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ABSTRACT

MDD is a severe and debilitating disorder that is associated with a growing global economic burden due to reduced workplace productivity along with increased healthcare resource utilization. Furthermore, depression markedly enhances the risk for suicide, mortality that is especially worrisome given that 30% of depressed individuals have an inadequate response to current antidepressants. This inadequacy of antidepressants necessitates the discovery of a better understanding of the pathobiology of MDD. Most current antidepressants work through monoamine neurotransmitters, and their relative efficacy in depression led to the now dated monoamine-deficiency hypothesis. The limited usefulness of antidepressants has led to a reinvigorated search for other pathologies in depression that might yield clues for the development of better drug treatments. In this regard, a strong association has been found between oxidative stress and MDD. Our lab recently found increased DNA oxidation and elevated poly(ADP)ribose polymerase (PARP1) gene expression in the brain from donors that had MDD at the time of death. Besides DNA damage repair, PARP1 mediates several downstream inflammatory effects that may contribute to pathology in MDD. In fact, our lab has demonstrated that PARP-1 inhibition produces antidepressant-like effects in rodents, suggesting that PARP-1 inhibitors hold promise as a novel antidepressant drug. While our lab had previously demonstrated elevated PARP1 gene expression in the frontal cortex in MDD, whether PARP1 protein levels were also increased in depression had not been verified. My thesis research was performed to determine whether PARP1 protein expression was also elevated in the brain in MDD. I studied primarily the hippocampus because it is part of the limbic (mediating emotion) system of the brain and because previous research has shown numerous other pathologies in the hippocampus. My study was carried out simultaneously as others in our lab were measuring PARP1 protein levels in frontal cortex in MDD. This latter work was important since the lab's previous work had observed elevated PARP1 gene expression in the frontal cortex, rather than in the hippocampus which was not previously studied. Hippocampal and frontal cortical brain sections were cut from frozen blocks of both MDD and psychiatrically normal control brain donors for these studies. PARP1 protein levels were estimated by assisted-imaging software. The findings herein demonstrate that levels of PARP1 immunoreactivity are significantly elevated in the frontal cortex of MDD donors as compared to control donors. However, there was no change in PARP1 immunoreactivity in the hippocampus in MDD.

Background:

Depression is an increasingly common disorder. Worldwide, the case count exceeds 120 million people and, in an average population, the lifetime prevalence of the disease ranges from 10% to 15%[1]. In the U.S. alone, 17.3 million adults are expected to experience at least one major depressive disorder within a year[2]. This prevalence does not come without due burden. The annual economic cost associated with depression in the U.S. is estimated at $83 billion dollars and individuals with increasingly severe depression have higher work productivity depletion alongside higher, costly healthcare resource utilization[3,4]. More directly, depression and contemplation of suicide are positively and significantly correlated with one another[5]. Thus, it is anticipated that better treatments for depression could reduce morbidity as well as reduce overall incidence of suicide. Better treatments are needed because an inadequate response to antidepressants is linked with suicidal behavior[6], and nearly 30% of individuals have an inadequate response to current antidepressants[7].

There have been a variety of theories introduced to explicate the pathophysiology of depression. The most common hypotheses to address this issue are those related to genetic vulnerabilities, the HPA axis, monoamine-deficiency, and neurotrophic dysfunction, amongst others[8]. Significant progress has been made in elucidating the role of each of these areas as well as their interactions with each other. However, the most widespread and common theory of depression is rooted in the pharmacological actions of the most common drugs that are used to treat it. Almost all current, established antidepressants regulate the monoamine neurotransmitter systems[9].

The monoamine-deficiency hypothesis suggests that the root pathophysiology of depression is in the reduced levels of monoamine neurotransmitters serotonin, norepinephrine, and/or dopamine in the central nervous system[10]. Almost all compounds that have been shown to reduce

monoamine uptake, which results in increased levels of monoamines in the synaptic cleft, have been observed as somewhat effective clinical antidepressants[11]. Prior to the 1980's, the "first generation" antidepressants which utilized this hypothesis were the monoamine-oxidase inhibitors (MAOI's) and tricyclic antidepressants (TCA's). MAOI's function by irreversibly inhibiting monoamine oxidases, which metabolize the monoamine neurotransmitters, while TCA's inhibit the transport and reuptake of norepinephrine and serotonin at nerve terminals[12,13]. However, both first-generation antidepressants, MAOI's and TCA's, have become significantly less common in therapeutic use due to varying intolerable side effects[14]. To combat these effects, in 1985, the first of the "second generation" antidepressants was introduced to the U.S. market. These "second generation" drugs also followed from the monoamine-deficiency hypothesis and are broadly categorized as selective serotonin reuptake inhibitors (SSRI's) and selective norepinephrine reuptake inhibitors (SNRI's). The second generation antidepressants have become more commonplace because of their better tolerability as compared to their first generation predecessors, however, questions of broad efficacy remain[15].

Although the second-generation antidepressants overcame the side-effect profile, the studies measuring their efficacy have not been promising. In a study measuring the SSRI citalopram, only 30% of patients showed full remission[16]. While yet another that utilized SSRI's in combination with other treatment modalities – this combination of treatments is often referred to as "antidepressant treatment" (ADT) – showed ineffectiveness in 20-30% of patients[17]. This treatment-resistant depression indicates incompleteness in the monoamine-deficiency hypothesis as the pathophysiological basis of depression and underscores the need for further research of depression pathobiology to identify new targets for the development of better drugs.

Recent research has alluded to the role of the immune system as a promising link between stress and depression[18,19]. One of these models has proposed that chronic stress activates the immune system such that inflammation ensues, and the resulting chronic inflammation leads to depressive symptomology[19]. More specifically, several studies have indicated elevated oxidative stress and inflammation in patients exhibiting MDD[20–33]. Extrapolating from this, studies have found oxidative damage to nucleic acids in MDD[34–36]. These discoveries led our lab to examine and find that DNA oxidation is elevated in white matter oligodendrocytes in the frontal cortex from MDD brain donors[37]. This damage is further associated with a reduced antioxidant enzyme gene expression in white matter oligodendrocytes[38]. Collectively, these findings have shown that oxidative stress and its role as a contributor to the development of MDD is promising.

In an MDD patient, reactive oxygen species (ROS) can alter several cellular molecules, including DNA. When an ROS attacks DNA, this activates the cell's base excision repair (BER) system. The BER pathway activates two specialized enzymes that are involved in the repair of oxidized nucleotides and/or deoxyribose moieties: poly(ADP-ribose) polymerase-1 (encoded by PARP1) and 8-oxoguanine DNA glycosylase (encoded by OGG1). The gene expression of both of these enzymes is induced by oxidative stress conditions[39]. OGG1 recognizes oxidized DNA and recruits the enzyme AP endonuclease I to form a single strand break (SSB) at the site of oxidation. PARP1 recognizes and binds the SSB using its DNA-binding domain and is subsequently activated. An activated PARP-1 begins synthesis of poly(ADP-ribose) (PAR) on itself ("PARylates" itself) and other local proteins. The formed PAR on PARP-1 at the site of the SSB serves as a signal to recruit other DNA repair enzymes: x-ray repair cross-complementing protein 1 (XRCC1), DNA polymerase-ß, and DNA ligase III. These recruited enzymes work

together to replace the excised base and conclude the base-excision repair process[40–42]. Par glycosylase (PARG) cleaves PAR from PARP1, restoring PARP1 to its original state.

Given its role described above, it is understandable that PARP-1 is upregulated under increased levels of oxidative damage to DNA and conditions of oxidative stress[43,44], i.e. it is upregulated in response to an increased need for DNA repair. Previous research from our lab demonstrated elevated PARP-1 gene expression in frontal cortical white matter from MDD brain donor patients[38]. These findings allude to a role of PARP-1 beyond base-excision repair. It is known that PARP-1 is involved in key inflammatory processes. PARP1 associates with and activates several transcription factors involved in inflammatory gene expression, most notably nuclear factor kappa B (NF-κB)[45]. Additionally, free PAR chains that have been cleaved by PARG from PARP1 and other proteins, serve as damage associated molecular patterns (DAMP) that are recognized by macrophages and lead to further inflammation[46]. On top of its role in driving inflammation, PARP1 activity leads to a depletion in cellular energy stores that results in cell death[47–51]. These additional effects of PARP1 coupled with the aforementioned findings indicate a potential role of PARP1 in MDD pathobiology.

Given this knowledge, it is possible that the downstream inflammatory effects of PARP1 upregulation mediate the disruption of emotional behaviors associated with MDD. An approach to test this association would be to pharmacologically inhibit PARP1 and produce the anticipated antidepressant effects in animal models of depression. Our lab tested this and, indeed, small molecule inhibition of PARP1 was found to produce antidepressant-like effects in two rodent models[52]. These findings indicate the promising role of PARP-1 inhibitors as possible antidepressant treatments for MDD patients, nevertheless, more research is needed to increase

confidence in PARP1's potential role as a critical mediator of behavioral disruption in human MDD patients.

The research herein had two goals. One goal was to determine whether elevated PARP1 gene expression in MDD is associated with elevated PARP1 protein levels. This is an important issue because elevated gene expression of a given enzyme is not always accompanied by elevated enzyme levels and/or elevated levels of enzyme activity. Hence, we used an antibody against human PARP1 to quantify PARP1 protein in MDD and normal control donors. A second goal was to achieve a better understanding of whether PARP1 upregulation is restricted to frontal cortical white matter. It is known that different areas of the brain have varying sensitivities to oxidative damage[53], with white matter oligodendrocytes being particularly susceptible. Studies have also indicated the hippocampus as an area of the brain containing neurons that are particularly susceptible to oxidative damage[54,55]. A previous study demonstrated elevated DNA oxidation in the hippocampus of depression-induced animals[56]. It is also noteworthy that the hippocampus is part of the emotional (limbic) brain, and a large number of studies have demonstrated other pathologies of the hippocampus in MDD. Hence, it is evident that the hippocampus is a promising area for observing changes in PARP1 levels in MDD patients. An accompanying study in the lab that paralleled my thesis project was the measurement of PARP1 levels in the frontal cortex, where PARP1 gene expression was previously found to be elevated. This parallel study was critical to the interpretation of my work since PARP1 protein levels had yet to be confirmed in the same brain region in MDD that gene expression changes were observed. The results of this study and the parallel study are reported here and provide clarity as to where in the brain the PARP1 pathobiology occurs and will contribute to further development of PARP1 inhibitors as possible antidepressant drugs.

Methods

Human Brain Tissue Sectioning and Storage

Frozen human hippocampal and frontal cortical (Brodmann area 10) brain sections were cut 20 µm thick in a cryostat at -17 ℃. These sections were transferred onto Superfrost Plus slides. These slides, now with the sections, were dehydrated in a vacuum desiccator overnight at 4 ℃. The next day, the slides were transferred from the desiccator to a -80 ℃ freezer for storage.

PARP1 Immunohistochemistry

The slides were fixed in cold acetone at -20 ℃ for 20 minutes. After fixing, they were washed 4 times (10 minutes each time) in phosphate-buffered saline (PBS) at room temperature (RT). The slides were then blocked and permeabilized in PTB buffer (PBS, 3% Triton X-100, 1% BSA) at RT for 1 hour. PARP-1 antibody (rabbit, 1:500 dilute in PTB) was used to incubate the slides at 4℃ overnight (*negative control is without PARP-1 antibody). The next day, the slides were washed in PBS 3 times (10 minutes each time) at RT. The slides were then incubated with the secondary antibody (donkey anti-rabbit Alexa Flour 594; dilute in PT; PBS, 3% Triton X-100) at RT for 1 hour. Next, the slides were washed in PBS 3 times (10 minute each time) at RT. A single drop of the mounting medium with DAP1 (fluorescent counterstain) was applied on each section, the drops were mounted with coverslips of which to the edges were applied clear nail polish to maintain placement. These were let to dry and later stored at 4 ℃. Slides were immuno-stained in batches of which contained equal numbers tissue sections of psychiatrically normal control and MDD donors.

Imaging with EVOS

An EVOS fluorescent microscope was used to take immunofluorescent images of the slides. The auto-imaging system of EVOS was utilized to scan the hippocampus or frontal cortex for future analysis. There are two stitching patterns for each region identified. One is with DAP1 fluorescent signaling (blue color; labels nuclei of cells), and the other is with PARP1 fluorescent immune activity signaling (red color). Negative control slides were used to set a parameter to exclude non-specific PARP-1 fluorescent immune-activity signaling.

MCID-Core Image Analysis

Both stitching patterns of a region (DAP1 and PARP1) were loaded onto 2 separate channels on MCID-Core image analysis software. Our lab decided on using the dentate gyrus (DG) region of the hippocampus to set a density parameter within a range of 3-5%. This parameter was then applied to find the PARP1 area fraction, i.e. the fraction of the analyzed area that was above background, in different hippocampal regions (CA1, CA4, DG, etc.) or frontal cortex subregions (layers 1-3, layers 4-6, white matter) within each experimental batch (containing MDD and control sections). For each batch, this same protocol was used: identify the first subsection, set the density parameter according to DG or cortical region of this subsection, apply parameters to find PARP1 area fraction of the hippocampal or cortical regions within the all the subsections of the batch. Each PARP1 area fraction was automatically stored by the MCID-Core software and these data points were later transferred to Microsoft Excel for organization and analysis. The analysis of images was done blind to whether hippocampal and cortical tissue sections came from MDD or control donors.

Statistical Analyses

Area fractions, as estimates of levels of PARP1 immunoreactivity, in brain regions from control and MDD donors were subjected to outlier analysis using ROUT (GraphPad Prism v.8.4.2). Corrected data were then analyzed using a one-way ANOVA. When the p value for the ANOVA was < 0.05, Sidak's multiple comparisons test was used to determine which brain areas demonstrated statistically significant differences between control and MDD donors. Again, a $p<0.05$ was considered statistically significant.

Results

Hippocampus

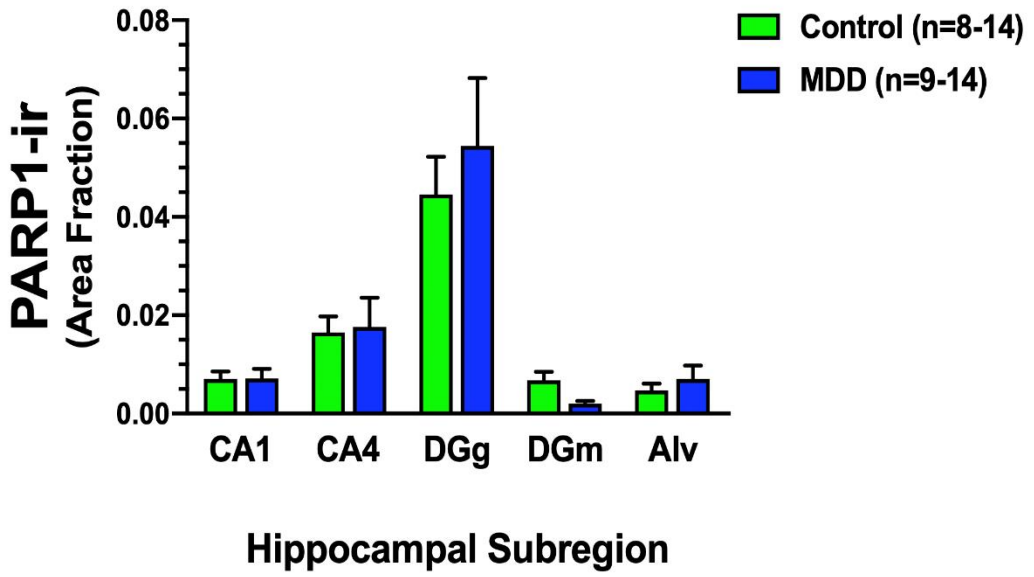

Figure 1. PARP1 area fraction in several hippocampal regions of both MDD and psychiatrically normal control groups gathered using the MCID-core imaging software

PARP1 area fraction collected in several hippocampal regions in both MDD and control groups is shown in Figure 1. PARP-1 area fraction levels across the different hippocampal regions were highly variable with the DGg region having the highest levels in both groups (MDD and control). However, for each of the CA1, CA4, DGg, DGm, and Alv hippocampal regions there was no significant difference between the PARP-1 area fractions of the MDD and control groups ($p<0.05$).

BA10 Frontal Cortex

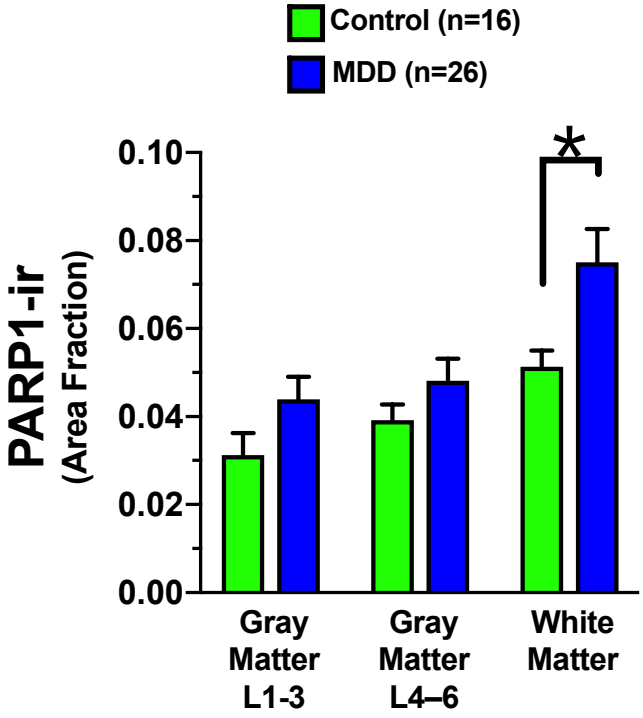

Figure 1. PARP1 area fraction in several BA10 frontal cortex regions of both MDD and normal control groups gathered using the MCID-core imaging software

PARP1 area fraction collected in regions of the BA10 frontal cortex in both MDD and non-MDD groups is shown in Figure 2. PARP1 area fraction varied across the BA10 frontal cortex regions with white matter having the highest levels. The white matter was also the only region that demonstrated significantly increased PARP1 area fraction in MDD donors as compared to control donors ($p<0.05$).

Discussion

In this study PARP1 protein levels were evaluated in the hippocampal and BA10 frontal cortex regions of both MDD and psychiatrically normal brain donors. This study was intended to elucidate whether the previously reported elevation of PARP1 gene expression in MDD is associated with elevated levels of PARP1 protein and to also determine if PARP1 upregulation in MDD is limited to the prefrontal cortex. The results demonstrated significantly elevated levels of PARP1 protein in the white matter of the frontal cortex of MDD donors, with no such increase of PARP1 protein levels in the hippocampus of MDD donors. The implications of these results are wide-ranging and will contribute to further understanding of the pathophysiology of depression as well as the potential development of PARP1 inhibitors as antidepressant drugs.

In previous studies, our lab tested the hypothesis that exposure to PARP1 inhibitors will lead to antidepressant-like effects. In order to measure this, rats were exposed to several psychological stressors, including combined social defeat and chronic unpredictable stress, which have both been proven to be effective models for measuring antidepressant effects[57]. Concurrently, the Porsolt swim test, which is commonly used to identify antidepressant drugs, was used[57,58]. To test the pharmacological ability of PARP1 inhibitors in producing antidepressant effects, rats were divided into four groups, including three treatment groups and one control. Treatment groups were given 3-aminobenzamide (3-AB; PARP1 inhibitor), fluoxetine (SSRI), and normal saline, and each treatment group was simultaneously exposed to psychological stressors. Control groups were given saline but were not exposed to psychological stressors. Antidepressant-like effects were measured using the sucrose preference and social interaction times test – an increase in each would suggest an effective antidepressant. The PARP inhibitor 3-AB increased both sucrose preference and social interaction times as compared to control rats, mediating effects

comparable to those produced by fluoxetine[52]. Moreover, 3-AB also demonstrated antidepressant-like activity in the Porsolt swim test, facilitating reduced immobility time and increased latency to mobility[52]. These results indicated that PARP1 inhibitors may produce antidepressant effects in MDD patients. This finding, coupled with the demonstrated upregulation of PARP-1 in BA10 frontal cortex white matter of both MDD and suicide patients[38], opened the door for the promising development of a truly novel antidepressant drug. A part of this development is gaining a better understanding of what area(s), if any, of the brain beyond the BA10 frontal cortex demonstrate PARP1 upregulation.

In one of our original studies, white matter oligodendrocytes from the frontal cortex (Brodmann area 10, BA10) were used to measure DNA oxidation and PARP1 gene expression in human MDD[38]. This is because white matter oligodendrocytes are particularly susceptible to oxidative damage and there is relative ease in collecting them from human postmortem brain sections. White matter oligodendrocytes were chosen from the frontal cortex because of previous studies associating increased activation of BA10 with depressive behaviors[59]. Another area of the brain that is especially sensitive to oxidative stress is the pyramidal neuron of the CA1 hippocampus[54,55]. The hippocampus is a part of the brain system responsible for memory consolidation and emotional responses[60]. Several studies have indicated hippocampal volume reductions, abnormal hippocampal gene expression, and reduced hippocampal neurogenesis in MDD and suicide[61–70]. Thus, we postulated that this region would show elevated PARP1 expression similar to frontal cortical white matter. Contrary to expectations, PARP1 protein expression was not significantly elevated in the hippocampus of MDD donors as compared to control donors. This doesn't necessarily suggest that there is no involvement of PARP1 in the

hippocampus in MDD, since it may be regulated in this brain area differently than in the frontal cortex.

The hippocampus is one of the regions of the brain with the highest PARP1 mRNA levels. In contrast, frontal cortical white matter is a brain region with very low levels of PARP1 gene expression. Following from this, it seems possible that PARP1 production by cells in the hippocampus may be near maximum, and that cellular stress (eg. DNA oxidation) putatively associated with MDD may not be accompanied by further increase in PARP1 expression. An increase of PARP1 protein levels in the hippocampus was anticipated because of strong associations of the hippocampus with MDD, and the noticeable upregulation of gene expression of PARP1 in the BA10 frontal cortex of MDD patients[38]. However, the activity of the PARP1 enzyme, as with any enzyme, is regulated at several cellular levels including changes in gene expression, translated protein levels, and in several post-translational modifications that can modify enzyme activity. A lack of increase in enzyme protein levels does not necessarily mean that there is no change in enzyme activity. A limitation of this study is that only translated PARP1 protein levels were measured and compared in MDD and control brain donors. It is possible that in MDD patients, PARP1 is post-translationally modified such that, even if the protein expression *levels* remain comparable to controls, protein *activity* could be elevated. The possibility that increased PARP1 activity occurs in MDD should be investigated before any conclusions are made about the role of PARP1 in hippocampal pathology associated with MDD.

Our lab's original study demonstrated significantly elevated PARP1 gene expression in white matter from the BA10 frontal cortex of MDD donors[38], and the present study found significantly elevated PARP1 protein levels from the same area in MDD donors. This indicates that PARP1 gene expression is positively correlated with protein levels. While this is promising, further

studies, as with the hippocampus, need to demonstrate how PARP1 protein expression is associated with protein activity in the BA10 frontal cortex. Collectively, these studies would make significant contributions in elucidating the promising PARP1 pathobiology model.

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