Expression of the Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) and MARCKS-Related Protein (MRP) in the Prefrontal Cortex and Hippocampus of Suicide Victims

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Background: Although suicide is a leading cause of death in the United States and represents a significant public health threat, little is known about the neurobiological or molecular factors that contribute to its pathophysiology. A number of studies now indicate that lithium has considerable efficacy in the prevention of suicide in patients with affective disorders, and accumulating evidence indicates that protein kinase C (PKC) and its substrates, in particular the myristoylated alanine-rich C kinase substrate (MARCKS), are primary targets of chronic lithium treatment. We therefore hypothesized that a dysregulation in MARCKS expression in key brain regions could contribute to the pathophysiology associated with suicide. To address this, we examined MARCKS, as well as the closely related MARCKS-related protein (MRP), mRNA expression in the hippocampus and dorsolateral prefrontal cortex of suicide victims and normal controls. ***Method:*** MARCKS and MRP mRNA expression was assessed by quantitative in situ hybridization histochemistry performed on postmortem hippocampal and dorsolateral prefrontal cortex sections from suicide (N = 9) and normal control (N = 10) brains. ***Results:*** In the normal hippocampus, both MARCKS and MRP mRNA expression were highest in the granule cell layer and low-moderate in CA1, CA3, and hilus. A high level of MRP mRNA expression was also observed in the white matter of the fimbria/fornix. Neither MARCKS nor MRP mRNA expression levels differed significantly in the granule cell layer, CA3, hilus, or CA1 in suicide victims relative to normal controls (1-way ANOVA, $p > .05$). In the normal prefrontal cortex, MARCKS was expressed exclusively in gray matter (layers I–VI), whereas MRP was expressed in both gray and white matter. Neither MARCKS nor MRP mRNA expression levels in the gray and white matter regions of the dorsal prefrontal cortex differed between suicides and normal controls (1-way ANOVA, $p > .05$). ***Conclusion:*** The present findings are the first to demonstrate the expression and distribution of MARCKS and MRP in the human hippocampus and dorsolateral prefrontal cortex, and their expression pattern within these regions bears strong resemblance to those observed in the adult rat brain. Comparison of MARCKS and MRP mRNA expression in the hippocampus and prefrontal cortex of suicide victims and normal controls indicates that these 2 mRNAs are not differentially regulated in these regions. However, differences in MARCKS and MRP protein expression and function cannot be ruled out by the present findings.

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Suicide is a leading cause of death in the United States and is commonly associated with mood disorders, particularly major depression and manic-depressive disorders.[1–3] This significant public health threat therefore necessitates an understanding of the risk factors that predispose a person to suicidal behavior so that better diagnostic criteria and effective treatment and prevention strategies can be developed. Studies over the past several years have provided increasing evidence for a neurobiological basis for suicidal behavior.[4] Altered regulation of serotonin neurotransmission in critical regions of the limbic system and prefrontal cortex is a prominent example of a biologically based risk factor for impulsive behaviors including suicide.[5,6] In addition, the results of family, twin, and adoption studies suggest a significant genetic contribution to

suicide[7] and provide an important rationale for employing molecular genetic approaches in the study of suicidal etiology. To date, only limited studies have used in situ hybridization of mRNA to examine gene expression in key regions of the postmortem brain of suicide victims (reviewed in reference 8).

Lithium is a first-line treatment for the prophylactic management of manic-depressive disorder and is becoming increasingly recognized for its antisuicidal properties not observed with other mood stabilizers.[9–11] Accumulating evidence indicates a role for protein kinase C (PKC) and its substrates in the actions of lithium (reviewed in references 12 and 13). PKC isozymes have been implicated in numerous cellular responses including neurotransmitter release and ion channel and gene regulation,[14,15] and recent studies have demonstrated that chronic exposure to lithium in vivo produces a significant down-regulation of PKCα in the rat hippocampus.[16] Moreover, our laboratory has demonstrated that chronic, but not acute, lithium treatment resulting in therapeutic brain levels (~1 mM) produces a significant reduction in the myristoylated alanine-rich C kinase substrate (MARCKS) protein levels in the rat hippocampus that persist beyond treatment discontinuation.[17] Furthermore, the reduction of MARCKS protein observed following chronic lithium treatment in an immortalized hippocampal cell line was also observed following chronic treatment with sodium valproate at therapeutic levels, but was not observed following chronic exposure to other psychotropic drugs.[18] These studies suggest that the regulation of MARCKS expression may play a significant role in mood stabilization and contribute to the antisuicidal actions of lithium.

The MARCKS and MARCKS-related protein (MRP) compose a small gene family of PKC substrates that have been implicated in cell signaling (e.g., neurotransmitter release) and neuroplastic events associated with cytoskeletal plasticity in both neurons and glia.[19,20] Both MARCKS and MRP are developmentally regulated in rat brain,[21] and homozygous mutant mice not expressing MARCKS exhibit abnormal brain development characterized by a reduction in brain size and cortical lamination abnormalities in neocortex and hippocampus.[22] A similar phenotype characterized by reduced brain size, callosal agenesis, cortical thinning, and exencephaly has also been observed in homozygous mutant mice not expressing MRP[23] (see also reference 24). Both MARCKS and MRP remain highly expressed in a number of regions in the adult rat brain, including the hippocampus, medial habenula, amygdala, piriform cortex, periventricular hypothalamic nuclei, neocortex, as well as white matter regions including the fimbria/fornix and corpus callosum,[21,25] indicating an ongoing role in neuroplastic events in brain. In the present study, we examined MARCKS and MRP mRNA expression and distribution in the postmortem hippocampus and dorsolateral prefrontal cortex of suicide victims and normal controls by quantitative in situ hybridization.

METHOD

Human Postmortem Brain Tissue

Postmortem brain sections were obtained from the Neuropathology Section, Clinical Brain Disorders Branch, Intramural Research Program, National Institute of Mental Health, of the National Institutes of Health Neuroscience Center at St. Elizabeth's Hospital. Slide-mounted sections (14 µm) were stored at –70°C until processing. Unfixed hippocampal and dorsolateral prefrontal cortex (area 9) sections from normal controls (N = 10) and nonpsychotic suicides (N = 9) were used in these studies. Normal control male-female ratios (6:4) were similar to those in the suicide group (7:2). The normal group was composed of 1 white and 9 African Americans, and the suicide group was composed of 8 whites and 1 African American. The mean age of normal controls (mean ± SEM = 43.5 ± 4.4) did not differ significantly from suicides (45.7 ± 6.5) ($p = .78$). The postmortem interval for normal controls (27 ± 4.3 h) and suicides (35 ± 6.3 h) did not differ significantly ($p = .28$).

Riboprobes

The MARCKS antisense riboprobe was synthesized from a 1.15-kb Xho II subclone of the murine MARCKS cDNA inserted into a pcDNAI/Neo vector flanked by T7 and SP6 promoters.[26] The 1.15-kb fragment spans bases 354–1505 of the murine MARCKS cDNA, which encompasses 32 bases of the 5' untranslated region (UTR), the entire 927 bases of the protein coding region, and 191 bases of the 3'-UTR. This MARCKS probe shared 82.3% sequence homology with the same region of the human MARCKS cDNA. The MRP antisense riboprobe was synthesized from a 1.2-kb fragment of the murine MRP cDNA[27] inserted into a Bluescript KS+ vector (Stratagene, La Jolla, Calif.) flanked by T7 and T3 promoters. The 1.2-kb fragment spans bases 1–1200 of the murine MRP cDNA, which encompasses 186 bases of the 5'-UTR, the entire 600 bases of the protein coding region, and 414 bases of the 3'-UTR. This MRP probe shared 92.7% sequence homology with the same region of the human MRP cDNA. Linearized DNA (1 µg) was labeled with α^{35}S-UTP (Amersham, Arlington Heights, Ill.) by in vitro transcription to a specific activity of > 10^8 cpm/µL. Previous studies have demonstrated the specificity of the MARCKS and MRP riboprobes.[21,25]

In Situ Hybridization Histochemistry

Mounted sections were first immersed in 4°C 4% paraformaldehyde (pH 7.4; 10 minutes), 0.1% DEPC (rinse), 0.1 M triethanolamine (TEA, pH 8, rinse), 0.1 M

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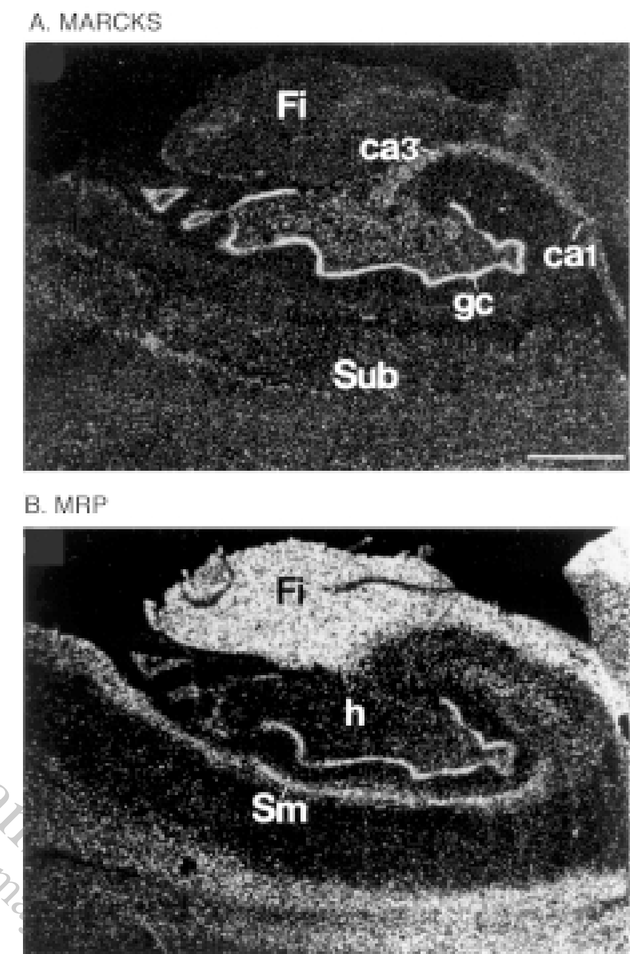
TEA + acetic anhydride (0.25%; 10 minutes), 2X SSC (rinse), ethanol series (50%, 70%, 95%, 100%, 3 minutes each), chloroform (5 minutes), 100% ethanol (2 minutes), and air-dried. Antisense riboprobes were added to slides (6×10^5 cpm/slide) in an 80 μL volume of hybridization buffer (50% formamide, 300 mM NaCl, 20 mM Tris; pH 8.0), 5 mM EDTA, 1X Denhardt's, 10% dextran sulfate (50% w/v), 10 mM dithiothreitol. Slides were then coverslipped, placed in a sealable container moistened with box buffer, composed of 50% formamide and 4X SSC, and incubated for 16 h at 55°C. Coverslips were then removed and slides washed in 2X SSC (2 × 10 minutes), pancreatic RNase A solution (20 μg/mL in 500 mM NaCl, 10 mM Tris, 30 minutes at 37°C), 2X SSC (2 × 10 minutes), 0.1X SSC (50°C for 2 h), 0.5X SSC (2 × 10 minutes), and ethanol (50%, 70%, 95%, 100%, each containing 0.3 M ammonium acetate, 2 minutes each). All wash solutions contained 10 mM 2-mercaptoethanol and 1 mM EDTA, except for the RNase A, 0.5X SSC washes, and ethanol solutions. Air-dried slides were apposed to X-ray film (Hyperfilm-βmax, Amersham) for 10 days. It was determined that the length of exposure did not alter the relative expression patterns. Hippocampal slides were then exposed to emulsion (NTB-2, Kodak) for 30 d, developed (D19, Kodak), fixed (Kodak), and counterstained with cresyl violet.

Quantitative Analysis

Hybridization signal was quantitatively measured from autoradiographic film (prefrontal cortex) or emulsion-dipped slides (hippocampus). Emulsion-dipped slides were examined under dark-field conditions with a Zeiss microscope (Axioscop 20) and a 10× lens. The video-imaging system consisted of a MicroComputer Imaging Device and image analysis program (Imaging Research Inc., St. Catharines, Ontario, Canada) interfaced via a video camera (Sony XC77) affixed to the microscope. On each brain section, grain density values were obtained from the following cell fields: CA1, CA3, hilus, and the granule cell layer. Background values obtained from regions of neuropil adjacent to the cell field being quantitated were subtracted from density values obtained from the target region. Sampling area parameters were set to encompass only the cell field being quantitated to avoid inclusion of adjacent regions. Nissl (cresyl violet) counterstain was examined under bright-field conditions to verify areal and laminar boundaries. Prefrontal cortex autoradiographic films were quantitated using a GS 700 imaging densitometer (Bio-Rad Laboratories, Hercules, Calif.), and both white and gray matter (layers I–VI) regions were quantitated. Background values obtained from adjacent regions of film were subtracted from density values obtained from both gray and white matter values. For both emulsion-dipped slides and autoradiographs, gray scale and light levels were adjusted both to minimize background detection and to illuminate grains to within the linear range and, once established, remained constant within each probe. All quantitative measures were obtained blindly. Following subject decoding, density values were grouped and compared statistically with normal controls using a 1-way analysis of variance.

Figure 1. Autoradiographs Illustrating MARCKS (A) and MRP (B) mRNA Distribution in the Normal Human Hippocampus[a]



[a]Abbreviations: Fi = fimbria/fornix, gc = granule cell layer, h = hilus, Sm = superficial medullary stratum of the subiculum, Sub = subiculum. Calibration bar = 2 mm.

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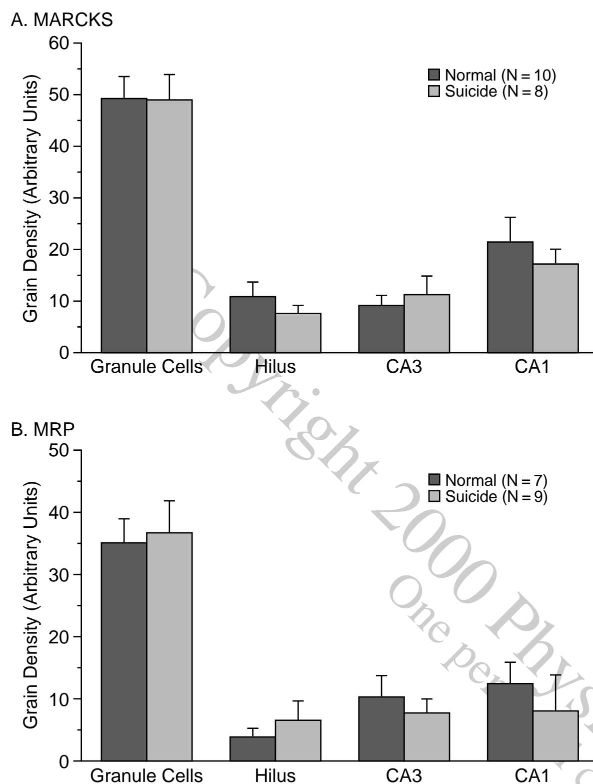
RESULTS

Hippocampus

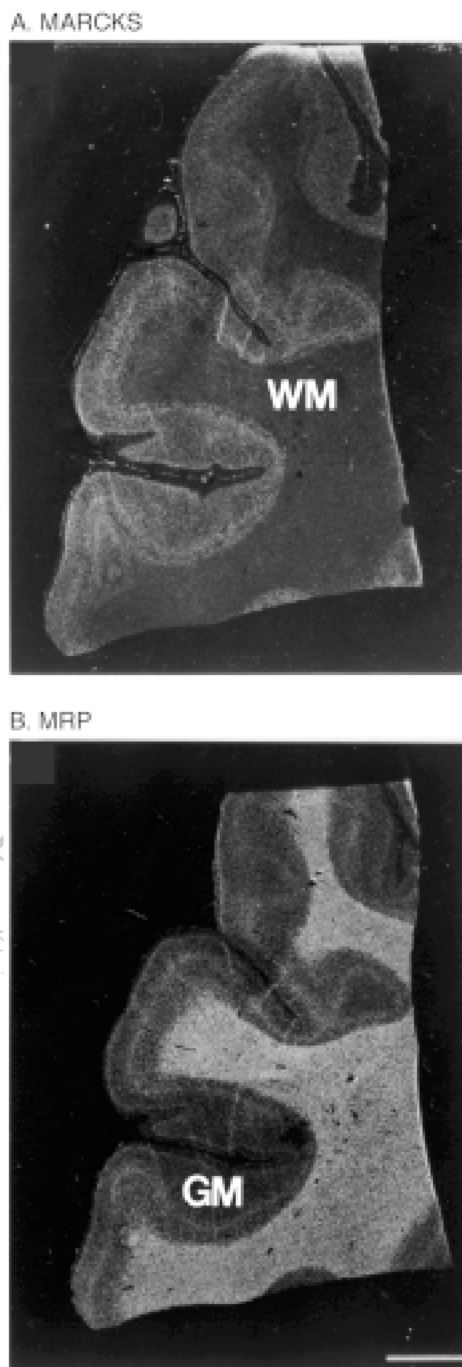
In the hippocampus of normal controls, MARCKS mRNA expression was highest in the granule cell layer and moderate-low in CA1, CA3, and hilus (Figures 1A and 2A). MARCKS mRNA expression in the granule cell layer, CA1, CA3, and hilus of normal controls did not differ significantly from that in suicide victims ($p > .05$), although a nonsignificant decrease in CA1 was observed in suicide victims (Figure 2A). In the normal hippocampus,

Figure 2. Quantitative Analysis of MARCKS (A) and MRP (B) mRNA Density in the Different Cell Fields of the Hippocampus of Suicide Victims and Normal Controls[a]



[a]Note that neither MARCKS nor MRP mRNA exhibits any alteration in expression in suicide victims.

Figure 3. Autoradiographs Illustrating MARCKS (A) and MRP (B) mRNA Distribution in the Normal Human Dorsolateral Prefrontal Cortex[a]



[a]Abbreviation: GM = gray matter. Scale bar = 6 mm. Note the high level of expression of MRP, but not MARCKS, mRNA in white matter (WM).

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MRP mRNA expression was highest in the granule cell layer and low in CA1, CA3, and hilus and was also observed to be highly expressed in the fimbria/fornix region (Figures 1B and 2B). The MRP mRNA expression levels obtained from normal controls did not differ significantly from values obtained from suicide hippocampal sections (all fields, $p > .05$) (Figure 2B). As with MARCKS, a nonsignificant reduction of MRP mRNA expression was observed in the CA1 region of the suicide hippocampus (Figure 2B).

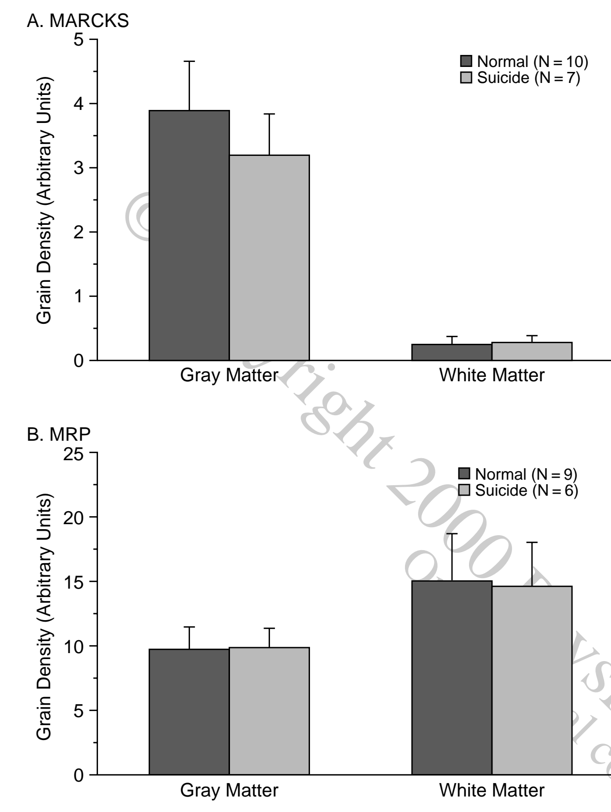
Dorsolateral Prefrontal Cortex

In the dorsolateral prefrontal cortex of normal controls, MARCKS mRNA was expressed in the gray matter, predominantly layers III/IV, and not in white matter (Figures 3A and 4A). MARCKS mRNA expression did not differ significantly between suicides and normal controls in either white or gray matter (Figure 4A). Unlike MARCKS, MRP mRNA was expressed in both white and gray matter (layers III/IV) of the normal dorsolateral prefrontal cortex ($p > .05$) (Figures 3B and 4B). The level of MRP mRNA expression did not differ significantly between suicides and normal controls in either white or gray matter (Figure 4B).

DISCUSSION

The present findings demonstrate for the first time the expression and distribution of the primary PKC substrates MARCKS and MRP mRNAs in the normal human hip-

Figure 4. Quantitative Analysis of MARCKS (A) and MRP (B) mRNA Density in the White and Gray Matter Regions of the Dorsolateral Prefrontal Cortex of Suicide Victims and Normal Controls



pocampus and prefrontal cortex. In the hippocampus, MARCKS mRNA expression was highest in the granule cell layer and moderate-low in CA1, CA3, and hilus, a pattern of expression that strongly resembles the pattern previously observed for MARCKS mRNA in the adult rat hippocampus.[25] Similarly, the high level of MRP mRNA expression in the normal human granule cell layer, but not in CA3 or hilus, resembles its distribution in the adult rat hippocampus, although MRP mRNA expression in human CA1 is lower relative to the moderate levels observed in the adult rat hippocampus.[21] In the dorsolateral prefrontal cortex, MARCKS was expressed exclusively in the gray matter, composed of layer I–VI neurons and absent from white matter, composed of axonal tracts. In contrast, MRP mRNA expression was observed in both gray and white matter, as well as in the white matter of the fimbria/fornix. This finding is consistent with our previous observations in the postnatal developing rat brain, in which MRP mRNA exhibited a dramatic increase in white matter (corpus callosum and fimbria/fornix) between postnatal day 14 and 21, and remained elevated in the adult rat brain.[21] Additionally, MRP, but not MARCKS, was highly expressed in the white matter of the cerebellar cortex, which is composed primarily of myelinated climbing and mossy fiber axons.[21] The elevation in MARCKS and MRP mRNA expression in white matter of the developing rat brain coincided with the differentiation of oligodendrocytes in the formation of the myelinated ensheathments and may play an ongoing role in maintaining the integrity of these ensheathments in both the adult rat and human brain. Moreover, the differential pattern of expression of MARCKS and MRP mRNA in cortical white matter provides additional evidence that different mechanisms regulate the expression of these 2 genes, which exhibit distinct promoter elements, displaying only 37% sequence homology over 407 bp of the promoter region, and possess different *cis*-acting elements within their promoters.[28]

In the present report, neither MARCKS nor MRP mRNA exhibited altered expression in the hippocampus or dorsolateral prefrontal cortex of suicide victims relative to normal controls. These data indicate that those mechanisms regulating normal MARCKS and MRP mRNA expression in these brain regions are not altered in suicide victims. However, the present data cannot rule out potential differences in MARCKS and MRP protein expression and/or function in the brains of suicide victims. It is noteworthy in this regard that the expression of the PKCα, which regulates in part the functional properties of MARCKS,[29] is also not altered in the prefrontal cortex of suicide victims with major depression.[30] However, an impairment of G protein–induced activation of phosphoinositide (PI) hydrolysis was observed in the prefrontal cortex (area 10) of suicide victims with major depression.[30] Since a primary product of PI hydrolysis is diacylglycerol, a principal activator of conventional and novel PKC isozymes,[14,15] reductions in PI hydrolysis would predict impaired PKC-mediated phosphorylation of MARCKS and MRP. Hence, future studies will additionally examine potential differences in the phosphorylation state of MARCKS and MRP in the postmortem brain of suicide victims.

A previous report has demonstrated preliminary morphometric evidence indicating a significant (17%) reduction in cortical thickness in the dorsolateral prefrontal cortex of suicide patients diagnosed with major depression relative to normal matched controls.[31] Previous data from our laboratory indicate that both MARCKS and MRP gene expression are developmentally regulated in the postnatal rat brain in both neocortex and hippocampus in association with neuroblast proliferation, migration, and differentiation,[21] and others have demonstrated cortical lamination abnormalities and cortical thinning in mutant mice not expressing MARCKS or MRP.[22,23] Therefore, the present findings suggest that such alterations in prefrontal cortical thickness are not secondary to alterations in MARCKS and MRP mRNA expression in the adult, although alterations in MARCKS and MRP mRNA and/or protein expression during cortical development may contribute directly to the morphological alterations observed in dorsolateral prefrontal cortex of adult suicide patients.

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Lithium is becoming increasingly recognized for its antisuicidal properties in affectively ill patients,[9–11] and a number of preclinical studies have implicated PKC and its substrates as molecular targets of chronic lithium.[13] Indeed, both PKCα[16] and MARCKS[17] proteins are significantly reduced in the hippocampus of rats chronically treated with lithium. While the contribution of the PKC signaling pathway to the antisuicidal actions of lithium remains to be determined, the present and previous findings[30] would suggest that neither MARCKS nor PKCα expression exhibits abnormalities in dorsolateral prefrontal cortex of suicide victims not administered lithium. However, lithium's interaction with the PI system may alter the functional activity of PKC and MARCKS/MRP without effecting their expression (reviewed in reference 13).

In summary, the present findings demonstrate for the first time the expression and distribution of MARCKS and MRP mRNA in the hippocampus and dorsolateral prefrontal cortex of suicide victims and normal controls. Our findings indicate that neither MARCKS nor MRP mRNA expression is altered in these regions of the suicide brain. However, future studies examining MARCKS and MRP protein expression and function will be required to determine the contribution of these proteins to the pathophysiology of suicide.

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