Serotonin 2A Receptor Autoantibodies Increase in Adult Traumatic Brain Injury In Association with Neurodegeneration

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Abstract

Objective

Traumatic brain injury (TBI) is associated with an increased risk of late neurodegenerative complications via unknown mechanisms. Circulating neurotoxic 5-hydroxytryptamine 2A receptor (5-HT2AR) autoantibodies were reported to increase in subsets of obese type 2 diabetes having microvascular complications. We tested whether 5-HT2AR autoantibodies increase in adults following traumatic brain injury in association with neurodegenerative complications.

Methods

 Plasma from thirty-five middle-aged and older adult veterans (mean 65 years old) who had suffered traumatic brain injury was subjected to protein-A affinity chromatography. The resulting immunoglobulin (Ig) G fraction was tested for neurotoxicity (acute neurite retraction, and accelerated cell death) in mouse N2A neuroblastoma cells or for binding to a linear synthetic peptide corresponding to the second extracellular loop region of the human 5-HT2A receptor.

Results

Nearly two-thirds of traumatic brain injured-patients harbored 5-HT2AR autoantibodies in their circulation. Active TBI autoantibodies caused neurite retraction in mouse N2A neuroblastoma cells and accelerated N2A cell loss which was substantially prevented by co-incubation with a two hundred and fifty nanomolar concentration of M100907, a highly selective 5-HT2AR antagonist. Antagonists of RhoA/Rho kinase and Gq11/phospholipase C/inositol triphosphate receptor signaling pathways blocked TBI autoantibody-induced neurite retraction. Following traumatic brain injury, autoantibody binding to a 5-HT2A receptor peptide was significantly increased in patients having co-morbid Parkinson's disease (n=3), dementia (n=5), and painful neuropathy (n=8) compared to TBI subsets without neurologic or microvascular complication (n=20). Autoantibody titer was significantly elevated in TBI subsets experiencing multiple neurotraumatic exposures vs. single TBI. Plasma white blood cell, a marker of systemic inflammation, correlated significantly (correlation coefficient r =0.52; P < 0.01) with, 5-HT2A receptor peptide binding of the TBI-autoantibody.

Conclusion

These data suggest that circulating neurotoxic 5-hydroxytryptamine 2A receptor agonist autoantibodies increase in adults following traumatic brain injury in association with late neurodegenerative complications.

Introduction

Type 2 diabetes and traumatic brain injury (TBI) are associated with an increased risk of late-onset neurodegeneration [1-3] via mechanisms involving increased peripheral and central inflammation, respectively. Visceral obesity-associated inflammation promotes activation of innate and adaptive immune mechanisms [4]. In older adult type 2 diabetic subsets having Parkinson's disease or dementia, circulating plasma immunoglobulin G (IgG) autoantibodies bound to the 5-hydroxytryptamine 2A receptor and mediated neurotoxicity in mouse neuroblastoma cells through activation of Gq11/inositol triphosphate receptor (IP3R)/Ca2+ and RhoA/Rho kinase signaling pathways [5]. Diffuse microvascular injury is an additional risk factor associated with the development of potent anti-endothelial, and neurotoxic IgG autoantibodies in subsets of long-standing, poorly controlled type 2 diabetes [6-8].

Since long-term recovery following traumatic brain injury depends (in part) on normal angiogenesis and restoration of blood brain barrier function, we set out to test whether circulating agonist 5-HT2AR autoantibodies increase in middle-aged and older adult veterans following traumatic brain injury and for possible association(s) between 5-HT2AR autoantibodies and neurodegenerative complications, or microvascular injury occurring in type 2 diabetes mellitus.

The 5HT2A receptor is highly concentrated in brain regions underlying cognition, memory, perception, and mood regulation [9]. Increased circulating 5-HT2AR IgG autoantibodies in traumatic brain injury might provide a biomarker (or be involved in the pathophysiology) of the later occurrence of neurodegenerative complications.

Participants and Methods

Patients

Thirty-five patients suffering prior traumatic brain injury (twenty-four with and eleven without co-morbid type 2 diabetes mellitus) were consecutively enrolled from the Endocrinology and Diabetes clinic of the Veterans Affairs New Jersey Healthcare System (VANJHCS) at Lyons and East Orange, New Jersey. Consent was obtained prior to blood drawing. For the comparison in Fig 2B, data in a previously reported cohort of forty-seven patients having type 2 diabetes mellitus but lacking TBI exposure were used to test for possible association between autoantibody binding and systemic inflammation.

Blood drawing

Blood was drawn in the morning after an overnight fast.Plasma or serum was either immediately subjected to protein-A affinity chromatography (to obtain the immunoglobulin G fraction) or was stored at -40 C for later use.

Protein-A affinity chromatography

Protein-A chromatography was carried out as previously reported [10]. Protein-A eluate fractions were stored at 0-4 C.

Acute neurite retraction

Mouse N2A neuroblastoma cells were maintained in Dulbecco's minimal essential medium with 10% fetal calf serum. Neurite retraction assay was carried out as previously reported [10]. Neurite retraction represents average neurite length-shortening after five minutes exposure to TBI autoantibodies (60-100 nM) in the presence or absence of various G-protein coupled receptor (GPCR) or signaling pathway antagonists.

Mouse neuroblastoma cell loss

N2A cell loss was quantified using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay after overnight incubation with TBI autoantibodies as previously reported [11].

Serotonin 2A receptor peptide

An 18-meric, linear synthetic peptide (QDDSK...N) having an amino acid sequence identical to that of the second extracellular loop region of the human 5-HT2A receptor was synthesized at Lifetein, Inc., catalog number 701781, and had 96.65% purity.

5-HT2A Receptor Peptide enzyme linked immunosorbent assay ELISA

A 60 micrograms per milliliter concentration of the 18-meric synthetic peptide (QDDSL...N) was use as the solid-phase antigen in an enzyme-linked immune absorbent assay (ELISA) performed as previously reported [11].

Pheochromocytoma (PC12)-cell derived heparan sulfate proteoglycan ELISA

An ELISA using immobilized rat PC-12 cell derived, purified heparan sulfate proteoglycan (HSPG) as the solid-phase antigen was carried out as previously reported [6]. Strongly anionic PC-12 cell-derived HSPG was purified from conditioned medium using diethylaminoethyl (DEAE)-cellulose chromatography as previously reported [6].

Chemicals

Y27632, U73122, spiperone, M100907, ketanserin, bosentan, losartan, prazosin was obtained from Sigma, Inc., (St. Louis, MO). YM254890 was obtained from Focus Biomolecules, (Plymouth Meeting, PA). All other chemicals were research grade.

Protein concentration

Protein concentration was determined using a bicinchoninic assay kit (Thermo Fischer, Inc,) as previously reported [6].

Statistics

Data are mean ± SD (Table 1) or mean ± SEM (Figure 1). Comparisons are with Student's t-test with a significance level of P = 0.05 for continuous variables, Pearson's correlation coefficient; or with Fischer's exact test for dichotomous variables.

Table 1: Baseline clinical characteristics in adult TBI patients	
TBI (N=35)	
Risk factor	Mean (SD)
Age (years)	65.2 + 8.6
BMI (kg/m2)	31.1 + 6.1
HbA1c (%)	8.6 + 1.6
BMI-body mass index; Hba1c-glycosylated hemoglobin	
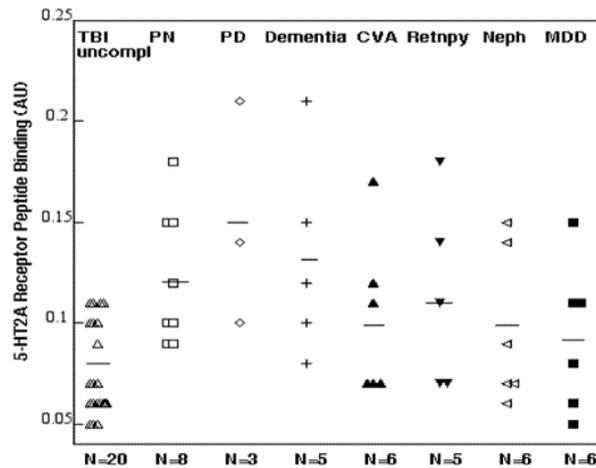
Figure 1: Enzyme-linked immunoassay using 5-HT2A receptor second extracellular loop region linear synthetic 18-mer; mean binding (indicated by horizontal lines) was significantly increased in autoantibodies (1/40th dilution) from TBI patients having co-morbid painful neuropathy (PN), Parkinson's disease (PD), dementia and diabetic retinopathy (Retnpy) compared to twenty TBi patients who lacked neurologic, diabetic microvascular complication (TBI uncompl). One non-diabetic TBI patient with highest binding had both Parkinson's disease and dementia. CVA-cerebrovascular accident, Retnpy-diabetic retinopathy, Neph(ropathy), MDD-major depressive disorder; Total N exceeds thirty-five because many of the same patients had more than one co-morbidity, e.g. dementia and retinopathy, and painful neuropathy and nephropathy.

Results

Baseline Clinical Characteristics in the Study Patients

The baseline clinical characteristics in the study patients is shown in Table 1. Twenty-four of thirty-five adult TBI patients

Table 2: Baseline prevalence of angiopathic and neurologic complications in TBI patients

| | TBI (n=35) | | | |
	Diabetes (N=24)		No Diabetes (N=11)	
Angiopathic complication	(N)	%	(N)	%
Diabetic Retinopathy	5	14	0	0
Diabetic Nephropathy	6	17	0	0
Painful neuropathy	7	20	1	9
Two or more angiopathies	7	20	0	0
Neurologic complication				
Parkinson's disease	2	6	1	9
Dementia	4	11	1	9
Cerebrovascular accident	5	14	1	0
Neuropsychiatric complication				
Major depressive disorder	5	14	1	9
No angiopathy	12	34	10	29
Uncomplicated TBI*	10	29	10	29

*excluding patients with co-morbid major depressive disorder

(69%) had co-morbid type 2 diabetes mellitus (Table 2). Mean autoantibody binding to the 5-HT2AR synthetic peptide was two-fold higher than background level in all 35 TBI patients tested (0.10 + 0.05). Mean 5-HT2AR peptide binding of the autoantibody among TBI patients with co-morbid type 2 diabetes mellitus was slightly higher (0.10 + 0.03 n=24 vs. 0.09 + 0.04; n=11; P =0.11) than in TBI without type 2 diabetes mellitus, but the difference was not statistically significant. An older man without type 2 diabetes mellitus who experienced TBI, and subsequently developed Parkinson's disease and dementia had autoantibody which displayed the highest level of 5-HT2AR peptide binding (0.21 AU, Fig 1). This finding is consistent with a prior report that neurodegenerative disease was associated with substantially increased 5-HT2A receptor peptide binding in the autoantibody from patients with or without co-morbid type 2 diabetes mellitus [11].

Association between TBI Autoantibody 5-HT2AR Peptide Binding and Baseline characteristics

Nearly two-thirds (22/35) of TBI patients demonstrated baseline presence of plasma 5-HT2A receptor autoantibodies (Fig 1), defined as receptor peptide binding 1.5-fold or greater, i.e. >/= 0.075 AU, than background (0.05 AU). Patient age, body mass index, or glycosylated hemoglobin was not significantly correlated with autoantibody 5-HT2AR peptide binding in all thirty-five TBI patients tested (data not shown) consistent with a prior report in 56 patients without TBI [11]. Significantly higher mean autoantibody binding (to the 5-HT2AR linear synthetic peptide) occurred in TBI subsets having painful neuropathy (mean 0.12;

P < 0.001; N=8), Parkinson's disease (mean 0.15; P< 0.001; N=3) dementia (mean 0.13; P =0.002;N=5) or diabetic retinopathy (mean =0.11; P =0.02; N=5) (Fig. 1) vs. TBI lacking neurologic or microvascular complications (mean = 0.0775; N=20). Mean autoantibody 5HT2AR peptide binding (1/40th dilution) was higher, but did not reach a statistically significant level of difference, in TBI subsets having stroke (mean =0.10; P=0.07; N=6), nephropathy (N=6; P =0.14), or major depressive disorder (MDD, mean 0.09; P=0.21; N=6) compared to TBI patients without neurovascular complications (Fig 1). Taken together, the data in the current and a prior study [11] indicate that autoantibody binding to a linear synthetic peptide corresponding to the second extracellular loop of the 5-HT2A receptor was significantly increased in 17/20 (85%) of patients having Parkinson's disease, i.e. suggestive of a possible disease specific association.

Association between 5-HT2AR peptide binding and systemic inflammation

We next tested for an association between 5-HT2AR autoantibody binding and white blood cell count (WBC), the latter is a marker of systemic peripheral inflammation which increases in a variety of chronic inflammatory conditions. There was a significant association (Pearson correlation coeff., R=0.52, N=35, P < 0.01) between WBC and autoantibody (1/40th dilution) to the 5-HT2AR synthetic peptide from all thirty-five adult TBI patients (Figure 2A). In an age-matched population of adult type 2 diabetes (n=47) without TBI previously described [11], there was a significant association (Pearson correlation coeff. R =0.49, P< 0.01) between WBC and autoantibody binding to the 5-HT2AR synthetic peptide (Fig 2B). Taken together, these data suggest systemic inflammation may contribute in part to the occurrence of 5-HT2AR autoantibodies in both TBI and non-TBI middle-aged and older adults, many of whom were affected by obese type 2 diabetes mellitus.
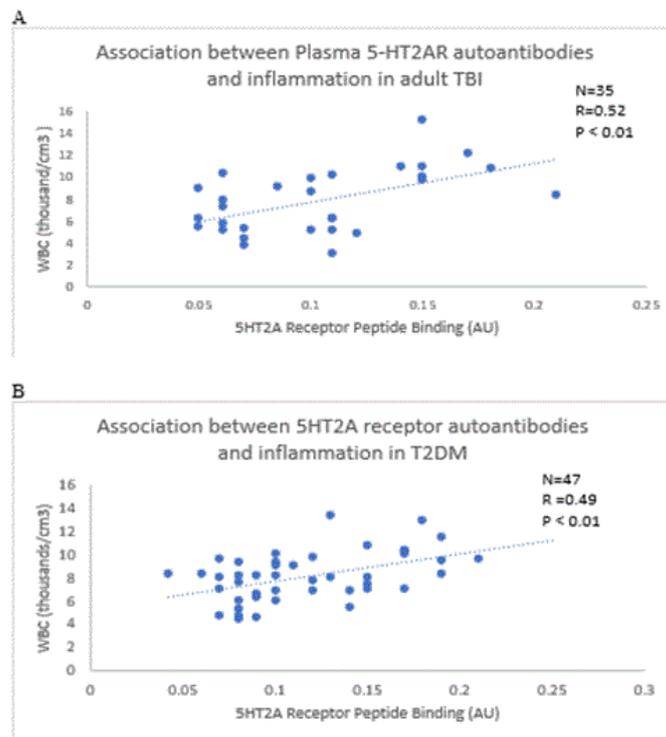
Figure 2: Correlation between white blood cell count and 5-HT2A receptor peptide binding in the protein A eluates from A) 35 patients with TBI or B) 47 patients with type 2 diabetes and no TBI

Pharmacologic specificity of 5-HT2AR autoantibodies neurotoxicity in N2A cells

Long-lasting agonist autoantibodies in subsets of obese type 2 diabetes having major depressive disorder, Parkinson's disease, or dementia were previously reported to cause acute N2A neuroblastoma cell neurite retraction and accelerated N2A cell loss by a mechanism involving activation of the 5-HT2A receptor which is positively coupled to Gq11/IP3R/Ca2+ signaling pathway [5,10]. Therefore, we next tested the pharmacologic specificity of TBI autoantibody-induced acute neurite retraction. The protein-A eluate fraction of adult TBI patient plasma caused dose-dependent acute neurite retraction in N2A cells. Acute neurite retraction was completely or nearly completed prevented

by co-incubation with a 250-500 nanomolar concentration of the highly selective 5-HT2AR antagonist M100907 (Table 3). It was also substantially blocked by co-incubation with a potent, less selective 5-HT2AR antagonist (spiperone) or a less potent, but specific 5-HT2AR antagonist, ketanserin (Table 3). Substantially higher (micromolar) concentrations of a 5-HT2BR antagonist SB204741 had no significant protective effect on autoantibody-induced neurite retraction (Table 3). Higher concentration of several different Gq11- subclass, GPCR receptor antagonists (losartan, bosentan, prazosin) selective for the (angiotensin II, type 1, endothelin 1, or alpha1 adrenergic) receptor, respectively had little or no significant protective effect on adult TBI autoantibody-induced neurite retraction (Table 3).

We next tested for involvement of Gq11- coupled, downstream signaling pathway mediators in the mechanism of TBI autoantibody induced acute N2A neurite retraction. In mouse neuroblastoma cells incubated with adult TBI autoantibodies (60 nanomolar concentration), the selective signaling pathway antagonists for Gq11 (YM254890), the inositol triphosphate receptor (2-APB) or phospholipase C (PLC) (U73122), afforded 89-100% protection against acute N2A neurite retraction (Table 4). Co-incubating N2A cells together with a 10 μM concentration of Y27632, a selective inhibitor of RhoA/Rho kinase (ROCK) signaling, provided near complete protection against TBI autoantibody-induced acute neurite retraction (Table 4). Taken together, these data suggest TBI autoantibody-induced neurite retraction activates RhoA/Rho kinase and Gq11/IP3R/Ca2+

Table 3: Results are expressed as % of TBI-induced neurite length-shortening….

Antagonist	Conc	GPCR	% of TBI-autoantibody Neurite Retraction
M100907	250 nM	5-HT2A	0%
Spiperone	400 nM	5-HT2A	20%
Ketanserin	1 μM	5-HT2A	26%
Ketanserin	2 μM	5-HT2A	18%
Prazosin	850 nM	A1-ADR	100%
SB204741	1 μM	5-HT2B	70%
Losartan	10 μM	Ang IIR	80%
Bosentan	10 μM	ET-1R	80%

A 60 nanomolar concentration of the IgG autoantibodies from a representative TBI patient was incubated with N2A cells in the presence or absence of the indicated concentration of the selective GPCR antagonist. Results are expressed as % inhibition of TBI-induced neurite length-shortening which varied by < 15%. Similar results were observed in the autoantibodies from four different TBI patients.

signaling downstream of 5-HT2A receptor binding consistent with prior reports in autoantibodies from patients with neurodegenerative disorders, but lacking TBI [5,10].

Adult TBI autoantibodies caused dose-dependent, accelerated N2A cell loss after 16-24 hours incubation (shown for a representative patient, Figure 3A). In eight of eight TBI patients tested, accelerated cell death was nearly completed prevented by co-incubation with a 250 nanomolar concentration of the highly selective 5-HT2AR antagonist, M100907 (Fig 3B). Co-incubation with a more than three-fold higher concentration (850 nM) of the selective alpha1 adrenergic receptor blocker prazosin had much less protective effect (n=5 patients tested, Fig 4B). M100907 (250 nM-500 nM) had no significant effect alone on N2A cell survival (not shown in Fig 3B).

Table 4: Results are expressed as % of TBI-induced neurite length-shortening….

Treatment	Conc	% of TBI Autoantibody Neurite Retraction
TBI Auto-AB	60 nM	100%
YM 254890	1 μM	11%
U73122	1 μM	10%
Y27632	10 μM	0%
2-APB	25 μM	0%

A 60 nanomolar concentration of TBI autoantibodies was incubated with N2A cells in the presence or absence of the indicated concentration of Gq11/PLC-gamma/IP3R antagonist or RhoA/Rho kinase pathway inhibitor. Results are expressed as % inhibition of TBI-induced neurite length-shortening which varied by < 15%. Similar results were observed in autoantibodies from two TBI patients.

Titer and potency of TBI autoantibodies

Titer and potency of plasma 5-HT2AR agonist autoantibodies were significantly increased in patients who had experienced repetitive athletic neurotrauma (n=2 patients) or two-three TBI episodes (n=2 patients) compared to a single uncomplicated TBI episode (n=4 patients) (Fig 4A). Although preliminary, these data suggest a possible dose-response relationship between the 5-HT2AR autoantibodies and higher number of neurotraumatic episodes. Among four patients who had experienced repetitive or two-three TBI exposures, one patient suffered with Parkinson's disease (n=1) and three had mild neurocognitive disorder (n=3) (according to criterion established in the St Louis University Mental Status test (SLUMS) [12]. All four patients with a single prior TBI (i.e. uncomplicated TBI; Figure 4A) were free of neurological sequelae or cognitive impairment.

Newly-recognized dementia (by SLUMS testing) occurred in a 70- year old man with long-standing type 2 diabetes mellitus complicated by nephropathy, painful neuropathy, and retinopathy. The patient's autoantibodies not only displayed high titer, potent binding to the 5-HT2A receptor linear synthetic peptide (Fig. 4B), but also similar (high-titer, high potency) binding to a
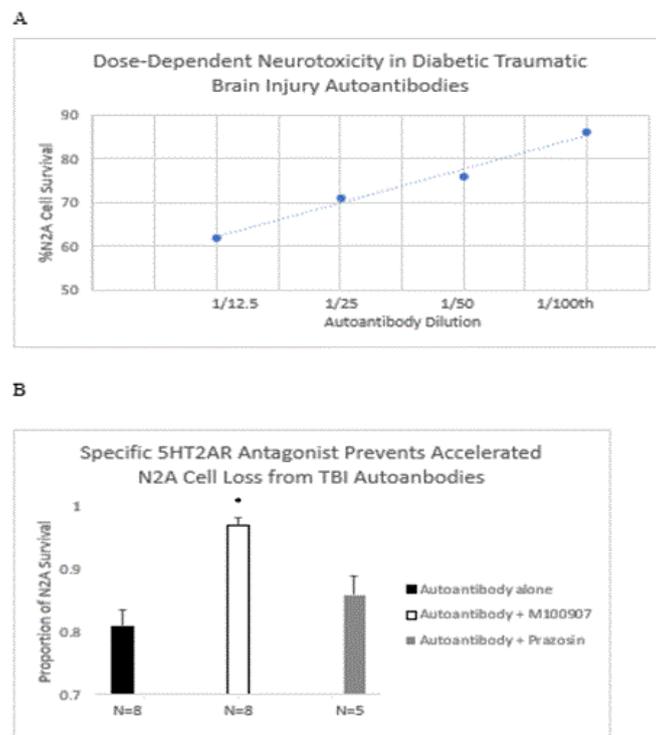
Figure 3: Protein-A eluate fraction in representative TBI patient was incubated with N2A cells for 24 hours at indicated dilutions. Cell survival was determined with MTT assay as described in Materials and Methods B)A one-fiftieth dilution of the protein-A eluate fraction from eight different TBI plasmas was incubated with N2a cells in the presence or absence of the highly selective 5-HT2AR receptor antagonist M100907 (250 nM) or the alpha-1 adrenergic receptor antagonist prazosin (850 nM)(n=5 patients). Cell survival (after 24 hours) was determined using MTT assay. * P< 0.01 compared to TBI autoantibodies alone.

neuronal-derived heparan sulfate proteoglycan purified from rat pheochromocytoma (PC12) cells (Fig.4B).

The 5-HT2A receptor and heparan sulfate proteoglycan are both concentrated on the cell surface of neurons and vascular cells; both receptors have anionic regions important for ligand binding. In thirty-one of thirty-five adult TBI patients tested, autoantibody (1/40th dilution) binding to the 5-HT2AR second extracellular loop region peptide was significantly correlated (P< 0.01; R =0.46) with binding to purified PC12-derived HSPG (Fig 5). These results suggest that TBI autoantibodies may target (in part) strongly anionic sites present on both the 5-HT2AR and neuronal HSPG.

Discussion

Late neurodegeneration increases substantially in middle-aged and older adults following TBI, however, the mechanism(s) are poorly understood and there is currently no biomarker to identify a high-risk subgroup [1]. The current data are the first to suggest that adult TBI is associated with a substantially increased prevalence of 5-HT2AR-targeting, agonist IgG autoantibodies.

Neurotoxicity mediated by the autoantibodies was significantly modulated by selective antagonists of the 5-HT2AR and by inhibitors of Gq11/phospholipase C/inositol triphosphate receptor/Ca2+ signaling consistent with prior reports in subsets of older obese type 2 diabetes mellitus having Parkinson's disease and/or dementia, but lacking TBI [5,10]. Systemic inflammation occurs in both obese type 2 diabetes and following traumatic brain injury. The present data suggest a possible link between systemic inflammation and the development of 5-HT2AR-targeting autoantibodies. In a rodent model of systemic inflammation induced by injection of lipopolysaccharide (LPS), 5-HT2AR mediated (in part) the effect of interleukin-1 on body temperature [13]. More study is needed to determine whether 5-HT2AR autoantibodies may have a role in mediating certain effects of inflammation on neurodegeneration.

Evidence that pools of TBI autoantibodies can target both 5-HT2AR and neuronal HSPG is of interest since HSPG abundant in basement membranes not only promotes cell binding, but contributes to blood: brain, blood: peripheral nerve, and blood: retinal barrier function. In prior reports in diabetic macular edema or painful neuropathy patients, autoantibodies which
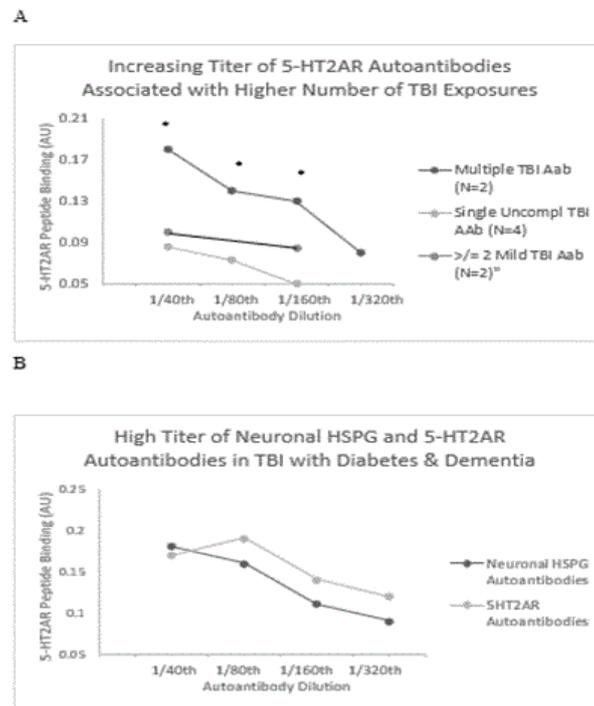
Figure 4: Multiple TBI exposure is associated with increased autoantibody titer and 5-HT2AR peptide binding potency compared to single uncomplicated TBI;* P< 0.01; Aab-autoantibody;   B) Protein-A eluate in type 2 diabetic dementia patient having multiple microvascular complications bind with high potency and titer to both 5-HT2AR peptide and to purified neuronal-cell derived heparan sulfate proteoglycan (HSPG).
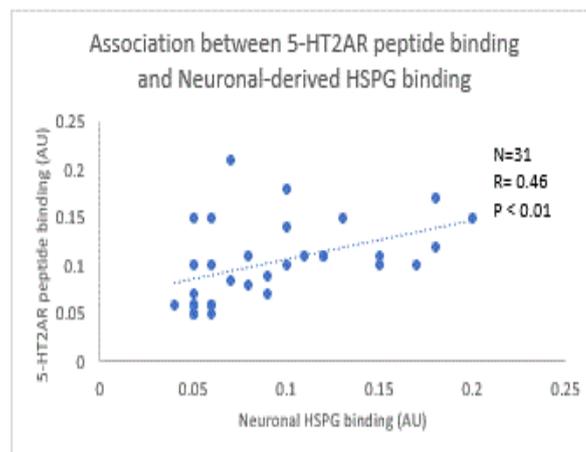
Figure 5: Correlation between 5-HT2AR peptide and neuronal HSPG binding in the protein-A eluates from thirty-one of thirty-five TBI patients.

displayed high affinity for heparin and purified neuronal-derived HSPG [7] caused contraction and apoptosis in endothelial cells via activation of RhoA/Rho kinase signaling [8]. Endothelial cell barrier disruption by HSPG-targeting autoantibodies could have a role in promoting increased access of 5-HT2AR-targeting autoantibodies to receptor binding sites in the central nervous system. Heparan sulfate proteoglycan is elaborated from basement membranes in poorly-controlled diabetes [14] via the action of heparanase, an enzyme which cleaves HSPG side chains. Endothelial cell heparanase expression increases under pro-inflammatory conditions [15] and HSPG is a known target of humoral immunity in subsets of systemic lupus erythematosus [16]. One possibility is that vascular remodeling following traumatic brain injury might cause targeting of neuronal HSPG (by the humoral immune system) especially in persons manifesting persistent inflammation and heightened adaptive immunity.

A limitation of the present study is that it is small and included only middle-aged and older men. Our use of a convenience sample of patients recruited from diabetes and endocrinology clinics might have led to an overestimation of the actual prevalence of 5-HT2AR-targeting autoantibodies since heightened inflammation which occurs in obese type 2 diabetes mellitus and thyroid autoimmunity are likely associated with diverse kinds of autoantibodies.

The 5-HT2AR is a highly druggable G-protein coupled receptor which is a known target for several FDA-approved medications useful in the treatment of diabetic painful neuropathy, major depression, schizophrenia, and Parkinson's disease-related hallucinations. More study is needed to determine whether agonist 5HT2A receptor autoantibodies which bind to the 5-HT2AR receptor might be a useful biomarker in identifying a subset of TBI at increased risk for late neurodegeneration.

Acknowledgements

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