Analysis of Primary Visual Cortex in Dementia with Lewy Bodies Indicates GABAergic Involvement Associated With Recurrent Complex Visual Hallucinations.

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ABSTRACT

A major clinical finding in dementia with Lewy bodies (DLB) is the presence of well-formed visual hallucinations and an associated finding is the presence of glucose hypometabolism and reduced perfusion on imaging of the primary visual cortex. We therefore sought to determine the underlying basis for these changes in order to aid understanding the involvement of the visual system in the generation of recurrent complex visual hallucinations (RCVH) which are a characteristic feature of DLB. Pathological investigation showed an absence of alpha-synuclein pathology or neurofibrillary tangles in primary visual cortex but the presence of mild alpha-synuclein pathology and tangles in the adjacent secondary visual cortex. This contrasted with Alzheimer's disease (AD) where both primary and secondary visual cortex showed significant neurofibrillary tangle burden. Stereological analysis of neurones in primary visual cortex showed no change in density or volume in DLB, but a reduction in neuronal volumes without density change in AD indicating atrophy. GAD65/67 immunohistochemistry showed no reduction in interneurone number in DLB suggesting that GABAergic neurones were essentially intact. However microarray analysis of primary visual cortex in DLB showed an altered neuropeptide profile and changes in GABAergic markers suggesting altered GABAergic neuronal function.. In addition loss of post synaptic GABA markers such as Gephyrin, GABARAP, and Kif5A were also present along with decreased synaptophysin suggesting altered synaptic activity within the primary visual cortex. Post synaptic glutamatergic neuronal signalling was also affected given a reduction in vesicular glutamate transporter protein expression and PSD-95 expression. The changes identified appear to support the concept that decreased inhibitory neurone activity and enhanced glutamatergic

neuronal excitability occur in the primary visual cortex in DLB and we speculate that this may contribute to the RCVH. If so, these results suggest it may be possible to reduce RCVH through targeting of GABA neurones using selective GABAergic agonists.

Introduction

Dementia with Lewy bodies (DLB) accounts for up to 20% of all dementia cases at autopsy [1]. Clinically DLB is associated with at least two of the following three core clinical features: recurrent complex visual hallucinations (RCVH), fluctuating cognition and parkinsonism [2] or one of these plus one supportive feature (from: neuroleptic sensitivity, abnormal dopaminergic imaging of striatum, or rapid eye movement sleep behaviour disorder) [3]. It is the presence of RCVH however, that has been suggested to be one of the most characteristic of the disorder and their treatment can be complicated by severe neuroleptic sensitivity [2] .

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The RCVH in DLB are described as being well formed and involve animals, people, and objects [4] and show some similarities with the visual hallucinations seen in Charles-Bonnet syndrome and those that occur following occipital infarction [5, 6]. Illusions and misperceptions are also experienced, and the occurrence of passage and presence hallucinations are also common, as with Parkinson's disease (PD) and Parkinson's disease with dementia (PDD) [7-9]. Whilst RCVH are one of the core clinical symptoms of DLB, their aetiological basis is essentially unknown. DLB subjects show, for example, hypoperfusion and glucose hypometabolism in the medial occipital lobe including the primary and secondary visual cortex [12-14] and this associates with the visuoperceptual problems which are common to both DLB and PDD [15]. RCVH have been suggested to be due to cortical atrophy, although most neuroradiological studies do not find significant atrophy of the medial occipital cortex in DLB or in PD or PDD [16-21]. Whilst there appears to be involvement of the lateral occipital cortex in DLB [13, 14], the only significant pathology reported is spongiform change and gliosis in the

medial occipital white matter [22], although this has not been observed in all studies [23].

Ocular pathology may also contribute but these are common in elderly individuals where clouding, increased central and peripheral corneal opacity, and macular degeneration being typical [10, 24]. Related to these ocular changes with age there are also changes in visual perception with decreased capacity to identify motion in spatial suppression tasks [25], and in motion perception [26, 27], along with reduced acuity [28] and ability to determine images in the peripheral fields [29]. These changes are accompanied by problems associated with visual image processing including altered saccadic eye movements. Visual imagery appears reduced in normal ageing with a decreased ability to respond to cues with decreased image generation as a result of decreased ability of cortical visual association areas [30]. This is also seen with simple image generation [31] and the prefrontal cortex may mediate this effect with atrophy leading to reduced image manipulation and generation [32]. The interaction between reduced higher cortical and altered proximal visual system mechanisms associated with ageing may therefore contribute to the RCVH seen in DLB.

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See Neil Archibald Brain review for PD stuff.

Overall I would shorten this paragraph and maybe focus on the pregeniculate data that exists for DLB/PD

The well-formed nature of RCVH in DLB suggests that the ventral visual stream [33, 34] is affected. The ventral stream from the primary visual cortex projects to the temporal lobe, including the amygdala. These areas contain relatively high numbers of LB and it has been speculated that these contribute to RCVH [35, 36] although no differences between hallucinating and non-hallucinating cases have been observed.

Nevetheless high densities in parahippocampal gyrus have been suggested to relate to early development of RCVH [23, 24]. Alpha-synuclein pathology in the retina [37] and cholinergic dysfunction [38] have been observed indicating that pathological changes in the eye and biochemical changes in the primary visual cortex may contribute to RCVH [39].

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Since the possible changes in DLB which could contribute to RCVH do not stand alone but are part of the highly complex visual system, the question arises as to what are the molecular substrates of RCVH in DLB? Given the paucity of information on pathology in the primary visual cortex in DLB, but with the presence of a marked and specific hypometabolic deficit in this region [13, 40], it is certainly possible that biochemical abnormalities in the visual cortex contribute to RCVH. We therefore explored this issue and describe here the analysis of the molecular changes in the primary visual cortex in DLB using a combination of pathology and stereology to identify pathological and neuronal involvement, coupled with transcriptomic analysis of RNA and complimentary protein determination to determine the biochemical systems involved.

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Clinical Samples

All procedures were approved by the Local Research Ethics Committee and tissue was obtained from the Newcastle Brain Tissue Resource (NBTR), a UK Human Tissue Authority approved research tissue bank. The DLB and AD cases were from a local prospective clinical study, with participants having clinical assessments repeated annually until death and agreeing to donate tissue for research purposes. Clinical diagnoses of DLB and AD were made according to international consensus criteria [3] and NINCDS ADRDA criteria [41] respectively. Healthy control subjects were obtained from NBTR and all such cases had had thorough case note review to confirm they did not have cognitive impairment and did have normal everyday function at time of death. All cases had neuropathological confirmation of either DLB, AD, or were neuropathologically normal according to established criteria [3, 42-44]. Cases of DLB had the presence of visual hallucinations documented or were without any documented RCVH (1 case), whilst AD cases showed an absence of any prior history of visual hallucinations (See Table 1). Tissue was obtained at post mortem, and the delay from death to freezing and fixation was noted. Tissue from the left hemisphere was rapidly frozen at -80^{o}C using pre-cooled copper blocks as approximately 1cm thick slices and stored at -80^{o}C until use. The right hemisphere was fixed in 10% formalin and processed by paraffin wax embedding. A 1cm-thick slab of fixed tissue of the occipital lobe was cut into a series of 25 x 40-µm-thick tissue slices from a pre-defined paraffin-embedded coronal block containing the relevant portion of the middle of the primary visual cortex. Three of the 25 sections were sampled at equally spaced intervals (every eight sections) and stained with cresyl fast violet. The quality of each section was checked for consistency and the slides coded (CMM) to ensure

that all analysis was carried out blind to diagnosis. The layers of the primary visual cortex were delineated in accordance with previous literature [45]. All measurements were taken from the cortical strip directly surrounding the Calcarine sulcus. Additional paraffin sections (6-10µm) were taken for pathological investigation and stained for myelin (Loyez stain), and with haematoxylin and eosin to show general structure. Immunohistochemical investigations of pathology used a standard ABC peroxidise method using anti-α-synuclein, anti-Amyloid β peptide , and anti-phosphorylated tau and quantified using a standardised morphometric approach [46, 47] (antibodies details given in Supplementary Table 3).

Stereology

Neuronal density and volume was estimated within layers of the primary visual cortex using analysis software (Stereologer, Chester, MD, USA) to apply the optical disector [48, 49] and rotator [50] methods to obtain the density and volume estimates respectively, as in our previous studies [51-53]. Neurones were identified using standardised criteria: the presence of a Nissl-stained cytoplasm, pale nucleus and single identifiable nucleolus in cells that were not spherical unlike glial cells. The sections were viewed in oil using a Zeiss Photomicroscope at x 100 objective and a numerical aperture of 1.25. The microscope was attached to a JVC colour video camera TK-C1360B (JVC UK Ltd, London, UK), a motorised *x*- and *y*-axis stage accurate to 1 µm (Optiscan ES110, Prior Scientific Instruments Ltd, Cambridge, UK) and a Heidenhain *z*-axis depth gauge accurate to 0.5 µm (Heidenhain GB Ltd, London, UK) to ensure accurate measurement of disector depth.

Based on our previous investigations [51-53], a random sampling strategy was used and estimates were conducted using one disector field per field of view and over 120 counts were made per layer examined in each subject to ensure precise estimates. Each disector frame measured 50 µm long, 50 µm wide and 15 µm deep, with a guard area of at least 4 µm taken (average µm) above and below the 3-D disector box, dependent on section thickness measurement. The mean coefficient of error (CE) for the overall neuronal and glial estimates were calculated using the Gundersen-Jensen method [54], as previously [51-53], and showed that the mean CE for neuronal volume and density reached an acceptable level of precision in all layers in all cases measured ($P<0.15$) [54]. There was no significant difference in demographic, clinical and histopathological information on the study sample, as summarised in Table 1. There were no significant differences between the groups in age, gender, tissue pH, duration of tissue fixation (d.f.= 25, $P<0.05$ for all measures), or post-mortem interval. Mean (±SD) section thickness across all layers was comparable (d.f. = 25, $p=0.70$) between control (31.82 [1.00] µm), AD (31.58 [0.79] µm) or DLB (31.89 [0.37] µm) groups after processing.

RNA isolation

The primary visual cortex was identified in blocks of snap frozen tissue and approximately 50mg of grey matter was dissected at -20^{o}C from a second series of 14 DLB, 13 control, and 15 AD cases rated as previously (see Supplementary Table 1) and placed in 5 – 10 volumes of pre-cooled RNA*later* solution (Ambion) and stored at -80ºC. Tissue was removed from RNA*later* and rapidly homogenised in TRI- Reagent

(Ambion) and stored at -80ºC. RNA was extracted using a spin column method as per the manufacturer's instructions (Ribopure, Ambion, Warrington, UK) and 1ug of RNA was DNase-treated (Turbo-DNAase free, Ambion). The RNA concentration was determined using a Nanodrop ND 1000 Spectrophotometer (Nanodrop Technologies) and RNA integrity number (RIN) examined with an Agilent 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies) according to the manufacturer's instructions.

Microarray Analysis

Control (n = 12) and DLB (n = 12) total RNA samples were selected from the second group on the basis of tissue pH (>6.0) and RIN value (>6.0) (see Supplementary Table 1) and were analysed using Illumina Human-6 v2 BeadChips with approximately 48,000 transcript probes per chip using a GLP certified facility (Aros, Brensby, Denmark). Data was imported into R, log2 transformed using a Variance Stabilizing Transformation [55] and Robust Spline Normalisation algorithm designed for Beadarray data [56]. Following removal of failed samples and outliers using hierarchical clustering, significantly altered transcripts were identified in the limma package [57] and differentially expressed genes identified by applying cut-offs of p-Value <0.05 (Benjamini-Hochberg FDR correction [58]), and an absolute fold-change of greater than 2. To identify systems that may be altered/associated with DLB in the primary visual cortex we used Gene Ontology (GO) [59] to identify biological process, molecular function and cellular component annotations using the GOstats package to test gene lists for over-represented terms [60] and also Kyoto Encyclopaedia of Genes and Genomes (KEGG) biochemical pathways [61].

q-RT-PCR

Reverse transcription of RNA was performed on 1µg of total RNA using oligo(dT)$_{12-18}$ primer (Invitrogen), SupeRNase Inhibitor (Ambion), and Superscript III Reverse Transcriptase at 50ºC and samples stored at -80ºC until use. Semi-quantitative RT-PCR was performed in triplicate using Taqman® assays (see Supplementary Table 2; Applied Biosystems) using Taqman® Universal PCR Master Mix (Applied Biosystems). A total of 35 different brain tissue samples with total RNA of RIN>6.0 were analysed by q-RT-PCR with 11 control, 12 DLB, and 12 AD subjects used. ABI PRISM® Sequence Detection System software was used to generate $2^{-\Delta\Delta CT}$ values based on the comparative CT method with GAPDH mRNA as a reference [62, 63]. To compare the relative gene expression, a Mann Whitney test was carried out using $2^{-\Delta\Delta CT}$ values with significance level of $p < 0.05$.

Protein Determination

Tissue blocks of the occipital lobe were brought to -20ºC in a freezing cabinet and the primary visual cortex identified. Primary visual cortex tissue and a small amount of underlying white matter were isolated using a pre-cooled scalpel (see case details, Supplementary Table 1). Extraction was performed by homogenising 100-150mg of tissue using a rotor stator type homogeniser in ice cold lysis buffer containing 0.2M tetraethyl ammonium bicarbonate, pH 7.2 (TEAB; Sigma), 1mM EDTA, and protease inhibitor tablets (Complete, Roche) and samples stored at -80°C. Samples were thawed, made to 0.02% sodium dodecyl sulphate and sonicated on ice in a sonicating water bath for 20 minutes before protein quantitation was performed using a BCA

Protein Assay kit (Pierce, Rockford, IL).

Proteins (5-20µg of protein per sample) were separated using NuPAGE 12% Bis-Tris gels (Invitrogen) with 1x SDS NuPAGE MOPS Running Buffer containing 0.248% NuPAGE Antioxidant (Invitrogen). Proteins in the gel were transferred to nitrocellulose membranes using an iBlot device (Invitrogen). Membranes were stained with Ponceau S solution (Sigma) to ensure equal protein loading, destained with 1x Tris-Buffered Saline (TBS) 0.2% Tween 20, and stored in 1x TBS 0.2% Tween 20 at 4^{o}C overnight. The membrane was incubated with 5% dried milk in 1x TBS 0.2% Tween 20 for 30 minutes at room temperature to block non-specific protein binding sites and incubated overnight with primary antibody (see supplementary Table 3) diluted in 5% dried milk in 1x TBS 0.2% Tween 20 at 4^{o}C with agitation. Membranes were then washed for 10 minute intervals 3 times with 1x TBS 0.2% Tween 20 and incubated a further 30 minutes with the appropriate horseradish peroxidase conjugated secondary antibody at room temperature. Membranes were washed for 1 hour with four washes of TBS 0.2% Tween 20 then proteins visualised by Enhanced Chemiluminescence (GE, Amersham, Bucks, UK) and detected using x-ray film (Fuji, Fisher Scientific, UK). Protein bands were subsequently quantified using Image-J (NIH). Target protein expression was determined by normalising to GAPDH protein expression.

Validated commercial ELISA assays against BDNF (Promega Corporation, Madison, WI, USA), somatostatin, and neuropeptide Y (Cambridge Bioscience, Cambridge UK) were

used according to the manufacturer's instructions. Samples of BA17 tissue homogenates were thawed on ice and centrifuged at 16,000 x g for 20 minutes at 4^{o}C and the supernatant collected and diluted 1:20-1:50 in assay diluent buffer and applied to prepared assay plates. Samples were read along with standards using a Biotek Synergy Plate Reader. Protein in supernatant samples was determined using a BCA Protein Assay kit (Pierce, Rockford, IL) and specific peptide levels expressed as ng or pg peptide/mg protein.

Statistical Analysis

Analysis of pathology and stereology data involved analysis of variance (ANOVA) with pairwise comparison of differences and Bonferroni correction for multiple comparisons . PCR data was analysed using Mann Whitney U tests since the data were not normally distributed and protein data were analysed initially using Levine's f-test to determine homogeneity of variance and differences using two-way unpaired t-tests. In all cases, $P<0.05$ was considered significant.

Results

Pathology

Pathological investigation of the primary visual cortex in relation to RCVH, showed no evidence of α-synuclein deposition as Lewy bodies or Lewy neurites within primary visual cortex in any DLB case, with some evidence of α-synuclein deposition in BA18 as Lewy neurites in DLB cases, and increased deposition as Lewy neurites and occasional Lewy Bodies in lateral occipital cortex (see figure 1). The mean intensity of α-synuclein staining in three occipital regions was recorded for primary visual cortex, secondary visual cortex and also lateral occipital cortex. No difference was seen in

staining intensity between DLB, AD and control cases for overall α-synuclein staining intensity. A difference in the intensity of staining between areas was seen for BA17, BA18 and BA37 within the DLB group (ANOVA: $F(2,42)=7.444$, $p=.002$) with significantly more staining in secondary visual cortex (114.49 ± 2.84, P=0.038) and in BA37 (119.10 ± 3.05, P=0.001) compared to primary visual cortex (104.99 ± 1.86). A similar significant difference between the areas was also seen in the AD group (ANOVA: $F=(2,147)=32.275$, $P<0.001$). There were no statistically significant differences between the staining in secondary visual cortex and lateral occipital cortex in AD (P=0.440). There was no statistically significant difference between the three areas in DLB compared to AD (ANOVA: $F=(2,147)=1.328$, $P=0.268$). Transcripts for α-synuclein mRNA were lower (37%) in DLB than controls although this was not significant, and similarly protein analysis of α-synuclein monomer (18kDa) showed no significant change in expression in DLB ($p = 0.16$), with comparable levels of expression in control and AD subjects (see Table 2), consistent with previous studies [64, 65].

Assessment of amyloid-β and Tau pathology in the primary visual cortex in AD showed a high level of amyloid-β pathology in the form of senile plaque and diffuse amyloid-β deposition, which was significantly higher than in controls and also higher than in DLB ($p<0.005$ vs control; $p<0.05$ vs DLB, see and Supplementary Figure 1). Amyloid-β pathology was found in the occipital cortex in DLB, but was not higher than in cognitively normal controls. No changes in APP mRNA expression were seen in the primary visual cortex using q-RT-PCR in either DLB or AD (see Table 2). Tau pathology as assessed by AT8 immunostaining in the occipital lobe in AD cases was present as

neurofibrillary tangles and neuropil threads and showed high densities in the primary visual cortex but was absent from control and DLB cases ($p<0.05$ vs control; $p<0.05$ vs DLB see Supplementary Figure 1). No changes in MAPT (Tau) mRNA expression were seen in the primary visual cortex using q-RT-PCR in DLB, although MAPT mRNA levels were higher in AD cases compared to controls though not significantly (see Table 2).

Stereology

Since neurodegenerative pathology in the primary visual cortex in DLB was not significantly different from normal elderly control individuals, neuronal densities were determined in the primary visual cortex. There was no significant difference in neuronal density in any layer of the primary visual cortex between control and DLB or AD groups after multiple comparison analysis (Figure 2). However, there was a significant reduction in neuronal volume in layer 4a in the AD group (P=0.042; see Figure 3) in comparison to the control group. Furthermore, a significant reduction in neuronal volume in the AD (P=0.043) versus control group was found across all layers when combined. No significant changes were found in neuronal volume in the DLB group against control or AD groups in any layer of the primary visual cortex. Similarly, since hypoperfusion and hypometabolism is a feature of DLB occipital cortex [13], we determined the presence of capillary density in primary visual cortex in DLB using Glut-1 staining. No alterations in capillary density were observed in either DLB or AD brain compared to age matched controls (not shown) suggesting hypoperfusion and hypometabolism is not associated with altered capillary density.

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Microarray

Owing to the lack of significant α-synuclein pathology or of major amyloid-β or Tau deposition in DLB and any significant cell loss in the primary visual cortex in DLB, whole genome microarrays were used to determine the changes that might underlie any biochemical abnormality associated with hypoperfusion and hypometabolism. Seventy three genes were seen to be significantly altered in the primary visual cortex using the described cut-offs (see Figure 4; Table 4). Several systems were identified as altered in the primary visual cortex and linked with DLB most notably in cellular signalling, synaptic transmission, and vesicle transport (see Supplementary Table 4). One finding associated with the microarray data was the presence of RNA changes for neuropeptide molecules such as proenkephalin (PENK), tachykinin (TAC1) and prodynorphin (PDYN) transcripts (see Table 4). These neuropeptides which are primarily expressed by specific interneurone populations suggests that there may be GABAergic dysfunction associated with the primary visual cortex in DLB, and this was underlined by the presence of other GABAergic markers in this patient group, most notably GAD2 (GAD65) and SLC32A1. Using GAD65/67 as a marker of total number of GABAergic cells, no alteration in total counts of cells was seen in the cortex for GAD65/67 stained neurons for DLB or AD in either primary visual (ANOVA: $F(2,23)=0.389$, $p=0.682$) or secondary visual cortices (ANOVA: $F(2,23)=1.184$, $p=0.186$; see Table 5) and this was paralleled by an absence of any significant change in *GAD1* mRNA transcripts for DLB relative to control, although a slight but significant reduction *GAD1* was seen in AD ($p = 0.0337$; Table 6), though AD and DLB groups were comparable. Similarly, there was no alteration in GAD65 or GAD67 protein determined by western blotting in either DLB or AD (see Table 6) suggesting that

GABAergic neurones are still present but potentially dysfunctional based on peptide markers.

To explore the possibility that GABAergic neurones were either degenerate or dysfunctional in specific neuronal subtypes, numbers of calcium binding protein containing neurones were quantified to determine if cell loss was selective. We studied the levels of parvalbumin (*PVALB*, mRNA; PVALB, protein), a marker of the fast spiking basket and chandelier neurones that provide major inhibitory inputs onto soma and axons of pyramidal neurones. *PVALB* mRNA was significantly reduced in DLB ($p = 0.0035$) but was also significantly increased in AD ($p = 0.0002$) (Table 6). These changes in *PVALB* mRNA were also complemented by changes in PVALB protein with a reduction of approximately 15% in DLB ($p=0.033$) and an increase in AD of approximately 25% ($p=0.0005$). Given the absence of any reduction in overall GABAergic neurones (see Table 5), Parvalbumin, D-28 Calbindin and Calretinin neuronal density in the primary visual cortex were investigated using stereological methods however, no significant change in the density of these neuronal markers in either DLB or AD were seen (see Figure 5) suggesting that whilst the neurones may show altered marker profiles, their density is unchanged. To further investigate this altered marker profile protein levels for other neuropeptides within the primary visual cortex in DLB were assessed. Neuropeptide Y levels were increased on ELISA analysis in DLB by 15% although not significantly ($p=0.22$) and in AD NPY was increased by approximately 20% ($p=0.027$) (see Table 6). Somatostatin levels in primary visual cortex were increased by 15% in DLB ($p=0.40$) and by 40% in AD ($p=0.09$).

To determine if these neuropeptide changes were associated with alterations in other GABAergic markers, levels of several proteins involved in GABA neurotransmission were determined. The GABAergic post-synaptic marker GABARAP involved in GABA (A) receptor clustering/transport [66, 67] showed reduced expression of the 17kDa GABARAP isoform of approximately 40% ($p< 0.001$) and of the 14kDa GABARAP isoform of approximately 30% ($p<0.001$) in DLB: in AD the 17kDa isoform was reduced to a lesser extent by about 15% ($p<0.001$) and the 14kDa isoform by 20% ($p<0.001$) when compared to controls (see Table 6). The GABA motor protein Kif5A showed a trend towards reduction in DLB ($p=0.06$), but no change in AD. Gephyrin, a post synaptic GABA-A associated protein which anchors GABA-ergic and glycinergic receptors to the post-synaptic cytoskeleton [68, 69], showed a significant reduction of approximately 25% in DLB ($p<0.001$) and in AD a reduction of about 20% ($p<0.0001$). Using ionotropic GABA A1 receptor subunit as an additional marker of receptor activity, showed no significant change in either DLB or AD (see Table 6).

Since alterations of GABAergic activity may lead to altered excitation, a series of glutamatergic synaptic markers were assessed (see Table 7). Using an anti-VGlut1 (SLC17A7) antibody as a marker of cortical input into the primary visual cortex, we demonstrated bands from 59-61 kDa, reflecting VGlut1 isoforms ranging from 59.3 to 61.3 kDa. DLB cases were not significantly different to controls in VGlut1 isoform 1 (61.3kDa; $p=0.14$), with AD cases showing a trend towards reduction ($p=0.08$). DLB cases were also not significantly different to controls in VGlut1 isoform 2 (59.3kDa; $p=0.72$) and AD cases were also not significantly different to controls ($p=0.78$).

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1)Previous data suggest limited structural change in DLB in primary visual cortex but this has not been studied in detail. However we expect that we will show similar findings

2)Previous studies demonstrated possible loss of inhibition in DLB + visual hallucination models (deafferentation) suggest loss of inhibition. There we hypothesed that we would see either an increase in excitatory markers and/or decrease markers of inhibitory activity. Changes would either come from altered bottom up input or top down input (as measured by synaptic markers)

3)compared and contrasted structure and function in a DLB hallucinator cohort vs. AD cohort and aged control cohort.

Similarly the metabotropic glutamate receptor mGluR1 was also unaltered in DLB. The reduction in glutamate synaptic markers in DLB was not associated with a loss of ionotropic AMPA glutamate receptor (GluR1/GRIA1) mRNA which was unchanged in DLB primary visual cortex. As a marker of neural activity we observed a selective reduction in BDNF mRNA in the primary visual cortex in DLB cases ($p<0.05$; Table 7) but were unable to detect BDNF protein in any samples using a specific ELISA. Whilst variable, there was a trend towards reduction of the post-synaptic excitatory scaffold protein DLG4 (PSD-95) mRNA in DLB, and in AD this reduction was significant ($p<0.05$; see Table 7). PSD95 protein determination however showed a marked 50% decrease in DLB compared to controls ($p=0.0002$), whilst AD cases showed no significant change in PSD95 ($p=0.96$) in primary visual cortex. In line with PSD-95 findings, we identified a selective reduction of 43% of the growth cone marker GAP43 in DLB primary visual cortex ($p=0.0005$) whilst AD cases showed no significant change in GAP43 ($p=0.11$). As markers of synaptic neurotransmission we determined synaptophysin as a marker for presynaptic vesicular membranes and SNAP25 levels as a marker of presynaptic vesicles. Synaptophysin showed a marked and significant 44% decrease in DLB cases ($p<0.00001$) and also a lesser 22% decrease in AD cases ($p=0.01$). DLB cases however, showed no significant difference in SNAP25 expression compared to controls ($p=0.43$), and similarly AD cases also showed no significant difference in SNAP25 expression compared to controls ($p=0.13$) indicating general exocytosis was unaltered.

Discussion

Visual hallucinations and other abnormal visual percepts have a complex aetiology with the potential involvement of multiple systems including the eye, primary visual

cortex, and higher order brain regions including the inferior and medial temporal lobe and frontal cortex [5, 6, 70]. A major feature of DLB is the presence of marked and well-formed RCVH [4] which may be a result of abnormalities in the ventral visual stream due to the object based nature of RCVH [34]. This study showed an absence of demonstrable neuropathological changes in primary visual cortex with no significant evidence of Lewy body or AD type pathology or change in neurone density or volume in DLB. This suggests that occipital hypoperfusion and hypometabolism seen in conjunction with RCVH are associated with dysfunction rather than neurodegeneration in the primary visual cortex. In AD, however, the presence of major pathology and also of neurone atrophy, may indicate that the primary visual cortex is simply less responsive to stimulation, and consequently RCVH in AD are not common findings due to neurodegeneration. Because of the absence of pathology in DLB, we used a transcriptomic approach to determine what gene expression changes underpin the possible changes in the primary visual cortex. The current findings indicate that there is an alteration in the profile of several GABA and neuropeptide markers which are suggestive of changes in the interneurone pool. Whilst the microarray finding shows GABAergic changes, the major interneurone population is still intact based on the calcium binding protein profile staining, underscoring that dysfunction rather than degeneration occurs in the primary visual cortex in DLB. Although the transcriptomic and morphological changes in the primary visual cortex point towards dysfunction, the underlying changes in neurotransmission that regulate this region require definition and here it would appear that specific synaptic changes in both inhibitory interneurones and also excitatory neurones were present.

Commented [JT7]: See earlier comment maybe framing things more along the a priori hypothesis

Commented [JT8]: Doesn't make sense

The changes in GABAergic interneurones and excitatory neurones suggest that there may be parallels with the changes seen in the primary visual cortex following dark rearing or reduced visual input in animals [71, 72]. Here, reduced input leads typically to changes in the function of inhibitory interneurones in the primary visual cortex with a reduced expression of parvalbumin in fast spiking interneurones [73]. There are also relatively specific changes in the primary visual cortex following reduced stimulation with an increased turnover of synaptic boutons of interneurones both acutely and chronically [74-77]. While much of this work has been conducted in the mouse, primate work also suggests a loss of GABAergic interneurone function following decreased input [76, 78] and acutely in man [79]and this degradation of function also occurs in primary visual cortex in normally aged monkeys and can be rescued by GABAergic agonists [80]. The reduced expression of GABAergic markers such as Gephyrin and GABARAP may be an indication of the eye pathology frequently found in DLB patients and ageing [10, 15]. DLB patients frequently show visuo-perceptual impairments and the presence of ocular pathology may reduce input to the primary visual cortex [15, 81]. Conversely, environmental enrichment in mice leads to enhanced BDNF expression in the primary visual cortex [82] which contrasts with the decreased BDNF mRNA expression seen in DLB cases in the current study (see Table 6, 7). Visual input regulates the expression of BDNF mRNA [83] in glutamatergic neurones with reduction of BDNF leading to altered GABAergic transmission [84] and development of ocular dominance columns [85] which may suggest that the reduced protein expression activity seen in the current study is a consequence of decreased visual input. This may be a stereotyped response of primary visual cortex in an attempt to increase excitation by decreasing inhibition,

and would support the suggestion of decreased inhibition as a factor in RCVH in DLB [86].

The results here suggest reduced connectivity in the primary visual cortex of DLB with a relatively selective reduction of synaptic proteins and genes, for example synaptophysin, α-synuclein, and PSD95, compared to the atrophy associated changes seen with AD. PSD95 is specifically associated with the post-synaptic membrane at excitatory synapses [87, 88] indicating that transmission from the pre-synaptic terminal to excitatory post-synaptic terminal is impaired along efferent connections (i.e. from the LGN to the primary visual cortex). Whilst the major input to the primary visual cortex is from the LGN, reciprocal connections do enter from adjacent visual cortical areas (secondary visual cortex, etc.) and through secondary visual pathways via the pulvinar and so reductions in PSD95 or synaptophysin may represent such defects. Using VGlut1 as a marker of cortico-cortical connections, we observed no significant reductions in VGlut1 in DLB but observed reductions in VGlut1 in AD where there was a general reduction along with atrophy in excitatory neurones (Fig 3) due to pathology (e.g. [89, 90]) and increased pathology in secondary visual cortex in these neurones [91]. This would indicate that intrinsic and cortical glutamatergic synapses are not lost in DLB but that subcortical input from the LGN to the primary visual cortex may be impaired. This supports the concept of reduced visual system input to the primary visual cortex as being part of the process of generation of RCVH in DLB as with other primary visual system lesions [70] and that "bottom up" changes are important in RCVH as has been noted for PD [92].

Commented [JT9]: What about top-down attentional input to V1 – evidence for this? Also maybe more needs to be made of the cholinergic system (so dysfunctional in DLB)– see Alex Thiele's work
http://www.ncbi.nlm.nih.gov/pubmed/18633352

Commented [JT10]: Again what about top-down?

These changes in input and GABA neurotransmission may have a clinical correlate within the symptomatology of RCVH in DLB. The effects of altered GABA neurotransmission may be seen in an increased excitability of pyramidal neurones, and this can be observed in DLB where the use of transcranial magnetic stimulation of the occipital cortex (occiput) is correlated with increased excitability and the generation of RCVH like phosphenes [93] and these changes may be due to decreased inhibition. Reduced levels of parvalbumin, such as seen in this study, have been suggested to cause altered gamma oscillations within cortical regions whereby the gain and modulation of firing of pyramidal neurones is affected [94, 95]. Desynchronisation of pyramidal neurones in the primary visual cortex and increased excitability may therefore lead abnormal outflow to higher order visual areas which appear to show changes in DLB [81] resulting in RCVH.

Commented [JT11]: So more noisy visual information which leads to "top-down" best guessing?

The presence of reduced visual input into the primary visual cortex in DLB whilst being part of the aetiology of RCVH, does suggest that further changes must contribute to RCVH. For example, RCVH do not necessarily occur in blind individuals, and this raises the possibility that in addition to changes in the primary visual cortex, other pathological or biochemical changes are required in order to result in RCVH [81]. Clearly there are changes in the retina in DLB and also in PD with loss of dopamine and of reduced retinal thickness found in some studies using optical coherence tomography which may contribute to the visual impairment and RCVH [11, 96-99]. Some studies indicate changes in processing associated with retinal changes may also occur in PD [100]. Considerable attention has however focussed on changes in temporal lobe structures in DLB and also PD in relation to visual hallucinations [7,

Commented [JT12]: Need to mention in introduction I think

81]. Pathological correlates have indicated that temporal lobe Lewy bodies correlate with the presence of RCVH [35, 101] and that, in PD, elevated Lewy body density in the basolateral nucleus of the amygdala and temporal lobe associate with RCVH [24, 102]. Structurally however, there is relative preservation of the temporal lobe in DLB [103] although this may not be the case in PD with RCVH [104]. This is accompanied in DLB with decreased connectivity of the temporal lobe [81, 105] and perfusion deficits, specifically in the ventral occipitotemporal junction following visual stimulation of DLB patients with frequent and more severe RCVH [106].

Our results therefore show that, whilst there is an absence of major pathological changes in the primary visual cortex in DLB, this is in the presence of functional changes associated with GABAergic neurones. This change may reflect a change to the visual input due to reduced visual acuity as a consequence of age related changes in the eye. These changes may therefore lead to an inhibitory/excitatory imbalance in the primary visual cortex and altered perception of visual stimuli due to changes in association cortex. One possibility is that by either treating eye disease or modulation of GABAergic inhibition, it may be possible to ameliorate the visual hallucinations perceived by DLB patients.

Commented [JT13]: And pathology of DLB

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