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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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 with 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 HDAC6 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 subjects, depressed suicides, and depressed nonsuicides (human males/females). While tissue homogenate showed no changes in tubulin acetylation between control, depressed suicides, and depressed nonsuicides, plasma membrane-associated tubulin showed significant decreases in acetylation from depressed suicides and depressed nonsuicides compared with 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.

Key words: antidepressant; cAMP; cytoskeleton; G-protein; GPCR; lipid-raft

Significance Statement

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

Introduction

Hallmarks of major depressive disorder (MDD) include persistent sad mood, anhedonia, changes in appetite, disturbed sleep, feelings of worthlessness, hopelessness, and suicidal thoughts. While various antidepressant drug therapies are available, the

biological underpinnings of their action as well as the molecular events leading to depression remain uncertain. Numerous suggestions about the biology of depression exist, and epigenetics (histone deacetylases [HDACs]) and HDAC inhibitors as novel antidepressants are a recent addition to this list (Tsankova et al., 2007; Covington et al., 2009; Gundersen and Blendy, 2009). The

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majority of the presently available antidepressants have, among their actions, prevention of monoamine uptake or degradation, and one consistent effect of antidepressant treatment has been a persistent increase in cAMP and an upregulation of the cAMP generating system (Nibuya et al., 1996; Malberg et al., 2000; Donati and Rasenick, 2003). Furthermore, PET studies from depressed subjects showed global decreases in brain cAMP and antidepressant drugs restored cAMP levels (Fujita et al., 2007, 2017). We have suggested that antidepressants achieve this by a gradual removal of G_{α_s} 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_{α_s} (Donati et al., 2008). G_{α_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_{α_s} may play an important role in its localization and function, and that chronic antidepressant treatment alters the membrane localization of G_{α_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 (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., 2015). 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 $\alpha\beta$ dimer, and these dimers are localized in membranes, and enriched in lipid rafts. Upon activation, G_{α_s} is released from the membrane, where it binds tubulin, activates tubulin GTPase, and increases microtubule dynamics (Roychowdhury and Rasenick, 1994; Davé et al., 2011; Sarma et al., 2015). These findings suggest that tubulin may act as an anchor for G_{α_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_{α_s} is complexed with tubulin.

The enzymes responsible for the regulation of acetylation status of α -tubulin are HDAC6 (deacetylating) and α -tubulin acetyltransferase-1 (ATAT-1: acetylating). There is emerging evidence for the role of HDAC in neuropsychiatric disorders, including MDD (Hobara et al., 2010; Guidotti et al., 2011). Altered levels of HDAC2, 4, 5, 6, and 8 mRNA have been observed in blood cells and postmortem brain from mood disorder subjects (Hubbert et al., 2002; Guidotti et al., 2011). HDAC6, localized in cytosol,

deacetylates α -tubulin (Verdel et al., 2000; Hubbert et al., 2002). 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. PFC tissue showed comparable tubulin acetylation in homogenates (Hs), but strikingly decreased acetylation in membranes prepared from depressed suicides (DSs) and depressed nonsuicides (DNSs). These data correspond well with a previous study showing increased G_{α_s} levels in lipid rafts, since acetylation of tubulin decreases its ability to bind G_{α_s} and anchor it to lipid rafts, resulting in less G_{α_s} available for adenylyl cyclase activation in the depressed brain. These findings also parallel those of G_{α_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_{α_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 DS subjects ($n = 15$), DNS 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. Tissues were collected only after a family member gave informed consent. All procedures were approved by the University of Maryland Institutional Review Board and by the University of Illinois Institutional Review Board.

All tissues from normal controls, DSs, and nonsuicide 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 previously (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, and the Structured Clinical Interview for the DSM-IV (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 (PFC) were resuspended and minced in TME buffer (10 mM Tris-HCl, 1 mM MgCl_2 , 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 H was saved to be run on Western blot along with other cell fractions. The rest of the H samples were centrifuged at $100,000 \times g$ for 1 h at 4°C , and the supernatant (cytosol) and pellet (plasma membrane [PM]) were

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Table 1. Demographic characteristics of suicide and control subjects^a

Group no.	Group	Diagnosis	Age (yr)	Race	Gender	PMI (h)	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	Female	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	MDD	27	White	Male	24	7	GSW	None	NO
2	Suicide	MDD	44	White	Female	11	7.2	Drug overdose	Nortriptyline	YES
3	Suicide	MDD	24	White	Male	7	7.1	GSW	Ethanol	NO
4	Suicide	MDD	43	White	Male	12	7	Drug overdose	Propoxyphene, acetaminophen	NO
5	Suicide	MDD	53	White	Male	23	6.9	Jumped 3rd floor	None	NO
6	Suicide	MDD	41	White	Female	27	7.1	Drug overdose	Amitriptyline, desipramine, diphenhydramine, nortriptyline	YES
7	Suicide	MDD	36	White	Female	18	7.2	GSW	None	NO
8	Suicide	MDD	38	White	Male	24	7	Drug overdose, Ethanol overdose	Ethanol, diphenhydramine	NO
9	Suicide	MDD	46	White	Female	16	6.8	Drug overdose, nortriptyline intoxication	Nortriptyline	YES
10	Suicide	MDD	30	White	Male	17	7.1	Hanging suicide	Venlafaxine	YES
11	Suicide	MDD	74	White	Female	27	7	Suicide by OD	Venlafaxine, ethanol	YES
12	Suicide	MDD	25	White	Male	14	6.8	Suicide by hanging, asphyxia	Ethanol	NO
13	Suicide	MDD	23	Black	Male	23	6.9	Hanging suicide	None	NO
14	Suicide	MDD	67	White	Male	22	7	GSW to chest	Fluoxetine, venlafaxine	YES
15	Suicide	MDD	40	White	Female	20	7	Suicide by OD	Acetaminophen, hydrocodone, diphenhydramine	NO
1'	Nonsuicide	MDD	65	White	Male	14	6.9	ASCVD	None	NO
2'	Nonsuicide	MDD	55	Black	Female	8	6.4	ASCVD	Fluoxetine, ethanol	YES
3'	Nonsuicide	MDD	71	White	Male	4	6.3	ASCVD	Bupropion, diltiazem	YES
4'	Nonsuicide	MDD	74	Black	Female	7	6.7	ASCVD	Paroxetine, thioridazine	YES
5'	Nonsuicide	MDD	14	White	Male	11	7	MVA	Sertraline	YES
6'	Nonsuicide	MDD	39	White	Male	36	6.8	Fatty liver	Thioridazine	NO
7'	Nonsuicide	MDD	46	Black	Male	20	7.1	Seizure disorder	Fluoxetine, risperidone	YES
8'	Nonsuicide	MDD	59	White	Male	20	7	ASCVD	Sertraline, atropine	YES
9'	Nonsuicide	MDD	46	White	Female	23	6.9	Mixed drug intoxication	Bupropion, lamotrigine, diphenhydramine	YES
10'	Nonsuicide	MDD	29	White	Female	22	6.9	Morbid obesity, cardiomegaly	Fluoxetine, norfluoxetine, nortriptyline	YES
11'	Nonsuicide	MDD	49	White	Male	24	7.1	ASCVD	desmethylsertraline	YES
12'	Nonsuicide	MDD	47	White	Female	26	6.5	DKA	Fluoxetine	YES

^aASCVD, Atherosclerotic cardiovascular disease; GSW, gunshot wound; MVA, motor vehicle accident. Values are Control: $\pm$ SD. Age, 45.07 $\pm$ 13.59 years; PMI, 18.40 $\pm$ 7.84 h; brain pH, 7.01 $\pm$ 0.15; Depressed suicides: Age, 40.73 $\pm$ 15.04 years; PMI, 19.00 $\pm$ 6.07 h; brain pH, 7.01 $\pm$ 0.12; Depressed Non-suicides: Age, 58.58 $\pm$ 29.99 years; PMI, 17.91 $\pm$ 16 h; brain pH, 6.8 $\pm$ 0.13.

saved. The crude membrane pellet was extracted with 0.75 ml of TME containing 1% Triton X-100 for 1 h 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 (TX-114) for 1 h 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) 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 H, PM, TX-100- and TX-114-soluble (TX-100-resistant) membrane fractions (12–15 μg) were analyzed by SDS-PAGE followed by Western blotting. The gels were transferred to nitrocellulose membranes (Bio-Rad) 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 TBS/Tween 20 and then incubated with an anti-acetyl- α -tubulin (Sigma Millipore, #T7451 clone 6-11B-1), α -tubulin (Sigma Millipore, #T9026), HDAC6 (Cell Signaling Technology, #7558S), ATAT-1 (Sigma Millipore, #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 Laboratories, catalog #115-036-072 for mouse, RRID:

Table 2. ANCOVA results

Dependent variable	df (model, error)	F	P > F
Acetyl- α -tubulin/total α -tubulin (H)	8,32	0.89	0.57
Acetyl- α -tubulin/total α -tubulin (PM)	8,32	2.17	0.04
Acetyl- α -tubulin/total α -tubulin (lipid rafts)	8,32	6.51	<0.0001

AB_2338525) and catalog #111-036-047 for rabbit, RRID: AB_2337945) for 1 h at room temperature, washed, and developed using ECL Luminata Forte chemiluminescent reagent (Millipore). Blots were imaged using Chemidoc computerized densitometer (Bio-Rad). The signal intensity of bands from each image were quantitated by densitometry using ImageJ software (National Institutes of Health) and the TX-100-resistant acetyl- α -tubulin/ α -tubulin (TX-114) was compared. The acetyl- α -tubulin/ α -tubulin were also observed in PM from control (NC), DS, and DNS samples as described (Toki et al., 1999; Donati et al., 2008). Additionally, HDAC6, ATAT-1 and GAPDH expression differences were analyzed between the three groups (C, DS, and DNS).

Normalization

To be consistent throughout the data collection, the same amount of starting material (H) was used for membrane isolation and lipid-raft extraction. Additionally, GAPDH was used as loading control for all three groups to account for expression differences in α -tubulin, HDAC6, and ATAT-1 among 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 (GraphPad). Data are mean $\pm$ 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, PMI, brain pH, antidepressant use, ethanol use, nonpsychotropic 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 and 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 and 3.

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 group (Table 1). The age range was 14–74 years, whereas the PMI was in the range of 5–30 h. There were no significant differences in age ($t = 0.83$; $df = 26$; $p = 0.29$) or PMI ($t = -0.23$; $df = 28$; $p = 0.82$) between suicides and normal control subjects. The mean brain pH values of NC, DS, and DNS were 7.01 ± 0.14 , 7.01 ± 0.12 , and 6.8 ± 0.13 , respectively, which were not different ($t = 0.14$; $df = 28$; $p = 0.89$).

PFC postmortem tissue from control, DS, and DNS subjects showed no changes in acetylation of α -tubulin in whole-tissue H

The whole-tissue H sample derived before PM and lipid-raft isolation from PFC tissue of control ($n = 15$), DSs ($n = 15$) and

Table 3. Bonferroni (multiple comparison) results

DV	Group comparison	Difference between means	Simultaneous 95% CI
Acetyl- α -tubulin/total α -tubulin (H)	NC vs DS	0.029	−0.7510 0.8091
	NC vs DNS	0.6143	−0.2115 1.44
	DS vs DNS	0.5853	−0.2277 1.3982
Acetyl- α tubulin/total α -tubulin (PM)	NC vs DNS	0.5592	0.1544 0.9640
	NC vs DS	0.5947	0.2124 0.9771
	DNS vs DS	0.0355	−0.3630 0.4340
Acetyl- α -tubulin/total α -tubulin (lipid rafts)	NC vs DS	3.9386	2.4972 5.3800
	NC vs DNS	4.0179	2.4920 5.5439
	DS vs DNS	0.0793	−1.5816 1.4230

DNSs ($n = 12$) showed no changes in acetylated- α -tubulin (Fig. 1A–D). The quantification of the results from all three groups (NC, DS, and DNS) showed no significant differences in the extent of tubulin acetylation or any significant effects of covariates, age, gender, PMI, brain pH, antidepressant use, ethanol use, nonpsychotropic medicine use, violent suicide, and hypoxia (Tables 2, 3).

DS brain PM-localized tubulin shows decreased acetylation of α -tubulin compared with that of normal controls

PMs isolated from PFC postmortem tissue of NC, DS, and DNS were compared for acetylation status of membrane-associated tubulin. Five samples from each group (NC and DS) were loaded on a single gel (Fig. 2A–C). Additionally, DNS samples (protein concentration equal to NC and DS group subjects) were loaded on a separate gel (Fig. 2D). SDS-PAGE analysis showed significant decrease in acetyl- α -tubulin in DS subjects (1–15) and DNS subjects ($n = 12$) compared with the NC subjects. Significant changes were observed between groups in acetyl- α -tubulin/ α -tubulin ($F_{(2)} = 8.79$, $p = 0.0009$). The tests from multiple comparisons showed significant differences at 95% CI between control versus DS (mean difference = 0.59, CI = (0.21, 0.97)) and NC versus DNS (mean difference = 0.56, CI = (0.15, 0.96)) (Fig. 2E; Tables 2, 3). There were no significant effects of age, gender, PMI, brain pH, antidepressant use, ethanol use, nonpsychotropic medicine use, violent suicide and hypoxia.

Detergent-resistant/lipid-raft membrane domains as well as TX-114-resistant/nonraft domains show decreased acetylation of α -tubulin in depressed subjects compared with normal control postmortem PFC

Using PM as the starting material (Fig. 2), we isolated lipid-raft fractions to determine whether the decrease in acetylated tubulin was localized to lipid rafts (Fig. 3A–E). The raft domains showed differences in levels of tubulin acetylation. The quantification of the results from all three groups (Control, DS, and DNS) showed significant differences between acetyl- α -tubulin/ α -tubulin levels in detergent-resistant lipid rafts ($F_{(2)} = 6.51$, $p < 0.0001$) (Fig. 3F). The multiple comparisons between control versus DS subjects and control versus DNSs showed significant differences between the extent of acetyl- α -tubulin/ α -tubulin in detergent-resistant lipid rafts [Control vs DS (mean difference = 3.94, CI = (2.49, 5.38)), Control vs DNS (mean difference = 4.02, CI = (2.49, 5.54))] as shown in Tables 2 and 3. There was a significant effect of hypoxia on lipid-raft tubulin ($t = -2.95$, $p = 0.01$). There was no effect of age, gender, PMI, brain pH, antidepressant use, ethanol use, nonpsychotropic medicine use, or violent suicide.

Figure 1. α -Tubulin acetylation status in postmortem brain PFC derived from normal and depressed subjects. PFC tissue from *A*) control (normal subjects), *B*) depressed suicide subjects, and *C*) Depressed non-suicide subjects was homogenized (H), run on SDS-PAGE gels, and transferred to nitrocellulose for detection with either acetyl- α -tubulin or α -tubulin antibodies. *D*) The signal intensity was quantified, and scatter plots were used to show the extent of tubulin acetylation in each group. ns, Nonsignificant compared with control.

Figure 2. Acetylated tubulin in PM prepared from PFC is decreased in suicides relative to control. PM was isolated from the samples presented in Figure 1 and analyzed in the same manner. PFC tissue from *A*) control (normal subjects) 1–5 and depressed suicide subjects 16–20, *B*) subjects 6–10 and 21–25, and *C*) Subjects 11–15 and 26–30 or *D*) Depressed non-suicide subjects 1'–12' were analyzed as in Figure 1. *E*) Scatter plots are used to show the extent of α -tubulin acetylation in both groups. ** p = 0.001; *** p = 0.0001.

Figure 3. Acetylated tubulin in lipid rafts prepared from postmortem PFC is decreased in depressed subjects relative to control. Plasma membrane was purified, and lipid rafts were prepared by TX-100-resistant (lipid rafts) and TritonX-114-resistant (nonrafts) micro-domain isolation. Samples were analyzed as in Figures 1 and 2. **A–D** Shows data for samples isolated from normal controls and depressed suicides. **E** shows samples isolated from depressed non-suicides. **F** Tubulin and acetylated tubulin were quantified as in Figures 1 and 2. Scatter plots were used to show the extent of tubulin modification. *** $p < 0.0001$

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 (Fig. 4A–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 nonsignificant among NC, DNS, and DS ($F_{(2)} = 0.96$, $p = 0.39$ (Fig. 4A–C,E). We investigated further the effect of GAPDH or any other covariates on HDAC6 and found no significant effect (Fig. 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 0.20 unit, but not significantly ($t = 0.47$, $p = 0.64$). Hypoxia ($t = -2.25$, $p = 0.03$) and violent suicide ($t = 2.44$, $t = 0.02$) have a significant effect on ATAT1. However, there are no group differences in the overall model ($F_{(2)} = 1.87$, $p = 0.17$). Most importantly, the ATAT1/HDAC6 ratio is not significantly different among the three groups (Fig. 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_{α_s} translocation from lipid rafts (Singh et al., 2018). Current findings in postmortem brain tissue suggest that acetylation status of tubulin may be important for sequestration of G_{α_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. 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 post-translational 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., 2013). Furthermore, proteomic studies from postmortem brain tissue of MDD subjects showed changes in proteins involved in cytoskeletal arrangement, neurotransmission, and synaptic function (Scifo et al., 2017). Chronic stress results in dendritic retraction and synaptic density loss causing regional atrophy in the hippocampus, amygdala, and PFC, as detected in MRI scans of psychiatric patients (McEwen et al., 2015). Finally, there is literature suggesting that microtubules might play a role in mood, memory, and consciousness (Cocchi et al., 2010; Craddock et al., 2012). Based on

Figure 4. Expression of tubulin acetylating and deacetylating enzymes in postmortem tissue. PFC tissue homogenates from *A*) control subjects, *B*) depressed suicide subjects and *C*) depressed non-suicide subjects were analyzed for presence of ATAT-1 (tubulin-acetylating) and HDAC6 (acetyl tubulin deacetylating) enzymes using GAPDH as a protein standard. Quantification of *D*) HDAC6/total protein, *E*) ATAT1/total protein and *F*) HDAC6/ATAT1 is shown. ns, non significant.

these data, altered tubulin and microtubules appear to be a common parameter for several neuropsychiatric disorders.

α -Tubulin undergoes acetylation and deacetylation at Lysine-40 (K40), catalyzed by acetyl transferase and deacetylase enzymes, respectively. HDAC6, a cytosolic HDAC, is known to deacetylate α -tubulin. HDAC6 enzyme is highly expressed in brain, where it is known to regulate emotional behaviors in rodents. HDAC6-deficient mice display hyperactivity, low anxiety, and low depressive-like phenotype, indicating that acetylation status maintains the cellular activity associated with control of emotions (Fukada et al., 2012). Similarly, pharmacological inhibition of HDAC6 in rodents using inhibitors with increased brain bioavailability (ACY738, ACY-775) shows increased anxiolytic and antidepressant-like effects in mice undergoing “depression-inducing” paradigms (Jochems et al., 2014). Furthermore, chronic stress in rodents has been shown to induce increased expression of HDAC6 in hippocampus (Jianhua et al., 2017). Decreased levels of acetylated tubulin are found in the hippocampus of rats following

social isolation (Bianchi et al., 2009). These studies further corroborated the microtubule roles, especially tubulin acetylation, in the pathophysiology of depression. Decreased dendritic spine density and reduced dendritic arborization are associated with neurologic diseases (Blanpied and Ehlers, 2004; Penzes and Vanleeuwen, 2011), including intellectual disability (Kaufmann et al., 2000), depression (Duman and Canli, 2015), and schizophrenia (Penzes and Vanleeuwen, 2011; Glausier and Lewis, 2013). Chronic stress induces atrophy in hippocampus and PFC, 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 among 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 among control, DSs and DNSs ($F_{(8,32)} = 1.04$, $p = 0.43$) (Fig. 4). Nonetheless, results in Figures 2 and 3 reveal that depressed subjects show decreased acetylated α -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 among 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 (Ishmael 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 $\alpha 3\beta 5$ region of $G\alpha_s$ and the GTP-binding pocket of α -tubulin (Layden et al., 2008; Davé et al., 2011). While the structural changes to α -tubulin resulting from modifying α -tubulin have not been established, it is clear that modifying α -tubulin has structural implications for the dimer (Nogales et al., 1998).

This study also is consistent with depression reducing availability of $G\alpha_s$ to activate adenylyl cyclase and a resultant decrease in cAMP production (Donati et al., 2008; Fujita et al., 2017). While those studies represent postmortem and PET imaging in human brain, human peripheral tissue from depressed subjects (platelets and lymphocytes) also shows diminished $G\alpha_s$ -stimulated adenylyl cyclase in depression (Pandey et al., 1985; Hines and Tabakoff, 2005; Mooney et al., 2013). Three of these studies above examined subjects before and after antidepressant treatment, and in those subjects responding to treatment, $G\alpha_s$ -stimulated cAMP production returned to levels seen in healthy controls (Pandey et al., 1985; Fujita et al., 2017; Mooney et al., 2013). Mice susceptible to stress show decreased cAMP and greater raft localization of $G\alpha_s$ in their nucleus accumbens and increasing cAMP in that brain region has an “antidepressant” effect (Zhang et al., 2020). Consistent with this, sustained treatment of cultured neuronal or glial cells with antidepressants translocates $G\alpha_s$ from lipid rafts and increases $G\alpha_s$ -activated cAMP (Donati and Rasenick, 2005; Czysz et al., 2015; Singh et al., 2018). Ketamine also has this effect, but on an accelerated time scale (Wray et al., 2019).

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

antidepressants commonly show a legion of behavioral, cellular, and neurophysiological effects.

This study strikes a thematic note in revealing that compounds with antidepressant activity show a consistent “bio-signature” 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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