Published in final edited form as:
Schizophr Res. 2014 April ; 154(0): 36–41. doi:10.1016/j.schres.2014.02.003.

Alterations of the myristoylated, alanine-rich C kinase substrate (MARCKS) in prefrontal cortex in schizophrenia

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Abstract

Abnormal synaptic plasticity has been implicated in the cognitive deficits seen in schizophrenia, where alterations have been found in neurotransmission, signaling and dendritic dynamics. Rapid rearrangement of the actin cytoskeleton is critical for plasticity and abnormalities of molecular regulators of this process are candidates for understanding mechanisms underlying these changes in schizophrenia. The myristoylated, alanine-rich C-kinase substrate (MARCKS) is crucial for many roles associated with synaptic plasticity, including facilitation of neurotransmission, dendritic branching and in turn cognitive function. Accordingly, we hypothesized that this protein is abnormally expressed or regulated in schizophrenia. We measured protein expression of MARCKS by Western blot analysis in postmortem samples of dorsolateral prefrontal cortex (DLPFC) from elderly schizophrenia patients (N=16) and a comparison group (N=20). We also assayed phosphorylated-MARCKS (pMARCKS), given the role of phosphorylation in reversing membrane association by MARCKS. We found decreased expression of both MARCKS and pMARCKS in schizophrenia. Altered myristoylation may be a mechanism that explains this down-regulation of MARCKS, so we also assayed expression of the two isoforms of the key myristoylation enzyme, NMT, and an enzymatic inhibitor of this enzyme, NMT-inhibitor protein (NIP71) by Western blotting in these same subjects. Expression did not change between groups for these proteins, suggesting a mechanism other than myristoylation is responsible for decreased MARCKS expression in schizophrenia. These data suggest a potential mechanism underlying aspects of altered synaptic plasticity observed in schizophrenia.

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Conflict of interest All authors declare that they have no conflicts of interest.

Contribution ALP and JHMW designed the study. ALP 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.

Keywords

Synaptic plasticity; cytoskeleton; neurotransmission; myristoylation; dendritic dynamics

1.0 Introduction

Cognitive dysfunction is a core feature of schizophrenia and is thought to be due to abnormalities in synaptic plasticity, where alterations have been found in trafficking, signaling, receptor localization, neurotransmission, and dendritic dynamics (Aoto et al., 2013; Arnsten et al., 2012; Glausier and Lewis, 2013; Jia et al., 2013). These synaptic processes all share the requirement for rapid rearrangement of the actin cytoskeleton at the synapse. The myristoylated, alanine-rich C-kinase substrate (MARCKS) is a novel protein that is widely distributed in the nervous system and well-known as an actin binding protein associated with dynamic cytoskeletal restructuring. MARCKS is important for a myriad of events at the plasma membrane; its roles in trafficking, regulation of the cell cycle, cellular motility, memory, dendritic morphology, and secretion all depend on dynamic rearrangement of the actin cytoskeleton (Aderem, 1992; Arbuzova et al., 1997; Calabrese and Halpain, 2005; Sheu et al., 1993). Studies have shown that alterations in MARCKS can impair learning and memory, neurotransmission, dendritic branching, and synaptogenesis (Calabrese and Halpain, 2005; McNamara et al., 2005; Okuda et al., 2010; Sasaki, 2003), abnormalities of all of which have been associated with schizophrenia.

MARCKS regulates actin dynamics through a myristoyl-electrostatic switch mechanism that creates a reversible association with biological membranes or phospholipid vesicles (McLaughlin and Aderem, 1995; Seykora et al., 1996). MARCKS membrane association is vital for many of its functional roles; its crosslinking of F-actin, lateral sequestration of phosphatidylinositol 4, 5-bisphosphate (PI(4,5)P2), proximity to kinases, and PKC-mediated vesicle transport from the Golgi, all require MARCKS membrane adsorption (Arbuzova et al., 2002; McLaughlin et al., 2002; Radau et al., 2000; Sasaki, 2003; Spizz and Blackshear, 2001; Wang et al., 2001; Wang et al., 2002). Regulating and reversing MARCKS membrane association is through phosphorylation and/or Ca^{2+}-calmodulin (CaM) binding to the effector domain (ED) of MARCKS (Arbuzova et al., 1997; Hartwig et al., 1992) following a depolarization-induced Ca^{2+} influx (Wang et al., 1988). The reversible membrane association of MARCKS and its role initiating calcium-dependent changes through dynamic rearrangement of the cytoskeleton are necessary for CNS development, balanced synaptic functioning and modification of brain circuitry.

Given the role of MARCKS in regulating cytoskeletal dynamics that are associated with synaptic abnormalities seen in schizophrenia, we hypothesized that this key protein is abnormally expressed and regulated in schizophrenia. Accordingly, in this study we measured protein expression of MARCKS in postmortem brain from elderly schizophrenia patients and a comparison group. To further characterize the regulation of this protein, we assayed phosphorylated-MARCKS (pMARCKS), as well as the two isoforms of the key myristoylation enzyme, N-myristoyltransferase (NMT), and an enzymatic inhibitor of this enzyme, NMT-inhibitor protein (NIP71).

2.0 Experimental/Materials and methods

2.1 Human subjects

Autopsy-obtained samples of dorsolateral prefrontal cortex (DLPFC, Brodmann areas 9/46) from schizophrenia and comparison subjects (Table 1) were obtained from the Mount Sinai Medical Center brain collection as previously described (Powchik et al., 1998; Rubio et al., 2012). Next of kin consent was obtained for each subject. The medical history of each subject was reviewed extensively. Subjects with previous drug or alcohol abuse, coma greater than 6 hours, or suicide were excluded from study. Neuropathological examination of all subjects was conducted, and none used for study had evidence of any degenerative diseases, including Alzheimer's disease. Subjects with schizophrenia all met DSM-III-R criteria for this illness, had documented psychosis before the age of 40, at least 10 years of hospitalization for schizophrenia, and were diagnosed by two clinicians. Comparison subjects were free of any psychiatric or neurological disorders.

2.2 Antipsychotic treated rats

Animal studies and procedures were performed according to UAB guidelines and approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (250g) were housed in pairs during the 9 month course of study. Treatment was either sesame oil (vehicle, N=10) or haloperidol decanoate (28.5mg/kg, N=10) administered via intramuscular injection every 3 weeks, for a total of 12 injections (Harte et al., 2005; Kashihara et al., 1986). The animals were sacrificed by decapitation, and brains were immediately harvested; the right frontal cortex was dissected on wet ice, snap frozen and stored at −80°C.

2.3 Sample preparation

Tissue samples were reconstituted in cold 5mM Tris-HCl pH 7.5, 0.32M sucrose with a protease inhibitor tablet and a phosphatase inhibitor tablet (Complete Mini, EDTA-free and PhosSTOP both from Roche Diagnostics, Mannheim Germany). A Power Gen 125 (Thermo Fisher Scientific, Rockford, Illinois) homogenizer was used at speed setting 5 for 60 seconds. Protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Rockford, Illinois). After homogenization, samples were stored at −80°C until used for assay.

2.4 Western blotting

Tissue homogenates were thawed on ice, denatured at 70°C for 10min under reducing conditions, and stored at −20°C until use. Samples were loaded in duplicate onto NuPAGE 4-12% Bis-Tris 1mm, 17 well gels (Invitrogen, Carlsbad, CA) and transferred to 0.45μm nitrocellulose membranes using a BioRad Semi-Dry Transblotter (Hercules, CA). Membranes were blocked for 1 hour at room temperature in LiCor blocking buffer (Lincoln, NE) before being probed with the primary antibody diluted in LiCor blocking buffer + 0.1% Tween-20, under the conditions indicated in Table 2. All antibodies were optimized for each protein to determine ideal conditions within the linear range of detection for the assay, and that the primary antibody was present in excess (Table 2). All antibodies were used for both human and rat experiments with the exception of MARCKS. In this case, the antibody used

for the human blots had inadequate cross-reactivity with the rat protein, and accordingly we used a different antibody for those blots. Additionally, two different valosin-containing protein (VCP) antibodies from separate host species were used; this was necessary to permit simultaneous quantification of both the protein of interest and VCP as an intralane normalizing control on the same blots. VCP has been shown to not be altered in subjects with schizophrenia in multiple brain areas and has been used as an intralane control in previous studies (Bauer et al., 2009; Stan et al., 2006). We used VCP in this work given that its molecular weight was sufficiently different from proteins we planned to study, thus could be used for each study experiment.

Membranes were washed 5X in cold Tris-buffered Saline + 0.05% Tween-20 (TBST) for 5 minutes each before being probed in IR-dye labeled secondary antibody diluted in LiCor blocking buffer + 0.1% Tween-20. Membranes were again washed 5X in cold TBST for 5 minutes, then twice in MilliQ water before being scanned with the LiCor Odyssey imager, and then stored in MilliQ at 4°C.

2.5 Data Analysis

LiCor Odyssey 3.0 analytical software (Lincoln, NE) was used to determine the expression of each protein. Integrated Intensity values were first normalized to the same-lane value of VCP (Bauer et al., 2009; Stan et al., 2006), then the duplicate values were averaged for each subject. All data were analyzed by one-way ANOVA using Statistica software (Statsoft, Tulsa, OK). Integrated intensity values for VCP were not different between schizophrenia and comparison groups, consistent with previous reports (Bauer et al., 2009; Stan et al., 2006). Correlation analyses indicated that no dependent measures were correlated with age, pH, or PMI. For all statistical tests, α=0.05.

3.0 Results

Both total MARCKS ($F(1,34)=7.79$, $p=0.009$) and pMARCKS ($F(1,34)=4.85$, $p=0.035$) were decreased in schizophrenia relative to the comparison group (Figure 1). To determine if the fraction of MARCKS that is phosphorylated is altered in schizophrenia, the ratio of protein expression of pMARCKS to total MARCKS was calculated. No significant difference in this ratio was found between schizophrenia and comparison subjects (Figure 2). In rats treated chronically with haloperidol, treatment did not affect the expression in frontal cortex of either total MARCKS or pMARCKS (Figure3).

Next, we assayed expression of the two isoforms of the key myristoylation enzyme, NMT1 and NMT2, to explore possible mechanisms to explain the observation of decreased MARCKS in schizophrenia. Neither NMT1 nor NMT2 protein levels were changed in schizophrenia (Figure 4). To further characterize this pathway, we also measured an endogenous inhibitor of NMT, NIP71, in these same subjects. As in the case of the NMT isoforms, no changes were seen in the levels of this protein between schizophrenia and comparison subjects (Figure 4).

4.0 Discussion

Abnormal cytoskeletal responses to synaptic stimuli may underlie multiple abnormalities associated with the pathophysiology of schizophrenia. MARCKS is an actin binding protein critical for dynamic cytoskeletal restructuring, thus we hypothesized that this key protein may be abnormally expressed or regulated in schizophrenia. We found decreased expression of both MARCKS and pMARCKS in the DLPFC in schizophrenia. On the other hand, we found no changes in the expression of the myristoylation enzymes NMT1 and NMT2, or the enzyme inhibitor protein NIP71. These deficits may underlie abnormalities of synaptic plasticity seen in schizophrenia (Aoto et al., 2013; Arnsten et al., 2012; Glausier and Lewis, 2013; Jia et al., 2013).

Schizophrenia is associated with multiple neurotransmitter systems which converge to produce synaptic dysfunction. MARCKS is central to these neurotransmitter pathways and is a potential molecular mechanism underlying synaptic plasticity, given the mediatory role of MARCKS in translating Ca^{2+} dependent kinase activity into dynamic cytoskeletal restructuring (Leenders and Sheng, 2005; Ramakers et al., 1999). Localized to axon terminals, dendritic spines and glial processes (Ouimet et al., 1990; Ramakers et al., 1999), MARCKS is highly expressed during development, and remains high in adulthood in neuronal populations with high degrees of neuroplasticity; these include the hippocampus, amygdala and multiple cortical regions (McNamara et al., 2005; McNamara and Lenox, 1997; Ouimet et al., 1990; Ramakers et al., 1999). Decreased MARCKS expression is consistent with changes in Ca^{2+} induced vesicular transport and synaptic vesicle cycling, via MARCKS interactions with PKC, PI(4,5)P2, and cellular membranes (Horn, 1998; Rose et al., 2001; Sasaki, 2003; Walaas and Sefland, 2000; Yang et al., 2002). Loss of MARCKS and consequent sequestration of PI(4,5)P2 at the cell membrane has been shown to impair cognition and LTP in mice (Trovo et al., 2013). Altered synaptic responses from dysregulated kinase-dependent MARCKS-PI(4,5)P2 mediated processes could underlie changes in the regulation of the neurotransmitter pathways implicated in schizophrenia.

Synaptic transmission is facilitated by actin-rich dynamic dendrites that alter shape, growth and retraction of spines in response to stimuli (Amaral and Pozzo-Miller, 2009). Dendritic morphology has been found to be abnormal in schizophrenia (Glausier and Lewis, 2013; Penzes et al., 2011). MARCKS has been shown to play a role in maintenance of spine morphology, filopodia formation, and dendritic branching (Calabrese and Halpain, 2005; Li et al., 2008; Matus, 2005). MARCKS-deficient mice have abnormalities in cortical lamination, increased ventricular volume, and decreased brain size (Stumpo et al., 1995). Taken together these data suggest that the decreased MARCKS protein expression we found in schizophrenia could be associated with abnormalities of diminished dendritic morphology (Garey, 2010; Glausier and Lewis, 2013), decreased grey matter (Glahn et al., 2008; Honea et al., 2008), and alterations in cortical volume (Fatemi and Folsom, 2009; Northoff et al., 1999) seen in schizophrenia. A recent study found that MARCKS transcription is regulated by Dysbindin (Okuda et al., 2010), which has been implicated as a susceptibility gene in schizophrenia (Papaleo et al., 2012) and involved in synaptic glutamate release and cognitive function (Chen et al., 2008; Jentsch et al., 2009; Saggu et al., 2013). Dysbindin

dysfunction could be associated with abnormal regulation of MARCKS-dependent actin reorganization.

MARCKS and pMARCKS have also been found to be changed in other psychiatric conditions associated with abnormal synaptic plasticity (Duman, 2013). MARCKS expression and its phosphorylation are abnormal in suicide (Le-Niculescu et al., 2013; Pandey et al., 2003), and pMARCKS levels decrease with lithium treatment (Fitzgerald et al., 2010; Szabo et al., 2009). Interestingly, the polysialylated neural cell adhesion molecule (PSA-NCAM) has been shown to be altered in the dorsolateral prefrontal cortex in schizophrenia and in the amygdala in major depression (Gilabert-Juan et al., 2012; Varea et al., 2012). A recent study found that interaction of MARCKS and extracellular polysialic acid (PSA) is involved in neurite outgrowth (Theis et al., 2013). PSA glycosylates neural cell adhesion molecule (NCAM); the polysialylated form, PSA-NCAM, plays an important role in synaptic plasticity and learning (Kochlamazashvili et al., 2010; Senkov et al., 2012; Varea et al., 2012). Given that PSA-NCAM is restricted to interneurons in frontal cortex (Gascon et al., 2007; Gomez-Climent et al., 2011; Guirado et al., 2013; Rutishauser, 2008), decreased MARCKS expression in that subpopulation of cells could affect PSA-NCAM-mediated membrane interactions and in turn the modulation of inhibitory cortical circuits. Altered MARCKS may thus contribute to the dendritic changes seen in both schizophrenia and depression.

MARCKS membrane interactions require a myristoyl-electrostatic switch mechanism facilitated by N-myristoylation and a highly basic effector domain (ED) (McLaughlin and Aderem, 1995; Murray et al., 2002; Murray et al., 1997; Vergeres et al., 1995). Myristoylation targets the subcellular location of MARCKS, thereby facilitating its actin-binding activity (Calabrese and Halpain, 2005; Ramsden and Vergeres, 1999; Tapp et al., 2005). Given the decreased expression of MARCKS we found, we sought to determine if this was as a result of abnormalities of the key myristoylating enzymes in brain. Therefore we measured the two isoforms of the key myristoylating enzyme, NMT, and the NMT inhibitor NIP71. We found protein expression of these was not changed in schizophrenia. These data suggest that a mechanism other than myristoylation is responsible for the down-regulation of MARCKS expression seen in schizophrenia.

The decreased MARCKS expression in schizophrenia could be due to an assay limitation; the antibodies we used may not detect pools of demyr-, unmyr-, or cleaved MARCKS. PKC phosphorylation of MARCKS protects it from cathepsin proteolysis (Spizz and Blackshear, 2001) and potential dendritic loss (Graber et al., 2004); decreased pMARCKS may suggest a larger pool of cleaved MARCKS undetected by our assay. Thus we sought to determine if the percentage of MARCKS phosphorylation differed in schizophrenia, and found the fraction of total MARCKS that is phosphorylated was not changed, further supporting our finding that MARCKS expression is reduced in schizophrenia.

There are several limitations to this work and all postmortem brain studies in schizophrenia. Our subjects were aged, and these results may not generalize to younger subjects, although we predict that similar changes in this plasticity-related protein will be found across the lifespan. This study was also limited to frontal cortex, thus it is not yet known if this change

is restricted to this region or is found in other areas of brain. Finally, the effects of chronic antipsychotic treatment are always of concern in these types of studies. Although lithium has been shown to alter MARCKS expression (Lenox et al., 1996; Pandey et al., 2003; Pandey et al., 2002; Watson and Lenox, 1996), these patients were not receiving this medication at the time of death. Most had received antipsychotics close to the time of death, however, but rats chronically treated with haloperidol did not exhibit this change in MARCKS expression. Thus, we feel it is likely that these changes are due to the illness and not antipsychotic treatment. Finally, although pH was well matched between diagnostic groups, PMI was appreciably longer in the schizophrenia patients than the comparison subjects. Neither MARCKS nor pMARCKS expression was significantly correlated with PMI (which was normally distributed) in the total sample ($r=-0.27$ for MARCKS and $r=-0.23$ for pMARCKS), or in just the schizophrenia group ($r=0.37$ and 0.33, respectively) or the comparison subjects ($r=-0.26$ and -0.25, respectively).

In summary, we found decreased MARCKS and pMARCKS in the frontal cortex in schizophrenia. Abnormal MARCKS expression is consistent with altered synaptic morphology and plasticity mediated by dysregulated cytoskeletal dynamics. These data suggest abnormalities of MARCKS as a possible mechanism underlying the altered synaptic plasticity observed in schizophrenia.

Acknowledgments

None

Funding This work was supported by MH53327 (JMW), and MH64673 and MH66392 (VH).

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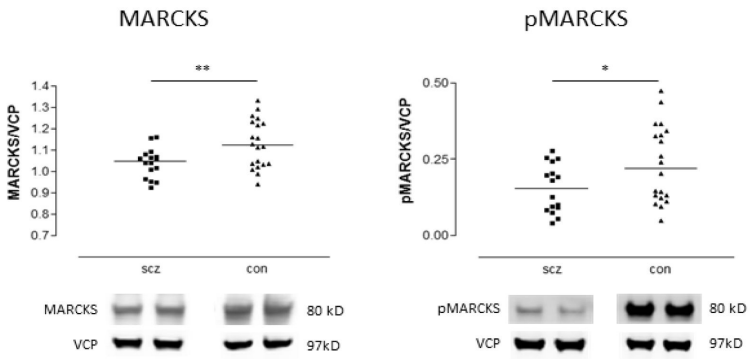

Figure 1.

Expression of MARCKS and pMARCKS in DLPFC from patients with schizophrenia (scz) and comparison (con) subjects. Data are expressed for each subject as the ratio of signal intensity for protein of interest divided by the intensity of valosin-containing protein (VCP) determined in the same lane from the same blot. Data are mean values of this ratio from duplicate samples. Both MARCKS and pMARCKS are reduced in schizophrenia. $p<0.05$*, $p<0.01$**.

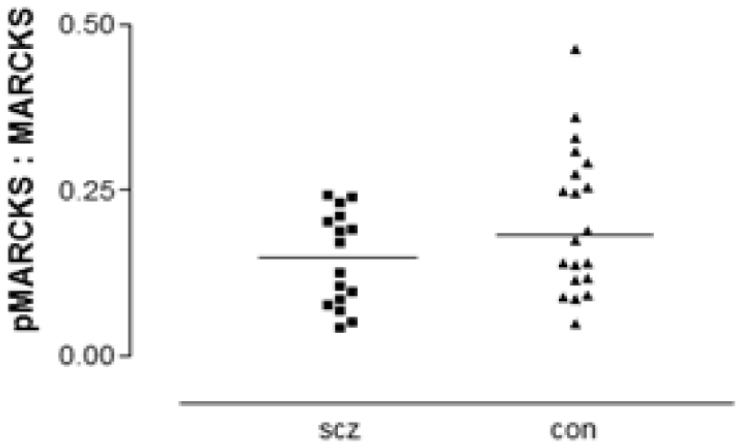

Figure 2.

Ratio of pMARCKS to total MARCKS in DLPFC in schizophrenia (scz) and comparison (con) subjects. The fraction of total MARCKS that is phosphorylated is not different between groups.

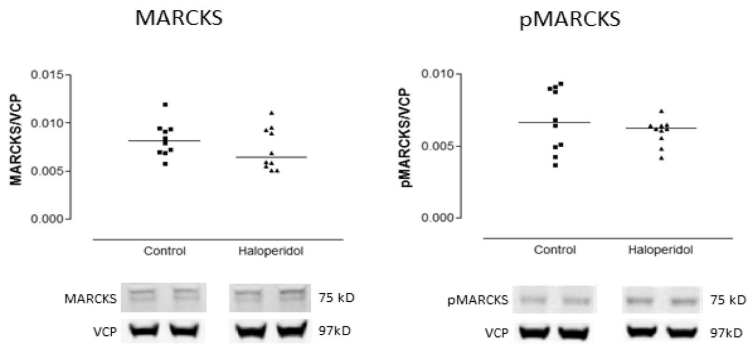

Figure 3.

Expression of MARCKS and pMARCKS in frontal cortex from adult male rats treated chronically with haloperidol decanoate (28.5mg/kg/3 weeks for 9 months) or vehicle (sesame oil), expressed as a ratio of signal intensity for each target protein to intensity of labeling for VCP. Each data point represents the mean value from duplicate lanes for each animal. Haloperidol treatment did not change expression of either MARCKS or pMARCKS.

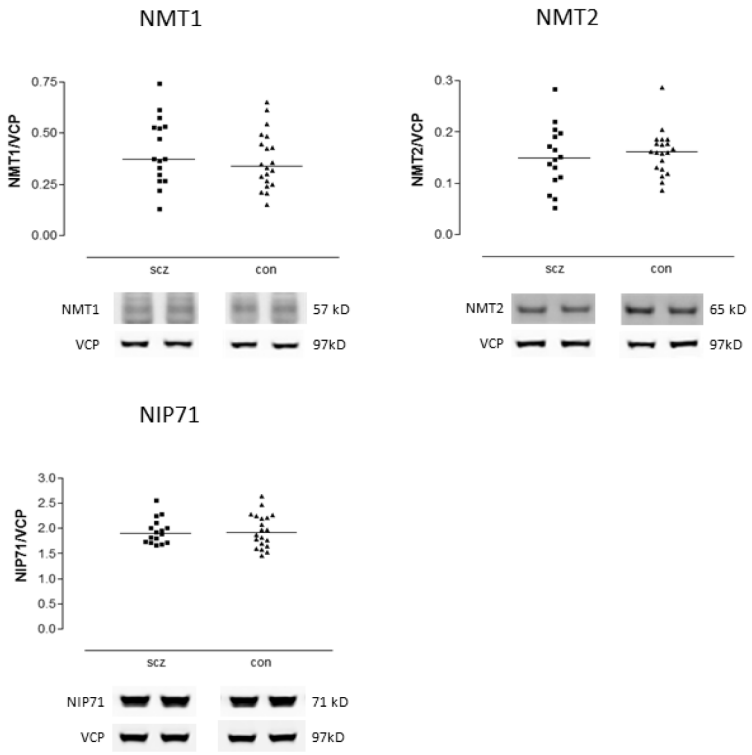

Figure 4.

Expression of NMT1, NMT2, and NIP71 in DLPFC from schizophrenia (scz) and comparison (con) subjects. Data are expressed for each subject as means from duplicate values first normalized to same-lane value of VCP. Expression did not differ between groups for any of these three proteins.

Table 1

Su bject Demographics

	Schizophrenia	Comparison
N	16	20
Sex	9M/7F	11M/9F
Tissue pH	6.5 ± 0.2	6.6 ± 0.3
PMI (hours)	15.5 ± 6.0	8.0 ± 5.9
Age (years)	78.2 ± 10.5	77.1 ± 10.4

Abbreviatio ns: F, female; M, m ale; PMI, p ostmortem interval Values presented as means ± standard deviation.

Table 2

Antibodies Used for Western Blotting

Antibody	Species	Dilution	Incubation	Company
Total MARCKS	Mouse	1:500	16hr 4°C	Abgent, San Diego, CA
Total MARCKS*	Rabbit	1:500	16hr 4°C	Abcam, Cambridge, MA
pMARCKS (S152/156)	Rabbi	1:250	16hr 4°C	Cell Signaling, Danvers, MA
NMT1	Rabbit	1:250	16hr 4°C	Abgent, San Diego, CA
NMT2	Mouse	1:250	16hr 4°C	BD Biosciences, San Jose, CA
NIP71 (HSC70)	Rabbit	1:3000	16hr 4°C	Novus, Littleton, CO
VCP	Mouse	1:25,000	1hr RT	Abcam, Cambridge, MA
VCP	Rabbit	1:25,000	1hr RT	Abcam, Cambridge, MA

Abbreviations: MARCKS, myristoylated, alanine-rich C kinase substrate; NMT, N-myristoyltransferase; NIP71, NMT protein inhibitor; RT, room temperature; VCP, valosin-containing protein.

*Antibody used for rat studies