The pro-apoptotic ARTS/Sept4 protein is significantly reduced in post-mortem brains from schizophrenic patients

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Received 29 March 2007; received in revised form 13 May 2007; accepted 14 May 2007

Abstract

Schizophrenic brains exhibit various neuro-pathological changes in size, volume and structure as compared to normal brains. These structural abnormalities could be the result of apoptotic cell death. ARTS/Sept4 protein plays an important role in induction and promotion of apoptosis. Though ARTS is highly expressed in the healthy human brain, most of tested schizophrenic brain samples showed no expression of ARTS protein. Specifically, using Western blot analysis with monoclonal anti-ARTS antibody we found that only 1 out of 14 schizophrenic samples (7%) showed a strong ARTS signal as compared to 10 out of 15 (66.6%) found in the normal controls group. Furthermore, using immunohistochemistry assay only 33.3% (5 of 15) (SE±12.5) of the schizophrenic patients samples showed any ARTS immunoreactivity as compared to (13 of 15) 87% (SE±9) of bipolar, (11 of 14) 78% (SE±11.3) of major depression and (10 of 14) 71% (SE±12.5) of normal controls. A four-fold reduction in apoptosis rate was measured in these schizophrenic samples as compared to average apoptosis rate found in all other samples. These data support the linkage between loss of ARTS expression and the loss of sensitivity towards apoptosis. Interestingly, levels of ARTS were significantly lower in male schizophrenic patients as compared to female schizophrenic patients, and males of all other control groups. We propose that ARTS may play an important role in the pathogenesis of schizophrenia and could be used as a marker for this disease.

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Keywords: ARTS; Apoptosis; Schizophrenia; Frontal cortex; Neurons

1. Introduction

Schizophrenic brains exhibit various neuro-pathological changes in size, volume and structure as compared to normal brains. The structural abnormalities that were reported include enlarged ventricles, reduced volume of temporal lobe structures at the hippocampus and amygdala, reduced anterior and posterior hippocampal volumes, unusual neuronal density, reduced cortical thickness and loss of cortical neuropil (Kaplan and Sadock, 1995; Selemmon et al., 1998; Rajkowska et al., 1998; Jeste and Lohr, 1995; Kubicki et al., 2002; Cecil et al., 1999; Weiss et al., 2005).

Apoptosis or programmed cell death serves as a major mechanism for the precise regulation of cell numbers and as a defense mechanism to remove unwanted and potentially dangerous cells (Yuan and Yankner, 2000; Hengartner, 2000; Hipfner and Cohen, 2004). The observed
structural changes in schizophrenic brains may be the result of aberrant cell death processes occurring during embryonic brain development in these patients (Sawa and Snyder, 2002; Margolis et al., 1994). Several studies have demonstrated reduced numbers of cortical neurons in schizophrenia post-mortem brains (Benes et al., 1999; Benes et al., 2001; Young et al., 2000; Popken et al., 2000). These structural changes and the observed loss of neurons could be the result of apoptotic cell death. Mechanisms such as NMDA receptor hypofunction and glutamate excitotoxicity have been suggested to contribute to the symptomatic features of schizophrenia (Olney and Farber, 1995; Goldstein et al., 1998; Coyle et al., 2000). Excitotoxicity caused by defects in NMDA glutamate receptors could lead to apoptotic cell death (Laruelle et al., 2003).

The involvement of several apoptotic genes has been studied on brain samples from schizophrenic patients. GSK3 is implicated in modulation of cell fate during development, and its levels were significantly lower in frontal cortex samples from schizophrenia patients (Kozlovsky et al., 2002). Similarly, Bcl-2 protein levels were demonstrated to be 25% less in cortex of schizophrenic patients (Jarskog et al., 2000) and Bax/Bcl-2 ratio increases in temporal cortex of schizophrenic patients (Jarskog et al., 2004). ARTS/Sept4 is a mitochondrial pro-apoptotic protein that can promote apoptosis in response to a variety of pro-apoptotic stimuli (Larisch et al., 2000; Gottfried et al., 2004a,b; Elhasid et al., 2004). Here forth referred to as ARTS. High levels of ARTS alone are sufficient to induce apoptosis in a large range of cancer cell lines (Lotan et al., 2005). Conversely, down-regulation of endogenous ARTS by antisense expression was shown to protect cells against apoptotic stimuli (Larisch et al., 2000). Because ARTS is implicated in a wide variety of apoptotic paradigms, it seems to function at a central apoptotic junction where different upstream apoptotic inputs converge to mediate caspase activation leading to cell death. ARTS induces caspase activation mainly through binding and antagonizing IAPs (Inhibitors of Apoptosis Proteins (Gottfried et al., 2004a)). ARTS functions as a tumor suppressor protein in leukemia (Elhasid et al., 2004) and is involved in the pathogenesis of astrocytomas (Gottfried et al., 2004b)). High levels of ARTS RNA were found in the human brain as compared to other tissues (Larisch et al., 2000), suggesting that ARTS may play an important role in the nervous system. ARTS is a member of the septin family of proteins derived by differential splicing from the human septin 4 gene H5/PNUTL2/CDCrel-2a (Sept4) (Macara et al., 2002; Larisch et al., 2000; Larisch-Bloch et al., 2000). Septins have diverse cellular roles which include polarity determination, cytoskeletal reorganization, membrane dynamics, protein scaffolds, vesicle trafficking, and exocytosis (reviewed by Hall and Russell, 2004).

In this study we show that ARTS protein levels are severely reduced in brains of schizophrenic patients, but not in normal controls, bipolar and major depression brains. Moreover, we demonstrate a parallel reduction in apoptosis rate measured in the same schizophrenic samples as compared to all other control groups. Finally, using cultured post-mitotic neuronal cells we could show that elevated levels of ARTS cause induction of apoptotic cell death in these cells. We therefore conclude that the absence ARTS in schizophrenic brains could be indicative for the role of apoptotic cell death and particularly of ARTS, in the pathogenesis of schizophrenia.

2. Materials and methods

2.1. Post-mortem brain sections

Sixty (60) samples of paraffin embedded human frontal cortex brains and sixty (60) samples of frozen human frontal cortex brains were kindly provided by the Stanley Foundation Brain Consortium. The sections included primarily the middle frontal gyrus (BA46). A small part of the superior frontal gyrus (BA9) was often included on the dorsal edge of the sections. The frozen samples were matched with the paraffin embedded samples. The samples were taken from 15 schizophrenic patients, 15 bipolar disorder patients, 15 with major depression and 15 normal comparison subjects. Immunohistochemistry assays were performed using the paraffin embedded sections. In these assays we used fifteen (15) samples from schizophrenic patients (nine men and six women) with average age of 44.5 years (range = 25–62), and mean post-mortem interval of 33.6 h (range = 12–61). Fifteen (15) bipolar disorder patients (nine men and six women) with average age of 42.3 years (range = 25–61), and mean post-mortem interval of 32.5 h (range = 12–61). Fourteen (14) major depression patients (eight men and six women) with average age of 46.5 years (range = 30–65), and a mean post-mortem interval of 27.5 h (range = 7–47). Fourteen (14) normal comparison subjects (eight men and six women) with average age of 48.3 years (range = 29–68), and mean post-mortem interval of 24.7 h (range = 8–48). For Western blots analysis and the RT-PCR assays we used the frozen samples of 14 schizophrenic patients and 15 normal controls. Two paraffin embedded samples (one major depression sample and one normal comparison sample), and one frozen schizophrenic sample were...
excluded from the assays (immunohistochemistry and Western blot analysis respectively) due to poor quality of the samples. The normal comparison subjects had no history of psychiatric disorder, did not receive any antipsychotic medication, and did not die as a result of suicide or a neurological disorder. Age, sex, post-mortem interval, and brain pH were matched between the four groups as follows: brain pH — using ANOVA test, no statistical difference was found among all four tested groups (mean 6.16–6.25, median 6.2–6.25). PMI — using ANOVA test – no statistical difference was found among all four tested groups (mean 24.71–33.67, median 26–32). Age — using Kruskal Wallis test – no statistical difference was found among all four tested groups (mean 42.33–48.36, median 44–52). In this case we also used the Bonferroni test to confirm that there was no significant difference between the tested groups. Gender — using Pearson Chi-Square – no statistical difference was found among all four tested groups (Asymp. Sig. = 0.997).

All experiments were done on coded slides in a blind manner, and allocation to the four diagnostic groups was done only after decoding data were sent from the Stanley Foundation Brain Bank.

2.2. Western blot analysis

Frozen sections were homogenized using Dounce tissue homogenizer and lysed with 300 µl of lysis buffer (50 mM Tris pH — 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40 protease inhibitors (mini complete, Roche). Protein concentration was determined by BCA Protein assay kit (PIERCE, Cat. Number 23225). Equal amounts of protein lysates (100 µg) were loaded into each well of the 12% SDS-gels, followed by Western blot analysis using monoclonal anti-ARTS antibodies 1:500 (Sigma, Cat. Number A4471), and anti-Actin antibody as loading control. Protein levels were quantified using a densitometer and compared with the level of β-Actin, using anti-Actin 1:1000 (Santa cruz Cat. Number sc-1615). Calculations were carried out using densitometry-analyzing software (Vilber Lourmat, Marne la Vallée, France).

2.3. Immunohistochemistry

Paraffin embedded slides were deparaffinized in xy- lene followed by alcohol gradient. Samples were treated with 0.1 M Na-citrate buffer, pH 6.8, microwaved (90 °C) for 5 min, cooled rapidly and washed twice with PBS. Slides were incubated with 3% hydrogen peroxide for 30 min followed by two 5 minute washes with PBS.

Blocking with 10% normal goat serum (Zymed) in PBS was done for 30 min, followed by incubation with polyclonal anti-ARTS antibody 1:100 (Sigma Cat. Number A 3720) for 90 min. After three washes with PBS the slides were incubated with biotinylated secondary antibody and developed using HRP-conjugated substrate according to manufacturer recommendations (Histostain®-plus bulk kit-Zymed). Each experiment contained a negative control slide incubated with normal rabbit serum. A total of ~1000 cells were counted for each sample. Samples containing any level of ARTS staining regardless of intensity or number of stained cells were considered “positive”. Samples were considered “negative” when no staining for ARTS was detected in the section.

2.4. Apoptosis and viability assays

TUNEL assay was performed on the parallel human frontal cortex samples as the ones used for immunohistochemistry and Western blot analysis. TUNEL assays were performed using the in situ cell death detection kit (POD, Roche) according to the manufacturer’s instructions. ~1000 cells were counted in each section. Samples containing any number of TUNEL positive cells were considered “positive”. Samples that did not show any TUNEL positive cells were termed “negative”. Apoptosis rate was defined as the number of positive or negative samples amongst a diagnostic group. Apoptosis was also evaluated by immunostaining with anti-active caspase-3, antibodies (R&D, Cat. Number: AF835). Cell viability was determined by staining the cells with DAPI and evaluating intact cells according to their nuclear morphology.

2.5. Cell line, transfection conditions and plasmids

The CNS catecholaminergic CAD cell line was a kind gift from Cogent Neuroscience Ltd. The cells were grown in Dulbecco’s modified Eagle F-12 medium (DMED-F12) with 4.5 g/l D-glucose, 8% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml) and glutamine (2 mM), medium (Biological Industries, Israel). The cells were grown at 37 °C in 5% CO2 atmosphere. CAD cells underwent differentiation following 5 days in serum free medium. The cells were transiently transfected using electroporation with PC-12 V Nucleofector kit (Amaxa), pEF1-AU5 control vector, and pEF1-AU5-ARTS constructs were used for all ARTS transient transfection experiments. AU5 tag was attached to the N‘ terminus of ARTS (Larisch et al., 2000).
2.6. Statistical analysis

Western blot densitometry measurements were analyzed using Mann–Whitney U test. ARTS immunostaining data were statistically analyzed using Fisher exact test. Age, sex, post-mortem interval, and brain pH were matched between the four groups. Kruskal Wallis test, Bonferroni test, Pearson Chi-Square and ANOVA test were used respectively to analyze these parameters. Significance was set at $p<0.05$.

3. Results

3.1. ARTS protein levels are significantly reduced in schizophrenic brain samples

To investigate the possible role of ARTS in schizophrenia, we analyzed ARTS protein levels in 14 schizophrenic samples versus 15 normal brain samples. SDS-PAGE and Western blot analysis were performed on frozen frontal cortex brain samples. Overall, ARTS expression levels in the schizophrenic samples were much lower than their levels in normal controls (Fig. 1A). Particularly, only 1 out of 14 schizophrenic samples (7%) showed a strong ARTS signal (more than 0.4 densitometer units) as compared to 10 out of 15 (66.6%) showing strong ARTS signal in the control group. Moreover, the average expression levels of ARTS (ARTS/Actin ratio) in the schizophrenic patients were 34% less than the average expression levels of ARTS in the control group (Mann–Whitney U: $p=0.02$) (Fig. 1B). Similar results showing lower ARTS RNA expression levels in schizophrenia frozen samples were found when compared to normal controls using RT-PCR assay with specific primers for ARTS (data not shown).

To further evaluate the expression levels of ARTS protein, we analyzed ARTS protein levels in 58 frontal cortex paraffin fixed brain sections by Immunohistochemistry assay. The samples were taken from 15 schizophrenic patients, 15 bipolar disorder patients, 14 major depression and 14 normal control subjects. Immunohistochemistry assay was performed using a specific anti-ARTS antibody, and a parallel section of each sample was stained with normal rabbit serum serving as a negative control. All experiments done on coded slides in a blind manner revealed a remarkably homogeneous and intense ARTS immuno-reactivity localized specifically in neuronal cell bodies (Fig. 2A and B). In contrast, negative samples did not show any detectable immunostaining for ARTS in their neuronal cell bodies. Fig. 2A, and B reflect the number of ARTS positive versus negative samples in each diagnostic group. While staining of axons was similar in all samples (data not shown) and served as an internal control, a striking difference in ARTS expression in neuronal bodies was seen among the various groups. Only 33.3% (5 of 15) (SE±12.5) of the schizophrenic patients samples showed any ARTS immunoreactivity as compared to (13 of 15) 87% (SE±9) of bipolar, (11 of 14) 78% (SE±11.3) of major depression and (10 of 14) 71% (SE±12.5) of normal controls (Fig. 2C, Table 1). The distinct absence of ARTS in neurons from schizophrenic patients was compared to all other diagnostic groups using the Fisher exact test. ARTS staining in the schizophrenic subjects was significantly different than the staining exhibited in the major depression subjects ($p=0.0253$) and the bipolar subjects ($p=0.0078$). A strong tendency for difference was found when compared to the normal control subjects ($p=0.06$). Interestingly, there were no significant differences in the average values of ARTS displayed among the three comparison groups (bipolar, major depression, normal controls). Thus, ARTS seems to play a specific role in the pathological process occurring in neurons of schizophrenic patients. Altogether, these data confirm the distinct loss of ARTS in brains of schizophrenic patients.
3.2. ARTS expression is significantly lower in male schizophrenic brains as compared to its expression in male brains of all other diagnostic groups

To determine whether ARTS is differentially displayed in male versus female patients in all four tested diagnostic groups, we analyzed ARTS immunoreactivity in neurons from paraffin sections. We found that sections from male schizophrenic patients showed a highly significant difference in ARTS expression as compared to the bipolar males, (Fisher exact test $p=0.0023$), the major depression males, (Fisher exact test $p=0.0152$) and a strong tendency for difference when compared to the normal control male subjects, (Fisher exact test $p=0.056$). In particular, only 22.2% (SE±11) of male schizophrenic brains were ARTS positive as compared to 50% (SE±14) of schizophrenic women (Fig. 3). Interestingly, males of all other diagnostic groups revealed the complete opposite phenomenon, consistently exhibiting more ARTS positive samples than women of the same diagnostic group. Numbers of ARTS positive samples among female sections were relatively equal across all four diagnostic groups (Fig. 3).

Fig. 2. Brain sections from schizophrenic patients exhibit significant reduction of ARTS immunostaining in neuronal cell bodies. Immunostaining using monoclonal anti-ARTS antibody was performed on post-mortem frontal cortex sections from schizophrenic, bipolar and major depression patients and matched normal controls. Slides were counterstained with Hematoxylin without Eosin (staining the nuclei in blue). ARTS-positive cells are stained in red. (A) Samples from a schizophrenic patients without any detectable ARTS-staining. (B) Normal controls sections contain large number of ARTS-positive neurons manifested as red staining in the cytoplasm of cells. (C) Number of brain samples containing ARTS-positive cells was considerably lower among the schizophrenic patients group as compared to their number in all other diagnostic groups. In particular, 33.3%, SE±12.5 of the schizophrenic patients slides contained ARTS positive cells as compared to 87%, SE±9 of the bipolar patients sections, 78%, SE±11.3 of the major depression patients sections and, 71%, SE±12.5 of the normal controls.

3.2. ARTS expression is significantly lower in male schizophrenic brains as compared to its expression in male brains of all other diagnostic groups

Please cite this article as: Gottfried, Y. et al. The pro-apoptotic ARTS/Sept4 protein is significantly reduced in post-mortem brains from schizophrenic patients. Schizophrenia Research (2007), doi:10.1016/j.schres.2007.05.031
3.3. Apoptosis rate is four times lower in schizophrenic patients as compared to all other diagnostic groups

Levels of ARTS protein were shown to be tightly linked to cells sensitivity towards apoptosis (Larisch et al., 2000; Gottfried et al., 2004a,b; Elhasid et al., 2004; Lotan et al., 2005). To assess the rate of apoptotic cell death taking place in the tested brain samples, a TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) assay was performed on parallel sections used for immunohistochemistry (Fig. 4A). The TUNEL assay detects DNA fragmentation process occurring in dying apoptotic cells. Interestingly, while the average TUNEL-positive samples (apoptosis rate) was 26% in all three control groups (28.5% in the normal controls, 28% in the major depression samples, and 20% of the bipolar samples) only 6.6% of the schizophrenic samples were TUNEL-positive (Fig. 4B). We suggest that the lower apoptosis rate observed within the schizophrenic group may be linked to the loss of ARTS in these schizophrenic post-mortem brains.

3.4. ARTS protein sensitizes post-mitotic neuronal cells towards apoptosis

To further explore the linkage between ARTS protein levels and apoptosis in neuronal post-mitotic cells, we used a CNS catecholaminergic cell line, CAD cells (Fig. 5A). CAD cells undergo differentiation to mature neurons following 5 days of serum deprivation (Qi et al., 1997; Wang and Oxford, 2000). Subsequently, these cells stop proliferating and develop a typical neuronal-like morphology (dCAD) (Fig. 5B). First, using Western blot analysis we showed that ARTS is present in both undifferentiated and differentiated CAD cells in relatively

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Table 1

<table>
<thead>
<tr>
<th>ARTS Immune-staining analysis</th>
<th>Total number of tested samples</th>
<th>ARTS positive samples</th>
<th>Percentage of ARTS positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schizophrenic subjects</td>
<td>15</td>
<td>5</td>
<td>33% (SE±0.12)</td>
</tr>
<tr>
<td>Normal controls</td>
<td>14</td>
<td>10</td>
<td>71% (SE±0.12)</td>
</tr>
<tr>
<td>Major depression subjects</td>
<td>14</td>
<td>11</td>
<td>78% (SE±0.11)</td>
</tr>
<tr>
<td>Bipolar subjects</td>
<td>15</td>
<td>13</td>
<td>86% (SE±0.09)</td>
</tr>
</tbody>
</table>

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Fig. 3. ARTS expression is significantly lower in male schizophrenic brains as compared to its expression in male brains of all other diagnostic groups. The number of samples containing ARTS positive cells was significantly lower among male schizophrenic patients as compared to their number among males of all other diagnostic groups. The difference as compared to the bipolar males; \( p = 0.007 \), compared to major depression males \( p = 0.0253 \) and compared to normal control male subjects \( p = 0.06 \). No significant difference in number of ARTS positive samples was seen among the female sections from all diagnostic groups.

Fig. 4. Schizophrenic brain samples show reduced levels of apoptosis. Levels of apoptosis were examined in all brain samples using the TUNEL assay. (A) An example of a frontal cortex section with several TUNEL-positive cells (arrows). (B) Amongst the different patient groups examined, the schizophrenic patient’s samples showed the lowest number of TUNEL-positive cells.

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similar amounts (Fig. 5C). Next, we transfected the dCAD cells with pEF1-AU5-ARTS expression vector or pEF1-AU5 empty vector using the Amaxa Nucleofector. Levels of active caspase 3 reflecting rate of apoptosis were measured in the transfected dCAD cells. Interestingly, in the transfected cells expressing high levels of ARTS, a three fold increase in caspase 3 activity was measured (Fig. 5D). In addition, we tested the survival rate of pEF1-AU5-ARTS transfected cells, as compared to cells transfected with pEF1-AU5 empty vector, by staining with DAPI and counting cells exhibiting normal nuclear morphology, 24 and 48 h posttransfection. The number of viable cells was significantly decreased upon ARTS transfection. Viability of ARTS transfected dCAD cells was reduced to approx. 60% after 24 h, and to approx. 25%, 48 h after transfection as compared to control transfected cells (Fig. 5E). These results confirm that increased levels of ARTS promote apoptosis in neuronal cells.

4. Discussion

In this study we explored the possible role of apoptotic cell death in the pathogenesis of schizophrenia, particularly through examining the expression of the pro-apoptotic ARTS/Sept4 protein in post-mortem neuronal cells.
from schizophrenic patients. Schizophrenic brains exhibit various neuro-pathological changes in size, volume and structure as compared to normal brains. These structural changes may be the result of aberrant cell death (Sawa and Snyder, 2002; Margolis et al., 1994). ARTS protein is highly expressed in healthy human brain (Larisch et al., 2000; Larisch-Bloch et al., 2000). In contrast, we found that ARTS protein was distinctly absent in neuronal cell bodies from schizophrenic brains. These results were especially convincing in light of the following reasons: First, all immunohistochemistry assays were performed in a blind manner, as all 58 brain sections were coded, and results were revealed only following decoding. Second, a strong staining of axons shown with anti-ARTS antibodies across all groups served as a reliable internal control. In addition, no staining was observed when samples were tested with normal rabbit serum. Finally, two independent methods for protein quantitation, Western blot analysis and immunohistochemistry, were used to confirm the significant reduction of ARTS in the schizophrenic samples. All these observations indicate that the loss of ARTS-immunoreactivity in schizophrenic brain samples was distinct and specific for these patients. Previous studies have shown that high levels of ARTS promote apoptosis, while lack of ARTS expression is correlated with lack of responsiveness to apoptosis (Larisch et al., 2000; Elhasid et al., 2004). Thus, we presume that the specific absence of ARTS in neurons from schizophrenic patients may be due to the selective loss of neuronal cells containing high levels of ARTS occurring prior to the full blown onset of the disease. Selective loss of neurons in schizophrenic subjects was reported by other groups. For example, Benes et al. described the loss of interneurons in schizophrenic brains (Benes et al., 1999), and Reynolds et al. found loss of a specific subtype of cortical GABAergic neurons in schizophrenic patients (Reynolds et al., 2002). Therefore, it is possible that apoptotic death of a specific population of neurons, presumably those containing high levels of ARTS, may contribute to the onset of schizophrenia. We hypothesize that genetic aberrations (either germ line or somatic mutations in the ARTS/Sept4 gene) or environmental effects may cause up-regulation of ARTS in certain neurons, and that this may promote the death of these cells in response to stress and/or other factors during the onset of schizophrenia. By the time the disease is manifested, few neurons with high levels of ARTS would remain, thereby explaining the observed reduction in ARTS-immunoreactivity. ARTS is a member of the septin family of proteins (Larisch et al., 2000; Larisch-Bloch et al., 2000). At least one other septin protein, Septin 5, has been implicated in neuronal cell death, since elevated levels of this protein were associated with increased cell death of dopaminergic neurons (Son et al., 2005). Many septin family proteins such as Sept8, Sept5 and Sept4 are shown to function together in heterotypic assembly (Martinez et al., 2004). Likewise, ARTS might act in a complex with other septins to maintain proper neuronal function and/or differentiation. Loss of ARTS might destabilize this complex leading to possible neuronal aberrations which could contribute to manifestation of schizophrenia. Therefore, it is possible that ARTS plays a role in brain physiology, and that the observed reduction of ARTS protein is correlated with this function. Reduced apoptosis in brain samples from schizophrenic patients has been reported by several other investigators (Benes et al., 2003; Jarskog et al., 2000). All these observations are consistent with the idea that brains of schizophrenic patients become depleted of those neurons that are particularly susceptible to apoptosis, leaving behind populations of relatively death-resistant brain cells. Another possible explanation is that the observed exceptional low levels of ARTS found in schizophrenic brains could be responsible for the particularly low apoptosis levels observed in these patients. The low protein expression of ARTS in brains of schizophrenic subjects could result from a range of genetic and/or molecular abnormalities such as; deletions/mutations, DNA methylation, or increased degradation of the protein through ubiquitin-proteasome mediated degradation. Indeed, loss of ARTS was demonstrated in the majority of Acute Lymphoblastic Leukemia (ALL) patients. One of the mechanisms found to be responsible for this loss of ARTS in these patients was DNA methylation (Elhasid et al., 2004). In addition, ARTS levels are shown to be tightly regulated through the ubiquitin proteasome system in normal non-apoptotic cells (Lotan et al., 2005). Thus, it is possible that aberrant excess degradation of ARTS could result in its specific loss in schizophrenic patients, which could also lead to reduced apoptosis levels seen in these post-mortem brains. Interestingly, a higher percentage of male (78%) compared to female (50%) schizophrenic patients showed no detectable expression of the ARTS protein (Fig. 3). Several studies indicate differences between schizophrenic males and females regarding onset of disease and severity of symptoms (Castle et al., 2000; Childers and Harding, 1990; Leung and Chue, 2000; Moriarty et al., 2001; Nasser et al., 2002; Roy et al., 2001; Tamminga, 1997). These differences may arise from the interplay of sex hormones, neurodevelopmental and psychosocial sex differences (Leung and Chue, 2000; Tamminga, 1997). It is possible that one of the factors affecting the severity of symptoms in male schizophrenic patients is the specific loss of ARTS-containing neurons (Fig. 3). The effect of medication on the observed low ARTS levels and/or low apoptosis rate could not be ruled out as a possible explanation for our...
findings. Yet, only two of the schizophrenic patients were medication free at the time of death, and this small number did not permit a comparison between medicated and unmedicated patients. In addition, very little and confusing data exists regarding the effect of antipsychotic drugs on apoptosis. Mainly, several reports show that antipsychotic drugs produce complex effects on apoptotic regulation in the CNS, activating both pro-apoptotic and anti-apoptotic signaling pathways (reviewed by Jarskog, 2006). Furthermore, the majority of our bipolar and depressed patients were on psychotropic medications, mainly antidepressants and mood stabilizers, but also antipsychotics in some cases. One would have to assume that only antipsychotic medications as a class (both old generation typical and new generation atypical antipsychotics) but not antidepressants or mood stabilizers could affect apoptosis. Furthermore, in our study though both male and female schizophrenic patients received similar treatment, significant reduction in ARTS levels was seen only among the schizophrenic male patients. This result could indicate that in our tested samples medication had little or no effect on ARTS post-mortem brain levels.

In summary, we show that ARTS-containing neurons are predominantly lost in schizophrenic patients. This may be explained by the preferential death of neurons in the frontal cortex of schizophrenic patients that contain high levels of ARTS. We interpret our findings to suggest that these particular neurons have increased susceptibility to environmental and/or other stresses leading to apoptosis, and are therefore at risk. According to our model, ARTS-positive neurons have higher probability to die at some point prior to or during the manifestation of the disease. Based on these results we propose that ARTS may play an important role in pathogenesis of schizophrenia and can be used as a marker for this disease.

Role of the funding source
This work was supported by the generous funding from the National Alliance for Research on Schizophrenia and Depression (NARSAD) and from the Israel Science Foundation (ISF). Support from these agencies allowed payment for a full time researcher and a PhD student that worked on this project and the materials and reagents needed to pursue the scientific experiments described in this manuscript. In addition we acknowledge the generous contribution of post-mortem frontal cortex brain sections supplied from the Stanley Foundation, that made this study possible.

Contributors
Yossi Gottfried — Performed the assays and their statistical analysis and participated in writing the manuscript. This work was done as part of his doctoral thesis at the Technion-Israel under the supervision of Dr. Sarit Larisch.

Asaf Rotem — Performed the assays and participated in writing the manuscript.

Ehud Klein — As the head of psychiatric department at Rambam Medical center, Prof. Klein provided important psychiatric evaluation regarding design of experiments, analysis of the data and important input regarding presentation of the results in the manuscript.

Sarit Larisch — Designed and supervised the work done in this study. Wrote the grants which provided funding of this work. The study was performed in her research lab.

Conflict of interest
All authors (Yossi Gottfried, Asaf Rotem, Ehud Klein and Sarit Larisch) state that there is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three (3) years of beginning the work submitted that could inappropriately influence, or be perceived to influence, their work.

Acknowledgments
The authors wish to deeply thank the Stanley Foundation Brain Consortium for post-mortem brain sections. We also thank Prof. Hermann Steller for fruitful discussions and thoughtful advice and Dr. Galila Agam from Ben-Gurion University, Beer-Sheva Israel for helpful comments on the manuscript. This manuscript is a part of Yossi Gottfried’s doctoral thesis at the Technion-Israel.

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Please cite this article as: Gottfried, Y. et al. The pro-apoptotic ARTS/Sept4 protein is significantly reduced in post-mortem brains from schizophrenic patients. Schizophrenia Research (2007), doi:10.1016/j.schres.2007.05.031