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Evidence of Altered Polyamine Concentrations in Cerebral Cortex of Suicide Completers

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Recent studies have implicated alterations in the expression of polyamine-related genes in the brains of suicide completers including widespread downregulation of spermidine/spermine N1-acetyltransferase, the key enzyme in polyamine catabolism, suggesting compensatory mechanisms attempting to increase brain levels of polyamines. Given the complexity of the polyamine system, quantification of the levels of the polyamines is an essential step in understanding the downstream effects of dysregulated gene expression. We developed a method using high-resolution capillary gas chromatography (GC) in combination with mass spectrometry (MS) for quantitation of polyamines from post-mortem brain tissue, which allowed us to accurately measure spermidine and putrescine concentrations in post-mortem brain tissues. Using this method, we analyzed putrescine and spermidine levels in a total of 126 samples from Brodmann areas 4, 8/9, and 11, from 42 subjects, comprising 16 suicide completers with major depression, 13 non-depressed suicide completers, and 13 control subjects. Both putrescine and spermidine levels fell within the expected nanomolar ranges and were significantly elevated in the brain of suicide completers with a history of major depression as compared with controls. These results were not accounted by possible confounders. This is the first GC–MS study to analyze the expression of putrescine and spermidine from post-mortem brain tissue and confirms the hypothesis raised by previous studies indicating alterations in putrescine and spermidine levels in suicide/major depression.

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INTRODUCTION

Suicide is a major public health issue. The risk for suicide is believed to be the result from a combination of social, environmental, and genetic factors. Neurobiological studies have implicated several neurotransmitter systems in suicide, and while the traditional focus has been on monoaminergic transmission, recent evidence also suggests a role for the polyamine system in suicide and other psychiatric disorders (Fiori and Turecki, 2008; Guipponi *et al*, 2008; Karssen *et al*, 2007; Sequeira *et al*, 2006).

Polyamines, including putrescine (1,4-diaminobutane, PUT), spermidine (*N*-[3-aminopropyl]-1,4-diaminobutane, SPD), and spermine (*N*,*N*-bis[3-aminopropyl]-1,4-diaminobutane, SPM), are low molecular weight aliphatic amines that have essential roles in living organisms (Casero and Pegg, 2009; Igarashi and Kashiwagi, 2009; Pegg, 2009; Wallace *et al*, 2003). In mammals, polyamines are involved in many physiological functions including cell proliferation and apoptosis (Igarashi *et al*, 1975; Marton and Pegg, 1995; Seiler and Raul, 2005), immunity (Seiler and Atanassov, 1994), and oxidative stress response. Brain polyamines are largely stored in astrocytes and synaptic vesicles and this distribution may confer polyamines regulatory activities to a variety of receptors located on the surface of glia and neurons (Belle *et al*, 2004; Lovaas and Carlin, 1991; Masuko *et al*, 2003; Takano *et al*, 2005). In particular, the ability of the polyamines to modulate transmission through *N*-methyl-D-aspartate (NMDA) receptors and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors have been well established (Kashiwagi *et al*, 1997; Kelly *et al*, 2009; Masuko *et al*, 2003; Rock and Macdonald, 1995). Polyamines have also been shown to influence the function of gamma amino-butyric acid (GABA) receptors (Gilad *et al*, 1992). Changes in the expression of the polyamines and their metabolic enzymes have been found in many pathological and psychiatric conditions, including schizophrenia (Ramchand *et al*, 1994; Svinarev, 1986), mood disorders (Genedani *et al*, 2001; Zomkowski *et al*, 2006), stress and anxiety (Gilad and Gilad, 2002; Sohn *et al*, 2002), and suicidal behaviors (Guipponi *et al*, 2008; Karssen *et al*, 2007; Sequeira *et al*, 2006, 2007).

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Studies by our group, using post-mortem brain tissues from suicide completers, have identified alterations in the expression of a number of polyamine-related genes using complementary methodologies, particularly spermidine/spermine N1-acetyltransferase (SAT1), which was found to be downregulated in suicide completers both with and without major depressive disorder (Klempan *et al*, 2009; Sequeira *et al*, 2006, 2007). This is consistent with data produced by other groups in both human and animal model studies (Guipponi *et al*, 2008; Karssen *et al*, 2007). As alterations in the expression of SAT1, as well as other polyamine genes, suggests compensatory changes aiming at increasing the availability of polyamines, we hypothesized that the levels of spermine, spermidine, and putrescine would be different in suicide completers compared with controls. To this end, this study aimed at directly measuring levels of two of these polyamines, specifically spermidine and putrescine, in brain tissues derived from suicide completers and controls.

Early analytical methods to measure polyamine concentrations were based on high performance liquid chromatography (HPLC) or gas chromatography (GC) alone. These methods have limitations regarding specificity, which becomes an important issue when only peak retention times are used for detection, as compounds with similar properties may co-elute or produce overlapping peaks. This is not a concern in mass spectrometry (MS), and when combined with GC, one of the most powerful analytical systems becomes available (GC–MS). In recent years, GC–MS has been used for polyamine analysis in simple biological samples, such as hair (Li *et al*, 2008), urine (Paik *et al*, 2006), and plasma (Paik *et al*, 2007). However, a GC–MS method for analyses of the major polyamines from brain samples was not available. We have recently developed a specific GC–MS method for measurement of spermidine and putrescine from post-mortem cerebral cortex (Chen *et al*, 2009). Although measurement of spermine by GC–MS has been shown from some biological samples, such as hair and urine (Li *et al*, 2008; Paik *et al*, 2006), GC–MS quantification of spermine simultaneously with putrescine and spermidine is methodologically challenging in post-mortem brain tissues, and requires a separate methodology. Therefore, this study aimed at quantification of putrescine and spermidine in cerebral cortex derived from suicide completers and controls.

MATERIALS AND METHODS

Subjects and Diagnostic Procedures

Our sample consisted of 42 male subjects of French-Canadian origin. We opted for an only-male sample to avoid gender-related heterogeneity. Controls died either in accidents (30%) or by natural death (70%), whereas suicides died by hanging (70%), carbon monoxide (12%), self-inflicted wounds (12%), or by other methods (6%). Post-mortem brain tissues were obtained from the Quebec Suicide Brain Bank (QSBB) (www.douglasrecherche.qc.ca/suicide), in which they were processed and dissected at 4 °C, and snap-frozen in liquid nitrogen before storage at −80 °C, following standard procedures (Bird and Vonsattel, 1993). All subjects collected by the QSBB have to have died suddenly without a prolonged agonal period. Brain tissue dissection was performed following standard procedures and anatomical landmarks (Mai *et al*, 2007; Nolte, 2002). Specifically, we focused on Brodmann areas (BA) 4, 8/9, and 11, which were the brain regions used in our original report indicating SAT1 differential expression in suicide (Sequeira *et al*, 2006). The following anatomical landmarks were used to identify these brain regions. BA4: precentral gyrus. As this BA region is located anterior to the central gyrus, the latter was used as landmark for the dissections. BA8–9: BA8 occupies mainly a portion of the superior frontal gyrus delimited, medially, by the cingulate sulcus, and laterally, by the superior frontal sulcus. BA9 occupies also part of the superior frontal gyrus, as well as part of the middle frontal gyrus. It is more anterior and ventral than BA8, extending medially from the cingulate sulcus, and laterally, to the inferior frontal sulcus. As the exact macroscopic boundaries between BA8 and BA9 are imprecise, we have opted to use tissue from both regions combined as BA8/9. In this study, we used tissue dissected from the superior frontal gyrus, dorsally to the frontal pole, to avoid dissecting tissue from BA10. BA11: this BA region occupies a large portion of the orbital gyrus and straight (rectus) gyrus. Tissue for this study was dissected from the orbital gyrus.

Psychiatric diagnoses were obtained using the psychological autopsy method with the Structured Clinical Interview for DSM-IV Axis I (SCID-I) (First *et al*, 2001), as described elsewhere (Dumais *et al*, 2005). Written informed consent was obtained for all subjects from next of kin. This study was approved by our local institutional review board.

Study Design

Putrescine and spermidine concentrations in post-mortem cerebral cortex tissues were analyzed using a specialized polyamine extraction and detection method, which involved high-resolution capillary GC in combination with MS (Chen *et al*, 2009). We collected putrescine and spermidine measurements from 126 brain samples obtained from 42 subjects across three different brain regions as indicated above. The 42 subjects consisted of 16 suicide subjects with major depression (SMD), 13 suicide subjects without major depression (NDS), and 13 control subjects (C). Cases and controls were matched for age, pH, and post-mortem interval (PMI). Methods of suicide were similarly distributed between both suicide groups. We considered individuals as having a history of active treatment if they had a 6-month history of psychotropic prescription and/or had a positive toxicology result.

GC–MS Measurement of Putrescine and Spermidine from Post-Mortem Cerebral Cortex

GC–MS method for analysis putrescine and spermidine. A dedicated GC–MS method was developed for extraction and quantification of putrescine and spermidine from post-mortem human brain tissue (Chen *et al*, 2009). This method is based on the ethoxycarbonyl (EOC) reaction of amino functions combined with subsequent trifluoroacetyl (TFA)

derivatization. Electron ionization GC–MS with selected ion monitoring (SIM) techniques were used with [2,2,3,3-2H4]-1,4-diaminobutane (putrescine-D4) as the internal standard for endogenous putrescine, and 1,7-diaminoheptane as the internal standard for spermidine. This method allows accurate quantitation of spermidine and putrescine down to nanogram levels per gram of wet brain tissue. The isolation methodology is robust and requires less work and time than many previous methods. Before analysis of brain samples, authentic polyamines used as calibration standards were measured, and calibration curves for each target analyte was plotted. A total ion current chromatogram for the derivatives of putrescine and spermidine as well as the internal standard 1.7-diaminoheptane, is given in Figure 1a. A selected ion chromatogram for putrescine and spermidine, extracted from the cerebral cortex of a normal brain and one from a suicide victim with spiked internal standards, 1,7-diaminoheptane, is shown in Figure 1b.

GC–MS conditions. An Agilent bench-top HP6890/MSD5973N Chemstation system (Agilent Technologies, Santa Clara, CA) was used for this work. Electron ionization (EI, 70 eV) was used in full scan and SIM modes for all measurements. The helium flow rate and column head pressure were set to 1.0 ml/min and 8.5 p.s.i., respectively. Sample aliquots of 2 μl were injected in splitless mode followed by a 5-min solvent delay. An HP-5MS capillary column (25 m, 0.25 mm i.d., and 0.25 μm thickness) was used for analysis and was programmed from 140 to 210 °C at 8 °C/min followed by a 2-min hold, then at 20 °C/min to 300 °C, followed by a 4-min hold. The column was baked out at 320 °C for 4 min. Instrument temperatures were: source 200 °C, quadrupole sector 150 °C, interface 250 °C, and injector 260 °C. The typical source pressure was 1.8×10^{-5} torr. Full scans at 2.0 s/scan were over the mass range m/z 10–700.

Data Analysis

GC–MS data analysis was carried out with Chemstation software (Agilent Technologies). Statistical analyses were performed using SPSS 15.0. Pearson correlations were used to assess relationships between sample characteristics and polyamine levels. Univariate two-way ANOVA tests with Bonferroni *post hoc* comparisons were used for the analysis of spermidine and putrescine differences. The two factors analyzed were group (C, NDS, SMD) and BA. Covariates considered were age, pH, PMI, comorbidity with substance, and toxicology/psychotropic treatment history. Age, pH, and PMI were entered in the ANOVA models as covariates. Comorbidity with substance was controlled for by running an ANOVA using this information as a grouping variable.

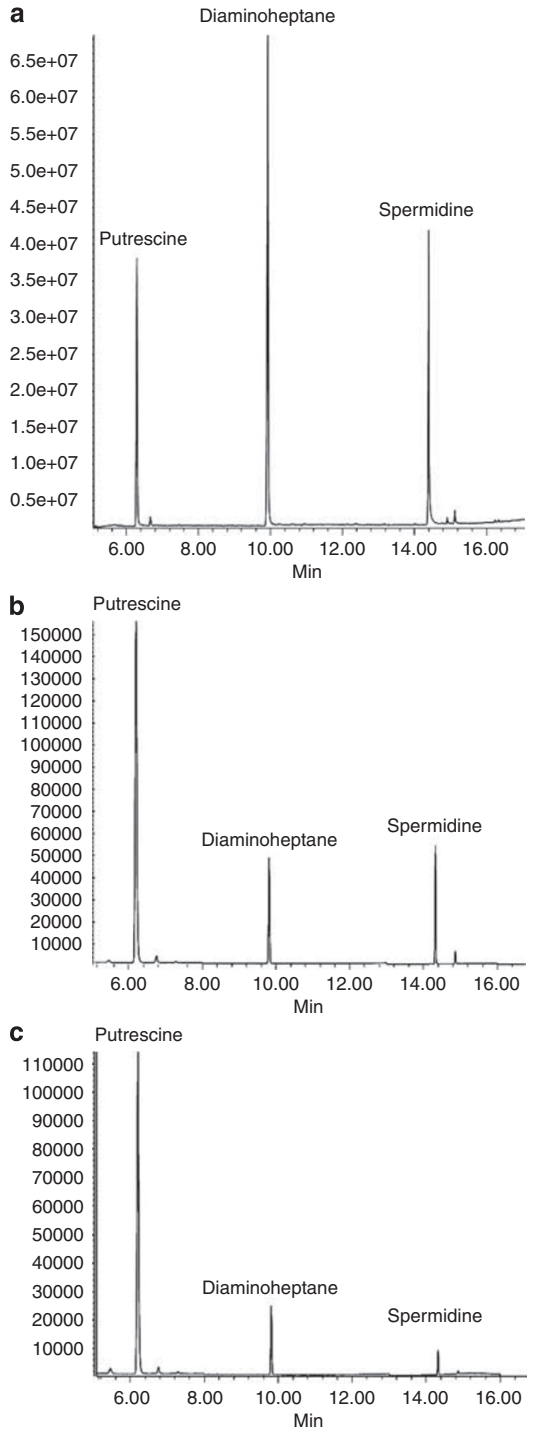

Figure 1 GC–MS spectra of polyamines and internal standards. (a) Total ion current (TIC) chromatogram for the derivatives of the authentic amines, putrescine, and spermidine, as well as the internal standard diaminoheptane, used in the study. (b, c) Composite selected ion chromatograms (SIM) for putrescine, spermidine, and the internal standard diaminoheptane isolated from the cortex of a suicide completer (b) and a control subject (c).

RESULTS

Clinical and Demographic Features

Table 1 lists the demographic and clinical characteristics of the subjects included in this study, according to groups. SMD and DNS cases, as well as controls, had similar demographic characteristics, with no significant differences in age, PMI, or pH.

Table 1 Demographic and Clinical Characteristics of the Subjects Included in this Study According to Group

Group	C	NDS	SMD
N	13	13	16
Age (years)	37.15 ± 11.48	31.92 ± 8.20	36.38 ± 11.58
PMI (h)	26.15 ± 4.43	34.38 ± 23.77	25.87 ± 6.75
pH	6.45 ± 0.30	6.48 ± 0.33	6.53 ± 0.25
MDD	—	—	100% (16/16)
Smoking	7.69% (1/13)	23.08% (3/13)	6.25% (1/16)
Alcohol dependence	23.08% (3/13)	30.76% (4/13)	37.5% (6/16)
Substance dependence	—	15.38% (2/13)	25% (4/16)
Positive toxicology for psychotropics	—	15.38% (2/13)[a]	18.75% (3/16)[b]
Rx	—	7.69% (1/13)[c]	6.25% (2/16)[d]

[a]Alcohol.
[b]Clomipramine, alcohol, and cocaine.
[c]Benzodiazepinic Rx.
[d]TCA and SSRI.

Analysis of Putrescine, Spermidine, and Spermine Levels

Representative mass spectra are shown in Figure 1. Levels of either polyamine were within the expected nanomolar ranges, with significant variability between brain regions. Although in principle spermine can be quantified using the method we developed (Chen *et al*, 2009), it is methodologically challenging to measure concurrently all three major polyamines. Spermine was at the limit of detection in the analyses conducted in this study and accurate measurement was not possible. Therefore, in this study, we focused on the analyses of putrescine and spermidine.

For putrescine, mean values (SD) were 1.84 (0.82), 3.59 (3.4), and 1.25 (0.33), respectively for BA 4, 8/9, and 11 ($F = 14.65$, $df = 2, 113$; $P < 0.001$); and for spermidine, mean values (SD) were 6.99 (6.27), 4.91 (4.90), and 4.39 (3.21), also respectively for BA 4, 8/9, and 11 ($F = 2.85$, $df = 2, 113$; $P = 0.06$). Levels for either putrescine or spermidine were not correlated with age, PMI, or pH, for any of the brain regions examined (r range from -0.095 to 0.017; all $P > 0.3$).

The results for putrescine and spermidine per group and brain region are depicted in Figure 2. We found significantly different levels of putrescine ($F = 6$, $df = 2, 113$; $P = 0.003$) and spermidine ($F = 3.0$, $df = 2, 113$; $P = 0.05$) between groups. Both putrescine ($P = 0.002$) and spermidine ($P = 0.05$) levels were significantly higher in Bonferroni adjusted *post hoc* comparisons between SMD and controls, but not between NDS suicides and controls or between the two suicide groups. None of these differences could be explained by the effect of pH, PMI, age, substance comorbidity, or psychotropic treatment, as none of these covariates were significant. For putrescine, there were no significant effects for region or region by group interaction. For spermidine, there were significant effects for region ($F = 2.85$; $df = 2, 113$; $P = 0.048$), but not for group by region interaction.

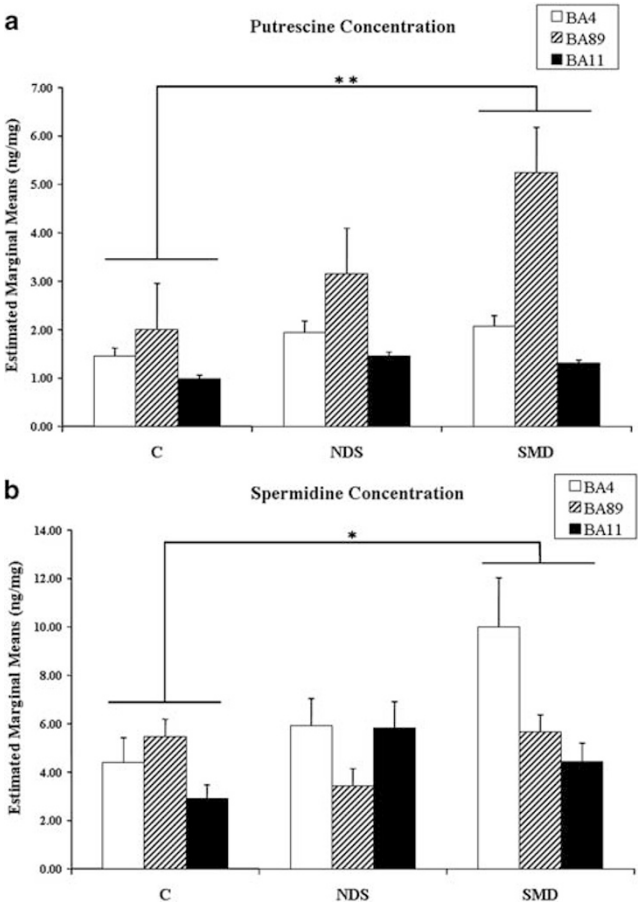

Figure 2 (a) Putrescine and (b) spermidine levels in post-mortem brain tissues. Concentrations (ng/mg tissue) + SEM were obtained from controls (C), suicide completers without major depressive disorder (NDS), and suicide completers with major depressive disorder (SMD). $*P \leqslant 0.05$; $**P \leqslant 0.01$.

DISCUSSION

Using a GC–MS method specifically designed for measuring polyamine concentrations in post-mortem brain tissues, we have successfully determined putrescine and spermidine levels in three different brain regions obtained from 42 suicide completers and control subjects. We found significant increases in putrescine and spermidine levels,

which were explained primarily by differences between the SMD suicides and controls. Although we found no significant difference between the two suicide groups (NDS *vs* SMD), this may be a result of insufficient power, and we therefore cannot rule out the possibility that differences do exist. Future experiments should be performed with more subjects to clarify this effect.

Our findings are in agreement with our previous studies showing decreased SAT1 expression in suicide completers at both mRNA and protein levels (Klempan *et al*, 2009; Sequeira *et al*, 2006). SAT1 is the rate-limiting enzyme in polyamine interconversion and a key regulator of cellular polyamine concentrations. Acetylation of spermine and spermidine by SAT1 is followed by oxidative deamination by polyamine oxidase (PAO) to produce spermidine and putrescine, respectively, as well as 3-acetamidopropanal and hydrogen peroxide (Bolkenius and Seiler, 1981). The increased levels of spermidine observed in this study may thus be a consequence of decreased SAT1 expression, resulting in the accumulation of spermidine, and possibly spermine. Elevated levels of the polyamines, particularly putrescine, are an important component of the polyamine stress response (PSR), which has been implicated in the detrimental effects of stress and anxiety, as well as the development of other psychiatric disorders (Gilad and Gilad, 2003; Sohn *et al*, 2002). Our findings of elevated putrescine and spermidine levels may therefore indicate a role for the PSR in the neurobiology of depression and suicide.

It has been shown that polyamines modulate learning and memory by interacting with the polyamine-binding site at the NMDA and AMPA glutamate receptors (Kashiwagi *et al*, 1997; Kelly *et al*, 2009; Velloso *et al*, 2009). NMDA receptors and AMPA receptors have distinct roles in controlling synaptic plasticity: AMPA receptors effect short-term changes in synaptic strength, whereas NMDA receptors regulate genes that are required for the long-term maintenance of these changes (Groc and Choquet, 2006; Rao and Finkbeiner, 2007). Dysfunction of NMDA and AMPA receptors have been implicated in a verity of neurological disorders, such as Parkinson's disease (Loschmann *et al*, 1997; O'Neill and Witkin, 2007), Alzheimer's disease (Carlson *et al*, 1993; Chang *et al*, 2006; Wang *et al*, 2009), schizophrenia (Bennett, 2009; Halene *et al*, 2009; Seillier and Giuffrida, 2009), anxiety and depression (Ireland and Abraham, 2009; Kielland and Heggelund, 2002; Li *et al*, 2004; Ryan *et al*, 2009; Szewczyk *et al*, 2009), and suicidal behaviors (Freed *et al*, 1993; Nowak *et al*, 2003; Tzschentke, 2002). It has been shown that different levels of the polyamines spermidine and spermine have distinct effects on NMDA receptor activity. Low levels of spermidine and spermine can attenuate glutamate-induced neurotoxicity by enhancing the binding of ion channel blockers; while high levels of spermidine and spermine have enhanced glutamatergic effects (Bence *et al*, 2000). AMPA receptors are a subtype of the ionotropic glutamate receptors that mediate fast synaptic transmission in the central nervous system and have key roles in memory formation (Sanderson *et al*, 2008). The mechanism of polyamine binding and action on AMPA receptors has been extensively studied using polyamine-based ligands (Jensen *et al*, 2006; Stromgaard and Mellor, 2004). Recently, it has been found that a subclass of AMPA receptors lacking the GluR2 subunit is susceptible to blockage by polyamines in a voltage-dependent manner, implicating polyamines in modulation of neurotransmission (Kelly *et al*, 2009). Therefore, the changes in polyamine content we observed in this study may have significant influences on neuronal activity, such as those related to synaptic plasticity and associated with anxiety, depression, and suicidal behaviors (Fiori and Turecki, 2008).

Analysis of brain polyamine levels in suicide completers has previously been reported by Gilad *et al* using HPLC (Gilad *et al*, 1995). They did not identify any significant differences in polyamine concentrations between suicide completers and control subjects. Many factors may have contributed to these differences. First, all our subjects are male and come from a genetically homogenous population, whereas the sample from the previous study comprised both males and females, and was derived from a general population. Our use of only male subjects may be particularly important as gender-specific differences in levels of polyamines and their metabolic enzymes, as well as responses to polyamine exposure have been observed (Barron *et al*, 2008; Bastida *et al*, 2007; Ferioli *et al*, 1999; Gilad *et al*, 2002). Our use of a more homogenous population may have made the group differences more easily detectable, or alternatively, our results may reflect differences in polyamine metabolism between populations. More importantly, however, the previous study used HPLC, which has inherent issues with specificity, as compounds with similar properties may elute at the same time or produce overlapping peaks with the compounds of interest. This problem is especially apparent in complex biological samples such as brain tissues. Indeed, we detected the presence of numerous other bioamines with similar properties to the polyamines. These bioamines may not have been distinguished from the compounds of interest had only peak retention times been used. Finally, differences in PMI or polyamine extraction procedures may also account for some of the differences in findings.

Although our GC–MS method allowed us to successfully quantify putrescine and spermidine, we were unable to measure spermine, the largest polyamine compound. Each GC–MS derivatization and analytical method can only quantify a limited number of compounds with similar properties. Concurrent quantification of the three major polyamines together was challenging in relatively simpler biological samples such as hair (Choi *et al*, 2000; Li *et al*, 2008). The difficulty for quantification of spermine is mainly because of the presence of hiding active hydrogen atoms in its two secondary amine groups. Although our method is capable of accurately and efficiently quantifying putrescine and spermidine, the ability to concurrently detect spermine is compromised because of sample complexity and the derivatization conditions used. Consequently, spermine was at the limit of detection and accurate measurement in the majority of brain samples analyzed. This is disappointing because spermine is present in mammalian brains at levels comparable to spermidine and it is a good substrate for SAT1. The spermine level in cerebral cortex of these subjects may be quantified by the development of a different GC–MS method specifically designed to measure this polyamine.

In addition to our inability to properly quantify spermine, other limitations are present in this study. As mentioned above, we used subjects from a homogeneous population, which may prevent us from extending our results to other populations. In addition, this method cannot distinguish between free and bound polyamines. The majority of polyamines are conjugated with other molecules in the cell to fulfill the diversified functions of polyamines (Gugliucci, 2004). Third, the brain tissue used for this study comprised both neuronal and glial cells. Although polyamines are present in both neurons and glia, they appear to be concentrated in astroglial cells (Laube and Veh, 1997). Given recent evidence suggesting astroglial dysfunction in major depression and suicide (Banasr *et al*, 2008; Ernst *et al*, 2009; Rajkowska and Miguel-Hidalgo, 2007) future experiments should quantify polyamine levels in different brain cell populations.

Taken together, our current findings of elevated putrescine and spermidine levels in suicide completers with a history of major depression provide evidence for important consequences of the downregulation of SAT1 observed in previous studies, and may indicate that stress responses may be altered in these individuals.

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DISCLOSURE

The authors declare no conflict of interest.

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