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Apoptosis-related proteins and proliferation markers in the orbitofrontal cortex in major depressive disorder

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Abstract

Background: In major depressive disorder (MDD), lowered neural activity and significant reductions of markers of cell resiliency to degeneration occur in the prefrontal cortex (PFC). It is still unclear whether changes in other relevant markers of cell vulnerability to degeneration and markers of cell proliferation are associated with MDD.

Methods: Levels of caspase 8 (C8), X-linked inhibitor of apoptosis protein (XIAP), direct IAP binding protein with low pI (DIABLO), proliferating cell nuclear antigen (PCNA) and density of cells immunoreactive (-IR) for proliferation marker Ki-67 were measured in postmortem samples of the left orbitofrontal cortex (OFC) of subjects with MDD, and psychiatrically-normal comparison subjects.

Results: There was significant increase in C8, a higher ratio of DIABLO to XIAP, lower packing density of Ki-67-IR cells, and an unexpected age-dependent increase in PCNA in subjects with MDD vs. controls. PCNA levels were significantly higher in MDD subjects unresponsive to antidepressants or untreated with antidepressants. The DIABLO/XIAP ratio was higher in MDD subjects without antidepressants than in comparison subjects.

Limitations: Qualitative nature of responsiveness assessments; Definition of resistance to antidepressant treatment is still controversial; Unclear role of PCNA.

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TUNEL detection of fragmented DNA: Details presented in the Supplemental Materials file.

Conclusions: Markers of cell vulnerability to degeneration are increased and density of Ki67-positive cells is low MDD, but accompanied by normal XIAP levels. The results suggest increased vulnerability to cell pathology in depression that is insufficient to cause morphologically conspicuous cell death. Persistent but low-grade vulnerability to cell degeneration coexisting with reduced proliferation readiness may explain age-dependent reductions in neuronal densities in the OFC of depressed subjects.

Keywords

Depression; prefrontal cortex; comorbidity; vulnerability,; postmortem

INTRODUCTION

Specific regions of the prefrontal cortex (PFC) undergo marked functional disturbances in subjects with major depressive disorder (MDD) (Drevets et al., 2008). Furthermore, PFC neurons and glial cells display significant morphological changes in MDD as compared to non-psychiatric control subjects. For instance, the packing density of astrocytes immunoreactive for glial fibrillary acidic protein (GFAP) and the levels of GFAP are lower in depressed subjects at relatively younger ages. However, GFAP values in older subjects with depression or subjects with prolonged duration of depression are not different from matched control subjects (Khundakar and Thomas, 2009; Miguel-Hidalgo et al., 2000; Si et al., 2004; Uranova et al., 2004). Lower densities of oligodendrocytes or their markers also occur in depression (Honer et al., 1999; Uranova et al., 2004), but mainly later in life (Vostrikov and Uranova, 2011).

In contrast to glia, neuronal packing density significantly declines later in life in one of the major subdivisions of the PFC, the orbitofrontal cortex (OFC), of subjects with MDD (Rajkowska et al., 2005). Also in the OFC, structural and functional neuroimaging studies have revealed significant abnormalities in depression (Drevets, 2007). Pathology in the OFC is relevant to the physiopathology of depression because the OFC is heavily involved in the regulation of emotion and decision-making, which are notoriously dysfunctional in major depression (Austin et al., 2001; Drevets, 2007).

Thus, there seems to be an age- or duration-related vulnerability to loss or reduced density of neurons and glial cells in the OFC of subjects with MDD. However, in contrast to classic neurodegenerative disorders, detecting cellular vulnerability in MDD has required the use of detailed microscopic cell counting and immunohistochemical staining techniques since there is no grossly noticeable loss of cells or brain tissue. Increased vulnerability of neural cells in some brain areas to progressive loss or degeneration could still derive from altered expression of proteins related to cell death and survival, and in glial cells, also of cell proliferation markers. An altered balance between these proteins in neocortical areas relevant to the pathology of depression could be a major immediate cause for increased cellular vulnerability to damage or slow depletion of cell numbers (Manji et al., 2001; Manji et al., 2000).

This study examined levels of apoptosis-promoting proteins caspase 8 (C8) and direct IAP binding protein with low pI (DIABLO), anti-apoptotic protein X-linked inhibitor of

apoptosis protein (XIAP), and proliferating cell nuclear antigen (PCNA), a marker for dividing cells, in subjects with depression. We also examined the presence of cell nuclei immunoreactive for Ki-67, another marker for cells undergoing the mitotic cycle, and of cells with fragmented DNA using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technique, an indicator of cells dying by apoptosis.

Caspase 8 (C8) is a major component in the initiation of the so-called extrinsic apoptotic pathway (Elmore, 2007; Lavrik et al., 2005), and it may be increased in vulnerable neurons and glia even if not immediately causing apoptosis (Kuranaga and Miura, 2007). Tumor necrosis factor α (TNF- α) and related cytokines, which act on receptors that activate C8, are elevated in the brain and plasma of depressed subjects (Dowlati et al., 2010) suggesting that increased C8 activation in neurons and glial cells (Berthold-Losleben and Himmerich, 2008) in specific brain areas may augment the risk for apoptosis-related cytopathology. C8 activates the apoptosis effector caspase 3, which cleaves various proteins essential for cell survival. Apoptosis can be blocked by XIAP, an inhibitor of caspase 3 (Deveraux et al., 1998; Holcik et al., 2001; Wang et al., 2004). XIAP is in turn inhibited by DIABLO, thus promoting apoptosis progression (Niizuma et al., 2010; Saito et al., 2003; Verhagen et al., 2000). Increases in the DIABLO/XIAP ratio reflect vulnerability to degeneration or apoptosis (Albeck et al., 2008; Scarabelli et al., 2004).

PCNA is a DNA-binding protein that acts as a cofactor of DNA polymerase delta, being highly expressed in the DNA replication step preceding cell division (Kurki et al., 1986; Muskhelishvili et al., 2003), but significantly reduced in non-dividing cells. PCNA is also heavily involved in DNA repair, (Essers et al., 2005; Maga and Hubscher, 2003). Ki-67 is another marker for cell nuclei involved in the mitotic cycle, but deemed more specific for cells engaged in that cycle than PCNA (Kee et al., 2002; Scholzen and Gerdes, 2000). Thus, we used Ki-67-labeling to determine *in situ* changes in the packing density of cells with proliferative potential.

METHODS

Human subjects

Human postmortem brain tissue originated from autopsies at the Cuyahoga County Coroner's Office in Cleveland, OH. Collection of postmortem materials was performed according to a protocol approved by the Institutional Review Boards at University Hospitals of Cleveland and the University of Mississippi Medical Center. Retrospective psychiatric diagnoses of the deceased, a validated technique (Dejong and Overholser, 2009), involved using information from knowledgeable informants (next-of-kin, significant others) and medical and toxicological records. Assessment of the presence or absence of psychiatric symptoms was based on the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)(Association, 1994). Subjects with head trauma or a neurological disease were excluded. Brain tissue from 46 subjects was included: 23 subjects met criteria for major depressive disorder (MDD) and 23 comparison subjects (COMP) did not meet criteria for an Axis I psychiatric disorder as defined in DSM-IV. However, due to limitations in tissue availability, experiments to detect DIABLO and XIAP included fewer subjects per group, as reflected in the degrees of freedom in statistical

data. Further summary information on subjects is presented in supplemental table 1. Estimates of responsiveness to antidepressant medication in subjects with MDD were obtained before experiments with the postmortem tissue started. The estimates were made according to antemortem clinical impressions and next-of-kin testimony. For only two subjects (one responder and one non-responder) was responsiveness estimated after the experiments had concluded.

Tissue—Human postmortem brain tissue from Brodmann's area 47 in the left OFC was examined. Groups were matched to minimize differences in age, postmortem interval, tissue pH, gender, and race, and there was no significant difference in any of those variables between the groups. The anterior part of Brodmann's area 47 was selected and located anterior to the transverse sulcus and lateral to the medial orbital sulcus based on cytoarchitectonic criteria and the pattern of orbital gyri and sulci (Duvernoy et al.; Uylings et al., 2010). For immunohistochemistry and Western blots, each frozen block was cut into alternating sections with a thickness of 20 μ m and 50 μ m, respectively.

Western blotting—Punch samples were taken from frozen 50- μ m sections. Each sample spanned the cortical gray matter from the pial surface to the boundary of gray and white matter. Samples were homogenized in 0.01M Tris-HCL containing 1% SDS, 2 mM EDTA, and protease inhibitor. After centrifugation, 40 μ g of supernatant protein were applied per gel lane and run with the Xcell II NuPAGE Bis-Tris Electrophoretic System (Invitrogen, Carlsbad, CA). Proteins were transferred to PVDF membranes that were probed with the following antibodies: rabbit polyclonal anti-C8 (Ab-4; Thermo Scientific, Fremont, CA), mouse monoclonal anti-PCNA (PC10; f Invitrogen, Camarillo, CA), mouse monoclonal anti-DIABLO at 1:2000 dilution (56/Smac/DIABLO, BD-Biosciences, San Jose, CA), and mouse monoclonal anti-XIAP at 1:4000 (48/hILP/XIAP, BD Biosciences, San Jose, CA). Some membranes were incubated overnight at 4 °C with the primary antibody to C8 and processed with alkaline phosphatase-conjugated secondary antibody. Chemiluminescent bands were imaged in a Kodak Image Station-440-CF. Antibodies were stripped from membranes and the membranes re-probed with anti-PCNA, followed by stripping, and incubation with anti- β -actin. In other experiments, membranes from a smaller number of subjects were incubated with anti-XIAP, processed for chemiluminescence, stripped, processed for anti-DIABLO chemiluminescence, further stripped and probed with anti- β -actin.

Samples were run in duplicate, altering gel positions to demonstrate replicability. The level of each protein was calculated as a ratio of the optical density of bands of interest to the band of β -actin. Two samples from two designated comparison subjects (internal control samples) that were approximately in the middle to low range of reaction intensities for comparison subjects were included in all blots. In this manner, relative levels of proteins were calculated by dividing the protein level (determined relative to actin, see above) in a particular subject by the level of the protein in the internal controls. The amounts of homogenate delivered were calculated so that blot densitometric measurements fell within the linear range of chemiluminescence intensities.

Immunohistochemistry—Frozen 20- μ m sections were mounted on slides, dried for 10–20 minutes and fixed 30 minutes in 4% paraformaldehyde. Triplicate sections were then washed in phosphate buffered saline (pH 7.4) and incubated with pre-diluted anti-Ki-67 antibody (supplied by Zymed). Three days later, sections were washed in 0.1 M Tris-HCl buffer (pH 7.4), incubated with biotinylated secondary antibodies, washed again and the bound antibody was detected using the ABC method (Vector) with 3,3'-diaminobenzidine (DAB) enhanced with nickel ammonium sulfate as chromogen. Omission of primary or secondary antibodies on sections or membranes prevented specific staining. Specific immunostaining was also absent after incubating the primary antibodies with the corresponding antigens before their use.

Three 20 μ m-thick sections of the OFC per subject were processed for DAB-based immunohistochemistry of Ki-67. The packing density of Ki-67-labeled nuclei (Fig. 4A-F) was measured using the optical disector function in the StereoInvestigator software (version 8, MicroBrightField Bioscience, VT) applied to a random sampling of counting frames (between 19 and 22 frames per section) within the gray matter, including all cortical layers of the gray matter.

Statistics—There were no statistically significant differences between groups in postmortem interval, age at the time of death, brain weight or brain pH. Thus, differences in protein levels or cell packing densities between groups were assessed by analysis of variance (ANOVA). Alternatively, when the number of subjects permitted, a two-way ANOVA was performed using diagnosis and gender as factors. Correlations of age with the various neurochemical (levels of proteins from western blots) or cell-counting parameters (Ki67-positive cells, TUNEL-stained cells) were studied for each diagnostic group using Pearson correlation matrices and applying the Bonferroni correction for multiple comparisons as needed. Particular attention was paid to the possible influence of PMI on the numbers of TUNEL-stained cells and the levels of proteins since there are reports in mice of possible effects of long PMI with techniques related to TUNEL such as in situ end-labeling (ISEL) when PMI is longer than 24 hours or fixation is long (in our samples fixation was 30 minutes) (Lucassen et al., 1995; Schallock et al., 1997). However, in our cohort of OFC samples, we did not find a correlation between PMI and TUNEL staining, which was further validated by the use of a positive control with PMI similar to the PMI in the experimental samples (see supplemental materials). In addition, PMI was not correlated with the level of any of the proteins in any of the diagnostic groups.

RESULTS

1.1 Caspase 8 levels

The antibody to C8 revealed a band at about 50 kDa known to represent procaspase 8 (C8) (supplemental fig. 1), a band at approximately 36 kDa, and a band at about 14–12 kDa that represents a small fragment of processed procaspase 8 (C8-14) (Fig. 1A). This latter band is considered to represent fragments of activated caspase 8 generated after interaction with death domain proteins (Schamberger et al., 2005). Studies indicate that higher levels of short fragments (18-kDa to 10 kDa) indicate activation of caspase 8 (Bredesen et al., 2006; Koski

et al., 2004; Medema et al., 1997). Comparison of the two diagnostic groups using a two-way ANOVA (disease and gender as factors) revealed a significant difference between the cohorts for levels of C8-14 ($F(1, 42)=15.708$, $p < 0.001$) (Fig. 1B), although there was no effect of gender. Levels of procaspase 8 (band at 50 kDa) were also higher in the MDD than in comparison subjects (Fig. 1A, Supplemental fig. 1) ($F(1,42) = 6.085$, $p = 0.018$).

1.2 DIABLO and XIAP levels

We determined the ratio of DIABLO to XIAP levels (DIABLO/XIAP) in the OFC in each of 15 comparison and 14 MDD subjects (Fig. 2A, B). The DIABLO/XIAP ratio was significantly higher in the MDD than in comparison subjects (two-way ANOVA, $F(1, 25) = 5.831$, $p = 0.023$) (Fig. 2B). While there was no significant effect of gender ($p=0.94$), there was a significant gender by diagnosis interaction ($F(1,25) = 5.216$, $p = 0.031$). In this interaction, depressed male subjects had the highest DIABLO/XIAP ratios. Comparison of DIABLO levels revealed no significant effect of depression ($F(1,25) = 2.455$, $p = 0.130$), although male subjects with MDD demonstrated the highest levels of DIABLO (Fig. 2C). There was no difference in XIAP levels between comparison and depressed subjects ($F(1, 25) = 0.780$, $p=0.385$) (Fig. 2D).

1.3 TUNEL staining in the OFC

TUNEL-positive cells were detected in only a few subjects per diagnosis, and the number of cells was not greater than 2 per sampling site. Thus, there was insufficient data to further evaluate this marker of apoptosis.

2.1 PCNA levels

In Western blots, the antibody to PCNA always revealed a single band at 36 kDa, common to all subjects (Fig. 3) and occasionally a weak band slightly above 36 kDa. Only the 36 kDa band was quantified (Fig. 3A). Two-way ANOVA revealed a significantly higher level of PCNA in subjects with MDD than in comparison subjects ($F(1,42)=4.243$, $p=0.046$) (Fig. 3B). There was no significant difference in PCNA levels between genders nor was there a gender by diagnosis interaction.

2.2 Packing density of Ki-67-positive cell nuclei

The density of Ki-67-IR nuclei (Fig. 4A-D) was significantly decreased in the OFC of depressed vs. comparison subjects (Two-way ANOVA $F(1,42) = 4.859$, $p=0.033$) (Fig. 4E). However, there was no significant effect of gender or gender by diagnosis interaction.

A significant negative correlation occurred between the density of Ki-67-IR cells and PCNA levels in comparison subjects ($r=-0.521$; $p=0.011$) (Supplemental Fig. 2A), but not in subjects with MDD.

3. EFFECT OF AGE

There was a positive correlation between c8-14 levels and age in COMP subjects ($r=0.422$, $p = 0.045$) (Fig. 5A) but not in subjects with MDD (Fig. 5B).

In the comparison group, there was a trend for a significant correlation between PCNA levels and age ($r = 0.405$, $p = 0.055$) (Fig. 6A). However, there was a significant positive correlation of PCNA level with age in the subjects with MDD ($r=0.551$, $p=0.006$) (Fig. 6B).

4. ANTIDEPRESSANTS AND RESPONSE TO TREATMENT

We split the subjects with MDD into those prescribed with antidepressants (Adep, 17 subjects), and those without a known Adep prescription (NAdep, 6 subjects). In addition, the markers of cell survival and proliferation were examined in Adep-treated subjects divided into those that were considered responders (7 subjects) or non-responders (10 subjects) to medication.

For c8-14, comparison of the three groups of subjects (COMP, MDD with Adep treatment, and MDD without Adep) still showed an effect of diagnosis, with depressed subjects having the highest levels of c8-14 (ANOVA $F(2, 43)=12.4$, $p < 0.001$) (Supplemental Fig. 3A). Subjects receiving Adep treatment were not significantly different from MDD subjects with no treatment, although there was a trend for higher c8-14 levels in subjects not under treatment ($p=0.064$). Comparison subjects differed significantly from non-responders ($p=0.044$) (Supplemental Fig. 3B).

The three groups (COMP, MDD with Adep, MDD without Adep) significantly differed in PCNA levels ($F(2,43) = 4.417$, $p = 0.018$). MDD with Adep were not significantly different from non-treated subjects, although they had significantly higher PCNA levels than COMP subjects (Supplemental Fig. 3C). PCNA level was higher in non-responders as compared to responders ($p = 0.023$), to MDD subjects without Adep. ($p = 0.029$) or to non-psychiatric comparison subjects ($p = 0.001$) (Supplemental Fig. 3D).

There were no significant differences between COMP and MDD subjects in the density of Ki-67 immunoreactive cells when considering either Adep treatment or response to treatment.

Due to the limited availability of tissue for determinations of DIABLO and XIAP, only two MDD subjects without Adep and 12 with Adep were processed, and no meaningful treatment-related comparison could be performed. Interestingly, the two subjects without treatment had the highest values for the DIABLO to XIAP ratio among subjects with MDD. Among the 12 subjects with Adep there were only three subjects that were deemed non-responders, but they were undistinguishable from the responders.

DISCUSSION

The present results suggest that levels of proteins crucial to mechanisms of cell degeneration, death or proliferation are significantly altered in the OFC in MDD. Both the C8-14 level and the DIABLO/XIAP ratio were increased in MDD. The depressed subjects also showed a significantly lower mean packing density of Ki-67 immunoreactive cells and, surprisingly, higher PCNA levels than the non-psychiatric comparison group.

INCREASED PRO-APOPTOTIC MARKERS

An increase in the C8-14 fragments indicates activation of C8, while an augmented DIABLO/XIAP ratio has been linked to an increased risk for cell death (Albeck et al., 2008; Scarabelli et al., 2004). Thus, an increase in C8-14 and the DIABLO/XIAP ratio in MDD would be consistent with increased vulnerability to cell death or degeneration of OFC cells. In turn, enhanced vulnerability to degeneration might explain decreased density of glial cells in young and middle-aged subjects with depression (Miguel-Hidalgo et al., 2000) or decreased density of neurons in older subjects with depression (Rajkowska et al., 2005). Indirect support for an involvement of C8 in the pathophysiology of depression is further provided by the ability of the antidepressant fluoxetine to up-regulate the expression of c-FLIP, a C8 inhibitor, in hippocampal neural stem cells (Chiou et al., 2006), suggesting that fluoxetine actions might be partly mediated by reducing the vulnerability of cortical cells to C8-mediated degeneration. In the absence of direct identification of the cell types with increased expression of C8 and DIABLO/XIAP ratio, we can only speculate that these increases affect both neurons and glia because packing density or numbers of both cell types in the prefrontal cortex are decreased in depression (Cotter et al., 2001; Rajkowska, 2000). Other research has shown that reduction of glia in depression appears to precede lowered neuronal density (Rajkowska and Miguel-Hidalgo, 2007). Thus, the present data may be either reflecting vulnerability in different cell types at different times or generalized expression of degeneration markers that, in the case of neurons, results in cell depletion only later in life or with longer durations of depression.

Low glial packing densities are seen in relatively young subjects with depression, raising the question of whether damage to or suppression of glial cells may characterize the initial stages of depression and lead to neuronal degeneration later in life (Miguel-Hidalgo et al., 2000). Consistent with an increased vulnerability of glial cells to degeneration, the present study revealed increased levels of markers of neurodegeneration in depression. However, in contrast to the increased levels of C8 and DIABLO/XIAP ratio, staining with TUNEL, which labels fragmented DNA in individual cells undergoing terminal cell death, was very low. This low number of TUNEL-positive cells suggests that a putative decline in neural cells either occurred around the onset of depression or that cell numbers slowly declined over a protracted period of time (so that very few of the dying cells are detectable at a given time). A plausible scenario could include increased C8 activation initially causing glial cell decline and, only later, resulting in neuronal depletion due to impaired glial support.

On the other hand, higher levels of C8 at all ages in depression may not be necessarily related to the induction of imminent cell death. Other cytological studies of depression have revealed few or no cells with signs of morphological apoptosis or necrosis. For instance, in the hippocampus and adjacent temporal brain regions of depressed subjects, there are very few signs of TUNEL staining even in areas where an increase in stress-induced glucocorticoids might elicit apoptosis (Lucassen et al., 2001). The very few to no cells showing DNA fragmentation (TUNEL staining) in the OFC, whether in control or depressed subjects, further speaks to the low rates of cell death in the brain even in depressed subjects (Lucassen et al., 2001).

The significant increase in C8 activation, rather than being a marker for impending cell death, might indicate the presence of cells at risk for apoptosis or other forms of cell degeneration, but not imminently dying. Increased vulnerability to apoptosis may be related to the activation of death receptors by TNF- α and related cytokines in neurons and glia (Berpohl et al., 2007; Choi and Benveniste, 2004; Choi et al., 1999; Segura et al., 2007). TNF- α and interleukin-6 are increased in MDD (Dowlati et al., 2010; Kulmatycki and Jamali, 2006). There is evidence that activation of the TNF- α receptor after traumatic brain injury, resulting in activation of cytoplasmic C8, can elicit cognitive dysfunction independently of cell death in mice. (Khuman et al., 2011). Similar effects of TNF- α and related cytokines may have cognitive effects accompanied by related markers of neurodegeneration, but not actual cell death, in the OFC of subjects with depression.

Scarce morphological evidence for cell death in MDD subjects would also be consistent with normal levels of XIAP, a factor that inhibits the progression of apoptosis. Thus, the observed levels of XIAP may have been sufficient in depressed subjects to prevent neurodegeneration conspicuous enough to be detected with the histological methods used. A sufficient supply of XIAP appears to be a major factor suppressing neuronal death even when axons (heavily involved in neuronal survival) themselves degenerate (Cusack et al., 2013).

The fact that MDD subjects not taking Adep medication and non-responders to Adep, had significantly higher C8-14 levels than comparison subjects would be consistent with a higher vulnerability to apoptosis caused by uncontrolled MDD. Recent experiments in animals suggest that antidepressants have significant effects on cell survival and proliferation (Manev et al., 2001; Sairanen et al., 2005), raising the question of whether effective antidepressant treatment results in a decrease in markers of cell degeneration in the brains of MDD patients.

MARKERS OF CELL PROLIFERATION

Our data showing a reduced density of Ki-67 immunoreactive nuclei in the OFC in MDD are consistent with research showing that stress-induced, depression-related behaviors in rats and humans may involve a reduced ability of glial cells and neurons to divide in the PFC (Banar et al., 2007). Toxin-induced glial cell damage in the PFC also results in depression-related behaviors (Banar et al., 2008). In the hippocampus, postmortem studies in depression have also found a decreased number of progenitor cells labeled with the MCM2 marker but without significant changes in cells labeled with proliferation markers PH3 or Ki-67 (Boldrini et al., 2009; Lucassen et al., 2010). Given that progenitor and dividing cells in the hippocampus mainly generate neurons, the comparison to the PFC (where precursors mainly result in glial cells) awaits more detailed studies that can explain the basis for differences in the fates of neural precursors between PFC and the hippocampus.

Ki-67 labeling in the cell nucleus is considered a faithful reflection of the readiness to divide by progenitor and precursor cells. PCNA is also considered a marker for dividing cells, although of lower specificity due to its varied functions. Thus, in view of the decreased density of Ki-67-positive cells in the OFC of MDD subjects, increased PCNA levels in MDD and their positive correlation with age are difficult to interpret solely on the basis of

PCNA's implication in cell proliferation. They are also in contrast to studies in human, non-human primate and other mammalian brains that rather suggest age-dependent declines in proliferative potential of neural precursors (Drapeau and Nora Abrous, 2008; Epp et al., 2009; Riddle and Lichtenwalner, 2007). Thus, increases in PCNA levels might not be fully related to increased proliferation, but might point to the well-known participation of PCNA in DNA repair (Essers et al., 2005; Maga and Hubscher, 2003). In fact, in the non-psychiatric comparison group, the density of Ki-67-immunoreactive cell nuclei, a sensitive index of readiness to cell proliferation, was negatively correlated with the levels of PCNA, suggesting that an important part of the detected PCNA might not have had a direct implication in the mitotic cycle.

If increases in PCNA level are related to DNA repair (Olesen et al., 2008; Scott and Hegyi, 1997) then the results presented here suggest that increased DNA damage might be more frequent in the OFC of subjects with depression as age increases. Until recently, DNA breaks have been associated with cellular pathology and neurodegenerative processes (Brasnjec et al., 2008). However, recent research is showing that an increase in specific forms of non-pathological brain activity results in significant increases in double-strand DNA breaks in neurons, which may be further enhanced in neurodegenerative disorders (Suberbielle et al., 2013). Interestingly, PCNA is directly involved in repairing double-strand DNA breaks and other forms of DNA damage (Ciccia and Elledge, 2010; Maga and Hubscher, 2003). Determining whether higher levels of PCNA in depression occur because of augmented DNA damage should be investigated.

Lack of perceived therapeutic response to Adep appeared to be a main factor associated with the low levels of PCNA among subjects with MDD. Non-responders, unlike responders, also had higher levels of c8-14 than non-psychiatric comparison subjects. Consistent with the contrast between Ki-67 labeling and PCNA levels, we did not detect an influence of Adep treatment or therapeutic response on the density of Ki-67-positive cells.

If the treatment-related differences are further confirmed by future studies, they would support the hypothesis that subjects resistant to treatment or with very severe depression present detectable molecular alterations in the prefrontal cortex that may influence their responsiveness to antidepressants and the vulnerability of prefrontal cells to degeneration (Merkl et al., 2011).

LIMITATIONS

The interpretations offered earlier must be moderated by the qualitative nature of the assessments of responsiveness to the antidepressant treatments in this study and the absence of proper quantification of the relationship between responsiveness and the levels of the various markers. It is also necessary to note the still controversial nature of the criteria to define resistance to antidepressant treatment (Kubitz et al., 2013; Souery et al., 1999). Another limitation is that, although Adep medications have effects on cell survival and proliferation (Manev et al., 2001; Sairanen et al., 2005), correlating Adep treatment with protein changes in human postmortem studies is challenging because of varied prescriptions, dosages, interaction with other medications, and compliance. The interpretation of results regarding PCNA changes in MDD is also challenging due to the contrasting function of this

protein in processes of cell proliferation and degeneration (as a DNA repair factor), so that further experimental studies are necessary to fully characterize the nature of the changes observed.

In conclusion, in the OFC of subjects with MDD there is an increased level of some markers related to the induction of apoptosis, but without significant decrease in XIAP, a major component of antiapoptotic mechanisms. Increase in molecules related to apoptosis induction but without major changes on antiapoptotic molecules may explain the low level of detectable cell death in the prefrontal cortex of subjects with MDD. However, this low level of cell demise combined with reduced cell proliferation, evidenced by lower-than-normal counts of Ki-67 positive cells, may explain reduced numbers of glial cells and late-life reductions in neuronal density previously noted in MDD. An age-dependent increase in PCNA in MDD, as compared to non-psychiatric controls, may be involved in mechanisms not related to the cell proliferative role of PCNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Western blot detection and quantification of caspase 8 (C8). A) Lanes from two different representative Western blots of OFC proteins (40 μ g per lane) from 8 different subjects with major depressive disorder (MDD, 4 in each blot) and 4 different comparison subjects (COMP, 2 in each blot). The blots were probed with an anti-caspase 8 antibody. Bands at the approximate molecular weights of 50, 36 and 14 kDa are singled out. Bands at about 14 and 50 kDa were quantified against β -actin bands. B) Bar graph representing the normalized levels of C8-14 in COMP (n=23) and MDD (n=23) subjects. Quantification of the 50 kDa band is presented in supplemental figure 1. Whiskers represent the standard error of the mean. * $p < 0.001$.

Fig. 2.

Western blot immunodetection and quantification of the proteins DIABLO and XIAP in the OFC from COMP and MDD subjects (abbreviations as in figure 1). A) Reactive bands in three different Western blot membranes, each including subjects of the two groups. Subjects in each blot were different from the subjects in the other blots. B) Bar graph of the ratio of the normalized levels of DIABLO to the normalized levels of XIAP in COMP (n=15) and MDD (n=14) subjects. C) and D) Bar graphs representing the mean normalized levels of DIABLO and XIAP, respectively, in the diagnostic groups. Whiskers represent the standard error of the mean. *p=0.023 relative to COMP.

Fig 3.

Western blot immunodetection and quantification of PCNA in the OFC the MDD and COMP subjects (abbreviations as in figure 1). A) Two representative Western blots with subjects from the two diagnostic groups; subjects in the left blot were different from the subjects in the right blot. B) Bar graph representing the normalized levels of PCNA in COMP (n=23) and MDD (n=23) subjects.. Whiskers represent the standard error of the mean. *p=0.046.

Fig. 4.

Immunohistochemistry and cell counting of Ki-67-immunoreactive cell nuclei in the gray matter of the OFC. A) and B) Low power micrographs of immunoreactive nuclei in the upper cortical layers in COMP and MDD subjects, respectively. C) and D) Higher power micrographs of Ki-67-immunoreactive cell nuclei in the gray matter of the OFC of a COMP subject. E) Bar graph of the average packing density of Ki-67-immunoreactive nuclei in MDD (n=23) and COMP (n=23) subjects. LI=cortical layer I; LII=cortical layer II. Other abbreviations as in figure 1. *p=0.033.

Fig. 5.

Scatter graphs illustrating the correlation between levels of the 14 kDa fragment of caspase 8 and age at the time of death in COMP (A) and MDD (B) subjects. There was a significant positive correlation in COMP, but not in MDD subjects. (Abbreviations as in figure 1)

Fig. 6.

Scatter graphs illustrating the correlation between levels of PCNA and age at the time of death in COMP subjects (A), MDD subjects (B). There was a significant positive correlation in MDD, but only a non-significant trend for a correlation in the COMP group. (Abbreviations as in figure 1).