Journal of Affective Disorders 158 (2014) 62–70

Contents lists available at ScienceDirect

Journal of Affective Disorders

journal homepage: www.elsevier.com/locate/jad

Preliminary communication

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

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ARTICLE INFO

Article history:
Received 23 January 2014
Accepted 3 February 2014
Available online 10 February 2014

Keywords:
Depression
Prefrontal cortex
Comorbidity
Vulnerability
Postmortem

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; and unclear role of PCNA.

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.

1. 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

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http://dx.doi.org/10.1016/j.jad.2014.02.010
0165-0327

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, 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.

2. Methods

2.1. 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) (American Psychiatric 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 Supplementary Table S1. 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.

2.2. 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., 1981; Uylings et al., 2010). For immunohistochemistry and Western blots, each frozen block was cut into alternating sections with a thickness of 20 μm and 50 μm, respectively.

2.3. Western blotting

Punch samples were taken from frozen 50-μm sections. Each sample spanned the cortical gray matter from the pial surface to the boundary of gray and white matter. Samples were homogenized in 0.01 M Tris–HCl containing 1% SDS, 2 mM EDTA, and protease inhibitor. After centrifugation, 40 μg 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