Temporal lobe proteins implicated in synaptic failure exhibit differential expression and deamidation in vascular dementia

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Running Title: VaD synaptome reveals potential mediators of cognitive decline in dementia.

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ABSTRACT

Progressive synaptic failure precedes the loss of neurons and decline in cognitive function in neurodegenerative disorders, but the specific proteins and posttranslational modifications that promote synaptic failure in vascular dementia (VaD) remain largely unknown. We therefore used an isobaric tag for relative and absolute proteomic quantitation (iTRAQ) to profile the synapse-associated proteome of post-mortem human cortex from vascular dementia patients and age-matched controls. Brain tissue from VaD patients exhibited significant down-regulation of critical synaptic proteins including clathrin (0.29 ; $p<1.0\cdot10^{-3}$) and GDI1 (0.51 ; $p=3.0\cdot10^{-3}$), whereas SNAP25 (1.6 ; $p=5.5\cdot10^{-3}$), bassoon (1.4 ; $p=1.3\cdot10^{-3}$), excitatory aminoacid transporter 2 (2.6 ; $p=9.2\cdot10^{-3}$) and Ca2+/calmodulin dependent kinase II (1.6 ; $p=3.0\cdot10^{-2}$) were substantially up-regulated. Our analyses further revealed divergent patterns of protein modification in the dementia patient samples, including a specific deamidation of synapsin1 predicted to compromise protein structure. Our results reveal potential molecular targets for intervention in synaptic failure and prevention of cognitive decline in VaD.

Key words: vascular dementia, synaptic failure, clathrin, deamidation, synapsin1, α/β-tubulins.

List of Abbreviations: iTRAQ, isobaric tag for relative and absolute quantitation; VaD, vascular dementia; AD, Alzheimer's disease; BA21, Brodmann area 21; PTM, posttranslational modification; SV, synaptic vesicle; SNARE, soluble NSF attachment protein receptors family; RMSD, root mean square deviation; SEM, standard error of the mean; PDB, protein database; LTP, long-term potentiation; IsoAsp, Iso-aspartic acid; CBF, cerebral blood flow; CNS, central nervous system; PTM, protein posttranslational modification; IsoAsp, isoaspartic acid; Asp, aspartic acid; Asn, asparagine; GLN, glutamine; ERLIC, electrostatic repulsion-hydrophilic interaction chromatography; TEAB, triethylammonium bicarbonate; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromathography tandem-mass spectrometry; MDLC, multidimensional liquid chromatography; TM-score, template modeling score; FDR, false discovery rate; CME, clathrin-mediated endocytosis.

INTRODUCTION

Vascular dementia is a potentially fatal condition that occurs following interruptions in blood supply to specific areas of the brain following a stroke or microinfarcts (Shih et al., 2013). VaD is the second most common form of dementia after Alzheimer's disease (AD) (Kalaria et al., 2008;Gorelick et al., 2011), and exhibits a pathology that is at least partially reversible (McVeigh and Passmore, 2006), indicating potential for new drug treatments if the underlying molecular mechanisms can be defined and suitable targets identified. At the cellular level, dementia syndromes are characterized by progressive synaptic failure amongst cortical neurons, which contributes to their common clinical manifestations; disorientation, defects in long-term memory, mood disorders and executive dysfunction (Herbert et al., 2014;Wesnes and Edgar, 2014). Synaptic degeneration begins with disrupted vesicular trafficking in presynaptic boutons, and progresses towards structural malformations in the postsynaptic dendrite arborization, preceding eventual loss of neurons in the affected cortical areas (Bolay et al., 2002;Mufson et al., 2012;Chang et al., 2013). While this process is in itself well documented, the molecular mediators of these events have yet to be identified (Yao, 2004;Muresanu et al., 2014). Synaptic failure strongly correlates with the cognitive impairments manifest in VaD and AD, even when the presence of characteristic senile plaques cannot be established (DeKosky and Scheff, 1990;Terry et al., 1991;Overk and Masliah, 2014). Moreover, synaptic failure is the pathological feature most commonly shared by VaD and AD patients (Kalaria, 2000;Kalaria, 2002;Iadecola, 2013), consistent with the concept that loss of synapse function is a key event in the pathogenesis of dementia syndromes.

Synaptic failure is promoted by a combination of oxidative stress, mitochondrial wreckage, neuroinflammation and excessive accumulation of misfolded proteins (Overk and Masliah, 2014). Recently, the role of the intracellular microenvironments and pH in particular has

received much attention as a potential contributor to the pathology of synaptic failure. Indeed, just small changes in pH can have profound consequences for the normal function of enzymes that mediate protein posttranslational modifications (PTMs) (Chaumeil et al., 2012). Abnormal patterns of PTMs can significantly alter protein structure and function, and accumulation of isoaspartic acid (IsoAsp) residues due to dysregulated deamidation has already been implicated in the pathogenesis of neurodegenerative diseases and dementia (Gaza-Bulseco et al., 2008;Desrosiers and Fanélus, 2011;Dunkelberger et al., 2012).

Numerous critical synaptic and neuronal structural proteins including synapsins, dynamins and α/β-tubulins, are highly susceptible to aspartic acid (Asp) isomerization and asparagine (Asn) deamidation events, while the Asn deamidation repair enzyme protein L-isoaspartyl methyltransferase (PIMT) is silenced in brain tissue (Qin et al., 2013). The clinical significance of these processes is illustrated by the prominent dementia-like symptoms seen in PIMT knock-out mice (Zhu et al., 2006;Yang and Zubarev, 2010). Synapsins play a critical role in neurotransmitter release via the unfastening of synaptic vesicles (SV) from the actin cytoskeleton, thus enabling their incorporation into reserve pools in close proximity to the active zone of axon terminals (Hilfiker et al., 1999;Bolay et al., 2002;Ferreira and Rapoport, 2002;Gitler et al., 2004a). In addition, synapsins are thought to be important regulators of SV fusion with presynaptic plasma membranes (Hilfiker et al., 1999;Humeau et al., 2001), hence the normal functioning of synapsin proteins is likely to be crucial for effective synaptic transmission. Modifications to other synaptic structural proteins have also been implicated in neuron dysfunction, including the isomerization of α/β-tubulins in human temporal lobe epilepsy and in mouse models of neurodegenerative disease (Lanthier et al., 2002;Zhu et al., 2006). The excess accumulation of IsoAsp residues in α/β-tubulins has also been linked with marked alterations in the structure of human cortical neurons (Lanthier et al., 2002), and may

contribute to the aberrant cerebral elongations apparent in PIMT null mice (Shimizu et al., 2002).

In the present study, we used an isobaric tag for relative and absolute quantification (iTRAQ) to profile the synaptic proteome in the temporal cortex of VaD patients. Our initial investigations revealed that the expression of PIMT enzyme is significantly dysregulated in VaD patients, hence we also performed an in-depth analysis of the deamidation profiles of synapsin1 and tubulin proteins in these samples. Using this approach, we identified numerous synaptic proteins that were differentially expressed and/or modified in VaD brain tissue compared with samples from age-matched control patients. These data shed new light on the molecular basis of VaD and may lead to the development of novel interventions that preserve synaptic function and prevent cognitive decline in human dementia patients.

MATERIALS AND METHODS

Subjects

Frozen post-mortem samples of temporal cortex (Brodmann area 21, BA21) were obtained from VaD subjects and aged-matched controls via the Newcastle Brain Tissue Resource (NBTR) Institute for Ageing and Health (demographic details in Supplementary Table 1). Informed consent was obtained from all study participants prior to donation of brain tissues after death. The average post-mortem delay was 24.5 and 36.5 hours for control and VaD subjects respectively.

VaD was diagnosed pathologically by the presence of features including lacunar infarcts, cortical infarcts, border-zone infarcts, micro-infarcts and small vessel disease in sub-cortical regions (Kalaria et al., 2004). None of the VaD samples displayed sufficient neuritic plaque or neurofibrillary pathology to meet the diagnostic criteria for AD. Age-matched controls had no evidence of neurological or psychiatric disease at the time of death, and samples showed either no or limited age-related Alzheimer's-type pathology. Neither VaD nor age-matched samples exhibited visible infarcts or any other lesions. Further details of the samples can also be found in (Datta et al., 2014b).

Tissue Preparation

All procedures were approved and performed in accordance with the ethical guidelines of the Nanyang Technological University ethics board. Ten VaD patients (mean age 84.0±8.5 years) and ten age-matched controls (mean 80.3±8.9 years) were used for the analyses, with all experiments performed in triplicate. In each group approx. 10 mg of brain tissue from each subject were pooled and homogenized before the proteins were purified by acetone precipitation: this is a common approach in quantitative proteomics due to the expense of

iTRAQ reagents, the limited amount of sample available, and the excessive use of instrument time (Gan et al., 2007;Bostanci et al., 2013). The sample pooling strategy also reduces the impact of biological variation by reporting the averaged quantity of proteins associated with disease-specific changes (Weinkauf et al., 2006;Datta et al., 2014a).

Two hundred micrograms of protein from each replicate from each experimental group were resolved by SDS-PAGE and visualized by Coomassie Brilliant Blue staining. Total protein was excised from the gel, cut into $1mm^2$ pieces and washed with 75% acetonitrile containing 25mM triethylammonium bicarbonate (TEAB) . Following de-staining, the gel pieces were reduced with Tris 2-carboxyethyl phosphine hydrochloride (5mM) and then alkylated with methyl methanethiosulfonate (10mM). Gel pieces were dehydrated using acetonitrile and subjected to protein digestion at 37°C in sequencing-grade modified trypsin (Promega, Madison, WI, USA). The peptides in gel were extracted using 50% acetonitrile, 5% acetic acid for three times under ultrasound sonication. The extracted peptides were concentrated using vacuum concentrators (Eppendorf AG, Hamburg, Germany) before labeling with iTRAQ reagents.

iTRAQ Labeling and LC-MS/MS Analysis

The iTRAQ labeling of dried peptides from the control and disease groups were performed using 4-plex iTRAQ reagent Multiplex kits (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. The control and VaD samples in each triplicate were labeled with 114 and 115 isobaric tags, respectively. Three independent iTRAQ replicates were performed to generate three sets of samples for each of the VaD and control groups. The labeled samples in each replicate were pooled together and concentrated using a vacuum centrifuge for subsequent electrostatic repulsion-hydrophilic interaction chromatography (ERLIC)

fractionation and LC-MS/MS analysis. The concentrated iTRAQ-labeled peptides from the three sets were then desalted separately using Sep-Pak C18 cartridges and then ERLIC fractionated by high-performance liquid chromatography (HPLC). The iTRAQ-labeled peptides were reconstituted in buffer A (10mM ammonium acetate, 85% acetonitrile, 0.1% formic acid) and fractionated on a PolyWAX LP column (4.6 × 200mm, 5μm, 300Å) (PolyLC, Columbia, MD, USA) adopting the HPLC system (Shimadzu, Kyoto, Japan) at a flow rate of 1ml/min (Hao et al., 2010). The 60min gradient HPLC mobile phases consisted of buffer A (10mM ammonium acetate, 85% acetonitrile, 0.1% acetic acid) and buffer B (30% acetonitrile, 0.1% formic acid) as follows: 100% buffer A for 5min, 0-36% buffer B for 25min, 36-100% buffer B for 20min, and 100% buffer B for 10min at 1ml/min flow rate. The ultra-violet (UV) absorption chromatograms were recorded at 280nm while 60 fractions were collected using an automated fraction collector. The collected fractions were then pooled into 26 fractions according to their peak intensities, concentrated using vacuum centrifuge, and reconstituted in 0.1% formic acid for liquid chromathography coupled with tandem mass spectrometry (LC-MS/MS) analysis.

The LC-MS/MS analysis of ERLIC-fractionated labeled samples was performed using a QSTAR Elite mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA) coupled with online nanoflow multidimensional liquid chromatography (MDLC) system. The iTRAQ-labeled peptides were separated on a custom-made nanobore C18 column with a picofrit nanospray tip (75μm ID × 15cm, 5μm particles). Each iTRAQ-labeled peptide fraction was sequentially injected in triplicate or more (three injections per fraction) and then analyzed by LC-MS/MS using a 90min gradient, thus allowing three technical replicates to be acquired for each iTRAQ experimental replicate. The QSTAR Elite was set to positive ion mode using Analyst QS 2.0 software for data acquisition (Applied Biosystems, Foster City,

CA, USA). The precursors with a mass range of 300-1600m/z and calculated charge from +2 to +5 were selected for fragmentation. Peptides above a 5-count threshold were selected for MS/MS and each selected target ion was dynamically excluded for 20s with a mass tolerance of 0.1Da. Smart information-dependent acquisition was activated with automatic collision energy and automatic MS/MS accumulation. The fragment intensity multiplier was set to 20, and the maximum accumulation time was 2s.

Mass Spectrometric Data Search and Data Analysis

MS/MS data analysis, including peak list generation, peptide and protein identifications and their respective quantifications, were performed using ProteinPilot software 3.0 (revision number 114732; Applied Biosystems, Foster City, CA, USA). The concatenated target-decoy Uniprot human database was used for the MS/MS data search. Peptide identification was performed with the Paragon algorithm (3.0.0.0, 113442) in ProteinPilot software, and further processed using the Pro Group algorithm, where isoform-specific quantification was implemented to trace the differences between expression levels of various isoforms. User-defined parameters in ProteinPilot software were: (i) sample type: iTRAQ 4-plex (peptide-labeled); (ii) cysteine alkylation: methyl methanethiosulfonate; (iii) digestion: trypsin; (iv) instrument: QSTAR Elite ESI; (v) special factors: urea denaturation; (vi) species: none; (vii) specify processing: quantitate, bias and background correction; (viii) identification focus: biological modifications and amino acid substitutions; and (x) search effort: thorough. The default precursor and MS/MS tolerance for the QSTAR ESI-MS instrument were adopted automatically by the software. The false discovery rates (FDR) of both peptide and protein identification were set at <1% (FDR = 2.0*(decoy hits/total hits)*100%) and an unused Protein Score value ≥2 was used as the qualification criteria (corresponding to a confidence limit of 99%).

The peptide for iTRAQ quantification was automatically selected by Pro Group algorithm in ProteinPilot according to the following criteria; i) the peptide was usable for quantitation (i.e. the iTRAQ reporter area was not zero), ii) the peptide was identified with good confidence, iii) the peptide was not shared by another protein identified with higher confidence. These criteria were used in the calculation of reporter peak area, iTRAQ reporter ratio, error factor (EF) and p-value. The Protein Pilot criteria for calculation of p-values are based on degrees of freedom for the average ratio and *t* value [*t* = (Weighted Average of Log Ratios – Log Bias) ÷ Standard Error of the Weighted Average of Log Ratios]. . Therefore the p-value evaluates the result based on the certainty of the identified ratio of being different from 1:1 between VaD and Controls.

All the above mentioned statistical criteria for analysis of iTRAQ data are described in the ProteinPilot v3 manual and were commonly used in reporting iTRAQ results (Dutta et al., 2014; Marneros, 2013; Ruppen et al., 2010; Unwin et al., 2005).

Furthermore, we have also calculated the % coefficient of variation (% CV) of the 1631 synaptic proteins evaluated in this study. The % CV average shown by the data was 14.1 and the 95 % of the proteins show a ratio % CV lower than 40% (Supplementary Figure 1), accordingly, we have set the regulation threshold of the proteins showing significant p-values at ≥ 1.4 and ≤ 0.6 for the determination of up-regulation and down-regulation changes respectively.

For further statistical performed analysis and plots (as the dependent t-test between same peptides from each experimental group for pathway associated proteins) the GraphPad Prism software version 5.00 for Windows (GrahPad Software, La Jolla. CA, USA) was used.

Identification of Synapse Proteome and Integrative Pathway Analysis

The proteins identified in the proteomic experiment (2551) were compared against those present in SynaptomeDB, a specialized database containing records of all known synaptic and related proteins (Pirooznia et al., 2012). The search criteria included presynaptic active zone, vesicles and postsynaptic categories, since our intent was to quantify all proteins involved in the normal functioning of presynaptic boutons and postsynaptic spines. A total of 1631 synaptic proteins were matched to those in the specialized database after searching. Only the significantly up-regulated and down-regulated proteins were shortlisted for further analysis (shown in Supplementary Tables 2 and 3). The synaptic proteins identified as significantly modulated in the VaD subjects were further studied using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system (Mi and Thomas, 2009; Mi et al., 2013) for identification of dysregulated molecular pathways.

All the synaptic proteins found in SynaptomeDB were then subjected to further bioinformatic analysis and screened for the presence of deamidation in any of their constituent residues. The extent of peptide deamidation between VaD and age-matched controls was determined by the iTRAQ reporter ratio.

Molecular Modeling

Synapsin1 and Tubulin β2A are known substrates of the PIMT enzyme in brain tissue (Qin et al., 2013), and these proteins displayed significantly up-regulated deamidation of N and Q residues in VaD compared with age-matched controls (fold-change >1.25 after exclusion of artifacts). We therefore conducted *ab initio* modeling using the I-TASSER server (Zhang, 2008;Roy et al., 2010;Yang et al., 2013) to simulate normal structure (WT) for comparison with the deamidated structure (Deam.) of each protein. The predicted structures of synapsin1

and tubulin β2A were aligned to related Protein Data Base (PDB) templates using TM-align (Zhang and Skolnick, 2005). TM-score and root mean square deviation (RMSD) parameters were used to analyze the similarity between protein structures and the distance between the backbones of the superimposed proteins, respectively (Jimenez-Lopez et al., 2010;de Carvalho and De Mesquita, 2013). A degree of mismatch in RMSD values between the predicted structures of >20 % was considered likely to adversely affect protein structure and function (Mistri et al., 2012). All protein structural images reported in this study were rendered in Pymol software (Bramucci et al., 2012).

RESULTS

Synapse-Associated Proteins are Up-Regulated in Human VaD Brain Tissue

A total of 52 synaptic proteins were found to be up-regulated in VaD brain tissue compared with samples from age-matched controls (Supplementary Table 2). Of these proteins, 35% were associated with presynaptic boutons, while the remaining 65% were involved in postsynaptic functions. Of the presynaptic proteins, 11% were related to vesicular trafficking and the remaining 24% were related to the terminal active zone (Figure 1 A).

The synaptic immunoglobulin-2 gamma subunits (IGHG2, 4.0; $1.4 \cdot 10^{-2}$) and lambda subunits (IGLC2, 3.8; $3.4 \cdot 10^{-2}$) were found under highly up-regulation in VaD post-mortem brain tissues. Next, the proteins identified as being modulated in VaD subjects were subjected to pathway analysis which indicated a role for synaptosomal-associated protein 25 (SNAP25) in almost 40% of the total integrated pathways identified (Figure 2). SNAP25 is a core protein in the stimulus-driven neurotransmitter release complex known as SNARE (soluble NSF attachment proteins receptor), and is universally present in both excitatory and inhibitory synapses (Tafoya et al., 2006). In our study, SNAP25 expression was found to be increased in VaD subjects relative to controls (1.6 ; $p=5.5 \cdot 10^{-3}$, Figure 1 C).

Another vesicle-related protein known as bassoon (BSN), was detected with increased abundance in VaD brain tissues compared with controls (1.4 ; $p=1.3 \cdot 10^{-3}$, Figure 1 C). We also observed VaD-associated modulation of several presynaptic proteins that were detected at increased levels, including the excitatory amino acid transporter 2 (EEAT2, 2.6 ; $p=9.2 \cdot 10^{-3}$), the Ca2+/calmodulin dependent kinase II (CAMK2A, 1.6 ; $p=3.0 \cdot 10^{-2}$) and dynein-1 (DYNC1H1, 3.2 ; $p=3.9 \cdot 10^{-5}$) (Figure 1 C).

The tubulin polymerization protein promoter 3 (TPPP3) (1.8 ; $p=4.1\cdot10^{-2}$) and the tubulin specific-chaperone A (TBCA) (1.6 ; $p=2.4\cdot10^{-2}$) were a further two postsynaptic proteins found to exhibit increased expression in VaD patients.

Finally, the enzyme PIMT was found to be up-regulated in VaD subjects compared with age-matched controls (2.0 ; $p= 1.7\cdot10^{-2}$). The PIMT has been proposed as a repair enzyme that protects against the accumulation of IsoAsp residues in proteins that undergo deamidation (Yamamoto et al., 1998).

Select Synapse-Associated Proteins are Down-Regulated in Human VaD Brain Tissues

We detected a total of 51 synapse-related proteins that displayed reduced abundance in VaD subjects compared with age-matched controls (Supplementary Table 3). Of these proteins, 27% were associated with pre-synaptic events, and the remaining 73% were associated with post-synaptic functions. Of the presynaptic proteins, 7% were involved in SV trafficking and storage, while the remaining 20% mediated disparate functions in terminal active zones (Figure 1 B).

Two essential synaptic proteins of mention were significantly down-regulated in VaD subjects; the clathrin heavy chain 1 (CLTC, 0.29, $p<1.0\cdot10^{-3}$) and the rab GDP dissociation inhibitor (GDI1, 0.51 ; $p= 3.0\cdot10^{-3}$) (Figure 1 C). The CLTC protein is responsible for SV recycling via the well-established 'clathrin-mediated endocytosis' pathway (CME). (Slepnev and De Camilli, 2000), in contrast, the GDI1 protein is known to be a direct initiator of SV exocytosis (Sanderson et al., 2008).

The integrative pathway representation of the VaD-associated proteins included several critical pathways with significantly down-regulated members, including the *ubiquitin proteasome pathway* and two *G-protein signaling pathways* (Figure 2).

Proteins Associated to the CME Pathway Show Expression Differences in Human VaD

To explore broadly the VaD proteome differences and add weight to the previous described findings, we have further analyzed the changes in expression of other proteins associated with the clathrin-mediated endocytosis pathway (CME). Initially some of these pathway-associated proteins have shown consistent changes according to their iTRAQ ratios between VaD and controls, however, none of them reached level of significance under the established criteria in Protein Pilot.

Therefore we performed a Student's dependent t-test analysis between the peak areas from the same peptides of the tags 114 and 115 respectively. This analysis revealed changes in the expression of several CME pathway constituents (Supplementary Table 3). Since the two main hub molecules of the CME pathway are the CLTC and the AP2 (Schmid and McMahon, 2007). The AP2-β subunit was revealed as decreased in VaD (AP2B1, 0.59; $p<1.0\cdot10^{-3}$) while the AP2-α subunit did not show any difference in expression. Furthermore, two more molecules associated to the CME pathway reached significance as down-regulated under the described analysis (β-arrestin-1, 0.54; $p=1.5\cdot10^{-2}$) and (auxilin, 0.69; $p=9.7\cdot10^{-3}$).

Accumulation of Deamidated Residues in Human VaD Synapse-Associated Proteins

1. Deamidation of Synapsin1

Total synapsin1 expression was not significantly altered in VaD, but when we analyzed the composition of the protein, 7 of the 489 detected synapsin1 tryptic peptides in the VaD group displayed up-regulation of deamidation at Asn (N) or Gln (Q) residues (Figure 3 and Table 1). The presence of abnormally deamidated residues may indicate an aberrant structural and

functional conformation of the parent protein in VaD temporal cortex synapses (Supplementary Figures 2 and 3).

In order to probe possible functional effects of the deamidated residues in synapsin1, we next used the improved I-TASSER prediction method to model how these modifications might alter protein structure. The Pymol-rendered model of wild-type synapsin1 (-2.63 C-score, 0.41±0.14 TM-Score, 14.7±3.6Å RMSD), did not exhibit any gross structural differences from that predicted for deamidated synapsin1 (-2.53 C-score, 0.42±0.14 TM-Score, 14.4±3.7Å RMSD) (Figure 4 A). However, when the two models were homologue-aligned to template structures in the protein database (PDB), the TM alignment scores indicated that the wild-type protein exhibited greater homology with the C-domain of rat synapsin1 (1pk8A; image not shown), while the deamidated protein displayed greater homology with the C-domain of rat synapsin2 (1I7LA) (Figure 5). The degree of RMSD mismatch between the deamidated and wild-type aligned structures exceeded the threshold set for the structural change to be considered pathological (>20% increase in RMSD index) and is indicated in the deamidated model of synapsin1 (Table 2).

Furthermore, 4 of the 5 domains comprising synapsin1 exhibited extensive peptide deamidation in VaD subjects when compared with age-matched controls (Figure 4 B). Strikingly, we also identified a Gln deamidation site (Q 564) adjacent to serine 566 (see amplification panel in Figure 4 A), that was in consensus with CAMK2A phosphorylation site 2 in the D domain of synapsin1 (Jovanovic et al., 2001;Gitler et al., 2004b) and suggests that deamidation may influence the phosphorylation profile of the protein.

2. Deamidation of α/β-Tubulins

Total expression levels of α-tubulin 1B (TUBA1B) and β-tubulin 2A (TUBB2A) proteins did not differ significantly between VaD subjects and controls, but 4 of the constituent peptides were highly modulated in VaD and exhibited multiple deamidations compared to controls (shown in Figure 6 and Table 3), precursor ion spectrums are shown in Supplementary Figures 4 and 5. The wild-type and deamidated structures of TUBB2A were subjected to I-TASSER modeling and PDB template alignment in TM-Align as previously described for synapsin1. The improved models generated for wild-type TUBB2A (1.80 C-score, 0.97±0.05 TM-Score, 2.3±1.8Å RMSD) and deamidated TUBB2A (1.82 C-score, 0.97±0.05 TM-Score, 2.3±1.8Å RMSD) did not exhibit any significant structural differences (<5% increase in RMSD index), hence no clear effect of this modification on protein function could be established.

3. Deamidation in Other Synapse-Associated Proteins

Differential regulated deamidation sites in the constituent peptides of diverse synapse-associated proteins have been detected for Asn (N#) and Gln (Q#) residues in VaD (Supplementary Table 4). The proteins superoxide dismutase 1 (SOD1) and the VAMP2 were those with highly up-regulated deamidation sites between VaD and controls for the Asn residues. On the contrary, the chaperone heat shock 70 (HSPA1A), the GDI1 and the calpain 1 (CAPNS1) showed lower ratios of deamidation in their Asn residue from a detected peptide in VaD compared to controls.

Regarding deamidation in Gln residues the proteins SNAP25 and Park7/DJ-1 a protein associated with Parkinson's disease have shown increased deamidation in their constituent peptides compared to controls. The protein GDI1 consistent with the Asn deamidated residues also showed decreased expression of Gln deamidation in a detected peptide in VaD compared to controls, other proteins such as the microtubule-associated protein TAU (MAPT) and the

neurosecretory protein VGF were also detected with a down-regulation of peptides containing deamidated Gln residues in VaD.

DISCUSSION

Impaired cerebrovascular blood flow (CBF) can disrupt the metabolism of brain neurons and is a normal feature of natural aging, but this process can also be exacerbated by vascular pathology in the CNS. Disruption of CBF in the temporoparietal cortex has previously been linked with synaptic decay and cognitive impairment in human patients affected by brain vascular pathology (Popa-Wagner et al., 2013). Although the biological consequences of brain vascular pathology are now well established, the protein mediators of vascular dementia (VaD) pathophysiology still remain largely unknown.

In the current study, we observed that 3 widely-recognized synapse-associated proteins were highly up-regulated in VaD temporal cortices compared with controls, namely SNAP25, BSN and EEAT2. It has previously been proposed that synaptic loss can induce compensatory mechanisms in the surviving synapses that may help to slow down cognitive decline (DeKosky and Scheff, 1990;Scheff, 2001;Sisková et al., 2010) and according to that hypothesis it would be expected that our findings regarding the mentioned up-regulated proteins could reinforce the existence of these mechanisms and would suggest that they are operational even at advanced stages of disease. Indeed, consistent with the data presented in the current report, a previous study of EEAT2 levels in temporal cortex tissue identified increased protein expression in VaD subjects (Kirvell et al., 2010). The up-regulation of the rodent brain homologs for human SNAP-25 and EEAT2 proteins has been recently described in a murine model of AD, thereby the increment in both proteins was linked to a compensatory response after the establishment of elevated extracellular glutamate levels aimed to reduce the manifested excitotoxicity and neuroinflammation in brain temporal regions (Brothers et al., 2013). The results from a recent study based on microRNAs quantitation in a mouse model of AD are also consistent with our findings in VaD (Barak et

al., 2013). These authors report the miRNAs up-regulation for the proteins mentioned in our study including the EEAT2, SNAP-25 and BSN in the hippocampus of AD mice. Finally, we propose that the observed up-regulation of the protein BSN could be part of these mentioned compensatory measures despite it is not directly linked to the excitotoxicity response hence BSN is mainly involved in the generation of new SV release sites in presynaptic terminals (Mendoza Schulz et al., 2014). It has previously been demonstrated that terminals lacking BSN are unable to sustain SV exocytosis (Frank et al., 2010), therefore, we would hypothesize according to our results that the prevailing synapses may increase the levels of this protein in VaD to compensate for the loss of functional synapses. Nevertheless, further studies need to be done to clarify whether the up-regulation of these proteins acts as compensatory mechanism in VaD or just as another pathological component of the disease.

In parallel, we detected that 2 other key synaptic proteins, CLTC and GDI1, were significantly less abundant in the temporal cortex of VaD subjects compared with age matched-controls. CLTC is a principal component of the pre-synaptic endocytotic and neurotransmitter vesicle-coated release machinery (Rizzoli, 2014). The extended clathrin pathway has been proposed as a significant contributor to synaptic decay in dementia and AD (Moreno et al., 2009). It has previously been shown that some of the CLTC pathway interactions are progressively altered during the progression of AD (Sze et al., 2000), with recent evidence confirming aberrant autoinhibition of CLTC function in that disease (Musunuri et al., 2014). Our results in VaD are consistent with previous findings reported in analyses of AD brain tissue which detected a -0.77-fold down-regulation of this protein in disease. The implication of CLTC modulation in synaptic failure is also supported by evidence from mice that lack this protein, since the animals exhibit a dramatic decrease in the amounts of SV quanta in the cortical presynaptic terminals (Reist et al., 1998). Imbalances in

CLTC level have also been linked with the enlarged SV shape evident in autopsied cortices from human AD subjects, since this protein may be singularly responsible for the restoration of aberrations in SV membranes after endocytosis (Cambon et al., 2000;Scheff et al., 2007). Similarly, a putative role for GDI1 in the pathology of dementia was reported in a previous study of knock-out mice that exhibited hippocampus-dependent memory deficits together with approximately a 50% of decrease in SV number at the presynaptic pools (D'Adamo et al., 2014).

Our study further reveals an excessive accumulation of deamidation-associated IsoAsp residues in the synapsin1 and tubulin proteins (TUBA1B/TUBB2A). Deamidation of Asn residues occurs up to 100 times faster than does the deamidation of Gln residues *in vitro* (Robinson and Robinson, 2001). Due to the rapid kinetics of Asn deamidation, this process is considered to be biologically important and is thought to have deleterious consequences for the affected proteins that may promote the development of degenerative diseases (Lanthier et al., 2002;Dunkelberger et al., 2012). In contrast, the spontaneous deamidation of Gln residues is a comparatively slow process *in vitro* (Wright, 1991), and seems to be mediated by glutaminase enzymes and perhaps also spontaneous chemical reactions *in vivo*. Excessive deamidation of synaptic proteins may suffice to induce functional impairment and promote synaptic failure and eventual dementia. Indeed, other investigators have reported that in the absence of altered protein expression, increased deamidation of certain residues can inhibit protein function and contribute to synaptic impairment in PIMT knock-out mice (Qin et al., 2013). Critical synaptic proteins including dynamin 1 and 2, synapsin1 and tubulins, have been identified as substrates of PIMT in mammalian brain tissue (Lanthier et al., 2002;Qin et al., 2013), and in our study we detected up-regulation of PIMT expression in VaD compared with age-matched controls. We therefore attempted to probe the potential effects of deamidation on some of the above mentioned PIMT substrates in brain tissue.

Since it is not possible to study the biological functions of IsoAsp-containing proteins using conventional molecular and cellular biology methods, we used structural modeling and alignment experiments to predict changes in protein conformation and function that might arise from the deamidations identified in our study. These data suggested that synapsin1 structure acquires pathological characteristics in VaD following the accumulation of deamidated sites, and that this accumulation likely affects protein function. Our alignment experiment was limited by the fact that only one domain could be aligned, since homologous templates for the other synapsin1 domains are not currently available. Accordingly, only the potential effect of the Gln deamidated site in the C domain of this protein could be examined here. It has previously been shown that the function of synapsin1 can be compromised by disruption of its phosphorylation profile (Jovanovic et al., 2001;Bolay et al., 2002;Gitler et al., 2004b), and interestingly, there are data to suggest a link between protein deamidation and altered patterns of synapsin1 phosphorylation in brain tissue from PIMT knock-out mice (Qin and Aswad, 2013). Our data are therefore consistent with the concept that the Gln deamidation site adjacent to serine 566 in the synapsin1 protein (phosporylation site 2) may alter S566 phosphorylation and promote increased activation/abundance of CAMK2A (Hilfiker et al., 1998).

VaD-associated deamidation sites were also observed in some of the tubulin residues in our study. We detected an increase in the presence of the tubulin polymerization promoter TPPP3 and tubulin-specific chaperone TBCA in VaD subjects. Increased expression of TPPP family proteins in the brain may induce aberrant conformations of neural tubulins (Ovádi and Orosz, 2009). Although the role of TPPP3 in the adult brain is currently unknown, TBCA has been shown to act as a molecular chaperone in neurons and can interfere with TUBB2A structure. In addition, the interaction between TBCA and tubulin subunits other than TUBB2A has not

yet been demonstrated (Nolasco et al., 2005;Tian and Cowan, 2013). However, it is tempting to speculate that the increased TBCA expression level identified in brain tissue from VaD subjects may promote abnormal conformations of TUBB2A, although definitive demonstration that the deamidated sites impact on protein function is currently lacking.

Conclusions

In the present study we identified multiple candidate proteins and deamidations of key synaptic mediators in brain tissue that may contribute to synaptic failure in human VaD. Altered abundance of synaptic proteins including SNAP25, BSN and EEAT2 could indicate that compensatory mechanisms are activated during progressive synaptic decay in dementia. Our observations also suggest that synaptic changes in VaD can occur in the absence of gross neurofibrillary pathology, consistent with a vascular basis for the cognitive defects that manifest in dementia syndromes and neurodegenerative diseases.

Author Contributions

Conceived and designed the experiments: SKS, XGP and CPC. Performed the experiments: JQ and XGP. Analyzed the data: XGP and AS. Contributed reagents/materials/analysis tools: SKS and CPC. Wrote the paper: XGP and SKS. Clinical Samples and analysis: RNK and CPC.

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Conflicts of Interest

None declared. The authors have no disclosures with regard to this report.

The study was not industry-sponsored.

Figure Legends

Figure 1. SynaptomeDB functional categorization of synapse-associated proteins that were differentially expressed in VaD temporal cortices compared with age-matched controls. **A.** Synapse-associated proteins with significantly increased abundance in VaD samples segregated according to their association with: presynaptic bouton active zone (light grey), synaptic vesicle release/endocytosis (black), or postsynaptic dendrites (medium grey). **B.** Synapse-associated proteins with significantly decreased abundance in VaD samples segregated according to their association with: presynaptic bouton active zone (light grey), synaptic vesicle release/endocytosis (black), or postsynaptic dendrites (medium grey). **C.** Percent increase/decrease in abundance of selected synaptic proteins in VaD relative to controls (mean percentage is expressed with SEM).

Figure 2. Integrative pathway representation including the proteins differentially regulated in VaD. The number of genes regulated (blue: up-regulated; red: down-regulated) in each pathway can be identified in the central graph, the proteins differentially regulated in each pathway are named together with the corresponding blue or red colors depending on their respective level of modulation.

Figure 3. A. 1. MS/MS spectrum of synapsin1 peptide including the asparagine (N#) deamidation site displaying differential expression in VaD and control subjects. The spectrum shows the peptide fragmentation pattern and is labeled with both *b* and *y* ions. **2.** Values in the table indicate the calculated theoretical fragments and the highlighted values refer to identified fragments. The row in the table corresponding to the identified deamidated fragment is framed in red. **3.** iTRAQ quantitative spectrum showing the label intensities for the selected peptide in control (114) and VaD (115) subjects. **B. 1.** MS/MS spectrum of

synapsin1 peptide, including the glutamine (Q#) deamidation site displaying differential expression in VaD and control subjects. The spectrum shows the peptide fragmentation pattern and is labeled with both *b* and *y* ions. **2.** Values in the table indicate the calculated theoretical fragments and the highlighted values refer to identified fragments. The row in the table corresponding to the identified deamidated fragment is framed in red. **3.** iTRAQ quantitative spectrum showing the label intensities for the selected peptide in control (114) and VaD (115) subjects.

Figure 4. Synapsin1 cartoon (horizontal view) showing the wild-type I-Tasser improved model (-2.63 C-score). The image includes the distribution of domains indicated by colors (blue: A domain; red: B domain; green: C domain; beige: D domain, and yellow: E domain); no apparent differences were detected between the wild-type synapsnin 1 structure **A** and the model including all VaD LC-MS/MS detected deamidation sites (rendering not shown). The position of Serine 566 is highlighted in red (CAMK2A phosphorylation site 2), the Threonine residue 565 in beige, and the deamidated Glutamine residue 564 in a combination of red, grey and blue are shown in ribbon. Detail in zoom of the mentioned residues is shown in the adjacent panel. Phosphorylation of phosphoserine 566 by CAMK2A is required for translocation of synapsin1 to the cytosol and subsequent liberation of SVs (Jovanovic et al., 2001). **B.** Illustration of synapsin1 domains. The numbers accompanying the N and the Q labels refer to the number of identified deamidated residues in each domain of the protein in VaD subjects.

Figure 5. Three dimensional alignment of the modelled D domain of human synapsin 1 to its homolog PDB template. The domain D of WT Synapsin1 is shown in green and its aligned homolog is shown in red. TM-score were used to assess the similarity between PDB

structures and the D domain of synapsin1, the PDB template with higher similarity with our target domain was used for the alignment and exhibited 0.437 of TM-score. The wild-type (WT) and deamidated [Deam.] modelled structures of the synapsin1 D domain were aligned to the same template structure (only the alignment of WT synapsin1 is shown in the figure as apparent visual differences between both performed alignment experiments cannot be found). The RMSD of the alignment product from WT and [Deam.] was assessed to reveal any structural difference between the WT and [Deam.] structures of Synapsin1.

Figure 6. A. 1. MS/MS spectrum of β-tubulin 2A peptide including the asparagine (N#) deamidation site displaying differential expression in VaD and control subjects. The spectrum shows the peptide fragmentation pattern and is labeled with both *b* and *y* ions. **2.** Values in the table indicate the calculated theoretical fragments and the highlighted values refer to identified fragments. The row in the table corresponding to the identified deamidated fragment is framed in red. **3.** iTRAQ quantitative spectrum showing the label intensities for the selected peptide in control (114) and VaD (115) subjects. **B. 1.** MS/MS spectrum of β-tubulin 2A peptide, including the glutamine (Q#) deamidation site displaying differential expression between VaD and control subjects. The spectrum shows the peptide fragmentation pattern and is labeled with both *b* and *y* ions. **2.** Values in the table indicate the calculated theoretical fragments and the highlighted values refer to identified fragments. The row in the table corresponding to the identified deamidated fragment is framed in red. **3.** iTRAQ quantitative spectrum showing the label intensities for the selected peptide in control (114) and VaD (115) subjects.

Supplementary Figure 1. Cumulative percent graph of the synaptic proteins (total 1631) plotted in the vertical axis against different %CV plotted in the horizontal axis. More than

ninety-five percent of the proteins showed less than 40% of %CV. Accordingly, the threshold of the ratio (115/114) was set at >1.4 and <0.6 to determine the difference in expression of VaD synaptic proteins.

Supplementary Figure 2. Precursor MS spectra of the synapsin1 peptides identified as being differentially expressed in VaD subjects, inclusive of any Asparagine (N#) or Glutamine (Q#) deamidated residues.

Supplementary Figure 3. Precursor MS Spectra corresponding to the non-deamidated synapsin1 peptides.

Supplementary Figure 4. Precursor MS spectra of the β-tubulin 2A peptides identified as being differentially regulated in VaD subjects, inclusive of any Asparagine (N#) or Glutamine (Q#) deamidated residues.

Supplementary Figure 5. Precursor MS Spectra corresponding to the non-deamidated β-tubulin 2A peptides.

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