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Membrane-associated α-tubulin is less acetylated in postmortem prefrontal cortex from depressed subjects relative to controls: cytoskeletal dynamics, HDAC6 and depression

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Conflict of interest

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Abstract

Cytoskeletal proteins and post-translational modifications play a role in mood disorders. Post-translational modifications of tubulin also alter microtubule dynamics. Furthermore, tubulin interacts closely with $G\alpha_s$, the G-protein responsible for activation of adenylyl cyclase. Postmortem tissue derived from depressed suicide brain showed increased $G\alpha_s$ in lipid-raft domains compared to normal subjects. $G\alpha_s$, when ensconced in lipid-rafts, couples less effectively with adenylyl cyclase to produce cAMP and this is reversed by antidepressant treatment. A recent *in-vitro* study demonstrated that tubulin anchors $G\alpha_s$ to lipid-rafts and that increased tubulin acetylation (due to HDAC-6 inhibition) and antidepressant treatment decreased the proportion of $G\alpha_s$ complexed with tubulin. This suggested that deacetylated-tubulin might be more prevalent in depression. This study, examined tubulin acetylation in whole tissue homogenate, plasma-membrane and lipid-raft membrane domains in tissue from normal control (NC) subjects, depressed suicides and depressed non-suicides (human males/females). While tissue homogenate showed no changes in tubulin acetylation between control, depressed suicides and depressed non-suicides, plasma-membrane associated tubulin showed significant decreases in acetylation from depressed-suicides and depressed-non-suicides compared to controls. No change was seen in expression of the enzymes responsible for tubulin acetylation or deacetylation. These data suggest that during depression, membrane localized tubulin maintains a lower acetylation state, permitting increased sequestration of $G\alpha_s$ in lipid-raft domains, where it is less likely to couple to adenylyl cyclase for cAMP production. Thus, membrane tubulin may play a role in mood disorders which could be exploited for diagnosis and treatment.

61

62 **Significance Statement**

63 There is little understanding about the molecular mechanisms involved in the development of
64 depression and in severe cases, suicide. Evidence for the role of microtubule modifications in
65 progression of depressive disorders is emerging. These postmortem data provide strong evidence
66 for membrane tubulin modification leading to reduced efficacy of the G protein, Gsα, in
67 depression. This study reveals a direct link between decreased tubulin acetylation in human
68 depression and the increased localization of $G\alpha_s$ in lipid-raft domains responsible for attenuated
69 cAMP signaling. The evidence presented here suggest a novel diagnostic and therapeutic locus
70 for depression.

71

72 **Introduction**

73 Hallmarks of Major depressive disorder (MDD) include persistent sad mood, anhedonia,
74 changes in appetite, disturbed sleep, feelings of worthlessness, hopelessness and suicidal
75 thoughts. While various antidepressant drug therapies are available, the biological underpinnings
76 of their action as well as the molecular events leading to depression remain uncertain. Numerous
77 suggestions about the biology of depression exist, and epigenetics (histone deacetylases-HDACs)
78 and HDAC inhibitors as novel antidepressants are a recent addition to this list (Tsankova et al.,
79 2007; Covington et al., 2009; Gundersen and Blendy, 2009). The majority of the presently
80 available antidepressants have among their actions, prevention of monoamine uptake or
81 degradation and one consistent effect of antidepressant treatment has been a persistent increase
82 in cAMP and an upregulation of the cAMP generating system (Nibuya et al., 1996; Malberg et al.,
83 2000; Donati and Rasenick, 2003). Furthermore, PET studies from depressed subjects showed
84 global decreases in brain cAMP and antidepressant drugs restored cAMP levels (Fujita et al.,
85 2007; Fujita et al., 2017). We have suggested that antidepressants achieve this by a gradual
86 removal of Gαs from lipid rafts and increasing association of that molecule with adenylyl cyclase

(Zhang and Rasenick, 2010; Czysz et.al 2015). Consistent with this, postmortem samples from depressed human subjects reveal increased $G\alpha_s$ (Donati et al., 2008). $G\alpha_s$ is the only heterotrimeric G protein undergoing translocation out of lipid-rafts in response to antidepressant treatment (Toki et al., 1999; Donati and Rasenick, 2005). Interestingly, antidepressant drugs have been shown to concentrate in lipid raft domains (Eisensamer et al., 2005; Erb et al., 2016). Together, these studies suggest that the lipid environment of $G\alpha_s$ may play an important role in its localization and function, and that chronic antidepressant treatment alters the membrane localization of $G\alpha_s$, resulting in augmented coupling to adenylyl cyclase (Allen et al., 2009; Zhang and Rasenick, 2010).

There is evidence for a role of cytoskeletal (microtubules) alterations in the pathology of several neuropsychiatric diseases (Perez et.al, 2009, Brown et al., 2013; Wong et al., 2013; Scifo et al., 2017) These disorders are associated with structural changes in brain including synaptic pruning defects and spine and dendrite atrophy (Glausier and Lewis, 2013). The development of depression is associated with exposure to triggering environmental factors such as chronic stress (Pittenger and Duman, 2008; Lin and Koleske, 2010; Schmitt et al., 2014) (McEwen et al., 2017). Most importantly, post-translational modifications such as acetylation of tubulin help to maintain cytoskeletal stability (Idriss, 2000; Westermann and Weber, 2003).

Lipid-raft domains are also associated with cytoskeletal elements such as microtubules. Tubulin is comprised of an αβ dimer, and these dimers are localized in membranes, and enriched in lipid-rafts. Upon activation, $G\alpha_s$ is released form the membrane, where it binds tubulin, activates tubulin GTPase and increases microtubule dynamics (Roychowdhury and Rasenick, 1994; Dave et al., 2011; Sarma et al., 2015). These findings suggest that tubulin may act as an anchor for $G\alpha_s$ within the lipid-raft domains. A recent *in vitro* study (Singh et al., 2018) shows that treatment with antidepressants reduces the extent to which $G\alpha_s$ is complexed with tubulin.

The enzymes responsible for the regulation of acetylation status of α-tubulin are histone deacetylase-6 (HDAC-6; deacetylating) and alpha-tubulin acetyl transferase-1 (ATAT-1:

acetylating). There is emerging evidence for the role of HDAC in neuropsychiatric disorders, including MDD (Guidotti A et al., 2011; Tsankova N et al., 2007; Hobara T et al., 2010). Altered levels of HDAC 2, 4, 5, 6, 8 mRNA have been observed in blood cells and postmortem brain from mood disorder subjects (Guidoti et al., 2011; Hubbert et al., 2002). HDAC-6, localized in cytosol, deacyates α-tubulin (Hubbert et al., 2002; Verdel et al., 2000). Peripheral white blood cells derived from MDD subjects showed altered HDAC6 mRNA levels (Hobara et al., 2010).

The current study compares the acetylation status of α-tubulin from postmortem human brain of depressed subjects and controls without known psychiatric histories. Prefrontal cortex (PFC) tissue showed comparable tubulin acetylation in homogenates, but strikingly decreased acetylation in membranes prepared from depressed suicides and depressed non-suicides. These data correspond well with a previous study showing increased $G\alpha_s$ levels in lipid rafts, since acetylation of tubulin decreases its ability to bind $G\alpha_s$ and anchor it to lipid rafts, resulting in less $G\alpha_s$ available for adenylyl cyclase activation in the depressed brain. These findings also parallel those of $G\alpha_s$ translocation from lipid-rafts by HDAC6 inhibitors (Singh et al., 2018). The data presented here and previous studies in model systems suggest that $G\alpha_s$ anchoring to lipid rafts is involved in both depression and therapies for that disease through modulation of the cAMP-generating system. These findings suggest a direct role of HDAC6 in maintaining acetylation status of α-tubulin, stabilizing/destabilizing microtubules during normal and depressive states. The data also suggest that tubulin acetylation may be relevant to depression and its treatment.

Materials and Methods

Human Subject Information

Tissue used in this study was from Brodmann area 9 obtained from the right hemisphere of depressed suicide subjects (n = 15), depressed non-suicide subjects (n = 12) and normal control subjects (n = 15). Both males and females are included and subject demographics are described

in Table 1. Brain tissues were obtained from the Maryland Brain Collection at the Maryland Psychiatric Research Center (Baltimore, MD). Tissues were collected only after a family member gave informed consent. All procedures were approved by the University of Maryland Institutional Review Board (IRB) and by the University of Illinois IRB.

All tissues from normal controls, depressed suicides and non-suicide subjects were screened for evidence of neuropathology. In addition, in each case, screening for the presence of HIV was done in blood samples, and all HIV-positive cases were excluded. Toxicology data were obtained by the analysis of urine and blood samples. pH of the brain was measured in cerebellum in all cases as described (Harrison et al., 1995). Psychiatric drugs in common use as well as drugs of abuse were screened for by using mass spectroscopy. Prescribed drugs were also screened for in interviews.

Control subjects with a known psychiatric illness or a history of alcohol or another drug abuse were excluded. However, alcohol or other substance abuse was present in the MDD subjects as indicated.

Diagnostic Method

Families were queried on all medications or drugs of abuse by trained interviewers. At least one family member, after giving written informed consent, underwent an interview based on the Diagnostic Evaluation After Death (DEAD) (Zalcman and Endicott, 1983 and the Structured Clinical Interview for the DSM-IV (SCID) (Spitzer et al., 1992). This was done as described in a previous study (Donati et al., 2008).

Sequential Detergent Extraction of Brain Membranes

Brain samples were dissected from the fresh brain and stored at −80°C or dissected from frozen brain tissue with a Stryker autopsy saw, repackaged, and stored at −80°C until use.

Brain samples (Pre-Frontal Cortex-PFC) were resuspended and minced in TME buffer (10 mm Tris-HCl, 1 mm MgCl2, 1 mm EDTA, pH 7.5; ~1 ml/100 mg tissue) followed by homogenization in a motorized Teflon glass homogenizer. Small amount of whole tissue homogenate (H) was saved to be run on western blot along with other cell fractions. The rest of the H samples were centrifuged at 100,000 × *g* for 1 h at 4°C, and the supernatant (cytosol) and pellet (Plasma membrane-PM) were saved. The crude membrane pellet was extracted with 0.75 ml of TME containing 1% Triton X-100 for 1h at 4°C followed by homogenization as above. This sample was centrifuged as above and both the supernatant (TX-100 extract) and pellet (TX-100-resistant membrane fraction) were saved. This pellet was extracted with 0.75 ml of TME containing 1% Triton X-114 for 1h at 4°C and homogenized as above. The sample was centrifuged as above and both the supernatant (TX-114 extract) and pellet (detergent-insoluble pellet) were saved. The detergent-insoluble pellet could not be efficiently solubilized to be quantified. Herein, the TX-100 extract will be referred to as the TX-100-soluble domain and the TX-114 extract will be referred to as the TX-100-resistant domain. All fractions were assayed for protein content (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA) and frozen at −80°C until further use. Frontal cortex was the only brain region available for these experiments (Donati et al., 2008).

SDS-PAGE and Western Blotting

Whole tissue homogenate (H), plasma membrane (PM), TX-100- and TX-114-soluble (TX-100-resistant) membrane fractions (12–15µg) were analyzed by SDS-PAGE followed by Western blotting. The gels were transferred to Nitrocellulose membranes (Bio-Rad, Hercules, CA USA) by Western blotting. The membranes were blocked with 5% nonfat dry milk diluted in TBS-T (10 mM Tris–HCl, 159 mM NaCl, and 0.1% Tween 20, pH 7.4) for 1 h. Following the blocking step, membranes were washed with Tris-buffered saline/Tween 20 and then incubated with an anti-

acetyl-α-tubulin (SIGMA-ALDRICH #T7451 Clone 6-11B-1), α-tubulin (SIGMA-ALDRICH #T9026), HDAC6 (Cell Signaling #7558S), ATAT-1 (SIGMA-ALDRICH #HPA046816), GAPDH (Proteintech #60004-1-Ig) overnight at 4°C. Membranes were washed with TBS-T and incubated with a secondary antibody [HRP-linked anti-mouse antibody IgG F(ab′)2 or HRP-linked anti-rabbit antibody IgG F(ab′)2 (Jackson ImmunoResearch, West Grove, PA, USA, catalog #115-036-072 for mouse, and catalog #111-036-047 for rabbit, RRID) for 1 h at room temperature, washed, and developed using ECL Luminata Forte chemiluminescent reagent (Millipore, Billerica, MA, USA). Blots were imaged using Chemidoc computerized densitometer (Bio-Rad, Hercules, CA, USA). The signal intensity of bands from each image were quantitated by densitometry using Image-J software (NIH) and the TX-100-resistant acetyl-α-tubulin/α-tubulin (TX-114) was compared. The acetyl-α-tubulin/α-tubulin were also observed in plasma membrane (PM) from Control (NC), depressed Suicide (DS) and depressed non-suicide (DNS) samples as described (Toki et al., 1999; Donati et al., 2008). Additionally, HDAC6, ATAT-1 and GAPDH expression differences were analyzed between the 3 groups (C, DS & DNS).

Normalization

To be consistent throughout the data collection, same amount of starting material (H) was used for membrane isolation and lipid-raft extraction. Additionally, GAPDH was used as loading control for all 3 groups to account for expression differences in α-tubulin, HDAC6 and ATAT-1 amongst groups. Additionally, this normalization procedure was repeated when comparing the amount of acetyl-α-tubulin/α-tubulin (normalized densitometry value = sample value/mean value). This allowed us to compare samples accurately among gels and their corresponding blots.

Statistical Methods

Western blot data were analyzed for statistical significance by unpaired, two-tailed Student's *t* test or one-way ANOVA using Prism 4.0 software package for statistical data analysis (Graph Pad, San Diego, CA). Means are ±SEM, and differences for all experiments were considered significant at $p < 0.05$ ($*p < 0.05$; $**p < 0.02$). The differences in TX-114 acetyl-α-tubulin/α-tubulin, age, gender, pH of the brain, and postmortem interval (PMI) between depressed and control subjects were analyzed using the independent-sample *t* test. The relationships between TX-114 acetyl-α-tubulin/α-tubulin and PMI, and age were determined by Pearson product-moment correlation analysis. Values of *p* were two-tailed. During data analysis, confounding variables such as age, PMI, gender, race, antidepressant exposure and pH of the brain were also used as covariates (Proc GLM)(SAS 9.4 statistical software package). A linear model was used to compare NC, DS, and DNS subjects simultaneously adjusting the effects of age, gender, postmortem interval (PMI), brain pH, antidepressant use, ethanol use, non-psychotropic medicine use, violent-suicide and Hypoxia. For post-hoc multiple comparisons, we used Bonferroni (Dunn) t Tests to adjust the type I error rates, and we reported mean differences (mean-diff) and confidence interval (CI) to test the significance at the 0.05 level. In addition, each outcome measure was tested for normality (Kolmogorov-Smirnov) before running the model. All results are included in tables 2. Table 2 shows the overall model

Results

There were 11 males and 4 females in the NC group, 9 males and 6 females in the DS group and 7 males and 5 females in the DNS groups (Table 1). The age range was 14-74 years, whereas the postmortem interval (PMI) was in the range of 5-30 h. There were no significant differences in age (t=.83; df =26; p =.29) or PMI (t=-.23; df = 28; p=.82) between suicides and normal control subjects. The mean brain pH values of NC, DS and DNS were 7.01± .14, 7.01± .12 and 6.8±.13 respectively, which were not different (t=.14; df =28; p=.89).

Prefrontal cortex postmortem tissue from control, depressed suicide and depressed non-suicide subjects showed no changes in acetylation of α-tubulin in whole tissue homogenate:

The whole tissue homogenate (H) sample derived before plasma membrane and lipid-raft isolation from prefrontal cortex tissue of control (n=15), depressed suicides (n=15) and depressed non-suicides (n=12) showed no changes in acetylated-α-tubulin (Figure 1A, B, C & D). The quantification of the results from all three groups NC, DS & DNS showed no significant differences the extent of tubulin acetylation or any significant effects of covariates, age, gender, postmortem interval (PMI), brain pH, antidepressant use, ethanol use, non-psychotropic medicine use, violent-suicide and Hypoxia (table 2).

Depressed suicide brain plasma membrane localized tubulin shows decreased acetylation of α-tubulin compared to that of in normal controls:

Plasma membranes isolated from prefrontal cortex postmortem tissue of NC, DS & DNS were compared for acetylation status of membrane-associated tubulin. Five samples from each group (NC & DS) were loaded on a single gel (Figure 2A,B, & C). Additionally, DNS samples (protein concentration equal to NC & DS group subjects) were loaded on a separate gel (Figure 2D). SDS-PAGE analysis showed significant decrease in acetyl-α-tubulin in DS subjects (1-15) and DNS subjects (n=12) compared to the NC subjects. Significant changes were observed between groups in acetyl-α-tubulin/α-tubulin ($F(2) = 8.79$, $p = .0009$). The tests from multiple comparisons showed significant differences at 95% confidence level between control vs DS (mean-diff = 0.59, CI = (0.21, 0.97)) and NC vs DNS (mean-diff = 0.56, CI = (.15, 0.96)) (Figure 1E, table 2). There were no significant effects of age, gender, postmortem interval (PMI), brain pH, antidepressant use, ethanol use, non-psychotropic medicine use, violent-suicide and Hypoxia.

Detergent-resistant/lipid-raft membrane domains as well as TritonX-114-resistant/non-raft domains show decreased acetylation of α-tubulin in depressed subjects compared to normal control postmortem prefrontal cortex:

Using plasma membrane as the starting material (Figure 2) we isolated lipid-raft fractions in order to determine whether the decrease in acetylated tubulin was localized to lipid-rafts (Figure 3A & B). The raft domains showed differences in levels of tubulin acetylation. The quantification of the results from all three groups Control, DS & DNS showed significant differences between acetyl-α-tubulin/α-tubulin levels in Detergent-resistant lipid-rafts ($F(2) = 6.51$, $P<.0001$) (Figure 3C). The multiple comparisons between control vs depressed suicide subjects and control vs depressed non-suicides showed significant differences between the extent of acetyl-α-tubulin/α-tubulin in detergent-resistant lipid-rafts [Control vs DS (mean-diff = 3.94, CI = (2.49,5.38)), Control vs DNS (mean-diff = 4.02, CI = (2.49, 5.54))] as shown in table 2. There was a significant effect of hypoxia on lipid-raft tubulin (t=-2.95, p=.01). There was no effect of age, gender, postmortem interval (PMI), brain pH, antidepressant use, ethanol use, non-psychotropic medicine use or violent-suicide.

Neither tubulin acetylating nor tubulin deacetylating enzymes show altered expression in depressed brain:

HDAC6 regulates deacetylation of α-tubulin and previous studies in blood cells and postmortem brain tissue derived from patients with mood disorders showed altered HDAC6 expression (Covington et al., 2009). We did not observe these changes. (Figure 4A, B, C, D). The enzyme ATAT-1 specifically acetylates α-tubulin at K-40, whereas HDAC6 deacetylates. Therefore, along with studying changes in HDAC6 expression levels, we investigated ATAT-1 enzyme level changes. ATAT-1 expression levels/GAPDH remain statistically non-significant

amongst NC, DNS & DS ($F(2) = .96$, $P=.39$ (Figure 4 A,B,C, E). We investigated further the effect of GAPDH or any other co-variates on HDAC6 and found no significant effect (Figure 4D). Similarly, we investigated whether GAPDH and other covariates have any effect on ATAT-1. For one unit increase in GAPDH, ATAT-1 is increasing by .20 unit but not significantly (t=.47, p=.64). Hypoxia (t= -2.25, p=.03) and Violent Suicide (t=2.44, t=.02) have a significant effect on ATAT1. However, there are no group differences in the overall model (F(2)=1.87, p=.17) Most importantly, the ATAT1/HDAC6 ratio is not significantly different amongst the three groups (figure 4F), suggesting that there is no meaningful change in the expression of the enzymes regulating tubulin acetylation.

Discussion

Postmortem results presented here dovetail well with results in a cellular model revealing that increased tubulin acetylation causes the antidepressant signature response of $G\alpha_s$ translocation from lipid rafts (Singh et al., 2018) Current findings in post-mortem brain tissue suggest that acetylation status of tubulin may be important for sequestration of $G\alpha_s$ in lipid rafts, as seen in depression (Donati et al, 2008). The findings lend a molecular rationale to antidepressant effects observed in HDAC6 depleted (Espallergues et al., 2012; Fukada et al., 2012; Lee et al., 2012) or pharmacological inhibitor treated animals (Jochems et al., 2014), where increased tubulin acetylation induced behavioral effects similar to that of traditional antidepressants. Note that, while any study relying on immunodetection is subject to variability, the ability to use the comparator of acetylated to total α tubulin lends stability to the data.

The tubulin posttranslational modifications observed in postmortem brain tissue from MDD subjects, evoke abnormal cytoskeletal organization and disruption of microtubule dynamics, resulting in disrupted neurite growth, synaptogenesis and dendritic arborization (Wong et al.,

316 2013). Furthermore, proteomic studies from postmortem brain tissue of MDD subjects showed
317 changes in proteins involved in cytoskeletal arrangement, neurotransmission and synaptic
318 function (Scifo et al., 2017). Chronic stress results in dendritic retraction and synaptic density loss
319 causing regional atrophy in the hippocampus, amygdala, and prefrontal cortex, as detected in MRI
320 scans of psychiatric patients (McEwen et al., 2015). Finally, there is literature suggesting that
321 microtubules might play a role in mood, memory and consciousness (Cocchi et al., 2010;
322 Craddock et.al, 2012). Based on these data, altered tubulin and microtubules appear to be a
323 common parameter for several neuropsychiatric disorders.

324 α-tubulin undergoes acetylation and deacetylation at Lysine-40 (K40), catalyzed by acetyl
325 transferase and deacetylase enzymes respectively. Histone deacetylase-6 (HDAC6), a cytosolic
326 HDAC is known to deacetylate ⟨-tubulin. HDAC6 enzyme is highly expressed in brain, where it is
327 known to regulate emotional behaviors in rodents. HDAC6-deficient mice display hyperactivity,
328 low anxiety, and low depressive like phenotype indicating that acetylation status maintains the
329 cellular activity associated with control of emotions (Fukada et al., 2012). Similarly,
330 pharmacological inhibition of HDAC6 in rodents using inhibitors with increased brain bioavailability
331 (ACY738, ACY-775) show increased anxiolytic and antidepressant-like effects in mice undergoing
332 “depression–inducing” paradigms (Jochems et al., 2014). Furthermore, chronic stress in rodents
333 has been shown to induce increased expression of HDAC6 in hippocampus (Jianhua et al., 2017).
334 Decreased levels of acetylated tubulin are found in the hippocampus of rats following social
335 isolation (Bianchi et al., 2009). These studies further corroborated the microtubule roles,
336 especially tubulin acetylation, in the pathophysiology of depression. Decreased dendritic spine
337 density and reduced dendritic arborization are associated with neurological diseases (Blanpied
338 and Ehlers, 2004; Penzes and Vanleeuwen, 2011), including intellectual disability (Kaufmann et
339 al., 2000), depression (Duman and Canli, 2015) and schizophrenia (Penzes and Vanleeuwen,
340 2011; Glausier and Lewis, 2013). Chronic stress induces atrophy in hippocampus and

prefrontal cortex, areas important for mood regulation. Reduced dendritic field size results in abrogated synaptogenesis (Gold, 2015). HDAC6 regulates deacetylation of α-tubulin and previous studies in blood cells and postmortem brain tissue derived from patients with mood disorders showed altered HDAC6 expression (Covington et al., 2009). Post-translational modifications in α-tubulin (acetyl-α-tubulin) result from either increased enzyme expression or increased enzyme activity. We did not observe any specific expression pattern within each group or amongst three groups when normalized to total α-tubulin (Control, DS, DNS). The enzyme ATAT-1 specifically acetylates the α-tubulin at K-40, acting as the "accelerator" to the "brake" represented by HDAC6. ATAT-1 expression levels show no significant difference amongst control, depressed suicides and depressed non-suicides ($F(8,32) = 1.04$, $P=.43$) (Figure 4). Nonetheless, results in figures 2 and 3 reveal that depressed subjects show decreased acetylated a tubulin in membrane fractions. This suggests that the activity of HDAC6 relative to ATAT1 is increased without any change in the expression of either enzyme. This could be explained by multiple factors. First, HDAC6 is regulated by nitrosylation (Okuda et.al, 2015). Perhaps more importantly, only membrane tubulin (particularly lipid-raft tubulin) was affected, as the total degree of tubulin acetylation was constant amongst all groups. Perhaps some membrane translocating mechanism is at play.

These findings are consistent with a link between decreased α-tubulin acetylation and increased localization of $G\alpha_s$ in lipid-rafts. Our *in vitro* studies in C6 cells showing HDAC6 inhibition induced α-tubulin acetylation results in disruption of tubulin-$G\alpha_s$ complex, specifically in the lipid-raft domain, bolster this (Singh et al., 2018). Furthermore, membrane tubulin appears to be associated, preferentially, with lipid rafts (Goudenege et.al, 2007), so "membrane tubulin and lipid-raft tubulin may be identical. While earlier studies showed that tubulin binding to $G\alpha_s$ was sensitive to $G\alpha_s$ conformation, the nucleotide status of tubulin was not important (Yu et al., 1999). The apparent binding site for $G\alpha_s$ on tubulin involves the α3β5 region of $G\alpha_s$ and the GTP-binding pocket of ®-tubulin (Layden et al., 2008; Dave et al., 2011) While the structural changes to ®-

366 tubulin resulting from modifying ⟨-tubulin have not been established, it is clear that modifying ⟨-
367 tubulin has structural implications for the dimer (Nogales et al., 1998).

368 This study also is consistent with depression reducing availability of $G\alpha_s$ to activate adenylyl
369 cyclase and a resultant decrease in cAMP production (Donati, et.al, 2008; Fujita et.al, 2016).
370 While those studies represent post-mortem and PET imaging in human brain, human peripheral
371 tissue from depressed subjects (platelets and lymphocytes) also shows diminished $G\alpha_s$-stimulated
372 adenylyl cyclase in depression (Hines and Tabakoff, 2006; Pandey et.al, 1985; Mooney et.al
373 2013). Three of these studies above examined subjects before and after antidepressant
374 treatment, and in those subjects responding to treatment, $G\alpha_s$-stimulated cAMP production
375 returned to levels seen in healthy controls (Fujita et.al, 2016; Pandey et.al, 1985; Mooney et.al,
376 2013). Mice susceptible to stress show decreased cAMP and greater raft localization of $G\alpha_s$ in
377 their nucleus accumbens and increasing cAMP in that brain region has an “antidepressant” effect
378 (Zhang et al., 2019). Consistent with this, sustained treatment of cultured neuronal or glial cells
379 with antidepressants translocates $G\alpha_s$ from lipid rafts and increases $G\alpha_s$-activated cAMP (Donati
380 and Rasenick, 2005; Czysz et.al, 2015; Singh et.al, 2018). Ketamine also has this effect, but on
381 an accelerated timescale (Wray et.al, 2018).

382 Several of the subjects on this study showed evidence of antidepressants in their blood.
383 Some subjects were prescribed these drugs and others may have ingested them, along with other
384 drugs, in the course of their suicide. Regardless, there was no effect of antidepressants on
385 tubulin acetylation (or $G\alpha_s$ in lipid rafts; Donati, et.al, 2008). Given the observation that, absent
386 therapeutic effect, antidepressants did not increase cAMP, the lack of effect on tubulin acetylation
387 or raft association of $G\alpha_s$ is consistent. Certainly antidepressant treatment translocates $G\alpha_s$ from
388 lipid rafts in cultured cells or rodents, While neither cells nor rodents were, necessarily,
389 “depressed”, antidepressants commonly show a legion of behavioral, cellular and
390 neurophysiological effects.

This study strikes a thematic note in revealing that compounds with antidepressant activity show a consistent "biosignature" in the release of $G\alpha_s$ from lipid rafts and the subsequent association of that molecule with adenylyl cyclase, evoking a sustained increase in cellular cAMP (Singh et al., 2018). We have also demonstrated that increased acetylation of tubulin can explain this, in part. Furthermore, the diminished tubulin acetylation seen in lipid rafts from depressed subjects might explain the increase in $G\alpha_s$ seen in their lipid rafts. Nevertheless, the ability of monoamine-centered antidepressants to mitigate $G\alpha_s$-tubulin association without altering tubulin acetylation (Singh et al., 2018) argues for the complexity of depression and its therapy.

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Figure Legends

Figure 1: α-tubulin acetylation status in postmortem brain prefrontal cortex derived from normal and depressed suicides: Prefrontal cortex tissue from control (normal subjects), depressed suicides and depressed non-suicides were homogenized (H), run on SDS-PAGE gel and transferred to nitrocellulose for detection with either acetyl-α-tubulin or α-tubulin antibodies. The signal intensity was quantified and scatter plots used to show the extent of tubulin acetylation in each group (ns=non-significant compared to control).

Figure 2: Acetylated tubulin in plasma membrane prepared from prefrontal cortex is decreased in suicides relative to control: Plasma membrane (PM) was isolated from the samples presented in figure 1 and analyzed in the same manner. Scatter plots are used to show the spread of tubulin modification in both the groups (*** p=.0001).

Figure 3: Acetylated tubulin in lipid-rafts prepared from prefrontal cortex plasma membrane is decreased in suicides relative to control: Plasma membranes were purified and lipid rafts were prepared by TritonX-100-resistant (lipid-rafts) and TritonX-114-resistant (non-rafts) micro-domain isolation. Samples were analyzed as in figures 1 and 2. Tubulin and acetylated tubulin were quantified and scatter plots were used to show the distribution of tubulin modification in both the groups (C) (*** P<0.0001)

569

570 **Figure 4: Expression of tubulin acetylating and deacetylating enzymes in postmortem**
571 **tissue**: Tissue homogenates were analyzed for presence of ATAT-1 (acetylating) and HDAC-6
572 (deacetyating) enzymes in postmortem homogenates (as in figure 1). Ratios of each pair were
573 calculated and plotted in G & H.

574

575 **Table 1: Demographic characteristics of suicide and control subjects**

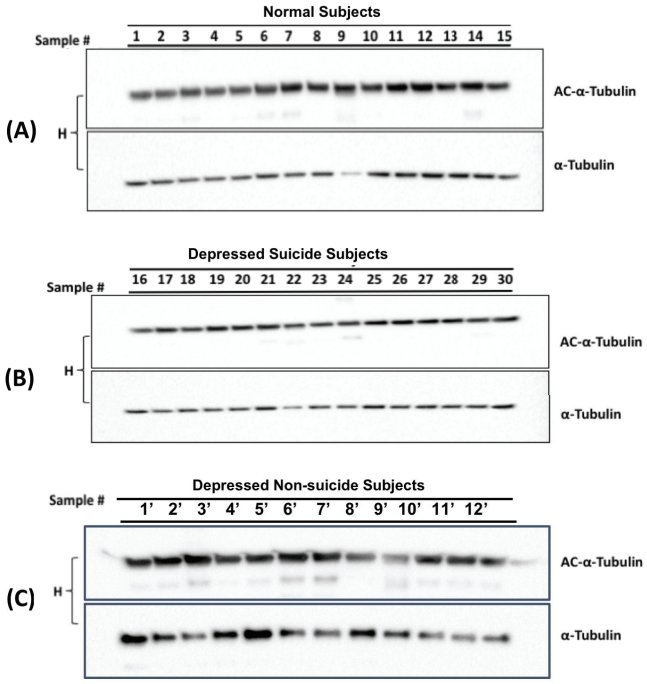
576

577 **Table 2: ANCOVA and multiple comparisons of results**

578

579

Figure 1



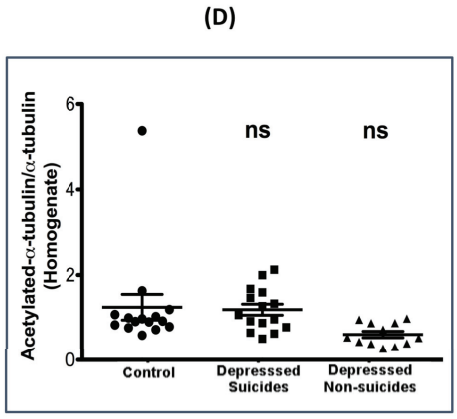
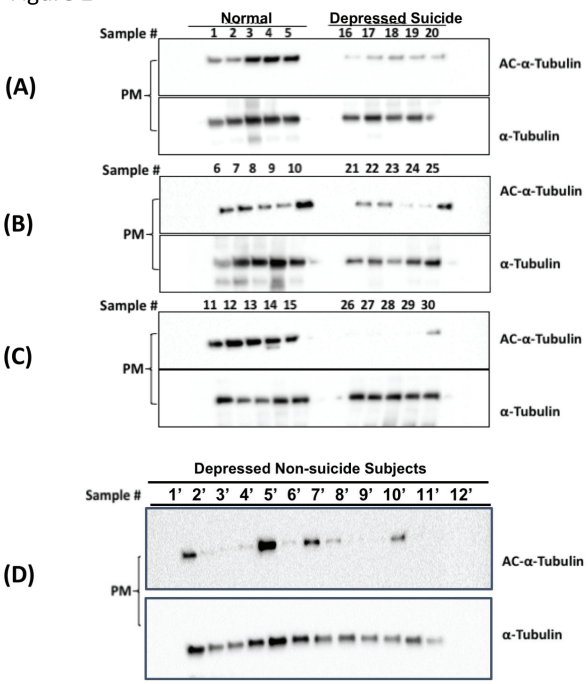


Figure 2



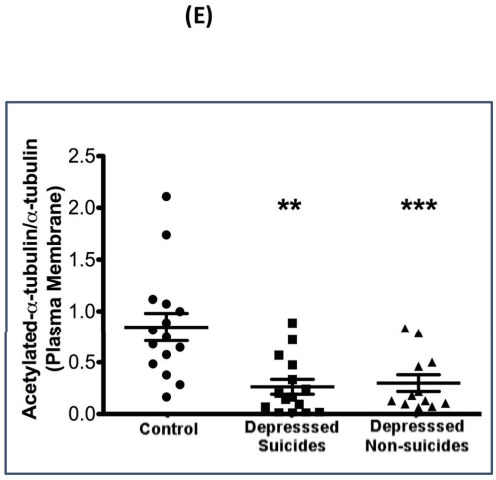


Figure 3

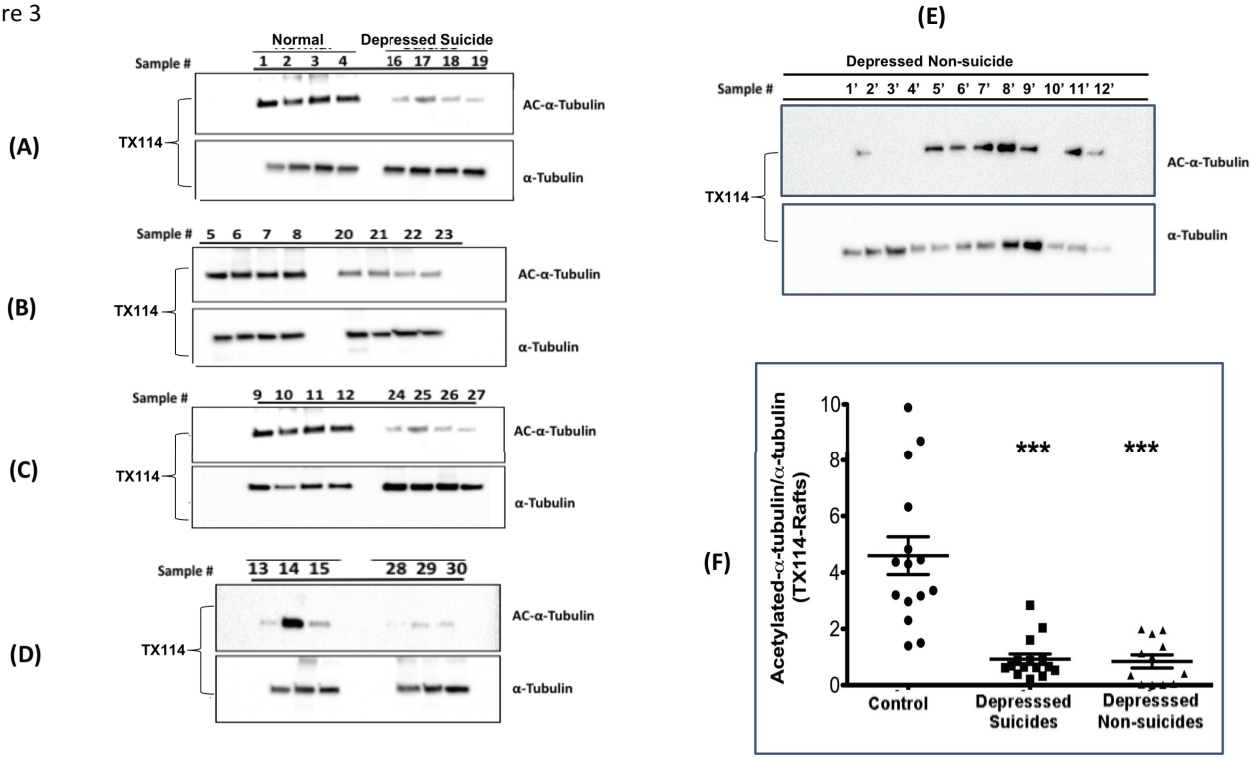


Figure 4

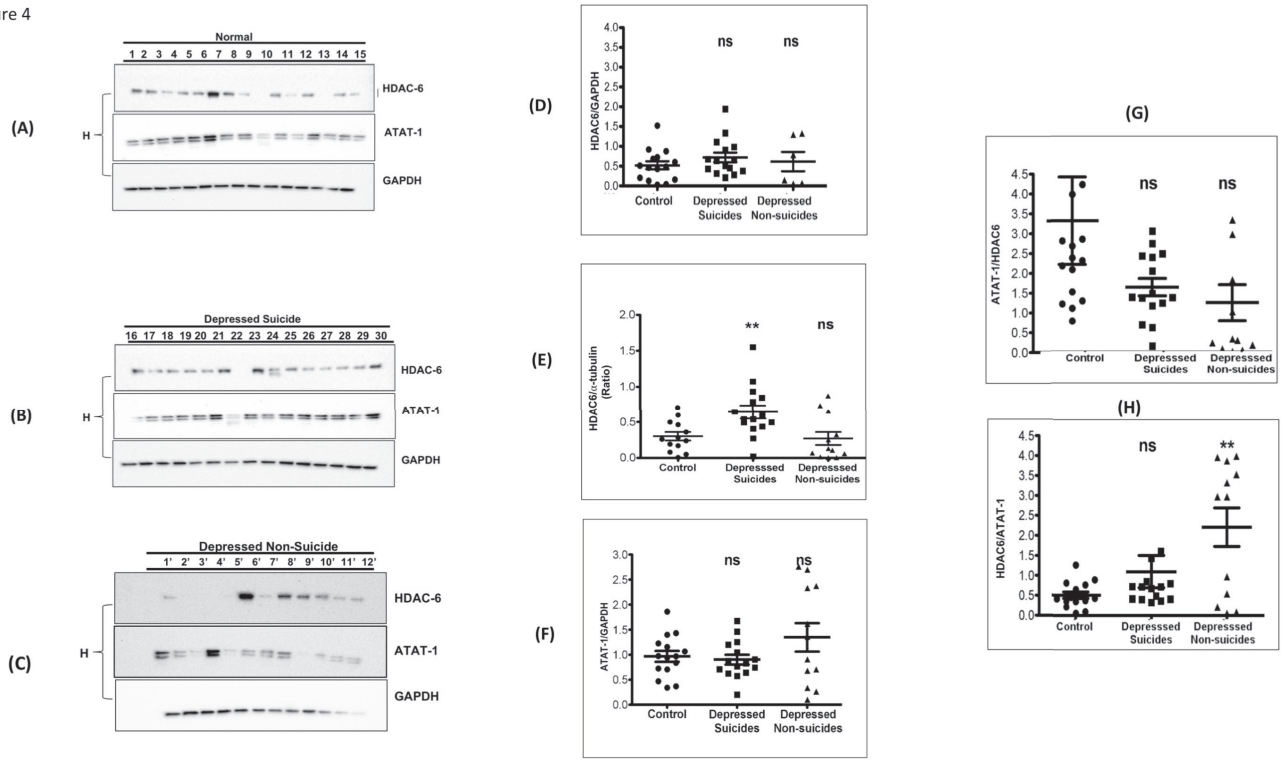


Table 1: Demographic characteristics of depressed suicide, depressed and control subjects

Group No.	Group	Diagnosis	Age	Race	Gender	PMI (hr)	Brain pH	Cause of Death	Drug Toxicity	Antidepressants Yes/No
1	CONTROL	NORMAL	37	Black	Male	5	7.1	ASCVD	None	No
2	CONTROL	NORMAL	31	Black	Male	8	7.2	GSW	None	No
3	CONTROL	NORMAL	46	Black	Male	9	7.1	Multiple injuries	None	No
4	CONTROL	NORMAL	33	White	Male	15	7	GSW	None	No
5	CONTROL	NORMAL	48	White	Male	26	6.9	ASCVD	None	No
6	CONTROL	NORMAL	38	Black	Male	16	6.9	Lung sarcoidosis	None	No
7	CONTROL	NORMAL	65	Black	Felame	23	6.9	ASCVD	None	No
8	CONTROL	NORMAL	52	White	Male	30	7.3	ASCVD	None	No
9	CONTROL	NORMAL	63	White	Female	30	7.1	Ovarian cancer	None	No
10	CONTROL	NORMAL	37	White	Male	24	7	ASCVD	None	No
11	CONTROL	NORMAL	72	White	Female	23	6.9	MVA	None	No
12	CONTROL	NORMAL	42	White	Female	23	6.9	Mitral valve prolapse	None	No
13	CONTROL	NORMAL	31	White	Male	16	7.2	MVA	None	No
14	CONTROL	NORMAL	28	White	Male	13	6.8	Electrocution	None	No
15	CONTROL	NORMAL	53	White	Male	15	6.9	ASCVD		
1	SUICIDE	Major depression, alc	27	White	Male	24	7	GSW	None	NO
2	SUICIDE	Major depression, alc	44	White	Female	11	7.2	Drug overdose	Nortriptyline	YES
3	SUICIDE	Major depression	24	White	Male	7	7.1	GSW	Ethanol	NO
4	SUICIDE	Major Depression, P	43	White	Male	12	7	Drug overdose	Propoxyphene, Acetaminophen	NO
5	SUICIDE	Major depression	53	White	Male	23	6.9	Jumped 3rd floor	None	NO
6	SUICIDE	Major depression, Al	41	White	Female	27	7.1	Drug overdose	Amitriptyline, Desipramine, Diphenhydramine, Nortrip	YES
7	SUICIDE	Major depression	36	White	Female	18	7.2	GSW	None	NO
8	SUICIDE	Major depression, Al	38	White	Male	24	7	Drug overdose, Ethanol overdose	Ethanol, Diphenhydramine	NO
9	SUICIDE	Major depression (29	46	White	Female	16	6.8	Drug overdose,Nortiptyline intoxication	Nortriptyline	YES
10	SUICIDE	Major depression (29	30	White	Male	17	7.1	Hanging suicide	Effexor	YES
11	SUICIDE	Major depression (29	74	White	Female	27	7	Suicide by Effexor OD	Effexor, Ethanol	YES
12	SUICIDE	Major depression (29	25	White	Male	14	6.8	Suicide by hanging, asphyxia	Ethanol	NO
13	SUICIDE	Major depression, N	23	Black	Male	23	6.9	Hanging suicide	None	NO
14	SUICIDE	Major depression (29	67	White	Male	22	7	GSW to chest	Prozac, Effexor	YES
15	SUICIDE	Major depression (29	40	White	Female	20	7	Suicide by OD	Acetaminophen, Hydrocodone, Diphenhydramine, X	NO
1*	Non-Suicide	Major depression, re	65	White	Male	14	6.9	ASCVD	None	NO
2*	Non-Suicide	Major depression, re	55	Black	Female	8	6.4	ASCVD	Fluoxetine, Ethanol	YES
3*	Non-Suicide	Major depression, re	71	White	Male	4	6.3	ASCVD	Bupropion, Diltiazem	YES
4*	Non-Suicide	Depression, NOS (31	74	Black	Female	7	6.7	ASCVD	Paroxetine, Thioridazine	YES
5*	Non-Suicide	Major depression, sin	14	White	Male	11	7	MVA	Sertraline	YES
6*	Non-Suicide	Major depression, re	39	White	Male	36	6.8	Fatty liver	Thioridazine	NO
7*	Non-Suicide	Major depression, re	46	Black	Male	20	7.1	Seizure disorder	Fluoxetine, Risperidone	YES
8*	Non-Suicide	Major depression, re	59	White	Male	20	7	ASCVD	Sertraline, Atropine	YES
9*	Non-Suicide	Major depression, re	46	White	Female	23	6.9	Mixed drug intoxication	Bupropion, Lamotrigine, Diphenhydramine	YES
10*	Non-Suicide	Major depression, re	29	White	Female	22	6.9	Morbid obesity, Cardiomegaly	Fluoxetine, Norfluoxetine, Norpropoxyphene	YES
11*	Non-Suicide	Major depression, N	49	White	Male	24	7.1	ASCVD	Desmethylsertraline	YES
12*	Non-Suicide	Major depression, re	47	White	Female	26	6.5	DKA	Fluoxetine	YES

ASCVD, atherosclerotic cardiovascular disease; GSW, gunshot wound; MDD, major depressive disorder; MVA, motor vehicle accident

Mean +/- SD age is 45.07 +/- 13.59 years; PMI is 18.40+/-7.84 hours; brain pH is 7.01+/- 0.15; 5 Black, 10 White; 11 Males, 4 Females

Mean +/- SD age is 40.73+/- 15.04 years; PMI is 19.00+/- 6.07 hours; brain pH is 7.01+/- 0.12; 1 Black, 14 White; 9 Males, 6 Females

Mean +/- SD age is 58.58+/- 29.99 years; PMI is 17.91+/- 16 hours; brain pH is 6.8+/- 0.13; 3 Black, 9 White; 7 Males, 5 Females

Table 2: ANCOVA and Bonferroni (multiple comparison) Results

ANCOVA

Dependent Variable	DF (Model, Error)	F Value	Pr > F
acetyl-□ tubulin/total □ -tubulin (Homogenate)	8,32	.89	0.57
acetyl-□ tubulin/total □ -tubulin (Plasma membrane)	8,32	2.17	0.04
acetyl-□ tubulin/total □ -tubulin (Lipid-rafts)	8,32	6.51	<.0001

Multiple Comparison Tests (Bonferroni)

Note: Comparisons significant at the .05 level are indicated by *

DV	Group Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
acetyl-□ tubulin/total □ -tubulin	NC - DS	0.029	-0.7510	0.8091	
(Homogenate)	NC - DNS	0.6143	-0.2115	1.44	
	DS - DNS	0.5853	-0.2277	1.3982	
acetyl-□ tubulin/total □ -tubulin	NC - DNS	0.5592	0.1544	0.9640	***
(Plasma Membrane)	NC - DS	0.5947	0.2124	0.9771	***
	DNS - DS	0.0355	-0.3630	0.4340	
acetyl-□ tubulin/total □ -tubulin	NC - DS	3.9386	2.4972	5.3800	***
(Lipid-rafts)	NC - DNS	4.0179	2.4920	5.5439	***
	DS - DNS	0.0793	-1.5816	1.4230	