Title: Decreased levels of VAMP2 and monomeric alpha-synuclein correlate with duration of dementia.

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Keywords: Dementia with Lewy bodies
Parkinson’s disease dementia
Alzheimer’s disease
Synaptic dysfunction
SNARE process
Alpha-synuclein
VAMP2
Munc18

Abstract: Alpha-synuclein (α-syn) aggregations are the key pathological hallmark of dementia with Lewy bodies (DLB) and Parkinson’s disease dementia (PDD), but are also frequently present in Alzheimer’s disease (AD). Yet much remains unknown about the role of α-syn in the synapse and the wider role of synaptic dysfunction in these dementias. Changes in concentrations of key ‘SNAP (Soluble N-ethylmaleimide Sensitive Factor Attachment Protein) Receptor’ (SNARE) proteins as a consequence of alterations in the aggregation state of α-syn may contribute to synaptic dysfunction in patients with DLB, PDD and AD and result in impaired cognition. We have studied a large cohort (n=130) of autopsy confirmed DLB, PDD, AD and control brains. Using semi-quantitative western blotting we have demonstrated significant changes across the diagnostic groups of DLB, PDD and AD in the SNARE and vesicle proteins syntaxin, Munc18, VAMP2 and monomeric α-syn in the prefrontal cortex, with a significant reduction of Munc18 in AD patients (p<0.001). This correlated to the final MMSE score before death (p=0.016). We also identified a significant negative correlation between the duration of dementia and the levels of the binding partners VAMP2 (p=0.0004) and monomeric α-syn (p=0.0002).

It is of particular note that this association was identified in people with AD. Our findings may indicate that an upregulation of SNARE complex related proteins occurs in the early stages of disease as an attempt at compensating for failing synapses, prior to widespread deposition of pathological α-syn.

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11/02/15

Dear Sir/Madame,

We would be grateful if the enclosed research article, ‘Decreased levels of VAMP2 and monomeric alpha-synuclein correlate with duration of dementia’, could be considered for publication by Neurobiology of Disease.

The Lewy body dementias are the second most common form of dementia in the elderly yet are chronically understudied, furthermore little is known about molecular mechanisms behind the development of dementia. Our study is founded upon one of the largest post-mortem cohorts of Lewy body dementia tissue assembled. We present evidence that reduced VAMP2 (a key SNARE protein) and monomeric alpha-synuclein relate to the duration of dementia and may represent an important early step in the aetiology of Lewy body dementia.

This manuscript is not under review by another journal, nor has any part of it been published before. The use of human tissue was approved by the National Research Ethics Service and consent was given by all tissue donors.

I take full responsibility for the data, the analyses and interpretation, and the conduct of the research; I have full access to all of the data; and the right to publish any and all data separate and apart from any sponsor.

Yours faithfully,

Dr David Whitfield
Decreased levels of VAMP2 and monomeric alpha-synuclein correlate with duration of dementia.

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Abstract

Alpha-synuclein (α-syn) aggregations are the key pathological hallmark of dementia with Lewy bodies (DLB) and Parkinson’s disease dementia (PDD), but are also frequently present in Alzheimer’s disease (AD). Yet much remains unknown about the role of α-syn in the synapse and the wider role of synaptic dysfunction in these dementias. Changes in concentrations of key ‘SNAP (Soluble N-ethylmaleimide Sensitive Factor Attachment Protein) Receptor’ (SNARE) proteins as a consequence of alterations in the aggregation state of α-syn may contribute to synaptic dysfunction in patients with DLB, PDD and AD and result in impaired cognition. We have studied a large cohort (n=130) of autopsy confirmed DLB, PDD, AD and control brains. Using semi-quantitative western blotting we have demonstrated significant changes across the diagnostic groups of DLB, PDD and AD in the SNARE and vesicle proteins syntaxin, Munc18, VAMP2 and monomeric α-syn in the prefrontal cortex, with a significant reduction of Munc18 in AD patients (p<0.001). This correlated to the final MMSE score before death (p=0.016). We also identified a significant negative correlation between the duration of dementia and the levels of the binding partners VAMP2 (p=0.0004) and monomeric α-syn (p=0.0002). It is of particular note that this association was identified in people with AD. Our findings may indicate that an upregulation of SNARE complex related proteins occurs in the early stages of disease as an attempt at compensating for failing synapses, prior to widespread deposition of pathological α-syn.

Keywords

Dementia with Lewy bodies
Parkinson’s disease dementia
Alzheimer’s disease
Synaptic dysfunction
SNARE process
Alpha-synuclein
VAMP2
Munc18

Abbreviations

Introduction

Dementia is defined as progressive cognitive decline of sufficient magnitude to interfere with normal social or occupational function. Alzheimer’s disease (AD) and the Lewy body dementias (LBD), incorporating dementia with Lewy bodies (DLB) and Parkinson’s disease dementia (PDD), collectively comprise over 70% of all dementia diagnoses (www.alzheimers.org.uk). LBD is pathologically characterized by α-synuclein (α-syn), with varying amounts of amyloid-β protein (Aβ) and hyperphosphorylated microtubule associated protein tau (tau) aggregates in addition to synaptic loss (Dickson, 2002). Clinical hallmarks include fluctuating and deteriorating cognition, hallucinations, and parkinsonism (McKeith et al., 2005; Aarsland et al., 2008). AD is characterised by Aβ and tau aggregates and gradual worsening of cognition – in particular episodic memory and executive function (Ballard et al., 2011; McKhann et al., 2011).

α-Syn, first identified as the main component of Lewy bodies (LBs) (Spillantini et al., 1997) – and thus the key pathological protein of DLB and PDD - is, under physiological conditions, located in the presynaptic terminal. Kramer and Schulz-Schaeffer (Kramer and Schulz-Schaeffer, 2007) reported accumulation of small aggregates of α-syn at the synapses of DLB post mortem brains. These aggregates were proposed to interfere with synaptic function. The authors also suggested that the formation of small aggregates of α-syn was a direct precursor to the development of LBs, and possibly represented a final cytoprotective attempt before cell death. Thus, focus on the events occurring at the synapse in advance of LB formation could be of greater potential therapeutic benefit. This is of relevance not just to the established synucleinopathies such as DLB and PDD but also AD. It is now recognised that a proportion of sporadic AD cases present with Lewy bodies; this has been reported to be as high as 51% (Jellinger, 2003), and is particularly observed within the amygdala where
Lewy bodies are often co-localised with neurofibrillary tangles (Schmidt et al., 1996; Uchikado et al., 2006; Fujishiro et al., 2008).

Some of the mutations in the SNCA gene known to give rise to α-syn disorders exert their pathogenic effect through promotion of aggregation or oligomerisation of α-syn (Brown, 2010). In addition to involvement in pathogenic mechanisms there has been increasing focus on the physiological role of α-syn. Current understanding indicates that α-syn plays a role in neurotransmitter release, synaptic plasticity and pre-synaptic vesicle pool size (Abeliovich et al., 2000; Murphy et al., 2000; Lee et al., 2014). More particularly it is thought that monomeric α-syn drives formation of the SNARE complex through a chaperone-like activity involving binding to phospholipids and VAMP2 (vesicle-associated membrane protein 2 – or synaptobrevin-2) (Burré et al., 2010). SNARE is an acronym for ‘SNAP (Soluble N-ethylmaleimide Sensitive Factor Attachment Protein) Receptor’. SNARE proteins play a key role in neurotransmitter-containing vesicle fusion to the presynaptic membrane thereby modulating neurotransmitter release (Jahn and Fasshauer, 2012). Interestingly, it was recently reported that large α-syn oligomers bind preferentially to VAMP2 (vesicle-associated membrane protein 2), resulting in inhibition of docking between donor and acceptor vesicles (Choi et al., 2013). Thus it is plausible that VAMP2 could represent a key component for mediating the impact of α-syn.

VAMP2 and syntaxin 1, both members of the SNARE protein family, are respectively localised to synaptic vesicles and the presynaptic membrane where they form part of the SNARE complex required for vesicle release (Jahn and Fasshauer, 2012). Munc18 binds to syntaxin 1, and is thought to assist in the first steps of SNARE assembly, as well as playing a role in later stages of the exocytosis (Jacobs et al., 2006; Han et al., 2010; Jorgacevski et al.,
Reductions in SNARE proteins have been previously reported in LBD and AD (Mukaetova-Ladinska et al., 2009; Mukaetova-Ladinska et al., 2013). This study aims to aid our understanding of the synaptic dysfunctions that may underlie the disease process observed throughout the development of LBD and AD. Our hypothesis was that changes in concentrations of key SNARE proteins as a consequence of alterations in the aggregation state of α-syn may contribute to synaptic dysfunction in people with LBD and AD and result in impaired cognition. We therefore determined the expression of the presynaptic proteins α-syn, VAMP2, syntaxin1A, Munc18, and the general synaptic marker synaptophysin (SPP) and investigated the potential correlations between these proteins and clinical data.
Materials and Methods

Participants, diagnosis and assessment

Table 1 shows the demographic details of the patients and controls. Post-mortem brain tissue was kindly supplied by the following Brains for Dementia Research Network brain banks: the MRC London Neurodegenerative Diseases Brain Bank, the Thomas Willis Oxford Brain Collection and the Newcastle Brain Tissue Resource, and from the University Hospital Stavanger (Norway). Informed consent was obtained for all tissue to be used in research and the study had ethics approval (08/H1010/4). Prefrontal cortex (Brodmann area, BA9) was used for all biochemical and histopathological analysis. BA9 was selected due to its proposed role in executive function and cognition (Fuster, 2001), decline of which is a cardinal symptom of DLB and PDD.

Neuropathological assessment was performed according to standardised neuropathological scoring/grading systems, including Braak staging, Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) scores, Newcastle/ McKeith Criteria for Lewy body disease, National Institute on Aging - Alzheimer’s Association (NIA-AA) guidelines and phases of amyloid-β (Aβ) deposition (Aβ-phases) (Mirra et al., 1991; Thal et al., 2002; McKeith et al., 2005; Braak et al., 2006; Montine et al., 2012). Controls were neurologically normal, with only mild age associated neuropathological changes (e.g., neurofibrillary tangle Braak stage ≤II) and no history of neurological or psychiatric disease.

Cognitive impairment data consisted of the last Mini-Mental State Examination (MMSE) scores a maximum of two years prior to death (Folstein et al., 1975). Patients and controls were categorised as previously described (Whitfield et al., 2014b). Final diagnoses for
patients are clinico-pathological consensus diagnoses incorporating the one-year rule to differentiate DLB and PDD (McKeith et al., 2005).

Immunohistochemistry

Semi-quantitative assessments of Aβ, tau and α-syn pathology were conducted as in our previous study (Howlett et al., 2014) blind to clinical diagnosis, by neuropathologists, using a scale of 0 (none), 1 (sparse), 2 (moderate) and 3 (severe/frequent) to score sections from BA9, BA24 and BA40. For detection of senile Aβ plaques sections were stained with an anti-Aβ 1E8 or 4G8 antibody at 1:1000. Tau immunohistochemistry (AT8 antibody (Innogenetics) at 1:200) and silver impregnation (Gallyas or modified Bielschowsky) were used to detect neurofibrillary tangles, neuritic plaques, dystrophic neurites and neuropil threads. α-syn pathology was detected using NCL-SYN antibody (Novocastra Laboratories) at 1:200.

Preparation of tissue samples for western blotting

Preparation of tissue for western blotting was as previously described (Kirvell et al., 2006). Briefly, grey matter was isolated from the tissue and homogenised in ice cold buffer containing 50mM tris-HCL, 5mM EGTA, 10mM EDTA, ‘complete protease inhibitor cocktail tablets’ (Roche, 1 tablet per 50ml of buffer), and 2μg/ml pepstatin A dissolved in ethanol:DMSO 2:1 (Sigma). Buffer was used at a ratio of 2ml to every 100mg of tissue and homogenisation performed using an IKA Ultra-Turrax mechanical probe (KIA Werke, Germany) until the liquid appeared homogenous.
Protein concentration was established using the Coomassie (Bradford) Protein Assay Kit (Thermo Scientific); 10μl of crude homogenate was diluted 1:50 and read in triplicate at 595nm using a FlexStation 3 (Molecular Devices). A BSA standard curve run at the same time as the samples was used to calculate the concentration.

**Western Blotting**

Crude brain homogenate was diluted 4:5 with 5x sample buffer (Genscript MB01015), boiled for 5 minutes then stored at -20°C. Samples were loaded at 20μg/ml total protein on 10% SDS-polyacrylamide gel for protein separation, transferred to nitrocellulose membrane (Hydrobond-C, Amersham) and probed with either anti-Syntaxin1 (abcam ab24731, 1:20000), anti-Munc18 (abcam ab75042, 1:2000), anti-VAMP2 (abcam ab70222, 1:10000), anti-alpha-synuclein monomeric (BD Transduction Laboratories 610787, 1:20000) or anti-synaptophysin (abcam ab8049, 1:10000) and the relevant secondary antibody (IRDye from LI-COR). Bands were detected using an Odyssey infrared fluorescent scanner, the integral of intensity quantified using Odyssey infrared imaging systems application software version 3.0.16 and expressed as ratios to rat cortex in arbitrary units.

**Statistical Analysis**

Statistical analysis of the biochemical data was undertaken as described previously (Whitfield et al., 2014a; Whitfield et al., 2014b); briefly synaptophysin values were significantly predicted by the years in storage and so a residual variable was created for this protein to statistically remove this effect. This variable was then normalised using a log10 transformation. Munc18 and VAMP2 ratio values were normalised using a square root
transformation. Syntaxin1 and monomeric α-syn ratio values were normalised using a LogE transformation. VAMP2 and monomeric α-syn ratio values were significantly predicted by age at death and so residual variables were created to compensate for this effect. VAMP2 ratio values were then normalised again using LogE.
**Results**

Synaptophysin (SPP) levels were significantly reduced in PDD cases compared to all other diagnostic groups but there was no significant difference between the levels of synaptophysin in control, AD or DLB cases (supplementary figure 1). As Munc18, syntaxin1, VAMP2 and monomeric α-syn are all synaptic proteins and we observed the aforementioned decrease in synapses, it was decided to express these proteins as ratios to the synaptophysin value, case by case. This established approach allows the actual change in these proteins to be removed from any general effect of a change in synapses (Weiler et al., 1990; Vawter et al., 1999).

In the Munc18:synaptophysin ratio, there was a reduction in AD cases of 33% compared to DLB cases, 26% compared to PDD and 25% compared to control cases (Fig. 1A). The significant changes in the syntaxin1:synaptophysin ratio were an increase of 28% in AD cases compared to PDD cases and 34% compared to control cases (Fig. 1B). The ratio of VAMP2 to synaptophysin was significantly higher in PDD cases compared to controls by 16%, to DLB cases by 23%, and to AD cases by 65%. Furthermore, the ratio of VAMP2 to synaptophysin was significantly lower in AD cases compared to DLB cases by 34% and compared to controls, by 42% (Fig. 1C). The ratio of monomeric α-syn to synaptophysin was significantly higher in PDD cases compared to controls, by 25% and to AD cases by 53%. Furthermore it was significantly lower in AD cases compared to DLB cases by 38% and compared to controls, by 22% (Fig. 1D).

Linear regression analysis showed the years of dementia to have a highly significant negative correlation with the ratios of VAMP2 to synaptophysin (figure 2A) and monomeric alpha-synuclein to synaptophysin (figure 3A) in the prefrontal cortex. Likewise, a significant
positive correlation was found between years of parkinsonism and both the ratio of VAMP2 to synaptophysin (figure 2B) and the ratio of monomeric α-syn to synaptophysin (figure 3B) in the prefrontal cortex. Both of these analyses included all individuals with AD, DLB and PDD, for whom data on the duration of dementia was available, but not controls. The n values for years of dementia for both VAMP2:SPP and α-syn:SPP were; PDD n=18, DLB=23 and AD=15. For years of parkinsonism they were; PDD n=18, DLB n=20 and AD n=15.

The ratio of Munc18 to synaptophysin correlated with the last MMSE score prior to death (Pearson’s r=0.282, p=0.016, n=73); the distribution and other details of the MMSE scores for this cohort have been reported previously (Whitfield et al., 2014b). There was no correlation between MMSE scores (the last score prior to death or the decline per year) and any of the other protein ratios (p>0.05). There was also no correlation between any of the protein ratios and the semi-quantitative pathology scores for Aβ, tau or α-syn (p>0.05).
Discussion

We report that a longer duration of dementia was strongly association with a decrease in
levels of both VAMP2 and monomeric α-syn. Furthermore VAMP2 and monomeric α-syn
appeared to be upregulated in PDD cases; cases that in general had low levels of α-syn
pathology compared to the DLB cases (see figure 4). This may indicate that an upregulation
of SNARE process related proteins occurs in the early stages of dementia as an attempt to
compensate for failing synapses, prior to widespread deposition of pathological α-syn.

α-syn aggregation is the pathological hallmark of DLB and PDD, appearing as Lewy bodies
and neurites throughout the cortex, but is also present in a significant proportion of people
with AD. Whilst evidence is emerging of the physiological roles of monomeric α-syn in
SNARE complex processes, less is known about the intermediate steps that lie between this
physiological role and the final pathological deposition seen in the end-stages of disease.

We saw a strikingly similar pattern of change between VAMP2 and monomeric alpha-
synuclein, which is not surprising given that the two proteins are binding partners (Burré et
al., 2010).

A benefit of expressing the biochemical data as a ratio to synaptophysin is that this provides
an index to the number of synaptic terminals, and importantly the results were not
substantially different when this ratio was not used (data not shown).

The accumulation of α-syn and SNARE proteins at synaptic nerve terminals in transgenic
mice and in Parkinson’s disease, in conjunction with impaired dopamine release in both
transgenic mouse brain and transfected PC12 cells, is suggested by García-Reitbock and
colleagues to indicate a gain of toxic function of α-syn at the synapse (Garcia-Reitbock et al.,
2010). This is consistent with previous findings in transfected cells, which showed that
overexpression of α-syn inhibits evoked neurotransmitter release by acting at a step between vesicle docking and fusion (Larsen et al., 2006). It could be that a balance between monomeric α-syn and SNARE proteins is necessary for proper SNARE assembly and function; alternatively, it could be that an increase in α-syn causes the formation of toxic oligomers that affect SNARE distribution and function. This causes a problem as an oligomer of α-syn can bind to many VAMP2 proteins on several different vesicles causing vesicle clustering (Choi et al., 2013). By clustering around oligomeric α-syn, vesicles cannot dock at the presynaptic terminal and therefore exocytosis of neurotransmitter cannot occur.

Here we observed a combination of increases in both monomeric α-syn and VAMP2 in PDD cases. A recent study reported increased expression of VAMP2 protein accompanied increased α-syn expression in the striatum of Snap25S187A/S187A mice (Nakata et al., 2012). These mice displayed a significant age-dependent change in the distribution of α-syn and its Ser129-phosphorylated form in hypertrophied glutamatergic nerve terminals in the striatum. Knowing that the binding of the C terminus of α-syn to the N terminus of VAMP2 primes the subsequent SNARE complex assembly (Burgoyne and Morgan, 2011), the increase in VAMP2 level might also reflect a compensatory response to the impaired synaptic vesicle release by enhancing SNARE complex formation in concert with the increased α-syn (Nakata et al., 2012). This increase in VAMP2 and monomeric α-syn could also relate to the inherent differences between PDD and the other dementias, such as the typically long period of parkinsonism prior to development of dementia, or to the relative lack of AD related pathology.

The positive correlation between MMSE prior to death and the ratio of Munc18 to synaptophysin underlines the importance of intact synaptic machinery to cognition. That we
did not see correlations with the other proteins is probably due to the prefrontal cortex not being a key region for memory – having a stronger role in executive function, a domain which the MMSE is poor at detecting (McKeith et al., 2005). We have previously shown that α-syn pathology in BA21 is associated with cognitive decline as assessed by the MMSE (Howlett et al., 2014), and so it would be interesting to examine these SNARE proteins in this region to determine if monomeric α-syn shows a similar relationship to pathological α-syn.

In the present study, we found that a concomitant deregulation of SNARE proteins and monomeric α-syn was strongly associated with the duration of dementia. It has been suggested that this process could represent an initial pathological event in DLB, eventually leading to the death and degeneration of neuronal cells (Orimo et al., 2008). Alterations in syntaxin and other SNARE proteins have been previously reported in AD and DLB (Minger et al., 2001; Jacobs et al., 2006; Mukaetova-Ladinska et al., 2009; Mukaetova-Ladinska et al., 2013); Minger et al only found a decrease in these proteins in the oldest dementia cases and the Mukaetova-Ladinska studies did not separate PDD and DLB. The toxicity of α-syn, in sporadic and familial disease, has been proposed to arise in several ways; through inhibition of histone acetylation, perforation of membranes and the consequent disruption of ionic balance and finally neuronal death via the inhibition of a neuronal survival factor MEF2D (Beyer et al., 2009). Beyer et al suggest that in fact there maybe multiple pathways involving α-syn and other proteins linked to LBs that culminate in the same end-stage pathology of Lewy bodies and neurites.

Targeting the toxicity of α-syn at the synapse, and its connection with SNARE proteins, could be an effective way to tackle the pathology and the progression of the disease at an early
stage. Further functional studies are warranted to provide insight into the chronology of the relationship between VAMP2 and monomeric α-syn: are changes in VAMP2 driving a loss of monomeric α-syn to other forms of α-syn? Or is the loss of monomeric α-syn impacting VAMP2, SNARE machinery and ultimately synaptic function? Finally, development of imaging ligands or an assay for CSF detection of VAMP2 or monomeric α-syn would be of great interest given the predictive association we show between duration of dementia and parkinsonism, and these proteins.

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References


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Table 1: Summary of subjects demographics

Values represent means ± SEM. There were no significant differences (according to one-way ANOVA) between diagnostic groups for any of these variables. It has been demonstrated that most human brain proteins are quite stable with respect to post-mortem factors and detailed analysis including large numbers of proteins covering the major cellular functions have not identified our proteins of interest as ‘at risk’ molecules being highly susceptible to post-mortem changes (Lewis, 2002; Hynd et al., 2003; Crecelius et al., 2008; Ferrer et al., 2008).
Figure 1: Synaptic proteins of interest expressed as a ratio to synaptophysin (SPP) and grouped according to clinical diagnosis.

One-way ANOVA and Bonferroni post-hoc tests were used to determine the differences in the protein ratios between diagnostic groups.

Graph A: Munc18; n=23,30,37,16. F=(3,102)11.995, p=0.000001. Bonferroni post-hoc tests showed AD cases to be significantly higher than; control p=0.001, PDD p=0.000273 and DLB p<0.000001.

Graph B: Syntaxin1; n=23,31,37,16. F(3,103)5.128, p=0.002. Bonferroni post-hoc tests showed AD cases to be significantly lower than control p=0.007 and PDD p=0.017.

Graph C: VAMP2; n=19,24,29,15. F=(3,83)25.814, p<0.000001. Bonferroni post-hoc tests showed; PDD cases to be significantly higher than; control p=0.01, DLB p=0.000019 and AD cases p<0.000001. Furthermore AD cases were significantly lower than control p=0.000004 and DLB cases p=0.000073.
Graph D: Mon α-syn; n=19,24,28,15. F(3,82)16.621, p<0.000001. Bonferroni PDD to control p=0.000463. AD to control p=0.049, PDD p<0.000001, DLB p=0.000027. The vertical bars represent means with SEM.
Figure 2: Associations between the monomeric α-synuclein:synaptophysin (α-syn/SPP) ratio and the duration of dementia and parkinsonism.

Linear Regression analysis showed the years of dementia and of parkinsonism to be predictors of the ratio of monomeric α-Syn to SPP in BA9. \( R^2=0.399 \). \( SE=0.371664 \). Years of dementia; \( B=-0.06 \), SE=0.015, \( t=-4.034 \), \( p=0.000188 \). Years of PD; \( B=0.21 \), SE=0.008, \( t=2.801 \), \( p=0.007 \). ANOVA; \( F=(2,50)16.599 \), \( p=0.000003 \).
Figure 3: Associations between the VAMP2:synaptophysin ratio and the duration of dementia and parkinsonism.

Linear Regression analysis showed the years of dementia and of parkinsonism to be predictors of the ratio of VAMP2 to SPP. $R^2=0.463$. SE = 0.17939. Years of dementia; $B=-0.028$, SE=0.007, $t=-3.833$, $p=0.000355$. Years of PD; $B=0.15$, SE=0.004, $t=4.032$, $p=0.000189$. ANOVA; $F=(2,50)21.595$, $p<0.000001$. 
Figure 4: α-synuclein pathology grouped by clinical diagnosis.

The percentage of cases in each of the categories of α-synuclein pathology score (0 = absent, 1 = sparse, 2 = moderate, 3 = severe/frequent) according to clinical diagnosis.