The role of the cingulate cortex in depression in dementia with Lewy bodies

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Abstract

Dementia with Lewy bodies (DLB) is a significant cause of dementia in the older population with a core set of clinical symptoms that help to distinguish DLB from other forms of dementia such as Alzheimer's disease (AD). The core symptoms of DLB include fluctuating cognition, recurrent complex visual hallucinations and parkinsonism. Additionally, depression is experienced in around half of DLB patients and is associated with faster rate of cognitive decline, higher mortality rates and a poor response to treatment. The subgenual anterior cingulate cortex (sgACC) is integral in mood regulation, displaying structural, functional and metabolic abnormalities in depression, and shows early and extensive pathological changes in DLB. However, little is known about how any pathological or neurochemical changes in sgACC contribute to the aetiology of depression in DLB.

Post-mortem tissue from cingulate cortex subregions was used to quantify neuropathological lesions in DLB cases with and without depression, and cognitively normal controls. Neurochemical analysis was performed to assess disease and depression specific changes in the sgACC in GABAergic, glutamatergic and monoaminergic transmission. Synaptic changes were assessed using confocal and stimulated emission depletion (STED) microscopy.

Neuropathological burden in cingulate subregions showed disease, but not depression specific changes in DLB. Abnormalities in GABAergic and glutamatergic neurotransmission were observed in DLB cases with depression, showing greater dysregulation compared to DLB cases without depression. Dopaminergic deficits were observed in sgACC in DLB cases, with a greater reduction in DLB cases with depression, whereas no major changes in serotonergic or noradrenergic neurotransmission were observed in DLB.

The results demonstrate that neurodegenerative neuropathological changes within the cingulate cortex do not appear to influence the development of depression in DLB. An imbalance in GABAergic/glutamatergic transmission within the sgACC was greater in DLB cases with depression, which may suggest greater dysregulation in excitation and inhibition, possibly contributing to the development of depression in DLB. This work also demonstrates the major role of dopaminergic neurotransmission in the aetiology of depression in DLB. Overall, this work indicates that treatment of depressive symptoms in DLB could benefit from modulation of dopaminergic, glutamatergic, or GABAergic transmission.

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Publications

Related to thesis

Patterson, L., Firbank, M. J., Colloby, S. J., Attems, J., Thomas, A. J. & Morris, C. M. 2019. Neuropathological Changes in Dementia With Lewy Bodies and the Cingulate Island Sign. *J Neuropathol Exp Neurol*, 78(8), 717–724.

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Declaration

I hereby declare that the research described within this thesis is my own work unless otherwise stated and that none of the material within this thesis has been previously submitted by me for a degree at this or any other university. All the contributions made that are not my own have been appropriately acknowledged and referenced.

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Abbreviations

5HT	Serotonin
5HTT	Serotonin transporter
5HTTLPR	Serotonin transporter linked polymorphic region
AA	Amino acid
Aβ	Amyloid-beta
ACC	Anterior cingulate cortex
ACh	Acetylcholine
AD	Alzheimer's disease
AGD	Argyrophilic grain disease
ANOVA	Analysis of variance
APP	Amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
CAA	Cerebral amyloid angiopathy
CB	Calbindin D-28K
CBD	Corticobasal degeneration
CBS	Charles Bonnet syndrome
CBT	Cognitive behavioural therapy
CIS	Cingulate island sign
CNS	Central nervous system
CR	Calretinin
CSDD	Cornell Scale for Depression in Dementia
CSF	Cerebrospinal fluid
CRYM	μ-crystallin
Cu	Cuneus
DA	Dopamine
DAB	Diaminobenzidine
DAT	Dopamine transporter
DB	Dot blotting
DBS	Deep brain stimulation
DLB	Dementia with Lewy bodies
DLPFC	Dorsolateral prefrontal cortex
DMN	Default mode network

DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, fourth edition
ECT	Electroconvulsive therapy
EDTA	Ethylenediamine tetra-acetic acid
EEG	Electroencephalographic
FDG	Fluorodeoxyglucose
FDR	False Discovery Rate
fMRI	Functional magnetic resonance imaging
FTD	Frontotemporal dementia
GABA	γ-aminobutyric acid
GAD	Glutamic acid decarboxylase
GDS	Geriatric Depression Scale
GR	Glucocorticoid receptor
HMPAO	Hexamethylpropyleneamine oxime
HPA	Hypothalamic–pituitary axis
LBD	Lewy body disorders
LB	Lewy bodies
LC	Locus ceruleus
L-DOPA	Levodopa l-3,4-dihydroxyphenylalanine
LN	Lewy neurites
LSD	Lysergic acid diethylamide
MADRS	Montgomery–Åsberg Depression Rating Scale
MAOI	Monoamine oxidase inhibitor
MCC	Mid-cingulate cortex
aMCC	Anterior mid-cingulate cortex
pMCC	Posterior mid-cingulate cortex
MCI	Mild cognitive impairment
MDD	Major depressive disorder
MDE	Major depressive episode
MIBG	Metaiodobenzylguanidine
MMSE	Mini-Mental State Examination
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MSA	Multiple system atrophy
MT	Microtubule

MTA	Medial temporal lobe atrophy
NA	Noradrenaline
NAC	Non amyloid-beta component
NAcc	Nucleus accumbens
NBM	Nucleus basalis of Meynert
NDRI	Norepinephrine-dopamine reuptake inhibitors
NFT	Neurofibrillary tangles
NGS	Normal goat serum
NMDA	N-methyl D-aspartate
NP	Neuritic plaques
NT	Neuropil threads
PBS	Phosphate-buffered saline
PCA	Posterior cortical atrophy
PCC	Posterior cingulate cortex
dPCC	Dorsal posterior cingulate cortex
vPCC	Ventral posterior cingulate cortex
PD	Parkinson's disease
PDD	Parkinson’s disease with dementia
PET	Positron emission tomography
PFC	Prefrontal cortex
PHG	Parahippocampal gyrus
PiB	Pittsburgh compound B
PiD	Pick's disease
pNPP	p-nitrophenyl phosphate
PPN	Pedunculopontine nucleus
Pr	Precuneus
PSG	Polysomnography
PSP	Progressive supranuclear palsy
PV	Parvalbumin
RBD	REM sleep behaviour disorder
REM	Rapid eye movement
ROI	Region of interest
RSC	Retrosplenial cortex
rTMS	Repetitive transcranial magnetic stimulation
SDS	Sodium dodecyl sulfate

SDS-PAGE	SDS - Polyacrylamide gel electrophoresis
sgACC	Subgenual anterior cingulate cortex
SN	Substantia nigra
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNCA	α-synuclein gene
SNDRI	Serotonin-norepinephrine-dopamine reuptake inhibitors
SNRI	Serotonin and norepinephrine reuptake inhibitors
spACC	Supragenual anterior cingulate
SPECT	Single photon emission computed tomography
SSRI	Selective serotonin reuptake inhibitors
STED	Stimulated emission depletion
STN	Subthalamic nucleus
STT	Somatostatin
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween20
TCAs	Tricyclic antidepressants
TDP-43	Transactivation response DNA binding protein 43 kDa
UPDRS	Unified Parkinson's disease rating scale
VaD	Vascular dementia
VEN	Von Economo neurone
VH	Visual hallucinations
VMAT-2	Vesicular monoamine transporter-2
WB	Western blotting
WMH	White matter hyperintensities

Chapter 1.
Introduction

1.1 Introduction

Dementia is a syndrome characterised by progressive decline in memory, thinking, behaviour and the ability to perform everyday activities. It is one of the major causes of disability worldwide, and has a physical, psychological, social and economic impact not only on patients, but also on their families, carers and society. Around 50 million people live with dementia worldwide, with nearly 10 million new cases diagnosed every year. The number of people living with dementia in 2030 is estimated to reach 82 million (WHO, 2019, Collaborators, 2019). In England and Wales, deaths due to dementia continue to increase and it is now acknowledged as the leading cause of death, accounting for 12.8% of all deaths registered (Office for National Statistics).

The major causes of dementia are the primary neurodegenerative dementias, which are progressive and irreversible due to neuronal dysfunction and neuronal death. The most common forms include Alzheimer's disease (AD), vascular dementia (VaD), dementia with Lewy bodies (DLB) and frontotemporal dementia (FTD) (WHO, 2019). Both clinically and pathologically AD is the most common form of neurodegenerative dementia, accounting for 60-70% of all dementia cases (World Alzheimer Report, 2019). AD is characterised by progressive cognitive decline and impaired ability to form recent memories (McKhann et al., 2011). The pathological characteristics of AD are intracellular neurofibrillary tangles, initially observed in the transentorhinal cortex, then temporal and insular cortex and eventually all cortical areas, as well as diffuse and neuritic extracellular amyloid plaques, that are initially observed in the neocortex, before spreading to the limbic, then subcortical brain areas and eventually the brainstem (Braak et al., 2006, Thal et al., 2002). Tau pathology is also associated with several other neurodegenerative disorders known as tauopathies, which include progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain disease (AGD) and Pick's disease (PiD) (Irwin, 2016).

Parkinson's disease (PD) is the most common neurodegenerative movement disorder (Dorsey et al., 2007), and is characterised by the classical motor features of Parkinson's, such as resting tremor, bradykinesia, rigidity, gait and postural instability, associated with the loss of dopaminergic neurones in the substantia nigra (SN) (Lang and Lozano, 1998). Dementia is often a late complication in PD (PDD), occurring in 75–90% of patients with a disease duration of 10 years or more (Buter et al., 2008). PD belongs to a group of neurodegenerative disorders called synucleinopathies, which have a common pathological substrate, α-synuclein protein,

which accumulates in Lewy bodies (LB) and dystrophic Lewy neurites (Trojanowski and Lee, 2001). Other synucleinopathies include dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) (Jellinger, 2003b).

In dementia, cerebral multi-morbidity is common and increases with age (Jellinger and Attems, 2011), with over 50% of cases exhibiting pathological lesions associated with more than one neurodegenerative disease (Kovacs et al., 2013). AD often presents with cerebrovascular disease, LB pathology, transactivation response DNA binding protein 43 kDa (TDP-43) pathology, argyrophilic grain disease (AGD) and hippocampal sclerosis (Nagy et al., 1997, Attems et al., 2014). Co-morbid AD pathology is also commonly observed in DLB (Walker et al., 2015).

1.1.1 Dementia with Lewy bodies

DLB is a common form of neurodegenerative dementia, second only to AD in terms of prevalence, accounting for approximately 15-20% of all neuropathologically defined cases (McKeith et al., 2005). The prevalence of DLB in the whole population over 65 is 0.36%, 1 in 270 people, or 4.2% of all dementia cases, with 7.5% observed clinically (Vann Jones and O'Brien, 2014). The incidence of DLB reaches 0.1% for the general population and 3.2% annually for all new dementia cases (Zaccai et al., 2005). Cognitive fluctuations, parkinsonian extra-pyramidal symptoms, recurrent complex visual hallucinations and rapid eye movement (REM) sleep behaviour disorder are the core clinical features of DLB (McKeith et al., 2017). Three indicative biomarkers are used clinically in the diagnosis of DLB: reduced dopamine transporter uptake in basal ganglia using single photon emission computed tomography (SPECT) or positron emission tomography (PET); abnormal ^{123}iodine meta-iodobenzylguanidine (MIBG) myocardial scintigraphy; as well as polysomnographic confirmation of REM sleep without atonia. A ‘probable DLB’ diagnosis can be made if two or more core symptoms, or one core symptom and one or more indicative biomarkers are present, whereas ‘possible DLB’ may be diagnosed if one core or one indicative biomarker is present in isolation (McKeith et al., 2017).

1.1.1.1 Core clinical symptoms of DLB

1.1.1.1.1 Cognitive fluctuations

Fluctuating cognition refers to spontaneous alterations in arousal, attention and cognition, which can be marked by periods of reduced awareness to external stimuli and changes in

responsiveness to one's surroundings, followed by normal, or near normal functioning (Ballard et al., 2001, McKeith et al., 2017). Cognitive fluctuations are a core symptom of DLB affecting 90% of cases, compared to 29% of PDD and 20% of AD cases (Ballard et al., 2002b, Walker et al., 2000, McKeith et al., 2005). DLB patients experience cognitive fluctuations more frequently than AD, where fluctuations are different in nature. These episodes of fluctuating cognition are typically associated with decreased executive and perceptual performance, confusion and difficulties in communication, whereas in AD they are primarily linked to memory impairment (Walker et al., 2000, Bradshaw et al., 2004).

Cognitive fluctuations are likely to arise from functional instability of specific neural networks, rather than structural abnormalities (Taylor et al., 2013). In DLB, cognitive fluctuations are closely related to attentional dysfunction (Ballard et al., 2001), with dysfunction of attention–executive networks being implicated in the aetiology of cognitive fluctuations in DLB (Peraza et al., 2014).

Cholinergic dysfunction in DLB may also contribute to manifestation of fluctuating cognition, and cholinergic abnormalities are more profound in DLB compared to AD (Perry et al., 1994). Cholinergic deficits often occur early in DLB, with the temporal cortex more susceptible to reductions in choline acetyltransferase activity, whereas AD patients experience cholinergic loss in the later stages of the illness, with the frontal and parietal cortices affected the most (Tiraboschi et al., 2002). In DLB patients, improvements in attention are observed following treatment with the cholinesterase inhibitor rivastigmine (McKeith et al., 2000), further supporting cholinergic deficiency in fluctuating cognition in DLB.

1.1.1.1.2 Parkinsonism

Parkinsonism is the predominant clinical feature of PD, which can manifest as resting tremor, bradykinesia, rigidity, mask-like face, stooped posture and shuffling gait (Gibb and Lees, 1988). Parkinsonian extra-pyramidal symptoms also affect about 70% of DLB patients (Aarsland et al., 2001a). Subtle differences in motor symptoms exist between DLB and PD patients. DLB patients mainly present with a motor phenotype primarily involving gait and postural difficulties, with very few DLB patients experiencing resting tremor compared to PD (Aarsland et al., 2001b, Burn et al., 2003). PDD patients often experience more severe motor features compared to DLB, with differentiation of DLB from PDD based on the timing of dementia and parkinsonian symptoms, whilst in PDD the presentation of extrapyramidal symptoms precedes the cognitive symptoms by at least a year (McKeith et al., 2005). AD

patients can also experience extrapyramidal symptoms, which often increase with disease progression (Tosto et al., 2015a). Parkinsonian features such as bradykinesia and rigidity are thought to be mediated by dopamine deficiency in PD and DLB (Zetusky et al., 1985). Gait and postural instability are more commonly observed in DLB patients than in PD, which are thought to be associated with cholinergic loss (Burn et al., 2003, Burn et al., 2006).

1.1.1.1.3 Visual hallucinations

Recurrent complex visual hallucinations (VH) occur in up to 80% of DLB cases (McKeith et al., 2017). The presence of VH in the early stages of the disease distinguishes DLB from other dementias (Fenelon et al., 2000). Recurrent complex VH in DLB manifest as well formed, complex vivid images of objects, animals and people (Burghaus et al., 2012). Gender differences in the context of VH have been observed, which might be related to personal experiences, with women more likely to hallucinate children, family members and pets, whereas men are more likely to hallucinate 'machines' (Urwyler et al., 2016). In AD, VH occur in 25% of patients (Ballard et al., 1999), which are often brief and simple, such as seeing shadows and patterns, and are often associated with greater cognitive impairment and in more advanced stages of the dementia (Hope et al., 1999). Around 20% of PD patients also experience VH, which are simple and non-threatening, involving the perception of movement or a presence in the peripheral visual field. PD patients can also experience auditory hallucinations, which are neutral in nature, unlike the threatening auditory hallucinations characteristic of schizophrenia (Fenelon et al., 2000). VH have also been observed in cognitively normal blind patients, known as Charles Bonnet syndrome (CBS). The content of VH in CBS often involves people, animals, plants and inanimate objects, which can occur in colour or black and white, more frequently occurring in the evening or at night, suggesting that sensory deprivation or low arousal (sitting or otherwise resting) are facilitating factors (Teunisse et al., 1996, Ffytche, 2005).

Although abnormalities in saccadic eye movements, visual perception and pupil reactivity are frequently observed in DLB patients (Armstrong, 2012), and may result from pathology in the visual system (Diederich et al., 2014), the absence of major pathological changes in the primary visual cortex (Khundakar et al., 2016), as well as preservation of the lateral geniculate nucleus (Erskine et al., 2016), suggests that VH in DLB might be due to an impairment in 'top down' visual processing. DLB patients with VH show higher burden of Lewy body pathology in the inferior temporal, parahippocampal gyrus and amygdala compared to PDD, involved in visual

perception (Harding et al., 2002). Impairment in perception and attention in DLB patients may stem from cholinergic dysfunction (Collerton et al., 2005), with cholinergic deficits in DLB strongly correlating with VH severity and frequency, and improvement of VH with cholinesterase inhibitor treatment (Marra et al., 2012).

1.1.1.1.4 REM sleep behaviour disorder

REM sleep behaviour disorder (RBD) is a sleep disturbance characterised by a loss of normal skeletal muscle atonia with prominent motor activity and dreaming (Ferman et al., 2002, McKeith et al., 2017). Patients with RBD act out their dreams, often with violent or injurious behaviour during sleep. It can result in loud vocalisations, screaming and talking, as well as thrashing, punching, kicking and even falling out of bed, which can cause self-injury or injury to their bed partners (Schenck et al., 2013).

RBD is often associated with α-synuclein accumulation and is considered a prodromal symptom of many synucleinopathies, such as DLB, affecting 76% of DLB cases, as well as PD, MSA and pure autonomic failure (Schenck et al., 2013, Boeve et al., 1998, Ferman et al., 2002). RBD is not as common in tauopathies, such as AD and PSP, and tends to occur concurrently with or after the onset of dementia or parkinsonism (Boeve, 2010).

Sleep-related brainstem nuclei, the peri–locus ceruleus, pedunculopontine nucleus (PPN) and laterodorsal tegmental nucleus, are key structures in modulating REM sleep (Chan et al., 2018). Cholinergic neurotransmission is known to be severely impaired in DLB due to dysregulation of cholinergic neurones in the PPN and nucleus basalis of Meynert (NBM), possibly contributing to the development of RBD (Kasanuki et al., 2018, Chan et al., 2018). LB related pathological changes and neuronal loss in the brainstem occurs early in DLB and PD (Braak et al., 2002), with post-mortem RBD cases showing predominant LB pathology in brainstem and limbic areas (Boeve et al., 2004). Early brainstem nuclei degeneration in synucleinopathies therefore might explain earlier occurrence of RBD in DLB and PD (Boeve et al., 2004).

1.1.1.2 Supportive symptoms of DLB

In addition to the core clinical features of DLB, there are several supportive features that may assist with a clinical diagnosis of DLB. Supportive symptoms of DLB, such as severe sensitivity to neuroleptics, repeated falls, syncope, postural instability, autonomic dysfunction, hypersomnia, hyposmia, delusions, apathy, anxiety and depression are very common, although they lack diagnostic specificity (McKeith et al., 2017).

DLB patients are particularly vulnerable to developing neuroleptic sensitivity, which occurs in 30-50% of patients, even when exposed to low doses (Ballard et al., 1998). This can result in sudden sedation, rigidity, immobility, confusion, as well as death (Aarsland et al., 2005). Severe neuroleptic sensitivity has also been observed in 27% of PD and 39% of PDD patients, although it is rare in AD (Aarsland et al., 2005). Neuroleptic drugs, also known as antipsychotics, are used to manage psychosis, principally in schizophrenia, as well as mania and delusions (Aarsland et al., 2005, Ballard et al., 1998), and typically act by inhibiting dopaminergic D2 receptors (Madras, 2013). DLB patients show a reduction in striatal D2 receptors, therefore neuroleptic medication may exacerbate striatal dopaminergic deficits further (Piggott et al., 1999).

Autonomic dysfunction is a common feature in DLB and PD, with abnormalities in autonomic function often observed in prodromal stages of the disease (Postuma et al., 2013). Autonomic dysfunction can present as urinary incontinence, observed in 80% of patients with LB disorders, sexual dysfunction, affecting 75% of patients with PD, and 30% with DLB, constipation, affecting 90% of PD and 83% of DLB patients, and orthostatic hypotension, affecting 30-50% of patients with PD, with a higher prevalence in DLB, reaching 50-60% (Allan et al., 2007, Palma and Kaufmann, 2018, Thaisetthawatkul et al., 2004, Horimoto et al., 2003). Repeated falls and syncope are also partly attributable to the presence of autonomic dysfunction (Stubendorff et al., 2012). The prevalence of falls in dementia is 8 times higher than in older people without dementia, with a prevalence of 90% in PDD, 77% in DLB, 47% in AD and 47% in VaD (Allan et al., 2009), and can cause significant injuries, institutionalisation, increased morbidity and mortality (Shaw, 2002). In DLB, syncopal attacks with complete loss of consciousness and muscle tone are thought to be associated with LB pathology in the brainstem (McKeith, 2004).

Depression, anxiety, apathy and delusions are the most common neuropsychiatric symptoms in DLB, which may assist in diagnosis. Depression affects 50-80% of DLB and 40% of PD patients (Stefanova et al., 2000, Yamane et al., 2011). The incidence of depression is much greater in DLB patients compared to AD (Chiu et al., 2017), with depression often present at prodromal stages of DLB (Fujishiro et al., 2015). Depression in dementia is associated with poor quality of life, increased morbidity and a more rapid progression of dementia (Barca et al., 2010, Lenze et al., 2005, Starkstein et al., 1992). Depression often goes undetected and undiagnosed (Baller et al., 2010) due to the overlapping symptoms of dementia and depression as well as other comorbid disorders (Kallenbach and Rigler, 2006). Anxiety and apathy are

frequent symptoms in DLB and PD patients, and often co-exist with depression (Brown et al., 2011, Shiba et al., 2000, Wen et al., 2016, Breitve et al., 2016). Delusions are also common neuropsychiatric symptoms in DLB, presenting in 65% of patients at some point during the illness, often occurring as recollections of hallucinations and perceptual disturbances that can have complex and bizarre content. In contrast, delusions in AD patients often present as mundane ideas based on forgetfulness and distorted or misinterpreted memories (McKeith et al., 2004, Tzeng et al., 2018).

1.1.1.3 DLB biomarkers

The revised DLB clinical diagnostic criteria refer to several indicative and supportive biomarkers for differential clinical diagnosis of DLB (McKeith et al., 2017). Dopamine transporter (DAT) imaging is used to detect nigro-striatal dopaminergic neurodegeneration associated with PD and DLB (Seifert and Wiener, 2013). DAT imaging shows 78% sensitivity and 90% specificity in differential diagnosis of DLB (McKeith et al., 2007), with DLB patients showing reduced dopamine transporter (DAT) uptake in the putamen compared to AD using SPECT imaging that correlates with autoradiographic evidence of reduced DAT in post-mortem tissue (Colloby et al., 2012b, Piggott et al., 1999). While DLB and PD show similar levels of striatal DAT loss, the differences arise in the subregional pattern of striatal dopamine depletion, where selective degeneration of ventrolateral SN neurones is seen in PD. This may explain the differences in clinical phenotypes between DLB and PD (Walker et al., 2004).

Cardiac postganglionic, presynaptic sympathetic denervation is common in Lewy body disorders (LBD), and can be detected using ^{123}Iodine-MIBG myocardial scintigraphy. DLB patients show extensive reduction in cardiac ^{123}Iodine-MIBG uptake, which is indicative of cardiac sympathetic neuronal loss (McKeith et al., 2017, Oide et al., 2003, Yoshita et al., 2015). It shows 69% sensitivity and 87% specificity for discriminating probable DLB from probable AD (Yoshita et al., 2015), as well as PD from MSA, which is associated with preganglionic sympathetic dysfunction (Druschky et al., 2000).

Polysomnography (PSG) is a sleep study methodology, used to diagnose sleep disorders, by recording the electroencephalogram (EEG), oxygen level in the blood, heart rate, breathing, eye and leg movements during sleep. PSG confirmation of REM sleep without atonia is a highly specific predictor of DLB (McKeith et al., 2017). A positive confirmation of PSG in a patient with dementia and a history of RBD is sufficient for a probable DLB diagnosis (Boeve et al., 2013).

Other supportive biomarkers can also aid in diagnostic evaluation but lack specificity in diagnosis of DLB. Structural magnetic resonance imaging (MRI) can be useful in detecting the medial temporal lobe atrophy (MTA) typically observed in AD, with DLB patients showing relative preservation of the medial temporal lobe (Burton et al., 2009). MTA represents loss of volume in the hippocampus and entorhinal cortex associated with neurofibrillary tangle pathology (Braak and Braak, 1991). Structural imaging can also be useful for the exclusion of other possible causes of dementia such as gliomas, subdural hematomas, vascular malformations and normal pressure hydrocephalus (Harper et al., 2014).

Functional imaging reflects cerebral metabolic activity and helps to identify characteristic patterns of hypometabolism associated with different neurodegenerative diseases using fluorodeoxyglucose (FDG)-PET and SPECT. Occipital metabolism/perfusion is a supportive biomarker in DLB diagnosis, showing reduced metabolism in DLB patients compared to AD (Ishii et al., 1998). Relative preservation of the posterior cingulate cortex (PCC) metabolism compared to precuneus and cuneus, called the cingulate island sign (CIS), is commonly observed in DLB compared to AD patients, and is another supportive biomarker in diagnosis of DLB (Graff-Radford et al., 2014). Lower pathological neurofibrillary tangle (NFT) burden in DLB compared to AD has been suggested to be a correlate of the CIS in DLB (Graff-Radford et al., 2014).

EEG changes have been reported in DLB, showing predictive value of 90% for the diagnosis of DLB compared with AD (Bonanni et al., 2008, McKeith et al., 2017). EEG provides a functional measure of neuronal and synaptic integrity. DLB patients show pronounced slow-wave activity, with increased delta and theta power in posterior occipital and parietal regions (van der Zande et al., 2018). These abnormalities positively correlate with severity of cognitive fluctuations, and may be useful in the differential diagnosis of DLB and AD even in the earliest stages of dementia (Bonanni et al., 2008, Ferman et al., 2011).

In vivo imaging of amyloid and tau has been useful in identifying early pathological changes, and can help predict disease progression and treatment response based on the presence of multiple pathologies (McKeith et al., 2017). The C^{11} Pittsburgh compound B (PiB), which demonstrates high affinity and selective amyloid-beta (Aβ) binding, has been suggested to predict the progression of mild cognitive impairment (MCI) to AD (Villemagne et al., 2011b). PiB retention is suggested to be lower in DLB than in AD patients and shows high specificity (Kantarci et al., 2019). Other novel fluorine 18–labelled (^{18}F) PET tracers, ^{18}F-florbetapir, ^{18}F-

florbetaben and ^{18}F-flutemetamol, which bind to fibrillar amyloid-β aggregates, have also shown high sensitivity and specificity for diagnosis of AD (Fleisher et al., 2011, Vandenberghe et al., 2010, Duara et al., 2013, Villemagne et al., 2011a, Schipke et al., 2012). PET ligands showing high affinity for NFT aggregates, such as ^{18}F-MK-6240 PET and ^{18}F-T807 show minimal off-target binding in the human brain, and may be useful in differential diagnosis of different tauopathies (Betthauser et al., 2019, Wooten et al., 2017), and can be useful in identifying DLB due to reduced retention compared to AD patients (Kantarci et al., 2017).

While cerebrospinal fluid (CSF) or blood α-synuclein biomarkers for DLB are not currently available, some studies have shown an increase in plasma oligomeric α-synuclein in DLB and PD (El-Agnaf et al., 2006), as well as total α-synuclein in PD and MSA compared to controls (Lee et al., 2006). Total α-synuclein levels in CSF were shown to be significantly lower in patients with PD (Tokuda et al., 2006) and DLB compared to controls (Wennstrom et al., 2012). CSF biomarkers of AD, including Aβ 1–42 (reflecting amyloid burden), total-tau (reflecting neuronal degeneration) and phospho-tau (reflecting neurofibrillary tangle density), can be useful in determining concomitant AD pathology or predicting cognitive decline in DLB (Hansson et al., 2006). Decreased levels of CSF Aβ 1-42 have been observed in PD patients (Alves et al., 2010), associated with faster cognitive deterioration and possible transition to PDD (Alves et al., 2014). Total tau CSF levels were shown to be higher in DLB patients compared to PD, PDD and controls, but lower than in AD (Parnetti et al., 2008, Parnetti et al., 2011).

1.1.1.4 Treatment of DLB

Due to the many facets of DLB, treatment is often complex and challenging, and while there are no treatments that slow the progression of disease, symptomatic treatments for motor, cognitive, neuropsychiatric, autonomic and sleep dysfunction can be effective in improving the patient's quality of life (Taylor et al., 2019). Non-pharmacological approaches can be used to improve the quality of life in patients with DLB, and exercise-based interventions have also been suggested to be beneficial in improving cognition and motor symptoms (Uc et al., 2014).

The efficacy of deep brain stimulation (DBS) in treating cognitive impairment in PDD has been shown using bilateral stimulation of the subthalamic nucleus (STN) and the nucleus basalis of Meynert (NBM), although has not been applied to DLB (Freund et al., 2009). Pharmacological treatment of cognitive symptoms with the cholinesterase inhibitors, rivastigmine and donepezil has been shown to improve cognition in DLB patients (McKeith et al., 2000, Rowan et al.,

2007), whereas memantine, an N-methyl D-aspartate (NMDA) receptor antagonist, has also shown efficacy in treating cognitive symptoms in DLB and PDD patients (Aarsland et al., 2009).

As in PD, levodopa (L-DOPA)/carbidopa treatment for motor symptoms in DLB has proven the most effective (Seppi et al., 2011). While L-DOPA compensates for the decrease in endogenous dopamine in the striatum, an increase in the levels of dopamine in the nucleus accumbens (NAcc), limbic regions and prefrontal cortex (PFC) can result in the exacerbation of neuropsychiatric and cognitive symptoms in DLB patients (Lucetti et al., 2010). Levodopa/carbidopa treatment may be most useful to patients with prominent parkinsonism, but few or no neuropsychiatric symptoms (Goldman et al., 2008). Dopamine agonists are less likely to be used in DLB, as they can induce compulsive behaviours (Hassan et al., 2011).

Cholinesterase inhibitors have also been shown to improve neuropsychiatric symptoms in LBD, such as VH and delusions (McKeith et al., 2000), whereas clozapine has proven to be effective in treatment of PD associated psychosis (Connolly and Fox, 2014, Seppi et al., 2011). The evidence for antidepressant efficacy in treatment of depression in DLB is sparse. The selective serotonin reuptake inhibitors (SSRI) and serotonin and norepinephrine reuptake inhibitors (SNRI) for treatment of depression in PD are well tolerated, although they lack efficacy (Starkstein and Brockman, 2017), whereas tricyclic antidepressants (TCAs), nortriptyline and desipramine, have been shown to be efficacious for the treatment of depression or depressive symptoms in PD (Liu et al., 2013). Reduction of depressive symptoms in LBD patients has been observed following treatment with electroconvulsive therapy (ECT), as well as repetitive transcranial magnetic stimulation (rTMS) (Takahashi et al., 2009). Symptoms associated with autonomic dysfunction, such as constipation and postural hypotension respond well to standard medications (Seppi et al., 2011).

1.1.2 Neuropathological features of DLB

1.1.2.1 α-synuclein

1.1.2.1.1 α-synuclein structure

α-synuclein is a 140 amino acid (AA) protein encoded by the *SNCA* gene. Alpha-synuclein has an N-terminal lipid-binding α-helix, a non amyloid-beta component (NAC) domain and a C-terminal acidic tail (Figure 1.1) (Chiba-Falek, 2017). The N-terminus of α-synuclein is a

positively charged region, with 11-AA repeats that contain a highly conserved KTKEGV motif, which is also present in the α-helical domain of apolipoproteins. These repeat sequences enable α-synuclein to form a helical structure, which is important in α-synuclein lipid interactions (Sode et al., 2006), with point mutations associated with synucleinopathies located in this region. The C-terminal domain of α-synuclein is an acidic tail of 43-AA residues, which is largely unstructured (Davidson et al., 1998, Ulmer et al., 2005) and a target of various post-translational modifications (Oueslati et al., 2010). The NAC domain (AA61-95) of α-synuclein was first observed in amyloid plaques associated with AD (Ueda et al., 1993), and is believed to be responsible for α-synuclein aggregation (Alexopoulos et al., 2013). The C-terminal interaction with the NAC region, especially the phosphorylation of serine 129 residue, is thought to be responsible for inhibition of α-synuclein aggregation (Esposito et al., 2007).

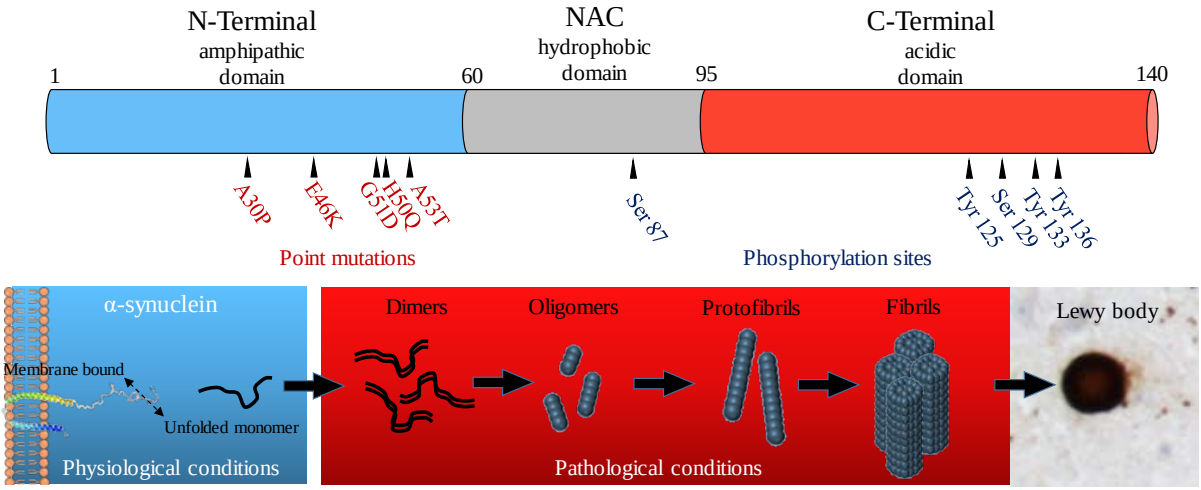

Figure 1.1 α-synuclein structure.
(Top) Schematic representation of α-synuclein protein. The N-terminal domain (amino acids 1–60) is responsible for α-synuclein-membrane interactions, with point mutations located in this region (A30P, E46K, G51D, H50Q and A53T), associated with PD. The central non-β amyloid component (NAC) hydrophobic region (amino acids 61-95) is required for the aggregation and formation of amyloid fibrils. The acidic C-terminal (amino acids 96–140) is largely unstructured and has a number of phosphorylation sites (tyrosine 125, 133, 136 and serine 129). **(Bottom)** α-synuclein aggregation. Under physiological conditions free cytosolic α-synuclein can exist as a monomer or conformation specific membrane bound. Under pathological conditions soluble α-synuclein monomers associate to form oligomers, which further generates protofibrils (immature fibrillar aggregates), then fibrils, which leads to the formation of large and insoluble aggregates, known as Lewy bodies and Lewy neurites. Adapted from (Jones et al., 2014).

The natively unfolded monomeric α-synuclein, approximately 14 kDa, is present in the cytosol, and on binding to phospholipids it acquires an α-helical structure, which suggests that α-

synucleins functions are based on different cellular locations (Ahn et al., 2002). α-synuclein has also been shown to exist as a helically folded tetramer when isolated from red blood cells (Bartels et al., 2011).

1.1.2.1.2 α-synuclein function

α-synuclein is predominantly expressed in the central nervous system (CNS) and synthesised in neuronal cell bodies. The physiological function of α-synuclein is poorly understood, with deletion of *SNCA* gene in mice showing no major abnormalities (Abeliovich et al., 2000a). While initially described as a nuclear protein (Mori et al., 2002, Yu et al., 2007), presynaptic localization of α-synuclein has become well established (Jakes et al., 1994), with studies also showing the presence of extracellular α-synuclein (El-Agnaf et al., 2003). α-synuclein facilitates exocytosis by pooling and trafficking synaptic vesicles and regulating SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein assembly (Garcia-Reitbock et al., 2010). The SNARE proteins assemble into a four-helix bundle to facilitate the exocytotic release of neurotransmitters into the synaptic cleft, through interaction with other proteins, including α-synuclein, which supports exocytosis by maintaining the SNARE complex assembly (Burre et al., 2010, Sharma et al., 2011). α-synuclein involvement in neurotransmitter release is dependent on the function of the SNARE protein complex (Bennett, 1994), via direct interaction with synaptobrevin II (Figure 1.2), a major component of the SNARE complex (Burre et al., 2010). α-synuclein knockout mice show a significant decrease in SNARE complex proteins (Burre et al., 2010), as well as exaggerated levels of dopamine release following stimulation, which suggests that α-synuclein is involved in modulation of dopamine neurotransmission (Abeliovich et al., 2000b). Overexpression of α-synuclein has been linked to changes in vesicular monoamine transporter-2 (VMAT-2) activity (Guo et al., 2008), an increase in oxidative stress (Chen et al., 2008) and degeneration of dopaminergic neurones (Gonzalez-Hernandez et al., 2004, Yamamoto et al., 2006). This is supported by a decrease in vesicular dopamine storage followed by degeneration of nigral dopamine neurones with α-synuclein overexpression (Ulusoy et al., 2012). α-synuclein has also been shown to be implicated in glutamatergic neurotransmission, where pathological α-synuclein oligomer application to hippocampal slices results in a decrease in long-term potentiation (Diogenes et al., 2012).

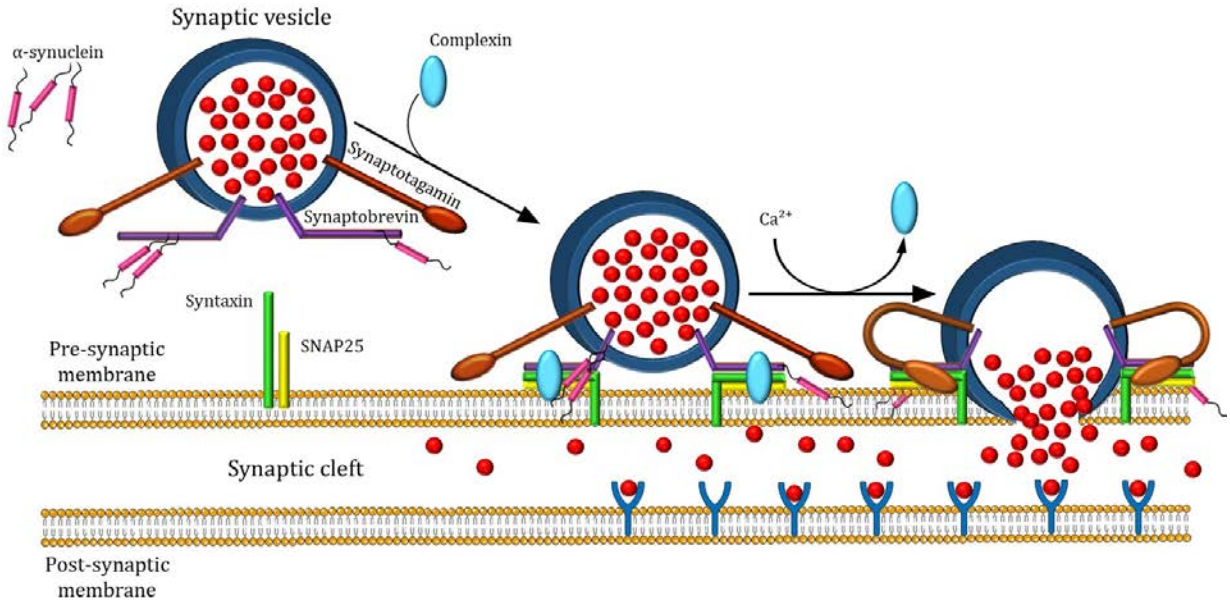

Figure 1.2 The SNARE complex.
The core SNARE complex is a four α-helical structure necessary in vesical fusion, which is facilitated by synaptobrevin, syntaxin, SNAP-25 and synaptotagmin, that come together to form a coiled-coil motif. Neurotransmitter release involves a series of events, that requires translocation of proteins in the membrane, disruption of the lipid bilayer, fusion pore formation, followed by neurotransmitter release into the synaptic cleft.

1.1.2.1.3 α-synuclein aggregation

Under pathological conditions, the unstructured soluble α-synuclein protein can assemble into amyloid aggregates, forming intracellular inclusions known as LB, which are characteristic of α-synucleinopathies (Spillantini et al., 1997). Several mechanisms have been suggested to be involved in α-synuclein aggregation and the pathogenesis of the α-synucleinopathies, and include changes in the ubiquitin-proteasome system (Emmanouilidou et al., 2010), mitochondrial dysfunction (Elkon et al., 2002) and production of reactive oxygen species (Junn and Mouradian, 2002), that can eventually lead to neuronal dysfunction and death (Cookson and van der Brug, 2008). The α-synuclein aggregation process follows a nucleation-dependent pattern, where monomers assemble to form aggregation nuclei, followed by the formation of oligomers, intermediates of the aggregation process that are thought to be the most potent neurotoxic species of α-synuclein (Karpinar et al., 2009, Winner et al., 2011), causing a wide range of damaging effects (Figure 1.1). Other studies have demonstrated that α-synuclein fibrils can also induce toxicity (Braak et al., 2002, Volpicelli-Daley et al., 2011b). The formation of oligomeric and insoluble fibrillar α-synuclein species may be initiated, enhanced and

accelerated by *SNCA* gene mutations, with point mutations of A30P, A53T and E46K implicated in familial PD (Zarranz et al., 2004, Kruger et al., 1998, Polymeropoulos et al., 1997). Post-translational α-synuclein modifications, i.e. phosphorylation of Ser-129 (Anderson et al., 2006), as well as overexpression (Yamada et al., 2004), can also promote α-synuclein aggregation.

1.1.2.1.4 α-synuclein pathology in DLB

The pathological hallmark of DLB is the loss of nigrostriatal dopaminergic neurones, induced by the aggregation of α-synuclein protein, which is a major component of LB and abnormal neuronal aggregates known as Lewy neurites (LN) (Spillantini et al., 1997). LB are typically intracytoplasmic eosinophilic inclusions with a dense core, consisting of dense granular material, which is surrounded by pale halo, composed of radiating filaments about 13 nm in diameter (Burkhardt et al., 1988, Galloway et al., 1992). Typically, LB are found in brainstem, limbic and neocortical brain regions, but also in the peripheral autonomic ganglia (Fumimura et al., 2007, McKeith et al., 2005). Compared to the brainstem LB, which are typically spherical, cortical LB are less defined and without a halo, with highest densities observed in the limbic regions, such as amygdala, cingulate, insula and entorhinal cortex (Burkhardt et al., 1988). The major components of LB and LN are α-synuclein (Spillantini et al., 1997), ubiquitin (Kuzuhara et al., 1988) and neurofilaments (Schmidt et al., 1991). Recent proteomic studies have revealed complex molecular composition of cortical LB, identifying just under 300 proteins, with 32% of identified proteins known to be involved in signal transduction and apoptosis, 19% in cytoskeletal function, 14% in metabolic activity, 10% in extracellular matrix formation, 8% in protein synthesis and degradation, 4% in neurotransmission and inflammation, with 11% of proteins of unknown function (Leverenz et al., 2007).

The progression of α-synuclein pathology in DLB is thought to correspond to the stages of PD pathology spread. According to the staging system proposed by Braak, the ascending progression of α-synuclein pathology is initiated in the lower brainstem (dorsal motor vagal nucleus and intermediate reticular zone) , which then spreads into upper brainstem (raphe, locus ceruleus (LC) and substantia nigra (SN)) and the basal forebrain, eventually affecting amygdala, thalamus, hypothalamus and ultimately neocortex (Figure 1.3) (Braak et al., 2002).

Pathological diagnosis of DLB requires the presence of limbic or neocortical LB pathology (McKeith et al., 2005). While the initial topographical spread of LB pathology in DLB and PDD patients may be different, with higher burden of LB pathology in the neocortical parietal

and temporal cortex observed in DLB cases, in the terminal stages of the disease they are indistinguishable (Ruffmann et al., 2016). DLB cases with higher neocortical compared to brainstem α-synuclein pathology often present with more severe dementia, whereas more severe presentation of parkinsonism has been associated with predominantly brainstem α-synuclein pathology (Yamamoto et al., 2005).

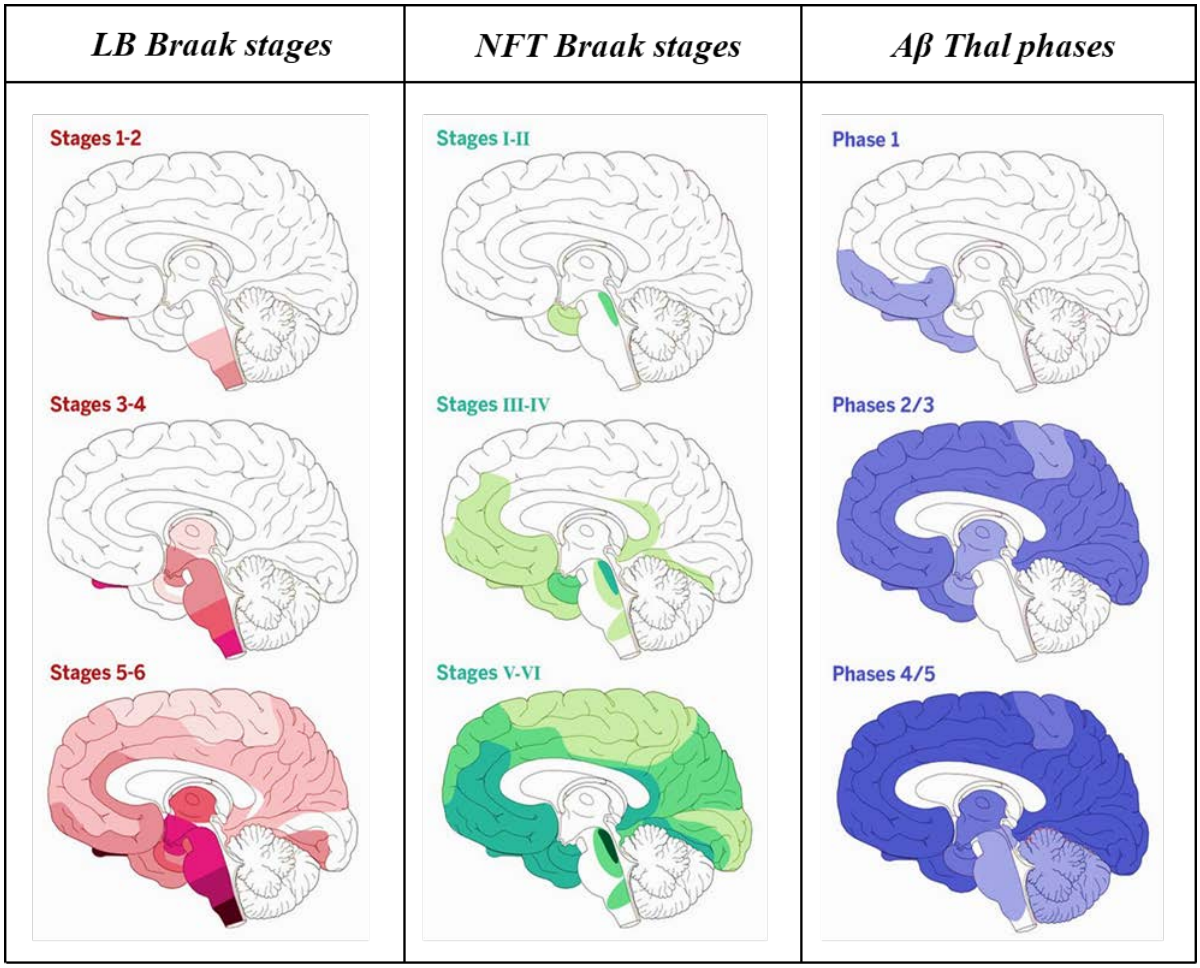

Figure 1.3 Propagation of α-synuclein, tau and Aβ in human brain
(**Left**) α-synuclein pathology ascends from the brainstem. LB and LN initially present in the olfactory bulb and the dorsal motor vagal nucleus and glossopharyngeal nerves of the medulla oblongata (stages 1 and 2), then spread to the pons, midbrain and basal forebrain (stages 3 and 4), followed by the neocortex (stages 5 and 6). (**Middle**) Tau pathology initially develop in the locus coeruleus, transentorhinal and entorhinal regions (stages I and II), followed by hippocampal formation and some parts of the neocortex (stages III and IV), eventually affecting the neocortex by large (stages V and VI). (**Right**) Aβ plaques are firstly observed in the basal temporal and orbitofrontal neocortex (phase 1), then in hippocampus, amygdala, diencephalon, basal ganglia and neocortex (phases 2 and 3), with severe AD cases showing Aβ plaques in mesencephalon, lower brainstem, and cerebellum (phases 4 and 5). Adapted from (Goedert, 2015).

Neurodegeneration along specific pathways is assumed to be due to prion-like spread of misfolded proteins through anatomically interconnected brain regions (Ahmed et al., 2016, Danzer et al., 2012, Hansen et al., 2011, Luk et al., 2012). Grafted foetal nigral neurones in PD patients show LB inclusions years after transplantation, suggesting that physical contacts between susceptible regions, axonal transport and trans-synaptic transmission of misfolded and aggregated α-synuclein might have a role in the pathogenesis and propagation of PD (Kordower et al., 2008, Li et al., 2008). Therefore, specific clinical symptoms in LBD patients may be due to accumulation of misfolded proteins in different brain regions within specific anatomical pathways.

1.1.2.2 Concomitant pathologies in DLB

Multiple pathologies are not uncommon in the aged brain (Kovacs et al., 2013), with mixed dementia diagnosis typically given when pathological diagnostic criteria for more than one disorder has been met (Jellinger and Attems, 2007, Walker et al., 2015). In addition to α-synuclein pathology, 50–80% of patients with DLB have concomitant AD pathology (Halliday et al., 2011) in the form of NFT, neuropil threads (NT), Aβ plaques and Aβ neuritic plaques (NP), (McKhann et al., 2011). NFT are intraneuronal aggregates in cell bodies, whereas NT present as axonal and dendritic segments containing aggregated and hyperphosphorylated tau (Braak and Braak, 1991). The primary site for NFT detection is the trans-entorhinal cortex, later showing abundancy in hippocampus and amygdala, with final stages of NFT spread reaching neocortex and ultimately involving primary motor and sensory areas (Hyman et al., 2012) (Figure 1.3). In AD, Aβ deposition during the disease course follows five stages, firstly affecting only neocortical brain regions, followed by allocortex, then basal ganglia, basal forebrain, and finally the cerebellum and brainstem nuclei (Figure 1.3) (Thal et al., 2002). While the presence of NFT or Aβ is not necessary for a neuropathological diagnosis of DLB (McKeith et al., 2005), concomitant AD type pathology is thought to contribute to faster cognitive decline in DLB (Howlett et al., 2015). α-synuclein, tau and Aβ have been shown to promote the accumulation of one another (Giasson et al., 2003, Kotzbauer et al., 2004), with induction of Aβ in vivo shown to enhance the aggregation of α-synuclein, suggesting that it may accelerate the progression of LBD (Masliah et al., 2001).

The frequency of vascular pathologies increases with age, and vascular pathology is also commonly observed in DLB cases, which show more severe cerebrovascular lesions compared to PD (Jellinger and Attems, 2008). In DLB cases, cerebral amyloid angiopathy (CAA) is more

commonly observed when compared with PD (De Reuck et al., 2013, Jellinger, 2010), and shows a significant increase in total mini-bleeds compared to control cases (De Reuck et al., 2013). DLB cases also present with more extensive white matter hyperintensities (WMH) compared to PDD, PD and controls (Joki et al., 2018). In PD, WMH were shown to be associated with more severe gait problems, bradykinesia and postural instability (Piccini et al., 1995).

The characteristic neuropathological substrate of FTD, TDP-43, has been observed in DLB, AD and control cases (McAleese et al., 2017). TDP-43 positive inclusions are observed in 45% of DLB cases, predominantly within the amygdala, hippocampus, dentate gyrus, entorhinal, occipito-temporal and inferior temporal cortices (Higashi et al., 2007). A similar distribution of TDP-43 has been observed in DLB and AD cases, with temporal cortex shown to be more severely affected in AD, whereas cingulate and insular cortices were unaffected in both groups (Higashi et al., 2007).

1.1.3 Major depressive disorder (MDD)

1.1.3.1 Diagnostic criteria

Depression is a leading cause of disability that impacts all aspects of everyday life (Steger and Kashdan, 2009). The lifetime prevalence of major depressive disorder (MDD) is 9.6% for men and 20.4% for women (Steffens et al., 2000). The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) defines MDD by one or more major depressive episodes and the lifetime absence of mania and hypomania. To meet the criteria for MDD, one of the symptoms must be depressed mood or anhedonia with, additionally, five of the nine symptoms present during the same two-week period and experienced ‘nearly every day’ (Table 1.1) (American Psychiatric Association, 2013). MDD defined as above is the type of depression for clinical research studies and trials, whereas broader definitions of depression are also frequently used based on symptom rating scales, which will be applied in this study.

Table 1.1 DSM-5 criteria for major depressive disorder

	DSM-5
A	Five or more out of nine symptoms (including at least one of depressed mood and loss of interest or pleasure) in the same 2-week period. Each of these symptoms represents a change from previous functioning. 1. Depressed mood (subjective or observed; *Most of the day, nearly every day*) 2. Loss of interest or pleasure (*Most of the day, nearly every day*) 3. Change in weight or appetite (*Weight: 5% change over month; Appetite: Nearly every day*) 4. Insomnia or hypersomnia (*Nearly every day*) 5. Psychomotor retardation or agitation (observed; *Nearly every day*) 6. Loss of energy or fatigue (*Nearly every day*) 7. Worthlessness or guilt (*Nearly every day*) 8. Impaired concentration or indecisiveness (*Nearly every day*) 9. Thoughts of death or suicidal ideation or attempt (*Thoughts: recurrent; Attempt: any*)
B	Symptoms cause significant distress or impairment.
C	Episode not attributable to a substance or medical condition [1, 2]
D	Episode not better explained by a psychotic disorder.
E	There has never been a manic or hypomanic episode [3]

[1]Criteria A–C represent a major depressive episode (MDE). [2]Clinical judgement is inevitably required to distinguish if MDE is present in addition to a normal response. [3]Exclusion E does not apply to a significant loss if (hypo)manic episode was substance induced or attributable to medical condition. Adapted from (American Psychiatric Association, 2013).

1.1.3.2 Treatment of depression

The first antidepressant drugs were discovered by accident and belong to a family of monoamine oxidase inhibitors (MAOI). Originally used to treat tuberculosis, the MAOI drug iproniazid was trialled in depressed patients, who showed substantial improvement in mood and increased interest in their surroundings following the treatment (Cole et al., 1959). Monoaminergic pharmacological agents are commonly used as a first line treatment option in moderate to severe depression and are based on the hypothesis that decreased mood and development of depression is due to reduced monoaminergic drive. The efficacy of early MAOI antidepressants was largely based on their serotonergic actions, which has led to the development of new generation of antidepressants, the SSRI (e.g. escitalopram, sertraline and fluoxetine). SSRI drugs proved to be much safer to use and showed reduced side effects compared to TCAs (Cryan and Leonard, 2000, de Montigny and Blier, 1984), and are still the most widely prescribed antidepressants (Dupuy et al., 2011). While SSRI antidepressants are better tolerated than TCAs, studies have shown no difference in their efficacy (Arroll et al., 2005, Geddes et al., 2000). Antidepressants with dual and triple action mechanisms, such as

SNRI (venlafaxine, levomilnacipran), norepinephrine-dopamine reuptake inhibitors (NDRI; bupropion), and serotonin-norepinephrine-dopamine reuptake inhibitors (SNDRI; amitifadine), are also used for treatment of depression (Papakostas et al., 2007).

Antipsychotic drugs have long been used in the treatment of MDD. The typical (first generation) antipsychotics were often used to treat severe depressive disorders accompanied with psychotic symptoms (Schatzberg and Rothschild, 1992a), but due to their side effects, such as extrapyramidal symptoms, tardive dyskinesia and neuroleptic malignant syndrome, are rarely used as a monotherapy. The atypical (second generation) antipsychotics show lower rates of side effects and less severe cognitive impairment, and in combination with other antidepressants have also been shown to improve the efficacy of treatment of psychotic depression, treatment resistant depression and as monotherapy for MDD (Chen et al., 2011, Schatzberg and Rothschild, 1992b, Kennedy et al., 2001).

In addition to pharmacological interventions, other treatments are effective in alleviating depression. Psychotherapies, especially cognitive behavioural therapy (CBT), are a safe and effective treatment for MDD (McMain et al., 2015), which is based on the premise that maladaptive information processing (repetitive negative thinking) has a causal role in depression. A study on neural mechanisms of CBT treatment for depression showed increased connectivity in attentional control networks following treatment (Yang et al., 2018).

ECT is a well-established treatment, proven to be effective in about 70% of patients with severe and treatment resistant depression (Kho et al., 2003). Restrictions to wide application of ECT in clinical settings are due to adverse effects on cognition and memory (Vasavada et al., 2017, Micallef-Trigona, 2014). rTMS is a non-invasive, safe neuromodulation therapy for MDD, supported by extensive clinical research (Janicak et al., 2008, Levinson et al., 2010, George et al., 2010). rTMS has been shown to have the same antidepressant efficacy as some antidepressants, with fewer adverse effects (Fregni et al., 2004). DBS was first used to treat movement disorders and recently has been extended to the treatment of psychiatric disorders. Neuroimaging has played an important role in identifying specific DBS targets. DBS has proved effective in producing antidepressant effects in treatment resistant depression patients (Lozano et al., 2008, Malone et al., 2009), with the subgenual anterior cingulate cortex (sgACC) (Holtzheimer et al., 2012, Choi et al., 2015, Berlim et al., 2014) and NAcc (Bewernick et al., 2010) being primary targets.

1.1.3.3 Late-life depression

MDD is very common throughout the life course (Kessler et al., 2005), but development of depression in later life may be a risk factor of early presentation of dementia, therefore the pathophysiological mechanisms that underlie depression in later life and neurodegeneration may differ greatly from MDD in early-life. Depression in later life has a high prevalence, with 1-5% of adults affected over 65 years of age (Fiske et al., 2009), and 17.1% of patients aged 75 years and older having been given a diagnosis of MDD (Luppa et al., 2012). MDD in later life shows a higher incidence of functional decline, decreased quality of life, morbidity and mortality (Fiske et al., 2009), and is associated with an increase in health care costs (Luppa et al., 2008). The presentation of depression in older adults differs somewhat from younger populations, with a higher prevalence of sleep disturbances, fatigue, loss of interest in daily activities, and a feeling of hopelessness about the future (Butters et al., 2004). Apathy is a common feature of late-life depression and a predictor of poor response to treatment and poor remission rates (Levkovitz et al., 2011).

Depression in neurodegenerative disorders is common, with variable prevalence depending on the screening method used (Barca et al., 2010, Vilalta-Franch et al., 2006). Studies using DSM-IV criteria showed that the prevalence of MDD in PD patients was 17% (Reijnders et al., 2008), in AD 12-42% (Chi et al., 2015, Vermeiren et al., 2015), whereas in DLB it is approximately 45-50%, and is more persistent than in AD (Fritze et al., 2011). Differences in depressive symptoms between DLB and AD have been observed, with pervasive anhedonia, sleep disruption and feelings of worthlessness being significantly higher in DLB patients (Chiu et al., 2017).

It is not clear whether depression is a risk factor for developing dementia or an additional feature of dementia. While early life depression has been associated with an increased risk of dementia, later life depression is suggested as a prodrome of dementia (Bennett and Thomas, 2014). MCI in later life may be associated with depression and shows underlying neuropathological changes (Tosto et al., 2015b). Cognitive deficits in later life depression are also prominent, which often persist despite remission of depressive symptoms (Kohler et al., 2010a). It is not clear whether impairments in memory, executive function and/or processing speed are the basis of cognitive impairment in later life depression. A study showed that information processing speed is impaired in patients with late onset depression, with a greater effect on executive functioning than on memory deficits (Kohler et al., 2010b).

1.1.3.4 Depression in DLB

Depression and anxiety are common prodromal psychiatric symptoms in DLB and also PD patients, and are associated with cognitive impairment (Fujishiro et al., 2015), faster cognitive decline (Boot et al., 2013), higher mortality rates and a poor response to treatment (Ritchie et al., 1998). These results are contradictory to a longitudinal study that found no clear association between anxiety and faster decline in cognition or dementia severity in DLB or AD patients (Breitve et al., 2016).

There are many challenges in detecting depressive symptoms in dementia patients. Depression in dementia can often go undiagnosed due to overlap of symptoms between the two disorders. Furthermore, dementia leads to reduced awareness of depression, therefore in the more advanced stages of dementia the use of self-reporting depression assessment scales, such as the Geriatric Depression Scale (GDS) and Montgomery–Åsberg Depression Rating Scale (MADRS) could be difficult. GDS has been shown to be less suitable for patients with severe dementia, but is considered to be applicable for patients with mild dementia (Korner et al., 2006). MADRS was developed for use in cognitively intact patients, where some symptoms can overlap with those found in dementia, and is also suitable for patients with mild dementia (Leontjevas et al., 2009). However, the Cornell Scale for Depression in Dementia (CSDD) is probably the most widely used tool to assess depression in dementia (Alexopoulos et al., 1988). It relies on an informant to confirm the responses of patients, and has been shown to be reliable and valid in various clinical settings (Barca et al., 2010, Towsley et al., 2012, Williams and Marsh, 2009, Jeon et al., 2016).

1.1.4 Pathophysiology of depression

1.1.4.1 Structural and functional abnormalities in depression

The neural circuits that mediate mood are not completely understood, although several brain regions have been identified. The clinical phenomena of MDD potentially involves many brain systems involved in the regulation of mood, anxiety, reward processing, attention, motivation and stress responses (Price and Drevets, 2012). Initial studies in MDD examined structural changes in the brain (size or volume), and identified relevant regions and networks potentially involved in the pathophysiology of depression. Certain regions, such as the medial, dorsal and orbitofrontal prefrontal networks are intimately involved in regulating different aspects of mood (Price and Drevets, 2012). The medial prefrontal network modulates visceral function in relation to emotion, and has extensive projections to the amygdala, hypothalamus and

brainstem. This prefrontal network also extensively overlaps with the anterior cingulate cortex (ACC), especially sgACC (Ongur et al., 2003). The dorsal prefrontal circuit is associated with regulating executive functions, including problem solving, organization and working memory, cognitive domains that are typically affected in MDD (Price and Drevets, 2010). The orbital prefrontal network has extensive connections with several sensory related cortical areas. Apart from sensory integration, it also appears to be critical in integration of mood and emotional information into behavioural response (Price and Drevets, 2012).

Patients with MDD show structural and functional abnormalities in several brain regions implicated in mood and emotional processing. Patients with late-life MDD show structural abnormalities in the dorsolateral prefrontal cortex (DLPFC), amygdala and cingulum (Knochel et al., 2015). Grey matter volume reductions are consistently observed in sgACC, orbital and ventrolateral PFC in MDD patients (Botteron et al., 2002, Drevets et al., 2008b, Price and Drevets, 2010). Reduction in glial density and neuronal size in layer VI, as well as GABAergic interneurone density in layer II was observed in the ACC in MDD (Cotter et al., 2002, Cotter et al., 2001). However, other studies found no changes in neuronal or glial cell density in orbitofrontal and ACC in later life depression (Khundakar et al., 2011a, Khundakar et al., 2011b). Reduced GABAergic interneurone density and pyramidal cell volume in DLPFC was observed in patients with depression (Rajkowska et al., 2007, Khundakar et al., 2009), which may suggest that loss of cortical projections from DLPFC to ACC may contribute to ACC dysfunction, as observed in MDD.

MDD patients show reduced cerebral blood flow and metabolism in PFC, ACC, PCC, amygdala, medial thalamus and parahippocampal cortex (Ongur et al., 2003). One functional imaging study has shown that apathy in late-life depression is associated with an impaired functional connectivity of the ACC with the DLPFC, as well as lower connectivity of the NAcc with amygdala and thalamus (Alexopoulos et al., 2013). This has been supported by another study showing altered functional and structural connectivity between prefrontal cortical and limbic regions in late-life depression (Yuen et al., 2014).

1.1.4.2 Neurobiological changes in depression

Several biological mechanisms have been implicated in the pathophysiology of MDD that potentially correlate with structural changes, including dysregulation of the hypothalamic–pituitary (HPA) axis (Vreeburg et al., 2009), neurotrophic growth factor loss (Molendijk et al., 2011) and inflammation in the periphery and the CNS (Vogelzangs et al., 2012). The HPA axis

regulates affective behaviours, with dysfunction of the HPA axis regarded as the neurobiological basis of affective disorders. Hyperactivity of the HPA axis results in an increase in corticosteroid levels that can be detected in patients with MDD (Holsboer and Ising, 2010). HPA axis dysfunction and chronic elevation of glucocorticoids are thought to promote hippocampal atrophy and functional deficits in late-life depression (Kim et al., 2006, Lupien et al., 1998, Tata et al., 2006). The impaired negative feedback of the HPA axis can cause impaired neurogenesis in the hippocampus (Vreeburg et al., 2009, Manji et al., 2003). Glucocorticoid receptor (GR) downregulation due to an increase in cortisol release was observed in PD, followed by a chronic inflammatory response (Ros-Bernal et al., 2011). Dysregulation of the HPA axis occurs early in AD, resulting in increased cortisol levels. Animal models of stress have shown that elevated levels of cortisol increase Aβ formation by increasing the levels of the amyloid precursor protein (APP), as well as accelerating the development of NFT (Green et al., 2006). In AD, the presence of a lifetime history of MDD corresponds with higher levels of NP and NFT in the hippocampus, suggesting an interaction between MDD and AD neuropathology (Rapp et al., 2006).

Brain-derived neurotrophic factor (BDNF) is thought to be involved in the pathophysiology of MDD, as well as the behavioural response to antidepressant medication (Molteni et al., 2009), and plays a role in neurogenesis, synaptic plasticity and neurotransmitter modulation. Reduced BDNF expression may result in deficits in neurogenesis (Jiang et al., 2017), as well as depressive behaviours (Jiang et al., 2017, Suri et al., 2013). Patients with MDD show altered expression of BDNF in the hippocampus and PFC in post-mortem studies (Pandey et al., 2003, Karege et al., 2005). BDNF has also been strongly correlated with dopaminergic neurotransmission (Hyman et al., 1991). Reduced levels of BDNF mRNA expression, as well as protein have been observed in the SN of PD patients (Mogi et al., 1999, Howells et al., 2000). Polymorphism of the BDNF gene (rs6265) has also been shown to be associated with cognitive impairment in PD (Wang et al., 2019), with α-synuclein shown to facilitate impairment of BDNF signalling in a mouse model of PD (Fang et al., 2017). AD patients also show reduced BDNF mRNA and protein levels compared with controls (Laske et al., 2007, Holsinger et al., 2000). Increased expression of BDNF was shown to be associated with slower rate of cognitive decline during life (Beeri and Sonnen, 2016), as well as reduced behavioural deficits, reduced neuronal loss and synaptic degeneration, and increased neurogenesis in P301L mice (Jiao et al., 2016).

Inflammation and depression are closely associated. An upregulation of various pro-inflammatory cytokines has been observed in MDD patients (Liu et al., 2012, Dowlati et al., 2010), and this is suggested to alter glutamate and monoamine neurotransmission, GR resistance and hippocampal neurogenesis (Liu et al., 2003, Zalcman et al., 1994). Patients with MDD display cognitive deficits, which have been shown to correlate with the plasma levels of pro-inflammatory cytokines (Wilson et al., 2002, Bobinska et al., 2017). Neuroinflammation also plays a major role the pathogenesis of neurodegeneration (Amor et al., 2014). α-synuclein aggregation has been shown to be involved in microglial activation and induction of an inflammatory response (Surendranathan et al., 2015). Increased levels of proinflammatory cytokines and reduced levels of neurotrophins has been observed in the SN and striatum in PD patients (Nagatsu et al., 2000). In PD, PET imaging shows early inflammatory response in the brainstem, with widespread microglial activation correlating with dopaminergic terminal loss and disease progression (Ouchi et al., 2005, Gerhard et al., 2006). DLB cases with mild disease showed significantly higher microglial activation compared to moderate/severe disease (Surendranathan et al., 2018), which may suggest its importance in an initial acute immune response, rather than chronic inflammation. Early microglial activation is also consistent with MCI and early AD (Okello et al., 2009), with an increase in peripheral inflammatory markers consistently observed in AD (Lai et al., 2017). Systemic inflammatory changes are also observed in DLB and PDD using blood and CSF samples, which are more pronounced than in PD (King and Thomas, 2017).

Neuroimaging correlates of cerebrovascular disease in patients with MDD in later life are high. The vascular depression hypothesis suggests that hyperintense white matter lesions (WMH) observed using MRI in the grey and white matter contribute to the pathogenesis of later life depression (Alexopoulos et al., 1997), especially in the form of ischaemic lesions (Thomas et al., 2002b). White matter structural differences in the ACC and corpus callosum have been observed in later life depression, which might suggest that altered neural connectivity may directly contribute to the pathogenesis of depression (Bae et al., 2006). White matter hyperintensities might also indicate disturbances to specific neural circuits or fibre tracts involved in mood regulation (Colloby et al., 2012a). Investigation of white matter microstructural changes in later life depression has also shown a loss of integrity in white matter fibres within limbic–orbitofrontal networks (Colloby et al., 2011). Microvascular changes in elderly depressed patients show decrease of capillary diameters in supragenual ACC (spACC) and sgACC in MDD (Sinka et al., 2012). An increase in intracellular adhesion

molecules on the cerebrovascular endothelium has been observed in post-mortem tissue of depressed elderly subjects, these being specific to the DLPFC (Thomas et al., 2002a). In contrast, other studies have found no significant increase in intercellular or vascular cell adhesion molecules in the serum of patients with late-life depression, and no correlation of serum levels and depression severity (Thomas et al., 2007).

1.1.4.3 Monoamine hypothesis of depression

The monoamine hypothesis of depression proposes that deficiency of monoamines, including serotonin (5-HT), noradrenaline (NA) and dopamine (DA) are the basis of depression. This was initially based on a number of observations, including investigation of the hallucinogen lysergic acid diethylamide (LSD) and its action on the serotonergic system, suggesting that serotonin may play a role in aetiology of mood disorders (Gaddum and Hameed, 1954, Woolley and Shaw, 1954). Furthermore, the use of the antihypertensive drug, reserpine, alleviated depression in a proportion of hypertensive patients (Muller et al., 1955). Reserpine is thought to interfere with vesicular storage of 5-HT and NA, which further reiterated the importance of monoaminergic transmission in depression. The MAOI drug iproniazid, originally used to treat tuberculosis, also has demonstrated efficacy in alleviating depressive symptoms (Cole et al., 1959), which has led to the development of the new generation of antidepressants, the SSRI, which are the most commonly prescribed antidepressants to this day. The monoamines are involved in the pathogenesis of several psychiatric disorders. DA neurotransmission changes are often associated with psychosis, 5-HT abnormalities associate with anxiety and panic disorders, whereas NA, 5-HT and/or DA are involved in depression (Moore and Bloom, 1979). While the biological approach to the treatment of depression has been dominated by the monoamine hypothesis for several decades, it does not provide an explanation as to why there is a delay in the therapeutic action of many antidepressants, which pharmacologically act via 5-HT and NA systems, possibly suggesting that the clinical effect is due to the adaptive changes in the brain, rather than the primary drug effect (Hirschfeld, 2000).

1.1.4.3.1 Serotonin

Serotonin, also known as 5-HT is synthesised from the amino acid tryptophan and is found in the brain and periphery. It is important in physiological responses, such as arousal, circadian rhythms, neuroendocrine function, sexual behaviour and feeding, as well as mood and cognition. The 5-HT synthesizing neurones are localised in raphe nuclei of the brainstem and midbrain, which project diffusely throughout the brain and spinal cord. A reduction of 5-HT is

suggested as being a major underlying cause of MDD, and elevation of 5-HT, or of 5-HT neurotransmission, as reversing or alleviating symptoms in MDD. Reduced 5-HT levels following acute tryptophan depletion supports a role for decreased 5-HT in depression in recovered depressed patients (Delgado et al., 1990, Jans et al., 2007). However, the low 5-HT hypothesis of depression has been challenged by a number of studies. SSRI efficacy may not be due to elevated 5-HT concentration or increased 5-HT neurotransmission in the brain, since serotonin transmission is elevated in multiple depressive phenotypes (Lacasse and Leo, 2005, Andrews et al., 2015). Tryptophan depletion, which reduces serotonin levels in the synaptic cleft, fails to induce depression in healthy subjects (Ruhe et al., 2007). The polymorphism in the promoter region of the serotonin transporter (5-HTT) gene (5-HTTLPR) can result in decreased expression of the 5-HTT (Greenberg et al., 1999, Heinz et al., 2000), and has been shown to interact with stressful life events to predict depression (Gillespie et al., 2005). However, another study has shown a polymorphism of 5-HTTLPR to be related with reduced risk of depression (Karg et al., 2011). To address some problems with monoamine hypothesis in the pathogenesis of MDD, the monoamine receptor hypothesis has been proposed, which asserts that downregulation of 5HT autoreceptors (such as 5-$HT1_A$) is a key mechanism of antidepressant efficacy. Since the 5-$HT1_A$ autoreceptor inhibits impulse flow of 5-HT neurones in the brainstem raphe nuclei, and the downregulation or desensitization of this receptor is induced by elevated 5-HT (resulting from antidepressant intake), this may increase impulse flow and 5-HT levels in axonal terminals (Liu et al., 2017). Downregulation of the 5-$HT1_A$ autoreceptor can take several days to weeks, which may explain the delayed onset of antidepressant efficacy.

Decreased serotonin 5-$HT1_A$ binding in ACC has been observed in depressed patients using PET imaging (Wang et al., 2016), as well as decreased 5-$HT2_A$ binding in ACC and DLPFC in treatment resistant depression (Baeken et al., 2012). Post-mortem studies have shown an increase in the density of 5-HT2 receptor binding sites, and a decreased number of 5-HTT binding sites in MDD (Owens and Nemeroff, 1994). Additionally, an increase in serotonin 5-$HT1_A$ autoreceptors in the dorsal raphe nucleus in MDD may suggest a compensatory response due to a decrease in extracellular 5-HT (Stockmeier et al., 1998). In patients with late-life MDD, PET imaging of 5-$HT2_A$ receptor showed no changes in the lateral orbitofrontal cortex, sgACC, pregenual ACC, striatum, amygdala, PFC, occipital and parietal cortices (Meltzer et al., 1999), suggesting that 5-$HT2_A$ receptors are differentially affected in late compared to early life depression.

Studies of the serotonergic system in neurodegenerative disorders in relation to depression has focused on both pathological and receptor changes. The largest serotonergic nucleus, the dorsal raphe, is often severely affected by LB pathology, which might explain the more severe loss of serotonergic innervation to the forebrain, resulting in a marked reduction of 5-HT levels in the striatum and PFC of DLB, PD and PDD patients (Ballard et al., 2013, Sharp et al., 2008). Serotonergic neurotransmission has been shown to be severely impaired in DLB compared to AD patients with depression, with reduced 5-HT and its metabolite concentrations observed in the PFC, temporal, limbic, occipital cortex and hippocampus (Vermeiren et al., 2015). A selective postsynaptic 5-HT1_A receptor upregulation was observed in depressed DLB patients, which may be a compensatory mechanism for the decreased serotonergic neurotransmission to neocortical and limbic projection areas (Vermeiren et al., 2015). Reduced 5-HTT levels were also observed in the raphe nuclei in PD patients compared to controls using PET imaging (Guttman et al., 2007, Murai et al., 2001, Politis et al., 2010a), whereas an increase in 5-HTT binding in raphe and limbic regions was observed in PD patients with depression (Politis et al., 2010b), possibly reflecting lower extracellular serotonin levels.

1.1.4.3.2 Noradrenaline

Noradrenergic neurones are found in the medulla oblongata and the dorsal vagal nucleus, with the highest density of noradrenergic cells bodies observed in the LC, which innervates the thalamus, dorsal hypothalamus, hippocampus, subcortical limbic regions, cerebellum, spinal cord and cortex. Peripherally, NA is part of the sympathetic nervous system and mediates responses of stress and acute anxiety, as well as the “fight or flight” response in animals and humans. Dysregulation of NA may also be involved in the pathogenesis of anxiety and depression (Goddard et al., 2010). An increase in firing rates of LC neurones was observed in animal models of stress compared to controls (Simson and Weiss, 1988), with reduction in firing rates observed following antidepressant treatment (West et al., 2009). LC lesions in animal models have shown to reduce depressive behaviours (Semba and Takahashi, 1988, Harro et al., 1999, Lin et al., 2011). This evidence suggest that increase in LC functional activity is associated with depressive behaviours.

In vivo PET studies have found elevated NA transporter levels in LC and thalamus in patients with MDD (Moriguchi et al., 2017), which is in line with animal studies, that found an increase in mRNA and protein levels of the NA transporter in rat models of depression (Miner et al., 2006, Chen et al., 2012). Post-mortem studies in MDD have shown reduced NA transporter

binding in the LC and other limbic areas, possibly due to a loss of NA axonal innervation (Ordway and Klimek, 2001, Klimek et al., 1997). Elevated α2-adrenoceptor levels in platelets have been observed in MDD patients (Kaneko et al., 1992, Garcia-Sevilla et al., 2004), with a decrease in α2-adrenoceptor density observed following antidepressant treatment with TCA (Gurguis et al., 1999). Chronic administration of TCA can also induce downregulation of α2-adrenoceptor, which results in the facilitation of NA release (Cottingham et al., 2015). These findings suggest that downregulation of α2-adrenoceptor is involved in the therapeutic antidepressant mechanism, possibly through arrestin-mediated internalization and downregulation of α2-adrenoceptors (Cottingham and Wang, 2012), which gives us a better insight into antidepressant pharmacology. However, it should be noted, that other neurotransmitter systems, including GABA and acetylcholine, may be involved in the altered NA transporter regulation (Huang et al., 2015, Mandela and Ordway, 2006).

Reduced NA concentrations are observed in the putamen, frontal, temporal and occipital cortices in DLB patients compared to AD and controls (Ohara et al., 1998). The loss of noradrenergic neurones in the LC is observed in DLB cases, as well as an increase in the mRNA for TH and sprouting of dendrites in the remaining neurones (Szot et al., 2006), which may be a result of the compensatory changes due to NA neuronal loss in the LC. The LC in DLB cases is severely affected by LB inclusions (Jellinger, 2003a), which may also play a major role in the NA neuronal loss in the LC. Degeneration of the LC is observed in PD (Paulus and Jellinger, 1991), which may play a role in depression in PD patients (Chan-Palay, 1993). This is supported by the lower catecholaminergic transporter binding (11[C]RTI-32 PET) in limbic regions and ventral striatum PD patients with apathy (Remy et al., 2005), and reduced noradrenergic innervation in ACC, amygdala and ventral striatum in PD patients with depression (Remy et al., 2005). Changes in the noradrenergic system, such as accumulation of tau pathology in LC, are also observed early in AD (Braak et al., 2006), with loss of noradrenergic innervation shown to be involved in AD pathogenesis and progression (Kalinin et al., 2007).

1.1.4.3.3 Dopamine

Dopamine (DA) is the most abundant catecholamine in the brain and is involved in regulating motor function, motivation, arousal, reward and pleasure (Salamone and Correa, 2012, Ben Zion et al., 2006, Berridge and Kringelbach, 2008). Dopaminergic projections give rise to four axonal pathways. The nigrostriatal projections of dopaminergic neurones extend from the SN

to the dorsal striatum, with the loss of these neurones most often associated with the motor symptoms of PD (Vallone et al., 2000). The mesolimbic dopamine pathway arises from the dopaminergic cells in the ventral tegmental area (VTA), which project to the NAcc, amygdala, hippocampus and other limbic regions, important for motivational function, reward of mood regulation (Nestler et al., 2002). The mesocortical pathway also originates in VTA and primarily projects to the PFC, as well as cingulate and perirhinal cortex, and is involved in cognitive control, motivation and emotional response. The tuberoinfundibular pathway connects the hypothalamus to the pituitary gland, where it influences the secretion of hormones such as prolactin (Vallone et al., 2000), which has been shown to be involved in the regulation of the stress responses through the inhibition of the HPA axis (Torner, 2016).

While in the past a considerable focus has been on serotonergic and noradrenergic systems in mood regulation, recently, the involvement of dopaminergic mechanisms in the pathophysiology of depression has gained increased interest (Nestler and Carlezon, 2006, Belujon and Grace, 2017). Dysregulation of the dopaminergic system has been linked to deficits in empathy (Kim et al., 2014), executive functioning (Lumme et al., 2007), as well as cognitive and emotional behaviour in MDD (Lacroix et al., 2003). Depressive symptoms, such as anhedonia and apathy have also been associated with dysfunction of the dopaminergic system (Wise, 2008), with PET imaging in anhedonic depressed patients showing significantly reduced DA transporter (DAT) binding compared with controls (Sarchiapone et al., 2006). In MDD patients increased striatal D2 receptor binding was observed using PET imaging (D'Haenen and Bossuyt, 1994), suggesting decreased DA turnover. Selective inhibition of DA neurones in the VTA was shown to induce a depression-like phenotype in mouse model, which was reversed by selective activation of the mesolimbic DA system (Tye et al., 2013).

Some depressive symptoms, including apathy, loss of pleasure and interest, are driven by dopaminergic degeneration, therefore antidepressants with an effect on the dopaminergic reward system may prove useful in treatment outcome. While seldom used as a first choice antidepressant treatment, some dopaminergic compounds, including DA agonists, have proven to be effective in treating MDD (Leentjens, 2011, Hori and Kunugi, 2013).

Selective dopaminergic neuronal loss occurs early in PD and DLB, which is associated with LB pathology and development of specific clinical symptoms (Schulz-Schaeffer, 2015). Higher α-synuclein burden in SN, VTA and NAcc was observed in LBD cases with depression (Patterson et al., 2018), which may suggest that α-synuclein pathology may be one of the main

factors driving these symptoms. α-synuclein interacts with DA metabolism and transmission (Galvin, 2006, Phan et al., 2017), and may play an important role in neuronal loss and development of depressive symptoms in LBD. The motor symptoms in PD and DLB stem from neurodegeneration of the nigrostriatal dopaminergic pathway, which can be demonstrated by DAT imaging, showing significant diagnostic value in differential diagnosis (Walker et al., 2004). A correlation between higher apathy scores and lower striatal DAT binding has also been observed in PD (David et al., 2008). The development of depressive symptoms in PD was shown to be associated with mesolimbic dopaminergic pathway dysfunction, as demonstrated by reduced volume in VTA, cingulate and amygdala, as well as reduced [11C]RTI-32 binding in several limbic regions in PD patients with depression (Remy et al., 2005), which may relate to the loss of DA projections. AD patients also show dopaminergic system abnormalities, including reduced levels of DA and its receptors (Gibb et al., 1989, Storga et al., 1996). VTA dopaminergic neurones project directly to hippocampus, with DA shown to modulate hippocampal synaptic plasticity (Broussard et al., 2016, Rossato et al., 2009). In an AD mouse model, VTA dopaminergic degeneration resulted in lower DA levels in the hippocampus, which may impact memory deficits and dysfunction of reward processing in AD (Nobili et al., 2017). An increase in subcortical and cortical LB pathology has been observed in MDD (Iritani et al., 2008, Tsopelas et al., 2011), as well as in amygdala in AD cases with a history of depression (Lopez et al., 2006), therefore LB pathology may be important in the aetiology of MDD, as well as depression in neurodegenerative disorders.

1.1.4.4 Glutamatergic and GABAergic dysfunction in depression

Glutamate is the primary excitatory neurotransmitter, whereas γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain. The synthesis of GABA and glutamate occurs through closely related metabolic pathways, with imbalance in excitatory/inhibitory neurotransmission suggested as greatly contributing to the development of mood disorders (Gabbay et al., 2012).

A growing body of evidence has directed focus onto the glutamatergic system as being involved in the pathogenesis of depression. Studies have demonstrated that certain antidepressant drugs, such as TCAs, interact with glutamate receptors (Reynolds and Miller, 1988), and modulate the release and/or reuptake of glutamate (McCaslin et al., 1992, Bouron and Chatton, 1999). Increased plasma and CSF glutamate levels have been observed in MDD patients, with reductions following antidepressant treatment (Kucukibrahimoglu et al., 2009,

Levine et al., 2000, Mitani et al., 2006). In vivo proton magnetic resonance spectroscopy (1H-MRS) studies have demonstrated reduced glutamate metabolite levels in the frontal and cingulate cortex of patients with MDD (Auer et al., 2000, Hasler et al., 2007). These regions have been identified as key structures for MDD, and show reductions in neuronal and glial cell densities (Cotter et al., 2001, Ongur et al., 1998), reduced expression of glial enzymes allowing for glutamate reuptake from the synaptic cleft (Choudary et al., 2005). This suggests that this may result in elevated levels of extracellular glutamate, which is potentially neurotoxic and can affect the efficiency of glutamate signalling. Glutamatergic deficits have been shown to be related to resting-state brain activity in depression (Engert and Bonhoeffer, 1999), with abnormal glutamatergic modulation related to the functional connectivity between spACC and anterior insula and depression severity (Horn et al., 2010). Glutamate plays a key role in neuronal plasticity, which is a fundamental mechanism of neuronal adaptation. Neuronal plasticity has been shown to be disrupted in mood disorders and in animal models of stress, resulting in enhanced glutamate release, altered synaptic transmission, dendritic remodelling, synaptic spine reductions and glial loss (Heldt et al., 2007, Bodnoff et al., 1995, Cook and Wellman, 2004, Liston et al., 2006). These data suggest that abnormal glutamatergic neurotransmission will result in the imbalance in excitation and inhibition, commonly observed in mood disorders.

Glutamatergic agents have shown antidepressant potential, with compounds such as ketamine, an NMDA receptor antagonist, proposed as a promising therapeutic intervention for treatment resistant depression, with other agents, including metabotropic glutamate receptor modulators, glycine transporter 1 inhibitors and glutamate release inhibitors, also being explored (Abdallah et al., 2015, Rotroff et al., 2016, Abdel-Magid, 2016). Ketamine has been shown to have clinical efficacy in antidepressant drug therapies producing rapid relief of depressive symptoms (Abdallah et al., 2015, Downey et al., 2016, Rotroff et al., 2016), acting by strengthening excitatory synapses in brain regions involved in the regulation of mood and reward in animal models (Zanos et al., 2016, Li et al., 2010). Esketamine, an isomer of ketamine, given intranasally, has been approved by the European Commission for use in treatment resistant MDD (Jauhar and Morrison, 2019).

A large body of clinical and preclinical evidence supports the role of GABA dysfunction in the pathophysiology of MDD (Marin, 2012, Brambilla et al., 2003). It has been hypothesised that MDD related epigenetic and stress-induced changes are due to deficits in GABAergic transmission, and currently available antidepressants involve downstream alterations in

GABAergic transmission. Reduced GABA levels are found in plasma and CSF of patients with MDD (Petty and Schlesser, 1981, Gerner and Hare, 1981), which may reflect decreased levels of extracellular GABA in the brain or decreased turnover. An increase in plasma GABA levels was observed in treated patients with MDD (Kucukibrahimoglu et al., 2009), which may indicate that monoaminergic compounds are directly involved in modulation of GABAergic neurotransmission. An MRS study has demonstrated reduced GABA concentrations in occipital and ACC in MDD (Gabbay et al., 2012), correlating with reduced amygdala and PFC mRNA for glutamic acid decarboxylase 67 (GAD67), a key enzyme for the synthesis of GABA, in post-mortem tissue (Cotter et al., 2002, Karolewicz et al., 2010). Reduced GABA and GAD67 levels are also consistent with reduced densities of specific subtypes of GABAergic interneurones in MDD, especially those containing specific calcium binding proteins, such as calbindin D-28K (CB), calretinin (CR) and parvalbumin (PV) (Berridge et al., 2000, Cotter et al., 2002, Beasley et al., 2006, Maciag et al., 2010). Significant reductions in CB neurone density in the ACC has been observed in schizophrenia, bipolar disorder and MDD (Cotter et al., 2002, Rajkowska et al., 2007, Maciag et al., 2010), which may suggest selective vulnerability of these neurones in affective disorders and involvement of GABA.

A downregulation of several GABA-related genes in sgACC has been reported in patients with MDD (Tripp et al., 2011), potentially affecting somatostatin (STT) dendritic targeting interneurones, with reduced SST expression in sgACC observed across all cortical layers in MDD cases (Tripp et al., 2011, Seney et al., 2015). GABAergic activity in sgACC is thought to be involved in isolating the memories of sad events and stopping them from 'over-participating' in conscious perception. Reduced levels of GABA in sgACC has been observed in unmedicated recovered depressed subjects (Bhagwagar et al., 2008), which may suggest that while some restoration of GABA levels in clinically recovered patients with depression occurs, detectable deficiency remains. This may be due to reductions in glial cell numbers in the ACC in MDD (Ongur et al., 1998), with astrocytes being an important source of the GABA precursor, glutamine, to GABA nerve terminals (Shulman et al., 2004).

As current monoaminergic pharmacological treatments for MDD display latency to achieve a therapeutic effect, with half of patients not responding to treatment, more effective and faster-acting therapies are needed. GABA acts through ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. $GABA_A$ receptors contain binding sites for ligands such as GABA agonists (e.g. muscimol), antagonists (e.g. bicuculline), barbiturates, benzodiazepines, anticonvulsants and neurosteroids (Sieghart, 1995). $GABA_B$ receptors have binding sites for $GABA_B$ receptor

agonists (e.g. baclofen), antagonists (e.g. phaclofen) and allosteric positive modulators (e.g. CGP7930, GS39783) (Urwyler et al., 2001, Urwyler et al., 2003)(Bowery et al., 2002)(Kerr and Ong, 1992). The use of GABA analogs (e.g. valproate, beta-phenyl-GABA), GABA agonists, and neurosteroids (e.g. allopregnanolone) reduces depression like behaviours in animal models (Frank and Sagratella, 2000, Gajwani et al., 2005, Lapin, 2001, Lloyd et al., 1983). Negative allosteric modulators of $GABA_A$ receptor α5 subunits (e.g. MRK-016) have been shown to reverse anhedonia in animal models by restoration of excitatory synaptic strength (Fischell et al., 2015). Similar to ketamine, MRK-016 induced a significant increase in coherent EEG γ frequency oscillations (Zanos et al., 2017). $GABA_A$α5 expression is less widespread compared to ketamine binding sites, predominantly in the hippocampus and frontal cortex in mouse brain (Malherbe et al., 1990), which may result in the fewer observed side effects. Negative allosteric modulators of $GABA_A$α5 have also been shown to have cognition-enhancing actions (Rudolph and Knoflach, 2011), and may be a target for memory-enhancing drugs (Ballard et al., 2009). $GABA_B$ receptors are highly expressed in the limbic system involved in regulating emotional behaviour (McDonald et al., 2004). Neurophysiological $GABA_B$ deficits have been shown to be involved in the pathophysiology of MDD in post-mortem tissue (Fatemi et al., 2005), with up-regulation of $GABA_B$ receptors shown to be essential for antidepressant drug action (Pilc et al., 2008).

1.1.5 The cingulate cortex

While there are multiple brain regions involved in the pathophysiology of depression, one region in particular, the cingulate cortex, plays a key role. The cingulate cortex lies immediately above the corpus callosum from which it is separated by the callosal fissure. It curves around anterior to the genu of the corpus callosum, extends along the dorsal surface of the callosal body, and turns ventrally behind the splenium. The cingulate cortex is a heterogeneous brain structure that has a number of structurally and functionally distinct subregions involved in cognitive, emotional and motor processes, as well as attention, pain and autonomic responses (Vogt et al., 1995, Vogt et al., 2006). There are multiple nomenclatures for the cingulate cortex, which was originally divided by Brodmann (1909) into precingulate (areas 24, 25, 32, and 33) and postcingulate (areas 23, 29, 30, and 31) divisions. Distinct subregions within the cingulate cortex differ in terms of cytoarchitecture, receptor organization, anatomical and functional connectivity (Palomero-Gallagher et al., 2008, Shackman et al., 2011, Vogt et al., 1995). A four-region neurobiological model of the cingulate cortex was proposed by (Vogt, 2005), which encompasses the ACC, mid-cingulate cortex (MCC), PCC and retrosplenial cortex

(RSC). The ACC can be further subdivided into sgACC and supragenual anterior cingulate (spACC) areas, the MCC into anterior (aMCC) and posterior (pMCC) regions, whereas the PCC can be divided into dorsal (dPCC) and ventral (vPCC) areas (Figure 1.4) (Gittins and Harrison, 2004, Palomero-Gallagher et al., 2009).

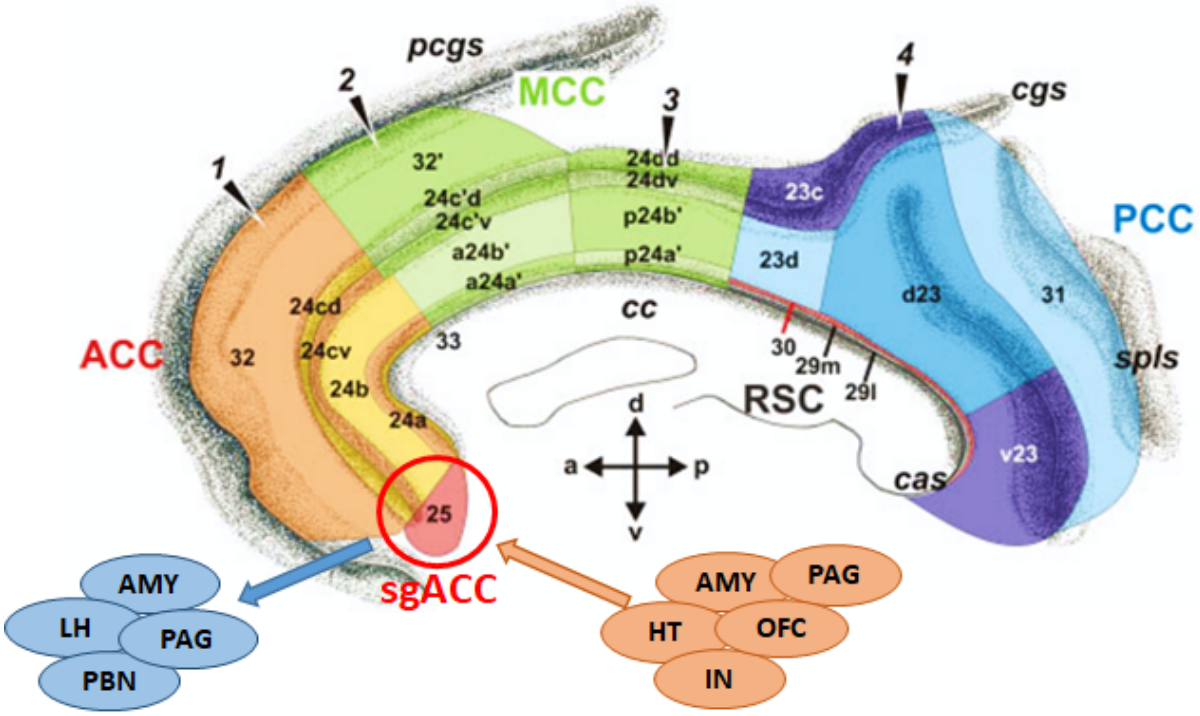

Figure 1.4 Cingulate subregions.
The major cingulate subdivisions are based on regional differences in microanatomy, connectivity and physiology. A four-region neurobiological model of the cingulate cortex includes the ACC, MCC, PCC and RSC areas. Anterior and ventral to the corpus callosum is the ACC, which is divided into sgACC (BA25) and spACC (BA24). The MCC has anterior (BAa24') and posterior (BAp24') subdivisions, whereas the PCC has dorsal (BAd23) and ventral (BAv23) subdivisions. The sgACC receives primary afferent projections from amygdala (AMY), hypothalamus (HT), insula (IN), orbitofrontal cortex (OFC) and periaqueductal grey (PAG), with primary efferent projections to AMY, PAG, lateral hypothalamus (LH) and parabrachial nucleus (PBN) (Palomero-Gallagher et al., 2009).

1.1.5.1 Anterior cingulate cortex

The ACC is a structurally and functionally heterogeneous brain region, which has motor, cognitive and affective subdivisions. In the ACC, pyramidal cells are more spinous than in PCC, which is important in functional specialization of the cortex, as higher branching and spine density may result in an increase of excitatory inputs (Elston et al., 2005a). This might suggest that pyramidal cells in the ACC are involved in more complex information processing

(Elston et al., 2005b). The ACC is an agranular cortex, with two major functional subdivisions – sgACC and spACC (Barrett and Simmons, 2015).

1.1.5.1.1 Subgenual anterior cingulate

Anatomically, early research defines the term ‘‘subgenual’’ to describe the entirety of the ventral anterior cingulate cortex, whereas more recent research defines the term ‘subgenual’ to include only a small volume of the posterior ventral ACC (BA25), and the anterior section of the ventral ACC (BA24) termed ‘subcallosal’ (Baeken et al., 2015). The cytoarchitecture in the human sgACC varies along the rostrocaudal extent. The more rostral area 25 has a narrower and less prominent layer II compared to caudal area 25, with an undifferentiated layer III, and with layer V pyramidal cells larger than in layer VI. The more caudal area 25 has a dense and broad layer II and the thinnest layer III of all cingulate areas, with sparsely distributed small pyramidal cells, with layers V and VI almost indistinguishable (Palomero-Gallagher et al., 2008). The neurones in layer II are globular rather than elongated in shape with small basal dendrites and receive primary inputs from the amygdala (Vogt and Pandya, 1987).

Neurotransmitter binding presents distinct laminar patterns throughout the sgACC. $GABA_A$, $GABA_B$, nicotinic, α1, α2 and 5-HT2 receptors are homogeneously distributed in all the layers (Varnas et al., 2004). Muscarinic M3 and 5-$HT1_A$ receptor densities are however higher in the superficial layers of caudal area 25, whereas kainate and M2 receptors show highest densities in the deep layers. 5-$HT1_A$, $GABA_A$ and $GABA_B$ receptor density also appears to be significantly higher in sgACC than in pregenual ACC regions, suggesting regional and possibly functional differences in cingulate subregions (Palomero-Gallagher et al., 2009). AMPA and NMDA receptors are located preferentially in the superficial layers of sgACC, and it is suggested these represent the glutamatergic input from the amygdala (Zhuo, 2007). Furthermore, the high density of 5-$HT1_A$ receptors in layers I–II suggests that serotonin may amplify the signal from the amygdala and further drive output from layer III neurones (Palomero-Gallagher et al., 2009). The thin layer III in sgACC suggests that cortico-cortical projections are relatively weak in comparison to cortico-cortical connections of other cingulate regions (Palomero-Gallagher et al., 2008).

The sgACC has direct projections to autonomic regulatory centres, including the lateral hypothalamus, periaqueductal grey and parabrachial nucleus, and is implicated in the modulation of autonomic responses to stress, including heart rate, digestion and respiratory rate. It also receives direct input from the amygdala, involved in “fight or flight” response

(Drevets et al., 2008b, Vogt, 2014). Functional magnetic resonance imaging (fMRI) studies have shown sgACC to be activated during feelings of sadness (Smith et al., 2011), suggesting its pivotal role in the emotional processing of negative stimuli (Gotlib et al., 2005), and involvement in MDD (Kumano et al., 2007). The sgACC may be a key regulator of amygdala activity and autonomic responsivity, important in emotional conflict resolution (Etkin et al., 2011). Under physiological conditions, activation of the amygdala during emotional stimuli is followed by activation of the sgACC, which exerts an inhibitory effect on the amygdala, resulting in suppression of the amygdala response (Etkin et al., 2006). This may suggest that dysfunction of the sgACC may result in hyperactivity of the amygdala, which is commonly observed in depression (Kumari et al., 2003), therefore the inability to deal with emotional conflict might be due to deficient amygdala inhibition by the sgACC.

1.1.5.1.2 Supragenual anterior cingulate

The spACC (area 24) lacks a granular layer IV, and has three subdivisions (24a, 24b and 24c) (Vogt et al., 1995). Area 24a has differentiated layers II and III, with layer II much broader and more cell-dense compared to the sgACC, whereas layer III is wider than layer II, with evenly distributed medium-sized pyramidal neurones, with a relatively thin layer V. Area 24b has a more dense layer II, and a broad and neurone dense layer V composed of small and large pyramidal neurones. Area 24c is cytologically one of the most heterogeneous areas in the cingulate cortex, and has a broader layer II compared to area 24b, with layer III containing medium-sized and uniformly distributed pyramidal neurones, and much thinner layer V (Palomero-Gallagher et al., 2008). $GABA_B$ and D1 receptor densities are significantly higher in layers I–III, whereas α1 adrenoreceptor densities are higher in all layers of area 24c (Palomero-Gallagher et al., 2009). Area 24c sends direct projections to the facial motor nucleus (Morecraft et al., 1996) and plays a role in facial expression and in the expression of emotion. Layer III of spACC provides the primary output to other cortical areas including DLPFC, orbitofrontal and ventral PCC (Vogt and Pandya, 1987), whereas layer V is the origin for spinal and brainstem motor output (Dum and Strick, 1991). Dopamine receptors are highly expressed throughout the ACC, with dopaminergic innervation of the spACC arising from the cell bodies of the SN and VTA. Dopaminergic modulation of GABAergic transmission in layer V pyramidal cells in the ACC has been shown to be through D1 receptors (Satoh et al., 2018). Low DA levels have been shown to act via D1 receptors, resulting in the enhanced GABAergic response, whereas high extracellular DA levels preferentially activate D2 receptors and reduce

GABA (Trantham-Davidson et al., 2004), which may suggest that this bidirectional modulation of inhibition is dependent on DA levels in the brain.

The spACC has the highest von Economo neurone (VEN) density in the cingulate cortex (Santillo et al., 2013). VEN are large bipolar neurones, characterized by a large spindle-shaped soma that gradually tapers into an apical axon in one direction, and a single large basal dendrite facing opposite, distinguishing them from pyramidal neurones. VEN cell bodies in the ACC are over four times larger than the layer V pyramidal neurones (Nimchinsky et al., 1995). These neurones have fewer dendrites, which could make them particularly vulnerable to synaptodendritic pathology, as well as fewer dendritic spines, which might suggest that they receive and integrate fewer inputs (Watson et al., 2006). The large, rapidly firing axons of VEN may also suggest increased metabolic demands (Watson, 2008). As VEN are not labelled by calcium binding proteins, which are normally co-localised with GABA, and highly express non-phosphorylated neurofilament protein epitopes, much like large pyramidal cells, they are not considered to be inhibitory neurones, but rather excitatory glutamatergic projection neurones (Nimchinsky et al., 1995). VEN also express higher levels of D3 receptors compared to typical pyramidal neurones, with D3 receptors being implicated in reward seeking behaviour. VEN also express high levels of serotonin 1b and 2b receptors involved in decision making specific to social interactions. High levels of vasopressin 1a receptors, which have been strongly linked to the formation of social bonds, are also seen on VEN (Viskontas et al., 2007a). They are found in layer V of the ACC and anterior insula cortex in humans and great apes, but not other primates, which may support complex higher-order cognitive functions of VEN, including formation of social and emotional bonds (Watson et al., 2006). VEN appear later in brain development than other pyramidal neurones, which suggest that they may also play a role in social and emotional behaviour(Viskontas et al., 2007b).

The spACC plays a key role in reward circuitry, particularly in reward based decision making and learning, suggesting substantial influence by dopaminergic circuitry, due to extensive connections with the VTA (Bush et al., 2002). The spACC has strong interconnections with the DLPFC, hippocampus, insula, NAcc and orbitofrontal cortex (Fujihara et al., 2015), and plays a role in cognitive and emotional processing. It is part of the default mode network (DMN) due to the high neuronal activity in the resting state, showing predominant deactivation in response to tasks (Enzi et al., 2012). The spACC, along with the insula, amygdala and medial thalamus comprise the fear and anxiety network (von Leupoldt et al., 2009), and patients with lesions in the spACC show an increase in anxiety and panic attacks (Shinoura et al., 2011). The

spACC is activated by a conditioned fear stimulus (Milad et al., 2007), as well as in response to strong threat (Straube et al., 2009), which suggests that spACC is highly important in mood disorders.

1.1.5.2 Mid-cingulate cortex

The MCC is an agranular cortex, with distinct morphological differences observed between anterior MCC (aMCC) and posterior MCC (pMCC) (Vogt et al., 2003). The aMCC has a poorly differentiated layer III with few neurofilament protein expressing neurones. The pMCC in comparison has a very dense layer V, with more neurofilament protein expressing neurones in layers III and V compared to the aMCC (Palomero-Gallagher et al., 2009). The MCC has high densities of glutamate, muscarinic M1, serotonin and α-adrenoceptors, compared with, for example, the supplementary motor areas (Zilles et al., 1995).

The MCC is involved in skeletomotor regulation and response selection (Devinsky et al., 1995), and is also one of the most frequently activated regions during acute pain (Derbyshire et al., 1994). The MCC has two separate cingulate motor areas that directly project to the spinal cord and motor cortices. This dichotomy suggests that two circuits differentially regulate the two cingulate motor areas. More nociceptive inputs from the midline and intra-laminar thalamic nuclei terminate in the aMCC than in the ACC or pMCC (Vogt, 2005). Activation of the aMCC by nociceptive inputs from the thalamus induces fear and memories of similar events, which drives the aMCC to enhance avoidance responses via layer V projections to motor structures (Bush et al., 2002). The aMCC has the greatest numbers of connections with the dorsal striatum, DLPFC, primary, premotor and supplementary motor areas, and has been strongly implicated in motor control in response to emotional stimuli (Asemi et al., 2015). The aMCC is also involved in processing cognitive information (Matthews et al., 2004), possibly playing an important role in integrating cognition and action (Wenderoth et al., 2005). The pMCC has strong connections with parietal cortex, DLPFC and motor regions (Beckmann et al., 2009). The pMCC circuit is not significantly driven by emotion and is poorly engaged by noxious stimulation, which may be due to less input from thalamic nuclei that receive spinothalamic and spinoreticular inputs (Vogt et al., 2003). The pMCC is more closely linked to motor output, as it projects to a part of the striatum that also receives primary motor input (Takada et al., 2001) and may be involved in the avoidance of potentially painful or harmful stimuli (Bentley et al., 2003).

1.1.5.3 Posterior cingulate cortex

Unlike other cingulate cortex divisions, the PCC is a granular cortex, which has six differentiated layers, with layer IV containing granule excitatory spiny stellate and small pyramidal neurones that amplify and distribute thalamo-cortical inputs (Barrett and Simmons, 2015). The structural dichotomy of PCC is derived from anatomical and functional studies, demonstrating the unique cytological organization of the dorsal (dPCC) and ventral (vPCC) subdivisions. The vPCC has a neurone dense layer III and IV, and more neurofilament-expressing layer V neurones compared to the dPCC (Vogt et al., 2006). The dPCC contains higher $GABA_B$, M3 and 5-$HT1_A$, but lower M1 receptor densities than its ventral counterpart (Palomero-Gallagher et al., 2008, Palomero-Gallagher et al., 2009).

PCC is a highly anatomically interconnected brain region with strong reciprocal connections to the entorhinal cortex, parahippocampal gyrus, parietal cortex, precuneus, thalamus, caudate, orbitofrontal and ACC. It has a high baseline metabolic rate and is a central part of the default mode network (DMN). It also interacts with executive, attentional, motor and language networks (Leech et al., 2012). The PCC is part of a network that mediates visuospatial orientation and related memories, which together with the parietal operculum, is involved in visual motion stimuli, and may play a role in visual motion perception (Antal et al., 2008). The PCC involvement in motion onset and processing of eye movement related signals has been observed in human and non-human primates (McCoy et al., 2003, Stebbins et al., 2004). The inputs to the dPCC and vPCC are associated with the dorsal and ventral visual pathways, where dPCC receives inputs from the mediodorsal, ventral anterior and lateral thalamic nuclei and is involved in visuospatial and body orientation (Vogt et al., 2006). The dPCC, although it is also part of the DMN, becomes less integrated with the DMN as the attentional load increases, and more associated with cognitive control networks involved in the executive control of behaviour (Leech et al., 2011, Leech and Sharp, 2014). The vPCC is integrated with the DMN and is activated during tasks that require an internal focus of attention, such as autobiographical memory retrieval, showing greater temporal lobe functional connectivity. The vPCC shows strong interaction with sgACC (Shibata and Yukie, 2003, Vogt et al., 2006), showing reciprocal and monosynaptic connections in monkey (Vogt and Pandya, 1987). The vPCC is activated during simple emotions driven by faces or scripts (Mayberg et al., 1999, Vogt et al., 2003), although it does not have direct autonomic connections. It is proposed that vPCC evaluates emotional content of visual information through direct interaction with the sgACC (Vogt et al., 2006).

1.1.6 The role of cingulate cortex in depression and dementia

The cingulate gyrus is intimately involved with emotional processing. The ACC is integral in mood regulation, as well as in the symptomatic expression of depression, displaying structural, functional and metabolic abnormalities (Drevets et al., 2008b, Rodriguez-Cano et al., 2014). The sgACC is activated during simple emotions, such as sadness, and DBS has been shown to partially alleviate the symptoms of sadness (Mayberg et al., 2005). DBS of the sgACC has significant antidepressant effects in the short and long term (Berlim et al., 2014), although its mechanism of action is unknown. It has been hypothesised that DBS works by restoring the overall network function (Berlim et al., 2014, Rizvi et al., 2011). One study argued that DBS works by altering conscious perception of the symptoms of depression, such as sadness or anhedonia, but does not address the basic disease process, having only temporary effect (Eggers, 2014), which may suggest the role of multiple systems in the aetiology of MDD.

Age and gender differences have been reported within different cingulate cortex divisions. With ageing, a significant grey matter loss occurs in the ACC, whereas the PCC shows relative structural preservation. Therefore, susceptibility of the ACC to age related grey matter loss may potentially contribute to cognitive decline with age (Vaidya et al., 2007). The prevalence of MDD is higher in women than in men, with women having higher number of symptoms, a more severe type of depression, and greater risk of recurring episodes (Perugi et al., 1990). Women have a relatively larger ACC grey matter volume compared to men (Brun et al., 2009, Mann et al., 2011). Gender differences have also been observed in structural and functional connectivity of the sgACC. The sgACC shows structural connections to the periaqueductal grey, amygdala, hypothalamus, thalamus, MCC and anterior insula in both genders. Structural connectivity of sgACC with the hypothalamus was greater in men than in women (Wang et al., 2014). Women show stronger functional connectivity of the sgACC with the affective regions, such as periaqueductal grey, raphe and MCC compared to men, whereas sgACC functional connectivity in men is stronger with anterior insula and temporoparietal junction compared to women (Wang et al., 2014). Therefore, the higher prevalence of MDD in women may be due to stronger functional connectivity of the sgACC with other affective regions.

Decreased functional connectivity has been reported in the spACC in elderly individuals with subthreshold depression using resting state fMRI (Li et al., 2014b). An abnormally elevated resting state activity in spACC was shown in patients with MDD (Enzi et al., 2012). Changes in resting state activity and connectivity, as well as glutamatergic modulation in spACC have

been linked to a number of psychiatric disorders, including MDD (Duncan et al., 2011). The high density of glutamatergic receptors in spACC suggests a relationship between glutamate and resting state activity (Bozkurt et al., 2005). Increased metabolism in the sgACC has been shown to be involved in MDD (Greicius et al., 2007, Mayberg et al., 2005), with decreased activity in the sgACC observed in response to antidepressant medication and ECT in treatment resistant depression (Baeken et al., 2015).

VEN are distinct population of cells characteristic to the ACC and insula, and are implicated in several neuropsychiatric conditions, including schizophrenia (Brune et al., 2010) and autism (Allman et al., 2010, Allman et al., 2005). A significant reduction in VEN has been observed in agenesis of the corpus callosum, which has a major effect on social and emotional behaviours (Kaufman et al., 2008). Studies have shown that in individuals with a clinical diagnosis of psychosis who die following self-harm, greater densities of VEN in the ACC are seen (Brune et al., 2011), and in autistic individuals (Uppal et al., 2014), which possibly underlies heightened anxiety and avoidance behaviours seen in these disorders. Selective, but variable loss of VEN within the ACC has also been shown in behavioural variant of FTD patients, who show behavioural and personality changes (Tan et al., 2014). This suggest that selective vulnerability of specific cell types within the ACC may be important in emotional and behavioural function.

Grey matter atrophy in the ACC has been observed in prodromal DLB patients compared to controls (Blanc et al., 2016). Decreased grey matter volume of the ACC was associated with neuropsychological impairment in DLB compared to PDD patients (Sanchez-Castaneda et al., 2009). The PCC shows metabolic and perfusion deficits in early AD (Lim et al., 2009). PCC hypometabolism has been shown to be a useful marker of AD pathology, independent of atrophy or volume loss (Graff-Radford et al., 2014). FDG and PiB PET scans of AD and DLB brains shows preservation of PCC metabolism in DLB, and this preservation is not associated with Aβ load, but rather lower Braak NFT stage (Graff-Radford et al., 2014, Iizuka et al., 2017). This could be partly attributed to functional deafferentation, where primary pathological lesion sites in AD, especially the entorhinal cortex, affect other distinct, but associated brain regions due to functional connectivity (Yoshida et al., 2011). The PCC is involved in memory retrieval, therefore the hypometabolism in this region seen in early stages of AD, may play an important role in the progression of the disease (Valla et al., 2001). It has been hypothesised that the MTA seen in early AD and MCI partially explains the PCC hypofunction due to the degeneration of the cingulum bundle that connects the PCC with the MTA (Choo et al., 2010). Additionally,

increased functional connectivity of PCC with frontal cortex may be as a result of a compensatory mechanism due to DMN dysfunction (Bai et al., 2009). Decreased functional connectivity of PCC and MTA has been shown in PD patients, with PDD patients showing decreased PCC and parahippocampal gyrus functional connectivity, which may lead to an increased vulnerability to early cognitive impairment and progression from PD to PDD (Chen et al., 2015).

In DLB, the ACC is a predilection site for LB formation showing early and extensive pathology (Thal et al., 2004, Toledo et al., 2016). However, it is not known how pathological or neurochemical abnormalities in cingulate cortex may relate to clinical symptoms in DLB. Severe cholinergic deficits are observed in DLB patients, often associated with neuropsychiatric symptoms (Perry et al., 1990). An increase in M2 muscarinic receptor binding in the cingulate cortex has been shown in DLB cases (Teaktong et al., 2005), which may be due to a decrease in acetylcholine (ACh) release, with upregulation of M2 receptor being a compensatory response to preserve ACh function from on surviving cholinergic nerve terminals. The increase in M2 receptor binding in cingulate cortex in DLB was shown to be associated with neuropsychiatric symptoms, including delusions and hallucinations (Teaktong et al., 2005). This may suggest that downregulation of specific neurotransmitters within the cingulate cortex may directly contribute to neuropsychiatric symptoms in DLB.

1.1.7 Summary and overview

Depression is a complex phenomenon, affecting multiple systems and pathways. Depressed individuals display structural and functional brain abnormalities, as well as changes in multiple neurotransmitter systems. Although the diversity of depressive symptoms suggests widespread alterations in brain function, as supposed to specific regional dysfunction, the sgACC has been shown to be integral in mood regulation, with abundant evidence of its involvement in MDD, with the DBS of the sgACC showing good clinical outcomes as a treatment for long standing refractory MDD (Mayberg et al., 2005). The ACC divisions show early and extensive pathology, as well as biochemical changes in MDD, although little is known how these abnormalities contribute to the aetiology of depression. Depression is a common psychiatric symptom in DLB, often observed in prodromal stages of the disease (Fujishiro et al., 2015), and is associated with faster cognitive decline and a poor clinical outcome (Ritchie et al., 1998). The basis of depression in DLB is therefore of major importance in understanding the underlying cause and identifying treatments.

1.1.8 Research aims

To investigate pathological and neurochemical changes within sgACC, in order to understand its role in depression in DLB, with a direct relevance for treatment.

1.1.8.1 Hypotheses

The hypothesis is that there are biochemical and pathological changes in specific anatomical locations within the cingulate cortex, notably the sgACC, which lead to depressive symptoms in DLB.

1.1.8.2 Objectives

- To assess pathological changes of α-synuclein, tau and Aβ in cingulate subregions overall, as well as the sgACC in DLB cases with and without depression, as well as controls, using quantitative immunohistochemistry and biochemical analysis.

- To investigate GABAergic and glutamatergic changes in sgACC by assessing:
    a) Cell density using adapted stereological analysis.
    b) Synaptic changes using confocal analysis.
    c) Protein changes using biochemical analysis.

- To investigate monoaminergic changes in sgACC by assessing:
    a) Fibre densities using adapted stereological analysis
    b) Synaptic changes using STED analysis
    c) Protein changes using biochemical analysis.

Chapter 2.

Methods and Materials

2.1 Methods and Materials

2.1.1 Post-mortem human brain tissue

All post-mortem human brain tissue was obtained from the Newcastle Brain Tissue Resource (NBTR), a UK Human Tissue Authority regulated research tissue bank. The ethical approval for the study was granted by Newcastle and North Tyneside National Health Service (NHS) Research Ethics Committee (ref: 09/H0908/42). All participants had received detailed clinical assessments during their life and had consented to the use of their brain tissue for research purposes. The consent for the donation of brain tissue was also confirmed by their next of kin following death.

2.1.2 Study cohort

DLB and control cases were selected based on clinical and neuropathological criteria. Neuropathological assessment was carried out according to standardised neuropathological diagnostic procedures, which together with clinical data was used to make a clinico-pathological diagnosis (Braak et al., 2006, McKeith et al., 2005, Thal et al., 2002). Of note, 4 DLB cases also fulfilled the neuropathological criteria for high AD neuropathological change and could therefore be classified as neuropathologically mixed AD/DLB with a Lewy body disease (LBD) clinical phenotype (Montine et al., 2012, Walker et al., 2015). All cases were as closely matched as possible for age, sex and post-mortem delay. For immunohistochemical analysis the cases were selected with shorter time in fixative, and for biochemical analysis with higher pH (as a surrogate for agonal state). The case selection for this study was based on the availability of clinical information, including sequential Mini-Mental State Examination (MMSE) and Unified Parkinson's Disease Rating Scale (UPDRS) scores. The inclusion criteria for depression diagnosis was made using the Cornell Scale for Depression in Dementia (CSDD) (≥8), as a validated rating scale for depression in dementia (Alexopoulos et al., 1988). Alternatively, the Geriatric Depression Scale (GDS) (≥10) was used (Figure 2.1), which while less sensitive, is shown to retain acceptable qualities when applied to a population of demented elderly patients (Korner et al., 2006). In the absence of clinical diagnosis of depression by a consulting psychiatrist, retrospective analysis of clinical records was used to verify the presence or absence of depression. The size and characteristics of each cohort is detailed in individual chapters.

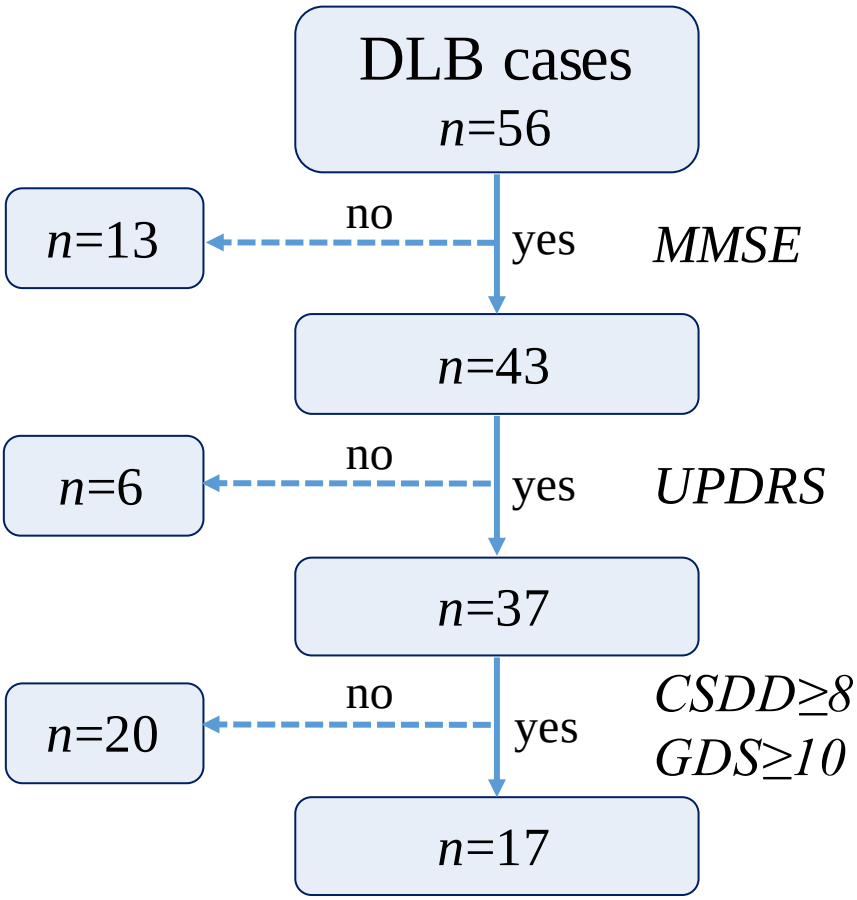

Figure 2.1 Case selection criteria.
DLB case selection for this study was based on the availability of the Mini-Mental State Examination (MMSE), Unified Parkinson's Disease Rating Scale (UPDRS), Cornell Scale for Depression in Dementia (CSDD) and the Geriatric Depression Scale (GDS).

2.1.3 Formalin fixed tissue acquisition and preparation

At autopsy, the brain weight and post-mortem delay were recorded. The right hemisphere was fixed in 10% neutral-buffered formalin for 4-6 weeks. Following fixation, the right hemisphere was cut into 7 mm coronal slices, then sub-dissected into blocks for diagnostic and research purposes. Paraffin-embedded blocks selected for analysis corresponded to the sgACC (block U, coronal level 9), spACC (block V, coronal level 10), MCC (block AB, coronal level 16) and PCC (block AE, coronal level 20; Figure 2.2). The sgACC (Brodmann area: BA25) was sampled at the level of the rostrum of the corpus callosum, spACC (BA24) at the level of the genu of the corpus callosum, MCC (BA24') at the level of the primary motor cortex and PCC (BA23) at the splenium of the corpus callosum. Blocks containing the regions of interest were

cut into 10µm sections using a HM340E rotary microtome with a z-axis monitor (Thermo Scientific, MA, USA) and mounted onto charged slides (Superfrost Plus, Thermo Scientific, UK) using a 45°C water bath. The sections were dried for 48 hours at 37°C, followed by 30 minutes at 60°C, then cooled and stored at room temperature prior to staining.

2.1.4 Histological staining

To assess cell density, sections from sgACC were stained with Cresyl Fast Violet (CFV), used to stain Nissl substance in the cytoplasm of neurones, which appears dark blue due to staining of ribosomal RNA. The 10µm sections were heated in a 60°C oven for 30 minutes, dewaxed in xylene for 15min, then rehydrated through a graded series of ethanol solutions (absolute and 95% ethanol). The sections were then immersed in 1% hydrochloric acid in 70% ethanol for 5min, washed in distilled water, then incubated in pre-heated CFV solution in a 60°C oven for 5min. Following a 5min cooling period in CFV solution, the sections were differentiated in 95%, then absolute ethanol, cleared in xylene and mounted using DPX (CellPath, Newtown, UK).

2.1.5 Immunohistochemistry

Formalin-fixed 10µm tissue sections were heated in a 60°C oven for 30 minutes. Following paraffin wax removal through xylene, the sections were rehydrated with decreasing concentrations of ethanol (100%, 95%, 70%, 50% and deionised water). To unmask epitopes cross-linked as a result of fixation, antigen retrieval was carried out according to optimised protocols for specific antibodies. Citrate buffer heat-induced antigen retrieval (using trisodium citrate pH 6.0) was carried out by pre-heating the buffer for 10 minutes in an 800 watt microwave, then heating the slides on full power for 10 minutes, followed by a cooling period of 20 minutes at room temperature. Ethylenediamine tetra-acetic acid (EDTA) heat-induced antigen retrieval pH 8.0 was carried out by immersing slides in boiling buffer and pressure cooking for two minutes. Formic acid antigen retrieval was carried out by immersing slides in absolute formic acid at room temperature for the optimised time period.

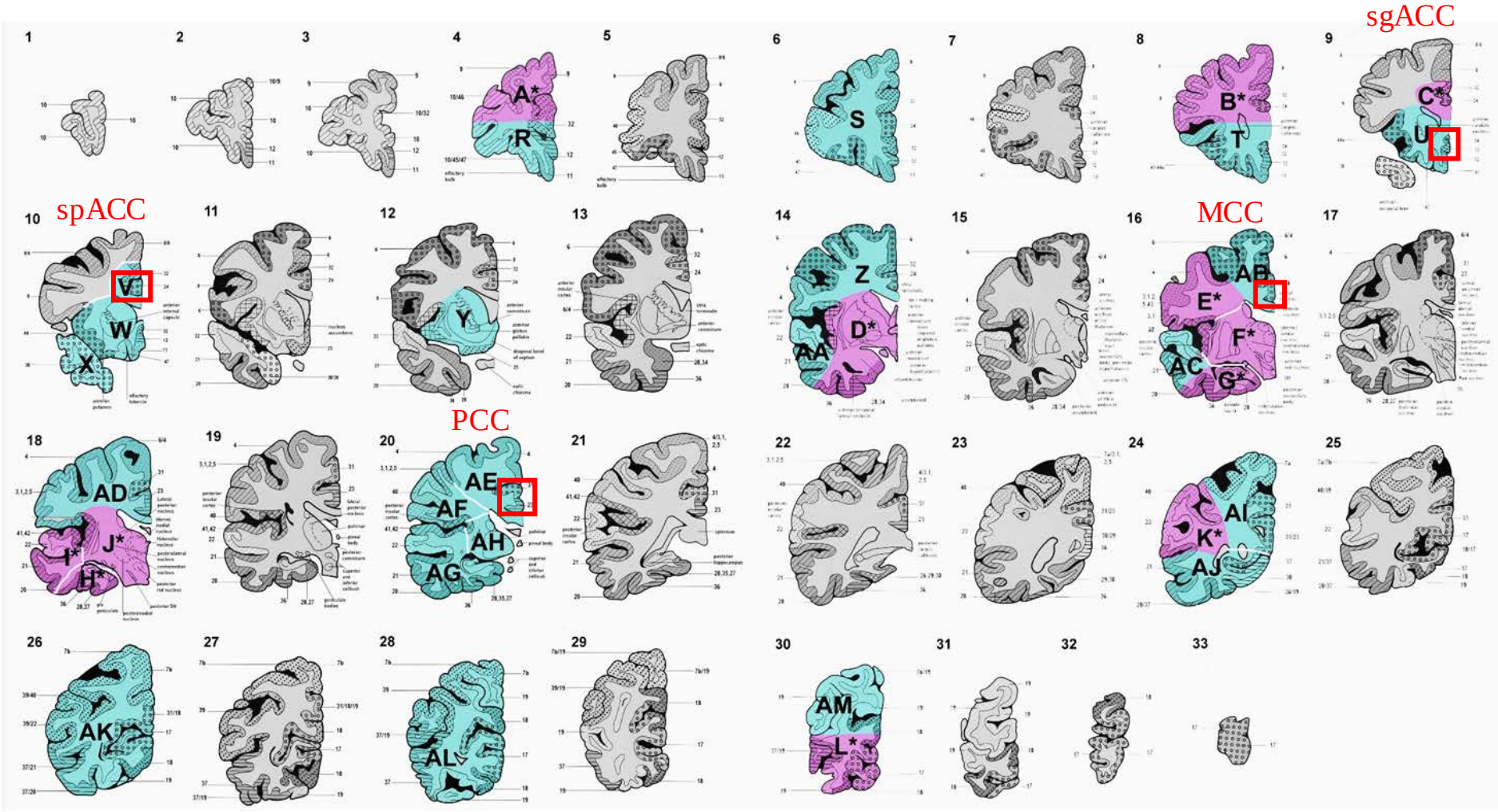

Figure 2.2 NBTR protocol for brain dissection.
Coronal dissection map of the right hemisphere. Slice 1 corresponds to the frontal pole and slice 33 to the occipital pole. The brain regions with the purple coloured blocks are used for diagnostic and the blue blocks for research purposes. The sgACC corresponded to block U, spACC to block V, MCC to AB and PCC to block AE. Adapted from (PERRY, 1993).

2.1.5.1 Chromogenic immunohistochemistry

Following antigen retrieval, sections were incubated with three percent hydrogen peroxide in deionised water for 20 minutes to block endogenous peroxidase activity. After quenching, the sections were washed in water, followed by three washes in 10mM tris-buffered saline containing 0.1% v/v Tween 20 (TBST) pH 7.6. An ImmEdge hydrophobic barrier pen (Vector Laboratories, Burlingame, CA, USA) was used to contain primary antisera by outlining tissue sections. Tissue sections were incubated with the primary antibodies diluted in TBST for one hour at room temperature. Following three washes in TBST, the sections were incubated with horseradish peroxidase (HRP) polymer conjugated universal probe (MenaPath, Menarini Diagnostics UK) for 30 minutes at room temperature. After three washes in TBST the tissue sections were incubated for 30 minutes with HRP reagent, followed by three TBST washes. Visualisation of the antibody was attained by using the Menarini X-Cell-Plus HRP Detection Kit (Menarini, UK), using Diaminobenzidine (DAB) substrate for two minutes. The sections were then washed in running water for five minutes, counterstained with Mayer's haematoxylin solution for 30 seconds, differentiated with 1% hydrochloric acid in 70% ethanol, blued with 1% ammonia in water and dehydrated through increasing concentrations of ethanol (90%, 95% and 100%). The sections were then cleared in xylene and mounted using DPX (Cell Path, UK).

2.1.5.2 Fluorescent immunohistochemistry

Following antigen retrieval, the sections were washed in water and blocked in TBS containing 10% normal goat serum (NGS: Sigma G9023) for one hour at room temperature. The tissue sections were then incubated with the primary antibodies diluted in TBS and 10% NGS overnight at 4°C. After three washes in TBS for 5 minutes, the sections were incubated with secondary fluorescent antibodies in TBS and 10% NGS for 1 hour at room temperature, followed by three TBS washes. To stain the nuclei, the sections were incubated with TO-PRO-3 Iodide (1 mM Solution in DMSO, Thermo Fisher, UK) for 15 min, then washed three times in TBS. Prior to and following an incubation in Sudan Black B (0.3%w/v in 70% ethanol in deionised water) for 10 minutes to quench autofluorescence, sections were immersed in 70% ethanol for 5 seconds, then washed in deionised water. Sections were mounted using ProLong Diamond Antifade Mountant (Thermo Fisher, UK) and stored at -30°C to preserve the fluorescent signal prior to imaging.

Adjustments were made to the previously outlined protocol for stimulated emission depletion (STED) microscopy. Higher primary and secondary antibody concentrations (2-3 fold) were

used to ensure optimal labelling density. The Sudan Black B step was omitted. The sections were mounted with ProLong Glass Antifade Mountant (Thermo Fisher, UK) using high precision coverslips (0.170 ± 0.01 mm thick; Roth, Germany, LH25.1) to enhance image quality.

2.1.6 Imaging

2.1.6.1 Light microscopy

Images were captured using a Zeiss Z1 microscope with a motorised stage and MRc camera (Zeiss, Germany) coupled to a PC. Stereologer software (Stereologer, Bethesda, MD, USA) was used to ensure adequate and unbiased sampling. The region of interest was drawn at 1.25X magnification and a randomly oriented point grid was superimposed over the observed image (Figure 2.3 A). Within the delineated region of interest 10-15 frames were captured at 10X magnification for the densitometric analysis, whereas 25-30 frames were used for the estimation of neuronal cell number using a 63X oil immersion objective.

2.1.6.2 Confocal imaging

Fluorescent quadruple labelled sections were imaged using Nikon A1R confocal microscope and NiS elements software using 60X oil immersion objective. The settings for laser power, gain and offset were optimised for each fluorophore (405nm, 488nm, 546nm and 647nm). Ti ZDrive construction of the Z-stacks was attained by determining the lower and upper thresholds of the section depth, with multiple images captured at 0.17μm intervals through the depth of the tissue. Image averaging was set to 16X to reduce background interference and give a more accurate depiction of the tissue stain. Four images were acquired per case within the region of interest, and images analysed using IMARIS software (Bitplane, Oxford Instruments).

2.1.6.3 STED Imaging

Stimulated emission depletion microscopy (STED) creates super-resolution images by using two laser beams, an excitation laser and a doughnut-shaped STED laser beam. STED is a point scanning method that uses an excitation laser being focused on a sample to a diffraction limited volume. The second depletion laser modulates fluorescence emission in the periphery, which produces a restricted central emission area that is smaller than the diffraction limited volume (Figure 2.4). STED 3D images were acquired using Leica SP8 STED microscope and Application Suite X software (LAS X; Leica Microsystems; Figure 2.5) with 100x oil immersion objective. Images of 256×256 pixels were obtained using 35x optical zoom,

resulting in a pixel size of 13 × 13 nm. The gating parameters were set to 1.5-6.0 for the 532nm laser, and 0.5-6.0 for the 594nm and 647nm lasers. Atto647N was imaged with 652nm excitation and 750nm depletion wavelengths, Alexa Fluor 532 with 505nm excitation and 581nm depletion wavelengths, whereas Alexa Fluor 594 with 533nm excitation and 591nm depletion wavelengths. All images were deconvolved using Huygens Essential Software (Scientific Volume Imaging, Netherlands).

2.1.7 Imaging analysis

2.1.7.1 Densitometric analysis

Densitometric analysis was used to assess the percentage area stained of immunoreactivity within the region of interest. The images were captured at 10X magnification and imported into Fiji Image J analysis software (Windows 64-bit: https://fiji.sc) for densitometric analysis (Figure 2.6Figure 2.3). The Red-Green-Blue (RGB) thresholds were adjusted manually for each antibody to eliminate the detection of non-specific background staining. The percentage area stained for each antibody was quantified overall, as well as in cortical layers II, III and V. The mean percentage area stained per case was calculated from the mean values obtained across all images taken.

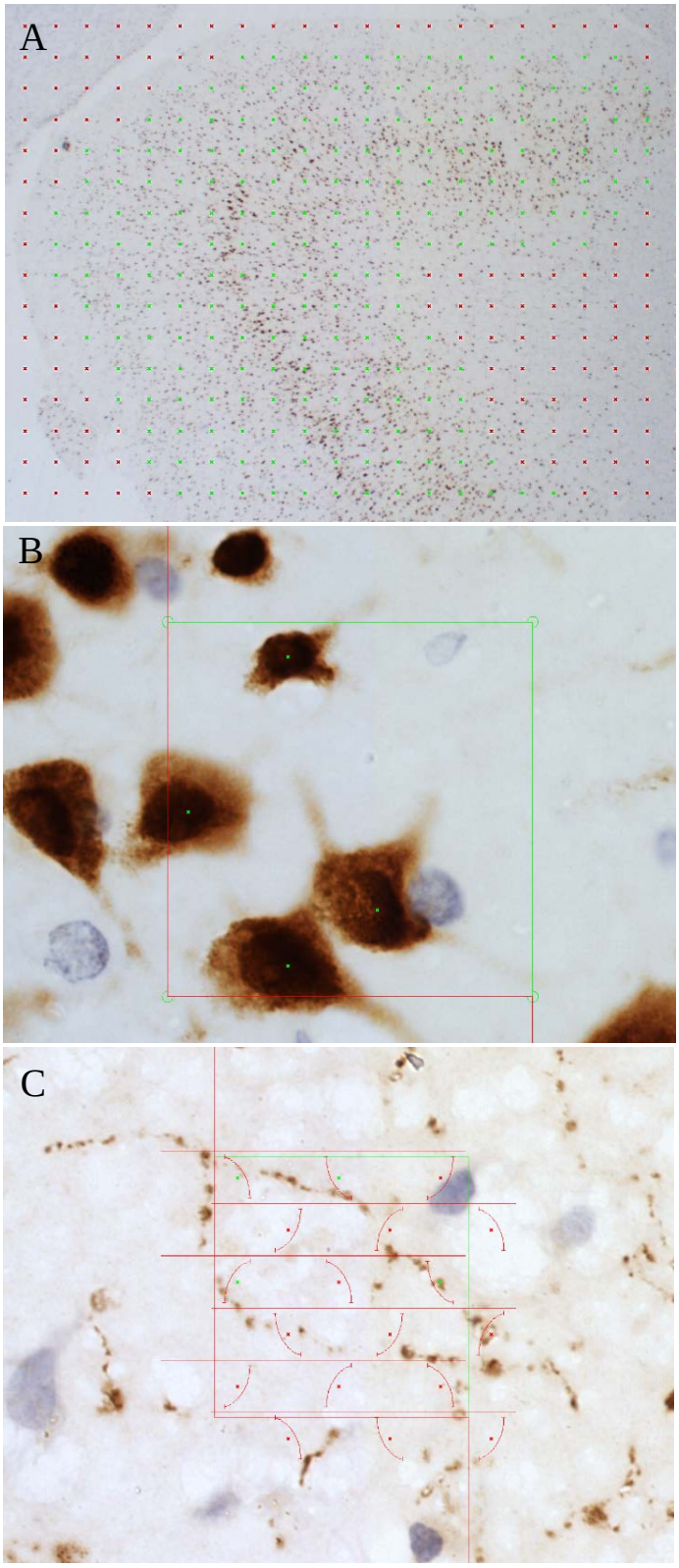

Figure 2.3 Stereological sampling.
A) A region of interest was drawn at 1.25X. A point grid was superimposed over the area, with green points representing the coordinates of sampling. B) Randomly sampled frames in the x-y axis taken at 63X, with neurones counted within a disector frame of known dimensions. C) For the estimation of monoaminergic fibres within the outlined reference space, the fibres intersecting a grid of randomly orientated cycloids are marked green.

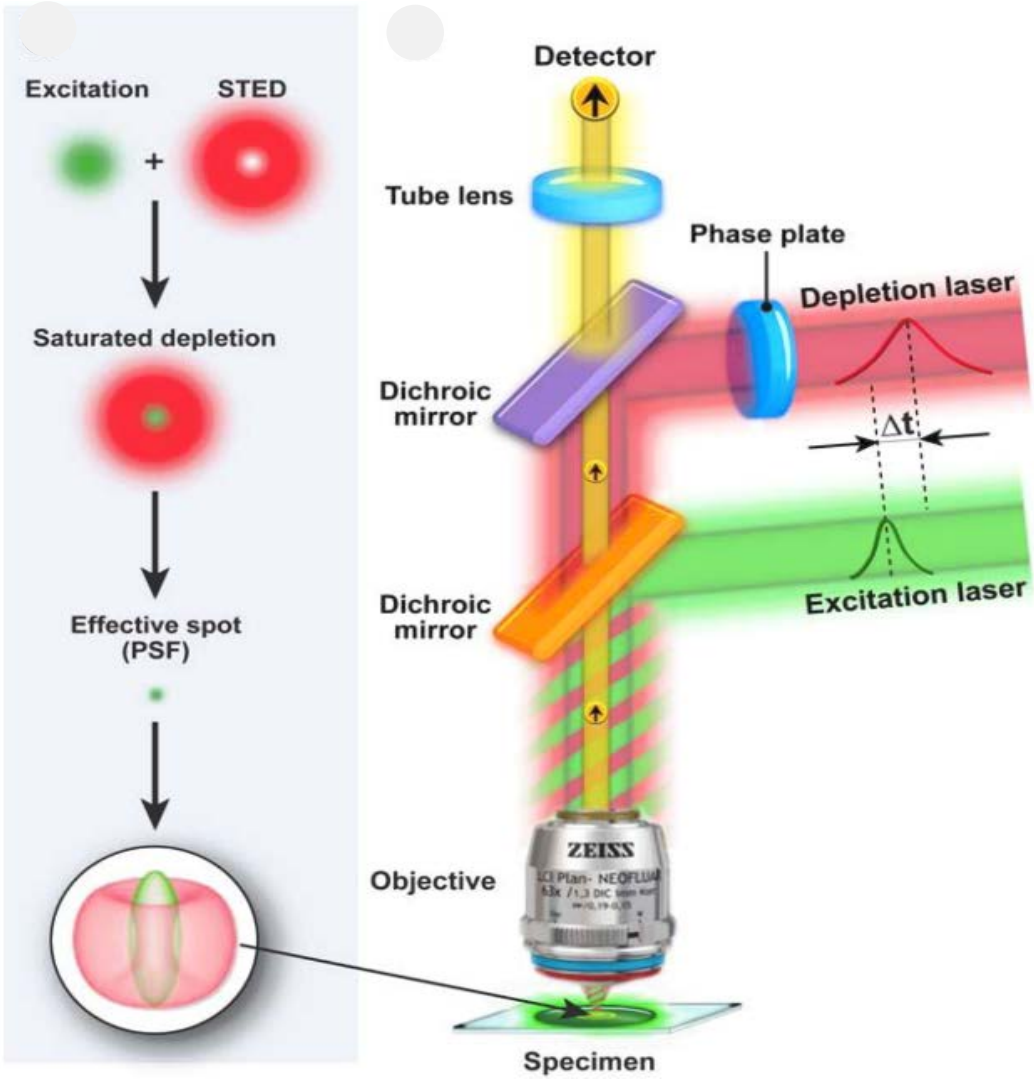

Figure 2.4 The principle of STED microscopy.
STED functions by depleting fluorescence in specific regions of the sample while leaving a central focal spot active to emit fluorescence. The depletion laser has a doughnut shaped intensity distribution, with zero intensity in the middle and high intensity in a circular region around the central position. Superimposing this depletion beam on the excitation beam prevents spontaneous emission (fluorescence) anywhere except in a very small central region (PSF), therefore the information can be acquired at a much higher resolution compared to standard confocal imaging (Ishikawa-Ankerhold, *et at.*, 2012).

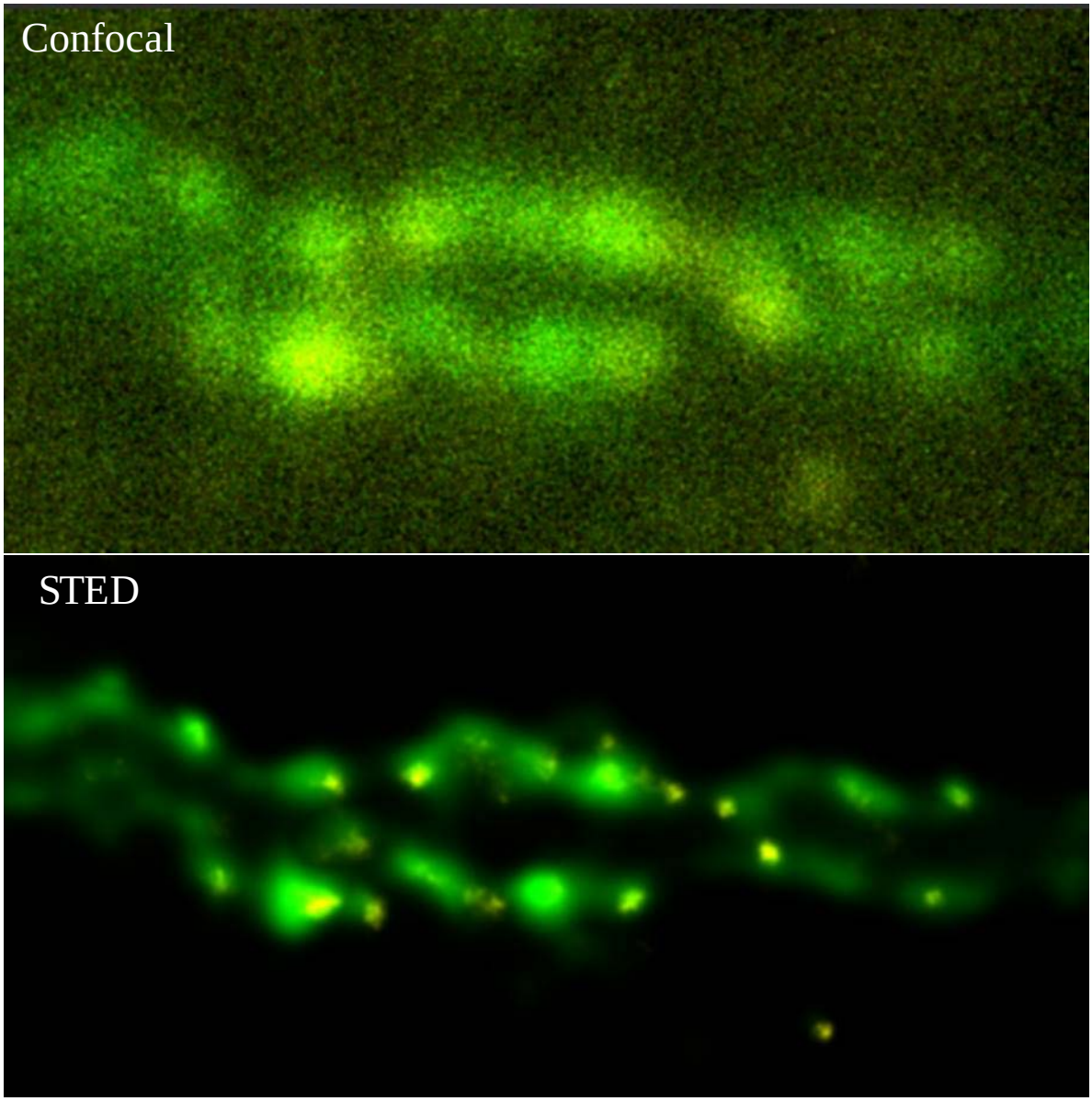

Figure 2.5 STED imaging.
3D confocal and super-resolution STED image of dopaminergic fibres (DAT; green) and presynaptic terminals (SNAP25; yellow).

2.1.7.2 Analysis of neuronal density

An adapted stereological method was used to estimate the number of neurones within the sgACC. Within each coronal section, a region of interest was drawn at low magnification (1.25X) using the Zeiss Z1 microscope with a motorised stage. A randomly oriented point grid was superimposed over the observed image ensuring the sampling of the structure in a systematic and unbiased manner through its x-y axis. The distance between points on the grid was determined based on the pilot study. The neurones were counted within the disector frame of known dimensions using 63X oil immersion objective (Figure 2.3 B). The neuronal density

within the sgACC was determined automatically by the Stereologer software (cells per μm^2), with values converted to cells per mm^2.

2.1.7.3 Analysis of monoaminergic fibers

Estimation of serotonergic (5HTT) and dopaminergic (DAT) fibres within sgACC was assessed using stereology software, by counting the number of intersections between the linear probe and lines that represent the surface feature within the dissector frame of known dimensions. The isotropic interaction between the linear probes and the surface feature was achieved through the use of VUR (vertical uniform random) sections in combination with sine-weighted line probes called cycloids (West, 2013). The green colour coded points indicate selected points where cycloids intersect with the objects of interest (Figure 2.3 C).

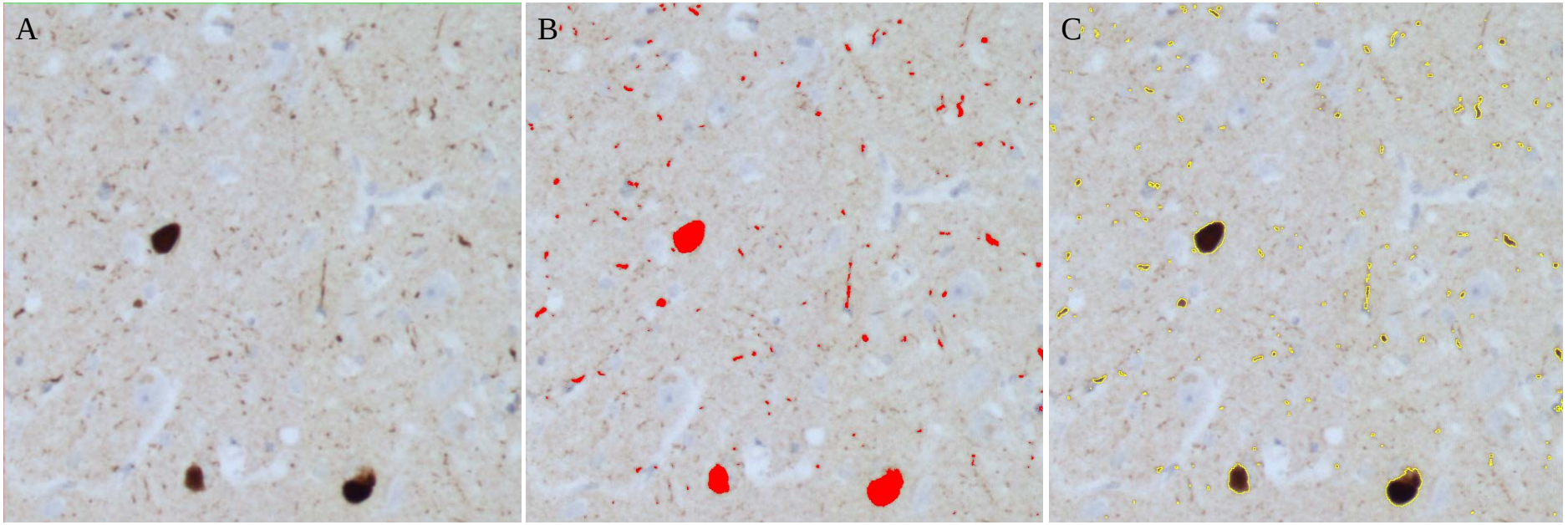

Figure 2.6 Densitometric analysis.
A) Image of α-synuclein (KM51) stained section from sgACC at 10X magnification; B) A colour threshold applied using Image J software to label Lewy body and Lewy neurite immunoreactivity; C) The selected threshold applied to quantify the percentage area stained.

2.1.7.4 Confocal analysis

To analyse GABAergic and glutamatergic synaptic volumes in relation to α-synuclein, IMARIS software was used to generate 3D representative images from the imported Z-stacks, with individual surfaces created for each fluorescent signal (Figure 2.7). 3D surfaces were created for Nuclei (647nm, purple), GAD65/67 positive GABAergic surfaces (488nm, green) or VGLUT1 positive glutamatergic surfaces (488nm, green), SNAP25 positive pre-synaptic terminals (546nm, red) and s129 positive α-synuclein surfaces (405nm, blue; Figure 2.7). The staining intensity boundaries were optimised for each laser channel (405nm 500-3500, 488nm 600-3500, 546nm 400-3000), whereas background subtraction of 1.0 and smoothness of 0.1 was applied to all the channels respectively. The number and the average volume of individual surfaces for different laser channels was calculated and averaged across four Z stacks. The number and average volume of GABAergic and glutamatergic synapses was determined based on the co-localisation between GAD65/67 or VGLUT1 positive surfaces and SNAP25. The GABA and glutamate positive surfaces were masked on to SNAP25, creating the new surface representative of a synapse. The synaptic surface was then masked onto α-synuclein, which allowed synapses containing α-synuclein to be selected, creating surfaces for the α-synuclein positive synapses.

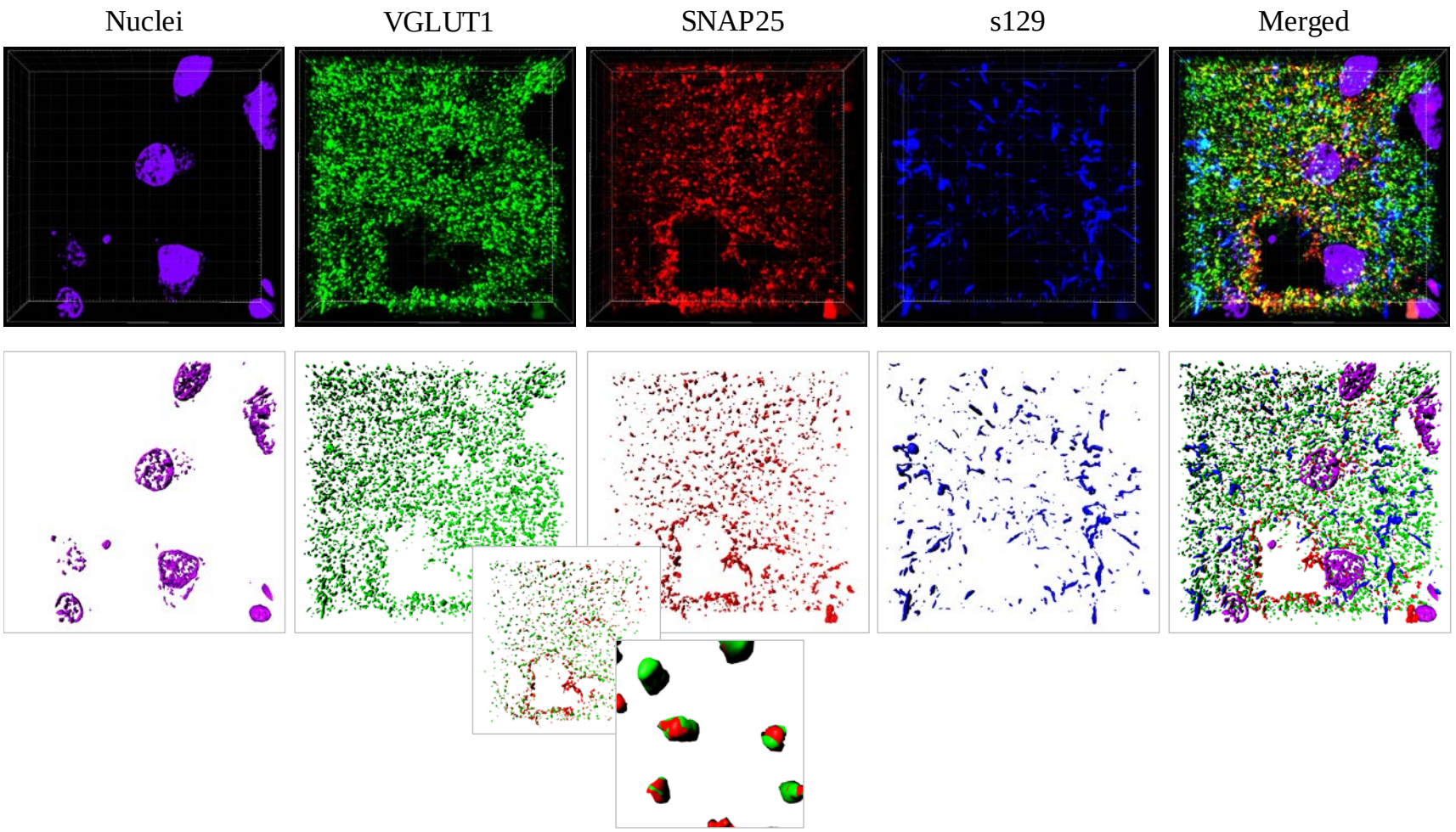

Figure 2.7 Confocal analysis.
Confocal images were acquired at 60X magnification with each fluorophore representing different laser channels. Cell nuclei (TO-PRO-3 Iodide) imaged using 647nm, glutamatergic (VGLUT1) and GABAergic (GAD65/67) surfaces with 488nm, presynaptic terminals (SNAP25) with 546nm and phosphorylated α-synuclein (s129) with 405nm. 3D surfaces were created from imported Z stacks for each laser channel using Imaris software. The surfaces for different fluorophores were overlaid to assess co-localisation.

2.1.7.5 STED analysis

3D STED images were deconvolved using Huygens Essential Software. Deconvolution is a combination of optical and computational techniques used to improve the contrast and resolution of images. Deconvolution settings were optimised to increase the final quality of the image. The number of iterations were set at 40, and the signal to noise ratio at 7. The quality threshold was set at 0.05, which is a restoration parameter that makes deconvolution stop when the change in the quality criterion between two consecutive iterations is below its value. The Object Analyzer Advanced tool was used to create 3D surfaces for each channel and obtain the quantitative measures of individual particles. A size threshold was applied to remove objects that were too small, with "garbage" volume set at 10 voxels. The seed and threshold criteria were optimised for individual channels. For the 488nm channel the seed was set at 5% and threshold at 30%, for 594 the seed was set at 5% and threshold at 15%, and for 647nm the seed set at 10% and the threshold at 25%. Colocalisation measurements were used to assess spatial overlap between structures in different data channels.

2.1.8 Protein isolation and quantification

2.1.8.1 Frozen tissue acquisition and protein extraction

The left hemisphere was sliced into coronal slices at approximately 1cm intervals, sealed in plastic bags, frozen at -120°C and stored at -80°C. Frozen tissue slices were brought to -20°C in a freezing cabinet, and cingulate subregions corresponding to sgACC, spACC, MCC and PCC were removed. Approximately 50mg of grey matter tissue was homogenised using a rotor-stator homogeniser in ice cold lysis buffer containing 0.2M triethylammonium bicarbonate (TEAB) pH 7.2 (Sigma-Aldrich, MO, USA), and 1X EDTA free protease inhibitor cocktail (Complete, Roche, UK). The crude samples were stored at -80°C.

2.1.8.2 Tissue fractionation

Total protein homogenate was fractionated to extract soluble and insoluble proteins (Culvenor et al., 1999). The tissue from sgACC was homogenized in 0.2M TEAB buffer containing EDTA free protease inhibitors with no detergent. The soluble protein fraction containing supernatant (containing proteins such as GAPDH) was extracted following high speed centrifugation (20,000 x g for 45min at 4°C), and the pellet re-suspended in 500µl of 0.1% Tween20 in 0.2M TEAB. The soluble membrane bound protein fraction (containing proteins such as SNARE proteins) was stored at -30C°. The remaining sample was centrifuged to extract

Tween20 soluble membrane bound proteins (containing proteins such as APP). The pellet was re-suspended in 500µl of 2% sodium dodecyl sulphate (SDS), and the insoluble membrane bound protein fraction (including GPCR coupled receptors) was stored at -30C°. The remaining sample was centrifuged further to extract SDS soluble membrane bound proteins, and the pellet containing highly insoluble aggregated proteins including aggregated α-synuclein, was re-suspended in 500µl of 6M Urea. The highly insoluble protein fraction was taken before further centrifugation to extract insoluble aggregated proteins (Figure 2.8). Due to the small amounts of material remaining in the Urea fraction, it was not possible to run all antibodies for this fraction. All protein fractions were stored at -30C° prior to use.

2.1.8.3 Protein quantification using Bradford assay

Two percent of SDS was added to the samples prior to sonication on ice in a sonic bath for 40min. Protein concentration was determined using Bradford assay (Bradford, 1976). The standards of known protein concentration ranging from 0 µg/ml to 1 mg/ml were prepared using bovine serum albumin (BSA; Sigma-Aldrich, UK), diluted in homogenising buffer (1:4 in MilliQ water). Sonicated samples were diluted 1:20 in MilliQ H_2O prior to analysis. Five µl of BSA standards and samples were pipetted into wells in triplicates to a flat-bottom 96-well plate. Two hundred and fifty microliters of Bradford reagent (Sigma-Aldrich, UK) was added to each well and incubated for 10min at room temperature. The absorbance was measured at 595nm using a microplate reader (TECAN, UK). The protein concentration of unknown samples was quantified from a standard curve produced from the absorbance of the BSA standard concentration.

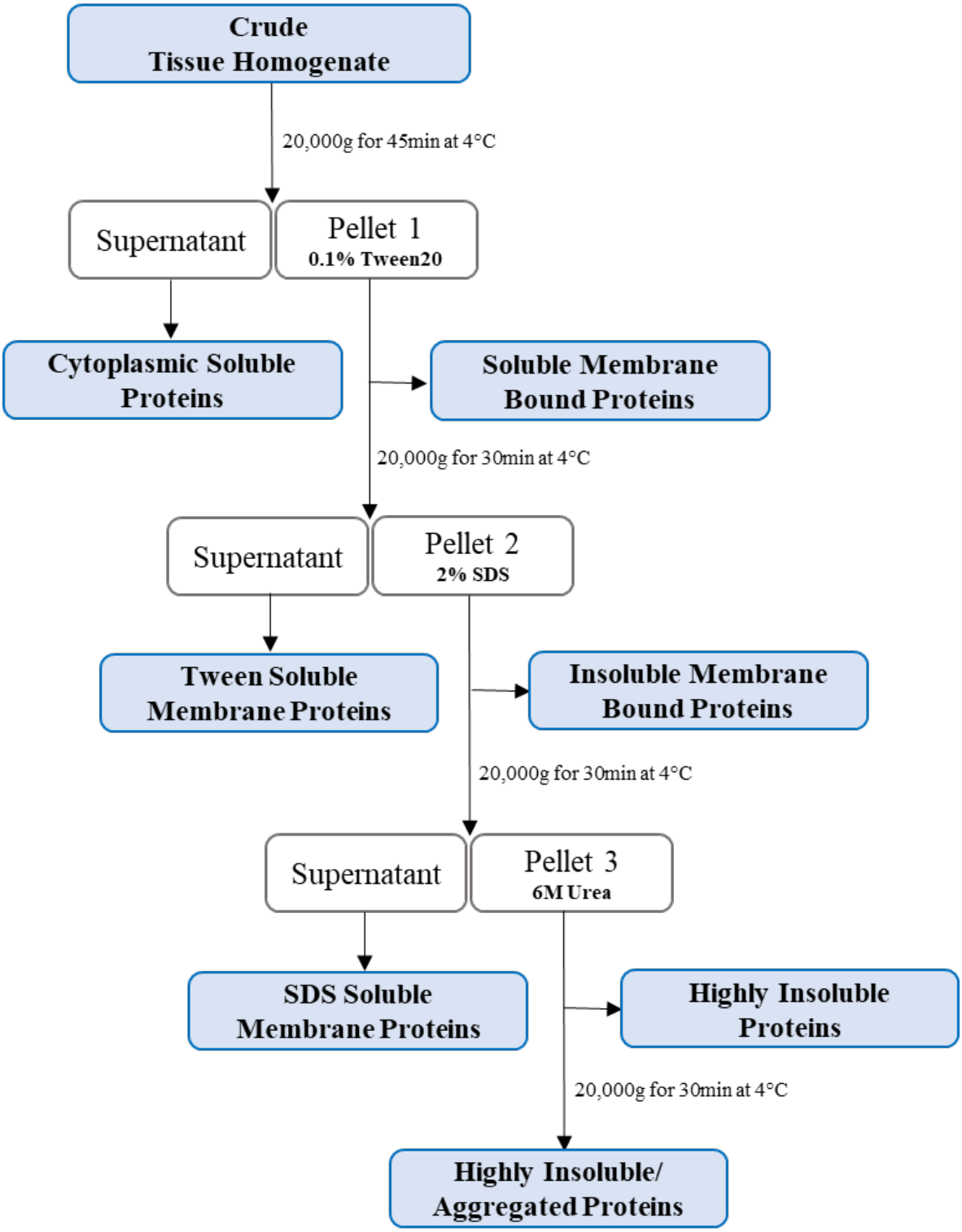

Figure 2.8 Tissue fractionation.
Crude tissue homogenates were centrifuged to extract cytoplasmic and membrane bound protein fractions using high speed centrifugation. The pellet was gradually dissolved by sequentially using 0.1% Tween20, 2% SDS and 6M Urea to extract insoluble protein fractions. Adapted from (Culvenor et al., 1999).

2.1.8.4 Western blotting

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their molecular weight and to measure changes in protein expression. Protein samples were prepared at 1 µg/µl with 4X NuPAGE LDS Sample Buffer, 10X NuPAGE Sample Reducing Agent and homogenising buffer. Denaturation of proteins was achieved by heating the samples at 70°C for 10min on a heated block, followed by brief spinning. Ten microliters of denatured samples were loaded into wells of a NuPAGE 4-12% Bis-Tris Gel (Invitrogen) alongside Chameleon 800 pre-stained protein ladder (LI-COR). The gel was electrophoresed in 1X NuPAGE MOPS SDS running buffer with each chamber containing 435µl of antioxidant (Invitrogen) at 120V for 20 minutes, followed by 160V for an hour. The transfer of proteins from the polyacrylamide gel to a nitrocellulose membrane was performed using a semi-dry electro-transfer using an iBlot2 device (Invitrogen) at 20V for 1min, 24V for 4min and 27V for 5min.

2.1.8.5 Dot blotting

The dot blot technique was used as a quantitative and semi-quantitative method for detection of proteins, with samples applied directly onto a membrane. The samples are pulled through the membrane by a vacuum, where proteins bind to the membrane and the other sample components pass through. Protein samples for dot blot were prepared at 1 µg/µl with 4X Orange G Loading Buffer, 10X NuPAGE Sample Reducing Agent and homogenising buffer. The standards ranging from 0 µg/µl to 120 µg/µl were prepared using pooled samples from all disease groups. The samples were denatured at 70°C for 10min on a heated block prior to loading. Vacuum-assisted 96-well Bio-dot Microfiltration apparatus (BioRad) was used to blot the samples. The nitrocellulose membrane (Amersham^{TM}, 0.2µm NC) was assembled on top of thick filter paper (0.5cm) in between a sealing gasket and sample template with attached sealing screws. A tight seal between the sample wells ensured maximum suction force and that there was no dispersion of samples into adjacent wells. During continuous suction, the membranes were washed with 100µl of TBS per well delivered using a multichannel pipette. Fifty µl of samples and standards were pipetted into each well in duplicate. The membrane was then washed with TBS (100µl per well), left to dry on a flat surface for 15min, fixed in 70% methanol for 20min under agitation, rinsed in MilliQ water and washed in TBS prior to blocking.

2.1.8.6 Near Infrared (NIR) Western and Dot blot detection

The proteins on nitrocellulose membrane were stained with Revert Total Protein Stain (LI-COR) for 2min under agitation. The membrane was washed twice with MilliQ water and imaged using near-infrared (NIR) fluorescence detection at 680nm for 2 min using LI-COR Odyssey Fc imaging system. After 3X 5min washes with TBS to remove the stain, the membranes were blocked using Odyssey blocking buffer (LI-COR) for 1 hour at room temperature under light agitation, then incubated with primary antibodies diluted in Odyssey blocking buffer and 0.2% Tween20 (Sigma-Aldrich) overnight at 4°C. To remove excess primary antibody the membranes were washed in TBS with 0.1% Tween20 3X for 5min on a platform shaker at room temperature, then incubated with IRDye 800WC secondary antibodies diluted in Odyssey blocking buffer and 0.2% Tween20 for 1 hour at room temperature while protected from light. Following 3X TBST washes the membranes were incubated with IRDye conjugated GAPDH 680nm (Santa Cruz Biotechnology) diluted in Odyssey blocking buffer and 0.2% Tween20 for 1 hour at room temperature. Prior to imaging, the membranes were washed 3X in TBST for 5min, followed by 2X 5min washes with TBS to remove residual Tween20 and imaged while wet.

2.1.8.7 Near Infrared (NIR) imaging analysis

The membranes were imaged using an Odyssey NIR imaging system (LI-COR Odyssey FC). Each membrane was scanned at 700nm (GAPDH) and 800nm (protein of interest) for 2 min. The protein intensity bands were quantified using Image Studio Lite (LI-COR; Figure 2.9). The brightness and contrast for each scanned image was adjusted to produce the best image quality (this does not alter the fluorescent signal). The Lane Normalisation Factor was calculated by dividing GAPDH signal for each lane by the highest GAPDH signal. The signal for protein of interest was then divided by the Lane Normalisation Factor to normalise the protein of interest.

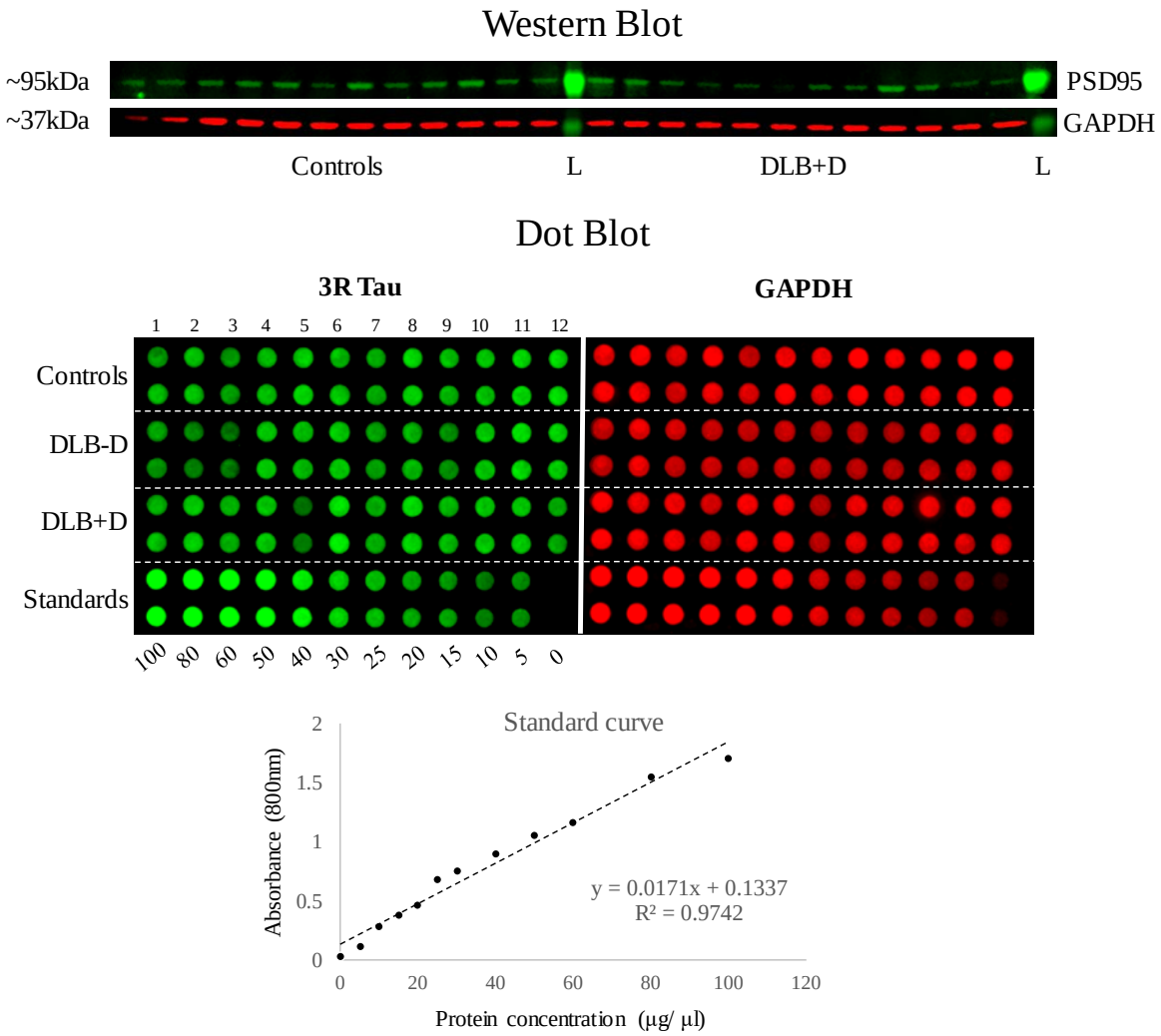

Figure 2.9 Near Infrared (NIR) imaging.
(**Top**) Western blot membrane imaged at 700nm (GAPDH) and 800nm (PSD95 and Chameleon 800 ladder) using control and DLB cases with depression. (**Middle**) Dot blot membrane imaged at 700nm (GAPDH) and 800nm (3R Tau) using controls and DLB cases with and without depression, as well as pooled standards. (**Bottom**) Plot of total protein value against absorbance (800nm) of the protein of interest to provide a standard curve for the pooled standards.

2.1.8.8 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to measure the levels of total and fibrillar α-synuclein in a fractionated sgACC tissue samples from DLB cases with and without depression and normal controls. For both total and fibrillar α-synuclein "Sandwich" ELISA (Figure 2.10), a 96 well plate (IMULON, Thermo Fisher Scientific) was coated (100µl per well) with 10D2 α-synuclein antibody (2.9 mg/ml stock concentration; Analytic Jena, Germany; 1:2000) diluted in Carbonate Bicarbonate Buffer (50mM; pH 9.6; Sigma Aldrich, UK), and incubated overnight at 4°C. Phosphate-Buffered Saline with 0.1% Tween20 (PBST) was used for all the washes (200µl per well). Following 3X

PBST washes, the plates were blocked with 1% BSA in PBST (200µl per well) for 1 hour at room temperature on an orbital shaker. Standards ranging from 0-60 µg/µl for fibrillary and 0-40 µg/µl for total α-synuclein were prepared using serially diluted recombinant proteins (Proteos, USA) in 1% BSA in PBST, with samples of 2.5 µg/µl protein prepared in PBST. Following a single wash with PBST, the standards and samples were pipetted in triplicate (100µl per well), and incubated for two hours on a shaker at room temperature. After 3X PBST washes, 100µl of detection antibody (for total α-synuclein: α-β synuclein (Abcam, EP1646Y, 1:1500); for fibrillar α-synuclein: filament (Abcam, MJFR-14-6-4-2, 1:2000)) diluted in 1% BSA in PBST was pipetted in triplicate and incubated for 1 hour, followed by 3X PBST washes. The plate was incubated with alkaline phosphatase (AP) conjugated goat anti-rabbit secondary antibody 1:1000 in 1% BSA in PBST (100µl per well) for 1 hour, followed by 3X PBST washes. A 1 mg/ml AP substrate solution was prepared by dissolving p-nitrophenyl phosphate (pNPP) in a solution consisting of 0.05M Sodium Carbonate, 0.001M magnesium chloride and 0.05M Sodium Bicarbonate (Sigma Aldrich), which was prepared 30min before use and the plate incubated at room temperature. One hundred microliters of substrate solution was added to each well and the plate incubated at 37°C in an incubator. The plate was read at 412nm absorbance at 15, 30, 45 and 60min time points.

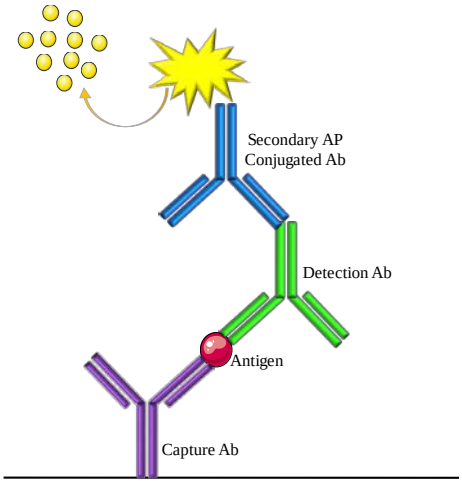

Figure 2.10 "Sandwich" ELISA.
The capture antibody 10D2 was used to coat a 96 well plate prior to incubation with antigen (recombinant non-aggregated or fibrillary α-synuclein), which was recognised by detection antibodies (α-β or fibrillar synuclein). The detection antibody was detected by the secondary AP conjugated antibody. The exposure to substrate resulted in the colour change, which was measure by absorbance at 412nm.

2.1.9 Statistical analyses

Statistical analyses were performed using SPSS Statistics version 22.0. The normal distribution across samples was assessed using Shapiro–Wilk test, with homogeneity of variance determined using Levene's test. If the assumptions of normality were met, analysis of variance (ANOVA) was used to analyse the data sets between the groups, followed by Bonferroni post-hoc analysis to correct for multiple comparisons. A paired-samples *t*-test was used for pairwise comparisons within groups. Where the normal distribution criteria was not fulfilled, a non-parametric Kruskal-Wallis test was used to compare multiple groups, with adjusted *p*-values to correct for multiple comparisons, so that the overall error rate remained at 5%. Friedman's ANOVA was used for pairwise comparisons within groups. The effect sizes for group differences was calculated using Wilcoxon test. Correlation analyses were carried out using Spearman's correlation coefficient ρ (rho). The relationship between imaging, pathological and clinical variables was assessed using linear regression analyses.

Chapter 3.

Pathological changes in the cingulate cortex

3.1 Introduction

In DLB, the cingulate cortex, and in particular the ACC, shows extensive LB pathology that can be observed at an early disease stage along with grey matter atrophy and metabolic abnormalities (Blanc et al., 2016, Braak et al., 2002, Yong et al., 2007). Decreased grey matter volume of the ACC was shown to be associated with neuropsychological impairment in DLB (Sanchez-Castaneda et al., 2009). Metabolic and perfusion deficits within the cingulate cortex are commonly observed in DLB patients, suggesting that the cingulate cortex is susceptible to functional changes (Lim et al., 2009). Co-occurring AD associated Aβ and tau pathological changes occur in 50-80% of DLB patients and may contribute to clinical features of disease (Halliday et al., 2011). The ACC is integral to emotional processing and symptomatic expression of depression (Drevets et al., 2008b), with the sgACC showing structural, functional and metabolic abnormalities in MDD (Li et al., 2014b). The sgACC is the primary target in the treatment of depression using DBS, producing an effective antidepressant effect in treatment resistant depression (Berlim et al., 2014). Depression is a common feature of DLB (Ballard et al., 2004), associated with higher mortality rates and a poor response to treatment (Ritchie et al., 1998). However, little is known about how pathological burden in cingulate subregions, particularly the sgACC, influences development of depression in DLB.

3.1.2 Aims

The aim of this study was to investigate pathological changes within the cingulate cortex and particularly in the sgACC in relation to depression in DLB.

3.1.2.1 Objectives

- To assess pathological changes of α-synuclein, p-Tau and Aβ within cingulate cortex subregions in relation to depression using *post-mortem* tissue from DLB cases with and without depression, and normal controls.

- To quantify overall and layer specific pathological changes of α-synuclein, p-Tau and Aβ in sgACC in relation to depression in DLB using post-mortem fixed tissue.

- To quantify protein levels of α-synuclein, p-Tau and Aβ markers in sgACC using fractionated frozen post mortem tissue samples.

- To assess neuronal cell numbers in sgACC in DLB cases with and without depression and controls.

3.2 Methods

The cohort for the neuropathological analysis consisted of 16 DLB cases with depression, 20 DLB cases without depression and 17 neurologically and psychiatrically normal controls (Table 3.1). Of note, 4 DLB cases also fulfilled the neuropathological criteria for high AD neuropathological change and could therefore be classified as neuropathologically mixed AD/ DLB with a Lewy body disease (LBD) clinical phenotype (Montine et al., 2012, Walker et al., 2015). The inclusion criteria for depression diagnosis was made using CSDD (>8) or GDS (>10). In the absence of clinical diagnosis of depression, retrospective analysis of clinical records was used to verify the presence or absence of depression including use of antidepressant medication. Ten micron thick sections were sampled from fixed tissue blocks corresponding to sgACC, spACC, MCC and PCC (Figure 3.1), and stained with antibodies against α-synuclein (KM51), p-Tau (AT8) and Aβ (4G8) (Table 3.2) for densitometric analysis (see Methods Section 2.7.1 for full details). The cohort for biochemical analysis consisted of 12 DLB cases with depression, 12 DLB cases without depression and 12 normal controls (Table 3.3). Cases were identified with relatively short PM delay and were selected for analysis, as autolysis and protein degradation is a potential concern when working with post mortem tissues. For the biochemical analysis, with the focus on sgACC, tissue samples were fractionated to extract soluble and insoluble proteins (see section 2.8.2 for fractionation method), which were then quantified using western blotting (WB) and dot blotting (DB) techniques (see sections 2.8.4 and 2.8.5).

Table 3.1 Demographic information for neuropathological cohort.

Case ID	Diagnosis	Depression	Gender	Age at death	PM delay	McKeith	NFT Braak stage	CERAD
1	DLB	yes	Male	71	22	Neocortical	3	Moderate
2	DLB	yes	Male	76	13	Neocortical	2	Sparse
3	DLB	yes	Female	91	10	Neocortical	3	Moderate
4	DLB	yes	Female	81	44	Neocortical	4	Moderate
5	DLB	yes	Male	77	29	Neocortical	2	Moderate
6	DLB	yes	Female	77	23	Neocortical	3	Moderate
7	DLB	yes	Male	84	72	Neocortical	4	None
8	DLB	yes	Female	73	99	Neocortical	3	None
9	DLB	yes	Male	72	89	Neocortical	3	None
10	DLB	yes	Male	78	83	Limbic	1	None
11	DLB	yes	Female	91	84	Limbic	5	Frequent
12	DLB	yes	Female	75	64	Diffuse	6	Frequent
13	DLB	yes	Male	77	65	Neocortical	3	Sparse
14	DLB	yes	Female	75	51	Limbic	5	Frequent
15	DLB	yes	Male	89	88	Neocortical	3	Sparse
16	DLB	yes	Female	57	40	Brain stem	0	N/A
17	DLB	no	Male	81	24	Neocortical	4	Sparse
18	DLB	no	Male	78	8	Neocortical	3	Moderate
19	DLB	no	Male	77	8	Neocortical	2	Moderate
20	DLB	no	Male	71	8	Neocortical	2	Sparse
21	DLB	no	Female	88	16	Neocortical	3	Moderate
22	DLB	no	Male	92	20	Neocortical	4	n/a
23	DLB	no	Male	74	60	Neocortical	2	None
24	DLB	no	Female	87	90	Neocortical	2	None
25	DLB	no	Male	73	47	Neocortical	3	None
26	DLB	no	Male	81	81	Neocortical	3	Moderate
27	DLB	no	Male	77	46	Neocortical	3	None

28	DLB	no	Male	74	42	Neocortical	4	Sparse
29	DLB	no	Male	71	68	Neocortical	3	Sparse
30	DLB	no	Female	75	78	Neocortical	6	Frequent
31	DLB	no	Female	78	96	Neocortical	3	Sparse
32	DLB	no	Female	78	120	Diffuse	5	Frequent
33	DLB	no	Male	84	56	Neocortical	3	Moderate
34	DLB	no	Female	84	127	Neocortical	2	None
35	DLB	no	Male	81	51	Neocortical	3	Sparse
36	DLB	no	Male	79	110	Neocortical	3	None
37	Control	no	Female	78	23	No LB pathology	2	Sparse
38	Control	no	Male	90	161	No LB pathology	3	Sparse
39	Control	no	Female	93	12	No LB pathology	3	None
40	Control	no	Female	93	101	No LB pathology	3	Sparse
41	Control	no	Male	94	39	No LB pathology	2	Sparse
42	Control	no	Male	71	25	No LB pathology	1	None
43	Control	no	Female	90	63	No LB pathology	2	None
44	Control	no	Female	76	86	No LB pathology	2	None
45	Control	no	Male	85	45	No LB pathology	1	none
46	Control	no	Male	96	16	No LB pathology	2	None
47	Control	no	Male	85	57	No LB pathology	3	sparse
48	Control	no	Male	99	5	No LB pathology	2	None
49	Control	no	Female	97	21	No LB pathology	2	Sparse
50	Control	no	Female	94	15	No LB pathology	2	None
51	Control	no	Female	85	95	No LB pathology	2	None
52	Control	no	Female	72	27	No LB pathology	1	sparse
53	Control	no	Female	58	39	No LB pathology	0	none

‘PM delay’ represents the interval between death and post-mortem examination; ‘Braak NFT stage’ evaluates the density and distribution of NFT pathology (Braak *et al*., 2006); ‘McKeith’ criteria represents global LB pathology stage (McKeith *et al.*, 2005), ‘CERAD’ consortium to establish a registry of AD, assesses neuritic plaques (Gearing *et al*., 1995). N/A, data not available.

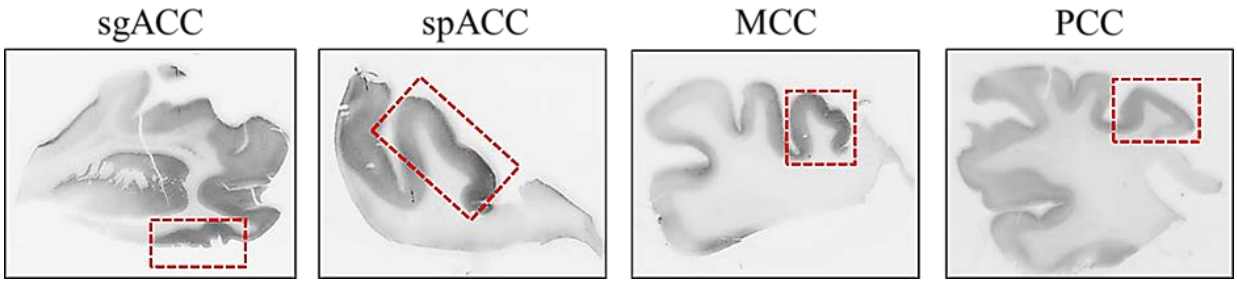

Figure 3.1 Cingulate subregions.
Scanned coronal cingulate sections of a DLB case with depression (case ID-14) stained with α-synuclein (KM51). The red boxes demonstrate the regions of interest.

Table 3.2 Antibodies used for pathological study.

Antigen	Host/IgG	Dilution	Source	Product Code
α-synuclein fillament	mRb (IgG)	1:500 (DB)	Abcam	ab209938
α-synuclein (KM51)	mMs l (IgG1)	1:500 (DB) 1:50 (IHC)	Leica Biosystems	ASYN-L
α-synuclein (5G4)	mMs (IgG1)	1:500 (DB) 1:4500 (IHC)	Analytik Jena	847-0102004001
α-synuclein (s129)	pRb (IgG)	1:500 (DB)	Abcam	ab168381
α-synuclein (α-β)	mRb (IgG)	1:500 (DB)	Abcam	ab51252
α-synuclein (pY125)	mMs (IgG2b)	1:500 (DB)	BD Biosciences	558246
Amyloid-beta (4G8)	mMs (IgG2b)	1:500 (DB) 1:12000 (IHC)	BioLegend	800701
Amyloid-beta (1-40)	mMs (IgG)	1:500 (DB)	Sigma-Aldrich	05-799
Amyloid-beta (1-42)	mMs (IgG1)	1:500 (DB)	Sigma-Aldrich	05-831-I
Tau (AT8)	mMs (IgG1)	1:500 (DB) 1:8000 (IHC)	Thermo Fisher	MN1020
Tau (3R)	mMs (IgG)	1:500 (DB)	Sigma-Aldrich	05-803
Tau (4R)	mMs (IgG)	1:500 (DB)	Sigma-Aldrich	05-804
NeuN	mRb (IgG)	1:1000 (IHC)	Abcam	ab207279

DB – dot blot; IHC – immunohistochemistry; m – monoclonal; p – polyclonal; Ms – mouse; Rb – rabbit.

Table 3.3 Demographic information for sgACC biochemical cohort.

Case ID	Diagnosis	Depression	Sex	pH	PM delay	Age at death
1	DLB	yes	Male	6.09	22	71
2	DLB	yes	Male	6.12	13	76
3	DLB	yes	Female	6.51	10	91
4	DLB	yes	Female	6.14	44	81
5	DLB	yes	Male	6.36	29	77
6	DLB	yes	Female	6.24	23	77
7	DLB	yes	Male	6.06	19	69
8	DLB	yes	Female	6.07	21	75
9	DLB	yes	Male	6.45	18	75
10	DLB	yes	Male	6.36	29	77
11	DLB	yes	Female	6.64	48	70
12	DLB	yes	Female	5.77	40	57
13	DLB	no	Male	6.66	26	79
14	DLB	no	Male	5.7	22	71
15	DLB	no	Female	6.27	10	72
16	DLB	no	Male	6.54	31	66
17	DLB	no	Male	6.15	24	81
18	DLB	no	Male	6.4	8	78
19	DLB	no	Male	6.41	8	77
20	DLB	no	Male	6.33	8	71
21	DLB	no	Female	5.92	16	88
22	DLB	no	Male	6.19	20	92
23	DLB	no	Male	6.29	18	72
24	DLB	no	Female	6.02	4	70
25	Control	no	Female	6.47	23	78
26	Control	no	Female	6.39	21	88
25	Control	no	Male	6.24	24	85
26	Control	no	Male	6.78	30	66
29	Control	no	Male	6.89	22	67
30	Control	no	Male	6.13	17	72
31	Control	no	Female	6.35	34	78
32	Control	no	Male	6.45	25	73
33	Control	no	Female	6.2	29	96
34	Control	no	Female	6.57	26	82
35	Control	no	Male	6.66	9	66
36	Control	no	Male	6.57	35	57

Pathological protein levels were assessed in 12 DLB cases with, 12 DLB cases without depression and 12 normal controls. The cases that were also included in immunohistochemical analysis are in **bold**. 'PM delay' represents the interval between death and post-mortem.

3.3 Results

3.3.1 Pathological changes in cingulate subregions

There was no significant difference in gender (p=0.109), post-mortem delay (p=0.790) or age at death (p=0.464) between controls or either of the DLB groups. No α-synuclein immunoreactivity was observer in control cases in all cingulate subregions. The KM51 α-synuclein pathological burden was highest in spACC, followed by sgACC in DLB cases, with MCC and PCC showing relatively low levels of LB and LN pathology (Figure 3.2). In DLB cases, 5G4 α-synuclein immunoreactivity in cingulate subregions was higher compared to KM51, with spACC and sgACC showing highest, and MCC and PCC moderate pathological burden (Figure 3.3). A significant main effect of diagnosis on α-synuclein pathological burden (KM51 and 5G4) was observed in sgACC, spACC, MCC and PCC, with significant increase found in all cingulate subregions in DLB cases with and without depression compared to controls (p<0.001, Figure 3.4). Using paired within subjects analysis, DLB cases with depression showed significantly higher α-synuclein (KM51) burden in spACC compared to the PCC (p=0.001) and MCC (p=0.039), whereas DLB cases without depression had higher α-synuclein levels in spACC compared to the PCC (p=0.001). No significant regional differences in α-synuclein (5G4) burden were observed between the groups.

The control cases showed p-Tau immunoreactivity in the form of NFT and NT in sgACC, with low levels of NT observed in the spACC, whereas MCC and PCC showing the absence of p-Tau pathology. DLB cases without depression had highest p-tau levels in sgACC, moderate levels of pathology in spACC and MCC, whereas low levels in PCC. The highest p-Tau burden in DLB cases with depression was observed in MCC and PCC (Figure 3.5). Tau pathological burden was significantly different in sgACC (H(2)=7.899, p=0.019), spACC (H(2)=15.414, p<0.001), MCC (H(2)=13.837, p=0.001) and PCC (H(2)=6.849, p=0.033) between the groups. DLB cases with depression showed significantly higher p-Tau burden in spACC (p=0.001), MCC (p=0.001) and PCC (p=0.027) compared to controls, however DLB cases without depression showed significant changes only in sgACC (p=0.028) and spACC (p=0.005) compared to controls (Figure 3.6 A). Within group paired analysis showed higher tau burden in sgACC compared to the MCC (p=0.045) and PCC (p=0.008) in controls, as well as in sgACC compared to PCC in DLB cases with depression (p=0.027), but no regional differences in tau distribution in DLB cases with depression.

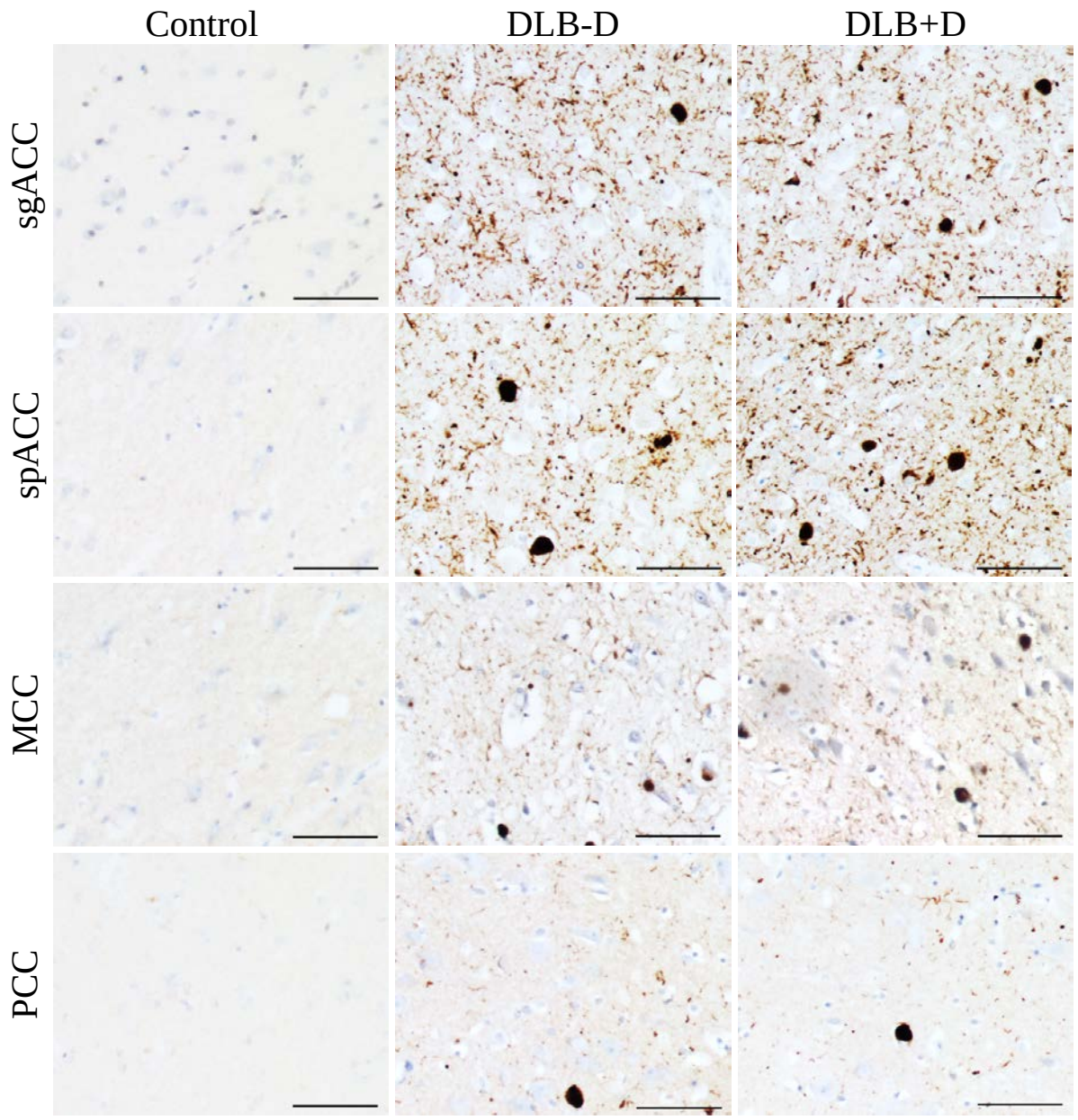

Figure 3.2 α-synuclein (KM51) pathology in cingulate subregions.
Photomicrographs of α-synuclein (KM51) pathology in sgACC (BA25), spACC (BA24), MCC (BA24') and PCC (BA23) in controls, DLB cases without (DLB-D) and DLB cases with depression (DLB+D); magnification X10; scale bars represent 100μm.

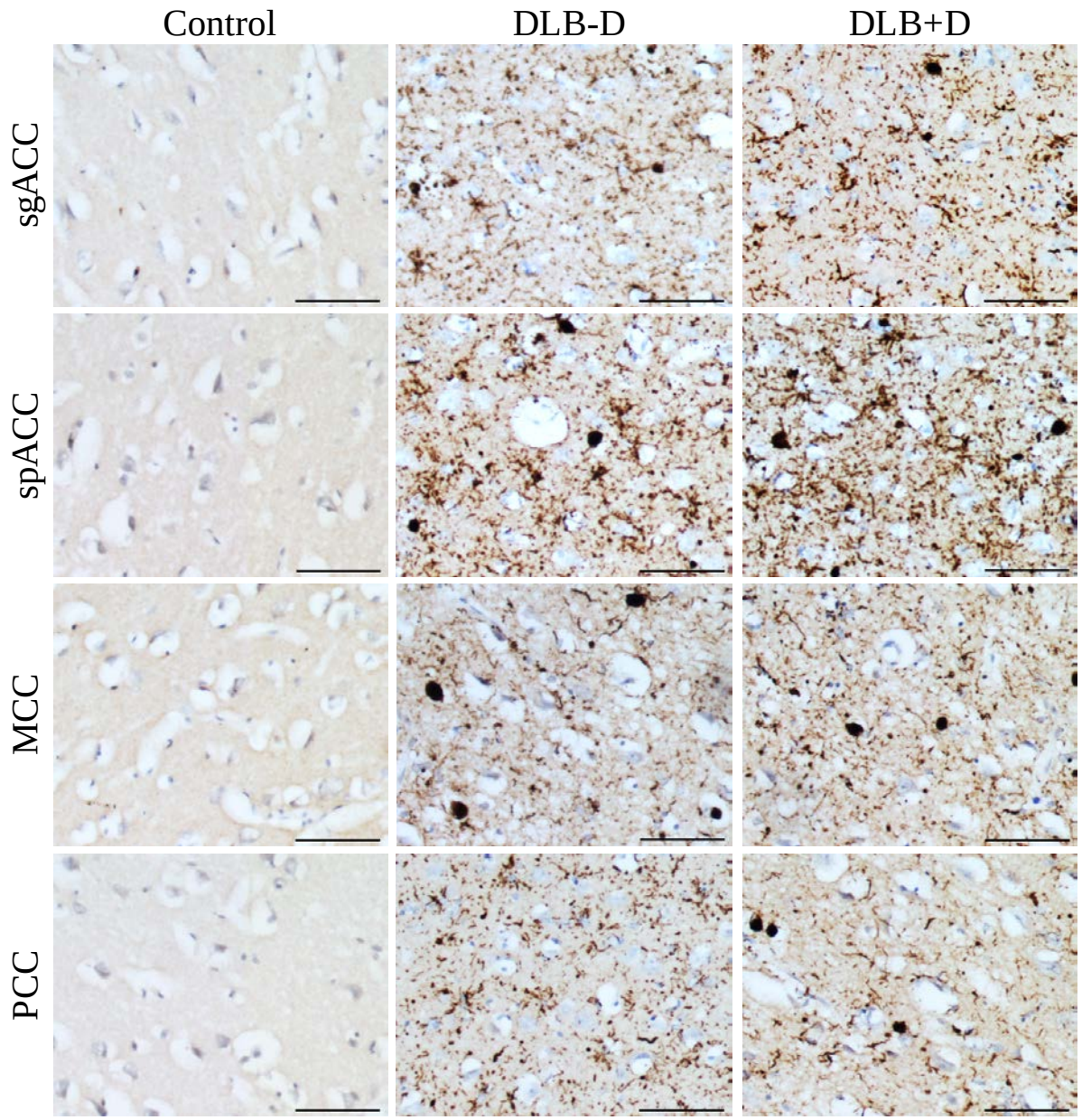

Figure 3.3 α-synuclein (5G4) pathology in cingulate subregions.
Photomicrographs of α-synuclein (5G4) pathology in sgACC (BA25), spACC (BA24), MCC (BA24') and PCC (BA23) in controls, DLB cases without (DLB-D) and DLB cases with depression (DLB+D); magnification X10; scale bars represent 100μm.

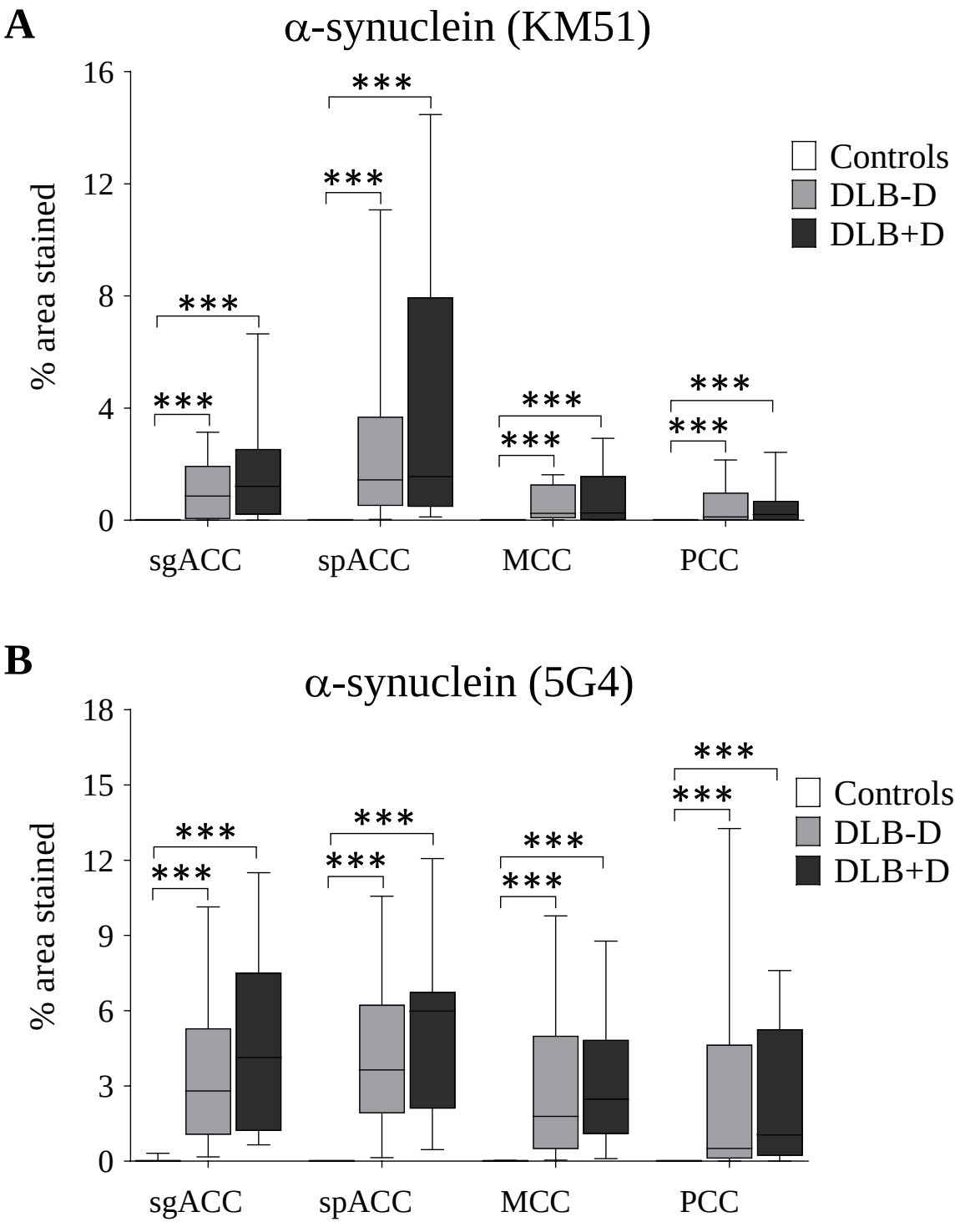

Figure 3.4 Densitometric analysis of α-synuclein pathological burden within cingulate subregions.

α-synuclein (A) KM51 and (B) 5G4 pathology (% area stained) was assessed within sgACC, spACC, MCC and PCC between DLB cases with and without depression and controls; (*** $p<0.001$, compared to control group).

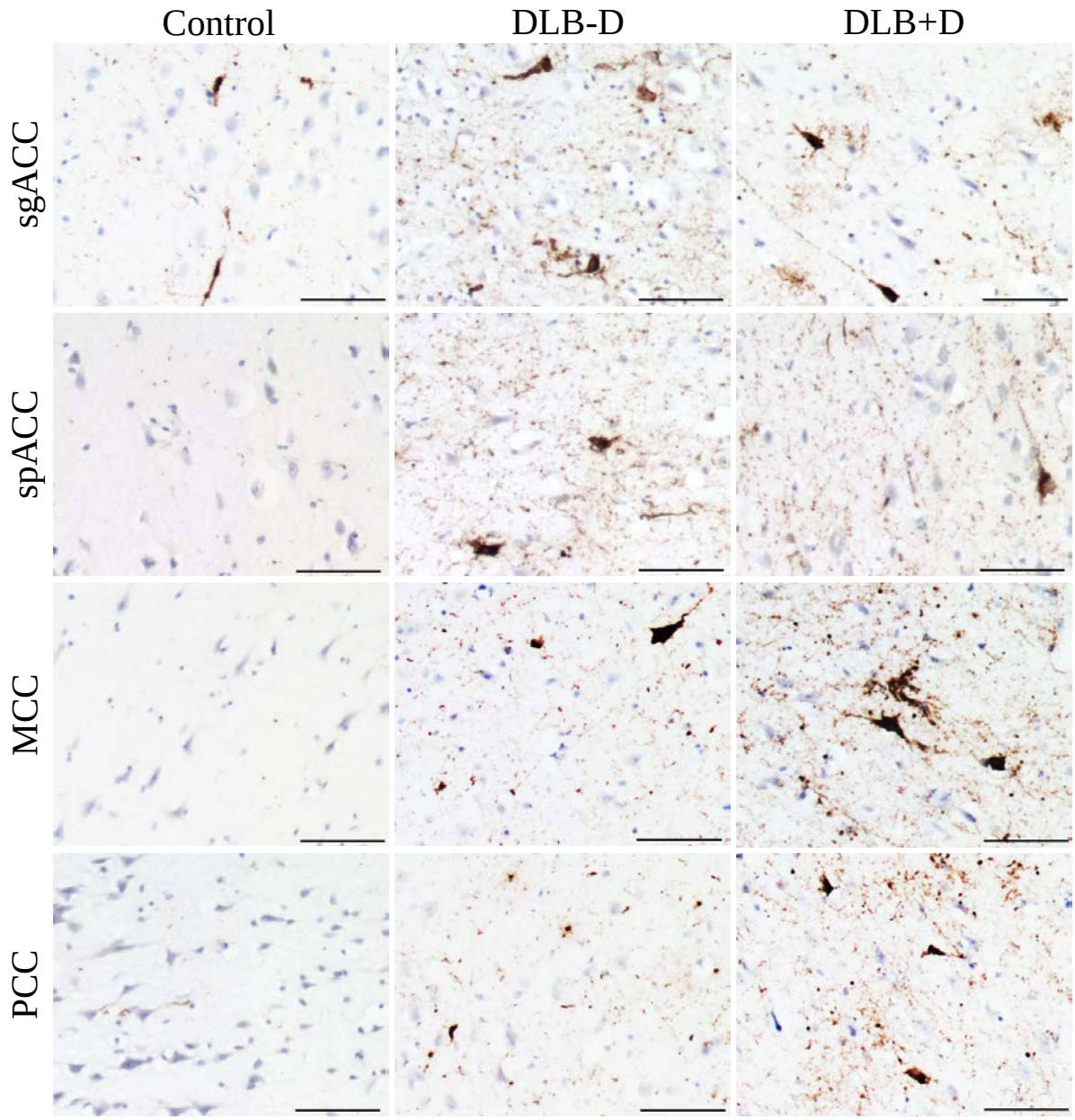

Figure 3.5 Tau (AT8) pathology in cingulate subregions.
Photomicrographs of tau (AT8) pathology in sgACC (BA25), spACC (BA24), MCC (BA24') and PCC (BA23) in controls, DLB cases without (DLB-D) and DLB cases with depression (DLB+D); magnification X10; scale bars represent 100μm.

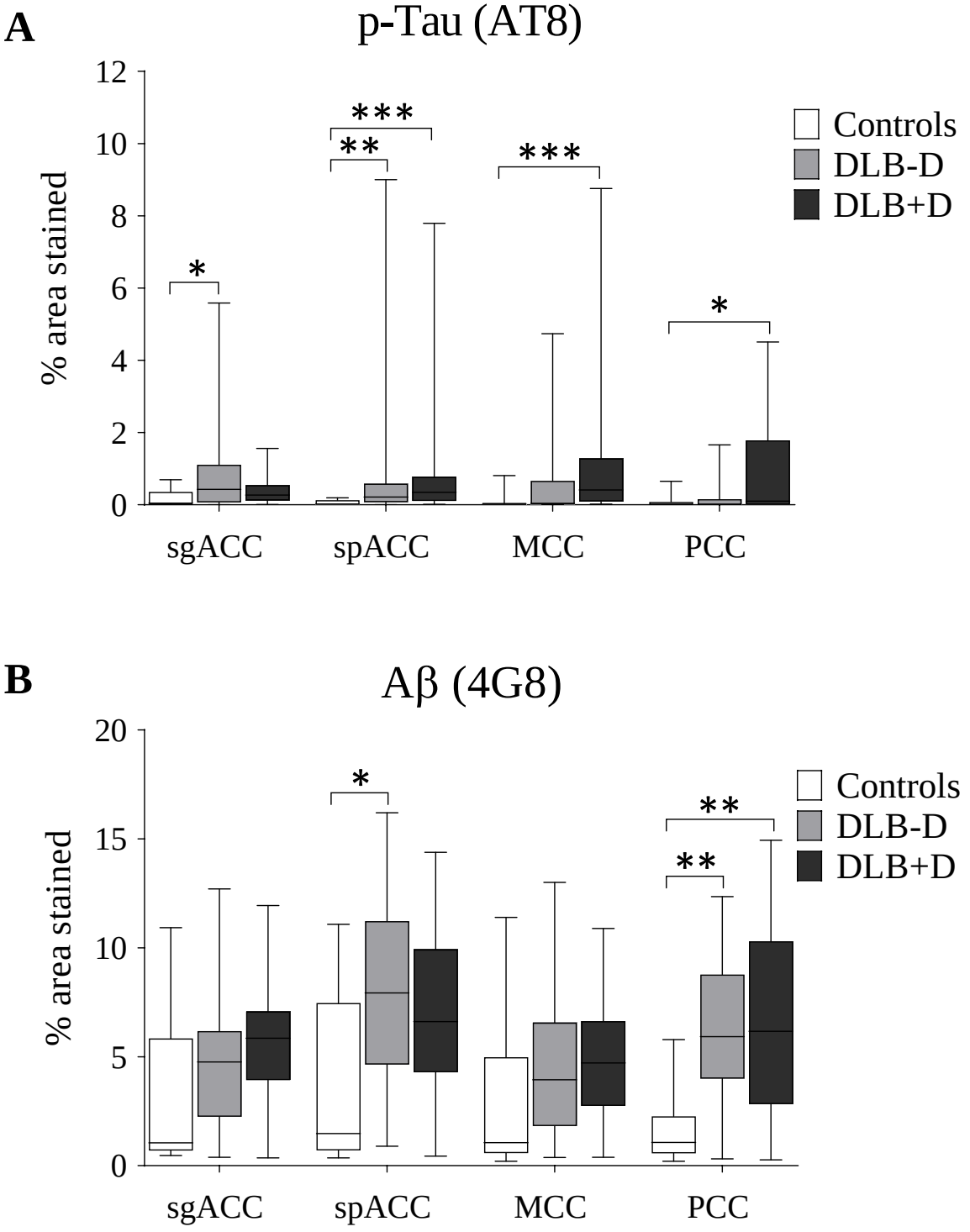

Figure 3.6 Densitometric analysis of tau and Aβ pathological burden within cingulate subregions.

(**A**) Tau (AT8) and (**B**) Aβ (4G8) pathology (% area stained) was assessed within sgACC, spACC, MCC and PCC between DLB cases with and without depression and controls; (**p*<0.05, ***p*<0.01 and *** *p*<0.001, compared to control group).

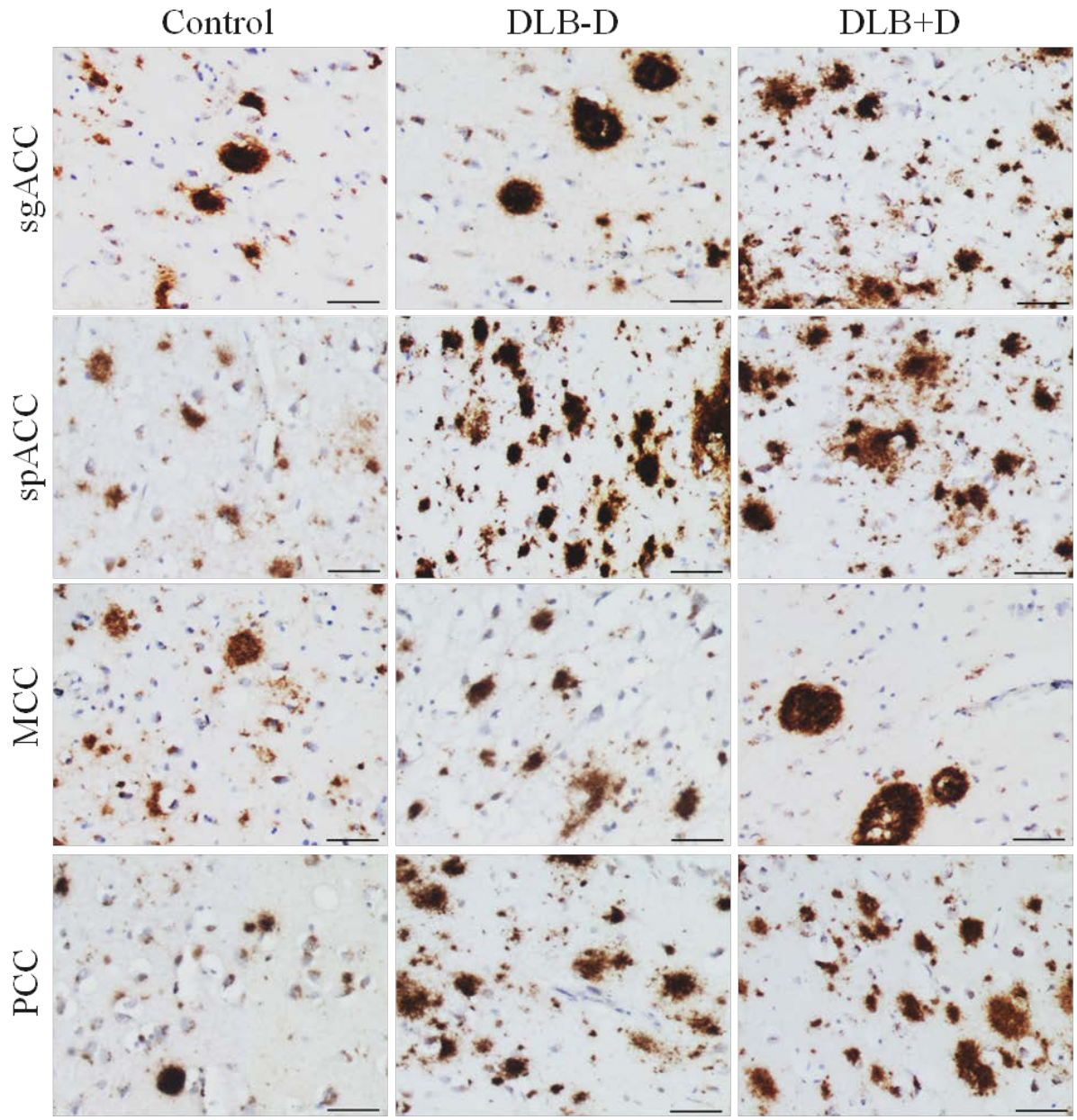

Figure 3.7 Aβ (4G8) pathology in cingulate subregions.
Photomicrographs of Aβ (4G8) pathology in sgACC (BA25), spACC (BA24), MCC (BA24') and PCC (BA23) in controls, DLB cases without (DLB-D) and DLB cases with depression (DLB+D); magnification X10; scale bars represent 100μm.

All groups showed Aβ immunoreactivity in all cingulate subregions. The highest Aβ pathological burden in DLB cases was observed in spACC and PCC (Figure 3.7). Aβ burden was significantly different between the groups in spACC ($H(2)=9.203$, $p=0.010$), and PCC ($H(2)=12.803$, $p=0.002$). A significant increase in Aβ pathology was found in DLB cases

without depression compared to controls in spACC (p=0.012) and PCC (p=0.009). Significantly elevated Aβ burden was observed in PCC in DLB cases with depression compared to controls (p=0.005) (Figure 3.6 B). Paired analysis showed higher Aβ burden in spACC compared to the MCC in DLB cases without depression (p=0.023), but no regional differences in Aβ distribution in DLB cases with depression or controls. No significant difference in α-synuclein, tau or Aβ burden was observed between DLB cases with and without depression in any cingulate subregions analysed.

3.3.2 Pathological changes in sgACC in relation to depression in DLB

3.3.2.1 Immunohistochemical changes

Using immunohistochemical analysis, pathological changes within main cortical input and output laminae (layers II, III and V) were assessed in sgACC. In DLB cases KM51 α-synuclein pathology was present in all laminae, with moderate LN pathology observed in layers II and III, and sparse in layer V. LB in layer III were less frequently observed and were smaller in size compared to layer V LB. Severe LN pathology was observed in all layers using 5G4 α-synuclein, with medium/large LB in layers III and V. Non-parametric Kruskal-Wallis analysis was used to assess overall and layer specific pathological changes between the groups in sgACC. Both KM51 and 5G4 α-synuclein pathological burden was significantly different between the groups, with higher α-synuclein immunoreactivity observed in DLB cases with and without depression compared to controls (p<0.001) in all cortical layers within sgACC, but no differences were seen between depressed and non-depressed DLB cases (Figure 3.8 A-B).

While NFT were primarily observed only in layer V, moderate NT distribution was seen in all the layers. Tau pathology in sgACC was significantly different between the groups overall (H(2)=9.936, p=0.007), layer II (H(2)=9.106, p=0.011), layer III (H(2)=10.130, p=0.006*)* and layer V (H(2)=8.798, p=0.012), with higher tau immunoreactivity observed in DLB cases without depression compared to controls overall (p=0.008), layer II (p=0.017), layer III (p=0.005) and layer V (p=0.011) (Figure 3.8 C). Aβ plaques were present in all three layers, with similar topographical distribution across all laminae. No significant difference in overall or layer specific Aβ pathology burden was observed between disease subgroups in sgACC (Figure 3.8 D).

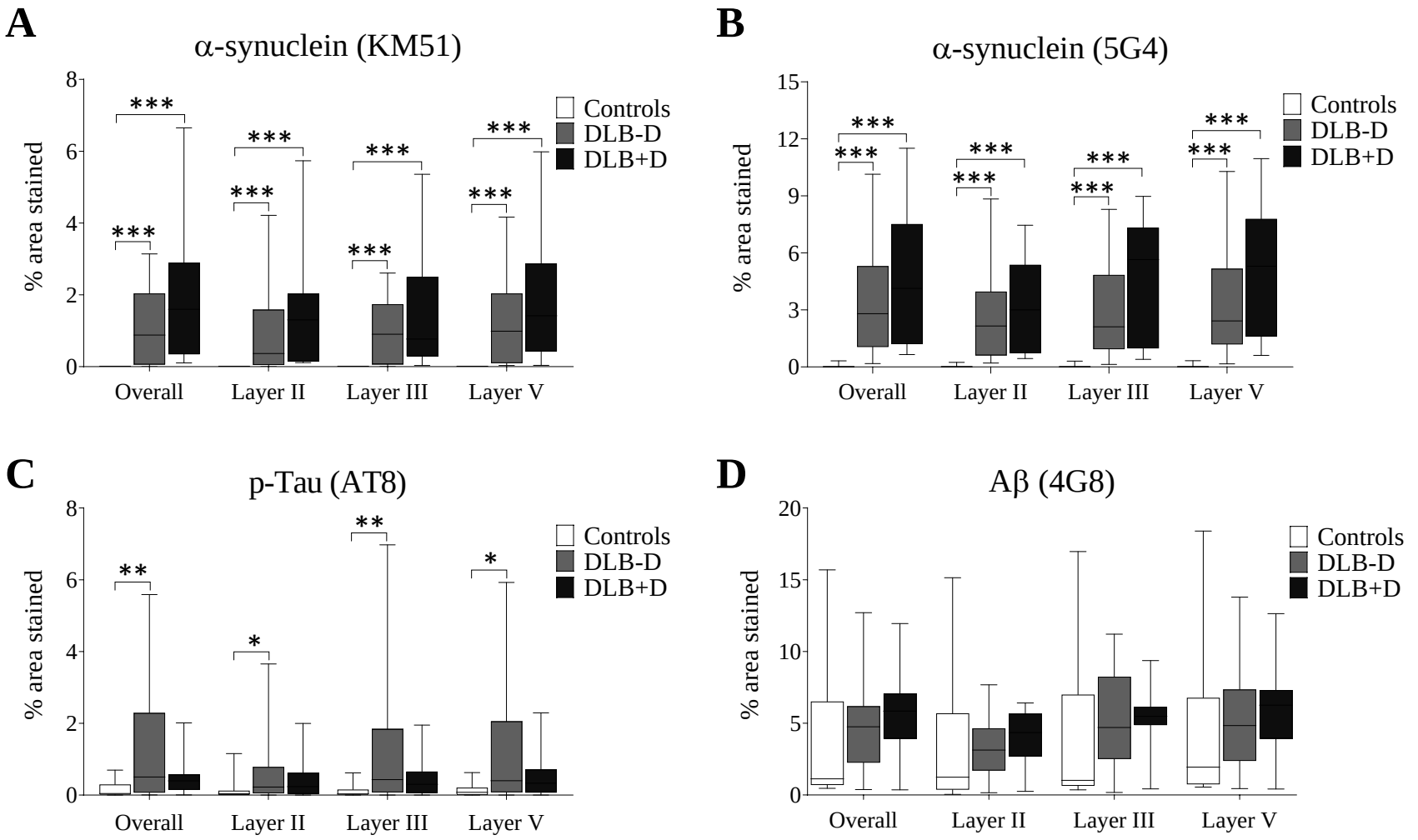

Figure 3.8 Densitometric analysis of pathology within sgACC.
Overall and layer specific pathological changes of α-synuclein (**A**) KM51 and (**B**) 5G4, (**C**) p-Tau (AT8) and (**D**) Aβ (4G8) (% area stained) were assessed within sgACC between DLB cases with (DLB+D) and without (DLB-D) depression and controls; (**p*<0.05, ***p*<0.01 and ****p*<0.001, compared to control).

The neuronal specific marker (HuD) raised against amino acids 1-300 of human HuD (Szabo et al., 1991) was used to assess neuronal cell density within sgACC between the groups. There was no significant difference observed in neuronal density overall ($F(2, 48)=0.393$, $p=0.677$), as well as layers II/III ($F(2, 48)=0.018$, $p=0.982$) and layer V ($F(2, 48)=1.183$, $p=0.315$) of the sgACC between the groups (Figure 3.9).

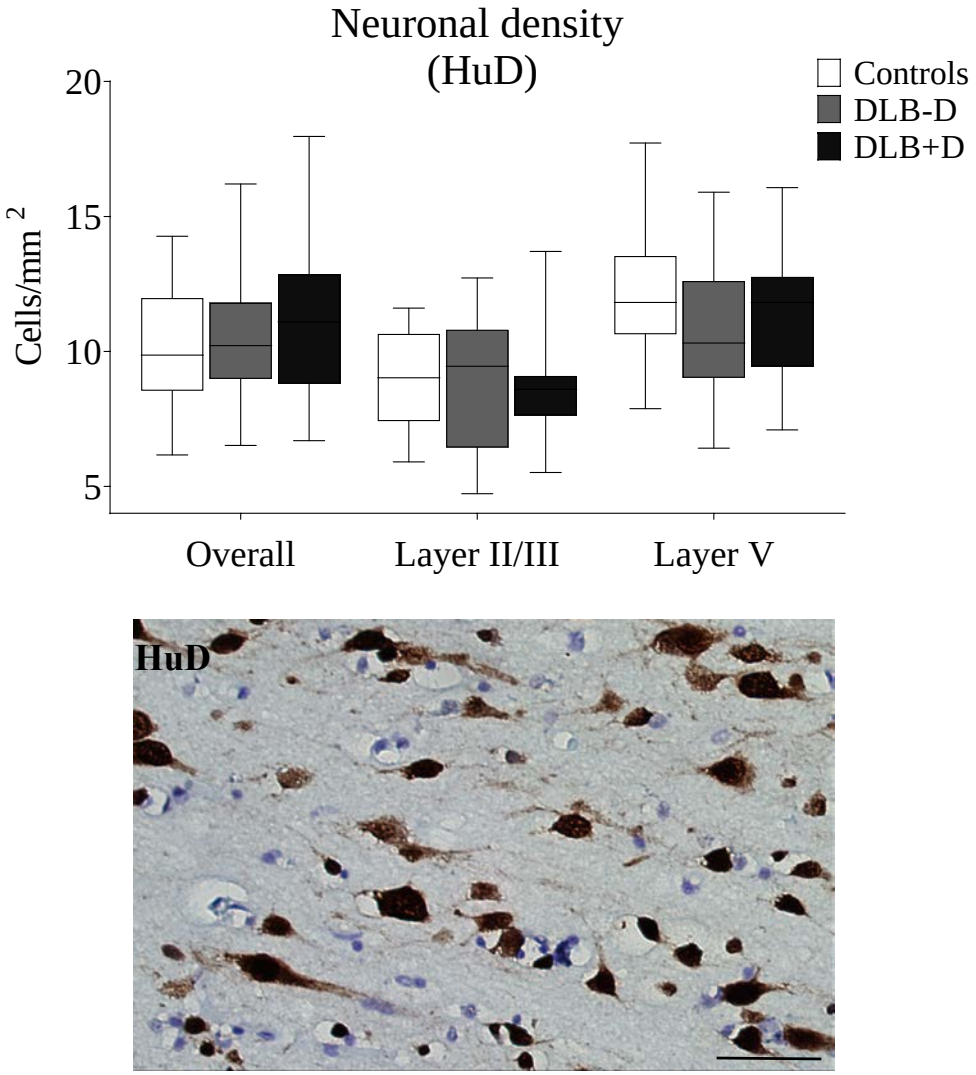

Figure 3.9 Neuronal cell density in the sgACC.
Overall, layer II/III and layer V cell numbers (cells per mm^2) within sgACC were assessed in DLB cases with and without depression, as well as controls. Tissue sections were stained with HuD (neuronal specific marker) and analysed using adapted stereological method. The micrograph above was taken at 20X magnification. The scale bar represents 100μm.

3.3.2.2 Biochemical changes

As pathological changes demonstrated using immunohistochemical approaches shows structural pathology, biochemical analysis of pathological proteins was used to determine differences, and if these could be correlated with clinical findings. These biochemical changes might include partitioning of proteins within specific subcellular compartments, such as synapses, reductions in the level of endogenous or normal protein species representing loss of normal function, or different pathological species such as oligomeric or fibrillar forms (Culvenor et al., 1999, Li et al., 2014a, Espindola et al., 2018). The sgACC was chosen as the region of interest owing to its role in depression and the high pathology burden in LBD, particularly DLB. Several α-synuclein (Figure 3.10), tau and Aβ antibodies were used to assess protein levels in the sgACC using fractionated tissue.

The biochemical analysis of α-synuclein pathological burden in sgACC generally supports the immunohistochemical data, which shows no significant difference in α-synuclein levels in sgACC between DLB cases with and without depression. Biochemical total (α-β) synuclein burden was determined using an antibody mapped to amino acids 1-70 of α-synuclein (Li et al., 2019). Significant differences between the groups were observed in the crude sample ($H(2)=17.780$, $p<0.001$), supernatant ($H(2)=10.925$, $p=0.004$), 0.1% Tween pellet ($H(2)=20.820$, $p<0.001$), 0.1% Tween supernatant ($H(2)=23.227$, $p<0.001$), 2% SDS pellet ($H(2)=19.117$, $p<0.001$) and 2% SDS supernatant fraction ($H(2)=21.520$, $p<0.001$). DLB cases with and without depression showed significantly higher total α-synuclein burden compared to controls in crude, 0.1% Tween pellet, 2 % SDS pellet and supernatant ($p<0.001$). DLB cases with depression also showed higher total α-synuclein immunoreactivity compared to controls in supernatant ($p=0.003$) and 0.1% Tween supernatant ($p<0.001$), whereas DLB cases without depression had higher total α-synuclein burden compared to controls in 0.1% Tween supernatant fraction of tissue homogenate ($p=0.002$; Figure 3.11).

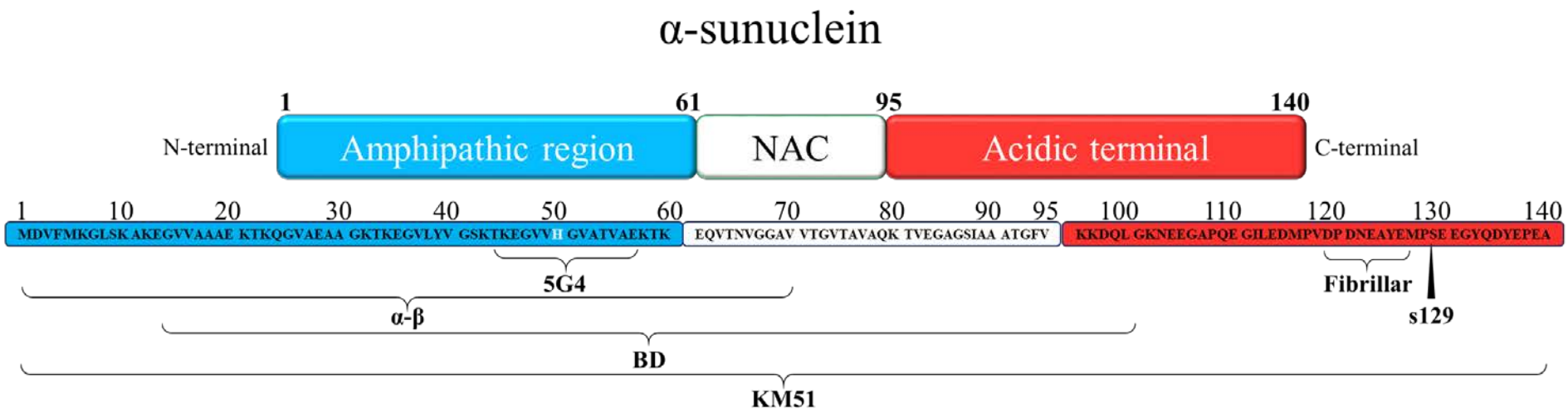

Figure 3.10 α-synuclein structure and antibody reagent mapping.
Several α-synuclein antibodies were used to detect pathological changes within the sgACC. Each antibody recognises specific amino acid (aa) sequence of the α-synuclein protein: fibrillar (120-126 aa), α-β synuclein (1-70 aa), s129 α-synuclein (129 aa), KM51 α-synuclein (full length protein– epitope not mapped), BD α-synuclein (15-100 aa) and 5G4 α-synuclein (45-58 aa).

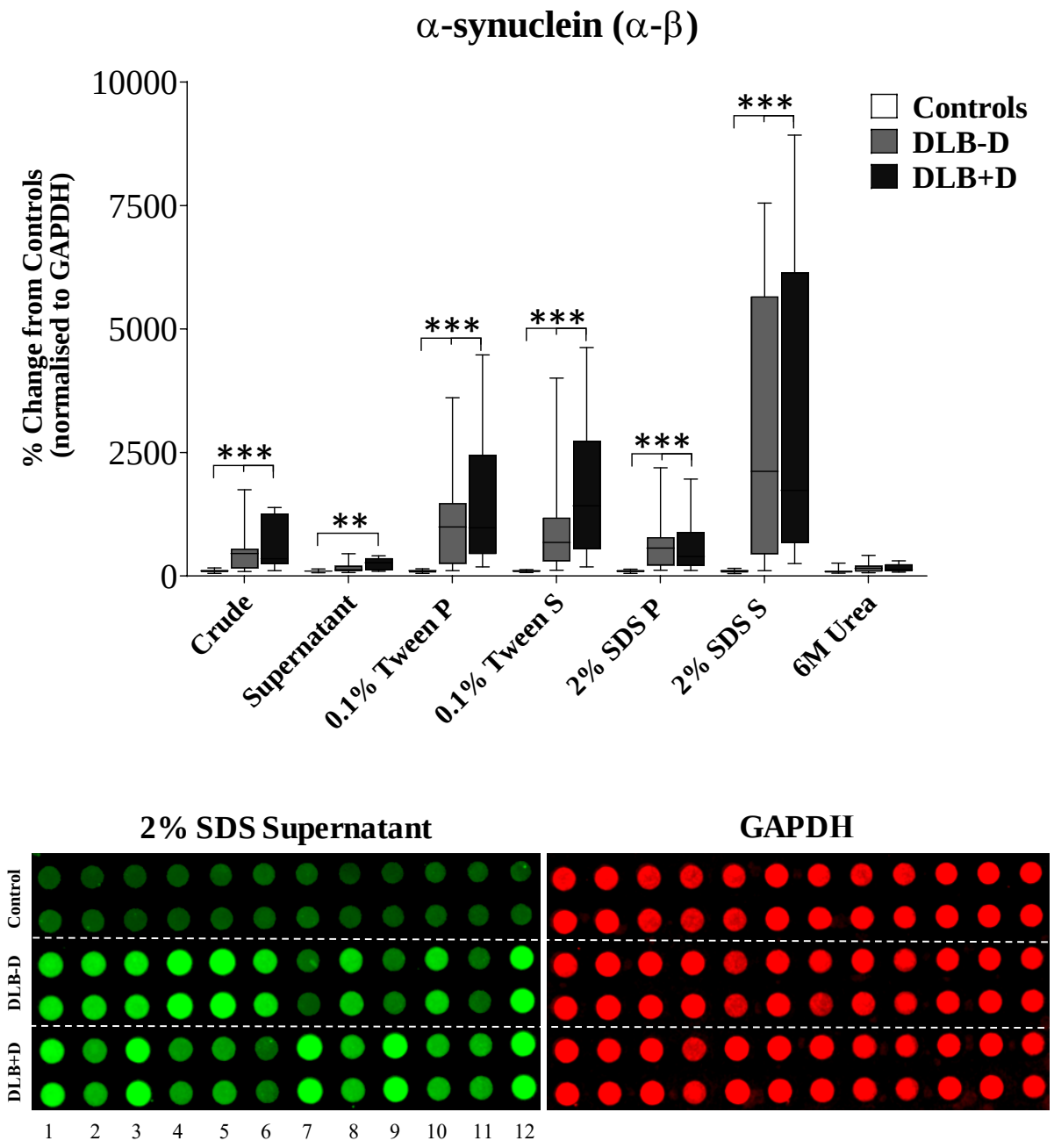

Figure 3.11 Total α-synuclein protein levels in tissue fractions of sgACC.
Dot blotting was used to assess changes in total α-synuclein protein levels in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins), 2% SDS supernatant (SDS soluble membrane proteins) and 6M Urea supernatant (highly insoluble proteins). (**p<0.01 and *** p<0.001, compared to control).

Using the fibrillar α-synuclein antibody, which recognises a conformational epitope within the 120-126 amino acid (aa) sequence of α-synuclein protein (Lassen et al., 2018), demonstrated significantly different changes between the groups in the crude tissue sample ($H(2)=7.265$, $p=0.026$), supernatant ($H(2)=9.015$, $p=0.011$), 0.1% Tween supernatant ($H(2)=17.350$, $p<0.001$) and 6M Urea fraction of tissue homogenates ($H(2)=8.437$, $p=0.015$). Significantly higher fibrillar α-synuclein reactivity was found in DLB cases with depression compared to controls in the crude ($p=0.041$), supernatant ($p=0.010$), 0.1% Tween supernatant ($p<0.001$) and 6M urea ($p=0.013$) fraction (Figure 3.12).

Analysis of tissue fractions using an antibody against α-synuclein phosphorylated at residue serine 129 (s129) (Delic et al., 2018) showed significantly altered levels between the groups in the crude sample ($H(2)=16.826$, $p<0.001$), supernatant ($H(2)=7.115$, $p=0.029$), 0.1% Tween pellet ($H(2)=11.988$, $p=0.002$), 0.1% Tween supernatant ($H(2)=16.122$, $p<0.001$), 2% SDS pellet ($H(2)=6.504$, $p=0.039$), 2% SDS supernatant ($H(2)=9.159$, $p=0.028$) and urea fraction of tissue homogenates ($H(2)=20.523$, $p<0.001$). DLB cases with depression showed significantly higher s129 α-synuclein burden compared to controls in crude ($p=0.001$), supernatant ($p=0.029$), 0.1% Tween pellet ($p=0.003$), 0.1% Tween supernatant ($p<0.001$), 2 % SDS supernatant ($p=0.020$) and urea ($p=0.001$). DLB cases without depression showed higher s129 α-synuclein immunoreactivity compared to controls in the crude ($p=0.002$), 0.1% Tween pellet ($p=0.045$), 2% SDS pellet ($p=0.010$) and 2% SDS supernatant ($p=0.035$) and 6M Urea tissue fraction ($p<0.001$). There was no significant difference between DLB cases with or without depression in s129 immunoreactivity (see Figure 3.13).

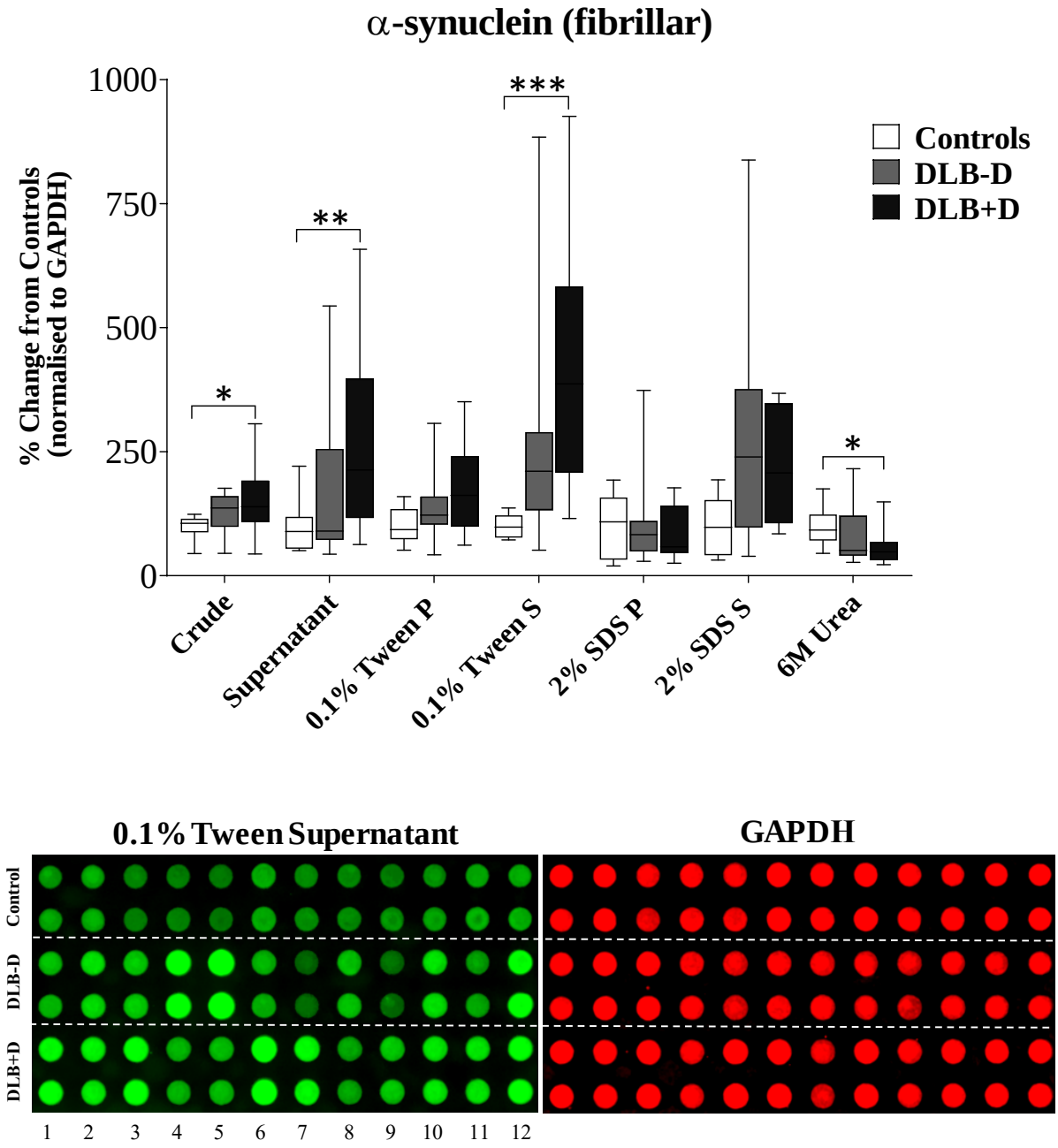

Figure 3.12 Fibrillar α-synuclein protein levels in tissue fractions of sgACC.
Dot blotting was used to assess changes in fibrillar α-synuclein protein levels in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins), 2% SDS supernatant (SDS soluble membrane proteins) and 6M Urea supernatant (highly insoluble proteins). (*p<0.05, **p<0.01 and *** p<0.001, compared to control).

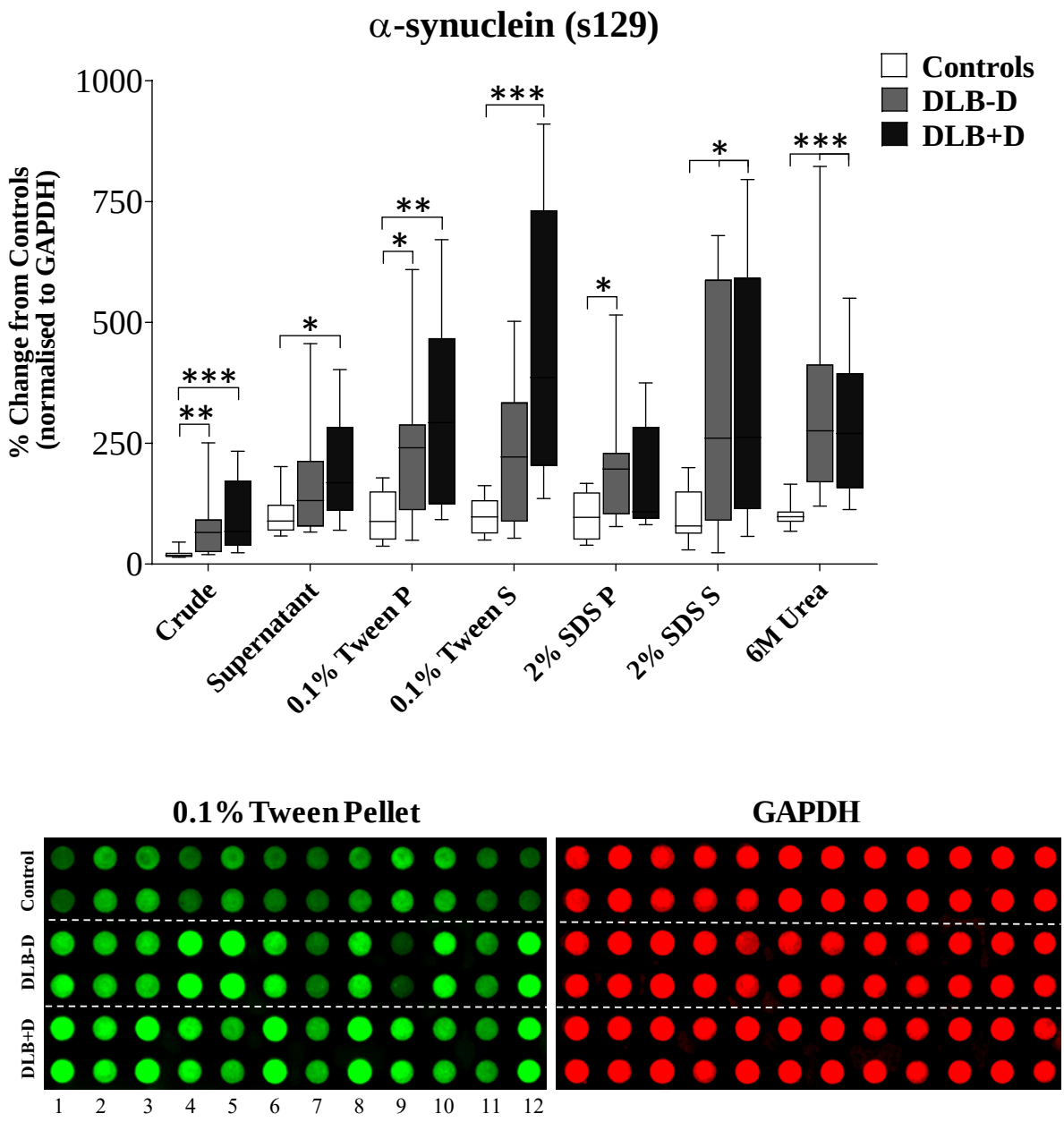

Figure 3.13 s129 α-synuclein protein levels in tissue fractions of sgACC.
Dot blotting was used to assess changes in protein levels of α-synuclein phosphorylated at position serine 129 in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins), 2% SDS supernatant (SDS soluble membrane proteins) and 6M Urea supernatant (highly insoluble proteins). (*p<0.01, **p<0.01 and *** p<0.001, compared to control).

Two antibodies used in immunohistochemical analysis, KM51 and 5G4, were also used to probe tissue fractions. A significant difference in KM51 (raised against full length recombinant α-synuclein – epitope not mapped) immunoreactivity was observed between the groups in 2% SDS pellet ($H(2)=16.290$, $p<0.001$) and 2% SDS supernatant ($H(2)=19.668$, $p<0.001$) fractions of tissue homogenate. Control cases showed significantly higher KM51 α-synuclein burden compared to DLB cases with depression in the 2% SDS pellet ($p=0.001$) and supernatant ($p<0.001$), as well as compared to DLB cases without depression in 2% SDS pellet ($p=0.002$) and supernatant ($p=0.021$; Figure 3.14). No changes in KM51 reactivity were seen between DLB cases with and without depression. A significantly higher 5G4 (mapping to amino acids 45-58) (Kovacs et al., 2012) α-synuclein immunoreactivity between the groups was only observed in the 0.1% Tween supernatant ($H(2)=6.132$, $p=0.047$) fraction of tissue homogenates, with higher a 5G4 burden in DLB cases without depression compared to controls trending on significance ($p=0.054$; Figure 3.15).

No significant difference was observed using the BD α-synuclein (raised against amino acids 15-99 of α-synuclein and mapping to amino acids 15-100 (Lassen et al., 2018) in any of the tissue fractions from control or DLB (Figure 3.16).

Data generated from ELISA determination of total and fibrillar α-synuclein analysis in total and fractionated samples showed similar, but non-significant changes, to dot blot approaches (data not shown in this thesis).

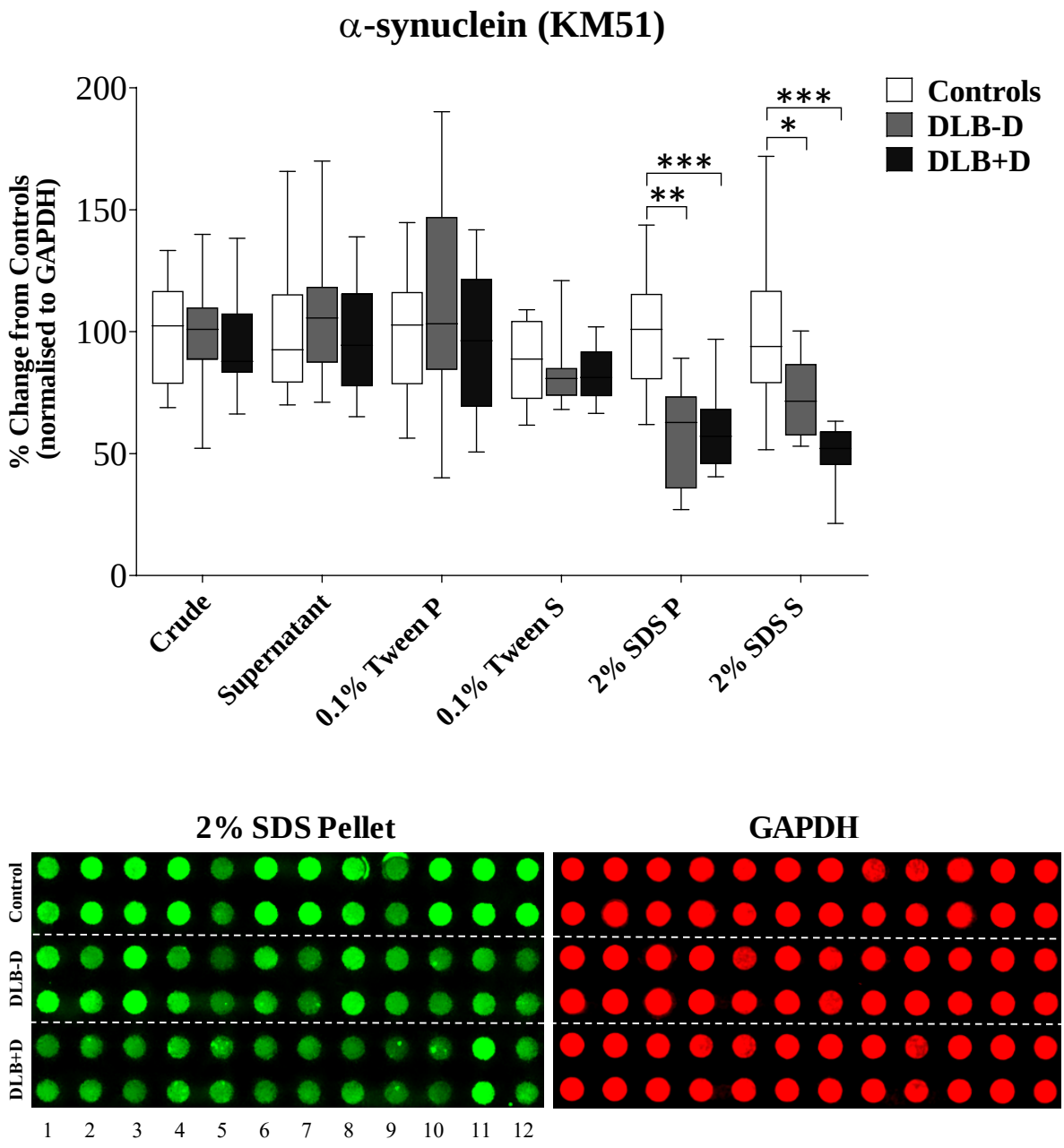

Figure 3.14 α-synuclein (KM51) protein levels in tissue fractions of sgACC.
Dot blotting was used to assess changes in KM51 α-synuclein immunoreactive protein levels in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins) and 2% SDS supernatant (SDS soluble membrane proteins). (Significance levels set at *p<0.01, **p<0.01 and *** p<0.001, compared to control).

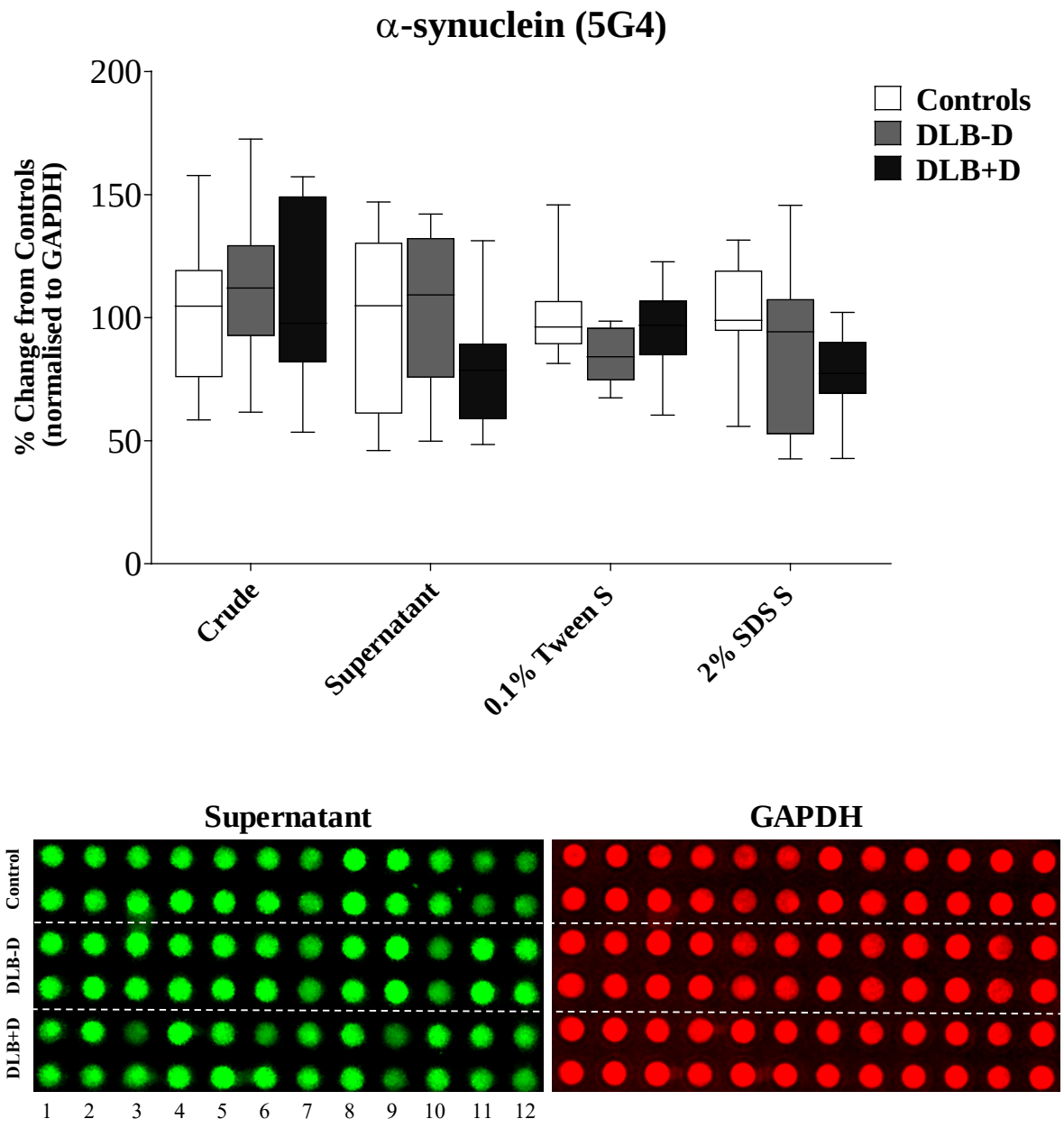

Figure 3.15 α-synuclein (5G4) protein levels in tissue fractions of sgACC.
Dot blotting was used to assess changes in α-synuclein (5G4) immunoreactive protein levels in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween supernatant (Tween soluble membrane proteins) and 2% SDS supernatant (SDS soluble membrane proteins).

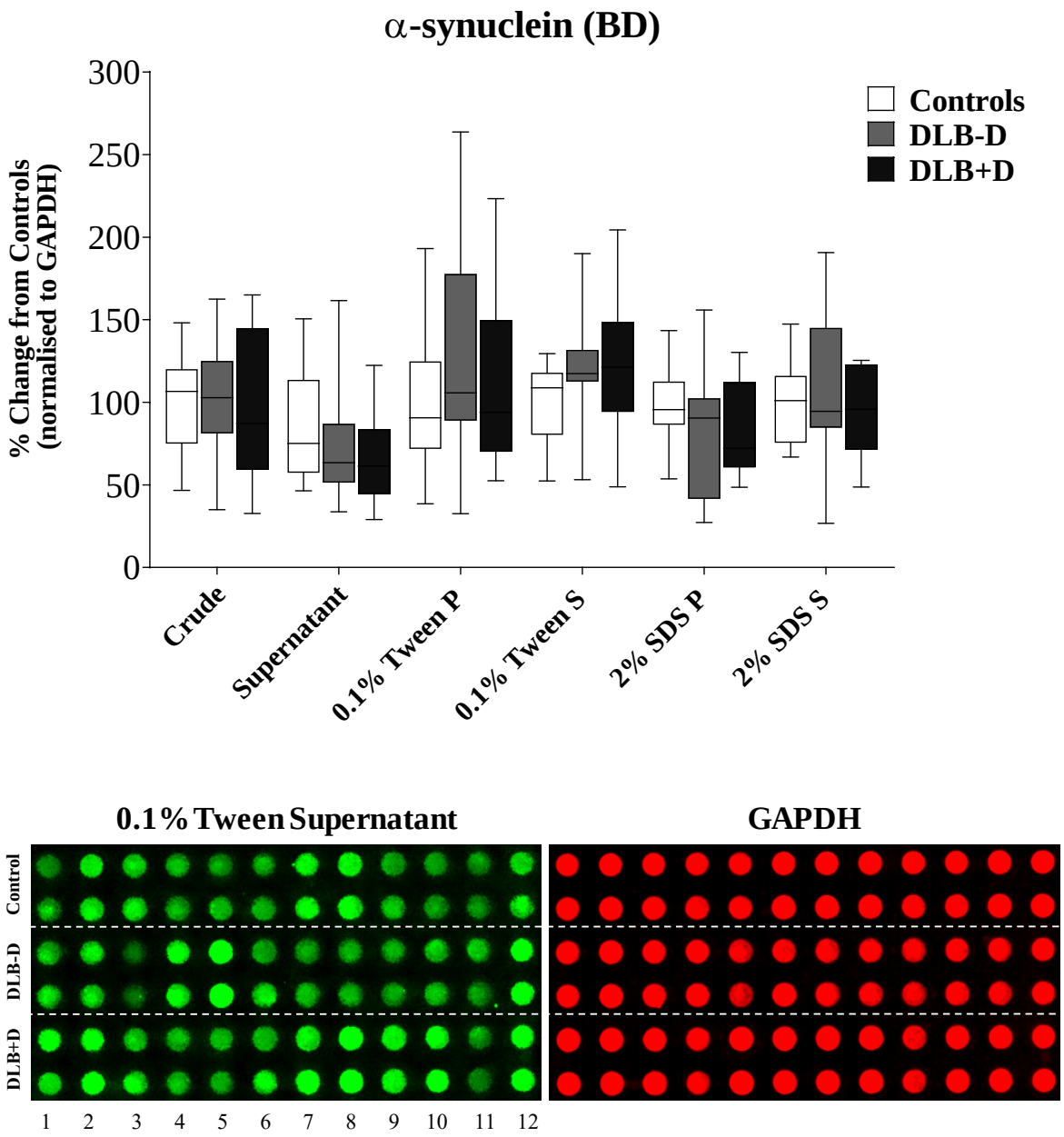

Figure 3.16 α-synuclein (BD) protein levels in tissue fractions of sgACC.
Dot blotting was used to assess changes in α-synuclein protein levels in sgACC using an antibody raised against amino acids 15-123 of α-synuclein (BD Biosciences) within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins) and 2% SDS supernatant (SDS soluble membrane proteins).

The microtubule (MT) associated protein tau regulates the axonal transport of proteins, vesicles and organelles and is the principal component of neurofibrillary tangles in AD and related neurodegenerative disorders. The alternative splicing of exon 10 in the human MAPT gene

gives rise to tau isoforms with three (3R) or four (4R) MT-binding repeats (Goedert et al., 1989). These isoforms are expressed at similar levels within the brain (Goedert and Jakes, 1990). The 3R/4R isoform imbalance is thought to play a role in axonal transport impairment and neuronal dysfunction in many neurodegenerative disorders. In AD, both isoforms are present in the Tau fibrils (Goedert et al., 1992), whereas in PSP only 4R Tau is present (Flament et al., 1991). Therefore, p-Tau, 3R, 4R expression and 3R/4R ratios were assessed in soluble and insoluble fractions of sgACC samples in relation to depression in DLB.

The AT8 antibody is routinely used to demonstrate NFT pathology in tissue sections and recognises a phosphorylated epitope in Tau at amino acids 202–205 (Goedert et al., 1995). A significantly higher AT8 p-Tau pathological burden was observed in the supernatant ($H(2)=6.892$, $p=0.032$), 0.1% Tween supernatant ($H(2)=9.701$, $p=0.008$) and in the 2% SDS pellet fraction ($H(2)=7.049$, $p=0.029$) between the groups. DLB cases with depression showed significantly higher AT8 p-Tau burden compared to controls in supernatant ($p=0.037$) and 0.1% Tween supernatant ($p=0.046$), whereas DLB cases without depression showed higher p-Tau immunoreactivity compared to controls in 0.1% Tween supernatant ($p=0.010$) and the 2% SDS pellet ($p=0.044$; Figure 3.17).

Antibodies to the repeat domains of Tau are available, with amonoclonal antibody against the 3R region of Tau used to demonstrate immunoreactivity in tissue fractions (de Silva et al., 2003). A significantly higher 3R Tau pathological burden was observed in the crude ($H(2)=6.695$, $p=0.035$) and 2% SDS supernatant fraction ($H(2)=7.101$, $p=0.029$) between the groups. Control cases shower significantly higher 3R Tau burden compared to DLB cases with depression in crude ($p=0.033$) and 2% SDS supernatant fractions ($p=0.024$; Figure 3.18).

Using a specific 4R Tau antibody (de Silva et al., 2003), a significantly higher 4R p-Tau pathological burden was observed in the 2% SDS pellet ($H(2)=11.866$, $p=0.003$) and 2% SDS supernatant ($H(2)=9.747$, $p=0.008$) between the groups. Control cases shower significantly higher 4R p-Tau immunoreactivity compared to DLB cases with depression in 2% SDS pellet ($p=0.005$) and 2% SDS supernatant ($p=0.038$), as well as compared to DLB cases without depression in 2% SDS pellet ($p=0.017$) and 2% SDS supernatant ($p=0.013$; Figure 3.19).

A significantly higher 3R/4R p-Tau ratio was observed in the 2% SDS pellet ($H(2)=9.869$, $p=0.007$) and 6M Urea fraction ($H(2)=9.165$, $p=0.010$) between the groups. DLB cases with depression had significantly higher 3R/4R p-Tau ratio compared to controls in 2% SDS pellet

(p=0.005) and 6M Urea fractions (p=0.025), whereas DLB cases without depression showed higher 3R/4R p-Tau ratio in 6M Urea (p=0.032; Figure 3.20).

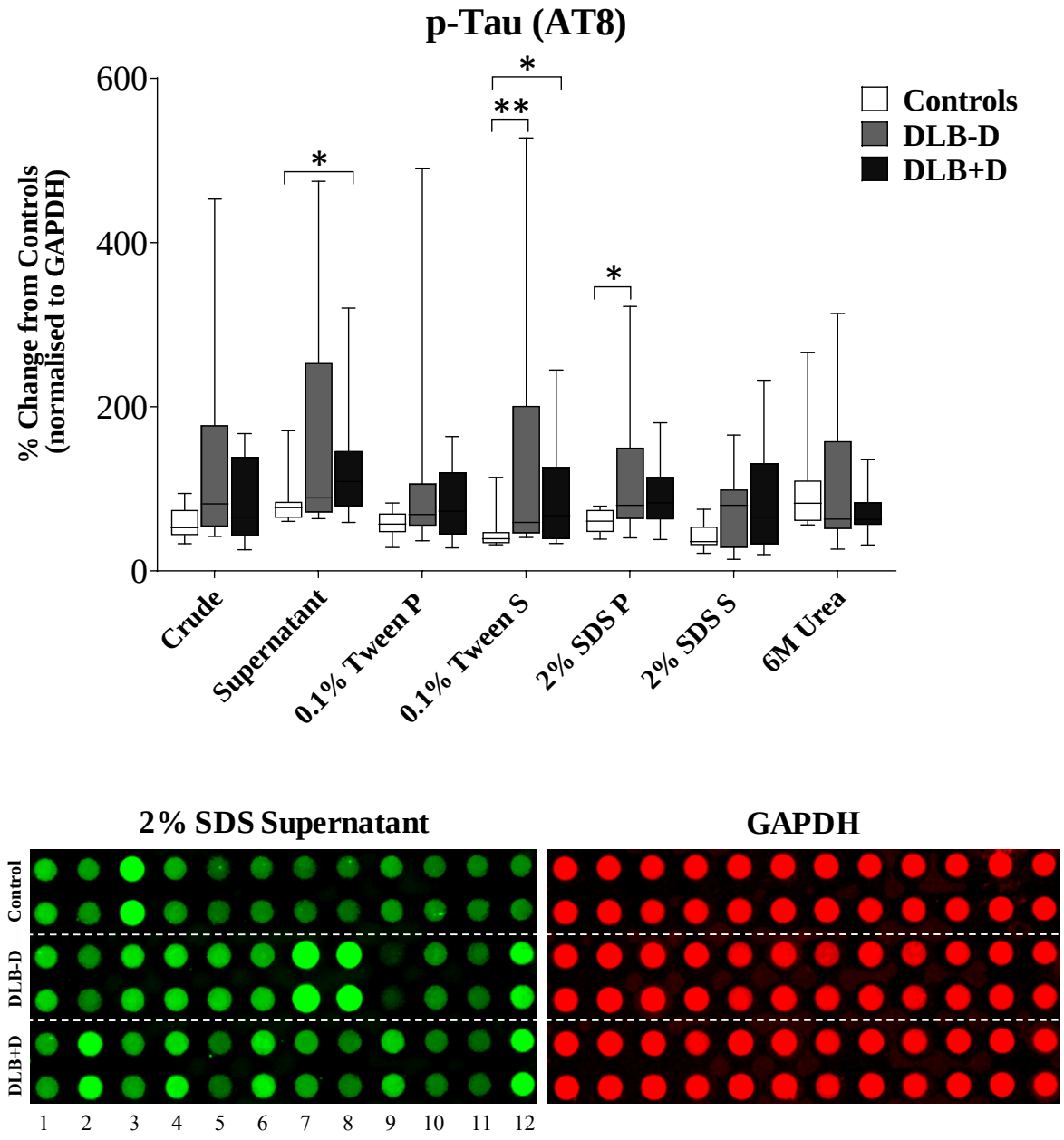

Figure 3.17 AT8 p-Tau protein levels in tissue fractions of sgACC.
Dot blotting was used to assess changes in AT8 p-Tau protein levels in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins), 2% SDS supernatant (SDS soluble membrane proteins) and 6M Urea supernatant (highly insoluble proteins). (*p<0.01 and **p<0.01, compared to control).

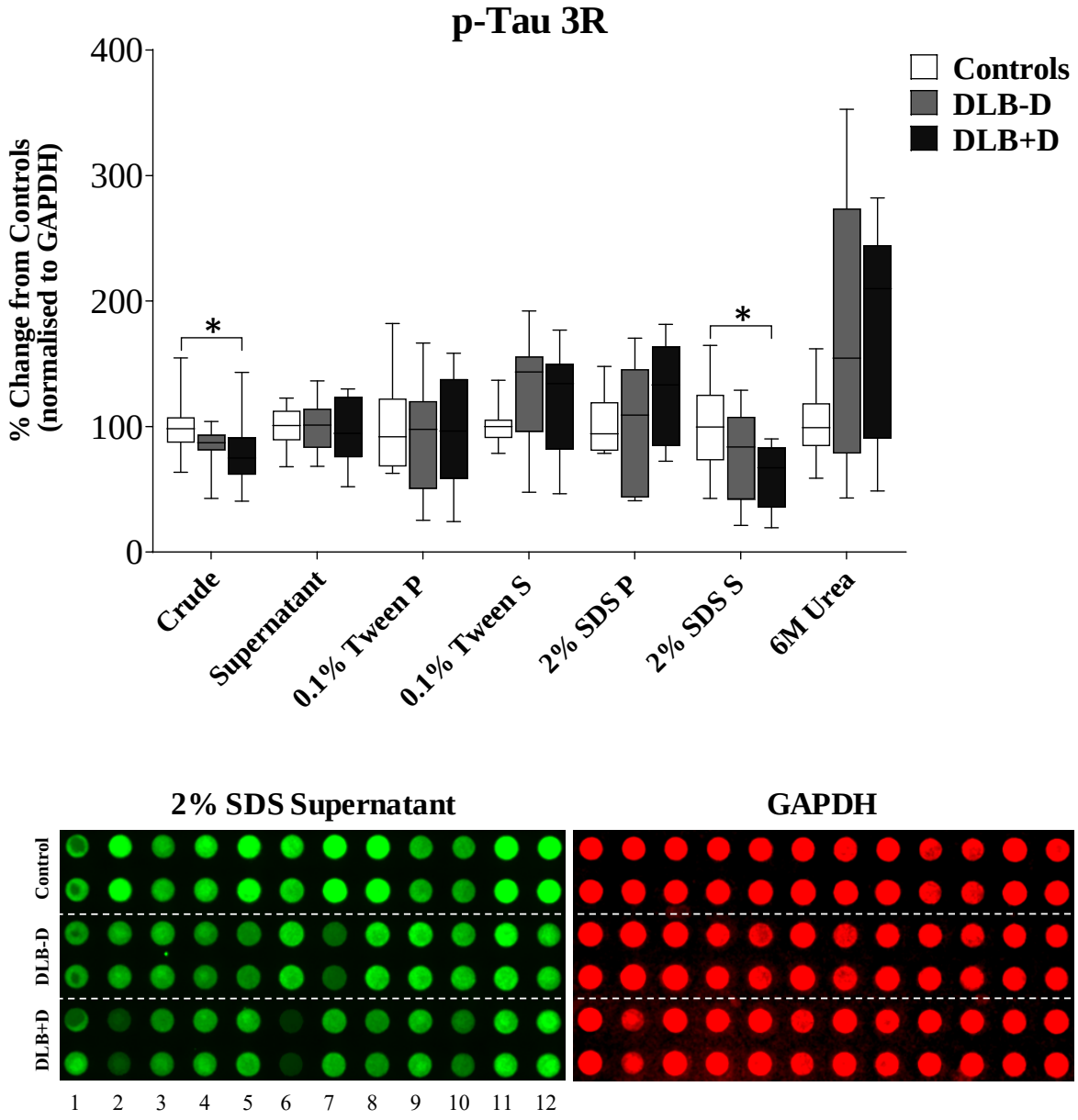

Figure 3.18 3R p-Tau protein levels in tissue fractions of sgACC.
Dot blotting was used to assess changes in 3R p-Tau protein levels in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins), 2% SDS supernatant (SDS soluble membrane proteins) and 6M Urea supernatant (highly insoluble proteins). (*p<0.05, compared to control).

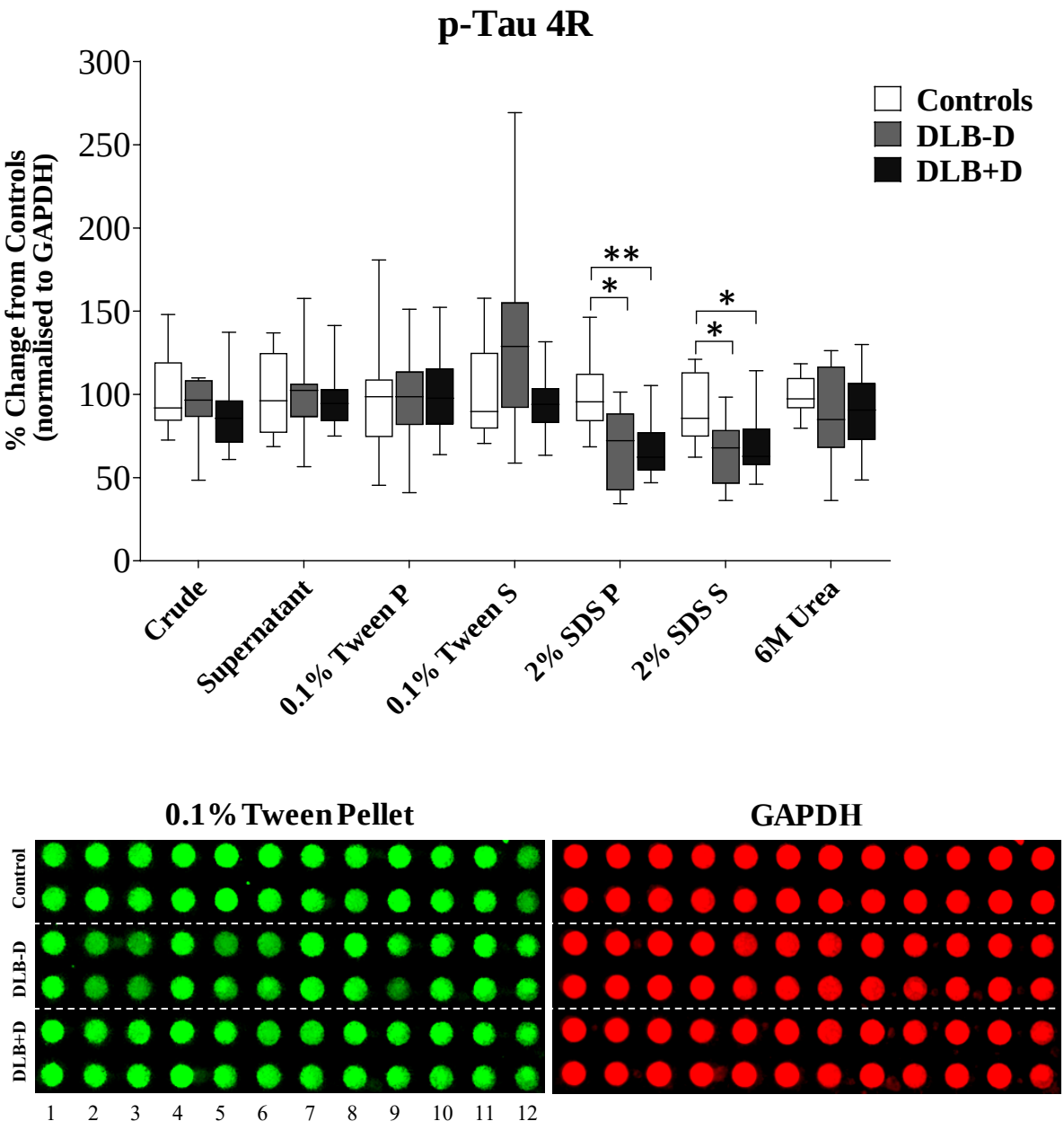

Figure 3.19 4R p-Tau protein levels in tissue fractions of sgACC.
Dot blotting was used to assess changes in 4R p-Tau protein levels in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins), 2% SDS supernatant (SDS soluble membrane proteins) and 6M Urea supernatant (highly insoluble proteins). (*p<0.05 and **p<0.01, compared to control).

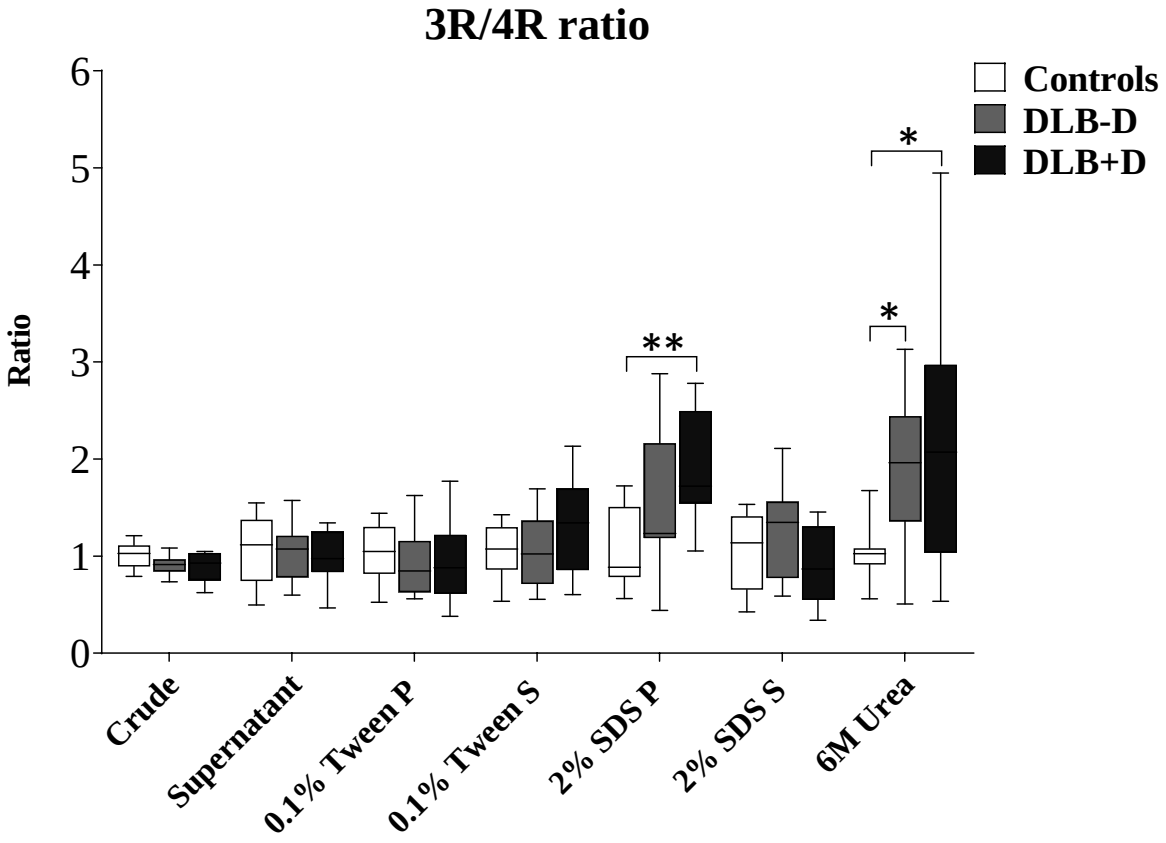

Figure 3.20 3R/4R p-Tau ratios in tissue fractions of sgACC.
Dot blotting was used to assess changes in 3R/4R p-Tau ratio in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins), 2% SDS supernatant (SDS soluble membrane proteins) and 6M Urea supernatant (highly insoluble aggregated proteins). (*p<0.05 and **p<0.01, compared to control).

The Aβ peptide is the major component of senile plaques in AD and related disorders and represents a core feature of AD pathology. A significantly higher level of Aβ immunoreactivity was observed in the 2% SDS supernatant fraction between the groups (H(2)=8.488, p=0.014), with significantly higher Aβ levels in DLB cases with depression compared to controls (p=0.010; Figure 3.21).

Using an epitope specific Aβ antibody, a significantly higher Aβ (1-40) level was observed in 2% SDS pellet (H(2)=12.565, p=0.002) and 2% SDS supernatant between the groups (H(2)=6.173, p=0.046), with significantly higher Aβ (1-40) levels in the 2% SDS pellet found in controls compared to DLB cases with depression (p=0.006) and without depression (p=0.007). A significant increase in Aβ (1-40) was also observed in the 2% SDS supernatant

in DLB cases with depression compared to controls (p=0.041; Figure 3.22). Material from the Urea fractions was not available for analysis.

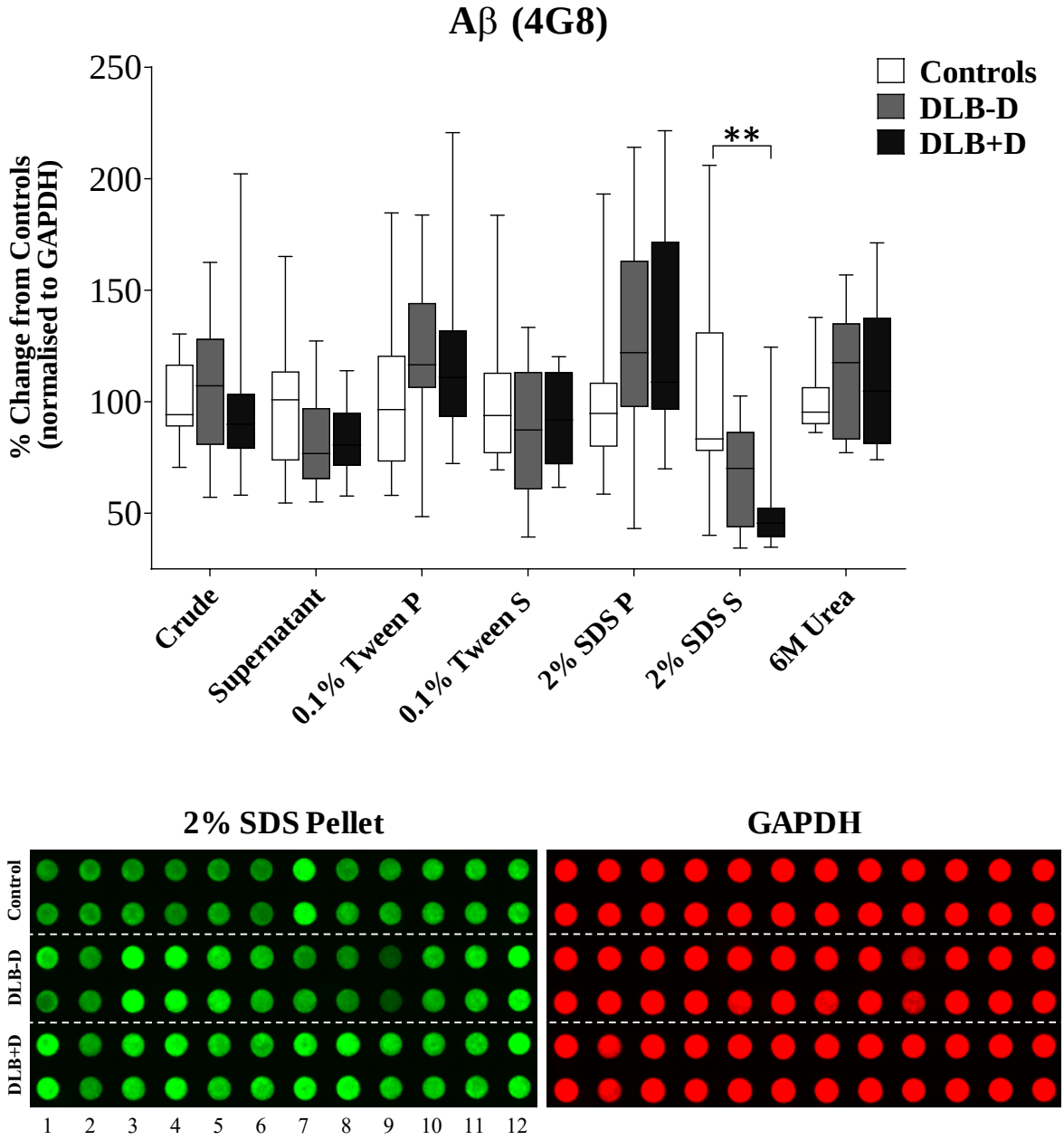

Figure 3.21 Aβ peptide levels in tissue fractions of sgACC.
Dot blotting was used to assess changes in Aβ peptide levels in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins), 2% SDS supernatant (SDS soluble membrane proteins) and 6M Urea supernatant (highly insoluble proteins). (**p<0.01, compared to control).

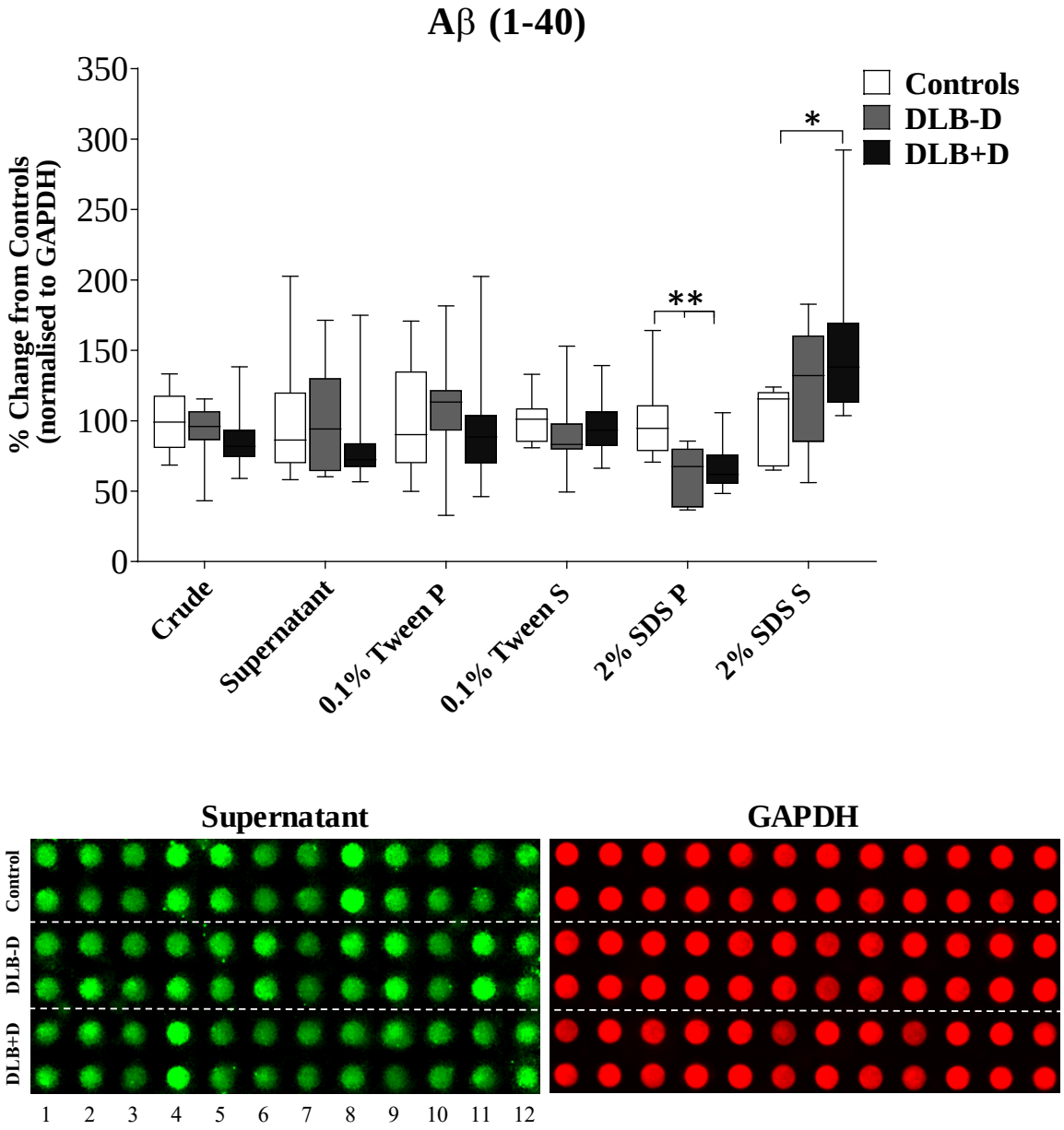

Figure 3.22 Aβ(1-40) peptide levels in tissue fractions of sgACC.
Dot blotting was used to assess changes in Aβ (1-40) peptide levels in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins), 2% SDS supernatant (SDS soluble membrane proteins). (*p<0.05 and **p<0.01).

A significantly higher Aβ (1-42) pathological burden was observed in 0.1% Tween supernatant (H(2)=12.375, p=0.002) and 2% SDS pellet (H(2)=11.210, p=0.004) between the groups. In 2% SDS pellet fraction significantly higher Aβ (1-42) levels were observed in controls

(p=0.006) and DLB cases with depression (*p*=0.007) compared to DLB cases without depression. Control cases had significantly higher Aβ (1-42) burden in 2% SDS pellet fraction compared to DLB cases with depression (p=0.007; Figure 3.23). Urea fractions were not available for Aβ (1-42) analysis.

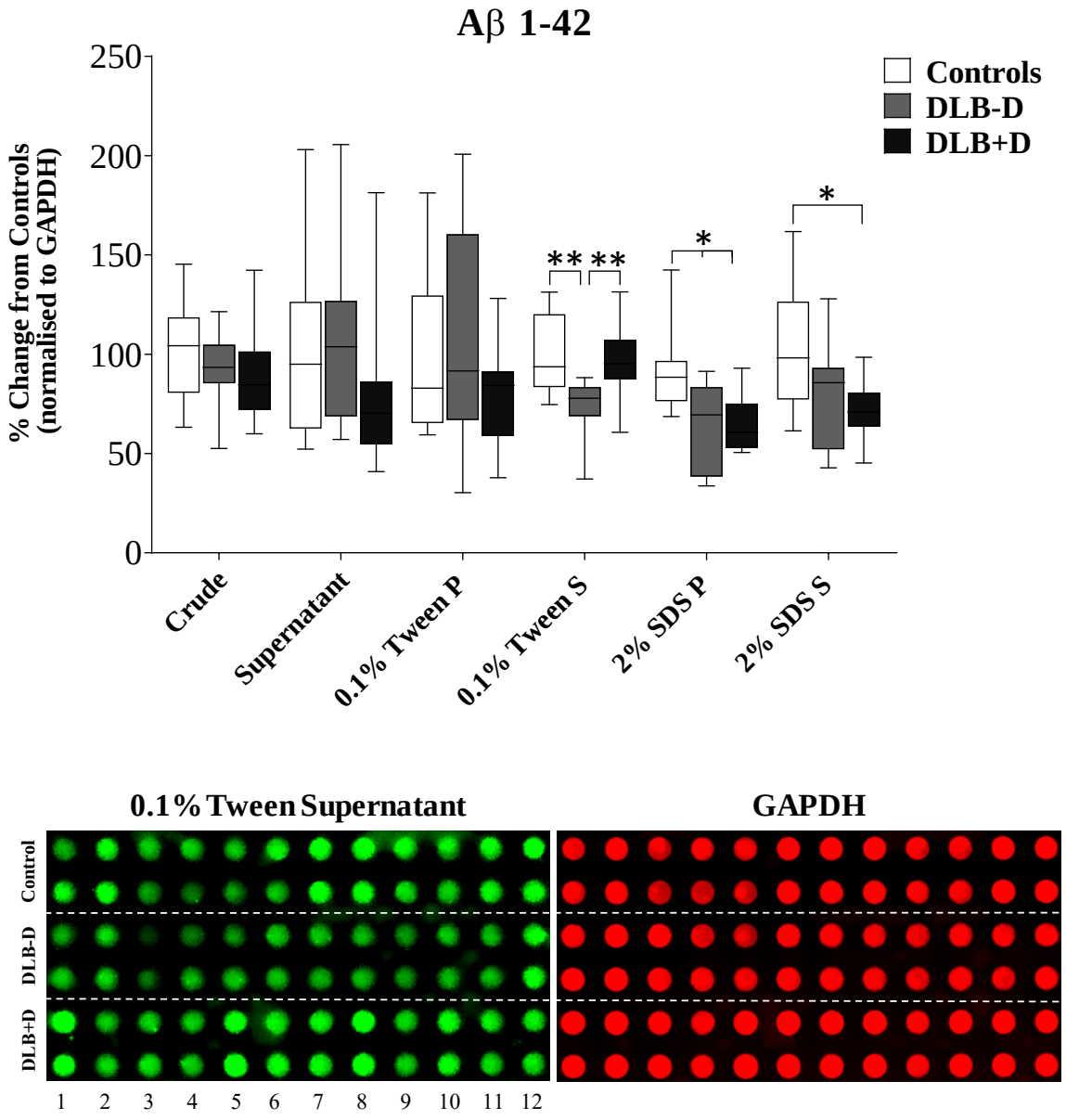

Figure 3.23 Aβ(1-42) peptide levels in tissue fractions of sgACC.
Dot blotting was used to assess changes in Aβ (1-42) protein levels in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins), 2% SDS supernatant (SDS soluble membrane proteins). (Significance set at *p<0.05 and **p<0.01).

A significantly higher Aβ 1-40/1-42 ratio was observed in the 2% SDS supernatant between the groups ($H(2)=19.352$, $p<0.001$). DLB cases with depression had significantly higher Aβ 1-40/1-42 ratio in 2% SDS supernatant compared to controls ($p<0.001$) and DLB cases without depression ($p=0.013$), whereas DLB cases without depression showed higher Aβ 1-40/1-42 ratio compared to controls ($p=0.004$; Figure 3.24).

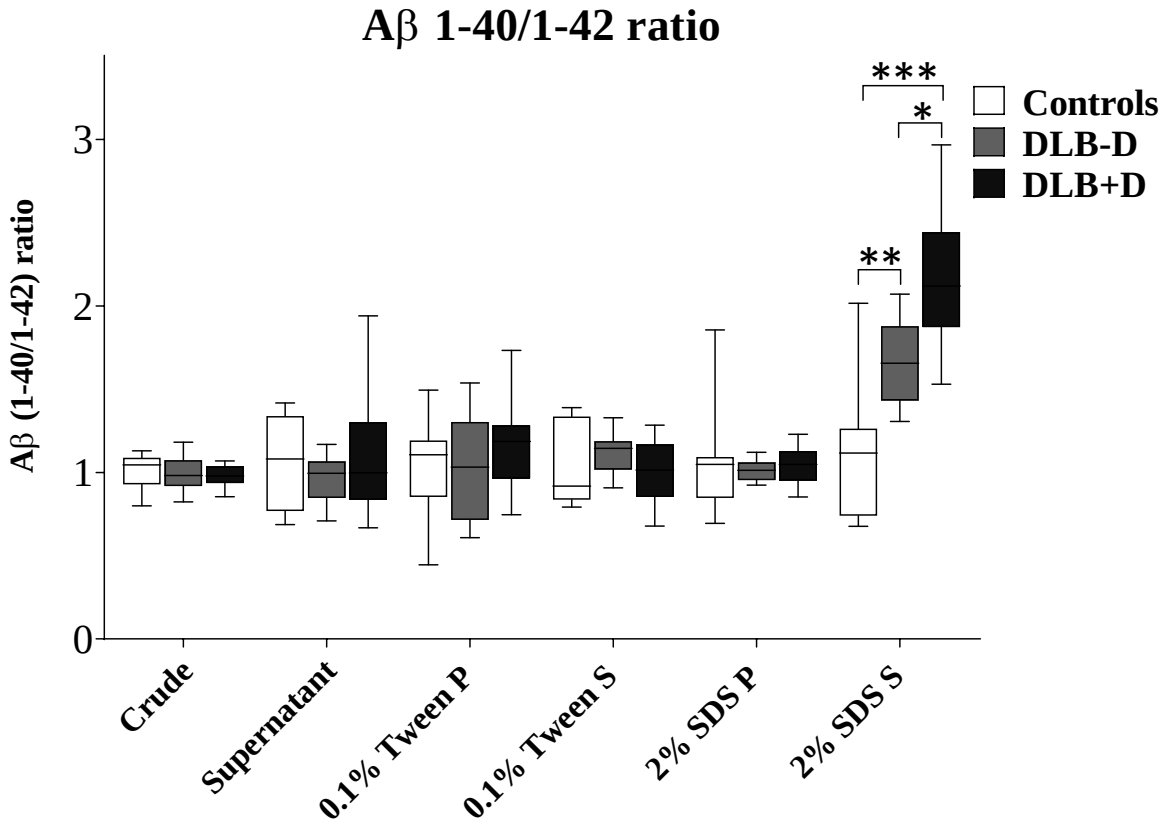

Figure 3.24 Aβ 1-40/1-42 ratio in tissue fractions of sgACC.
Dot blotting was used to assess changes in Aβ 1-40/1-42 ratio in sgACC within different tissue fractions of DLB cases with (DLB+D) and without (DLB-D) depression and controls: crude, supernatant (cytoplasmic soluble proteins), 0.1% Tween pellet (soluble membrane bound proteins), 0.1% Tween supernatant (Tween soluble membrane proteins), 2% SDS pellet (insoluble membrane bound proteins), 2% SDS supernatant (SDS soluble membrane proteins). (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

3.5 Discussion

Depression is a common psychiatric symptom in DLB, associated with faster cognitive decline and a poor clinical outcome. The association between depression and dementia, which is not mutually exclusive, needs further clarification. It is not clear whether depression and dementia are manifestations of the same underlying neuropathology, if depression is a consequence of dementia, or if there is an independent association between these two conditions. The present study assessed pathological changes within four main functional divisions of the cingulate cortex to understand its role in depression in DLB, with a focus on the sgACC given its role in depression. DLB cases without depression were included in this study in order to separate those pathological changes, which are specifically due to depression, from those that are 'disease' related. This is the first post-mortem study to assess neurodegenerative pathology in cingulate subregions using a large cohort.

The present findings indicate that neurodegenerative pathology in the cingulate cortex does not directly relate to depression in DLB. Studies examining neuropathological changes in relation to depression in patients without dementia or in AD are scarce, often presenting contradictory results. In line with the present findings, one study found no association between depressive symptoms in DLB or AD and severity of cortical or subcortical LB pathology (Samuels et al., 2004). Similarly, a large scale longitudinal study demonstrated an association between depressive symptoms and cognitive decline, but this was independent of any neuropathological hallmarks of dementia such as Aβ plaques, NFT and LB (Wilson et al 2014). Previous studies have suggested the involvement of neurodegeneration in the pathogenesis of MDD (Burgut et al., 2006). The presence of LB in amygdala has been shown to be associated with psychiatric morbidity and increased risk for MDD in AD (Lopez et al., 2006). Higher levels of NP and NFT were found in the hippocampus in AD patients with depression compared to non-depressed AD patients (Rapp et al., 2006), as well as a positive correlation between depressive states and NP density in AD (Meynen et al., 2010). Later life MDD is frequently associated with cognitive impairment and has been linked to AD pathological changes (Sweet et al 2004), suggesting interactions between MDD and AD lesions (Rapp et al 2006). The current results do not however support these findings in DLB for the cingulate, and it is possible that the substrates of depression in DLB are independent of pathology, with neuropathology being an initiator of this cascade, which once activated is self-sustaining and relatively independent of amyloid and tau pathology.

Several studies have shown that the cingulate cortex is severely affected by α-synuclein pathology compared to other cortical regions in DLB (Colom-Cadena et al., 2013). The present findings show that the pathological burden of α-synuclein is most severe in ACC compared to MCC and PCC in DLB cases, although despite the considerable pathological burden, no major changes in neuronal density were observed in ACC. While some studies have observed cortical thinning and grey matter atrophy of the ACC in DLB based on MRI (Blanc et al., 2016, Blanc et al., 2015), other studies have shown no marked reduction in grey matter volume in ACC in DLB (Patterson et al., 2019, Harper et al., 2017). This may suggest that higher pathological burden may not necessarily result in neuronal loss, but rather dysfunction. The current results support this, showing an absence of significant neuronal loss despite considerable pathological changes. Furthermore, imaging studies show reduced functional activity in the ACC based on PET, SPECT and fMRI in depression (Fu et al., 2018, Ongur et al., 2003). Reduced metabolic activity in the ACC has also been observed in DLB patients compared to MCC and PCC, which is thought to relate to higher α-synuclein burden in ACC (Patterson et al., 2019). While that study found no direct relationship between α-synuclein pathology and reduced perfusion in the ACC, this may indicate that there is a threshold effect, once certain levels of pathology are reached, metabolism begins to decline, and metabolic imaging changes can be observed. Occipital hypometabolism is also a common feature in DLB (Ishii et al., 1998, Colloby et al., 2002). This region does not show significant atrophy (Burton et al., 2004), and typically, does not show major pathological changes (Khundakar et al., 2016), which may suggest that factors other than pathological burden, cell loss or atrophy play a role in the reduced metabolism in specific brain structures. More direct markers of metabolism that are downstream consequences of pathological change will be required to observe these metabolic changes and allow the relationships between pathology and metabolism to be explored. Metabolic changes in the cingulate cortex may therefore better reflect the presence or absence of depression. Similarly, given that there is an extensive literature indicating neurochemical changes associated with depression (see section 1.4.3-1.4.4), direct determination of neurochemical changes such as monoaminergic, glutamatergic, or GABAergic markers in the cingulate may be more relevant indicators of depression.

The biochemical analysis of pathology within the sgACC largely reiterate the immunohistochemical results, showing no association between pathology in sgACC and depression in DLB. In sgACC the highest level of α-synuclein was detected in insoluble

membrane bound fractions, which may suggest that the majority of α-synuclein in DLB is sequestered in insoluble pathological aggregates. Similar observations were made regarding tau pathology in sgACC, with no differences observed in tau expression between DLB with and without depression. Higher 3R/4R ratios were observed in DLB cases compared to controls. Tau can be expressed with three repeat domains (3R) or four (4R), which arise from alternative splicing of MAPT exon 10. The abnormal increase in 4R tau expression is observed in many tauopathies, such as FTD, PSP and AD, with 4R tau present in NFT and NT (de Silva et al., 2003, Arai et al., 2001). The increases in the 4R to 3R ratio have been described in many neurodegenerative disorders, including FTD, PSP and AD (Ginsberg et al., 2006, Ingelsson et al., 2007). The higher 3R/4R ratios in DLB may indicate dysfunction in axonal transport, which may contribute to neuronal dysfunction in sgACC. The altered 3R/4R ratio may also represent the shift in tau expression associated with tau related neurodegeneration in AD considering that many DLB cases have a considerable burden of tau related neuropathology (Ginsberg et al., 2006, Ingelsson et al., 2007).

In AD, a reduced CSF level of Aβ42 is thought to be associated with the deposition of Aβ42 in senile plaques, with studies showing a strong correlation between low CSF Aβ42 and high density of plaques in the neocortex and hippocampus (Strozyk et al., 2003). This is also demonstrated by a high retention of PIB that reflects plaque pathology in the brain (Forsberg et al., 2008). Some studies have also suggested that the ratio of Aβ42/Aβ40 may be more important to the neurobiology of AD than the absolute level of Aβ42 (Kumar-Singh et al., 2006, Bentahir et al., 2006). In this study, higher Aβ40 and lower Aβ42 levels were observed in the SDS soluble fraction of sgACC in DLB cases with depression compared to controls, as well as higher Aβ40/Aβ42 ratio in DLB cases with depression compared to controls and also DLB cases without depression. Many studies have examined plasma levels of Aβ in AD with mixed results (Irizarry, 2004), although decreased CSF Aβ42 levels were observed in DLB patients (Kanemaru et al., 2000, Tschampa et al., 2001), with higher Aβ40/Aβ42 ratios in DLB patients compared to AD (Lewczuk et al., 2015). Some studies have reported lower plasma concentrations of Aβ42 in depression in the elderly, leading to a higher Aβ40/Aβ42 ratio (Sun et al., 2008), whereas others have found higher Aβ42 plasma concentrations in late onset depression (Blasko et al., 2010, Moon et al., 2011). Other studies have also shown higher plasma Aβ40, but not higher Aβ42 nor Aβ40/Aβ42 ratio in depressed patients with MCI, which was associated with parahippocampal atrophy and poor cognitive prognosis (Yamazaki et al.,

2017). Aβ levels appear therefore, to be variably changed depending on both disease state and sampling region.

In conclusion, these results demonstrate that neurodegenerative neuropathological changes within the cingulate cortex do not appear to influence appreciably the development of depression in DLB. While the ACC is a predilection site for the development of α-synuclein pathology, pathological changes including neuronal loss do not directly associate with depression. The underlying biochemistry of LB, NFT, and Aβ pathology also do not associate with depression. These findings do however suggest that, since there are metabolic changes associated with depression and neurodegeneration in the cingulate cortex (Drevets et al., 2008a, Drevets et al., 2008b, Rodriguez-Cano et al., 2014), that study of metabolic changes or neurochemical imbalance in the cingulate gyrus may be more relevant in relation to depression in DLB.

Chapter 4.

GABAergic and glutatergic changes in the sgACC

4.1 Introduction

The monoamine hypothesis is well established and has dominated the field of research into depression for some time, although increasing attention has been placed on other potential changes. Neurobiological abnormalities responsible for depression, such as dysregulation of the HPA axis, neurotrophins and multiple neurotransmitter systems may suggest that depression is not a single entity, with a variable underlying cause. This may explain why no single treatment approach, such as enhancement of monoaminergic transmission, would be effective in all patients. With recent advances in the development of fast-acting antidepressants that can overcome current therapeutic limitations of monoaminergic drugs, there is a clear indication that other neurochemical mechanisms are significant in MDD, and increasing interest has been placed on the development of more selective glutamatergic and GABAergic agonists, antagonists and modulators, which appear to be effective.

A growing body of evidence supports an impairment of glutamatergic and GABAergic neurotransmission in the pathophysiology of depression (Sanacora et al., 2012, Lener et al., 2017, Cryan and Kaupmann, 2005). The majority of cortical neurones and synapses use glutamate as a neurotransmitter, therefore it has been suggested that the glutamatergic system is a primary mediator of depression, and may represent a common pathway for the therapeutic action of antidepressant drugs (Sanacora et al., 2012). Evidence from clinical studies demonstrates abnormal glutamatergic transmission in limbic and cortical areas involved in emotional processing in depressed individuals (Zanos et al., 2016, Downey et al., 2016). Abnormal glutamatergic signalling has also been observed in post mortem and MRI studies in patients with MDD, contributing to cytoarchitectural and volumetric changes (Konarski et al., 2008, Lorenzetti et al., 2009, Koolschijn et al., 2009). Enhanced glutamate release, altered synaptic transmission, structural and morphological changes in limbic brain regions have also been observed in animal models of stress (Gorman and Docherty, 2010, Pittenger and Duman, 2008, Musazzi et al., 2011). Glutamatergic abnormalities have additionally been identified in plasma (Mitani et al., 2006, Bocchio-Chiavetto et al., 2010), and CSF in MDD, indicating widespread glutamatergic changes (Levine et al., 2000, Frye et al., 2007).

Functional and structural alterations of GABAergic circuits have been shown to contribute to the pathophysiology of anxiety and MDD (Marin, 2012, Brambilla et al., 2003). Reduced GABA levels in ACC in patients with MDD have been observed using PET imaging (Gabbay et al., 2012, Godfrey et al., 2018). A downregulation of several GABA related genes in sgACC

has also been reported in patients with MDD, demonstrating altered GABAergic circuitry in key regions associated with depression (Tripp et al., 2011). Reduced GABA levels have also been shown in plasma (Petty and Sherman, 1984) and the CSF (Gerner and Hare, 1981, Gold et al., 1980) in patients with depression. There is therefore significant evidence implicating GABAergic changes in MDD.

Disrupted balance in excitatory and inhibitory (E/I) neurotransmission is thought to contribute greatly to the development of mood disorders (Gabbay et al., 2012), possibly through impaired synaptic function (Zhao et al., 2012, Feyissa et al., 2009). Abnormalities of glutamatergic and GABAergic neurotransmission in the sgACC in MDD are well established (Tripp et al., 2012, Tripp et al., 2011, Ongur et al., 1998), but how these changes relate to depression in DLB is unknown.

4.2 Aims

The aim of this study was to investigate changes in glutamatergic and GABAergic neurotransmission in the sgACC in relation to depression in DLB.

4.2.1 Objectives

- To assess glutamatergic and GABAergic cell numbers in sgACC using an adapted stereological method in post-mortem tissue from DLB cases with and without depression and in normal controls.

- To assess whether the presence of aggregated phosphorylated α-synuclein (s129) induces changes in glutamatergic and/or GABAergic synapses in the sgACC in relation to depression in DLB using confocal analysis.

- To quantify protein levels of pre- and postsynaptic markers associated with glutamatergic and GABAergic synaptic function and neurotransmission in the sgACC using frozen post mortem tissue samples from DLB cases with and without depression and in normal controls.

- To assess mitochondrial changes in sgACC in DLB cases with and without depression and normal controls using frozen post mortem tissue.

4.3 Methods

The cohort for confocal and stereological analysis consisted of 17 DLB cases with depression, 20 DLB cases without depression and 17 neurologically and psychiatrically normal controls (see section 3.3; Table 3.1). The cohort for protein analysis consisted of 12 DLB cases with depression, 12 DLB cases without depression and 12 normal controls (see section 3.3; Table 3.3).

Fixed tissue sections corresponding to the sgACC were stained with GABA (GAD67) and pyramidal (CRYM) cell markers for assessment of cell density and analysed using an adapted stereological method (see section 2.1.7.2). Quadruple immunofluorescence was used to stain sgACC tissue sections for confocal analysis of glutamatergic and GABAergic synapses (see sections 2.1.5.2, 2.1.6.2 and 2.1.7.4). To quantify changes in neurotransmitter receptors and pre- and postsynaptic proteins involved in GABAergic and glutamatergic neurotransmission, western blot and dot blot techniques were employed, using Near Infrared (NIR) detection (see sections 2.1.8.4-2.1.8.6). The proteins were quantified using Image Studio Lite (see section 2.1.8.7) and normalised against GAPDH. All of the antibodies used in this study can be found in Table 4.1.

Table 4.1 Antibodies used for GABAergic and glutamatergic study.

Antigen	Host/IgG	Dilution	Source	Product Code
α-synuclein (s129)	pRb (IgG)	1:500 (DB) 1:500 (F-IHC)	Abcam	ab168381
Donkey Anti-Sheep, 546 conjugate	Sheep (IgG)	1:200 (F-IHC)	Life Technologies (AF)	A21098
GABARAP	mRb(IgG)	1:500 (DB)	Abcam	ab109364
GABAR3	pRb (IgG)	1:500 (DB)	Abcam	ab33299
GABAARα5	mMs (IgG1)	1:500 (DB)	Santa Cruz Biotechnology	sc393921
GABRG1	pRb (IgG)	1:500 (DB)	GeneTex	GTX66238
GAD 65/67	mMs (IgG1)	1:500 (DB)	Enzo Life Sciences	ADI-MSA-225-E
GAD 67	mMs (IgG1)	1:500 (DB) 1:1000 (IHC)	Santa Cruz Biotechnology	sc58531
GAP-43	pRb (IgG)	1:1000 (DB)	GeneTex	GTX1015355
Gephyrin	mMs (IgG1)	1:1000 (DB)	Synaptic Systems	147011
GLUR1/5	mMs (IgG1)	1:500 (DB)	Neuromab	N75/3
GLUR5	pRb (IgG)	1:500 (DB)	Abcam	ab27190
GLUR6/7	mRb(IgG)	1:500 (DB)	Millipore	MAB5683
Goat Anti-Mouse, 488 conjugate	Gt (IgG1)	1:200 (F-IHC)	Life Technologies (AF)	A21121
Goat Anti-Mouse, 488 conjugate	Gt (Ig2b)	1:200 (F-IHC)	Life Technologies (AF)	A21141
Goat Anti-Mouse, 546 conjugate	Gt (IgG1)	1:200 (F-IHC)	Life Technologies (AF)	A21123
Goat Anti-Rabbit, 405 conjugate	Gt (IgG)	1:200 (F-IHC)	Life Technologies (DyLight)	35550
μ-crystallin (CRYM)	mMs (IgG2b)	1:500 (DB) 1:1000 (IHC)	Santa Cruz Biotechnology	sc376687
MTCO2	mMs (IgG2a)	1:1000 (DB)	Abcam	ab110258
NDUFB8	mMs (IgG1)	1:500 (DB)	Abcam	ab110242
Neuroligin-2	pRb (IgG)	1:250 (DB)	Santa Cruz Biotechnology	sc50394
NMDAR2B	mMs(IgG2b)	1:500 (DB)	BD Biosciences	610416
Parvalbumin	pRb (IgG)	1:1000 (DB)	Abcam	ab11427
PSD-95	mMs (IgG2a)	1:500 (DB)	EMD Millipore	MAB1598
SNAP25	mMs (IgG1)	1:500 (WB) 1.500 (F-IHC)	Santa Cruz Biotechnology	sc20038
SNAP25	pSheep (IgG)	1:500 (F-IHC)	LifeSpan BioSciences	LS-C94834
Synaptogamin I	mMs (IgG1)	1:500 (DB)	Santa Cruz Biotechnology	sc136480
Synaptophysin	mMs (IgG1)	1:1000 (DB)	EMD Millipore	MAB368
Syntaxin Ia	pRb (IgG)	1:1000 (DB)	Abcam	ab41453
Ubiquinol-Cytochrome C Reductase Core Protein I	mMs (IgG1)	1:1000 (DB)	Abcam	ab110252
VDAC1/Porin	mMs (IgG2b)	1:500 (DB)	Abcam	ab14734
VGLUT1	mMs (IgG1)	1:500 (DB) 1:500 (F-IHC)	Sigma-Aldrich	AMAb91041
VGLUT2	mMs (IgG2b)	1:250 (DB)	EMD Millipore	MAB5504

DB – dot blot; IHC – immunohistochemistry; m – monoclonal; p – polyclonal; Ms – mouse; Rb – rabbit, Gt – goat.

4.4 Results

4.4.1 GABAergic and glutamatergic cell density in the sgACC

A pilot study was carried out to verify μ-crystallin (CRYM) as a reliable pyramidal cell marker (Maruoka et al., 2011, Arlotta et al., 2005). Temporal cortical sections from six controls were stained using dual immunofluorescence (Figure 4.1). The captured images (15-20 frames per case) were analysed by counting CRYM and GAD67 positive neurones. The co-localisation between CRYM and GAD67 was less than two percent, which suggested CRYM to be a reliable pyramidal cell marker.

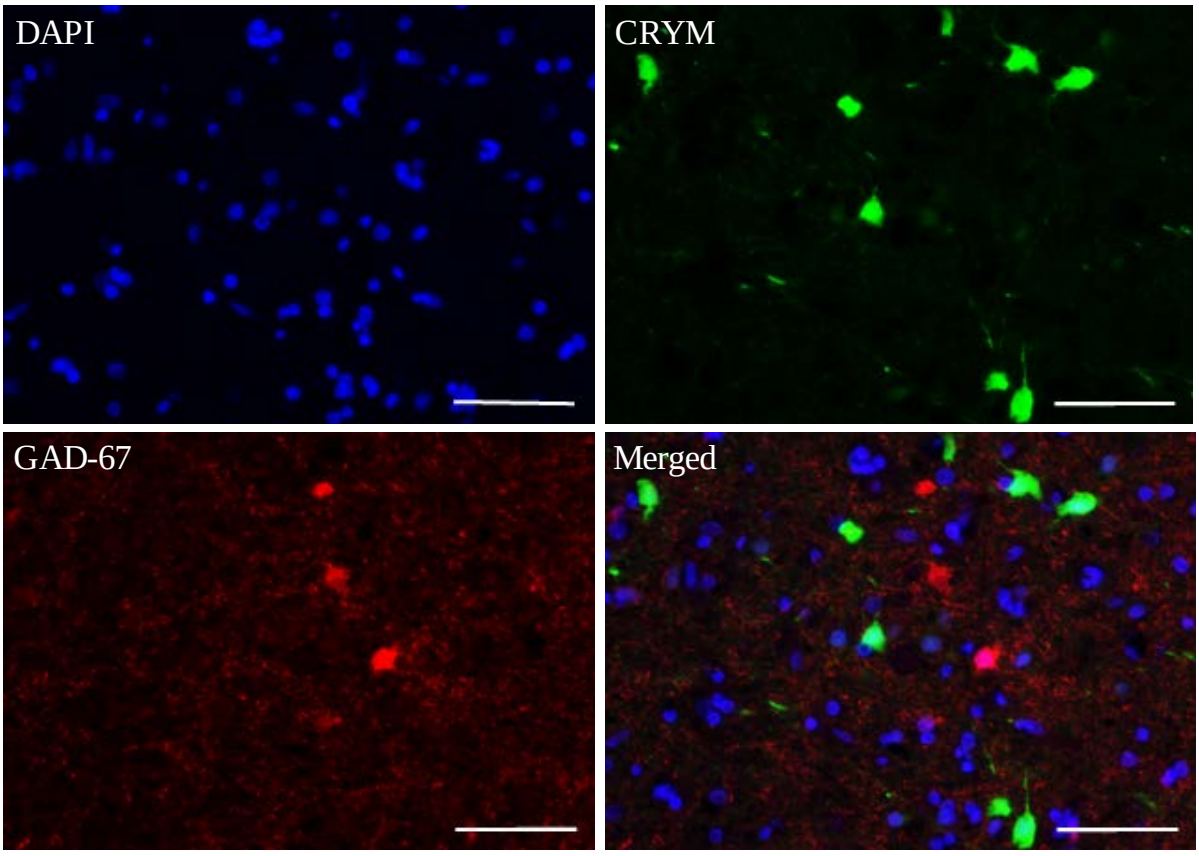

Figure 4.1 Co-localisation of CRYM and GAD 67 markers in the temporal cortex.
The nuclei were labelled using DAPI (405nm). Co-localisation of CRYM (pyramidal cell marker; 488nm) and GAD67 (GABAergic cells marker; 594nm) was assessed in the temporal cortex in six control cases (20x magnification). Scale bar represents 100μm.

Overall ($F(2, 48)=1.038$, $p=0.362$) or layer V ($F(2, 48)=1.452$, $p=0.244$) pyramidal cell density in the sgACC was not significantly different between the groups. The pyramidal cell density in

layers II/III was significantly different between the groups ($F(2, 48)=3.940$, $p=0.026$), and was higher in controls compared to DLB-D ($p=0.022$; Figure 4.2 A). No significant difference was observed in overall GABAergic cell density ($H(2)=2.997$, $p=0.223$), or in layers II/III ($H(2)=4.473$, $p=0.107$). GABAergic cells density in layer V was significantly different between the groups, with significantly higher cell density observed in controls compared to DLB+D ($p=0.004$; Figure 4.2 B).

4.4.2 Glutamatergic synaptic changes in the sgACC

Glutamatergic synapses were assessed in the sgACC in controls, DLB cases with and without depression (Figure 4.3). The total number of glutamatergic surfaces (VGLUT1 positive) was significantly different between the groups ($F(2, 40)=4.887$, $p=0.013$), and was higher in DLB cases with depression compared to controls ($p=0.048$) and DLB cases without depression ($p=0.019$). The average volume of glutamatergic surfaces was also significantly different between the groups ($F(2, 40)=3.965$, $p=0.040$), with significantly higher volumes observed in DLB cases with depression compared to controls ($p=0.049$; Figure 4.4 A-B). The number ($F(2, 40)=1.781$, $p=0.181$), as well as total volume of presynaptic surfaces (SNAP25 positive) ($F(2, 40)=1.799$, $p=0.178$) was not significantly different between the groups (Figure 4.4 C-D). The total number of α-synuclein surfaces (s129 positive) was not significantly different between the groups ($H(2)=5.660$, $p=0.059$), although it was trending towards significance. The average volume of α-synuclein surfaces was significantly different between the groups ($H(2)=27.099$, $p<0.001$), with DLB cases with and without depression showing higher s129 average volume compared to controls ($p<0.001$; Figure 4.4 E-F).

The number of glutamatergic synapses (VGLUT1 and SNAP25 positive) was not significantly different between the groups ($F(2, 40)=1.789$, $p=0.180$), whereas changes were observed in the synaptic volume between the groups ($F(2, 40)=7.592$, $p=0.002$), which was significantly higher in DLB cases with depression ($p=0.002$) and DLB cases without depression ($p=0.011$) compared to controls (Figure 4.5 A-B). The percentage of glutamatergic synapses containing s129 α-synuclein surfaces was significantly different between the groups ($H(2)=13.529$, $p=0.001$), and was significantly higher in DLB cases with depression compared to controls ($p=0.001$; Figure 4.5 C). The average volume of glutamatergic synapses containing s129 α-synuclein surfaces was significantly different between the groups ($F(2, 40)=7.228$, $p=0.002$), and was significantly higher in DLB cases with depression compared to controls ($p=0.002$; Figure 4.5 D).

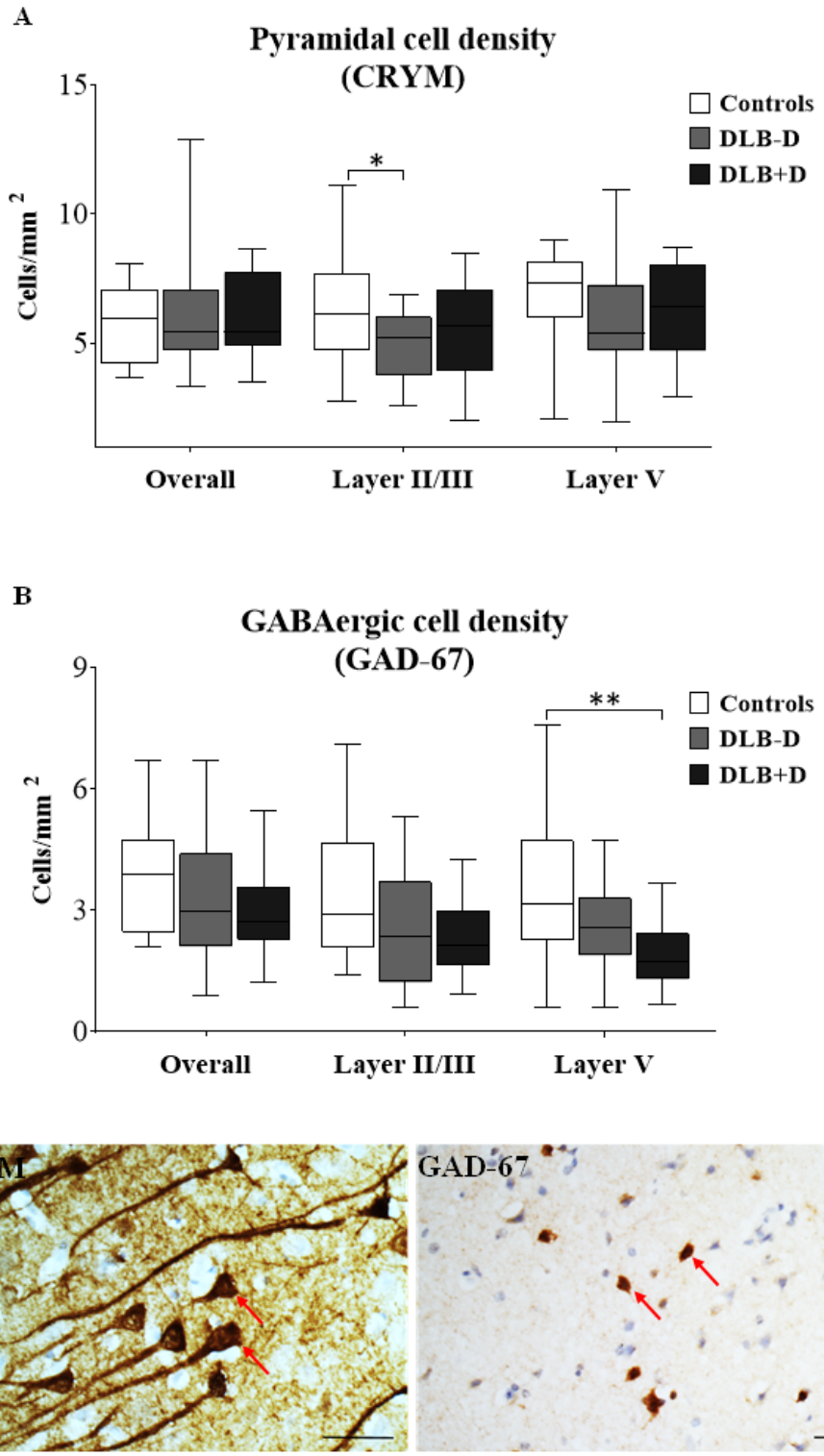

Figure 4.2 Glutamatergic and GABAergic cell density in sgACC.
(**A**) CRYM positive pyramidal neurones and (**B**) GAD-67 positive GABAergic cells were assessed in sgACC overall, as well as in layers II/III and layer V in controls (n=17), DLB cases without depression (DLB-D; n=20) and DLB cases with depression (DLB+D; n=17). The images were captured at 20X magnification. *p<0.05 and **p<0.01compared to control. Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values. Scale bar represents 100μm.

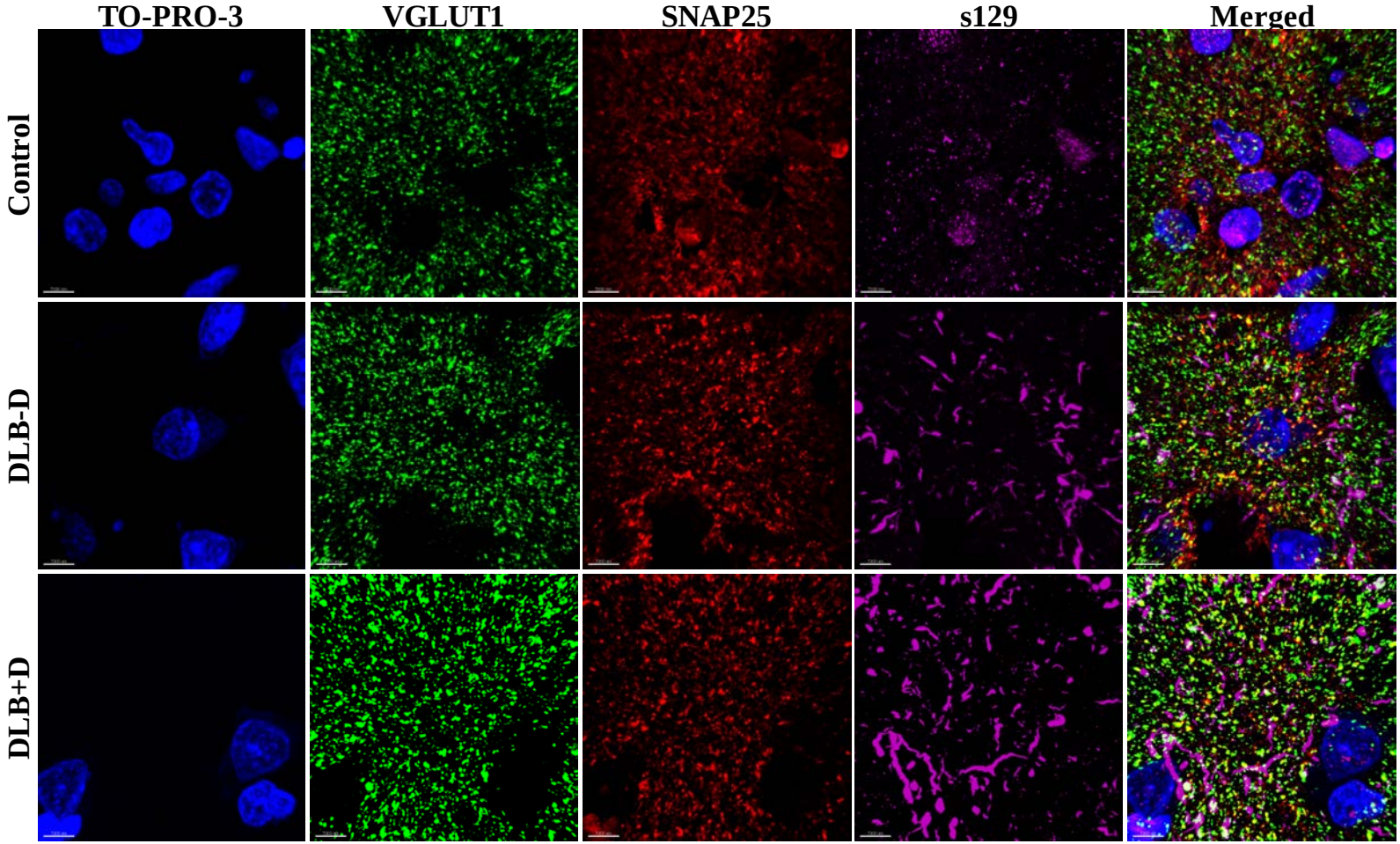

Figure 4.3 Confocal imaging of glutamatergic synapses.
Representative images of glutamatergic synaptic immunoreactivity (VGLUT1+SNAP25) in relation to s129 α-synuclein in the sgACC in age matched controls and DLB cases with (DLB+D) and without (DLB-D) depression. The nuclei were visualised using TO-PRO-3 Iodide (blue, 647nm), glutamatergic synaptic terminals were visualised using VGLUT1 (green, 488nm), presynaptic terminals demonstrated using SNAP25 (red, 546nm) and α-synuclein immunoreactivity shown using s129 (purple, 405nm). Scale bar represents 7μm.

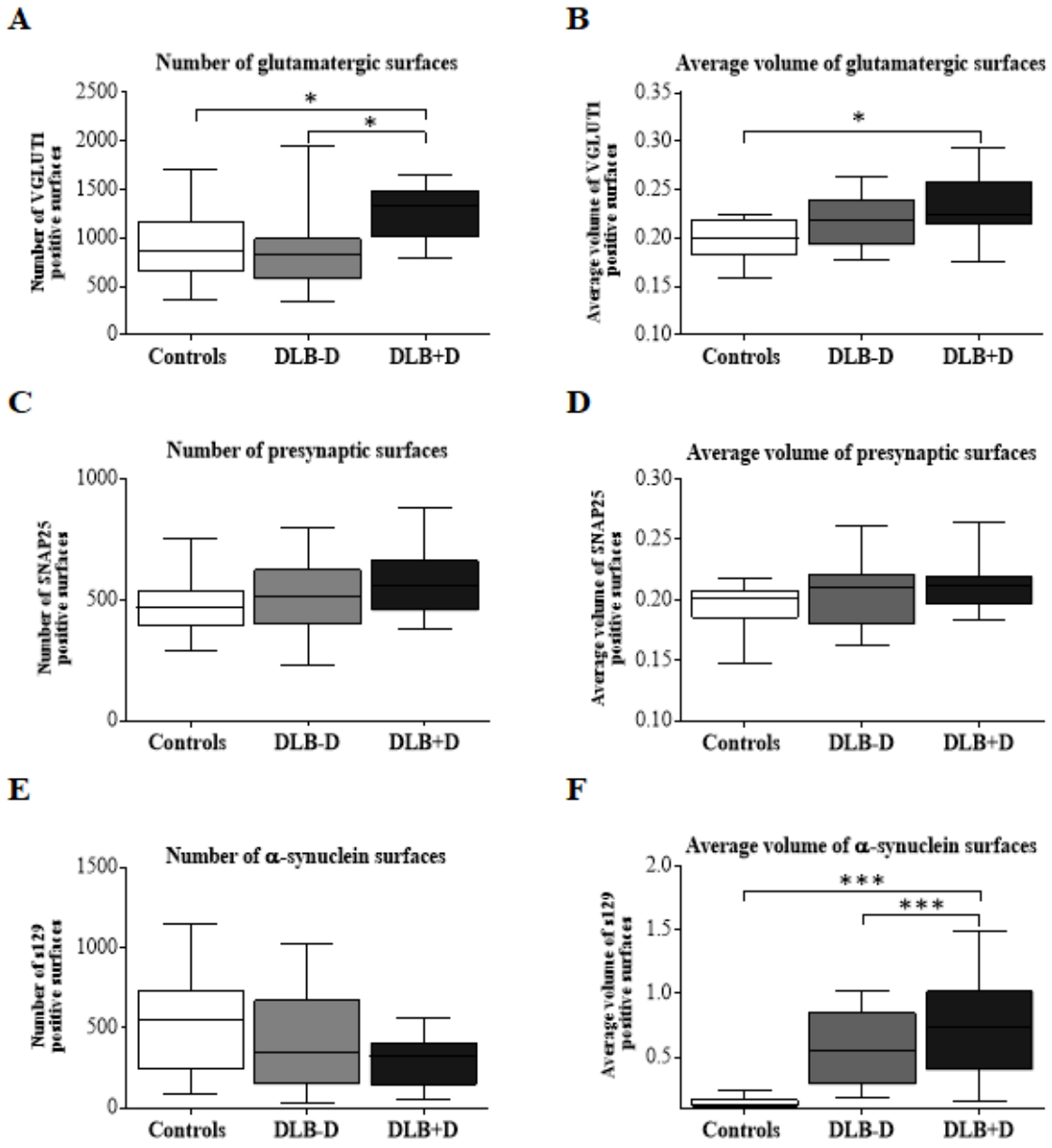

Figure 4.4 Analysis of VGLUT1, SNAP25 and s129 positive surfaces.
The number and the average volume (μm³) of glutamatergic (VGLUT1; **A-B**), presynaptic (SNAP25; **C-D**) and α-synuclein (s129; **E-F**) immunoreactive structures in the sgACC was assessed in age matched controls and DLB cases with (DLB+D) and without (DLB-D) depression. There was a significant increase in the number and average volume of glutamatergic surfaces in DLB cases with depression compared to controls, as well as the number of glutamatergic surfaces in DLB cases with compared to without depression. The average volume of α-synuclein was significantly higher in DLB cases compared to controls. (*p<0.05 and ***p<0.001, compared to appropriate group). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

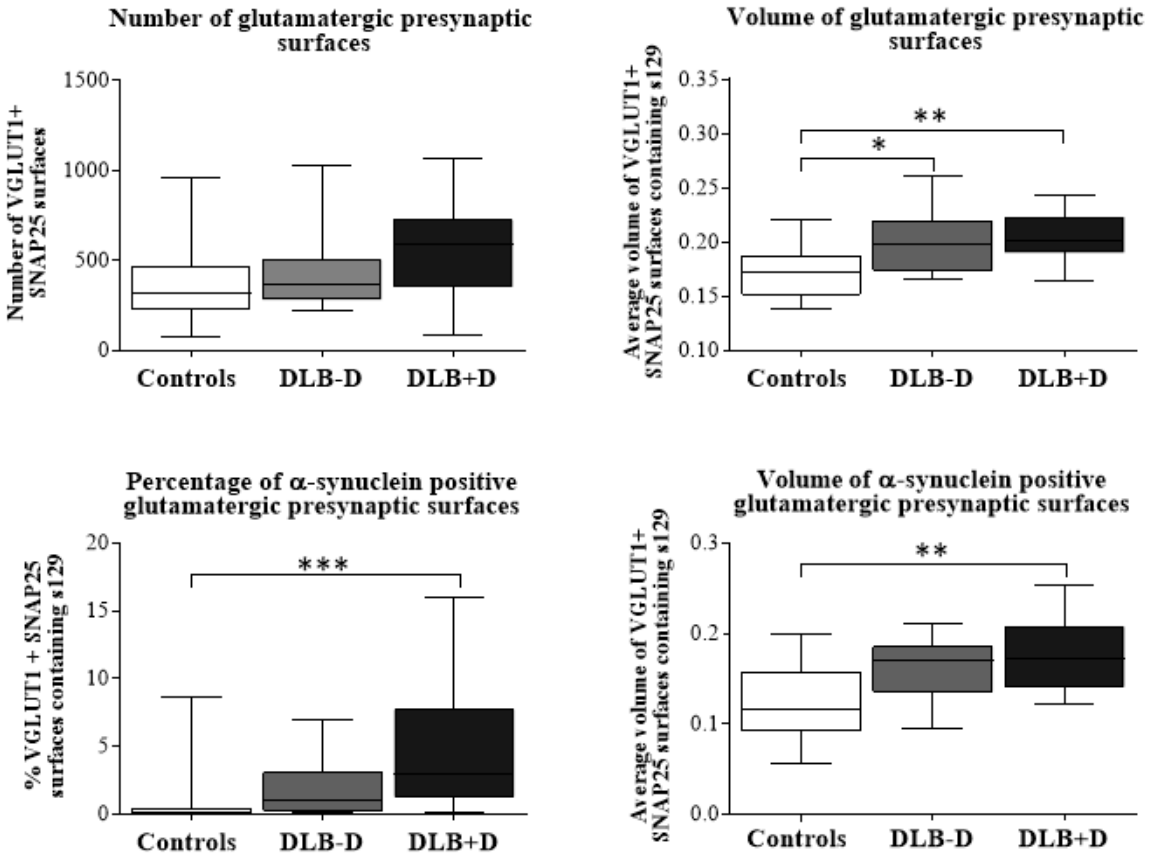

Figure 4.5 Analysis of glutamatergic synapses.
Analysis of the number and volume of glutamatergic synapses (VGLUT1 + SNAP25; **A**-**B**), as well as the percentage and volume of glutamatergic synapses containing α-synuclein (s129; **C**-**D**) in sgACC in age matched controls, DLB cases with (DLB+D) and without (DLB-D) depression. (*, $p<0.05$, **, $p<0.01$, or *** $p<0.001$, compared to control). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

4.4.3 Biochemical analysis of glutamatergic changes in the sgACC

To complement histological assessment of glutamatergic synapses, various biochemical markers of glutamatergic function were assessed. CRYM protein level in sgACC was significantly different between the groups ($H(2)=17.754$, $p<0.001$), with significantly lower CRYM levels detected in DLB cases with ($p<0.001$) and without depression compared to controls ($p=0.012$; Figure 4.6 A). Vesicular glutamate transporter is a marker of vesicular glutamate uptake, with VGLUT1 primarily expressed in the cortex, hippocampus and cerebellum, and VGLUT2 in brainstem and deep cerebellar nuclei (Fremeau et al., 2001). VGLUT1 levels in the sgACC were significantly different between the groups ($F(2,$

34)=13.984, $p<0.001$), with significantly lower VGLUT1 levels observed in DLB cases with ($p<0.001$) and without depression compared to controls ($p=0.001$; Figure 4.6 B). No significant difference between the groups was observed in VGLUT2 levels in sgACC ($F(2, 34)=2.104$, $p=0.139$; Figure 4.6 C).

Postsynaptic density protein 95 (PSD95), a marker of postsynaptic glutamate receptors, was significantly different between the groups ($F(2, 34)=6.620$, $p=0.004$), with a significant decrease in levels observed in DLB with ($p=0.008$) and without depression ($p=0.014$) compared to controls (Figure 4.7 A). Neuroligin-2 is a postsynaptic protein primarily located at inhibitory synapses, which interacts with PSD-95 and regulates E/I ratio. Neuroligin-2 was significantly different between the groups ($H(2)=12.069$, $p=0.002$), with significant increase in levels observed in DLB cases with ($p=0.012$) and without depression ($p=0.006$) compared to controls (Figure 4.7 B). The PSD95/Neuroligin-2 (E/I) ratio was significantly different between the groups ($H(2)=15.193$, $p=0.001$), with a significant decrease in the ratio observed in DLB with ($p=0.003$) and without depression ($p=0.002$) compared to controls (Figure 4.7 C).

Ionotropic ligand gated NMDA receptor 2B subunit protein concentration in sgACC was significantly different between the groups ($F(2, 34)=4.800$, $p=0.015$), with a significant decrease in levels observed in DLB with depression ($p=0.018$) compared to controls (Figure 4.8 A). No significant change in metabotropic glutamate receptor levels in sgACC was observed between the groups, including GLUR 1/5 ($F(2, 34)=0.021$, $p=0.979$), GLUR 5 ($F(2, 34)=2.351$, $p=0.112$) and GLUR 6/7 ($F(2, 34)=2.552$, $p=0.093$; Figure 4.8 B-D).

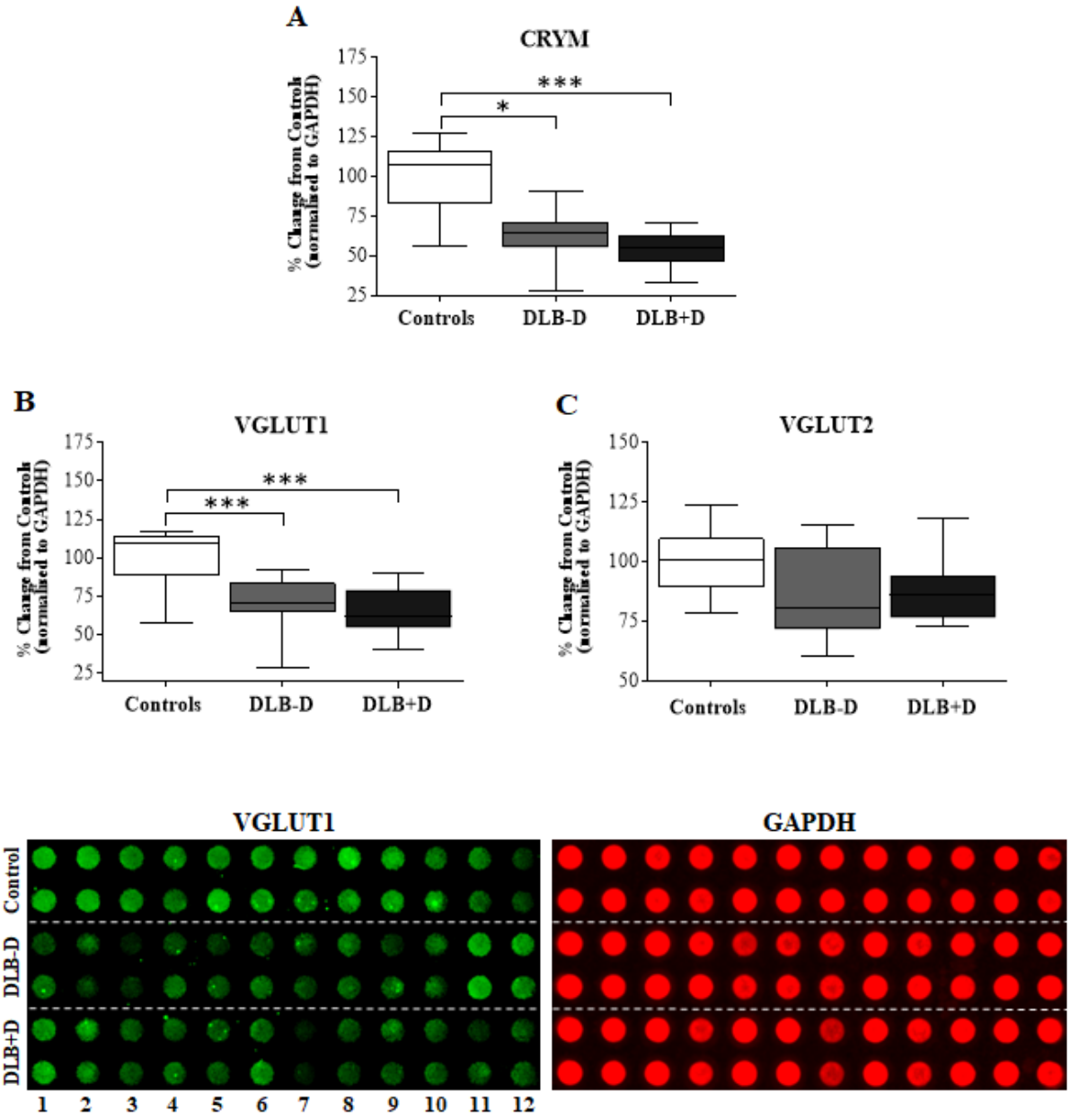

Figure 4.6 Dot blot analysis of CRYM, VGLUT1 and VGLUT2 glutamatergic markers. μ-Crystallin (CRYM; **A**), vesicular glutamate transporter 1 (VGLUT1; **B**) and 2 (VGLUT2; **C**) protein concentrations in sgACC were assessed in age matched controls, DLB cases with (DLB+D) and without (DLB-D) depression. (*, $p<0.05$ and ***, $p<0.001$ compared to control). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

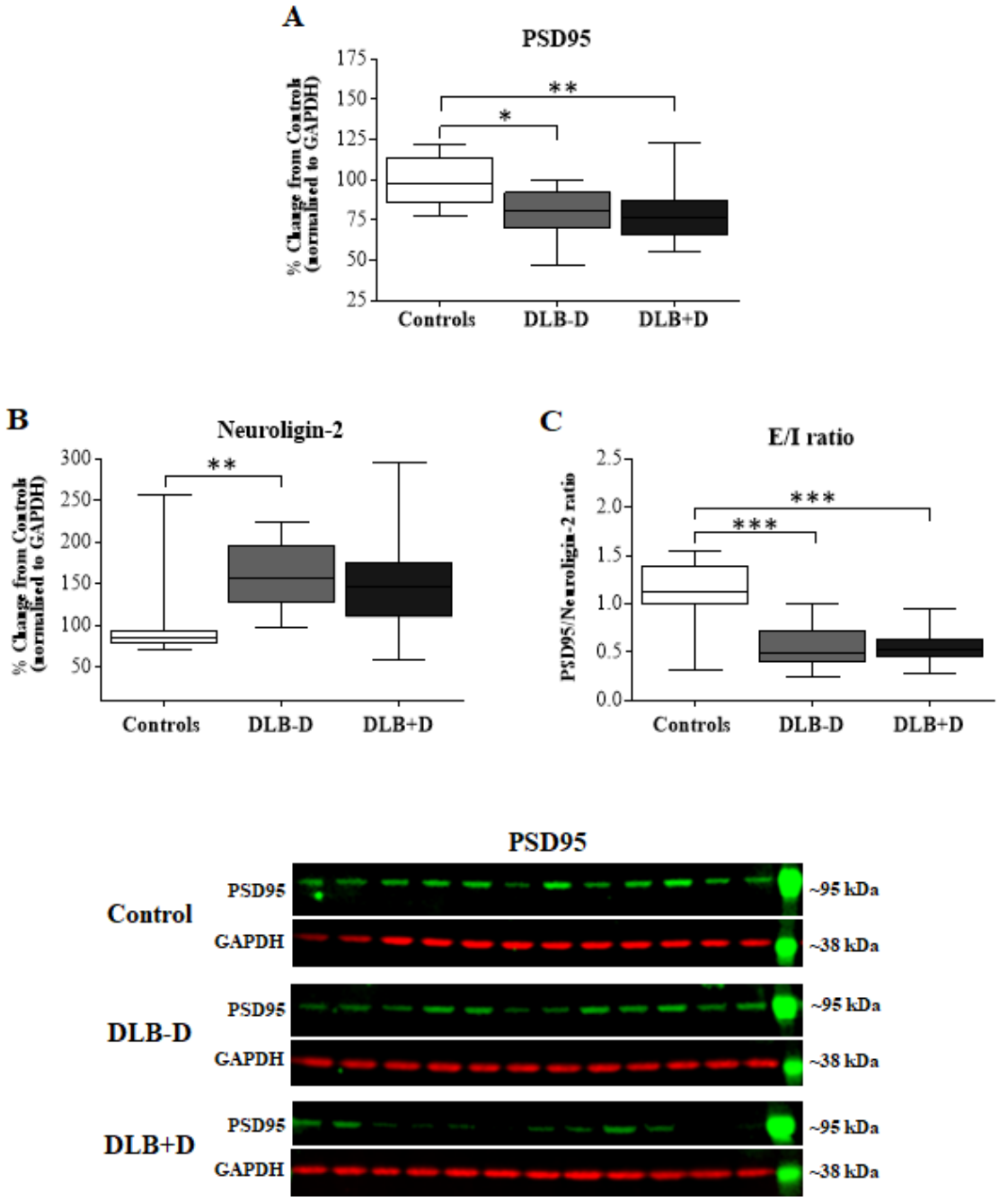

Figure 4.7 Dot blot analysis of PSD95, Neuroligin 2 and E/I ratio.
Postsynaptic density protein 95 (PSD95; **A**) and Neuroligin 2 (**B**) protein levels were assessed using in sgACC in age matched controls, DLB cases with (DLB+D) and without (DLB-D) depression. PSD95/Neuroligin ratio (**C**) was used to assess changes in excitation and inhibition (E/I) (*p<0.05, **p<0.01 and ***p<0.001 compared to control). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

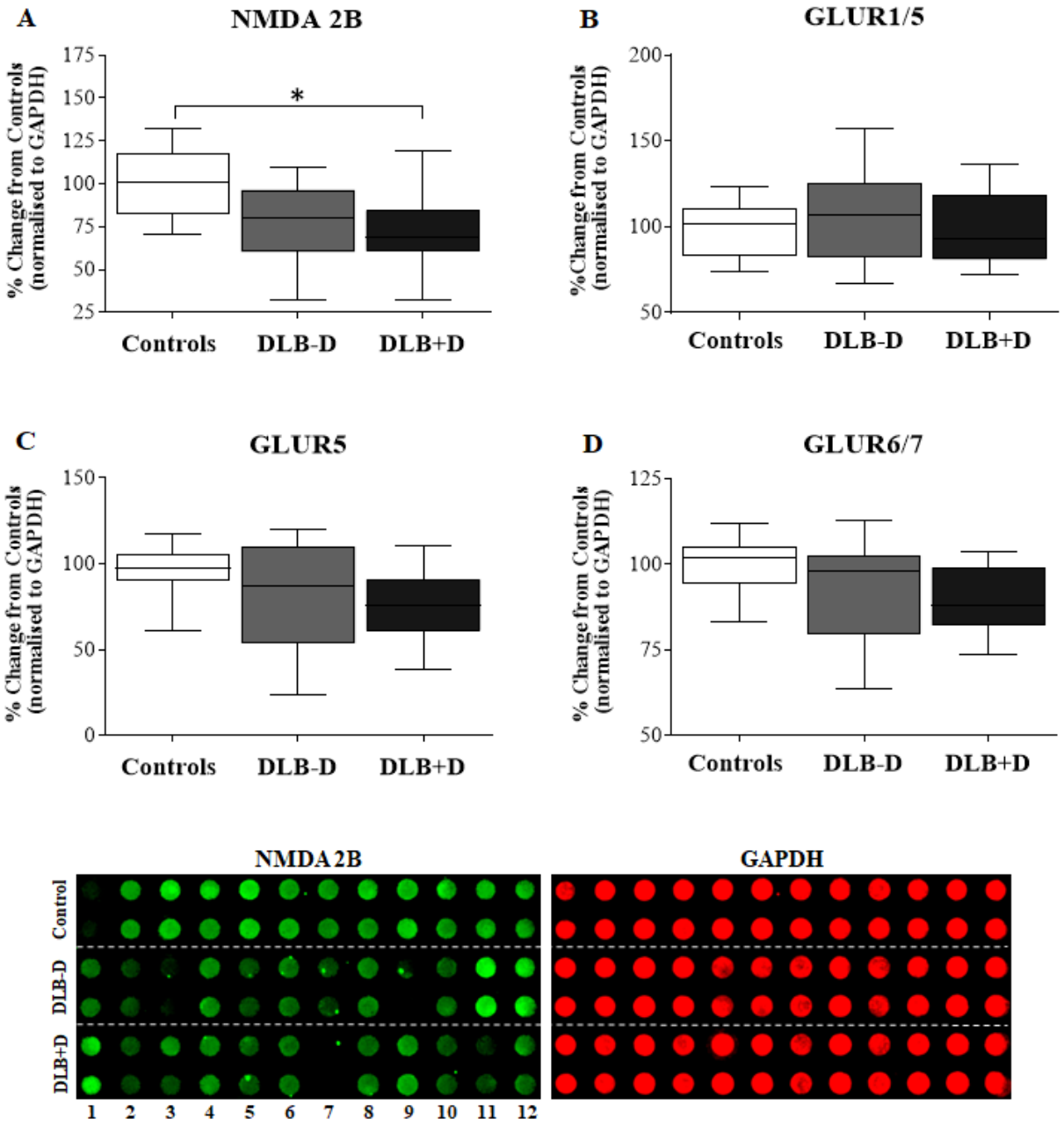

Figure 4.8 Dot blot analysis of NMDA2B, GLUR1/5, GLUR5 and GLUR6/7 glutamatergic receptors.

Ionotropic (NMDA 2B; **A**) and metabotropic (GLUR 1/5, GLUR 5 and GLUR 6/7; **B-D**) glutamatergic receptor levels were determined using Near Infra-Red dot blots in sgACC in age matched controls, DLB cases with (DLB+D) and without (DLB-D) depression. (* $p<0.05$ compared to control). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

Table 4.2 Summary Table for Glutamatergic Markers.

	Controls	*DLB-D*	*DLB+D*
CRYM	—	*ns*	↓ *p<0.001* [b]
VGLUT1	—	↓ *p=0.001* [a]	↓ *p<0.001* [b]
VGLUT2	—	—	—
PSD95	—	*ns*	*ns*
NMDA2B	—	—	*ns*
GLUR1/5	—	—	—
GLUR5	—	—	—
GLUR6/7	—	—	—

Summary of significant glutamatergic changes after post hoc Bonferroni corrections; ns - no significant difference after corrections for multiple comparisons; DLB-D<Controls[a] and DLB+D<Controls[b].

4.4.4 GABAergic synaptic changes in the sgACC

GABAergic synapses were assessed in the sgACC in controls, DLB cases with and without depression using four colour confocal microscopy (Figure 4.9). The total number of GABAergic surfaces (GAD65/67 positive) was significantly different between the groups ($F(2, 40)=3.513$, $p=0.040$), although no significant differences were seen after post hoc analysis (Figure 4.10 A). The average volume of GABAergic surfaces was significantly different between the groups ($F(2, 40)=5.216$, $p=0.010$), with a significant reduction in average volume observed in DLB cases with depression compared to controls ($p=0.008$; Figure 4.10 B). No changes were observed in the number ($F(2, 40)=0.250$, $p=0.780$) or the average volume of presynaptic surfaces (SNAP25 positive) between the groups ($F(2, 40)=0.54$, $p=0.579$; Figure 4.10 B-C). The total number of α-synuclein surfaces (s129 positive) was significantly different between the groups ($F(2, 40)=24.759$, $p<0.001$), with control cases having a significantly higher number of individual α-synuclein surfaces compared to DLB cases with or without depression ($p<0.001$; Figure 4.10 E). The average volume of individual α-synuclein surfaces was significantly different between the groups ($F(2, 40)=15.695$, $p<0.001$), with DLB cases with or without depression showing higher s129 average volumes compared to controls ($p<0.001$; Figure 4.10 F).

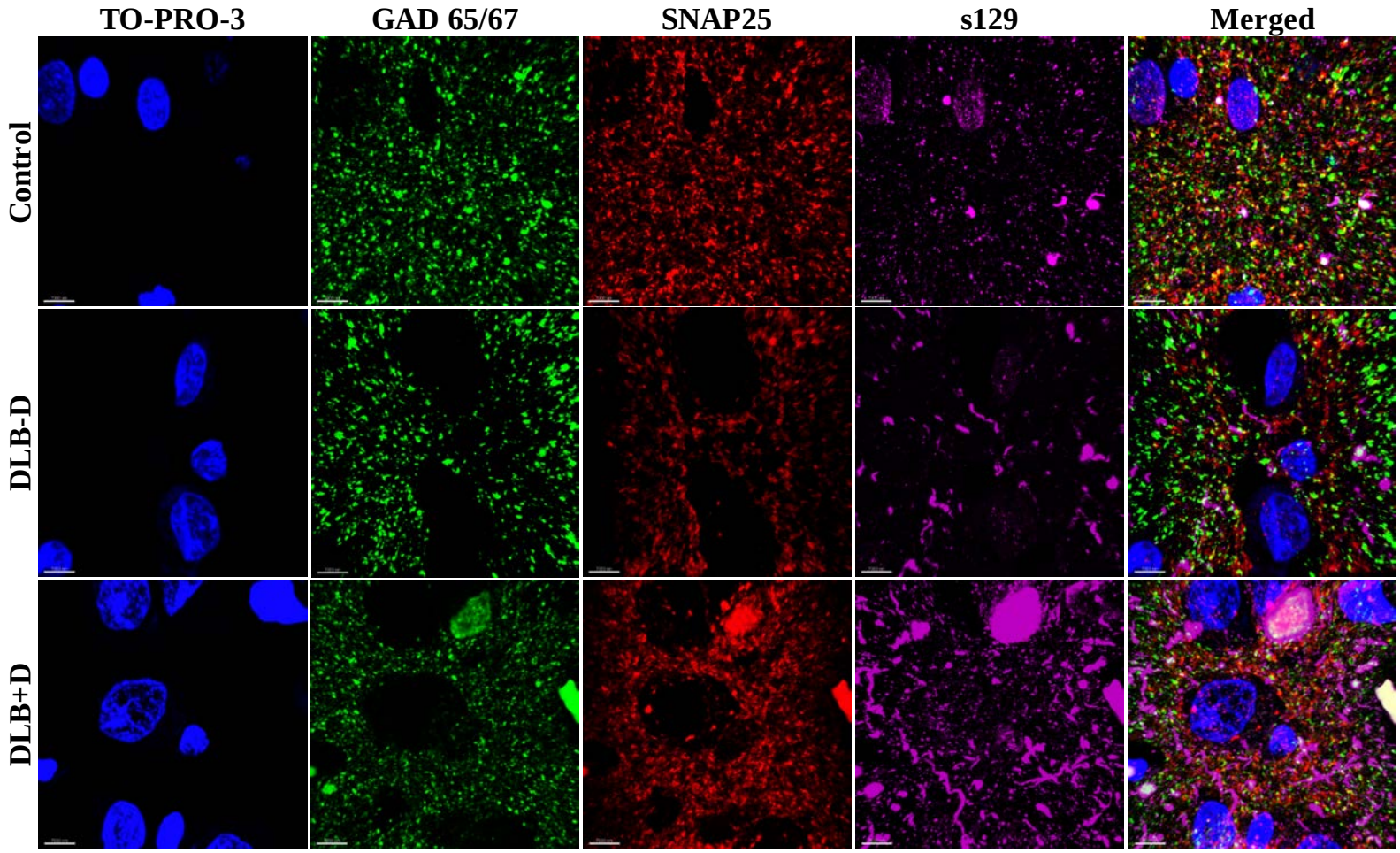

Figure 4.9 Confocal imaging of GABAergic synapses.
Representative images of GABAergic synaptic (GAD65/67+ SNAP25) immunoreactivity in relation to s129 α-synuclein in the sgACC in age matched controls and DLB cases with (DLB+D) and without (DLB-D) depression. The nuclei were visualised using TO-PRO-3 Iodide (blue, 647nm), GABAergic synaptic terminals were visualised using GAD 65/67 (green, 488nm), presynaptic terminals identified using SNAP25 (red, 546nm) and α-synuclein immunoreactivity using s129 (purple, 405nm). Scale bar represents 7μm.

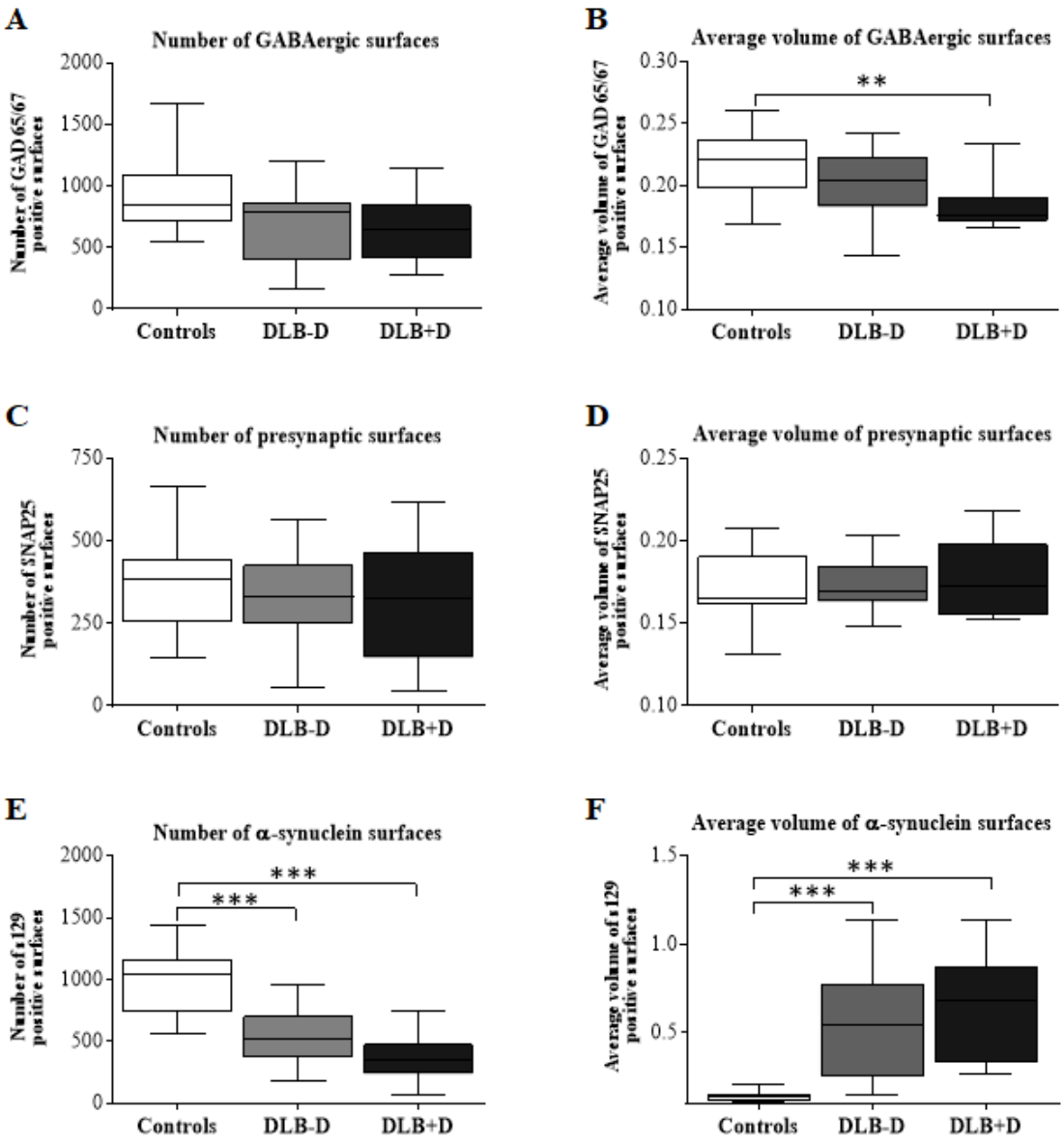

Figure 4.10 Analysis of GAD67, SNAP25 and s129 positive surfaces.
Analysis of the number and the average volume (μm³) of the GABAergic (GAD 65/67; **A-B**), presynaptic (SNAP25; **C-D**) and α-synuclein (s129; **E-F**) immunoreactive structures in the sgACC in age matched controls and DLB cases with (DLB+D) and without (DLB-D) depression. (**p<0.01 and ***p<0.001, compared to control group). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

The number ($F(2, 40)=2.082$, $p=0.139$) and average volume ($F(2, 40)=0.812$, $p=0.451$) of GABAergic synapses was not significantly different between the groups (Figure 4.11 A-B). The percentage of GABAergic synapses containing 129 α-synuclein was significantly different between the groups ($H(2)=13.881$, $p=0.001$), and was significantly higher in DLB cases with ($p=0.003$) or without depression compared to controls ($p=0.005$; Figure 4.11 C). The average volume of GABAergic synapses containing 129 α-synuclein surfaces was significantly different between the groups ($F(2, 40)=14.755$, $p<0.001$), and was significantly higher in DLB cases with ($p=0.001$) or without depression ($p<0.001$) compared to controls (Figure 4.11 D).

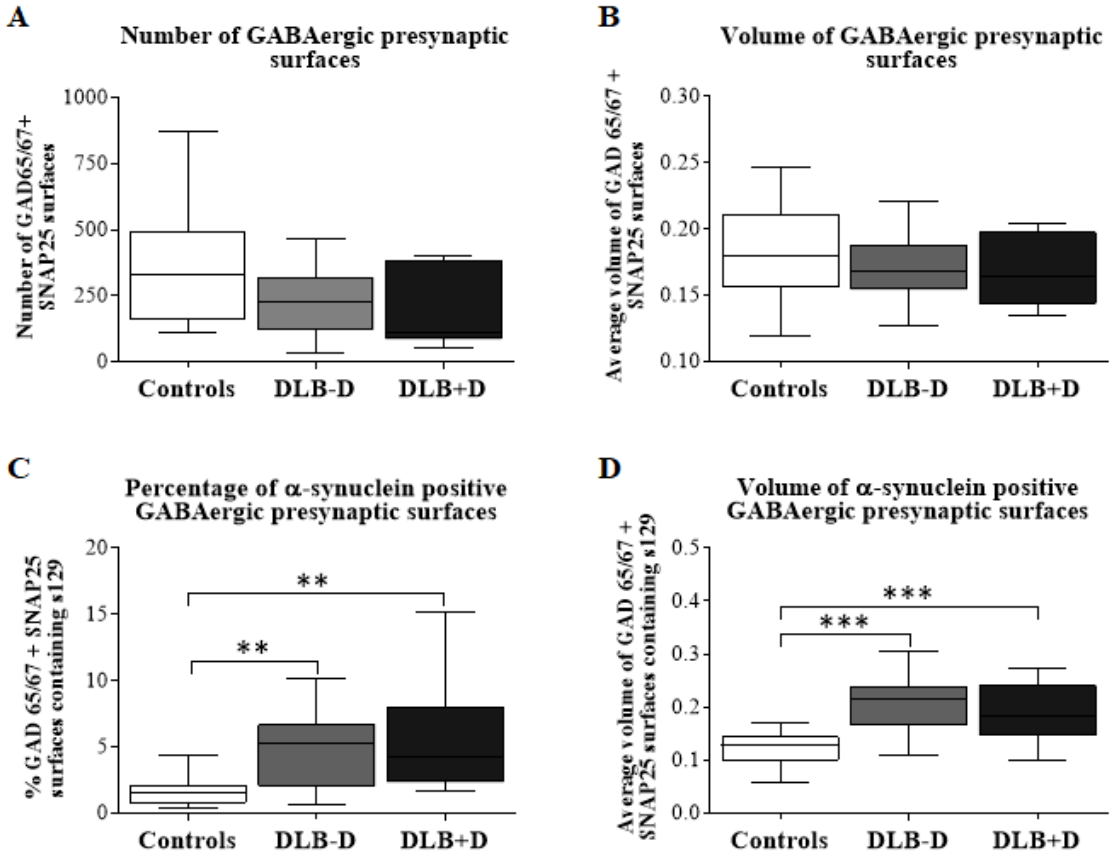

Figure 4.11 Analysis of GABAergic synapses.
Analysis of the number and volume of GABAergic synapses (GAD 65/67 + SNAP25; **A-B**), as well as the percentage and volume of GABAergic synapses containing α-synuclein (s129; **C-D**) in sgACC in age matched controls, DLB cases with (DLB+D) and without (DLB-D) depression. (*, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$, compared to control). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

4.4.5 Biochemical analysis of GABAergic changes in the sgACC

To complement confocal analysis of GABergic synapses, biochemical analysis of GABAergic proteins in post mortem tissue was determined using western blotting or dot blotting approaches. No significant change in GAD 65/67 ($H(2)=0.920$, $p=0.631$) was detected in sgACC between the groups (Figure 4.12 A). Gephyrin levels were significantly different between the groups ($F(2, 34)=16.277$, $p<0.001$), with a significant decrease observed in DLB cases with ($p<0.001$) and without depression compared to controls ($p=0.001$; Figure 4.12 B). Parvalbumin levels in sgACC were significantly different between the groups ($F(2, 34)=10.035$, $p<0.001$), with a significant decrease in levels observed in DLB with ($p=0.003$) or without depression ($p=0.001$) compared to controls (Figure 4.12 C). Calbindin levels were significantly different between the groups ($F(2, 34)=4.118$, $p=0.025$), with a significant decrease observed in DLB cases with depression compared to without ($p=0.027$; Figure 4.12 D).

$GABA_A$ receptor α3 subunit levels were significantly different between the groups ($F(2, 34)=4.185$, $p=0.025$), with a significant reduction observed in DLB cases with depression compared to controls ($p=0.024$; Figure 4.13 A). $GABA_A$ receptor α5 subunit levels in sgACC was significantly different between the groups ($F(2, 34)=7.265$, $p=0.002$), with a significant decrease observed in DLB cases with depression compared to controls ($p=0.002$; Figure 4.13 B). No significant change was observed between the groups in $GABA_A\gamma1$ ($F(2, 34)=1.695$, $p=0.199$) or GABARAP proteins ($F(2, 34)=1.115$, $p=0.340$; Figure 4.13 C-D).

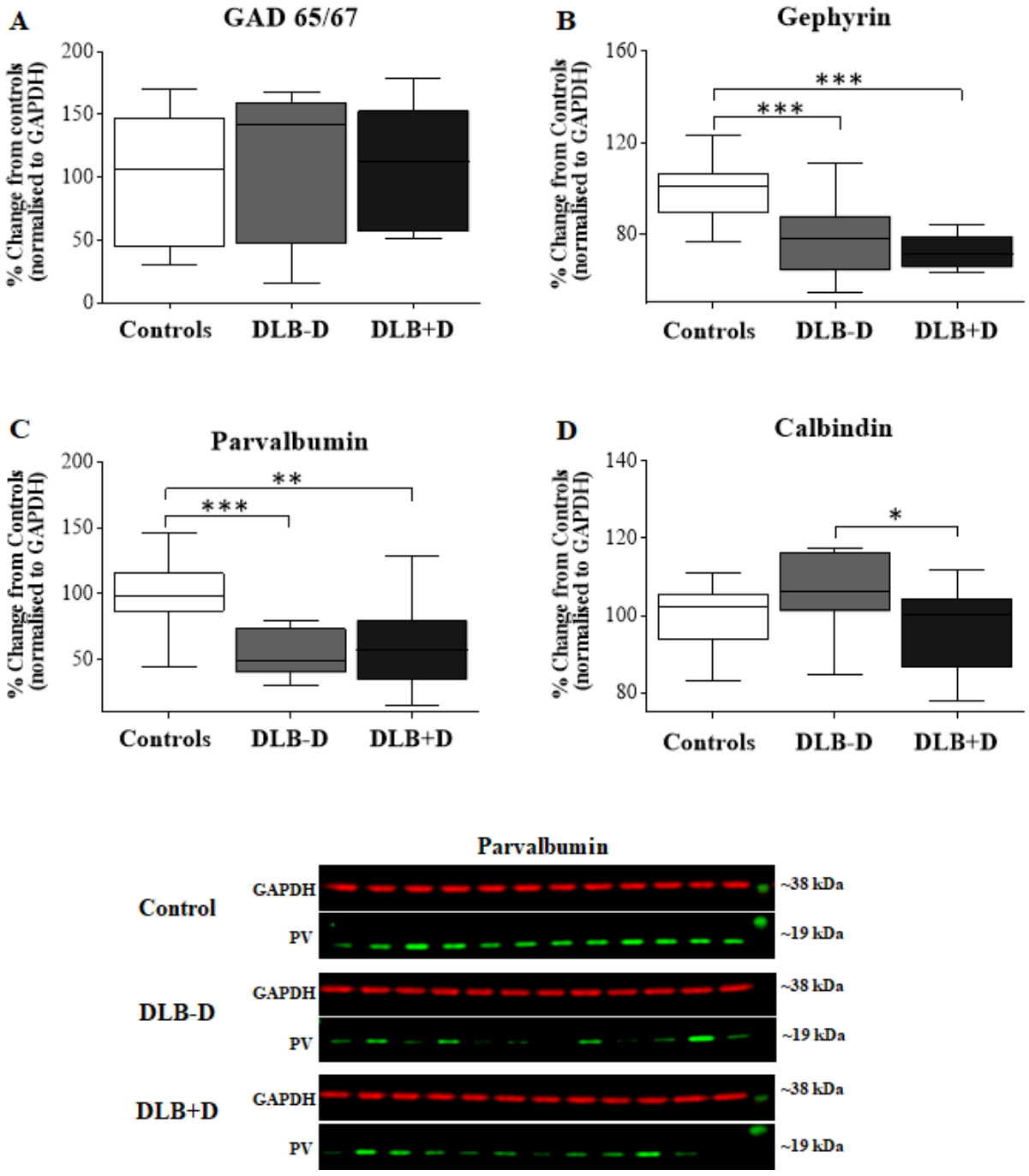

Figure 4.12 Dot blot analysis of GAD65/67, Gephyrin, Parvalbumin and Calbindin GABAergic markers.

GABAergic markers were assessed using NIR blotting in sgACC of age matched controls, or DLB cases with (DLB+D) or without (DLB-D) depression, including GAD 65/67 (**A**), Gephyrin (**B**), Parvalbumin (**C**) and Calbindin (**D**); (*p<0.05, **p<0.01 and ***p<0.001 comparing appropriate groups). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

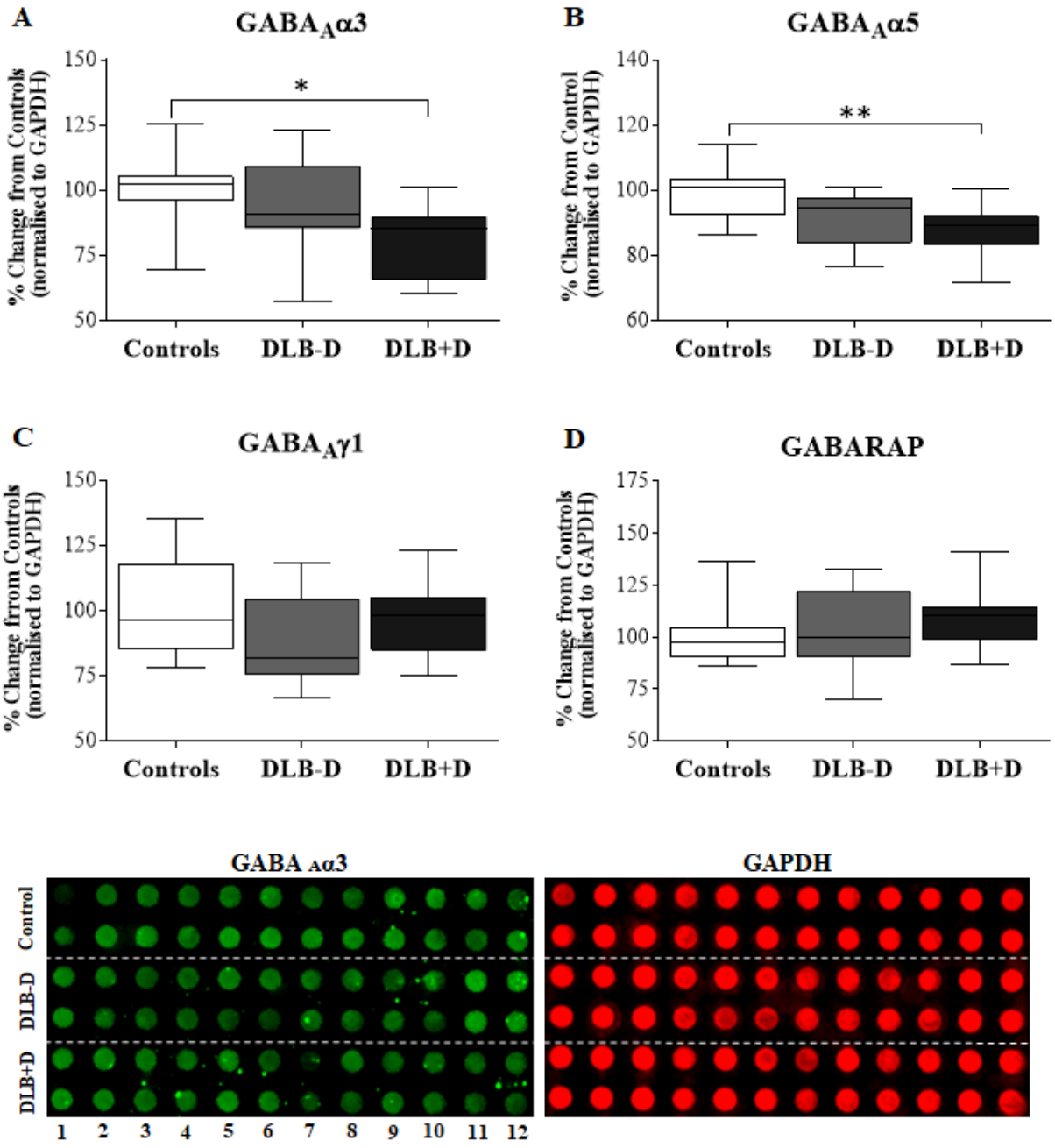

Figure 4.13 Dot blot analysis of GABA$_A$α5, GABA$_A$α3, GABA$_A$γ1 and GABARAP GABAergic markers.

GABAergic receptor markers were assessed in sgACC in age matched controls, DLB cases with (DLB+D) and without (DLB-D) depression, including GABA$_A$α3 (**A**), GABA$_A$α5 (**B**), GABA$_A$γ1 (**C**) and GABARAP (**D**); (*p<0.05 or ***p<0.001, compared to control). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

Table 4.3 Summary Table for GABAergic Markers.

	Controls	*DLB-D*	*DLB+D*
GAD65/67	—	—	—
PV	—	↓ $p=0.001^{a}$	↓ $p=0.003^{b}$
CB	—	—	ns
Gephyrin	—	↓ $p=0.001^{a}$	↓ $p<0.001^{b}$
GABARAP	—	—	—
GABAAα5	—	—	↓ $p=0.002^{b}$
GABAAα3	—	—	ns
GABAAγ1	—	—	—

Summary of significant GABAergic changes after post hoc Bonferroni corrections; ns - no significant difference after corrections for multiple comparisons; DLB-D<Controls[a], DLB+D<Controls[b].

4.4.6 Biochemical analysis of synaptic and mitochondrial changes in the sgACC

Since changes were identified in both glutamatergic and GABAergic synapses using confocal microscopy, overall levels of synaptic marker proteins, known to be involved in synaptic function were determined in sgACC. SNAP25 protein level was significantly different between the groups ($F(2, 34)=18.214$, $p<0.001$), with a significant decrease observed in DLB cases with depression ($p<0.001$) and also DLB cases without depression ($p=0.010$) compared to controls. SNAP25 was also significantly reduced in DLB cases with depression compared to DLB cases without depression ($p=0.021$; Figure 4.14 A). Synaptophysin levels in the sgACC were significantly different between the groups ($F(2, 34)=4.762$, $p=0.015$), with a significant decrease observed in DLB cases with depression compared to controls ($p=0.018$), whereas a trend towards significance was observed in DLB cases without depression compared to controls ($p=0.081$; Figure 4.14 B). No significant changes were observed in Synaptogamin I ($F(2, 34)=3.217$, $p=0.053$), Syntaxin Ia ($H(2)=2.190$, $p=0.335$) or Synapsin IIa ($F(2, 34)=1.229$, $p=0.306$) between the groups (Figure 4.14 C-E). GAP-43, a synapse/growth cone

marker, was also significantly different between the groups ($F(2, 34)=9.868$, $p<0.001$), with a significant reduction observed in DLB cases with depression ($p<0.001$) and also DLB without depression compared to controls ($p=0.024$; Figure 4.14 F).

Synaptic activity is a highly energy dependent process and intimately linked to functional metabolism, therefore, select mitochondrial markers as indicators of energy metabolism associated with synaptic activity were determined in sgACC. No significant change was observed in NDUFB8, a marker of Complex I ($F(2, 34)=2.008$, $p=0.150$; Figure 4.15 A) or in Ubiquinol-Cytochrome C Reductase Core Protein I expression, a marker of Complex III, in sgACC between the groups ($F(2, 34)=2.740$, $p=0.079$; Figure 4.15 B). MTCO2, a complex IV cytochrome oxidase subunit marker, determined in sgACC was significantly different between the groups ($F(2, 34)=15.446$, $p<0.001$), with a significant decrease observed in DLB cases with depression ($p<0.001$) and also DLB cases without depression compared to controls ($p<0.001$; Figure 4.15 C). VDAC1/Porin, a mitochondrial mass marker, was significantly different between the groups ($F(2, 34)=9.248$, $p=0.001$), with significant reduction observed in DLB cases with depression ($p=0.001$) and in DLB without depression ($p=0.020$) compared to controls (Figure 4.15 D).

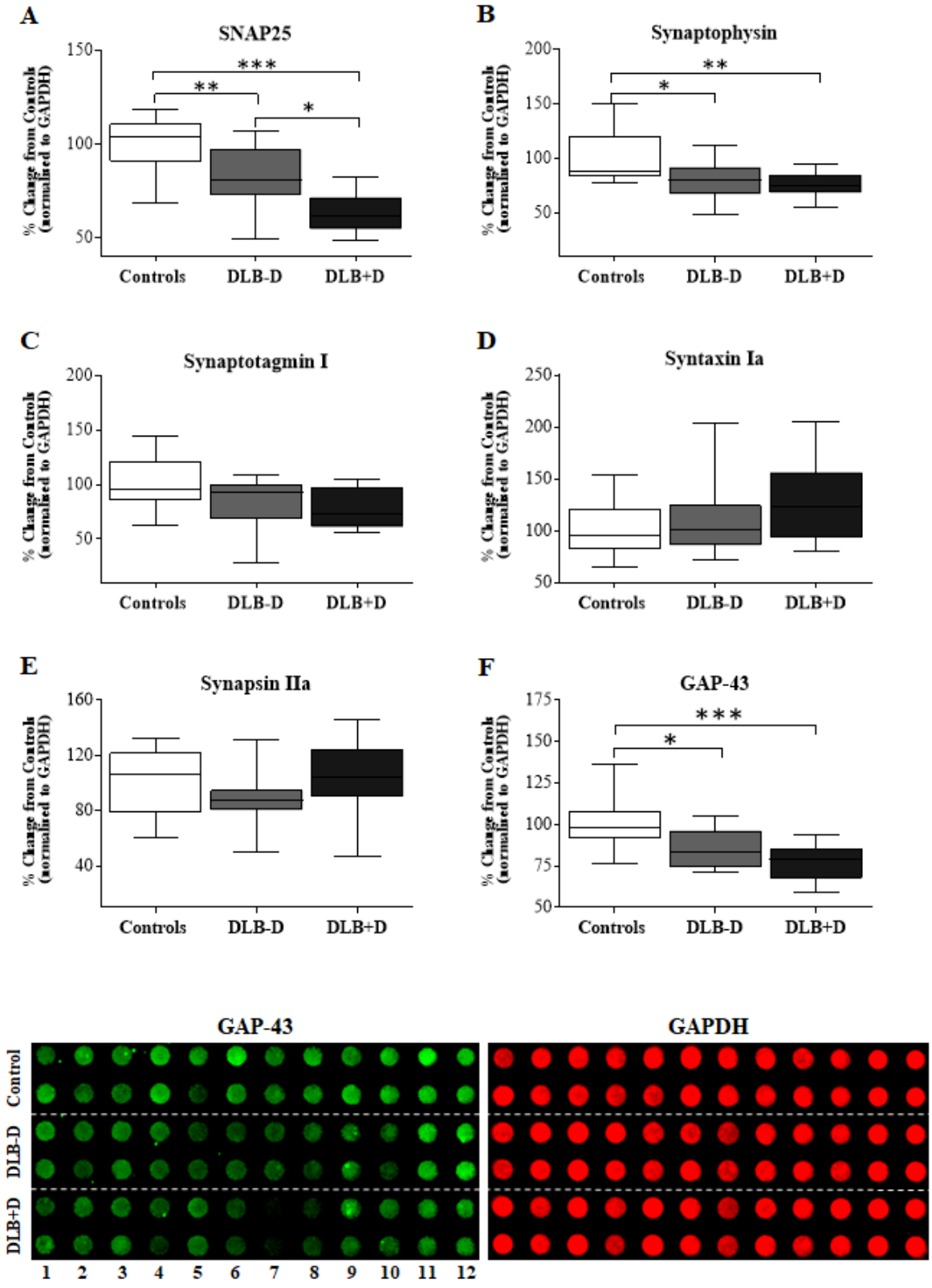

Figure 4.14 Dot blot analysis of synaptic markers.
Synaptic changes were assessed in sgACC in age matched controls, DLB cases with (DLB+D) and without (DLB-D) depression, using NIR based dot blots for SNAP25 (**A**), Synaptophysin (**B**), Synaptotagmin I (**C**), Syntaxin Ia (**D**), Synapsin IIa (**E**) and GAP-43 (**F**) markers; (* $p<0.05$ and *** $p<0.001$ compared to appropriate group). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

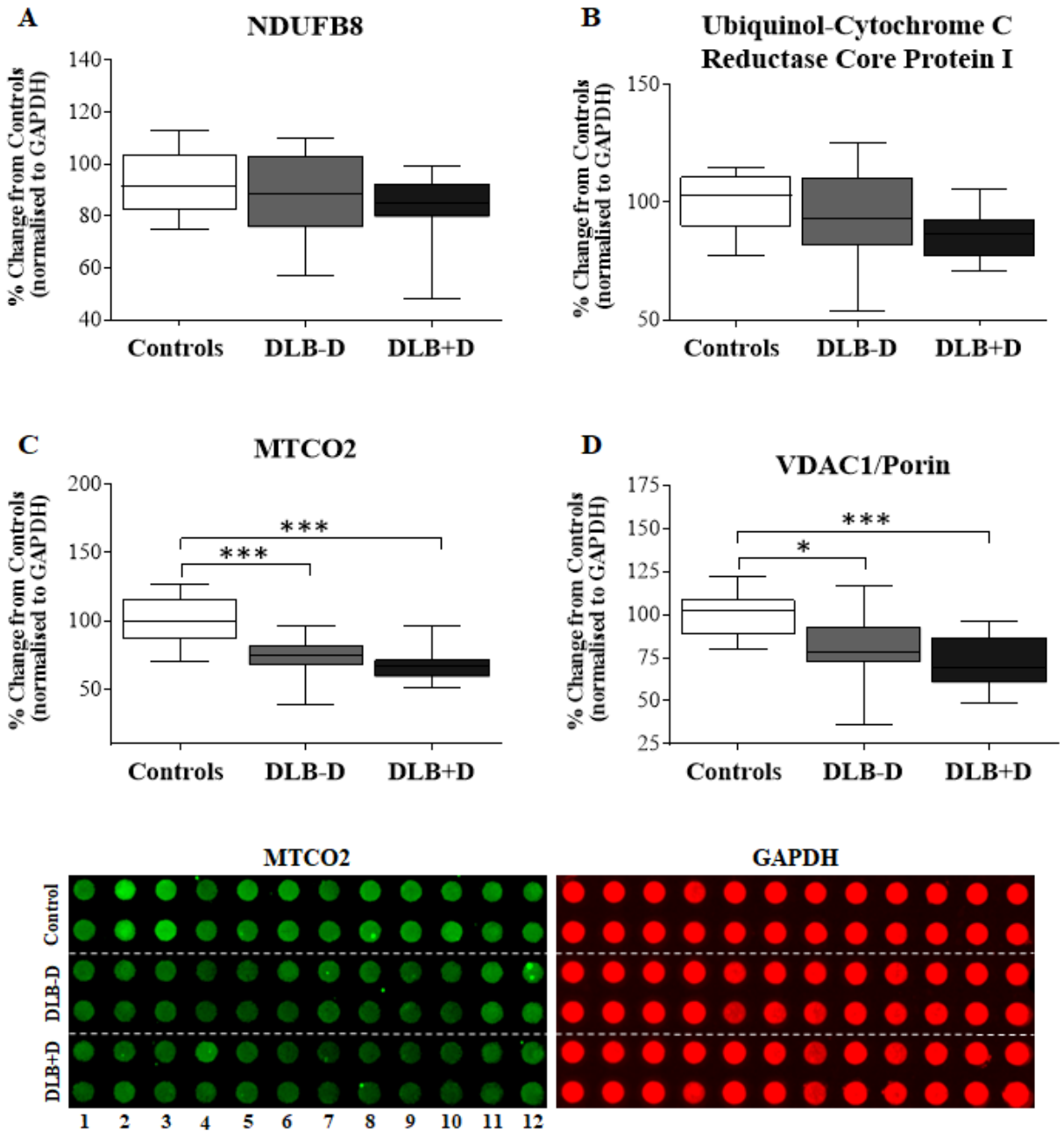

Figure 4.15 Dot blot analysis of mitochondrial markers.
Mitochondrial protein levels in sgACC were assessed in age matched controls, DLB cases with (DLB+D) and without (DLB-D) depression, using complex I (NDUFB8; **A**), complex III (Ubiquinol-Cytochrome C Reductase Core Protein I; **B**), complex IV marker (MTCO2; **C**), and general mitochondrial marker (VDAC1/Porin; **D**); (*p<0.05 and ***p<0.001 compared to control). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

4.5 Discussion

Abnormalities of glutamatergic and GABAergic neurotransmission in the sgACC in MDD are well established (Tripp et al., 2012, Tripp et al., 2011, Ongur et al., 1998), but it is unclear how these changes relate to depression in DLB. This study investigatedglutamatergic and GABAergic changes in the sgACC in relation to depression in DLB. The present findings suggest that an imbalance in excitatory and inhibitory neurotransmission may contribute to the development of depression in DLB. Furthermore, metabolic, neurotransmitter receptor, and synaptic protein changes in the sgACC may contribute to synaptic dysfunction in DLB, possibly due to accumulation of aggregated α-synuclein within presynaptic terminals.

Imaging studies in MDD have provided consistent evidence of a reduced volume of cortical and limbic regions involved in the regulation of emotions, mood and cognition (Hercher et al., 2009, Price and Drevets, 2010), with structural, functional and metabolic abnormalities observed particularly in the sgACC (Li et al., 2014b, Coryell et al., 2005). At a cellular level, these imaging changes are consistent with the finding of reduced neuronal soma size and increased neuronal density in layer V of the ACC in MDD (Chana et al., 2003, Cotter et al., 2001), although other studies have found no such changes (Ongur et al., 1998, Hercher et al., 2009, Bouras et al., 2001). A reduction in the size of pyramidal neurones has also been observed in the PFC in MDD (Rajkowska et al., 1999), with altered dendritic branching in the ACC in MDD (Hercher et al., 2010). PFC superficial layer II/III pyramidal neurones have been shown to be critical for moderation of stress in the context of depressive behaviours, since deletion of wolframin (the protein encoded by WFS1 gene), which is exclusively expressed in superficial layer pyramidal cells causes depression-like behaviour in mice (Shrestha et al., 2015). In this current study, no pyramidal cell loss was observed overall or in layer V in the sgACC in DLB. A small but significant reduction in pyramidal cell numbers was however observed in layers II/III in DLB cases without depression compared to controls. These findings, although superficially similar to those seen in MDD, where reduced cell volume was seen (Rajkowska et al., 1999), differ in that significant loss of layer II/III pyramidal neurones was observed in DLB cases without depression. Whilst it is not clear why this would be the case, it is tempting to speculate that in cases with more severe neurodegenerative pathology, there is reduced ability to demonstrate depressive behaviours or that these are more difficult to communicate. Indeed, in this case series, higher (although not significantly) levels of AT8 (phospho-tau) reactivity are seen in non-depressed DLB donors in the sgACC (see chapter 3).

As a correlate of pyramidal neurone density, a blotting approach was used to determine the protein levels of μ-crystallin (CRYM) (Figure 4.6), a pyramidal cell marker, and this was significantly reduced in DLB cases overall compared to controls and was slightly more severe in DLB cases with depression. CRYM depletion is suggested to make neurones more vulnerable to mutant huntingtin protein (Francelle et al., 2015), and it is possible that loss of CRYM in DLB patients may make neurones more vulnerable to cellular stress. Therefore, while mild pyramidal cell loss was observed in DLB without depression, molecular changes may suggest *functional* pyramidal cell abnormalities. This is also seen with other glutamatergic pyramidal neurone markers including the vesicular glutamate transporter isoforms VGLUT1 and VGLUT2 that mediate glutamate uptake into synaptic vesicles of excitatory neurones, and are differentially expressed in the brain, with VGLUT1 more abundantly expressed in the cortex (Fremeau et al., 2001). In this study, as with CRYM expression, a significant reduction in VGLUT1 was observed in the sgACC in DLB cases with depression compared to controls, similar to the findings in PD with reduced VGLUT1 expression (Kashani et al., 2007). VGLUT levels have been shown to be critical in determining the balance between excitation and inhibition (Erickson et al., 2006), and have been shown to be implicated in mood disorders, with reduced VLGUT1 expression in the hippocampus observed in animal models of depression (Tordera et al., 2007, Zink et al., 2010). In PD, an increase in VGLUT1 and VGLUT2 expression has been shown in putamen, whereas a decrease in VGLUT1 has been seen in the temporal cortex and PFC, suggesting the existence of profound alterations of glutamatergic transmission in PD (Kashani et al., 2007). The findings of the current study may suggest a more pronounced glutamatergic deficits in DLB cases with depression, possibly contributing to a greater imbalance in excitation/inhibition (E/I).

To further explore a potential change in excitatory neurone activity in DLB, PSD-95 and Neuroligin-2 levels were assessed in sgACC, which provide a possible measure of the E/I balance. Postsynaptic density 95 (PSD-95) protein, is a major glutamatergic receptor scaffold protein present at excitatory synapses, and has been shown to be elevated in the lateral amygdala of MDD patients (Karolewicz et al., 2009). This may be a specific change in MDD since other studies have shown no changes in PSD-95 levels in the striatum (Kristiansen and Meador-Woodruff, 2005), hippocampus and orbitofrontal cortex in depressed patients (Toro and Deakin, 2005). In DLB, disease, but not depression specific changes were observed in PSD-95 levels in sgACC, which may suggest that PSD-95 involvement in depression in DLB,

as with MDD (Karolewicz et al., 2009), may be limited to specific brain regions involved with emotional processing, such as the amygdala. PSD-95 has been shown to be involved in modulating Neuroligin-2 localisation at the synapse, and regulation of the E/I ratio (Levinson and El-Husseini, 2005). An increase in the excitation/inhibition synaptic current was observed in neurones overexpressing PSD-95, whereas knockdown of PSD-95 reduced the E/I ratio (Prange et al., 2004), therefore, PSD95 may directly affect E/I balance and synaptic strength. The PSD95/Neuroligin-2 ratio was significantly reduced in DLB in this study, although again disease, but not depression changes were observed. This change in the E/I ratio would appear to favour abnormal inhibition in DLB. Pre- and postsynaptic proteins involved in anchoring receptors and adhesion molecules are important in maintaining the E/I balance, which is disrupted in neuropsychiatric disorders (Yizhar et al., 2011). Abnormalities in Neuroligin-2 expression have been shown to be one of the factors contributing to chronic changes in the E/I balance in PFC and behavioural impairments in neuropsychiatric patients (Liang et al., 2015). Neuroligin-2 is a cell adhesion protein on the postsynaptic membrane, that interacts with β-neurexins located presynaptically, mediating the formation of synapses between neurones. Neuroligin-2 is primarily located at inhibitory synapses and is important in function of GABAergic synapses (Sudhof, 2008). Neuroligin-2 knock-out mice show increased anxiety-like behaviour (Blundell et al., 2009), with Neuroligin-2 overexpressing mice also showing anxiety-like behaviour, as well as enlarged synaptic contact size and reduction in the excitation/inhibition (E/I) ratio. In this study, elevated levels of Neuroligin-2 were found in DLB cases with and without depression in sgACC, whereas decreased Neuroligin-2 levels were observed in the NAcc in a mouse model of depression (Heshmati et al., 2018). This may suggest that brain regions involved in regulating emotional behaviour may be more susceptible to changes in Neuroligin-2 expression and that even subtle changes in Neuroligin-2 might lead to dysregulation of the balance between excitation and inhibition.

A decrease in GABAergic interneurone density has been reported in the DLPFC in patients with MDD (Rajkowska et al., 2007). In this study no overall changes in GABAergic cell density within sgACC were observed, although a significant reduction in GAD67 positive cells in layer V was observed in DLB cases with depression compared to controls. This may suggest reduced inhibition of layer V pyramidal neurones, followed by an increase or imbalance in excitation (Naka and Adesnik, 2016). GABA is synthesized by two isoforms of the enzyme glutamic acid decarboxylase (GAD), GAD65 and GAD67, and reductions in GAD67 have been shown to decrease inhibitory network activity in cortical regions (Lazarus et al., 2015). In this

study no significant changes in the protein levels of GAD65/67 were found between DLB cases and controls. Some studies have also found no changes in GAD65 or GAD67 cell numbers and mRNA levels in DLPFC in MDD (Sibille et al., 2011, Gilabert-Juan et al., 2012), whereas others found reduced GAD67 protein and gene expression in the DLPFC and sgACC in MDD (Karolewicz et al., 2010, Tripp et al., 2012). PV expressing GABAergic neurones constitute the largest group of layer V interneurones (Xu and Callaway, 2009), whereas CB expressing interneurones are primarily expressed in layer II (Moon et al., 2002). While a decrease in PV gene expression in the sgACC was found in post-mortem tissue of depressed patients (Tripp et al., 2012), other studies failed to observe any robust changes (Sibille et al., 2011, Beasley et al., 2002). Reduction in CB positive neurones have also been observed in layer II of the ACC in MDD (Woo et al., 2008, Cotter et al., 2002). In this study, reduced PV protein levels were observed in DLB cases with depression compared to controls, as well as reduced CB levels in DLB patients with depression compared to DLB cases without depression, therefore selective vulnerability in specific populations of interneurones may play a role in development of depression in DLB.

Alpha-synuclein

Unlike in AD, DLB patients show no major atrophy in the brain (Burton et al., 2002), therefore dysfunction of synapses may play a role in the pathophysiological mechanism of the disease. Synaptic abnormalities in DLB have been suggested to precede neuronal loss and LB formation (Chung et al., 2009, Calo et al., 2016), and are closely related to clinical symptoms (Bereczki et al., 2016). The aggregation of α-synuclein at synapses is thought to be a major factor in the pathogenesis of DLB (Calo et al., 2016). Recent evidence suggests a close relationship between differential expression profiles of α-synuclein and selective vulnerability of certain neuronal populations (Taguchi et al., 2019, Li et al., 2002), with aggregation of α-synuclein thought to be dependent on its endogenous expression (Volpicelli-Daley et al., 2011b, Erskine et al., 2018).

Presynaptic α-synuclein aggregation in cortical brain regions in DLB cases was shown to correlate with a reduction of dendritic spines, possibly contributing to synaptic loss (Kramer and Schulz-Schaeffer, 2007). Phosphorylated α-synuclein has been detected in synaptic-enriched fractions in post-mortem DLB cases (Walker et al., 2013, Muntane et al., 2008), with synaptic α-synuclein induced toxicity observed in animal models (Nemani et al., 2010, Garcia-Reitbock et al., 2010). Glutamatergic neurones are known to develop LB in DLB. The

inhibitory neurones in the cortex show low expression of endogenous α-synuclein in the mouse brain (Taguchi et al., 2019), with hippocampal GAD positive inhibitory neurones suggested to show an absence of LB aggregates (Taguchi et al., 2014). Cortical PV interneurones are also suggested to show sparing of LB pathology in DLB patients (Gomez-Tortosa et al., 2001). However, overlap of α-synuclein expression in GAD positive inhibitory synapses is observed within the globus pallidus, SN and olfactory bulb, despite the suggestion of α-synuclein not being present in interneurones of the cerebral cortex, hippocampus or thalamus (Taguchi et al., 2019). It is unclear therefore if only specific GABAergic neurones develop α-synuclein pathology.

Synaptic α-synuclein and VGLUT1 in the current study appears to show similar patterns of expression within cortical regions and similarly, the majority of striatal α-synuclein is also localised to glutamatergic terminals, possibly derived from corticocortical and corticostriatal input (Emmanouilidou et al., 2016, Taguchi et al., 2019). There is potentially a differential expression and also differential susceptibility of specific neuronal populations to LB and α-synuclein pathology.

To address this issue of differential neuronal susceptibility, confocal microscopy was used to identify α-synuclein in neurochemically defined synapses. An increase in the average size of glutamatergic synapses was observed in DLB with or without depression compared to controls. Phosphorylated α-synuclein was observed in glutamatergic, but also GABAergic synapses. A significant increase in the number and size of glutamatergic synapses containing phosphorylated α-synuclein was found in DLB cases with depression compared to controls. No significant changes in the number or the average volume of GABAergic synapses were observed between the groups in sgACC. Similar to the findings with glutamatergic synapses, the size and number of GABAergic synapses containing α-synuclein was higher in the DLB group in general compared to controls, but showed no selective increase in relation to depression.

While phosphorylated α-synuclein was present in both GABAergic and glutamatergic synapses, the latter were more affected. Synaptic phosphorylated α-synuclein in the form of small aggregates was observed in DLB cases, which has previously been associated with structural changes in presynaptic terminals (Colom-Cadena et al., 2017). Around 20% of phosphorylated α-synuclein co-localized with presynaptic terminals in the cingulate cortex (Colom-Cadena et al., 2017), which is similar to the observations made in the current study,

with about 8% of GABAergic and 15% of glutamatergic synapses containing phosphorylated α-synuclein. The same study also observed larger presynaptic terminal volumes in the presence of phosphorylated α-synuclein (Colom-Cadena et al., 2017), again in line with the present results, although only enlargement of glutamatergic synapses in DLB was observed in the present study. Enlargement of VGLUT1 terminals has been observed in SNAP25 knock-in mutant mice bearing a SNAP25 S187A mutation, with synapses also demonstrating concomitant accumulation of α-synuclein and phosphorylated α-synuclein (Nakata et al., 2012). Other studies have shown relative preservation of presynaptic terminals, but found retraction of dendritic spines (Kramer and Schulz-Schaeffer, 2007, Schulz-Schaeffer, 2010), which may suggest synaptic dysfunction is induced by reduction of key neurotransmitters in DLB. While no synaptic loss in the sgACC was observed in this study, the enlarged glutamatergic and GABAergic synapses may indicate possible dysfunction and may be a result of a response involving the accumulation of pathological phosphorylated α-synuclein.

Further investigation of synaptic protein expression in the sgACC was carried out in DLB to determine if the presence of α-synuclein may have a detrimental effect on synaptic function. Alpha-synuclein is normally localised in the presynaptic terminals and directly interacts with SNARE complex proteins (Burre et al., 2010). SNAP25 and syntaxin proteins are integral in SNARE complex function. These SNARE protein complexes are expressed on the presynaptic membrane, and play a role in vesicle fusion and exocytosis (Zhang et al., 2002). Deletion of SNAP25 in the mouse results in profound dysfunction in calcium-mediated neurotransmitter exocytosis (Washbourne et al., 2002). SNARE complex dysfunction has also been shown to result in presynaptic accumulation of endogenous α-synuclein, possibly representing the initial pathological event in DLB, and eventually leading to cellular dysfunction and death (Nakata et al., 2012). Abnormalities in SNAP25 and syntaxin expression have also been shown to be involved in a wide range of neuropsychiatric disorders (Houenou et al., 2017, Nakamura et al., 2008). Using confocal analysis, no changes were found in the number and size of presynaptic terminals in sgACC, whereas overall SNAP25 protein level was significantly reduced in DLB cases with depression compared to DLB cases without depression and controls. Syntaxin Ia is also part of the SNARE complex, and has been shown to interact with SNAP25 facilitating exocytosis (Liang et al., 2013). In the present study, no changes in syntaxin Ia protein levels were observed between the groups. One study demonstrated that syntaxin Ib, but not syntaxin Ia is necessary for the regulation of synaptic vesicle exocytosis (Mishima et al., 2014), which may suggest functional differences in two isoforms. Synaptophysin, another presynaptic

protein that associates with the pre-synaptic SNARE complex to facilitate synaptic vesicle exocytosis (Hansen et al., 1998), is involved in regulating neurotransmitter release and improving efficiency of exocytosis (Gordon et al., 2016). A reduction in synaptophysin levelwas observed in DLB cases with depression compared to controls in the present study. These results suggest that both the machinery involved in synaptic vesicle exocytosis, and presynaptic proteins involved in efficient neurotransmitter release are altered, and this may play a role in the pathogenesis of depression in DLB, possibly due to the accumulation of α-synuclein pathology. Growth associated protein 43 (GAP43), which is predominantly expressed on presynaptic membranes and involved in axonal growth, although does not directly associate with the SNARE complex, was reduced in DLB. This finding may also suggest that presynaptic proteins that regulate function may be affected by α-synuclein.

Mitochondrial activity is intimately linked to synaptic function and is vital in neurotransmitter release given the high energy requirements to maintain vesicle exocytosis and recycling and membrane polarisation (Lee et al., 2018). Mitochondrial dysfunction has been observed in neuropsychiatric, as well as neurodegenerative disorders,therefore, the investigation of expression of mitochondrial markers in DLB was carried out in the current study, to determine if any changes may relate to synaptic function or neuropsychiatric symptoms. Reduced rates of mitochondrial respiration in MDD (Karabatsiakis et al., 2014), and reductions of respiratory chain complexes I, III and IV in cerebral cortex and cerebellum has been reported after chronic exposure to stress (Rezin et al., 2008). Mitochondrial enzyme activity within complex I-V in the frontal cortex shows significant reductions in DLB compared to controls (Garcia-Esparcia et al., 2017). Similarly, mRNA expression levels of mitochondrial complex V subunit are downregulated in DLB cases compared to controls (Garcia-Esparcia et al., 2017). Protein expression levels of several complex I subunits were shown to be significantly reduced in DLB compared to controls (Garcia-Esparcia et al., 2017), which was not observed in the present study based on NDUFB8 expression. Protein levels of complexes II-V have also been shown to be significantly decreased in DLB cases compared to controls (Garcia-Esparcia et al., 2017), however this is in contrast to the present results, which showed no change in the Complex III marker and only showed a decrease in the Complex IV marker MTCO2. The voltage dependent anion channel (VDAC), which is used as a marker of mitochondrial mass has been shown to be reduced in DLB cases compared to controls (Garcia-Esparcia et al., 2017), which is in agreement with present results, and may suggest a reduced numbers of mitochondria or reduced mitochondrial size. Regional deficits in mitochondrial function, particularly in the ACC, appear

to consistently differentiate patients with mood disorders from controls (Konarski et al., 2008). Since α-synuclein can interfere with mitochondrial function (Vicario et al., 2018), the presence of synaptic α-synuclein may be one factor driving alterations in mitochondrial function in the synapse. The current findings suggest certain mitochondrial deficits as a potential factor in metabolic changes in DLB, although it does not seem to influence depressive symptoms in DLB.

Glutamatergic and GABAergic Synapses

Changes in glutamate and GABA receptors have been observed in many psychiatric and neurodegenerative disorders. While downregulation of glutamatergic and GABAergic receptors is often observed in MDD, receptor deficits in DLB may be due to a loss of intrinsic neurones and synapses that express these receptors. Based on clinical and preclinical evidence, several drugs and compounds with high affinity for glutamatergic receptors have been identified as being effective in providing rapid relief of depressive symptoms. Ketamine, a non-competitive high affinity NMDA receptor antagonist has been shown to have clinical efficacy in antidepressant drug therapies by strengthening excitatory synapses in the brain regions involved in the regulation of mood and reward (Zanos et al., 2016, Li et al., 2010, Zarate et al., 2006). Negative side effects due to NMDA receptor inhibition however, pose limitations for the use of ketamine as an antidepressant. However, esketamine, an isomer of ketamine, has been approved for use in treatment resistant MDD (Jauhar and Morrison, 2019). Selective targeting of the NMDA receptor 2B subunit, by using the antagonists MK-0657 and CP-101,606, has also been shown to be effective in treating depression without serious side effects (Preskorn et al., 2008, Ibrahim et al., 2012). A significant reduction in NMDA NR2A and NR2B subunit expression was observed in the PFC of patients with MDD (Feyissa et al., 2009), with a decrease in NMDA, but not AMPA receptors also seen in patients with depression (Scarr et al., 2003). These findings are compatible with present results, showing reduced NMDA 2B subunit levels in the sgACC in DLB cases with depression compared to controls, suggesting the NMDA receptor may mediate some of the depressive symptoms found in DLB.

Metabotropic glutamate receptors (mGluR) mediate many intracellular metabolic processes, particularly within neurones. The mGluR5 receptor is a potential target for novel drug development in the treatment of MDD (Singh and Singh, 2011, Knopfel et al., 1995). mGluR5 knockout animals and also mGluR1 knockout animals show deficits in sensorimotor gating (Brody et al., 2003, Brody et al., 2004), which is impaired in some psychiatric disorders, and can be reversed by certain mood stabilizer agents, such as the sodium channel blocker

lamotrigine. GluR7 exhibits a wide distribution throughout the entire brain, with mGluR7 knockout mice showing anxiety and depression type behaviours (Cryan et al., 2003, Stachowicz et al., 2008). No disease or depression specific changes in the mGluR in sgACC were observed in this study, therefore this possibly suggests selective receptor changes, implicating dysfunction of glutamatergic ion-channel receptors in the pathophysiology of depression in DLB.

Postsynaptic ionotropic $GABA_A$ receptors are targets of many psychotropic agents, such as benzodiazepines, ethanol and barbiturates (Engin et al., 2018), whereas pre- and post-synaptic metabotropic $GABA_B$ receptors function as autoreceptors, inhibiting GABA release (Cryan and Kaupmann, 2005). Decreased expression of $GABA_A$ receptor α1, α3, α4, γ1, β2 and ρ1 subunit genes (Luscher et al., 2011, Klempan et al., 2009, Merali et al., 2004, Sequeira et al., 2007), and increased expression of α5, γ2, β3 and δ subunits is observed in MDD (Klempan et al., 2009, Merali et al., 2004, Sequeira et al., 2007). This may suggest distinct roles of $GABA_A$ receptor subunits in the aetiology of MDD. In this study, a significant reduction in $GABA_A$ α5 and $GABA_A$ α3 receptor subunits was seen in DLB cases with depression compared to controls.The evidence from preclinical, clinical or post-mortem studies show GABA receptors to be a promising target for MDD. Selective agonists or positive modulators of $GABA_A$α3 subunit such as TPA023 and eszopiclone, have been recently proposed as potential antidepressants (Atack, 2010, Atack, 2011, Vollenweider et al., 2011). Studies have shown that when co-administered with SSRIs, the preferential $GABA_A$ α3 positive modulator eszopiclone induced a faster onset of efficacy and greater treatment response of first line antidepressants (Fava et al., 2006, Fava et al., 2011). The $GABA_A$ α5 subunit has been shown to be upregulated in the cortex and hippocampus in human and rodent depression studies (Matsumoto et al., 2007, Xiong et al., 2018), with preclinical studies showing that both positive and negative allosteric modulators of $GABA_A$ α5 receptors produce rapid antidepressant-like effects (Xiong et al., 2018, Zanos et al., 2017). The $GABA_A$ α5 negative allosteric modulator L-655,708 can restore alterations in hedonic behaviours (Fischell et al., 2015) by increasing the expression of the GluA1 subunit of the AMPA receptor, suggesting that it produces an indirect potentiation of excitatory synapses. Another $GABA_A$ α5 negative allosteric modulator MRK-016 has been shown to reverse anhedonia in animal models by restoration of excitatory synaptic strength (Fischell et al., 2015). Similar to ketamine, MRK-016 induced a significant increase in coherent EEG γ frequency oscillations (Zanos et al., 2017). $GABA_A$ α5 expression is less widespread compared to the target of ketamine the NMDA receptor, being found predominantly in the

hippocampus and frontal cortex in mouse brain that may result in the reduced side effects seen with $GABA_A\alpha5$ modulation (Malherbe et al., 1990). Negative allosteric modulators of $GABA_A\alpha5$ also were shown to have cognition-enhancing actions (Rudolph and Knoflach, 2011), and may be a target for memory-enhancing drugs (Ballard et al., 2009). Similar antidepressant effects are observed after acute and chronic increases of $GABA_A$ α5 activity by a positive modulator, although this was exclusively restricted to female mice, suggesting sex-specific effects (Piantadosi et al., 2016). This may suggest that $GABA_A$ α5 positive modulators interact with steroid hormones to produce an antidepressant response (Maggi and Perez, 1986, Maguire and Mody, 2007). The $GABA_A$ α5 subunit is therefore one possible mediator of depression in DLB.

Gephyrin is a scaffolding protein, essential for $GABA_A$ receptor clustering at the post-synaptic sites (Tyagarajan and Fritschy, 2014). Abnormalities in gephyrin functions have been associated with many neuropsychiatric disorders (Lionel et al., 2013, Fritschy and Brunig, 2003). Lithium and valproic acid have been widely used in treatment of epilepsy and bipolar disorder, and show enhancement of gephyrin clustering (Wang et al., 2012, Andre et al., 2001). A global reduction of gephyrin expression has been observed in AD (Agarwal et al., 2008), which may mean a reduced neuronal inhibition and increased glutamatergic excitotoxicity, possibly contributing to synaptic degeneration and neuronal death. Downregulation of gephyrin levelwas observed in DLB cases with and without depression compared to controls, and this was more pronounced in depressed DLB cases. Gamma-aminobutyric acid receptor-associated protein (GABARAP) directly interacts with gephyrin (Kneussel et al., 2000), and is important in intracellular transport of $GABA_A$ receptors (Kittler et al., 2001). However, no reduction in GABARAP was observed in this study, suggesting that a downregulation of gephyrin may not directly result in the loss of other associated proteins at synapses.

Pharmacological treatments using SSRIs, as well as transcranial magnetic stimulation in treatment of depression have shown to restore plasma and cortical GABA levels (Karolewicz et al., 2010, Kucukibrahimoglu et al., 2009), and act directly as an allosteric modulators of $GABA_A$ receptors (Robinson et al., 2003). Direct interactions between the GABAergic and serotoninergic systems have been reported (Llado-Pelfort et al., 2012, Celada et al., 2001), with the majority of serotoninergic cells in the raphe nucleus expressing $GABA_B$ receptors, which control serotoninergic cell firing (Abellan et al., 2000, Serrats et al., 2003). Studies have shown PV positive cells to express serotoninergic receptors (mainly $5HT1_A$ and $5HT2_A$) (Santana et al., 2004), with 8-OH-DPAT, a $5HT1_A$ receptor agonist, preferentially targeting GABA

interneurones, which results in pyramidal neurone disinhibition and enhancement of cell firing (Llado-Pelfort et al., 2012). The inhibition of pyramidal neurones has been observed by activation of GABA interneurones through $5HT_3$ receptors (Puig et al., 2004), and compounds, that are high affinity $5HT_3$ receptor antagonists such as vortioxetine, have shown antidepressant efficacy in treating MDD (Thase et al., 2016, Artigas et al., 2018). This evidence that normalization of a GABA mediated imbalance in excitation and inhibition through the action of antidepressants, provides further support for the involvement of GABAergic dysfunction in the aetiology of MDD. While GABAergic dysfunction in MDD may reflect downregulation of specific receptors, GABAergic deficits in DLB may be due to a selective (e.g. PV) loss of intrinsic neurones and synapses that express these receptors. Therefore, selective targeting of GABA receptors might lead to more effective psychotherapeutics for mood disorders in which there is a high and unmet medical need.

To conclude, a reduction in postsynaptic proteins was observed depending on neurochemical composition, as well as selective loss in presynaptic proteins involved in SNARE complex facilitated exocytosis. This may indicate neuronal degeneration, although only mild and very selective neuronal loss in sgACC was observed in DLB cases with depression. Reduction in presynaptic proteins involved in regulation of neurotransmitter release and efficient exocytosis may indicate altered inputs to the sgACC, possibly due to degeneration or cell loss in the brainstem, amygdala and NAcc, which directly project to sgACC (Vogt et al., 1995, Vogt and Pandya, 1987). Patients with MDD show hyperexcitability in the sgACC (Greicius et al., 2007), which may suggest decreased inhibition and this correlates with the finding of changes in GABAergic and glutamatergic neurotransmission observed in this study, suggesting dysregulation in excitation and inhibition, contributing to the abnormal activity in sgACC in DLB. While the present findings did not show clear changes specific to DLB patients with depression, depressed patients show more pronounced dysfunction in GABAergic and glutamatergic neurotransmission, suggesting higher imbalance in excitation and inhibition compared to DLB cases without depression. There is therefore the potential to use selective targeting of glutamatergic and GABAergic receptors in the treatment of depression in DLB.

Chapter 5.

Monoaminergic changes in the sgACC

5.1 Introduction

The monoamine hypothesis of depression is well established, suggesting that changes primarily in serotonergic, but also noradrenergic and dopaminergic neurotransmission are the basis of depression (Stahl et al., 2003). There is considerable evidence in support of the hypothesis of abnormal serotonergic transmission in depression, with reduced concentrations of 5-HT in serum and reduced 5HIAA in CSF in patients with MDD (Bot et al., 2015, Asberg et al., 1976), along with decreased serotonin 5HT1A and 5HT2A binding in the ACC in depressed individuals (Wang et al., 2016, Baeken et al., 2012). Serotonergic neurotransmission is also impaired in DLB compared to AD patients with depression, with reduced 5-HT and metabolite concentrations in limbic regions (Vermeiren et al., 2015). However, reductions in levels of 5-HT as a basis of depression have been challenged by several studies, primarily based on low and delayed response to 5-HT based therapies (Lacasse and Leo, 2005, Andrews et al., 2015, Ruhe et al., 2007, Karg et al., 2011).

The noradrenergic system is involved in mediating stress responses and has been shown to be involved in the pathogenesis of depression (Goddard et al., 2010). Post-mortem studies in MDD have shown reduced noradrenaline transporter binding in the LC and other limbic regions, possibly due to a loss of noradrenergic innervation (Ordway and Klimek, 2001, Klimek et al., 1997). Elevated α2-adrenoceptor levels in platelets are seen in MDD patients (Kaneko et al., 1992, Garcia-Sevilla et al., 2004), with a decrease in α2-adrenoceptor density observed following antidepressant treatment with TCAs (Gurguis et al., 1999). The LC in DLB cases is severely affected by LB pathology (Jellinger, 2003a), which may also play a major role in the noradrenergic neuronal loss in the LC. Reduced noradrenergic innervation in ACC, amygdala and ventral striatum has been observed in PD patients with depression using [11C]-RTI-32 PET imaging (Remy et al., 2005), as well as in DLB cases (Szot et al., 2006). This associates with an increase in the mRNA for TH and sprouting of dendrites in remaining neurones, potentially as a result of the compensatory changes due to noradrenergic neuronal loss in the LC.

Dopamine plays a role both in reward and stress (Pignatelli and Bonci, 2015), with dysfunction of dopaminergic neurotransmission within the mesolimbic and mesocortical system contributing to anhedonia and loss of motivation in depressive disorders (Chaudhury et al., 2013, Ben Zion et al., 2006, Berridge and Kringelbach, 2008). A significant reduction in DAT binding using [^{123}I]-FP-CIT binding in the caudate and putamen was shown in anhedonic

depressed patients (Sarchiapone et al., 2006), as well as increased striatal D2 receptor binding in patients with MDD (D'Haenen and Bossuyt, 1994), which may suggest decreased dopamine turnover, as indicated by low HVA levels in the CSF in depressed patients (Reddy et al., 1992). The development of depressive symptoms in PD has been shown to be associated with mesolimbic dopaminergic pathway dysfunction, as demonstrated by reduced volume in VTA, cingulate and amygdala on MRI, as well as reduced [^{11}C]RTI-32 dopamine and noradrenaline transporter binding in several limbic regions in PD patients with depression (Remy et al., 2005), which may relate to the loss of dopamine projections from the VTA.

The sgACC is important in mood regulation, as well as in the symptomatic expression of depression, displaying structural, functional and metabolic abnormalities in depression and MDD (Drevets et al., 2008b, Rodriguez-Cano et al., 2014). However, little is known about how changes within monoaminergic neurotransmission in the sgACC might relate to development of depression in DLB, despite the high levels of α-synuclein pathology in the sgACC (see chapter 3).

5.2Aims

The aim of this study was therefore to investigate potential changes in monoaminergic neurotransmission in the sgACC in relation to depression in DLB.

5.2.1 Objectives

- To assess serotonergic and dopaminergic innervation in the sgACC using stereological analysis in post-mortem tissue from DLB cases with and without depression, and in normal controls.

- To assess whether phosphorylated α-synuclein (s129) induces changes in monoaminergic synapses in the sgACC in relation to depression in DLB using STED microscopy.

- To quantify protein levels of serotonergic, dopaminergic and noradrenergic markers and receptors in the sgACC using frozen post-mortem tissue samples from DLB cases with and without depression and in normal controls.

5.3 Methods

Cases with as short a post mortem interval as possible were selected from the total DLB and control population for STED and stereological analysis, including 11 DLB cases with depression, 12 DLB cases without depression and 11 neurologically and psychiatrically normal controls (see section 3.3; Table 3.1). The cohort for protein analysis consisted of 12 DLB cases with depression, 12 DLB cases without depression and 12 normal controls with similar post mortem interval (see section 3.3; Table 3.3).

Fixed tissue sections of the sgACC were used to estimate the number of serotonergic (5HTT) and dopaminergic (DAT) fibres between the groups using an adapted stereological method (see section 2.7.3). Noradrenergic fibres were not assessed as the available primary antibody did not provide cell or fibre labelling of the required specificity. Triple-colour STED microscopy was applied to assess DAT or 5HTT co-localisation with a presynaptic terminal marker (SNAP25) and phosphorylated α-synuclein (s129) in relation to depression in DLB (see sections 2.5.2, 2.6.3 and 2.7.4). Immunohistochemical findings were also complemented by biochemical assessment of neurotransmitters synthesizing enzymes and receptors involved in monoaminergic neurotransmission. A dot blot technique was employed using Near Infra-Red (NIR) detection for protein determination (see sections 2.8.4-2.8.6). The specific proteins were quantified using Image Studio Lite (see section 2.8.7) and normalised against GAPDH. All of the antibodies used in this study can be found in (Table 5.1), and showed bands of the correct molecular weight on western blotting.

Table 5.1 Antibodies used in monoaminergic study.

Antigen	Host/Ig	Antibody Dilution	Source	Product Code
5HT1A	pRb (IgG)	1:1000 (DB)	Abcam	ab227165
5HT2A	pRb (IgG)	1:100 (DB)	Abcam	ab66049
5HT3B	pRb (IgG)	1:500 (DB)	Abcam	ab39629
5HTT	mMs (IgG1)	1:1000 (DB), 1:500 (STED)	MAb Technologies	ST51-2
s129	pRb (IgG)	1:50 (STED)	Abcam	ab168381
D2DR	pRb (IgG)	1:500 (DB)	Abcam	ab130295
D3DR	mRb (IgG)	1:1000 (DB)	Abcam	ab155098
D4DR	mMs (IgG1)	1:500 (DB)	Santa Cruz	sc136169
Donkey Anti-Sheep 594	Dk (IgG)	1:50 (STED)	Life Technologies	A11016
DDC	mMs (IgG1)	1:500 (DB)	Atlas Antibodies	AMAb91089
DBH	pRb (IgG)	1:500 (DB)	Atlas Antibodies	HPA002130
DAT	mMs (IgG1)	1:250 (DB), 1:20 (STED)	Atlas Antibodies	sc32258
Goat Anti-Rabbit Atto 647N	Gt (IgG)	1:50 (STED)	Sigma-Aldrich	40839
Goat Anti-Mouse 532	Gt (IgG)	1:50 (STED)	Life Technologies	A11002
NET	mMs (IgG1)	1:500 (DB)	Atlas Antibodies	AMAb91116
SNAP25	pSh (IgG)	1:100 (STED)	LifeSpan BioSciences	LS-C94834
TPH-2	mMs (IgG1)	1:500 (DB)	Atlas Antibodies	AMAb91108
TH	pRb (IgG)	1:500 (DB)	Abcam	ab112

DB – dot blot; IHC – immunohistochemistry; m – monoclonal; p – polyclonal; Ms – mouse; Rb – rabbit, Gt – goat, Dk – donkey.

5.4 Results

5.4.1 Dopaminergic Projections

The sgACC receives dopaminergic afferent projections from the VTA (Berridge and Kringelbach, 2008), and therefore the assessment of the number of pigmented dopaminergic

neurones in the VTA, as well as DAT positive fibres in the sgACC between the three experimental groups was carried out in this study. Dopaminergic fibres compared to serotonergic were less abundant in the sgACC. DAT positive processes showed numerous "swellings" or varicosities (Figure 5.1 A). The number of DAT positive dopaminergic fibres in the sgACC was significantly different between the groups ($F(3, 43)=9.440$, $p<0.001$), with significantly lower fibre density observed in DLB cases with depression compared to controls ($p<0.001$; Figure 5.1 B). The number of neurones in the VTA was significantly different between the groups ($F(3, 81)=3.027$, $p=0.034$), with significantly lower neuronal counts observed in the DLB group overall compared to controls ($p=0.043$), with DLB cases without depression showing a trend towards significance ($p=0.067$; Figure 5.1 C). A weak negative correlation between the number of dopaminergic neurones in VTA and the number of dopaminergic fibres in sgACC was observed in DLB cases with depression ($r=-0.580$, $p=0.048$), whereas no correlations were observed in controls or DLB cases without depression (Figure 5.1 D).

The above gross structural findings were extended by assessment of dopaminergic synapses (DAT and SNAP25 positive) in relation to phosphorylated α-synuclein (s129) in the sgACC using super-resolution STED microscopy (Figure 5.2). Due to the high resolution analysis provided by STED microscopy, a stereological approach to image analysis was not possible. No significant differences were observed in the percentage of dopaminergic synapses ($p=0.582$), dopaminergic axonal synapses ($p=0.578$), or dopaminergic synapses containing s129 between the groups ($p=0.823$; Figure 5.3). Since α-synuclein is known to interact with synaptic vesicle proteins (Burre et al., 2010), the volume of s129 α-synuclein was correlated with volumes of SNAP25 within synapses, to determine if the presence of pathological α-synuclein affected synapses. A significant positive correlation between the volume (μm^3) of DAT and SNAP25 was observed in DLB cases with depression ($r=0.379$, $p=0.007$), but not in DLB cases without depression or controls (Figure 5.4). A significant positive correlation was also observed between the volume (μm^3) of s129 negative DAT and SNAP25 in DLB cases with depression ($r=0.216$, $p=0.021$), but not in DLB cases without depression or controls (Figure 5.5).

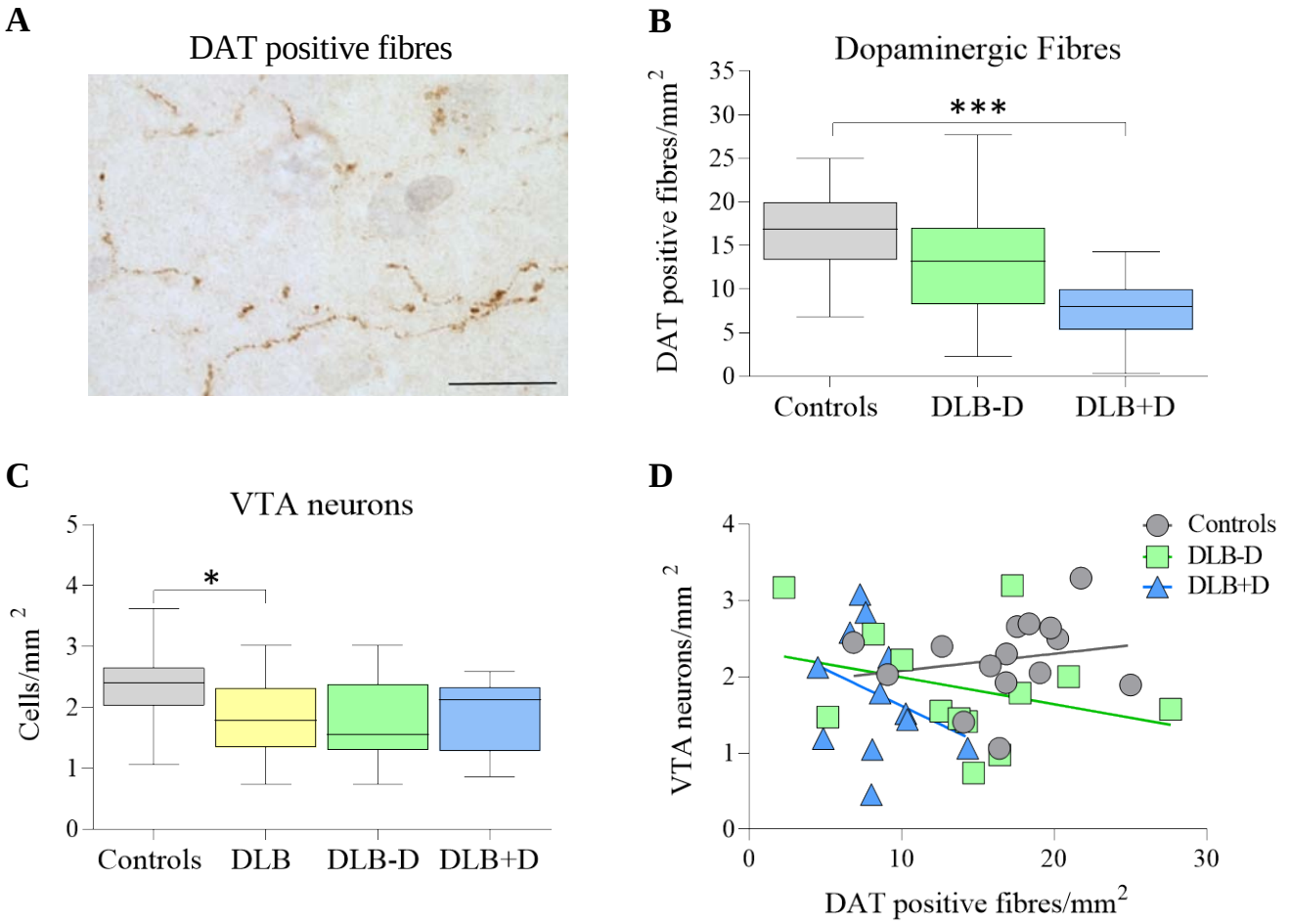

Figure 5.1 Stereological analysis of dopaminergic fibres and dopaminergic cells in sgACC.
(**A**) Dopaminergic fibres (DAT positive) in sgACC and (**B**) pigmented dopaminergic neurones in the VTA were assessed using stereological approaches in controls, DLB cases overall, DLB cases without (DLB-D) and DLB cases with depression (DLB+D) (Significant at * $p<0.05$ and ***$p<0.001$). (**C**) The relationship between neuronal number in the VTA and dopaminergic fibre density in the sgACC was assessed using Spearman's correlation and was significant for DLB+D. (**D**) A micrograph of dopaminergic fibres in sgACC (X63 magnification; the error bar represents 50μm). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

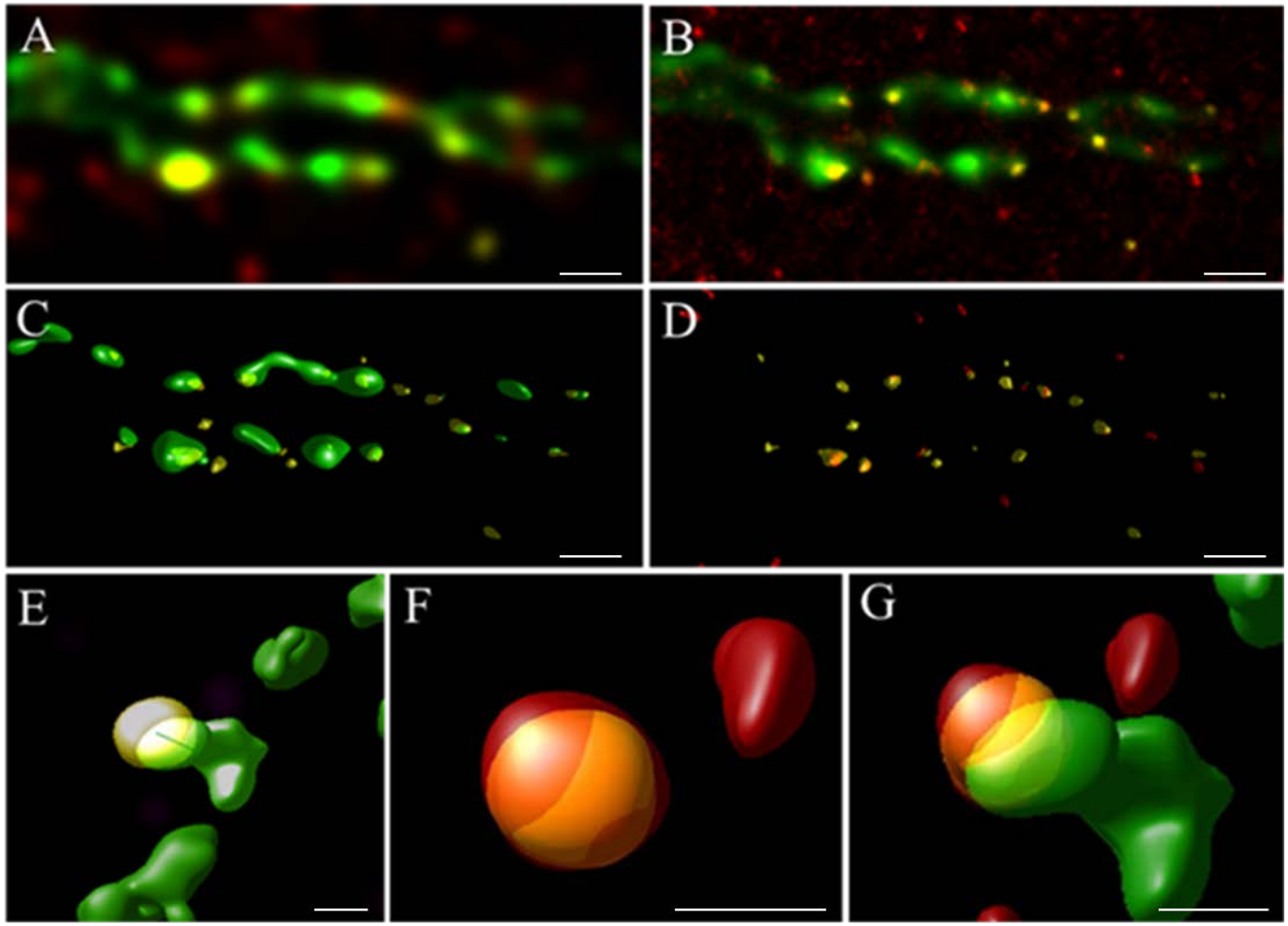

Figure 5.2 STED imaging of dopaminergic synapses.
A - STED 3D image of dopaminergic fibres (DAT; green), presynaptic terminals (SNAP25; yellow) and phosphorylated α-synuclein (s129; red); **B -** Deconvolved STED image; **C** and **E -** 3D surfaces of dopaminergic fibres and presynaptic terminals; **D** and **F -** 3D surfaces of presynaptic terminals and α-synuclein; **G -** 3D surfaces of co-localisation of all 3 channels. Scale bars represent (**A-D**) 0.5μm, (**E-G**) 0.1μm.

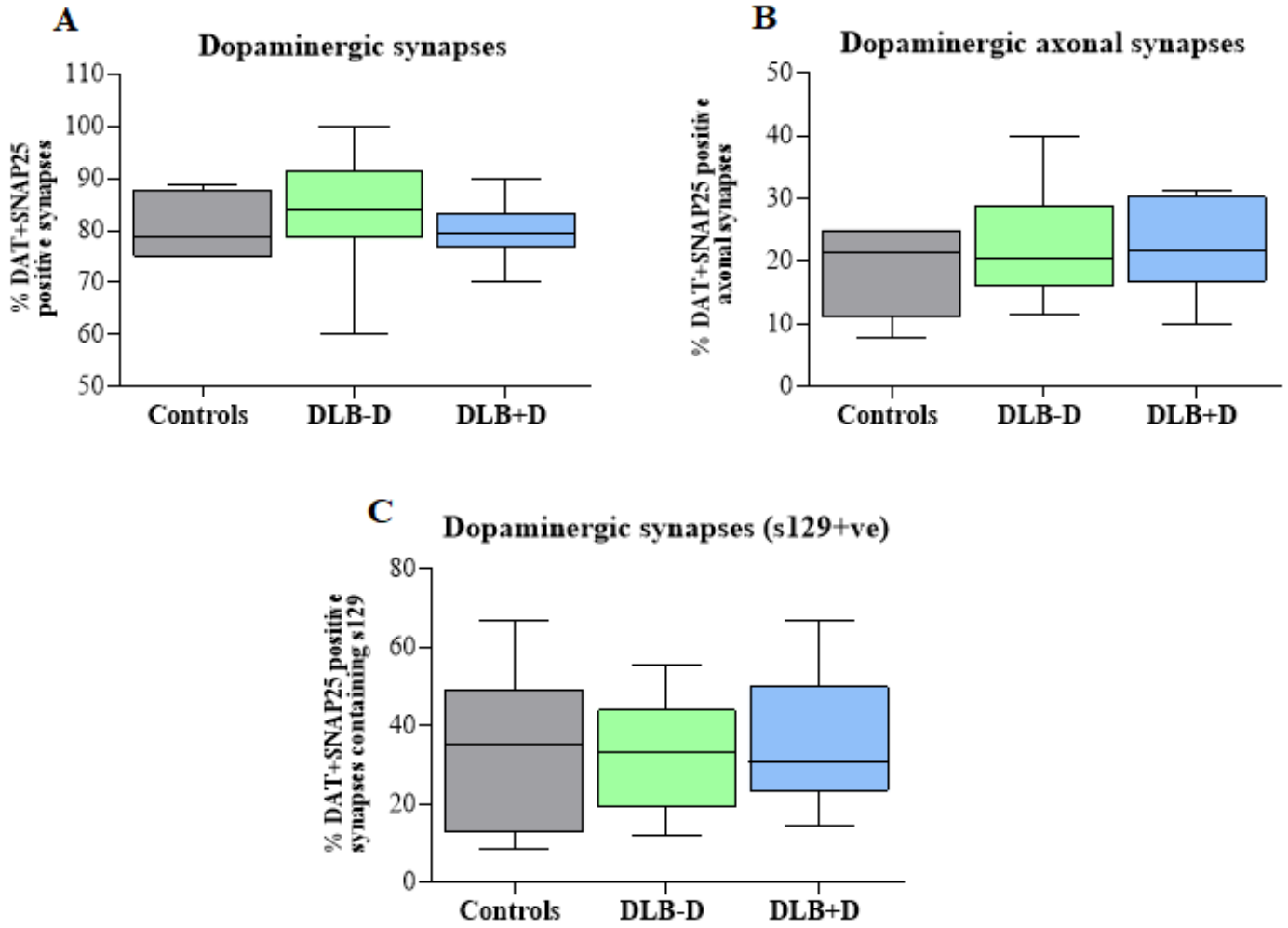

Figure 5.3 Analysis of dopaminergic synapses using Stimulated Emission Depletion (STED).
The percentage of dopaminergic synapses present at synaptic terminals, synapses adjacent to dopaminergic axons, and synapses containing s129 were assessed in the sgACC in age matched controls, DLB cases with (DLB+D) and without depression (DLB-D). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

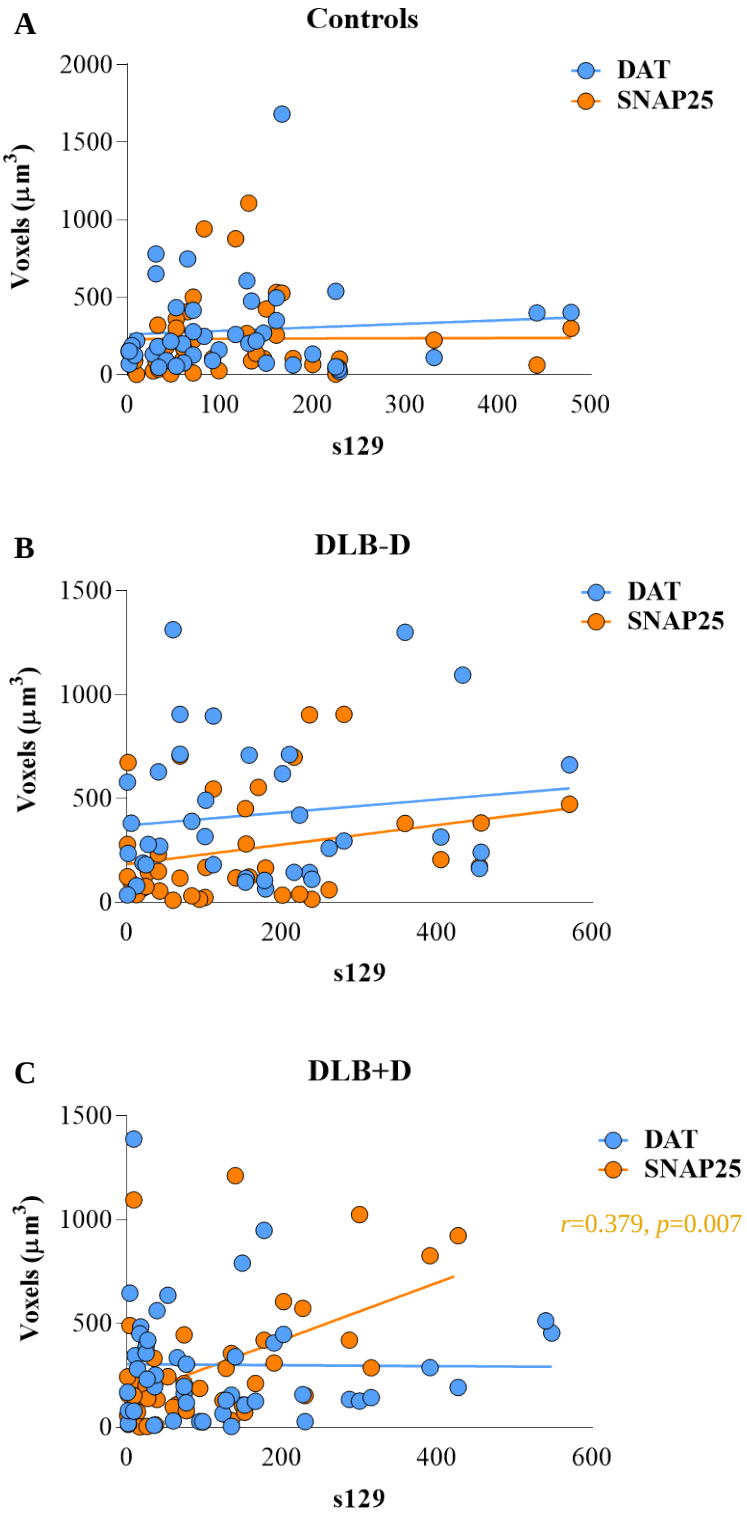

Figure 5.4 Dopaminergic Synapse Correlation Analysis.
The relationship between DAT and SNAP25 within synapses in the sgACC was assessed using STED microscopy data and Spearman's correlation analysis in controls, DLB cases with (DLB+D) and without (DLB-D) depression. Significant correlations were observed between volume of s129 α-synuclein and SNAP25 in DLB+D.

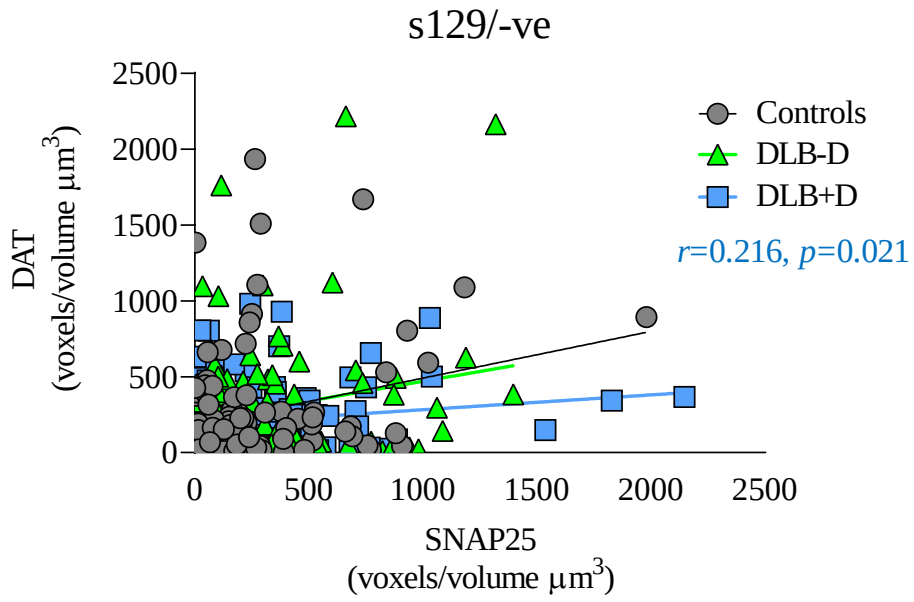

Figure 5.5 Effect of Phosphorylated Alpha-Synuclein (s129) Expression on Dopaminergic Synapses.
A relationship between s129 negative DAT and SNAP25 volumes (μm^3) within sgACC were assessed using Spearman's correlation analysis in controls, DLB cases with (DLB+D) and without (DLB-D) depression. A significant correlation was observed between synapse DAT volume and SNAP25 volume in s129 containing synapses in DLB+D cases.

To determine the impact of DLB on dopaminergic neurotransmission, a dot blot approach was used with validated antibodies against specific dopaminergic markers. TH ($F(2, 34)=10.493$, $p<0.001$), DAT ($F(2, 34)=6.409$, $p=0.005$) and DDC levels ($F(2, 34)=4.224$, $p=0.023$) were all significantly different between the groups in the sgACC. Significantly lower levels of TH ($p=0.001$), DAT ($p=0.003$) and DDC ($p=0.033$) were observed in DLB cases with depression compared to controls, as well as in DLB cases without depression compared to controls ($p=0.002$; Figure 5.6 A, C, E). No significant difference was observed in the levels of dopamine D2 receptors in the sgACC between the groups ($F(2, 34)=0.704$, $p=0.502$; Figure 5.6 B). Dopamine D3 receptors ($F(2, 34)=4.162$, $p=0.024$) and dopamine D4 receptors ($F(2, 34)=3.338$, $p=0.048$) were significantly different between the groups in the sgACC. A significantly lower dopamine D3 ($p=0.034$) and D4 receptor level ($p=0.043$) was observed in DLB cases with depression compared to controls (Figure 5.6 D, F).

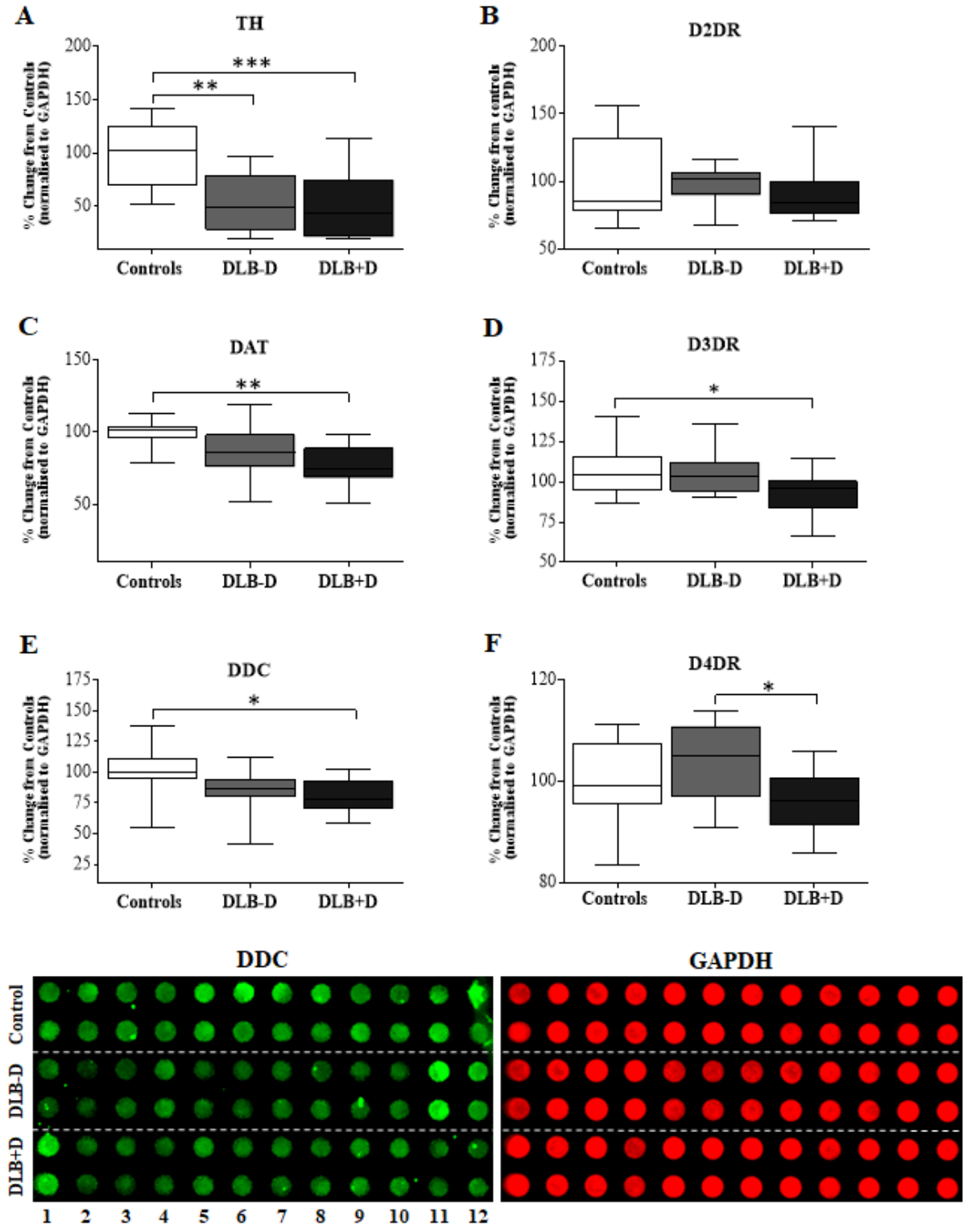

Figure 5.6 Dot blot analysis of dopaminergic markers and receptors.
Dopaminergic markers, including (**A**) tyrosine hydroxylase (TH), (**C**) dopamine transporter (DAT) and (**E**) dopamine decarboxylase (DDC), as well as dopamine D2, D3 and D4 (**B, C, F**) receptors were assessed in sgACC in age matched controls, DLB cases with (DLB+D) and without (DLB-D) depression. (*p<0.05, **p<0.05 and ***p<0.001, compared to appropriate group). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

5.4.2 Serotonergic Projections

Since serotonin neurotransmission abnormalities are thought to underpin much of the neurobiology of depression, serotonergic biomarkers were assessed in depression within DLB. The number of serotonergic fibres in the sgACC assessed using a stereological approach were not significantly different between the groups ($F(2, 39)=1.694$, $p=0.197$) (Figure 5.7). Using STED microscopy to analyse serotonergic markers, no significant difference in the percentage of presynaptic serotonergic terminals ($F(2, 22)=1.258$, $p=0.299$) or the proportion of serotonergic synapses containing α-synuclein was observed between the groups ($F(2, 22)=0.057$, $p=0.944$). A significant difference was observed in the proportion of synapses adjacent to serotonergic axons between the groups ($F(2, 22)=3.444$, $p=0.0465$), with significantly higher proportion of axonal synapses in DLB cases with depression compared to DLB cases without depression ($p=0.016$; Figure 5.8). Using STED data, a significant positive correlation was observed between the volume of s129 α-synuclein within presynaptic terminals in DLB cases without depression ($r=0.313$, $p=0.016$) and DLB cases with depression ($r=0.316$, $p=0.030$) (Figure 5.9). A significant positive correlation was also observed between the volume (μm^3) of s129 negative 5HTT and SNAP25 in DLB cases with depression ($r=0.285$, $p=0.020$), and DLB cases without depression ($r=0.307$, $p=0.028$) (Figure 5.10).

Using the dot blot approach in frozen post mortem tissue from the sgACC, no significant changes in 5HTT ($F(2, 34)=0.098$, $p=0.907$), TPH-2 ($F(2, 34)=2.149$, $p=0.133$), 5HT1A receptor ($F(2, 34)=0.375$, $p=0.690$) was observed between the groups (Figure 5.11 A-C). A significant difference in 5HT2A receptor protein levels was observed in the sgACC between groups ($F(2, 34)=5.093$, $p=0.012$), with significantly lower 5HT2A receptor protein levels observed in DLB cases with depression compared to controls ($p=0.017$; Figure 5.11 D). 5HT3B receptor level was significantly different between the groups ($F(2, 34)=3.838$, $p=0.032$), with significantly lower 5HT3B receptor levels observed in DLB cases with depression compared to without ($p=0.028$; Figure 5.11 E).

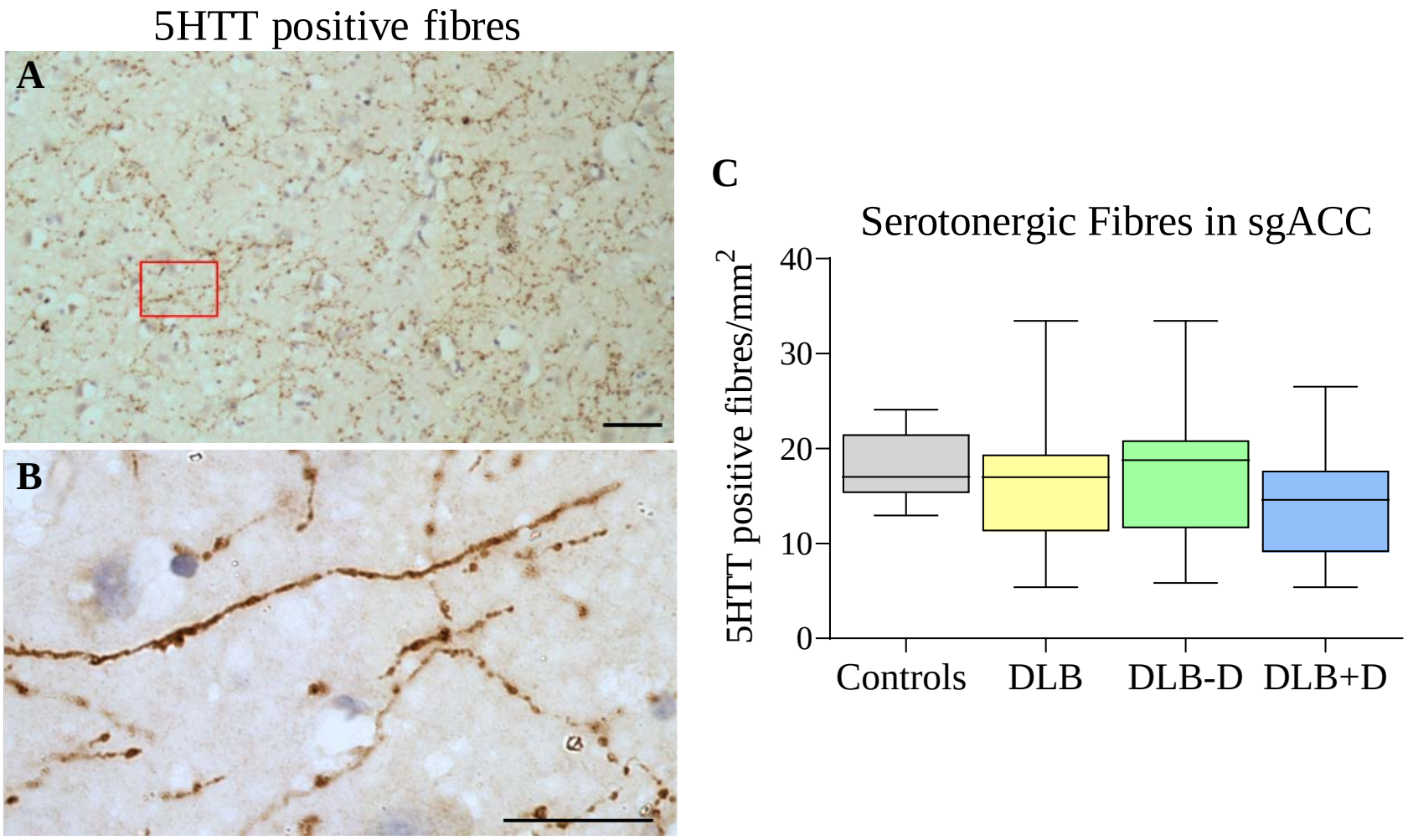

Figure 5.7 Stereological analysis of serotonergic fibres in sgACC.
Serotonergic fibres (5HTT positive; (**A**) – X10 magnification, error bar represents 100μm; (**B**) – X63 magnification, error bar represents 50μm) were assessed using a stereological approach in the sgACC in controls, DLB cases overall, cases without (DLB-D) and DLB cases with (DLB+D) depression (**C**). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

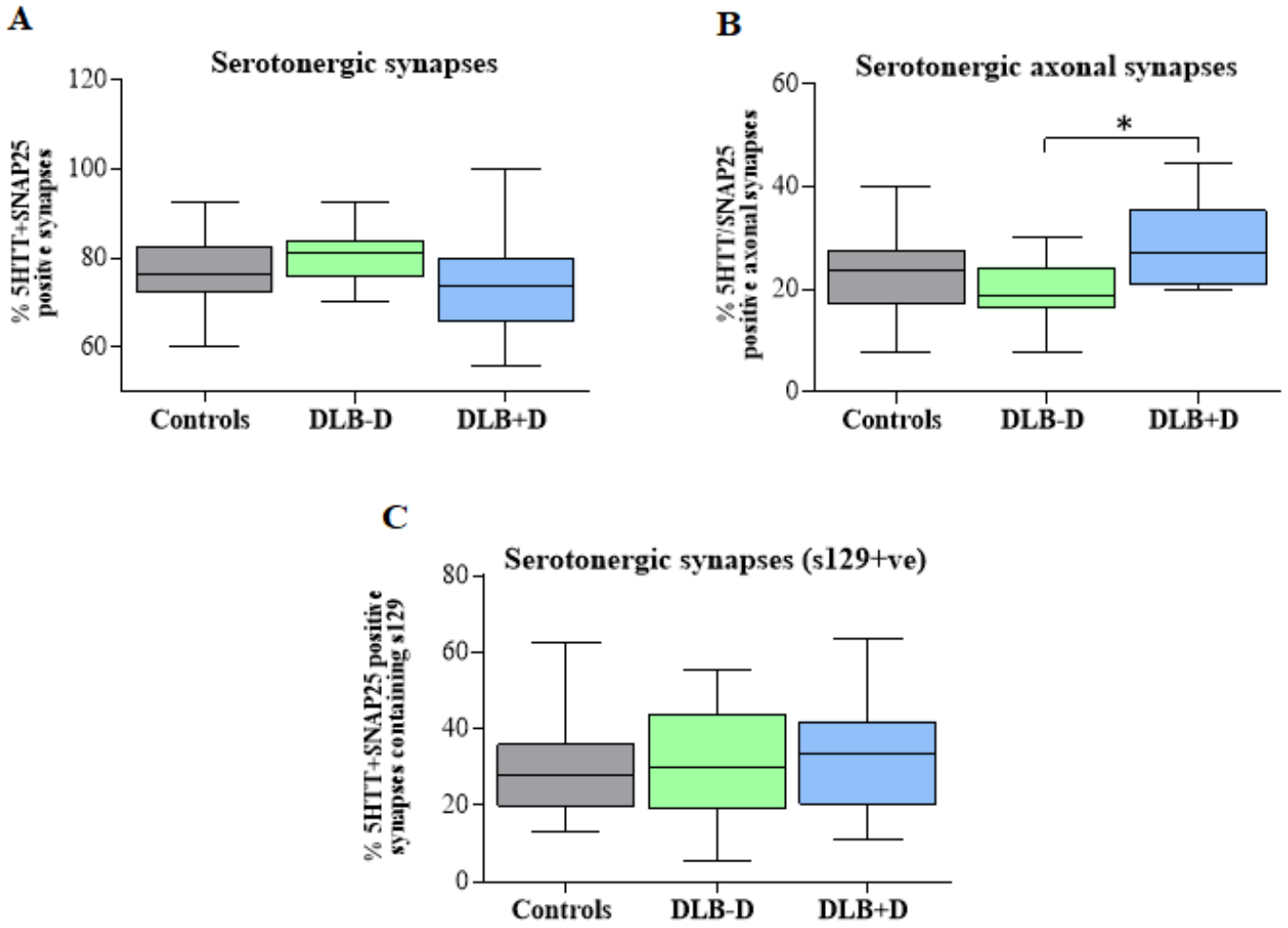

Figure 5.8 Analysis of serotonergic synapses using Stimulated Emission Depletion (STED).
The percentage of (**A**) serotonergic synapses present at synaptic terminals, (**B**) synapses adjacent to serotonergic axons, and (**C**) synapses containing s129 α-synuclein were assessed in the sgACC in age matched controls, DLB cases with (DLB+D) and without (DLB-D) depression. (*, Significant at $p<0.05$). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

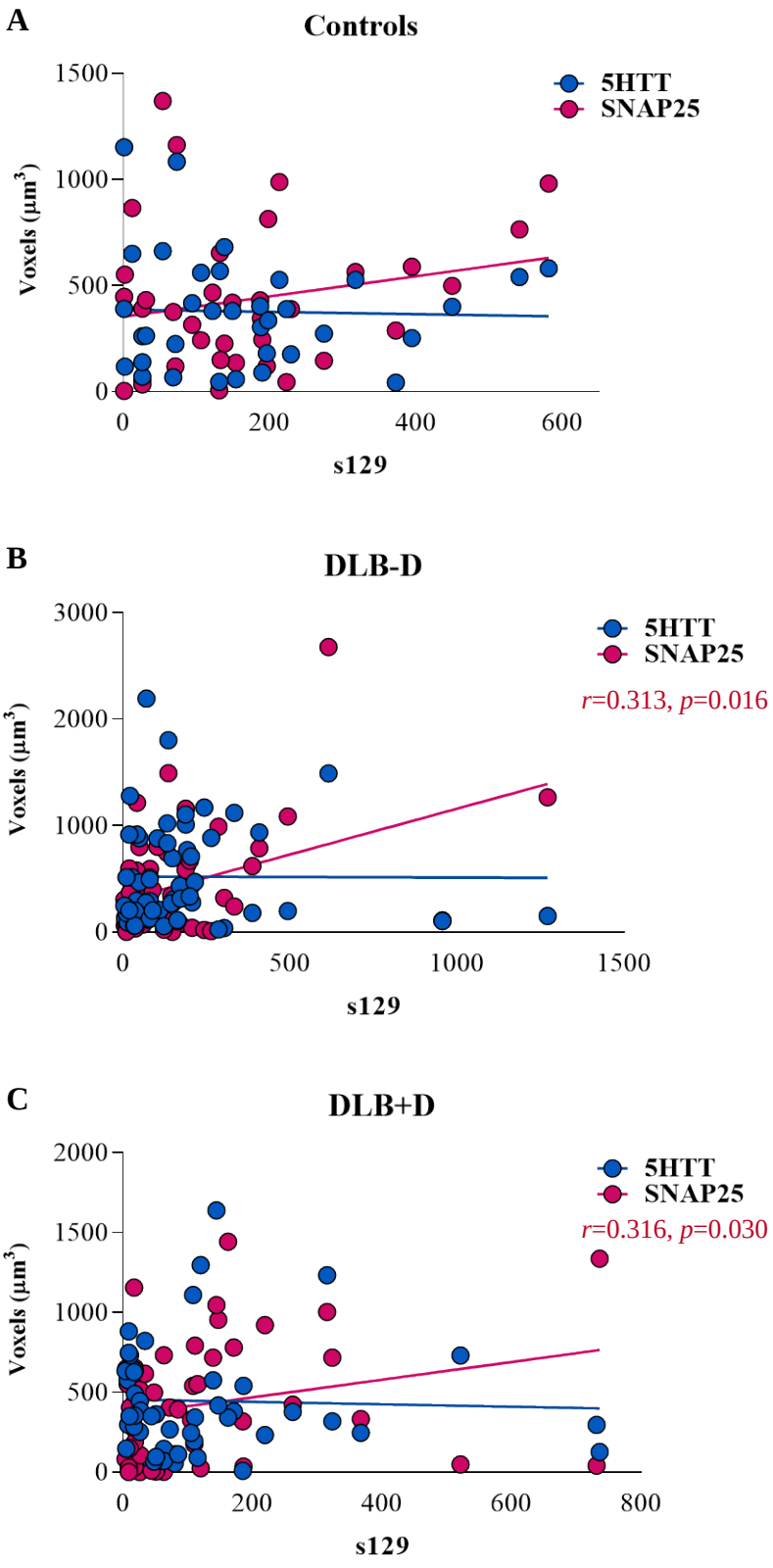

Figure 5.9 Effect of phosphorylated α-synuclein (s129) on Serotonergic Synapses.
The relationship between 5HTT and SNAP25 within synapses in the sgACC was assessed using STED microscopy data and Spearman's correlation analysis in controls, DLB cases with (DLB+D) and without (DLB-D) depression. Significant correlations were observed between volume of s129 α-synuclein and SNAP25 in DLB+D and DLB-D cases.

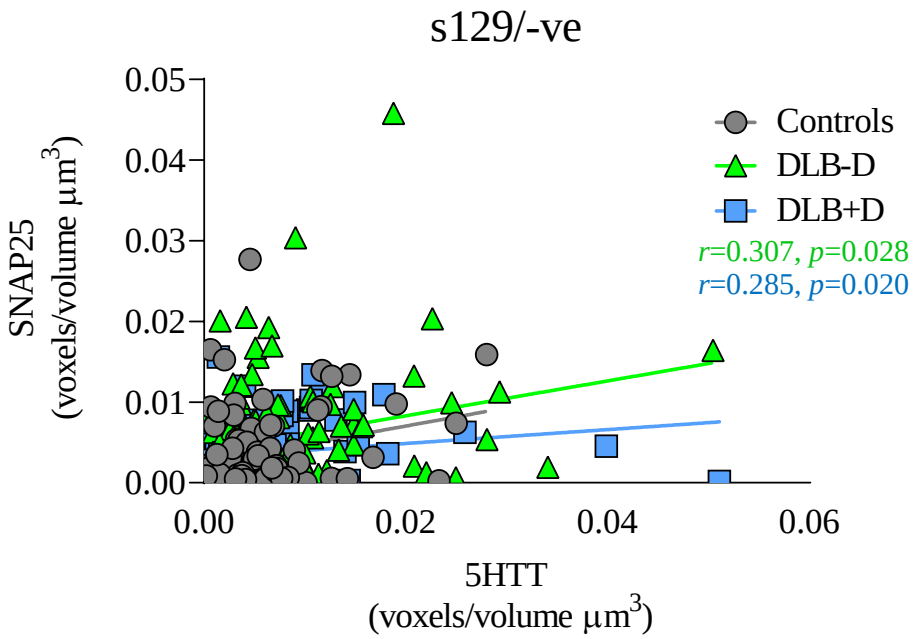

Figure 5.10 Correlation analysis of α-synuclein negative serotonergic synapses.
The relationship between s129 negative 5HTT and SNAP25 volumes (μm^3) within sgACC was assessed using Spearman's correlation analysis in controls, DLB cases with (DLB+D) and without (DLB-D) depression. Significant correlations were observerved between synapse 5HTT volume and SNAP25 volume in s129 containing synapses in DLB+D and DLB-D cases.

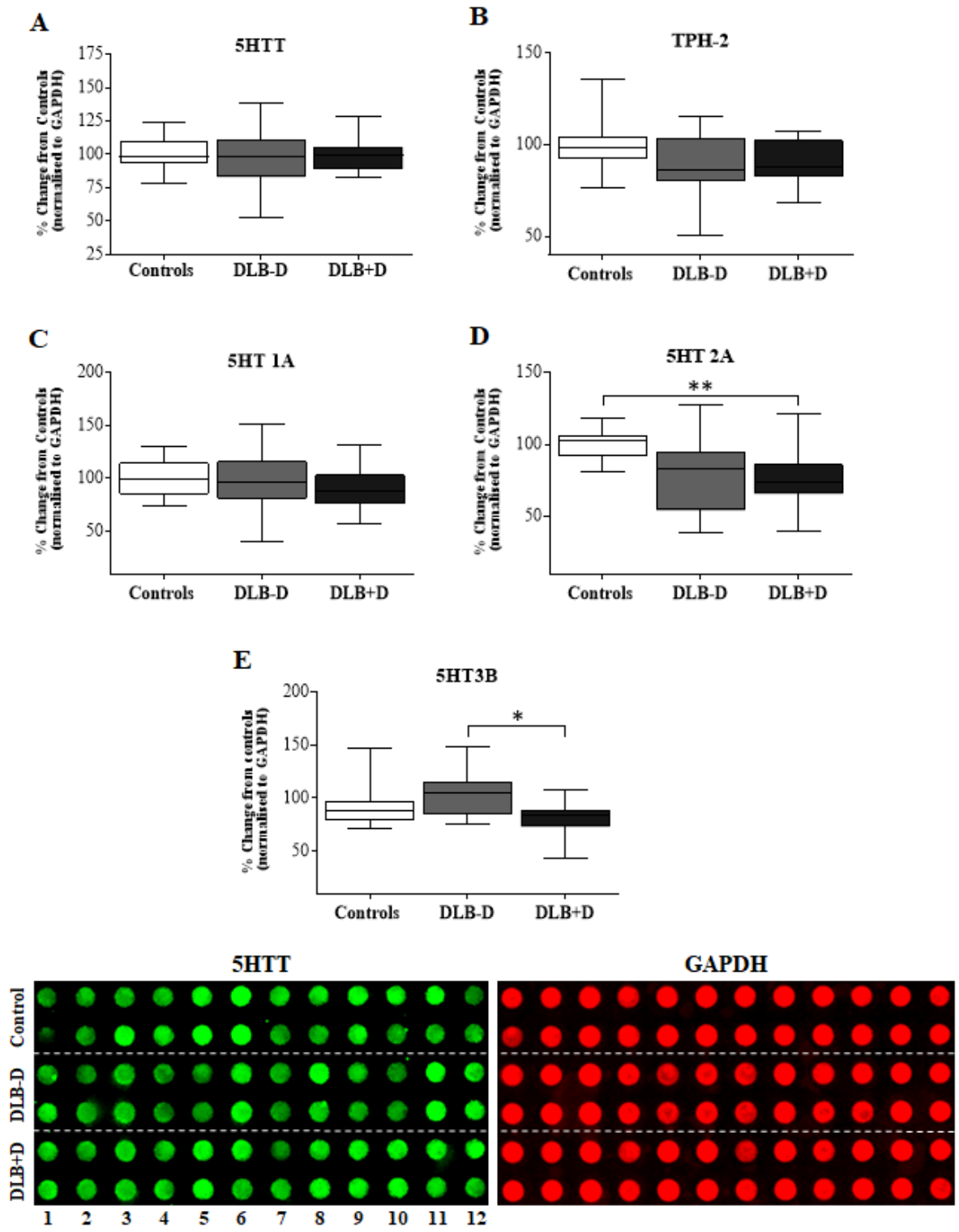

Figure 5.11 Analysis of serotonergic markers and receptors in the sgACC.
Serotonergic markers, including (**A**) serotonin transporter (5HTT), (**B**) tryptophan hydroxylase-2 (TPH-2), and serotonin receptors 5HT1A, 5HT2A and 5HT3B (**C-E**) were assessed using a dot blot approach with validated antibodies in post mortem frozen tissue from the sgACC of age matched controls, DLB cases with (DLB+D) and without (DLB-D) depression. (**p<0.01, compared to control group). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

5.4.3 Noradrenaline

Due to a lack of specific staining for NET, no immunohistochemical analysis of noradrenergic innervation into the sgACC was possible. Using dot blotting, no significant changes were observed in DBH levels between the groups in the sgACC ($F(2, 34)=2.642$, $p=0.087$). A significant difference in NET levels was observed between the groups ($F(2, 34)=7.842$, $p=0.002$), with significantly lower NET levels observed in DLB cases with depression ($p=0.008$) and DLB cases without depression compared to controls ($p=0.003$; Figure 5.12).

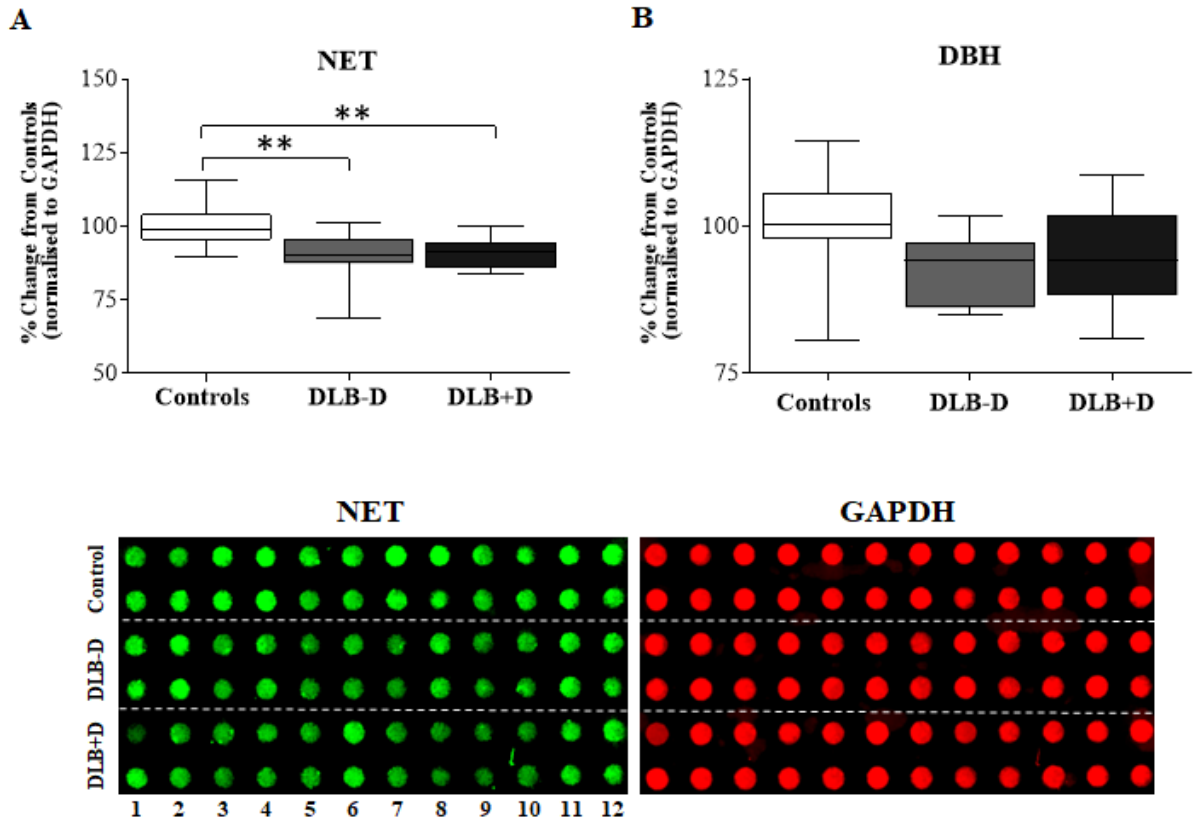

Figure 5.12 Analysis of noradrenergic markers in the sgACC.
Noradrenergic markers, including (**A**) noradrenaline transporter (NET) and (**B**) dopamine beta-hydroxylase (DBH) were assessed in sgACC in age matched controls, DLB cases with and without depression. (**$p<0.010$, compared to control group). Box plots represent the median (midline) and quartile ranges, with error bars representing the minimum and maximum values.

5.5 Discussion

Monoaminergic abnormalities have been a mainstay of the neurobiology of depression with altered serotonin neurotransmission being a major theory in the aetiology and treatment of

depression. The sgACC shows structural and functional abnormalities in depression (Drevets et al., 2008b, Rodriguez-Cano et al., 2014), as well as high LB pathological burden in DLB. Little is known however, about how changes within monoaminergic neurotransmission within the sgACC relate to development of depression in DLB. The present results suggest that primarily there is a reduced dopaminergic drive associated with depression in DLB, with reduced dopaminergic innervation in the sgACC in DLB cases with depression, along with reduced levels of dopaminergic markers and receptors, as well as changes in dopaminergic synaptic function. This contrasts with little change in serotonin markers in depression in DLB, although noradrenergic markers are reduced.

The mesolimbic and mesocortical dopamine pathway sends projections from the VTA to various cortical and subcortical regions including the ACC. Dopaminergic neurones in the VTA play a role in both reward and stress (Pignatelli and Bonci, 2015), with dysfunction of dopaminergic neurotransmission within the mesolimbic system contributing to anhedonia and loss of motivation in depressive disorders (Chaudhury et al., 2013). In this study, a subtle decrease in VTA dopaminergic neurone number was found in the DLB group overall compared to controls. This was not however significant when separated into DLB with and without depression, which may suggest that due to low numbers in each group there is insufficient statistical power to detect the difference. Compared to the SN, the VTA is suggested to show a relative preservation in terms of the cell loss in PD (Alberico et al., 2015, Gibb and Lees, 1988). This may be due to co-expression of calbindin on VTA dopaminergic neurones, which is suggested to be neuroprotective and may potentially explain why VTA neurones are resistant to α-synuclein (Pan and Ryan, 2012, Maingay et al., 2006), but also that a subgroup of calretinin expressing dopaminergic neurones are protected in both the VTA and SN in PD (Mouatt-Prigent et al., 1994). Studies have shown that counting SN neurones in PD using a single section provides reliable estimates of cell loss (Ross et al., 2004b, Ma et al., 1995). It is unclear if the similar counting approach of using a single section used in this study provides reliable estimates of VTA neuronal loss. Supporting present findings in DLB, some studies indicate loss of VTA neurones in PD (Torack and Morris, 1992, Seidel et al., 2015, Uhl et al., 1985), indicating that VTA neurones are not necessarily protected in Lewy body disorders, and that in DLB, VTA neurones are lost.

In the nonhuman primate the sgACC dopaminergic innervation gradient is denser within the superficial cortical layers (Williams and Goldman-Rakic, 1993), with DAT positive processes showing numerous "swellings" or varicosities similar to the findings of this study (see Figure

5.1). In this study, dopaminergic innervation in all cortical layers in the sgACC, and reduced dopaminergic fibre density was observed in sgACC in DLB cases with depression compared to controls, which may indicate reduced innervation from the VTA, corresponding with present finding of reduced VTA neurone numbers in DLB overall. In PD, the first motor symptoms appear when about 50% of SN dopamine neurones are lost (Ross et al., 2004a), whereas the extent of striatal damage at the time of the onset of motor symptoms is much more severe, showing about 80% of dopamine loss (Bernheimer et al., 1973). The axons and their terminals are suggested to be the main site of pathology, with the neurodegenerative process in PD thought to follow a retrograde pathway originating in the striatal terminals (Hornykiewicz, 1998). This may explain the present finding of significant dopaminergic fibre loss in DLB cases with depression in this study, without significant VTA neuronal loss in depressed DLB patients. These findings of reduced dopaminergic fibre density in DLB cases with depression are similar to the finding of decreased dopamine metabolites in cortical regions in PD (Scatton et al., 1982) and levels of dopamine (and noradrenaline) transporter determined using [^{11}C]-RTI-32 PET imaging in the ACC, that has been observed in depressed PD patients (Remy et al., 2005).

In this study,a decrease in dopaminergic neurotransmission markers was observed, almost selectively in DLB cases with depression compared to controls, including DAT, TH and DDC. This is in line with other studies suggesting a decrease in extracellular dopamine levels to be implicated in depression (Lambert et al., 2000), with some antidepressants shown to enhance neurotransmission in dopamine within the mesolimbic system (Maj et al., 1987). Reduced DAT binding in the striatum was observed in depressed patients with anhedonia (Sarchiapone et al., 2006), as well as in PD and DLB, with DLB cases showing more severe loss of DAT binding in caudate compared to PD (Walker et al., 2004).

STED based analysis showed the presence of α-synuclein within dopaminergic synapses in the sgACC (see Figure 5.3). In depressed DLB cases, the presence of α-synuclein showed a positive correlation with SNAP25, where elevated α-synuclein corresponded significantly with elevated SNAP25 (Figure 5.4), a finding also seen within serotonergic terminals (Figure 5.9). One possibility is that pathological α-synuclein causes sequestration of SNAP25 within synapses leading to synaptic dysfunction. Alpha-synuclein is a presynaptic protein that modulates synaptic activity through effects on vesicle recycling and release (Burre et al., 2010, Burre et al., 2014, Diao et al., 2013). Endogenous α-synuclein in particular, promotes synaptic vesicle generation by assisting with the formation of SNARE complexes (Chandra et al., 2005, Burre et al., 2014, Sun et al., 2019). Pathological fibrillar α-synuclein can however rapidly

promote aberrant synaptic activity following application to neurones (Wu et al., 2019, Volpicelli-Daley et al., 2011a). In forming macromolecular aggregates within synapses, fibrillar α-synuclein may lead to an effective depletion of the endogenous pool of functional SNARE proteins including SNAP25, reducing formation of a functional synaptic vesicle pool. Fibrillar and oligomeric α-syn also has direct effects on synaptic machinery with further depletion of SNARE complexes (Choi et al., 2013, Larson et al., 2017, Rockenstein et al., 2014). This may underscore the observed increase in SNAP25 seen within α-synuclein containing synapses, with SNAP25 increased, but non-functional due to being sequestered in α-synuclein aggregates (Choi et al., 2018).

Dopamine exerts its action by binding to dopaminergic receptors with dopamine D2 and D1 receptors typically expressed at high levels in the striatum (Ng et al., 1994, Bates et al., 1991). D1 receptors are highly expressed in the striatum, as well as in mesolimbic and mesocortical areas (Cortes et al., 1989). PET studies on D1 receptors in affective disorders are scarce, however, patients with MDD show lower D1 receptor binding in frontal cortex and striatum (Suhara et al., 1992, Dougherty et al., 2006). A novel allosteric potentiator of the dopamine D1 receptor (DETQ) was shown to amplify the effects of endogenous dopamine transmission improving when and where dopamine is released, showing promise in applications in treatment of PD and neuropsychiatric disorders (Bruns et al., 2018). The highest levels of D2 dopamine receptors are expressed in the striatum, NAcc and the olfactory tubercle, with significant levels in the SN, VTA, hypothalamus, amygdala, hippocampus and cortical areas. In PD, progressive loss of dopaminergic neurones in SN results in reduced dopamine levels in the striatum. An increase in D2 receptor density in the striatum has been observed in PD (Rinne et al., 1995), which may suggest an involvement of compensatory mechanism in response to low dopamine levels. Increased dopamine D2/D3 receptor binding and lower DAT activity has been shown in depression, possibly reflecting compensatory changes due to the impairment in mesolimbic dopaminergic neurotransmission (Klimek et al., 2002, Allard and Norlen, 2001).

Dopamine D3 receptors are expressed on postsynaptic and presynaptic dopaminergic neurones, and have the highest affinity for dopamine and dopamine agonists (Sokoloff et al., 2006). The dopamine D3 receptor is a primary target within the mesolimbic dopamine system, with highest level of expression observed in the limbic areas (Sokoloff et al., 1992). In the amygdala, dopamine D3 receptor expression is primarily observed on target glutamatergic neurones as a postsynaptic modulatory receptor (Li and Kuzhikandathil, 2012). The dopamine D3 receptor has been shown to be clinically relevant as a therapeutic target for depression. A number of

relatively non-specific agonists (aripiprazole, pramipexole and ropinirole) and selective antagonists (SB-277011-A) of the dopamine D3 receptor have been developed that have been shown to have antidepressant and anxiolytic effects in humans and animals (Tadori et al., 2011, Zarate et al., 2004, Rogoz et al., 2004, Leggio et al., 2013, Reavill et al., 2000). Downregulation of the expression of dopamine D3 receptors has been shown in MDD (Leggio et al., 2013). While no changes were identified in dopamine D3 receptor density in the striatum in a monkey model of PD following loss of dopaminergic innervation (Hurley et al., 1996), a reduction in dopamine D3 receptors, particularly in ventral striatum, has been observed in PD cases (Joyce, 1993). Furthermore, L-DOPA treatment has been shown to alter expression of dopamine D3 receptors in the striatum and SN of parkinsonian primates (Sanchez-Pernaute et al., 2007). Other studies have shown elevated dopamine D3 receptor binding in non-demented PD cases, but with reduced dopamine D3 receptor binding in PD cases with dementia (Joyce et al., 2001), which may suggest that dopamine D3 receptors are also involved in modulating cognitive function. The present results suggest that reduced dopamine D3 receptor levels may play a role in depression in DLB patients. Therefore, the dopamine D3 receptor could be target for the development of more specific dopamine based treatments for depression. Dopamine D3 receptor agonists have been used in treatment of dyskinesia in PD and the dopamine D2/D3 agonist piribedil has been shown to reduce apathy in PD indicating potential benefits that could be applied to DLB (Thobois et al., 2013).

The dopamine D4 receptor has the lowest levels of expression in the brain, with higher receptor densities in anterior regions of the limbic and cortical forebrain, which may suggest its function is in modifying executive and behavioural regulation (Rondou et al., 2010). Dopamine D4 receptor polymorphisms in FTD have been shown to be associated with greater behavioural apathy and atrophy in the ACC, suggesting involvement in mood (Butler et al., 2019). The findings of the present study demonstrated reduced dopamine D4 receptor levels in the sgACC in DLB cases with depression compared to without, suggesting a possible role in depression in DLB. The antidepressant Roxindole is a relatively selective dopamine D4 receptor agonist with a known clinical safety profile and while Roxindole has been used in PD for treatment of dyskinesia, effects on mood were not noted (Bravi et al., 1993).

Studies of the serotonergic system in neurodegenerative disorders and in relation to depression has focused on both pathological and receptor changes. The dorsal raphe, the major cell group involved in forebrain serotonin innervation, is severely affected by LB pathology in DLB and PD, which might explain the loss of serotonergic innervation to the forebrain (Ballard et al.,

2013, Sharp et al., 2008). Neurones in the raphe are also affected by NFT pathology and neurone loss observed in AD, although there does not appear to be any appreciable difference in neuronal numbers using stereological approaches between depressed and non-depressed AD subjects (Hendricksen et al., 2004). This also correlates with findings in late onset depression where dorsal raphe neurone pathology or neurone counts do not relate to symptoms (Syed et al., 2005). Serotonergic neurotransmission has been suggested to be severely impaired in DLB compared to AD patients with depression, with reduced 5-HT and its metabolite concentrations observed in the PFC, temporal, limbic, occipital cortex and hippocampus (Vermeiren et al., 2015). In the present study, no changes in serotonergic innervation in the sgACC was observed in DLB cases overall or in relation to depression, and additionally no changes in protein levels of serotonin transporter. Other studies have observed reduced 5HTT levels in the raphe nuclei in PD patients compared to controls using in vivo PET imaging (Guttman et al., 2007, Murai et al., 2001, Politis et al., 2010a), with an increase in 5HTT binding in the dorsal raphe and limbic regions in PD patients with depression (Politis et al., 2010b). An increase in 5-HT concentrations in the ACC in depressed DLB patients has been suggested (Vermeiren et al., 2015), which may be a compensatory mechanism for the decreased serotonergic neurotransmission overall in DLB in neocortical and limbic projection areas. No changes in the levels of $5HT1_A$ receptor were observed in the sgACC between the groups, which is in contrast to a previous study that found decreased serotonin $5HT1_A$ binding in ACC in depressed patients using PET imaging (Wang et al., 2016). The present finding does correspond with previous studies in depression in DLB/PDD where there was no significant change in [^{3}H]-8OH-DPAT binding in the inferior temporal gyrus (BA20), although a significantly increased $5HT1_A$ level was found in the fusiform gyrus (BA36) in depressed DLB/PDD cases (Sharp et al., 2008). A decrease in $5HT2_A$ binding in ACC and DLPFC was observed in treatment resistant depression (Baeken et al., 2012), which is in line with current results, showing reduced levels of $5HT2_A$ receptors in sgACC in DLB cases with depression compared to controls, which may suggest selective and region specific serotonergic receptor changes. This also correlates with ligand binding studies of the $5HT2_A$ receptor in DLB, PD, and AD, where reduced binding was observed across all disease states (Cheng et al., 1991).

Whilst studies have shown loss of neurones from the dorsal raphe in AD (Hendricksen et al., 2004) and the presence of LB and LN pathology in Parkinson's disease (Seidel et al., 2015), there appears not to be any significant neuronal loss from the dorsal raphe in PD (Frisina et al., 2009, Cheshire et al., 2015). This absence of neuronal loss in PD is despite alterations in the

levels of 5-HT metabolites (Cheshire et al., 2015, Kish et al., 2008). In one large study using PET imaging with [^{123}I]-FP-CIT in PD, reduced 5HTT activity in PD was not associated with depression or anxiety (Qamhawi et al., 2015). In early de novo PD, 5HTT was unaltered in cortical regions including sgACC, as measured using [^{11}C]DASB PET (Strecker et al., 2011), suggesting that changes in 5HTT may be a late event, since in established PD, 5HTT levels were altered predominantly in subcortical structures (Guttman et al., 2007). Treatment may also play a role in 5HTT expression (Strecker et al., 2011), since L-DOPA administration may change 5HTT levels in PD (Beucke et al., 2011). Similarly, DLB cases may not have reduction in 5HTT in cortical regions based on [^{123}I]-FP-CIT binding (Joling et al., 2018). In one post mortem study, 5HTT measured using [3H] cyanoimipramine binding was preserved in DLB cases with depression, but reduced in DLB cases without depression (Ballard et al., 2002a). 5-HT levels are suggested to be reduced in cortical regions in DLB (Ohara et al., 1998, PERRY, 1993), suggesting that despite a potential lack of change in raphe neurone number, there may be altered activity of serotonergic systems in DLB. Loss of other serotonergic neurones in PD has been described, including from the pontine and medullary raphe (Halliday et al., 1990) along with α-synuclein pathology (Seidel et al., 2015). These neuronal groups however project to subcortical, cerebellar and brainstem structures rather than neocortical regions. It is possible therefore, that changes in serotonergic function are apparent in DLB, as with PD, but that these do not relate the dorsal raphe, but to other midline serotonergic nuclei, and may explain the finding of changes in striatal 5HTT activity in PD (Qamhawi et al., 2015). In the current study, due to the complexity of the dorsal raphe anatomy and its different projection sites (Ren et al., 2018) and the lack of evidence of changes in established PD, the dorsal raphe neurone numbers were not assessed in this DLB cohort. Future studies should determine, using appropriate stereological approaches and anatomical markers, the effects of α-synuclein pathology in DLB in the brainstem and midbrain serotonergic system.

Noradrenergic LC projections innervate multiple brain areas involved in the pathophysiology of depressive states, with dysregulation of noradrenaline involved in the pathogenesis of anxiety and depression (Goddard et al., 2010). The noradrenaline transporter (NET) is responsible for the rapid reuptake of noradrenaline into presynaptic terminals, and is involved in mediating behavioural responses to stress (Haenisch and Bonisch, 2011). The present results show reduced NET levels in the sgACC in DLB patients with and without depression compared to controls, which may indicate reduced noradrenergic innervation in the sgACC due to noradrenergic neuronal degeneration of the LC, commonly observed in DLB and PD, and also

in AD (Szot et al., 2006, Paulus and Jellinger, 1991, Ordway and Klimek, 2001, Klimek et al., 1997, Seidel et al., 2015, Haglund et al., 2016). As no reduction in DBH protein levels were observed in DLB, this may limit the findings of reduced NET. One suggestion is that surviving LC neurones may increase innervation into various regions as a compensatory mechanism leading to preserved levels of certain noradrenergic markers (Leverenz et al., 2001).

The LC in DLB cases is however, severely affected by α-synuclein pathology (Seidel et al., 2015, Jellinger, 2003a), which may be a major contributor to the neuronal loss observed in the LC. Reduced noradrenergic innervation in the ACC, amygdala and NAcc has been shown in PD patients with depression based on PET studies using a DAT/NET ligand (Remy et al., 2005), suggesting noradrenergic system involvement in both depression and neurodegeneration. Similarly, in AD LC neurone loss correlates with depression (Forstl et al., 1992), although depression does not appear to relate to LC neuronal pathology despite marked neuronal loss (Hoogendijk et al., 1999). A limitation of the current study is the lack of investigation of detailed pathology of noradrenergic systems in DLB. As the available antibody to NET did not provide suitable staining, the NET fibre density in the sgACC in DLB or LC neurone numbers could not be determined. Further investigation, using neuronal and fibre counting in an analogous manner to those used for dopaminergic and serotonergic systems, along with investigation in AD and PD cases for comparison would be beneficial in determining any role of noradrenaline in depression in DLB. LC cell counts in DLB show similar reductions to those found in AD (Leverenz et al., 2001), however, the LC has a widespread projection area to cortical, subcortical, cerebellar and brainstem structures. Using markers that identify specific cortically projecting neurones, or those LC neurones that innervate structures such as the VTA involved in depression (Isingrini et al., 2016) would provide suitable tools to accurately investigate the role of the LC and noradrenaline in depression in DLB.

In summary, a major effect of dopaminergic system degeneration of the VTA was observed in relation to depressive symptoms in DLB. Changes in serotonergic and noradrenergic markers appear to be less involved in the aetiology of depression in DLB. Careful treatment with selective dopaminergic agonists or positive allosteric modulators may therefore be of benefit in alleviating depressive symptoms in DLB.

Chapter 6.

Summary Discussion

6.1 Introduction

Depression is a common illness, with more than 264 million people affected worldwide (Disease et al., 2018), and is a leading cause of disability that impacts all aspects of everyday life (Steger and Kashdan, 2009). Almost one in five people will experience depression at some point in their lifetime (Malhi and Mann, 2018), with depression arising from a complex interaction of social, psychological and biological factors.

The clinical symptoms associated with major depressive disorder (MDD; as defined by DSM-IV criteria) implicates many neural systems involved in the regulation of mood, anxiety, reward processing, attention, motivation and stress responses (Price and Drevets, 2012). Structural and functional studies in MDD have identified several brain regions that are changed and potentially involved in the pathophysiology of depression, such as the prefrontal cortex (PFC), orbitofrontal cortex, amygdala, hypothalamus, various brainstem nuclei and the anterior cingulate cortex (ACC) (Price and Drevets, 2012, Ongur et al., 2003, Price and Drevets, 2010). The ACC, especially the subgenual region (sgACC), is integral in mood regulation, as well as in the symptomatic expression of depression, displaying structural, functional and metabolic abnormalities (Drevets et al., 2008b, Rodriguez-Cano et al., 2014). The sgACC is activated during simple emotions, such as sadness, and deep brain stimulation (DBS) of the sgACC has been shown to partially alleviate the symptoms associated with depression (Mayberg et al., 2005). Furthermore, increased metabolism in the sgACC has been shown to be involved in MDD (Greicius et al., 2007, Mayberg et al., 2005), with decreased sgACC activity observed in response to antidepressant medication or ECT in treatment resistant depression (Baeken et al., 2015) underscoring the importance of the sgACC as a region intimately involved in the pathophysiology of depression.

Depression is a common psychiatric symptom in DLB, observed in around half of DLB patients, often occurring in prodromal stages (Fujishiro et al., 2015). Depression in DLB is also associated with faster cognitive decline, higher mortality rates and a poor response to available treatment (Ritchie et al., 1998). The basis of depression in DLB is therefore of major importance in understanding the underlying cause of this major symptom and identifying treatments that would lead to improved quality of life in patients. In DLB, the ACC is a predilection site for the formation of LB, showing early and extensive α-synuclein pathology (Thal et al., 2004, Toledo et al., 2016). However, it is not known how pathological or

biochemical abnormalities in the ACC and in particular the sgACC may relate to depression in DLB.

6.2 Key findings

- Pathology does not play a direct role in depression in DLB
- Imbalance in excitatory and inhibitory systems more pronounced in DLB with depression
- Dopaminergic drive of depression in DLB

6.2.1 No relationship between pathology and depression in DLB

This study showed no direct relationship between pathological burden in the sgACC and depression in DLB. Studies assessing neuropathological changes in relation to depression in patients without dementia or in AD are scarce. While some studies have suggested the presence of LB in amygdala to be associated with increased risk for MDD in AD (Lopez et al., 2006), others have indicated that higher levels of NP and NFT burden in the hippocampus in AD patients associates with depression (Rapp et al., 2006). These studies investigating pathology and depression in AD have analysed regions that are anatomically associated with depression such as the amygdala and hippocampus, and therefore these findings may have direct relevance. In line with current findings however, one study found no association between depressive symptoms in DLB or AD and severity of cortical or subcortical LB pathology (Samuels et al., 2004), with again a focus on regions associated with the biology of depression. It is possible that, while pathology in AD may associate with depression, differences in the underlying neural systems affected in DLB will mean that AD neuropathological changes in DLB have less of an effect in promoting depression.

The present findings also showed that α-synuclein pathological burden was most severe in ACC compared to other cingulate subregions in DLB cases, including within the sgACC. Although considerable pathological burden was observed in the ACC and sgACC, no major changes in neuronal density were observed in DLB. This may suggest that pathology may be initiating neuronal dysfunction rather than cell death. The substrates of depression in DLB appear therefore to be independent of gross pathology. Changes to different aspects of neuronal function, such as synaptic abnormalities, may be caused initially by pathological deposition of, for example, α-synuclein. Once these changes in function occur however, an essentially

autonomous process is activated where pathology can increase over a background of already established dysfunction. Furthermore, since depression is caused by changes to specific neuronal systems, using gross pathological measures may simply obscure the fine detail needed to observe only depression specific pathological changes (cf. monoaminergic studies).

The biochemical analysis of pathological changes within the sgACC largely reiterated immunohistochemical results, showing no association between pathology in sgACC and depression in DLB. In sgACC the highest level of s129 α-synuclein, a suggested marker of pathological α-synuclein, was detected in insoluble membrane bound fractions of the tissue. This may suggest that the majority of α-synuclein is sequestered in insoluble pathological aggregates. These pathological aggregates while demonstrating presence of disease, may lack sufficient specificity to show changes only associated with depression, which may be in a subset of neurones.

6.2.2 Imbalance in GABAergic/glutamatergic neurotransmission.

The sgACC shows increased metabolism in patients with MDD (Greicius et al., 2007) and this may suggest dysregulation in excitation and inhibition. In this study, DLB patients with depression show more pronounced dysfunction in GABAergic and glutamatergic neurotransmission than DLB patients without depression, suggesting a greater imbalance in excitation/inhibition (E/I).

Reduction in postsynaptic proteins involved in GABAergic and glutamatergic neurotransmission, as well as selective loss in presynaptic proteins involved in SNARE complex facilitated exocytosis in the sgACC may indicate altered inputs to the sgACC. Changes to pre- and postsynaptic markers may be due to dysfunction or degeneration of other brain regions involved in emotional processing, with afferent projections to the sgACC, including brainstem, VTA, amygdala and NAcc (Vogt et al., 1995, Vogt and Pandya, 1987). Certainly, reduced VTA neurone numbers may be a feature of DLB based on current investigations. Synaptic dysfunction within the sgACC is however supported by observation of unchanged numbers of GABAergic or glutamatergic synapses but with an accompanying increase in the average size of glutamatergic synapses in the DLB group. This increase in size may be a result of a compensatory response to the accumulation of pathological phosphorylated α-synuclein. The present results are in line with other studies, showing significant increase in the size of glutamatergic synapses and presynaptic terminals containing phosphorylated α-

synuclein (Nakata et al., 2012, Colom-Cadena et al., 2017). The increase in synapse size may be simply due to the presence of a physically large protein aggregate, however other compensatory mechanisms, such as hypertrophy to increase declining synaptic activity may occur (Reeve et al., 2018). Further work to determine the effects of α-synuclein at a synaptic level would therefore be of interest.

In this study, no overall changes in GABAergic cell density within the sgACC and no changes in GAD65/67 protein levels was observed between the groups. However, DLB cases with depression showed reduced GABAergic cell density in layer V, and reduced parvalbumin (PV) and calbindin (CB) protein levels in the sgACC, indicating highly selective changes affecting only certain populations of interneurones. These findings are consistent with other post mortem studies showing reduced CB neurone density in occipital and PFC in MDD (Rajkowska et al., 2007, Maciag et al., 2010), as well as reduced CB neurone density in layer II of the ACC in schizophrenia and bipolar disorder patients (Cotter et al., 2002). A decrease in PV gene expression in sgACC post-mortem tissues has also been observed in MDD (Tripp et al., 2012), with PV interneurones shown to contribute to regulation of E:I within the PFC, influencing emotional responses (Ferguson and Gao, 2018). GABAergic interneurones belong to a very heterogeneous group of cells that differ based on their morphological phenotypes (basket cells, Martinotti cells, chandelier cells, Cajal-Retzius cells, double-bouquet cells, long-range GABAergic projecting cells) (DeFelipe et al., 2013), neurochemical properties (PV, somatostatin (STT) and 5-HT3A receptor-expressing interneurones) (Lee et al., 2010, Rudy et al., 2011), and varying electrophysiological properties (fast-spiking cells, non-fast-spiking, adapting cells, accelerating cells, irregular-spiking cells, intrinsically bursting cells) (Riedemann, 2019). STT expressing interneurones constitute around 30% of all GABAergic interneurones. Downregulation of STT gene expression has been observed in DLB (Khundakar et al., 2016) and MDD (Tripp et al., 2011) and this may correlate with present observation of a loss of CB in the sgACC in DLB, since CB identifies SST expressing Martinotti cells within the cortex. Reduced SST expression in the sgACC has been observed across all cortical layers in sgACC in MDD cases (Tripp et al., 2011, Seney et al., 2015) and how SST neurones are affected in DLB would be of major interest. This intrinsic vulnerability of SST positive neurones in MDD and also DLB might result in increased excitation of pyramidal cells, and further dysregulation in E/I balance. Therefore, a more in depth assessment of specific loss or dysfunction of certain types of interneurones such as CB positive Martinotti cells or PV

expressing chandelier cells may provide clues to their role in neurodegeneration, as well as neuropsychiatric diseases.

Downregulation of glutamatergic and GABAergic receptors is often observed in MDD (Feyissa et al., 2009, Luscher et al., 2011, Sequeira et al., 2007). Selective reduction in NMDA2B and $GABA_A$α5 and α3 receptor subunits was seen in DLB cases with depression in the present study, therefore, selective targeting of specific receptors might lead to more effective psychotherapeutics for treatment of depression in DLB. Recent advances in the development of fast-acting antidepressants to overcome current therapeutic limitations of monoaminergic drugs has resulted in the development of more selective glutamatergic and GABAergic agents. Esketamine, a non-competitive NMDA receptor antagonist has been recently approved for use in treatment resistant MDD (Jauhar and Morrison, 2019). Selective targeting of the NMDA2B receptor by using the antagonists MK-0657 and CP-101,606, has also been shown to be effective in treating depression without serious side effects (Preskorn et al., 2008, Ibrahim et al., 2012). The use of NMDA antagonists such as memantine which is effective in the treatment of mild to moderate DLB could therefore be explored for specific effects on depression (Emre et al 2010). Similarly, positive modulators of $GABA_A$α5 (Piantadosi et al., 2016) and α3 receptor subunits (Fava et al., 2011) have shown antidepressant efficacy in animal and human studies and also cognitive enhancing properties (Jacob, 2019). $GABA_A$α5 negative allosteric modulators such as MRK-016 and L-655,708 have also shown promise in alleviating depressive symptoms in animal models (Fischell et al., 2015, Zanos et al., 2017), and also demonstrate cognitive enhancing properties in preclinical models. Using $GABA_A$α5 modulation of synaptic or extrasynaptic receptors might be a therapeutic target for use in DLB. The GABAergic dysfunction in MDD may be due to downregulation of specific receptors, whereas GABAergic deficits in DLB may reflect a loss of intrinsic neurones and synapses that express these receptors.

The present findings provide a platform for much needed future research in depression in DLB. While α-synuclein pathology in glutamatergic and GABAergic synapses was observed in the sgACC, possibly contributing to synaptic dysfunction and development of depression in DLB, given the wide diversity of cortical interneurones, it is not known which specific interneurone populations are the most affected by pathological changes. To address this, further neuropathological, biochemical and transcriptomic studies are much needed. To determine the effects of α-synuclein on specific interneurone populations, future studies should use targeted

single cell nuclei RNA sequencing to identify the molecular changes caused by α-synuclein in vulnerable cell groups.

6.2.3 Dopaminergic drive of depression in DLB

Monoaminergic abnormalities in depression are well established (Moore and Bloom, 1979). DLB patients show abnormalities in serotonergic (Vermeiren et al., 2015, Ballard et al., 2013, Sharp et al., 2008), noradrenergic (Ohara et al., 1998) and dopaminergic neurotransmission (Walker et al., 2004). Little is known however, of how changes within monoaminergic neurotransmission within the sgACC relate to development of depression in DLB. These results suggest that primarily there is a reduced dopaminergic drive associated with depression in DLB, with reduced dopaminergic innervation in the sgACC, along with reduced levels of dopaminergic markers and receptors, and changes in dopaminergic synaptic function. Changes in serotonergic and noradrenergic markers appear to be less involved in the aetiology of depression in DLB. STED based analysis also revealed the presence of phosphorylated α-synuclein within dopaminergic and serotonergic synapses in the sgACC, directly linking a major pathological change in DLB, that of aggregated α-synuclein, with monoaminergic dysfunction in DLB. These α-synuclein changes in monoaminergic synapses in DLB also showed a positive correlation with SNAP25 in DLB cases with depression. One explanation may be that pathological α-synuclein causes sequestration of SNAP25 within synapses leading to synaptic dysfunction (Choi et al., 2018). How α-synuclein interacts with synaptic components in DLB would be a useful area of future study.

Recent evidence suggests a close relationship between differential expression profiles of α-synuclein and selective vulnerability of certain neuronal populations (Taguchi et al., 2019, Li et al., 2002), with aggregation of α-synuclein thought to be dependent on its endogenous expression (Volpicelli-Daley et al., 2011b, Erskine et al., 2018). While the effect of phosphorylated α-synuclein on monoaminergic synapses was explored, future studies should focus how different α-synuclein species affect synaptic function in DLB using approaches such as cell culture and aggregation based assays (Lau et al., 2020).

6.3 Study strengths and limitations

6.3.1 Study strengths

All cases included in this study were well characterised based on clinical and neuropathological criteria. The case selection criteria was based on the availability of clinical information, including MMSE, UPDRS, CSDD and GDS scores. The cases for immunohistochemical analysis were selected with shorter time in fixative, and to ensure the optimal biochemical analysis the cases were chosen with higher pH (as a surrogate for agonal state). One of the strengths of this study was the ability to assess pathological changes within cingulate subregions, and more importantly the sgACC, using immunohistochemical and biochemical techniques. Furthermore, by using fractionated tissue samples and multiple pathological markers, the analysis of pathological changes and its relationship with clinical findings was carried out much more in depth. The use of dot blot analysis enabled comparison of a wide range of proteins, considerably adding greater breadth to the study.

Another strength of this study was using super-resolution stimulated emission depletion (STED) microscopy. STED achieves spatial resolution in the ~20-80 nanometer range (Vicidomini et al., 2018), revealing molecular features of synapses beyond the diffraction limit, approaching the gold standard of electron microscopy (Spuhler et al., 2016, Hell, 2007). This improved imaging technique was used in the present monoamine study to assess dopaminergic and serotonergic synapses.

6.3.2 Study limitations

One of the limitations of this study is that we only looked at the overall GABAergic cell density within the sgACC. This rather generalised approach may prevent us from identifying subtle changes that are important in the aetiology of depression in DLB. The limbic system also expresses high levels of GABAB receptors (McDonald et al., 2004) indicating their role in regulating emotional behaviour. A limitation of the current study was the lack of assessment of GABAB receptors, as the available antibody for GABAB2 receptor did not provide a band of the right molecular weight. Recently, there has been greater emphasis on antagonism of GABAB receptors as a potential therapeutic strategy for depression (Bowery et al., 2002). Chronic treatment with the GABAB receptor antagonist CGP36742 has shown antidepressant-like responses in rats (Nakagawa et al., 1999), and one GABAB receptor antagonist

CGP56433A showed an increase in the BDNF concentration in the hippocampus and cortex (Heese et al., 2000), which might contribute to their antidepressant-like effects (Shirayama et al., 2002). Further investigations of the role of GABAB receptors in DLB and selective targeting of GABAB receptors might therefore lead to more effective psychotherapeutics for mood disorders in which there is a high and unmet medical need.

The methodological approach to imaging of glutamatergic and GABAergic synapses is one possible limitation of this study. Although confocal microscopy is useful in imaging individual synapses in cortical tissues (Schoonover et al., 2014), the diffraction limit of conventional optical microscopy (~200nm) precludes accurate analyses of such information. When imaging very small cellular structures, such as synapses, confocal imaging does not achieve the required resolution, and multiple synapses that are very close together could be seen as one single synaptic unit. This may result in the distortion of quantitative measures, such as size and volume.

6.4 Further Considerations

The clinical diagnosis of depression in DLB cases was based on symptom rating scales, primarily CSDD or GDS. These patients were also diagnosed with depression at different stages of the disease and had variable duration of depression and antidepressant treatment. The lack of a unifying depression scale in assessment of depression in DLB, variability in timing, duration and treatment of depression, thus precluded comparison of the pathological and biochemical findings with severity and frequency of depression in DLB. Studies with larger donor cohorts, with more uniform assessment of depression would be useful to show the effects of pathology, biochemistry and structure on depression in DLB. In a similar way, the role of anxiety in DLB, which is typically present in DLB and closely mirrors the presence of depression has not been assessed (McKeith et al., 2017). Accurate indicators of anxiety within this DLB population would allow us to determine how, for example, GABAergic changes might contribute to the development of anxiety in DLB, over and above the occurrence of depression.

The use of DLB cases with and without depression has allowed us to identify disease and depression specific changes in DLB. The incidence of depression is also high in PD, PDD and is common in AD patients and comparison between other disease groups with depression may

help us understand similarities and differences in depression in neurodegenerative diseases. It is likely that given the high incidence of depression in PD/PDD and the dopaminergic changes that dominate this disorder, abnormal dopaminergic activity may underpin depression in PD/DLB in a similar way to depression in DLB. In AD, due to the changes in regions such as the dorsal raphe, serotonergic changes may be more likely to contribute to depression, although studies so far have not shown this (Hendricksen et al., 2004). Nevertheless, comparing AD and DLB cases using similar methods to those used in the current studies would be able to show the differences and similarities between the two disorders.

While the sgACC has been shown to be highly important in depression (Drevets et al., 2008b, Rodriguez-Cano et al., 2014), other brain regions, such as PFC, orbitofrontal cortex, amygdala and hypothalamus, also have been shown to play a role in regulation of mood, anxiety, reward, attention, motivation and stress responses (Price and Drevets, 2012). Considering the interconnected nature of the brain, further studies involving other brain regions would likely further current understanding in the pathophysiology of depression in DLB. Similarly, the individual components of the sgACC were assessed essentially in isolation, and there is likely to be a considerable interplay between glutamatergic, GABAergic, and monoaminergic systems throughout the brain. For example, noradrenergic input to the dorsal raphe from the LC promotes arousal (Szabadi, 2013), and input from the dorsal raphe onto VTA GABAergic neurones is anxiolytic (Kimmey et al., 2019). An integrated approach to the anatomy and biochemistry of depression in DLB would undoubtedly prove beneficial.

Due to time restrictions, other neurotransmitter systems were not explored that are also important in regulation of mood, including cholinergic neurotransmission (Janowsky et al., 1972). Cholinergic dysfunction is profound in DLB patients (Perry et al., 1994), often observed early in the disease course, and possibly contributing to clinical symptoms such as cognitive fluctuations. Therefore assessing cholinergic changes may provide further insight in the aetiology of depression in DLB. Using STED microscopy to determine cortical synaptic changes in cholinergic neurones would be able to show how specific changes contribute to depression in DLB.

Gender differences in relation to depression have been considered, but not explored in the present study due to the relatively small sample size and should be the focus of the future studies. The prevalence of MDD is higher in women than in men, with women having higher number of symptoms, a more severe depression, and greater risk of recurring episodes (Perugi

et al., 1990). Gender differences in volumetric changes in the ACC (Brun et al., 2009, Mann et al., 2011), as well as structural and functional connectivity of the sgACC have been observed (Wang et al., 2014), and the higher prevalence of MDD in women may be due to greater functional connectivity of the sgACC with other affective regions. Sex differences were also observed in pharmacological treatment of depression, with females shown to respond better to serotonergic antidepressants than males, which may be related to antidepressant metabolism (Sramek et al., 2016). Therefore, a better understanding of anatomical, functional, metabolic and neurochemical changes associated with gender differences in relation to depression may result in identifying novel targets and improved treatment response.

6.5 Conclusion

Overall, these results demonstrate that in DLB, depression appears to associate with a reduction in dopaminergic markers in the sgACC, most likely derived from mild loss of VTA neurones. This would suggest that selective treatment with agents that improve cortical dopaminergic function may provide treatment for depression in DLB. Pathological changes at a gross level do not appear to associate with depression or imaging changes in DLB, although synaptic accumulation of pathological α-synuclein within synapses may play a role. Additional changes in glutamatergic and GABAergic markers associated with specific neuronal groups occurs in DLB cases with depression. Further investigation of specific glutamatergic and GABAergic neurone classes might provide additional treatment options for depression in DLB.

Chapter 7.

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Appendix A

Clinical data

Table A-1. Clinical data for control cases.

		Case ID	Diagnosis	Depression	Anxiety	VH	Fluctuations	REM sleep	L-DOPA	Other medication	Antidepressants	Depression scores	NPI	MMSE	UPDRS
		19890980	Control	no	no	n/a	n/a	n/a	no	n/a	n/a	n/a	n/a	n/a	n/a
		19960194	Control	no	no	n/a	n/a	n/a	no	n/a	n/a	n/a	n/a	n/a	n/a
		20100685	Control	no	no	no	no	no	no	n/a	n/a	n/a	n/a	30	n/a
		20050102	Control	no	no	no	no	no	no	Chemo	n/a	n/a	n/a	n/a	n/a
		20110891	Control	no	no	no	no	no	no	Trazodone	n/a	GDS-6	n/a	30	n/a
		20120854	Control	no	no	n/a	n/a	n/a	no	n/a	n/a	n/a	n/a	n/a	n/a
		19960188	Control	no	no	n/a	n/a	n/a	no	n/a	n/a	n/a	n/a	n/a	n/a
		19891035	Control	no	no	n/a	n/a	n/a	no	n/a	n/a	n/a	n/a	n/a	n/a
		20100742	Control	no	no	no	no	no	no	n/a	n/a	n/a	n/a	n/a	n/a
		20110272	Control	no	no	no	no	no	no	Carboplatin	n/a	n/a	n/a	n/a	n/a
		19930051	Control	no	no	n/a	n/a	n/a	no	n/a	n/a	n/a	n/a	n/a	n/a
		20152904	Control	no	no	no	no	no	no	Warafarin, digoxin, allopurinol, metoprolol, lisinopril, insulin, furosemide, amlodipine	n/a	GDS-3	0	27	n/a
		20152903	Control	no	no	n/a	n/a	n/a	no	n/a	n/a	GDS-7	0	29	n/a
		20151077	Control	no	no	n/a	n/a	n/a	no	Codeine phos, aspirin, lisinoprol, furosemide, atorvastatin, allopurinol, alendronic acid, paracetamol	n/a	GDS-3	0	MoCA-23	n/a
		20151023	Control	no	no	no	no	no	no	Frusemide, diltiazem, simavastatin, omeprazole, warfarin, latanoprost drops	n/a	GDS-4	n/a	22	n/a
		20150535	Control	no	no	n/a	n/a	n/a	no	Aspirin, Bispoprolol, ferrous fumarate, ISMN, levothyroxine, perindopril, simvastatin	n/a	n/a	n/a	n/a	n/a
		20150242	Control	no	no	n/a	n/a	n/a	no	Lisinipril, simvastatin, atenolol, aspirin	n/a	GDS-1	0	MoCA-27	7
		20140562	Control	no	no	n/a	n/a	n/a	no	Codeine, lansaprazole, paracetamol	n/a	GDS-1	0	30	0
		20140523	Control	no	no	n/a	n/a	n/a	no	Prednisolone/dexamethasone and oromorph	n/a	GDS-0	0	28	2
		20140155	Control	no	no	n/a	n/a	n/a	no	Alodipine, atorvastatin, atenolol, ISMN, lansaprazole, aspirin	n/a	GDS-5	n/a	28	n/a
		20131129	Control	no	no	no	no	no	no	Valsartan, bendroflumethiazide, tinzaparin	n/a	n/a	n/a	29	n/a
		20130656	Control	no	no	no	no	no	no	Alendronic acid, frusemide	n/a	GDS-4	1	27	n/a
		20130020	Control	no	no	no	no	no	no	Nitrazepam, atenolol, tramadol, paracetamol, risedronate	n/a	GDS-3	n/a	22/25	n/a
		20100359	Control	no	no	n/a	n/a	n/a	no	n/a	n/a	n/a	n/a	29	n/a
		20080014	Control	no	no	n/a	n/a	n/a	no	n/a	n/a	GDS-1	n/a	30	n/a
		20070049	Control	no	no	n/a	n/a	n/a	no	Steroids	n/a	n/a	n/a	n/a	n/a
		20060093	Control	no	no	n/a	n/a	n/a	no	Gaviscon, frusemide, ipatropium bromide, lansaprazole, simvastatin, losartan	n/a	n/a	n/a	n/a	n/a
		20060081	Control	no	no	n/a	n/a	n/a	no	n/a	n/a	n/a	n/a	n/a	n/a

Clinical information for pathological (**green**) and biochemical (**blue)** cohorts.

Table A-2. Clinical data for DLB cases without depression.

		Case ID	Diagnosis	Depression	Anxiety	VH	Fluctuations	REM sleep	L-DOPA	Other medication	Antidepressants	Depression scores	NPI	MMSE	UPDRS
		19980019	DLB	no	yes	n/a	n/a	n/a	n/a	n/a	n/a	Cornell-5	n/a	24	n/a
		19950019	DLB	no	no	yes	n/a	n/a	L-Dopa, Seleginine	Madopar, bromocryptine, selegeline	n/a	n/a	n/a	5	n/a
		19920202	DLB	no	no	yes	yes		n/a	Haloperidol	n/a	n/a	n/a	26/37 MTS	n/a
		19910172	DLB	no	no	yes	yes	poss	L-Dopa	Anticonvulsants, neuroleptics	n/a	n/a	n/a	23/37	n/a
		20121032	DLB	no	no	yes	yes		n/a	Donepezil, Clonazepam	n/a	n/a	n/a	25	n/a
		20020008	DLB	no	no	no	yes	n/a	n/a	-	n/a	n/a	n/a	27	n/a
		20152919	DLB	no	no	yes	yes	yes	L-Dopa	Rivastigmine, ropinirole, amitryptyline, cetrirzine, quinine, latanaoprost	Amitryptyline	Cornell - 2	5	29	53
		20150907	DLB	no	yes	yes	yes	no	n/a	Olanzapine, simvastatin, metaprolol, omeprazole	n/a	GDS - 6	24	25	26
		20150159	DLB	no	no	yes	yes	yes	Sinemet	Rivastigmine, Donepezil, ibuprofen, lansaprazole, latanoprost, brinzolamide, paracetamol, tolterodine, movicol, tramadol	n/a	n/a	n/a	8	26
		20111090	DLB	no	no	yes	yes	n/a	Sinemet	Rivastigmine, atorvastatin, tamulosin, alendronate, calcium carbonate & cholecalciferol, aspirin, lisinopril	n/a	GDS - 6	29	21	28
		20110932	DLB	no	no	yes	yes		L-Dopa/ Carbidopa	Donepezil, stalevo, pravastatin	n/a	GDS - 1	13	9	55
		20110645	DLB	no	yes	yes	yes	yes	n/a	Rivastigmine, clonazepam	n/a	n/a	n/a	20	n/a
		20070105	DLB	no	no	yes	yes	yes	Sinemet	Donepezil, rivastigmine, thyroxine, quetiapine	no	Cornell - 3	5	1	21
		20070017	DLB	no	yes	yes	yes	n/a	n/a	Donepezil, quetiapine	no	Cornell - 5	20	24	n/a
		20050098	DLB	no	no	yes	n/a	yes	n/a	Lorazepam, donepezil, lansaprazole, atenolol, ISMN, trazodone, aspirin, frusemide, clopixol	Trazodone	n/a	n/a	n/a	n/a
		20050040	DLB	no	no	yes	no	no	n/a	Donepezil, risperidone	no	Cornell - 0	4	15	33
		20050010	DLB	no	no	yes	yes	no	n/a	Donepezil, calcichew, chlormethiazole, midodrine, fludrocortisone, quetiapine, hydroxycobalamine, senna, lorazepam, prednisolone	no	Cornell - 5	5	9	14
		20030128	DLB	no	yes	yes	yes	no	Madopar	Donepezil, aspirin, calcium D3, sodium risedronate, GTN spray, madopar, atenolol	no	6	15	4	45
		20030007	DLB	no	no	yes	yes	no	Madopar	Clonazepam, donepezil	n/a	Cornell - 0	21	18	50
		20010080	DLB	no	no	yes	yes	n/a	Sinemet	-	n/a	n/a	n/a	n/a	n/a
		20010074	DLB	no	no	yes	no	yes	Sinemet	Rivastigmine	n/a	n/a	2	22	24
		20010026	DLB	no	no	yes	yes	n/a	n/a	Rivastigmine, ferrous sulphate, Vit B12, paracetamol	n/a	Cornell - 6	n/a	9	46
		20010025	DLB	no	no	yes	yes	n/a	L-Dopa	Rivastigmine	n/a	n/a	26	10	37
		20000064	DLB	no	no	yes	yes	n/a	L-Dopa	Rivastigmine	n/a	GDS - 2	n/a	15	37

Clinical information for pathological (**green**) and biochemical (**blue)** cohorts.

Table A-3. Clinical data for DLB cases with depression.

Case ID	Diagnosis	Depression	Anxiety	VH	Fluctuations	REM sleep	L-DOPA	Other medication	Antidepressants	Depression scores	NPI	MMSE	UPDRS
20152920	DLB	yes	no	yes	yes	n/a	Simemet	Donepezil, memantine, quetiapine	Fluoxetine	n/a	n/a	n/a prob 0	n/a
20141164	DLB	yes	no	yes	no	n/a	n/a	Donepezil, galanthamine, memantine, antipsychotics, benzodiazepines, candesartan, amlodipine, dabigatran	Citalopram	Cornell - 17	27	n/a prob 0	4
20110403	DLB	yes	yes	n/a	yes	n/a	Co-Careldopa	Donepezil	Venlafaxine, mirtazepine	GDS - 8	n/a	13	54
20110310	DLB	yes	no	yes	yes	yes	n/a	Donezepil, Rivastigmine	Sertraline, paroxetine	GDS-14	13	21	26
20100745	DLB	yes	no	yes	yes	yes	n/a	Donepezil 5mg	Citalopram	n/a	44	17	n/a
20100575	DLB	yes	yes	yes	yes	yes	n/a	Donepezil	Fluoxetine	Cornell - 5	23	8	21
20070009	DLB	yes	yes	yes	yes	yes	Sinemet	Clonazepam, donepezil, allopurinol, hydroxycobalmine	Fluoxetine, mirtazepine	GDS-10	13	12	31
20100082	DLB	yes	no	yes	yes	n/a	Sinemet	Memantine, rivastigmine, ropinerole, fludrocortisone, midodrine, amisulpiride	Venlafaxine, paroxetine, quetiapine, amitriptyline,	GDS-11, Cornell-9	n/a	23	27
20060025	DLB	yes	no	yes	yes	yes	Co-Careldopa	Donepezil, clonazepam, oxybutynin, paracetamol, aspirin	Citalopram	Cornell-11	29	2	36
20060019	DLB	yes	no	yes	yes	n/a	Sinomet		Citalopram	n/a	n/a	18	n/a
20050030	DLB	yes	yes	yes	yes	no	n/a	Olanzapine, donepezil, risperidone	Paroxetine	Cornell-13	103	0	24
20040085	DLB	yes	no	yes	yes	no	Sinomet	Donepezil, risperidone, midodrine, fludrocortisone	Citalopram	Cornell-4	0	4	58
20040034	DLB	yes	no	yes	yes	no	Sinemet	Donepezil, gabapentin, clonazepam	Citalopram	Cornell-16	49	14	20
20030113	DLB	yes	no	yes	yes	n/a	Sinemet	Donepezil	No anti deps	GDS-8, Cornell-9	20	12	51
20020079	DLB	yes	no	yes	n/a	n/a	n/a	Rivastigmine	Paroxetine, fluoxetine, venlafaxine,	Cornell-14	n/a	4	32
20020052	DLB	yes	no	yes	yes	n/a	n/a	Donepezil, chlormethiazole, lansaprazole, gaviscon	Citalopram	n/a	n/a	14	18
19950221	DLB	yes	no	yes	yes	n/a	Sinemet	Clozapine	Sertraline, dothiepin	n/a	n/a	0	42
19980060	DLB	yes	yes	yes	yes		Sinemet	Rivastigmine	Diazapam, trazedone	Cornell-8	n/a	19	n/a
19960131	DLB	yes	no	yes	yes	n/a	n/a	-	Paroxitine	Cornell-10	n/a	0	n/a
19980841	DLB	yes	yes				n/a	-	Lofepremine, lithium,	GDS-14	n/a	n/a	n/a
19870884	DLB	yes	no	n/a	n/a	n/a	Sinemet, L-Dopa, akineton, eldepryl	-	Diothiepin	Hamilton-11	n/a	n/a	n/a
20040063	DLB	yes	no	yes	yes		L-Dopa, Co-Carbedopa	Rivastigmine	Flouxetine	n/a	n/a	12	23
19960192	DLB	yes	no	n/a	n/a	n/a	n/a	Neuroleptics	Fluoxetine	Cornell-9	n/a	n/a	n/a

Clinical information for pathological (**green**) and biochemical (**blue)** cohorts.

Appendix B

Antibody validation

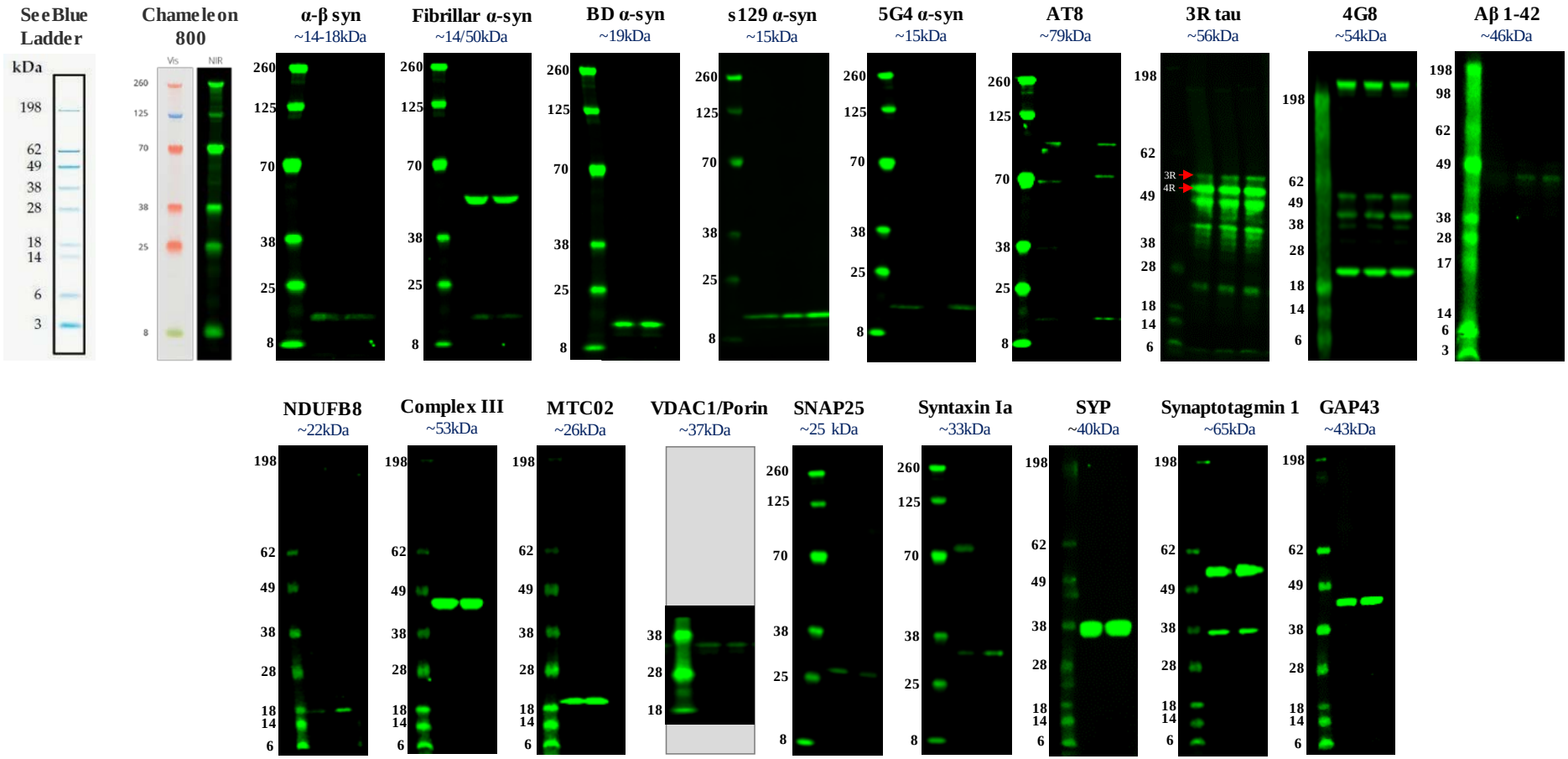

Figure B-1. Validation of pathology and metabolic markers.
The specificity of antibodies used for dot blot analysis was verified using western blotting. The proteins of interest were imaged using NIR Odyssey Fc system at 800nm together with SeeBlue pre-stained protein standard (Invitrogen) or Chameleon 800 (LI-COR) ladder.

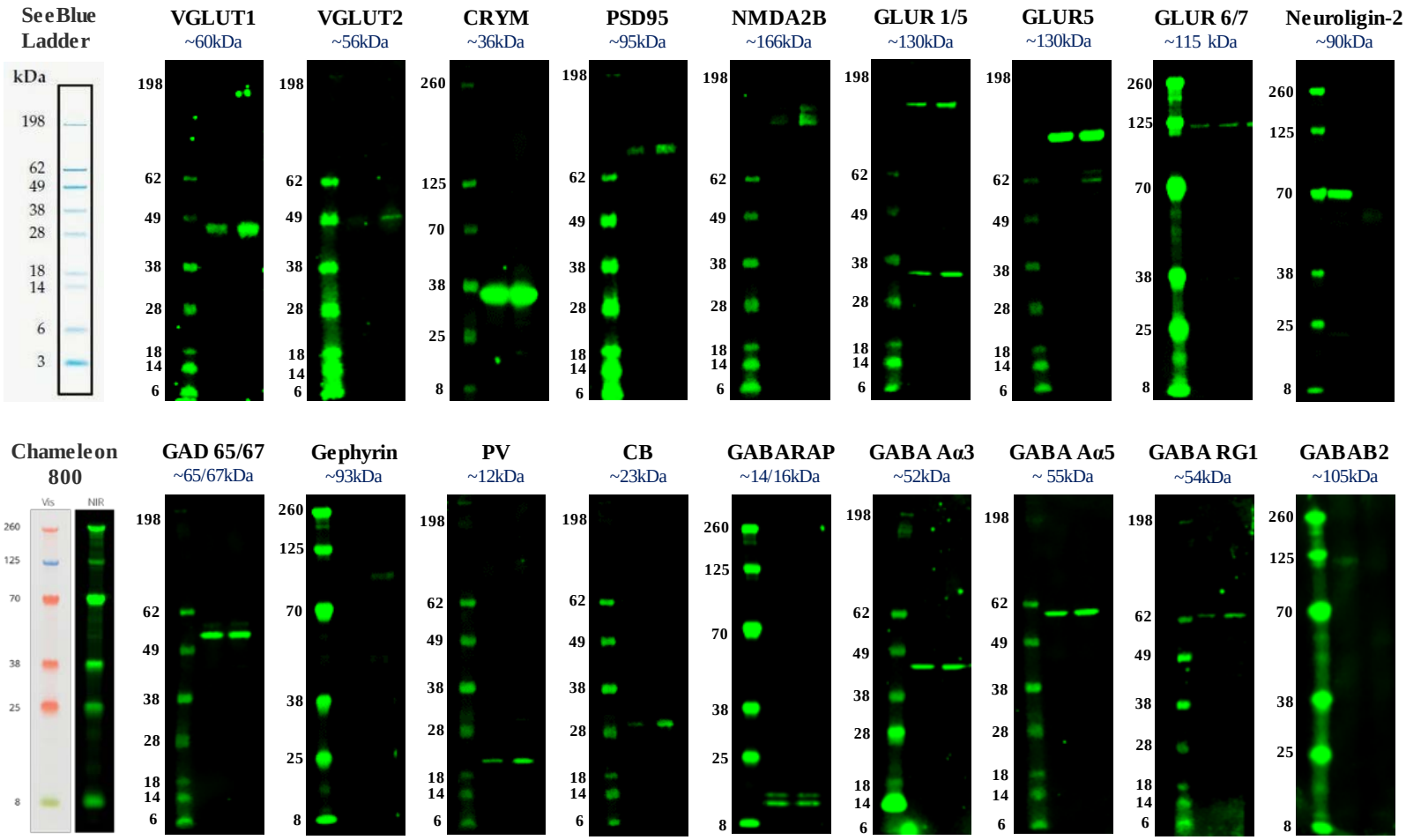

Figure B-2. Validation of glutamatergic and GABAergic markers.
The specificity of antibodies used for dot blot analysis was verified using western blotting. The proteins of interest were imaged using NIR Odyssey Fc system at 800nm together with SeeBlue pre-stained protein standard (Invitrogen) or Chameleon 800 (LI-COR) ladder.

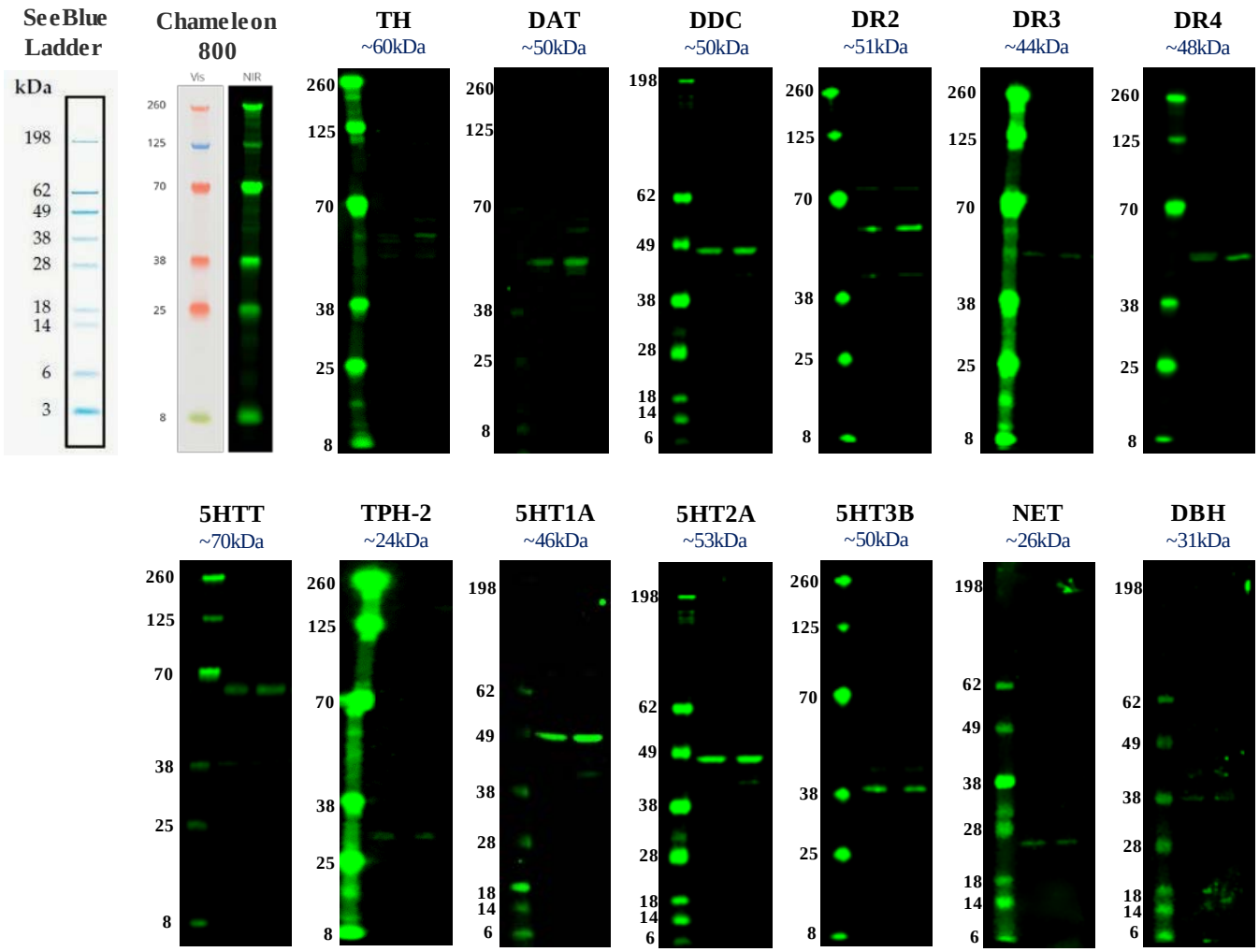

Figure B-3. Validation of monoaminergic markers.
The specificity of antibodies used for dot blot analysis was verified using western blotting. The proteins of interest were imaged using NIR Odyssey Fc system at 800nm together with SeeBlue pre-stained protein standard (Invitrogen) or Chameleon 800 (LI-COR) ladder.