room temperature.

DISCUSSION

Summary and Implications of Findings

Here, we report changes in AMPAR auxiliary proteins, TARPs and CNIHs, in frontal cortex in schizophrenia. In homogenates from anterior cingulate cortex (ACC) we found decreased transcript expression of TARP γ-8, and increased CNIH-1, CNIH-2, and CNIH-3 transcripts in dorsolateral prefrontal cortex (DLPFC) in schizophrenia. At the protein level, we found decreased γ-2, γ-4, γ-7, γ-8, and CNIH-2 but increased γ-3 and γ-5 in homogenates from ACC in schizophrenia. TARP γ-5 appears to have opposing effects on biophysical properties of AMPARs (decreasing channel activity instead of increasing activity), thus an increase in expression of this subunit in schizophrenia is consistent with the decreased levels of other TARP subunits. Knockout studies performed in mice have demonstrated that TARPs can interchangeably compensate for each other (see Jackson and Nicoll, 2011), and the increased expression of γ-3 may be a reflection of this capability in schizophrenia. These data are consistent with other reports of AMPAR-specific modulatory protein alterations in schizophrenia (see Table 4 in Introduction), and suggest that forward trafficking of AMPARs may also be dysregulated in this illness. We also measured AMPAR and NMDAR protein expression in total tissue homogenates from ACC and found decreased GluA1 and GluN2A in schizophrenia.

One way to evaluate forward trafficking of AMPARs in postmortem brain is by measuring AMPAR expression within subcellular compartments associated with the reg-

ulation and recycling pathways of AMPARs. A recent study from our laboratory found increased protein expression of the AMPAR subunit GluA1 in an isolated endosome fraction in DLPFC in schizophrenia (Hammond *et al*, 2010), suggesting that the pathophysiology of schizophrenia may be associated with altered AMPAR trafficking. Electrophysiological studies performed in mice with genetically altered glutamate receptor or auxiliary protein expression have also shown that changes in synaptic AMPAR responses correspond to changes in protein expression or localization in these animals (Table 2 Introduction; Jackson and Nicoll, 2011a). Taken together, these findings indicate that changes in trafficking may be specifically identifiable by studying protein expression within subcellular compartments involved with AMPAR regulation.

To address whether decreased TARP expression in schizophrenia was an indication of abnormal AMPAR forward trafficking, we first measured AMPARs in fractions enriched for ER from ACC. We found an 80% reduction of GluA1 protein expression, but no changes for the other AMPAR subunits, suggesting altered forward trafficking of the GluA1 subunit in schizophrenia. This change may be specific to the ACC, as a previous study found no change in GluA1 ER localization in dorsolateral prefrontal cortex in schizophrenia (Hammond *et al*, 2012). Next, we developed tools that would allow us to measure AMPAR expression at synapses. Since synaptic membranes are insoluble in Triton X detergent, we isolated and enriched synapses from postmortem ACC using a series of centrifugation and detergent extraction steps. We measured each AMPAR subunit in this fraction and found a 40% reduction of GluA1 protein at synapses, but no changes for GluA2-4 subunits in schizophrenia. The ratio of GluA1/A2 was also decreased in the

synaptic fraction, suggesting altered receptor stoichiometry may be occurring at synapses in schizophrenia.

Surprisingly, we did not detect any changes in expression for TARP or CNIH proteins in either fraction, or in total homogenates besides TARP γ-2 in schizophrenia. The discrepancy between these data and our previous report of altered TARPs in total tissue homogenates from ACC may be explained by the smaller number of subjects studied (for schizophrenia subjects, N = 18 versus N = 36 in previous study), as most TARPs displayed a trend towards downregulation in total homogenates in schizophrenia. However, ratios examining GluA1/TARP and GluA1/CNIH protein expression were mostly decreased in the ER and synaptic fractions, indicating that these auxiliary proteins may alternatively bind to GluA1 in schizophrenia, and in turn affect the forward trafficking of AMPARs to synapses. Consistent with the evolutionary conserved role for CNIHs as ER chaperones, and the specific role TARPs have in receptor stability at synapses via their direct binding to PSD95, it is interesting that the majority of GluA1/TARP ratios were altered in the synaptic fraction, whereas GluA1/CNIH ratios were mostly changed in the fraction enriched for ER. We also measured NMDAR subunits in ER and synaptic fractions and found no changes. Similar to AMPARs, however, the ratio of GluN1/2B was decreased at synapses, suggesting that subunit binding and stoichiometry may also be altered for synaptic NMDARs.

Taken together, these data support a model of altered forward trafficking and processing of GluA1-containing AMPARs in schizophrenia, and also suggest that synaptic remodeling via subunit reorganization may also occur at synapses in this illness. Since we found decreases in GluA1 localization at both ER and synpases, it is likely that decreased

synaptic GluA1 is not due to increased ER retention of this subunit, but rather accelerated ER exit. Hence, the ER may be dysfunctional in schizophrenia, and contributing to changes in receptor assembly and composition during early biogenesis and passage through the secretory pathway. Receptors that exit too quickly from ER but do not traffic to the synapse may become tagged for degradation. Thus, GluA1 could be trafficked to endosomes for lysosomal degradation, or could enter the ubiquitin-proteasome pathway rather than travel to the synapse, which could contribute to fewer synaptic and total levels of GluA1 subunits in schizophrenia. Considering the evidence suggesting NMDAR hypofunction in schizophrenia, a dysfunctional ER could also alter the conformations of NMDARs in a manner similar to AMPARs, therefore contributing to altered synaptic function of both types of glutamate receptors in this illness.

It is intriguing that our findings are specific to the GluA1 subunit, whose activity-dependent trafficking to synapses is widely accepted as an underlying mechanism behind learning and memory. Therefore, a second possibility is that decreased synaptic GluA1 is a direct reflection of decreased synthesis due to decreased synaptic activity in schizophrenia. Changes in activity can spur neurons to increase or decrease synthesis or trafficking of GluA1 to effectively scale up or down synaptic strength in response to changes in synaptic activity. Therefore, accelerated exit of GluA1 from ER could conceivably be an attempt to increase levels at the synapse, rather than a consequence of primary ER dysfunction. Regardless of the mechanism behind reduced GluA1 at synapses, our findings are consistent with previous studies from our laboratory suggesting altered mechanisms controlling forward trafficking for both AMPARs and NMDARs in schizophrenia.

These data are also in agreement with the glutamate hypothesis of schizophrenia, and may illuminate details underlying the pathophysiology of this illness.

Evidence Supporting Abnormal ER Function in Schizophrenia

Since AMPAR complexes are first assembled and modified in ER before being trafficked to the synapse (Bredt and Nicoll, 2003), and we observed a reduction of 80% in GluA1 expression in this compartment (compared to reductions of 37% and 40% in the synaptic fraction and homogenates, respectively), the ER may be the initial site of AMPAR dysregulation in schizophrenia. Decisions regarding subunit composition and receptor stoichiometry are made in the ER, along with posttranslational modifications that ensure proper assembly and folding of proteins. Hence, our findings of decreased GluA1 in ER, along with altered GluA1/auxiliary protein ratios in ER, suggest that GluA1 processing and subsequent ER exit may be abnormal in schizophrenia. We have previously reported abnormal N-linked glycosylation of GluA2 in schizophrenia (Tucholski *et al*, 2013), which may also be indicative of accelerated forward trafficking of GluA2-containing AMPARs in this illness, since N-linked sugars are important for early receptor biogenesis and trafficking.

Similarly for NMDARs, earlier reports from our laboratory have shown that GluN2B and PSD95 protein expression are decreased in a fraction enriched for ER (Kristiansen *et al*, 2010b) and that the GluN1-C2' splice variant, which traffics away from the ER with faster kinetics than the GluN1-C2 form, is increased in schizophrenia (Kristiansen *et al*, 2006). We have also found altered expression of several NMDAR-specific motor proteins (Kristiansen *et al*, 2010a) and interacting proteins (Funk *et al*,

2012) which serve to traffic NMDAR-containing vesicles away from ER or Golgi, and stabilize NMDARs at synapses, respectively. Taken together, these data are consistent with accelerated ER exit and forward trafficking of glutamate receptors in schizophrenia, and may also reflect abnormal assembly and processing of these proteins at ER.

We expected to find decreased subcellular GluA2 expression and ER-specific changes for the ratios of GluA1/A2 and GluA2/A3 in schizophrenia along with decreased GluA1. However, considering GluA2 primarily associates with GluA3 in ER and does not require activity for synaptic insertion (Greger *et al*, 2002), and that synaptic GluA2 is present in complexes containing GluA1 and to a lesser extent GluA3 (Lu *et al*, 2009; Shi *et al*, 2001; Wenthold *et al*, 1996) in brain, these data may reflect differential subunit trafficking mechanisms that are activity-specific. The GluA1 subunit possesses faster forward kinetics during early trafficking steps through the secretory pathway, but slower rates of insertion at synapses that is activity-dependent (Greger *et al*, 2002; Passafaro *et al*, 2001). Compared to GluA1, GluA2 has slower initial trafficking kinetics, and more rapid insertion at synapses driven by constitutive trafficking pathways in an activity-independent manner thought to underlie homeostatic regulation (Greger *et al*, 2002; Hayashi *et al*, 2000; Passafaro *et al*, 2001; Shi *et al*, 2001). Therefore, finding no change in GluA2 subcellular expression, or GluA1/A2 and GluA2/A3 ratios in ER, may support a role for activity-dependent dysregulation of GluA1 in schizophrenia.

If changes in subcellular expression of GluA1 were due to net decreases in total receptor number (40% decrease in tissue homogenates), then we would expect to see a similar reduction in ER. Since we observed an even greater reduction of 80% in ER in schizophrenia, we instead interpreted this difference as evidence of accelerated ER exit of

GluA1, consistent with abnormal forward trafficking of AMPARs. In agreement with this conclusion are the inconclusive changes in GluA1 transcript levels previously reported in schizophrenia (see Table 3 in Introduction), suggesting that decreased GluA1 may not be due to decreased gene expression. Further, studies of GluA1 subcellular distribution show primary localization at dendritic spines, and less presence within cell bodies (Greger *et al*, 2002), indicating that a change in total GluA1 number in schizophrenia could be a reflection of reduced dendritic localization rather than altered regulation at the soma. Although these findings point towards normal protein synthesis in schizophrenia, we cannot completely rule out the possibility of altered synthesis in ER, since NMDAR antagonists have been shown to alter the rate of dendritic GluA1 synthesis (Ju *et al*, 2004) in cell culture. Thus, an intriguing possibility is whether altered synthesis of GluA1 in schizophrenia could occur specifically at ER proximal to dendritic spines in this illness.

Altered ER function could also contribute to improper assembly or folding of GluA1-containing AMPARs, leading to increased degradation of this subunit in schizophrenia. Glutamate receptors are primarily degraded by the lysosome (Ehlers, 2000), but AMPARs can be directly ubiquitinated and degraded by both the proteasome and lysosome systems (Henley and Wilkinson, 2013). If GluA1 subunits become fated for degradation between exiting the ER or Golgi network and reaching the synapse in schizophrenia, the precise route towards degradation is unclear. However, for GluA1-containing AMPARs localized at synapses, Cdh1, an activator for the ubiquitin ligase anaphase-promoting complex, directly binds to GluA1and leads to degradation of this subunit by the ubiquitin-proteasome system (Fu *et al*, 2011). Conversely, activation by direct agonist binding can induce the ubiquitination of GluA1 by the E3 ligase Nedd4-1 and the subse-

quent endocytosis and lysosomal degradation of this receptor (Lin *et al*, 2011; Schwarz *et al*, 2010). Recent studies from our laboratory have shown altered protein expression of Nedd4-1 (Rubio *et al*, 2013), and increased GluA1 localization at early endosomes (Hammond *et al*, 2010), which lend support to the possibility that enhanced lysosomal degradation of GluA1 may be occurring in schizophrenia.

Since we did not detect accumulation of AMPAR subunits in ER in this study or in previous reports (Hammond *et al*, 2012), but rather found an 80% reduction of GluA1 in ER in schizophrenia, it is unlikely that GluA1-containing AMPARs would be increasingly degraded due to misfolding of this receptor. In most circumstances, misfolded proteins are retained in ER and undergo ER-associated degradation (ERAD), a quality control mechanism that acts to clear accumulated proteins from ER (Walter and Ron, 2011). However, a role for ERAD or the unfolded protein response (UPR) in the pathophysiology of schizophrenia cannot be ruled out since recent findings suggest that misfolded proteins are not the only targets of ERAD (see Ruggiano *et al*, 2014 for a review). Further, new evidence has emerged from our laboratory that indicates proteins involved in UPR, ERAD and ER quality control are upregulated in dorsolateral prefrontal cortex in schizophrenia (unpublished observations). While overactivation of these pathways is an intriguing mechanism that may help explain consistent reports of decreased protein expression in schizophrenia literature, it may not be applicable to our findings of GluA1 since we found no evidence of protein accumulation for any protein we examined in ER, nor any other changes in subcellular expression that would support the conclusion of degradation from misfolded glutamate receptors or auxiliary proteins in schizophrenia.

Future Directions

Abnormal protein expression as a function of altered ER activity in schizophrenia could provide an explanation for why the majority of studies of this illness report decreased protein expression. Hence, a primary ER problem may underlie the widespread consistency of these observations, including our current findings of decreased subcellular localization of GluA1, and decreased protein expression of GluN2A and auxiliary proteins in schizophrenia. In agreement with our hypothesis of accelerated ER exit and subsequent forward trafficking of AMPARs in schizophrenia, we predict that assembly and processing of AMPAR complexes is abnormal in schizophrenia and is contributing to fewer GluA1 subunits reaching the synapse. The following sets of experiments would attempt to address early AMPAR biogenesis in schizophrenia, and whether this may result in altered stoichiometry of GluA1-containing receptors that do traffic to the surface.

GluA1 Assembly

AMPARs form as heteromers consisting of mostly GluA1/A2 and GluA2/A3 in ER (Greger *et al*, 2002; Lu *et al*, 2009; Shi *et al*, 2001; Wenthold *et al*, 1996). These reports and others (He *et al*, 2009; Plant *et al*, 2006; Shi *et al*, 1999) have also found evidence for the existence of GluA1 homomers in brain, but their endogenous levels and functional roles remain unclear. Receptor stoichiometry can be measured by mass spectrometry (for a review of this technique see Gingras *et al*, 2007) or co-immunoprecipitation (CoIP) approaches to assess changes in the proportion of these complexes in ER and synapse fractions from schizophrenia and comparison subjects. Based on finding decreased GluA1 at ER, and decreased ratios of GluA1/A3 but not

GluA1/A2 in ER, we would predict these studies would demonstrate altered proportions of GluA1/A3 that would be consistent with more GluA2/A3 and less GluA1/A2-containing AMPARs targeted to synapses in schizophrenia. This would be in agreement with reports demonstrating that loss of synaptic GluA1 not only leads to reduced transmission, but also a residual pool of GluA2/A3 AMPARs at synapses (Herring *et al*, 2013; Zhu *et al*, 2000). It is also tempting to speculate that the changes we observed for GluA1 in schizophrenia may involve homomeric formations, which would potentially leave other stoichiometric AMPAR combinations relatively intact, and would offer an additional explanation as to why our findings are specific to the GluA1 subunit.

Similarly, these approaches could also be used to assess potential stoichiometry differences in schizophrenia between GluA1 and auxiliary proteins such as TARPs or CNIHs in ER. Although each TARP and CNIH protein can bind to GluA1, we would expect these analyses to reveal preferred binding partners, and thus provide additional insight into the machinery controlling GluA1 exit from ER and targeting to synapses. The results of these studies would also expand our view on AMPAR biogenesis in schizophrenia, and help elucidate the consequences of altered receptor stoichiometry in ER on synaptic integrity in this illness.

GluA1 Glycosylation in ER

N-linked glycosylation is the most common glycosylation modification, and adds gycan sugars to nascent proteins as they are being translated and transported into ER, and later as they traffic through the Golgi network. Glycosylation is important to protein integrity and stability, and can regulate the retention or exit of proteins from the ER. Of

particular relevance is the finding that cerebellar AMPARs in the *stargazer* mouse show immature glycosylation and increased ER retention, which suggests important roles for both glycosylation status and the presence of TARPs for efficient ER exit and synaptic targeting of AMPARs (Tomita *et al*, 2003). Compared to GluA2, GluA1 matures more quickly and has faster forward trafficking kinetics through the early secretory pathway (Greger *et al*, 2002). Considering these data, and our findings of decreased GluA1 and TARP expression in homogenates, and decreased GluA1 at ER and synapses in schizophrenia, it is possible that GluA1 is abnormally glycosylated in this illness. A previous study did not detect changes in glycosylation of GluA1 in brain homogenates in schizophrenia (Tucholski *et al*, 2013). However, our recent development of tools to isolate ER will enable more precise measurements of this modification within the ER in schizophrenia, and may potentially increase our threshold of detection. We would expect this measurement to reveal altered glycosylation of GluA1 consistent with our hypothesis of accelerated ER exit of GluA1, and our previous report of abnormally glycosylated GluA2 in schizophrenia (Tucholski 2013).

Synaptic Trafficking In Postmortem Brain

Trafficking to degradation pathways rather than the synapse after ER exit is one potential explanation of fewer GluA1 subunits at ER and synapses in schizophrenia. Thus, mechanisms regulating GluA1 targeting to, and trafficking within, the synapse may be abnormal in schizophrenia, resulting in fewer GluA1-containing AMPARs at the surface due to decreased insertion at synaptic or extrasynaptic sites. Since a blockade in this final stage of the secretory pathway would render this pool of GluA1 subunits as unnec-

essary, it is likely that they then traffic to endosomes for lysosomal degradation. The next sets of experiments aim to evaluate trafficking pathways and modifications specific to GluA1 that may facilitate fewer synaptic GluA1 subunits in schizophrenia.

GluA1 phosphorylation at synapses. Phosphorylation is a posttranslational modification that occurs by the addition of phosphate groups onto a target protein. Such an addition can change the conformational state of a protein, thus promoting its activation or subsequent deactivation through dephosphorylation. Altering the phosphorylation status of GluA1 has been shown to mediate delivery of GluA1-containing AMPARs to synapses. Specifically, phosphorylation at Ser845 can recruit GluA to extrasynaptic sites for subsequent trafficking to synapses (Oh *et al*, 2006). Conversely, mutation of this site can block delivery of GluA1 to synapses (Esteban *et al*, 2003; Roche *et al*, 1996). Another study found that mimicking phosphorylation at Ser831 or Ser845 was sufficient to increase GluA1 levels in dendrites, and that this appeared to protect GluA1 from lysosomal degradation (Kessels *et al*, 2009). Therefore, we predict that decreased GluA1 at synapses could be partly due to decreased phosphorylation of GluA1 and also increased degradation. Antisera that recognize Ser 831 and Ser845 are commercially available, and could be used for Western blot analysis experiments measuring phosphoGluA1 normalized to GluA1 expression in synaptic fractions from schizophrenia and comparison subjects. We expect that these studies will show decreased phosphorylation of GluA1 at synapses in schizophrenia, which would be consistent with our model of altered forward trafficking and increased degradation of GluA1 in schizophrenia.

GluA1 palmitoylation at synapses. Palmitoylation is another posttranslational modification that adds palmitate to cysteine residues of target proteins. Similar to the other modifications, GluA1 is palmitoylated (Hayashi *et al*, 2005; Kang *et al*, 2008), which in turn can promote binding of GluA1 to the 4.1N protein which facilitates insertion into the membrane (Lin *et al*, 2009). Interestingly, PKC phosphorylation of Ser816 and Ser818 of GluA1 can also enhance GluA1 binding to 4.1N, increasing GluA1 insertion at synapses (Lin *et al*, 2009). If decreased GluA1 at synapses in schizophrenia is due to reduced insertion, then corresponding decreases in GluA1 palmitoylation, Ser816 or Ser818, or 4.1N protein may all be indications of altered GluA1 synaptic insertion in schizophrenia.

New tools to evaluate GluA1 trafficking in postmortem brain. To further extend our snapshot of AMPAR forward trafficking through the secretory pathway in schizophrenia, it will be important to continue developing new tools that will allow for greater evaluation of subcellular localization. Isolating the Golgi network would expand our analyses and understanding of glycosylation status and AMPAR maturation in schizophrenia, and we predict changes consistent to those in ER. Similarly, developing a technique to isolate lysosomes or multivesicular bodies (MVBs) would allow direct measurement of GluA1 targeted to these compartments, and we would expect to find increased GluA1 protein expression here consistent with our findings of decreased GluA1 subcellular localization, and decreased total protein levels in homogenates in schizophrenia. This would also be in agreement with increased GluA1 protein expression found in isolated early endosomes in schizophrenia (Hammond *et al*, 2010). Isolating recycling and late

endosomes from postmortem tissue would also yield information regarding synaptic regulation of the GluA1 subunit. We would expect to find no change in recycling endosomes that traffic internalized cargo back to the synapse for reinsertion, and increased GluA1 expression in late endosomes which transport contents to lysosomes for subsequent degradation.

Since AMPARs may be initially trafficked to extrasynaptic membranes by TARPS and CNIHs before lateral translocation into the synaptic cleft, isolating extrasynaptic membranes would be useful to understand changes in surface trafficking dynamics in schizophrenia. A possibility is that lateral diffusion between extrasynaptic and synaptic sites is altered due to TARP or CNIH protein unavailability, or changes in phosphorylation of GluA1. Thus, we would predict that within extrasynaptic membranes, phosphorylation of GluA1 at Ser845 is decreased, and contributes to less activation and trafficking to synapses. We would also expect to find decreased expression of auxiliary proteins at extrasynaptic membranes in schizophrenia, consistent with decreased synaptic insertion or increased degradation of GluA1.

In summary, these studies aim to study the lifecycle of GluA1-containing AMPARs in schizophrenia, and may help elucidate precise points of the secretory pathway where dysregulation occurs. These sites would be potential new targets for future therapeutic interventions designed to restore, or partially restore, GluA1 levels at synapses in schizophrenia.

Excitatory/Inhibitory Balance in Schizophrenia

Studies that have characterized the *stargazer* mouse have found loss of synaptic and extrasynaptic AMPARs in cerebellar granule neurons due to loss of function of TARP γ-2 (see Jackson and Nicoll, 2011 for a review). Counterintuitively, these animals also exhibit a phenotype resembling absence epilepsy, implying overexcitation is occurring in these mice. These types of seizures have been proposed to originate from dysregulated excitability within corticothalamic networks (Huguenard and McCormick, 2007), and studies performed in *stargazer* cortex and thalamus have consistently found hyperexcitable layer V pyramidal cells (Noebels *et al*, 1990), and disrupted glutamatergic synapses onto inhibitory thalamic nucleus reticularis neurons (Menuz and Nicoll, 2008), respectively. Cerebellar stellate cells from *stargazer* mice, which are small interneurons that influence feedforward inhibition onto cerebellar Purkinje cells, also display dramatic loss of synaptic AMPARs, but interestingly maintain normal levels at extrasynaptic membranes (Jackson and Nicoll, 2011b). Together these findings suggest the potential for TARPs to affect AMPAR localization at inhibitory sites to effectively alter the inhibitory/excitatory balance in a cell-type specific manner.

Since inhibitory cells make up only 10-20% of cortical cells (Rudy *et al*, 2011), we predict that our finding of decreased synaptic GluA1 is specific to excitatory pyramidal neurons, and reflects reduced synaptic activity in schizophrenia. However, recent reports have demonstrated that GABAergic neurons are capable of expressing high levels of GluA1-containing AMPARs (Biou *et al*, 2008), raising the distinct possibility that fewer GluA1 subunits could also be present at inhibitory interneurons, which could increase activity levels. Similarly, TARPs are also expressed in cortical inhibitory cells

(Tao *et al*, 2013) and if reduced here, could also contribute to decreased AMPARs but increased activity. Seizures are not typically associated with schizophrenia, but the use of anti-epileptic drugs can alleviate some symptoms in treatment-resistant patients (see Hosák and Libiger, 2002 a review), suggesting the potential for alterations in excitatory and inhibitory transmission in this illness.

To address whether there are fewer GluA1 subunits localized to inhibitory cells in schizophrenia versus comparison subjects, laser-capture microdissection (LCM) could be used to isolate these two cell populations from the synaptic fraction for subsequent protein quantification of GluA1 and TARPs by Western blot analysis. These experiments would not only inform specific localization of GluA1 deficits, but would also differentiate between excitatory and inhibitory systems in our synaptic fraction. Preliminary studies from our laboratory have previously demonstrated the feasibility of protein detection from cells harvested in this fashion (unpublished observations). Since we detected reductions in total levels of expression, but no changes in subcellular compartments for TARP and CNIH proteins, one possibility is that they are highly localized to interneurons in schizophrenia, and were below the threshold of detection in our current experiments. Using the LCM method, it would be possible to enrich samples of interneuron cells, increasing the likelihood that we would find decreased TARP expression within these cells in schizophrenia. Such findings could potentially provide novel details regarding synaptic activity and AMPAR function in schizophrenia, and would point to cell-type specific differences in excitatory and inhibitory neurotransmission. This study could also be expanded to other relevant areas of the brain such as the hippocampus and thalamus, to gain an

even greater understanding of cell-mediated regulation of activity levels in schizophrenia, and highlight specific cells or brain regions to target for new therapeutic interventions.

Using Stem Cells to Model Schizophrenia

Stem cell technology is an exciting new avenue of biomedical research, and will likely become increasingly relevant towards the study of schizophrenia. The possibilities that arise from using live cells from human patients are endless, as they would theoretically enable the design of relevant new models of schizophrenia that could examine basic biological mechanisms over a developmental time course. Specifically, using stem cells as a model system would allow for direct study of AMPAR trafficking, as well as the study of glutamate receptor function in schizophrenia. These types of experiments will importantly allow researchers to study the etiology of proposed abnormal neurotransmission in this illness directly for the first time.

Given that previous studies have demonstrated that either accumulation or reduction of synaptic NMDARs is directly controlled by ER export (Mu *et al*, 2003), future studies examining if abnormalities in GluA1 localization might be due to changes in local dendritic synthesis or other distal dysregulation would be intriguing considering the importance of GluA1 availability at proximal sites of exocytosis for rapid response to synaptic stimuli. Increasing evidence supports that mRNA can be transported to dendrites for local translation (Guzowski *et al*, 2005; Martin and Zukin, 2006), including those of GluA1 and GluA2 (Ju *et al*, 2004). Testing proximal versus distal ER activity in schizophrenia could be achieved by culturing patient-derived induced pluripotent stem cells (iPSCs) that were transplanted into mouse neonatal brain. Previous studies have deter-

mined that these cells are capable of integrating with host neurons and that they can establish functional synapses within the host's circuitry (Espuny-Camacho *et al*, 2013). GluA1 could be transfected with a tetracysteine motif that is fluorescent upon application of biarsenical dyes shown previously to effectively distinguish preexisting AMPAR subunits from recently synthesized subunits in response to activity (Ju *et al*, 2004). Next, dendritic transection could be performed to separate dendritic processes for the specific analysis of protein synthesis in these locations.

In addition to synthesis, this system could also be used to directly measure AMPAR trafficking, synaptic insertion, and receptor activity in regards to schizophrenia. Additionally, if patient-derived cells demonstrated loss of synaptic GluA1 that was due to increases in degradation, then treatment with the lysosome inhibitor leupeptin, or the proteasome inhibitor lactacystin, would be expected restore GluA1 at synapses. As with all studies, experiments using stem cells are not without their limitations. However, with appropriate controls, it is reasonable that these types of studies may provide specific mechanistic details of GluA1 regulation and trafficking in schizophrenia, and other associated mechanisms underlying the pathophysiology of this illness that could be explored for future treatments and biomarkers.

Limitations of Postmortem Study

A common limitation to all studies involving postmortem tissue is postmortem interval (PMI), or the time that lapses between death and brain removal. While this can be closely controlled in animals, it is rarely possible in human subjects, and for many subjects the PMI can surpass 10 hours. To attempt to control for differences in brain in-

tegrity that may occur during this period, we evaluated our dependent measures for associations with PMI, as well as tissue pH. If associations were found, statistical corrections were performed using analysis of covariance (ANCOVA). Another approach is to closely match schizophrenia and comparison subjects for PMI, tissue pH, and age as part of the experimental design, although subjects are not always available to appropriately match.

A second limitation that is specific to studies of schizophrenia, however, is treatment with antipsychotic drugs that likely modulate the pathways being examined in our studies. To account for this in our TARP and CNIH experiments using brain homogenates, we performed parallel studies in brain tissue from rats treated chronically with a common antipsychotic, haloperidol. The benefits of these analyses are twofold, as they not only provide a measure of control for antipsychotic treatment, but can also suggest whether a dependent measure may be a novel target of antipsychotic drugs. In studies using either brain homogenates or subcellular fractions, we also performed posthoc statistical tests for each dependent measure to evaluate differences between schizophrenia subjects off antipsychotic medications for at least 6 weeks before death, and those who were actively receiving treatments at time of death. This approach is not ideal, however, as 6 weeks may not be long enough to reverse the molecular consequences of years of antipsychotic treatment.

Subject age is another limitation of our studies, and since it is well established that the severity of positive symptoms is lessened with age, our findings are likely specific to negative and cognitive symptoms that remain during end-stages of the illness. Although all experiments are normalized to comparison subjects, we cannot completely rule out that changes we found may be specific to the aging process rather than the illness.

Further, since most subjects are elderly, it is difficult to address etiology and specific molecular events that may occur during onset of symptoms. Availability of younger subjects for postmortem study would be valuable, but would not completely address this limitation, as working with postmortem tissue provides only a glimpse at molecular profiles at the moment of preservation. New methodologies will need to be employed for future investigations and characterizations of early-stage illness.

Since many symptoms of schizophrenia have been mapped to the frontal cortex, we chose to focus our studies on the anterior cingulate cortex. However, considering the complex and vast nature of schizophrenia-related symptoms, and the likely synergistic involvement of several different neurotransmitter systems, it is clear that this is not the only brain region with relevance to schizophrenia. A valuable future direction will be to expand these studies to other brain regions of interest, including other cortical regions, hippocampus, cerebellum, and thalamus.

Limitations Specific to Subcellular Fractionation Experiments

Although we have improved the specificity and implications of our results by investigating protein changes within subcellular fractions, another limitation is that these studies do not differentiate between excitatory and inhibitory cells. This is an important area to address, as the interpretation of decreased GluA1 expression would likely change if found to correspond specifically to inhibitory rather than excitatory neurons, or if we found altered proportions of excitatory, inhibitory, or silent (lacking functional AMPARs) synapses in schizophrenia.

Due to the various steps and methods used to achieve subcellular fractionation, it is possible that significant protein is lost throughout this process, and that final fractions only contain small proportions of representative protein. To attempt to address this concern in our experiments, we calculated percent recovery of GluA1 and found we could account for 84% of total GluA1 throughout the fractionation steps. This indicates that we were successful in recovering a vast majority of protein throughout the protocol, and that decreased expression of GluA1 is not simply due to changes in protein recovery.

Final Conclusions

Nearly all studies of schizophrenia performed to date consistently point to widespread disturbances in the regulation of basic biological processes. The findings we report here are in agreement with this overarching conclusion, and are novel in that they provide the first, to our knowledge, direct evidence of altered glutamate receptor expression and localization at synapses in schizophrenia. The technique we validated of synapse isolation in postmortem tissue may also help broaden the scope of discovery for additional synaptic disturbances in schizophrenia, and synaptic changes in other neurological disorders. Due to the proposed role of GluA1 in modulating synaptic strength and plasticity, decreased subcellular expression of GluA1 in schizophrenia may reflect decreased AMPAR-mediated glutamatergic neurotransmission. This may also play a mechanistic role in the presentation of negative and cognitive symptoms which are considered as the core of the illness, and their predominance during late stages of illness. New therapeutic interventions that can selectively bind and modulate GluA1 may help alleviate these symptoms in patients suffering from schizophrenia.

Altered expression and localization of GluA1 is also consistent with studies evaluating effects of stress, which is a risk factor for major depression, and is considered to be a main component underlying susceptibility to schizophrenia along with genetic predisposition. Interestingly, some reports show that decreased GluA1, but not NMDARs or GluA2, can occur at the same synapses under stress conditions, which suggests parallel roles for both a stressed system and excitatory synapse dysfunction in the development of neuropsychiatric illnesses. Since we also found decreased GluA1 at ER, stress may be particularly harmful to this organelle, and could conceivably drive many of the altered transcript and protein findings that have been reported in schizophrenia.

Moving forward, new models that can inform mechanisms contributing to the etiology of schizophrenia, and point towards the development of new therapeutic interventions and predictive biomarkers are desperately needed. In this regard, the importance and coincidence of the first psychotic break that occurs in late adolescence or early adulthood for most patients cannot be overlooked. Future studies involving patient-derived iPSCs may help elucidate specific mechanisms contributing to this devastating onset, and provide a closer look into the glutamate hypothesis of schizophrenia and the true functional capacity of glutamate receptors. In light of our current findings for GluA1-containing AMPARs, new questions emerge that could potentially be addressed in iPSC models, such as the developmental role AMPARs may have in the onset of schizophrenia, and whether decreased synaptic GluA1 could reflect a problem of AMPAR acquisition during development. These types of studies may show that new treatments aimed at GluA1 or other novel targets may require administration during a specific developmental window to provide an effective or preventative strategy.

In closing, schizophrenia is a devastating and complex illness surrounded by many challenges and limitations to its study. Although it is currently unclear from our current studies what role GluA1 may have on the development of schizophrenia, or the persistence of its symptoms, our findings of decreased subcellular localization of GluA1 supports a model of both AMPAR and NMDAR dysfunction in this illness that may be due, in part, to abnormal receptor exit from ER. Whether decreased synaptic GluA1 is due to accelerated ER exit and abnormal subunit assembly, or is a secondary consequence to altered synaptic activity, are questions that will be important to address in future studies examining subcellular pathophysiology in schizophrenia.

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