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Department of Biomedical Sciences

IDENTIFYING DYSFUNCTIONAL NEUROTRANSMISSION IN NEURODEGENERATIVE DISORDERS:

METHODOLOGICAL CONSIDERATIONS AND FUNDAMENTAL INSIGHTS

IDENTIFICATIE VAN DISFUNCTIONELE NEUROTRANSMISSIE IN NEURODEGENERATIEVE AANDOENINGEN:

METHODOLOGISCHE BESCHOUWING EN FUNDAMENTELE INZICHTEN

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Jana Janssens

Supervisors:
Prof. dr. Peter Paul De Deyn
dr. Debby Van Dam

Co-supervisor:
dr. Yannick Vermeiren

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COMPOSITION OF THE PHD EXAMINATION COMMITTEE

Supervisors

Prof. dr. Peter P. De Deyn
Neurochemistry & Behavior, Institute Born-Bunge (IBB)
Department of Biomedical Sciences, University of Antwerp

dr. Debby Van Dam
Neurochemistry & Behavior, Institute Born-Bunge (IBB)
Department of Biomedical Sciences, University of Antwerp

Co-supervisor

dr. Yannick Vermeiren
Neurochemistry & Behavior, Institute Born-Bunge (IBB)
Department of Biomedical Sciences, University of Antwerp

Internal members of the jury

Prof. dr. Stuart Maudsley, chair
Receptor Biology Lab (VIB)
Department of Biomedical Sciences, University of Antwerp

Prof. dr. Peter De Jonghe
Department of Neurology
University Hospital Antwerp
Neurogenetics group (VIB)
Department of Biomedical Sciences, University of Antwerp

External members of the jury

Prof. dr. Inge Mertens
Centre for Proteomics
Health Unit, VITO
Faculty of Medicine and Health Sciences, University of Antwerp

Prof. dr. María Ramírez
Department of Pharmacology
University of Navarra

Prof. dr. Patrick Santens
Department of Neurology
Ghent University Hospital

List of abbreviations

(C)NRQ	(calibrated) normalized relative quantity
(f)MRI	(functional) magnetic resonance imaging
3-HAA	3-hydroxyanthranilic acid
3-HK	3-hydroxykynurenine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine; 5-HT
5-HTP	5-hydroxytryptophan
A	Adrenaline
AA	Anthranilic acid
ACh	Acetylcholine
ACTB	Beta-actin
AD	Alzheimer's disease
ADRB3	β-adrenergic receptor 3 gene
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
APOE	Apolipoprotein E gene
APP	Amyloid precursor protein
AUC	Area under the curve
Aβ	Amyloid beta
B2m	Beta-2-microglobulin
BA	Brodmann area
BBB	Blood-brain barrier
BCA	Bicinchoninic acid assay
Behave-AD	Behavioral Pathology in Alzheimer's Disease Rating Scale
BG	Basal ganglia
bvFTD	Behavioral variant frontotemporal dementia
C1-5	Cerebrospinal fluid fraction
C9ORF72	Chromosome 9 open reading frame 72 gene
CA	Cornu ammonis
Chrm1	Muscarinic acetylcholine receptor 1 gene
CI	Confidence interval
CMAI	Cohen-Mansfield Agitation Inventory
CNS	Central nervous system
COMT	Catechol-o-methyltransferase
Cq	Quantification cycle
CSDD	Cornell Scale for Depression in Dementia
CSF	Cerebrospinal fluid
D1-5	Dopamine receptors 1-5
DA	Dopamine
DAPI	4′,6-diamidino-2-phenylindole

DAT	Dopamine transporter
DLB	Dementia with Lewy bodies
DOPAC	3,4-dihydroxyphenylacetic acid
DOPAL	3,4-dihydroxyphenylacetaldehyde
DSM	Diagnostic and Statistical Manual of Mental Disorders
ECD	Electrochemical detection
ELISA	Enzyme-linked immunosorbent assay
FAD	Familial Alzheimer's disease
FDA	Food and Drug Administration
FTLD	Frontotemporal lobar degeneration
FUS	Fused-in sarcoma
GABA	ɣ-amino-butyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDetS	Global Deterioration Scale
GDP	Guanine diphosphate
Gfap	Glial fibrillary acidic protein
Glu	Glutamate
GPCR	G protein-coupled receptor
Gpi/e	Globus pallidus pars interna/externa
Grm5	Metabotropic glutamate receptor 5 gene
GRN	Progranulin gene
GTP	Guanine triphosphate
Gusb	Beta-glucuronidase gene
HEM	Hemizygous
Hprt	Hypoxanthine guanine phosphoribosyl transferase gene
HTR1A	Serotonin 1A receptor gene
HTR7	Serotonin 7 receptor gene
HVA	Homovanillic acid
Iba1	Ionized calcium-binding adapter molecule 1
IDO	Indoleamine 2,3-dioxygenase
IQR	Interquartile range
ISAAC	In situ Ag/AgCl
KA	Kynurenic acid
KP	Kynurenine pathway
KYN	L-kynurenine
LB	Lewy bodies
LBD	Lewy body dementia
LC	Locus coeruleus
LC-MS/MS	Liquid chromatography tandem mass spectrometry
L-DOPA	L-3,4-dihydroxyphenylalanine

LID	L-3,4-dihydroxyphenylalanine-induced dyskinesia
LP	Lumbar puncture
M1-5	Muscarinic acetylcholine receptor 1-5
MAO	Monoamine oxidase
MAPT	Microtubule associated protein tau gene
MCI	Mild cognitive impairment
MESOR	Midline estimating statistic of rhythm
MFS	Middelheim Frontality Score
mGluR	Metabotropic glutamate receptor
MHPG	3-methoxy-4-hydroxyphenylglycol
MMSE	Mini-Mental State Examination
NA	Noradrenaline
NAD^+	Nicotinamide adenine dinucleotide
NDD(s)	Neurodegenerative disorder(s)
NeuN	Neuronal nuclear protein
NF	Normalization factor
NFTs	Neurofibrillary tangles
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association
NMDA	N-methyl-D-aspartate
NPS	Neuropsychiatric symptoms
PA	Picolinic acid
PAF	Pure autonomic failure
PD(D)	Parkinson's disease (dementia)
PET	Positron emission tomography
Pgk1	Phosphoglycerate kinase 1 gene
PNMT	Phenylethanolamine-N-methyltransferase
PPA	Primary progressive aphasia
PSEN1/2	Presenilin 1/2 gene
$P\text{-}Tau_{181P}$	Tau phosphorylated at threonine 181
PVDF	Polyvinylidene difluoride
QA	Quinolinic acid
RCG	Rostrocaudal concentration gradient
RG	Reference gene
RIPA	Radioimmunoprecipitation assay
RN	Raphe nuclei
Rpl13a	Ribosomal protein L13a gene
RP-(U)HPLC-ECD	Reversed-phase (ultra-) high performance liquid chromatography with electrochemical detection

RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SCN	Suprachiasmatic nucleus
SCNA	α-synuclein gene
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERT	Serotonin transporter
SINE	Short interspersed nuclear element
SNpc/pr	Substantia nigra pars compacta/pars reticulata
SOD	Superoxide dismutase
SPECT	Single-photon emission computed tomography
SSRI	Selective serotonin reuptake inhibitor
STN	Subthalamic nucleus
TBK1	TANK-binding kinase 1 gene
Tbp	TATA-box binding protein gene
TBS(-T)	Tris-buffered saline (supplemented with Triton X-100)
TDO	Tryptophan 2,3-dioxygenase
TDP-43 (*TARDP*)	Transactive response DNA-binding protein of 43 kDa (gene)
TH	Tyrosine hydroxylase
TRP	L-tryptophan
T-tau	Total tau
VCP	Valosin containing protein
VTA	Ventral tegmental area
WT	Wild-type
XA	Xanthurenic acid
Y-CONTR	Young controls
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta gene
ZNA	Ziekenhuis Netwerk Antwerpen

Summary

Neurodegenerative disorders (NDDs) pose an ever-increasing healthcare burden on society. Studying neurochemical disturbances in these diseases could help identify novel targets of interest, and pinpoint certain neurochemicals which might serve as biomarkers of the disease (process). A considerable proportion of drugs to treat NDDs are based on neurotransmitter changes in the central nervous system (CNS). However, some of these therapeutics may offer limited advantage and involve possible health risks. Therefore, this thesis has focused on the neurochemical characterization of Alzheimer's disease (AD), frontotemporal lobar degeneration (FTLD), dementia with Lewy bodies/Parkinson's disease dementia (DLB/PDD) and amyotrophic lateral sclerosis (ALS), primarily with respect to (nor)adrenergic, dopaminergic and serotonergic (monoaminergic) neurotransmitter systems and, to a lesser degree, the neuroinflammatory kynurenine pathway (KP). In **Chapter I**, these NDDs and monoaminergic pathways are presented.

Neurochemical research is sometimes hampered by methodological issues. Therefore, we looked at two problems related to the analysis of monoamines and their metabolites: the circadian rhythm in cerebrospinal fluid (CSF) and plasma composition and the rostrocaudal concentration gradient (RCG) in CSF, in **Chapter III**. We found that there was a 24-hour fluctuation in homovanillic acid (HVA), a dopaminergic metabolite, and a RCG for HVA and 5-hydroxyindoleacetic acid (5-HIAA). These findings thus underscored the importance of the standardization of pre-analytical procedures.
In addition, we also verified whether B elements, which are a type of short interspersed nuclear elements (SINEs), could be applied as an alternative normalization strategy in reverse transcription quantitative PCR (RT-qPCR) experiments. For this study, we used hippocampal and cortical tissue of the APP23 amyloidosis mouse model for AD, and found that B elements could be adopted as normalization factors in hippocampus, but less so in cortex. This was the first time these SINEs were implemented in a normalization approach for RT-qPCR in mice.

We further investigated the monoaminergic (dis)similarities between AD and behavioral variant frontotemporal dementia (bvFTD), belonging to the FTLD spectrum, in **Chapter IV**. Increased serotonin (5-hydroxytryptamine; 5-HT) and noradrenaline (NA) levels, in addition to lowered serotonergic and noradrenergic metabolite/neurotransmitter ratios in frontal, temporal and limbic brain regions, were indicative of bvFTD compared to AD. Thus, we hypothesized that a relatively higher degree of frontal atrophy in bvFTD compared to AD, with preservation of serotonergic afferents, could result in a relatively greater serotonergic inhibition of glutamatergic neurotransmission.

Next, we analyzed monoamines and metabolites in CSF and serum samples derived from patients with AD, FTLD, DLB/PDD and CONTR. Although many inter-group differences were identified, the most remarkable results were noted for 3-methoxy-4-hydroxyphenylglycol (MHPG) levels, as they were generally higher in CSF and lower in serum of DLB/PDD subjects compared to all other groups. Consequently, we investigated whether this compound could distinguish between AD and DLB/PDD, given that their neuropathological and clinical overlap considerably hampers the differential diagnosis. We found that CSF and serum MHPG indeed improved the AD versus DLB/PDD differential diagnosis, either combined with the core CSF biomarker panel for AD, or on their own. We attributed this finding to a heavier neuropathological burden in the locus coeruleus, which represents the main NA-producing nucleus within the CNS.
Since there is considerable clinical, genetic and neuropathological overlap between FTLD and ALS, we analyzed CSF and serum samples of patients with these NDDs and compared monoaminergic and kynurenergic contents. A general dopaminergic disturbance was found in the CSF and serum of FTLD and ALS subjects, possibly indicating a shared dysfunction of dopaminergic neurons. The KP did not appear to be altered in the ALS-FTD continuum. In spite of the lack of convincing serotonergic findings in ALS or DLB/PDD in this thesis, several direct and indirect serotonergic control mechanisms are at play to regulate other neurotransmitter systems. Therefore, we thought about a unifying theory explaining the glutamatergic and dopaminergic alterations in ALS and PD, respectively, which is included in a viewpoint on the matter.

Since changes in neurotransmitter levels do not offer fully conclusive information, we also characterized alterations in expression of neurotransmitter receptors, belonging to the G protein-coupled receptors (GPCRs), in **Chapter V**. Transcript expression of several serotonergic GPCRs and the β_3-adrenoceptor (*ADRB3*) was investigated using RT-qPCR in brain tissue of AD and CONTR subjects. Results indicated increased 5-HT_{1A} receptor mRNA levels (*HTR1A*) in limbic areas, increased *ADRB3* expression in hippocampus, and decreased *HTR7* levels in frontal cortex of AD subjects versus CONTR. Hippocampal *HTR1A* levels negatively correlated with the presence of anxieties and phobias, potentially implying a protective effect of 5-HT_{1A} on the occurrence of this behavioral disturbance.
We also looked at the expression of glutamatergic (*Grm5*) and cholinergic (*Chrm1*) GPCRs in the hippocampus of APP23 mice, as disturbances in these systems have long been identified in AD. We could not corroborate any genotype effects based on their expression levels, except at the age of 6 months.

Possibly, this finding can be linked to the first appearance of neuropathological lesions and/or behavioral disturbances at this age.
Protein-level analyses of GPCRs might confer even more information about (dys)functional neurotransmission. In the hippocampus of APP23 mice, we therefore studied 5-HT_6 density using western blot and immunofluorescence approaches. Finally, no age- or genotype effects were identified.

Taken together, we addressed several important methodological issues, and provided guidelines/advice about how to tackle these problems. Moreover, the work presented in this dissertation offers more insight into the neurochemical imbalance in several NDDs. Some monoaminergic compounds, such as MHPG, were found to differ significantly between NDDs, while other findings led to hypotheses regarding the neurochemical pathophysiology of these disorders. Based on these hypotheses, we formulated several suggestions regarding their therapeutic implication, such as the use of a 5-HT_{1A} receptor antagonist in FTLD and, possibly, ALS. However, more mechanistic studies identifying the pathological role of monoaminergic compounds, are necessary to provide more conclusive evidence regarding said hypotheses, as well as to define the biomarker potential of these neurochemicals.

Samenvatting

Neurodegeneratieve aandoeningen (neurodegenerative disorders; NDDs) vormen een steeds toenemende zorglast voor onze samenleving. Het identificeren van neurochemische stoornissen die deze ziekten kenmerken, kan helpen om nieuwe onderzoeksdoelen te formuleren en om bepaalde neurochemicaliën te identificeren die kunnen dienen als biomarkers voor de ziekte of het ziekteproces. Een aanzienlijk deel van de geneesmiddelen die ingezet worden om NDDs te behandelen, is gebaseerd op veranderingen in neurotransmitterconcentraties in het centrale zenuwstelsel (central nervous system; CNS). Sommige van deze geneesmiddelen bieden echter beperkt klinisch voordeel en houden bovendien mogelijke gezondheidsrisico's in. Daarom heeft dit proefschrift zich vooral gericht op de neurochemische karakterisering van de ziekte van Alzheimer (Alzheimer's disease; AD), frontotemporale lobaire degeneratie (FTLD), dementie met Lewy bodies/ziekte van Parkinson en Parkinson dementie (DLB/PDD), en amyotrofe laterale sclerose (ALS). Dit voornamelijk met betrekking tot (nor)adrenerge, dopaminerge en serotonerge verbindingen die behoren tot de monoaminerge neurotransmittersystemen en, in mindere mate, de neuroinflammatoire kynureninepathway (KP). In **Hoofdstuk I** worden deze NDDs en monoaminerge systemen kort voorgesteld.

Neurochemisch onderzoek wordt soms bemoeilijkt door methodologische problemen. Daarom hebben we in **Hoofdstuk III**, twee problemen onderzocht met betrekking tot de analyse van monoamines en hun metabolieten: het circadiane ritme in cerebrospinale vloeistof (cerebrospinal fluid; CSF) en plasma en de rostrocaudale concentratiegradiënt (RCG) in CSF. We ontdekten een 24-urenfluctuatie in homovanillinezuur (homovanillic acid; HVA), een dopaminerge metaboliet, en een RCG voor HVA en 5-hydroxyindolazijnzuur (5-hydroxyindoleacetic acid; 5-HIAA). Deze bevindingen toonden aldus het belang van standaardisatie in pre-analytische procedures aan.
Daarnaast zijn we ook nagegaan of B-elementen, een type korte verspreide nucleaire elementen (short interspersed nuclear elements; SINEs), konden worden toegepast als een alternatieve normalisatiestrategie voor experimenten die gebruik maken van reverse transcriptie kwantitatieve PCR (reverse transcription quantitative PCR; RT-qPCR). Voor deze studie gebruikten we hippocampaal en corticaal weefsel van het APP23 amyloidosis muismodel voor AD. We ontdekten dat B-elementen konden worden toegepast als normalisatiefactoren in hippocampus, maar in mindere mate in cortex. Deze studie was de eerste waarin deze SINEs werden geïmplementeerd als normalisatiestrategie bij RT-qPCR in muisweefsel.

Monoaminerge overeenkomsten en verschillen tussen AD en de gedragsvariant van frontotemporale dementie (bvFTD), behorend tot het FTLD-spectrum, werden onderzocht in **Hoofdstuk IV**. Verhoogde serotonine (5-hydroxytryptamine; 5-HT) en noradrenaline (NA) niveaus, gecombineerd met verlaagde serotonerge en noradrenerge metaboliet/neurotransmitter-verhoudingen in frontale, temporale en limbische hersengebieden waren indicatief voor bvFTD vergeleken met AD. Daarom opperden we dat een relatief hogere graad van frontale atrofie in bvFTD vergeleken met AD, samen met het behoud van serotonerge afferenten, zou kunnen resulteren in een relatief groter effect van serotonerge inhibitie op de glutamaterge neurotransmissie.
In een andere studie werden monoamines en metabolieten geanalyseerd in CSF- en serumstalen afkomstig van patiënten met AD, FTLD, DLB/PDD en CONTR. Hoewel een groot aantal verschillen tussen de groepen kon worden geïdentificeerd, werden de meest opmerkelijke resultaten genoteerd voor MHPG-niveaus, omdat ze over het algemeen hoger waren in CSF en lager in serum van patiënten met DLB/PDD in vergelijking met alle andere studiegroepen. Daarom hebben we onderzocht of deze metaboliet onderscheid kon maken tussen AD en DLB/PDD, gezien de neuropathologische en klinische gelijkenissen tussen deze twee NDD's de differentiële diagnose bemoeilijkt. We vonden dat MHPG in CSF en serum inderdaad de differentiële diagnose tussen AD en DLB/PDD verbeterde, hetzij in combinatie met het standaard CSF biomarkerpanel voor AD, hetzij op zichzelf. We schreven deze bevinding toe aan een verregaande neuropathologische aantasting van de locus coeruleus, die de belangrijkste NA-producerende kern in het CNS vertegenwoordigt.
Omdat er een aanzienlijke klinische, genetische en neuropathologische overlap is tussen FTLD en ALS, hebben we CSF- en serumstalen van patiënten met deze NDD's geanalyseerd en hun monoaminerge en kynurenerge inhoud vergeleken. Er was een algemene dopaminerge stoornis aanwezig in het CSF en serum van FTLD- en ALS-patiënten, wat potentieel duidt op een gemeenschappelijke disfunctie van dopaminerge neuronen. De KP leek niet te zijn gewijzigd in het FTD-ALS continuüm. Ondanks het ontbreken van overtuigende serotonerge bevindingen in ALS of DLB/PDD in dit proefschrift, spelen verschillende directe en indirecte serotonerge controlemechanismen toch een rol bij het reguleren van andere neurotransmittersystemen. Daarom hebben we nagedacht over een uniforme theorie die de glutamaterge en dopaminerge veranderingen in respectievelijk ALS en PD verklaart. Deze hypothese wordt voorgesteld in een hieraan gewijd opiniestuk.

Omdat veranderingen in neurotransmitterniveaus geen volledig sluitende informatie bieden, hebben we in **Hoofdstuk V** ook veranderingen in de

expressie van neurotransmitterreceptoren, behorende tot de G-eiwit-gekoppelde receptoren (GPCR's), gekarakteriseerd. De transcriptexpressies van verschillende serotonerge GPCR's en de β_3-adrenoceptor (*ADRB3*) werden onderzocht met behulp van RT-qPCR in hersenweefsel van AD- en CONTR-individuen, wat wees op verhoogde mRNA-niveaus van de 5-HT_{1A}-receptor (*HTR1A*) in limbische gebieden, verhoogde *ADRB3*-expressie in de hippocampus en afgenomen *HTR7*-niveaus in de frontale cortex van AD-proefpersonen versus CONTR. Hippocampale *HTR1A*-niveaus vertoonden een negatieve correlatie met de aanwezigheid van angsten en fobieën, hetgeen mogelijk een beschermend effect van 5-HT_{1A} op het optreden van deze gedragsstoornis impliceert.
We hebben ook de expressie van glutamaterge (*Grm5*) en cholinerge (*Chrm1*) GPCR's in de hippocampus van APP23-muizen bestudeerd, aangezien verstoringen in deze systemen reeds lange tijd gekend zijn in AD. Deze expressieniveaus leken onveranderd onder invloed van genotype, behalve op de leeftijd van 6 maanden. Wellicht kan deze bevinding worden gekoppeld aan de eerste neuropathologische laesies en/of gedragsstoornissen die verschijnen op deze leeftijd.
Eiwitanalyses van GPCR's kunnen nog meer informatie opleveren over (dis-) functionele neurotransmissie. In de hippocampus van APP23-muizen hebben we daarom de dichtheid van 5-HT_6 bestudeerd met behulp van western blot en immunofluorescentie. In deze studie werden geen leeftijds- of genotype-effecten geïdentificeerd.
Samengevat hebben we verschillende methodologische kwesties voorgesteld en richtlijnen/advies gegeven over hoe deze problemen kunnen worden aangepakt. Bovendien biedt het in dit proefschrift gepresenteerde werk meer inzicht in de neurochemische onbalans die diverse NDD's typeert. Sommige monoaminerge verbindingen, zoals MHPG, bleken aanzienlijk te verschillen tussen NDDs, terwijl andere bevindingen leidden tot hypothesen met betrekking tot de neurochemische pathofysiologie van deze ziekten. Op basis van deze hypotheses hebben we verschillende suggesties geformuleerd met betrekking tot hun therapeutische implicaties, zoals het gebruik van de 5-HT_{1A}-receptorantagonist in FTLD en mogelijk ook in ALS. Meer mechanistische studies die de pathologische rol van monoaminerge verbindingen identificeren, zijn echter nodig om meer overtuigend bewijs met betrekking tot deze hypothesen te leveren, evenals om het biomarkerpotentieel van sommige monoaminerge verbindingen te bewijzen.

Chapter I: General Introduction

1. NEURODEGENERATIVE DISORDERS

In June 2019, the global population was estimated at 7,713,468,000 people, with the proportion of people aged older than 65 estimated at around 10% and expected to double by 2100. In high-income regions such as Europe and North-America, this proportion is even expected to reach 32.5% [1]. Neurodegenerative disorders (NDDs) comprise age-related diseases characterized by progressive damage to or loss of neurons in the central nervous system (CNS). As age represents the major risk factor for NDDs, it is clear that our society will be faced with an enormous healthcare burden in the course of this century.

A couple of the most commonly occurring NDDs include Alzheimer's disease (AD), frontotemporal lobar degeneration (FTLD), dementia with Lewy bodies (DLB), Parkinson's disease (dementia) (PD(D)) and amyotrophic lateral sclerosis (ALS). These disorders all differ in their pathophysiological and clinical characteristics, although a certain degree of overlap between some of them can be observed, often complicating clinical diagnosis [2]. An important subset of NDDs are known as dementias. According to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-V) [3], dementia is a major neurocognitive disorder (**Table 1**).

Table 1. Diagnostic criteria for major neurocognitive disorders

A. Evidence of significant cognitive decline from a previous level of performance in one or more cognitive domains (complex attention, executive function, learning and memory, language, perceptual-motor, or social cognition) based on: **1.** Concern of the individual, a knowledgeable informant, or the clinician that there has been a significant decline in cognitive function; and **2.** A substantial impairment in cognitive performance, preferably documented by standardized neuropsychological testing or, in its absence, another quantified clinical assessment. **B.** The cognitive deficits interfere with independence in everyday activities (i.e., at a minimum, requiring assistance with complex instrumental activities of daily living such as paying bills or managing medications). **C.** The cognitive deficits do not occur exclusively in the context of a delirium. **D.** The cognitive deficits are not better explained by another mental disorder (e.g. major depressive disorder, schizophrenia)
Specification of 1. The etiological subtype (e.g. due to AD, FTLD or Huntington's disease) 2. Presence/absence of behavioral disturbance 3. Current severity (mild, moderate, severe)

Abbreviations: AD: Alzheimer's disease; FTLD: frontotemporal lobar degeneration. Table adapted from [3].

Apart from cognitive decline, representing the most important diagnostic criterion for dementia, patients suffering from this neurocognitive disorder also show

behavioral disturbances or neuropsychiatric symptoms, and disturbances in activities of daily living [4, 5]. Therefore, it is useful to note that the term "dementia" is a syndrome, i.e. a group of several clinically recognizable features, signs and symptoms, and does not refer exclusively to a cognitive disorder. A distinction is frequently made between early- (occurring in patients <65 years of age) and late-onset dementia [6], not to be confused with familial (caused by genetic mutations) and sporadic forms of dementia.

In several chapters of this thesis, research in the domains of distinct dementia types (AD, FTLD, DLB, PDD) and ALS will be presented. These NDDs will be introduced in following paragraphs, and an overview of their most important characteristics can be found in **Table 2**.

1.1. Alzheimer's disease

The leading cause of dementia is AD, accounting for 60-80% of all dementia cases [7]. This disorder is characterized microscopically by the deposition of misfolded proteins, amyloid-β (Aβ) and hyperphosphorylated tau, in the brain. The ABC scoring system [8] is used to provide a measure of the severity of these neuropathological lesions, in which 'A' stands for Aβ plaque score [9], 'B' for staging of neurofibrillary tangles (NFTs) consisting of aggregated hyperphosphorylated tau [10, 11] and 'C' for CERAD (Consortium to Establish a Registry for AD) neuritic plaque score [12]. These lesions are accompanied by synapse loss and neuronal death [13], and manifest macroscopically as a widespread pattern of brain atrophy, with severe atrophy of the temporal (hippocampal) cortex [14].
In most (typical) cases, the clinical presentation of AD entails progressive cognitive decline in two or more cognitive domains such as memory and executive functioning as initial symptoms, combined with behavioral dysfunction and functional dependence [15]. Atypical features on the other hand, may be characterized by non-amnestic signs, such as a frontal/executive, visuospatial or language presentation [15], complicating the clinical diagnosis. To account for this problem, the revised National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria are applied to establish a diagnosis of possible or probable AD, based on these clinical manifestations [16].
The large majority of patients with AD have a sporadic variant rather than a familial or genetic variant. In about 5% of all cases, the disease is linked to mutations in three major genes: amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*), which are all involved in the (patho-)physiological generation of Aβ [17]. Several risk factors have been identified in sporadic AD as well. In this regard, the *APOE* (apolipoprotein E) ε4 allele is believed to account for most of the genetic risk [18], while a recent genome-wide association study has identified nine

novel risk loci for AD, involved in gene transcription, APP processing and neuroinflammation [19, 20]. Moreover, non-genetic risk factors for the development of AD include a low degree of education, head injury and risk factors associated with vascular disease, such as hypertension and smoking [21, 22].
The molecular pathogenesis of AD is still not fully elucidated, nonetheless, several hypotheses have been proposed. The oldest of these theories is the cholinergic hypothesis, assuming that damaged groups of acetylcholine-containing neurons in the forebrain result in the downregulation of cholinergic markers which are associated with cognitive decline and abundance of NFTs [23, 24]. Nevertheless, the amyloid cascade hypothesis is probably the most prevailing. This theory states that the initial pathological process is caused by sequential cleavage of the transmembrane protein APP by β- and γ-secretases, instead of α- and γ-secretases. This results in the formation, extracellular secretion and oligomerization of Aβ peptides causing synaptic dysfunction and astrocytic and microglial activation, which in turn leads to oxidative injury and alterations in kinase and phosphatase activity [25]. The altered enzyme activities promote the (hyper)phosphorylation of tau, resulting in NFTs. Activated microglia and astrocytes secrete cytokines, nitric oxide and other deleterious substances and thus initiate a neuroinflammatory reaction. Additionally, they attempt to clear Aβ peptides and oligomers, although high cytokine levels decrease the phagocytic capability of microglia [26]. All abovementioned processes influence the activity and sensitivity of the glutamatergic neurotransmitter system, and N-methyl-D-aspartate receptors in particular, resulting in excitotoxicity and associated neuronal dysfunction, as well as cell death [27]. Together with neurotoxic Aβ peptides, NFTs are also proposed to mediate neuronal loss [28]. Eventually, Aβ oligomers are proposed to aggregate into insoluble Aβ plaques [29]. Despite being the dominant model of AD pathogenesis, evidence against this theory has accumulated over the years. Importantly, cognitive decline does not correlate well with Aβ neuropathology. Instead, the presence of NFTs in the medial temporal lobe seems to be the determining neuropathological hallmark for cognitive dysfunction and episodic memory in particular [30]. In addition, novel treatment strategies based on the amyloid cascade hypothesis have been proven ineffective, as some clinical studies investigating antibodies against Aβ were discontinued over the past couple of years [31]. However, several counterarguments in favor of this theory have also been published [29] and thus, the debate about the actual cause of AD is still ongoing.

1.2. Frontotemporal lobar degeneration

The terminology with respect to FTLD is often confusing. In general, FTLD denotes a heterogeneous syndrome comprising a variety of dementias which are all characterized by atrophy in frontal and temporal brain regions [32]. The term

'frontotemporal dementia' (FTD), is also commonly encountered in the literature accompanied by an indication of the meaning of this idiom, as it could mean the general syndrome featuring frontal and temporal lobe degeneration, or the specific behavioral variant of this syndrome (bvFTD).

Frontotemporal lobar degeneration is the second most common form of early-onset dementia [33]. Due to its heterogeneity, a considerable amount of different protein aggregates are observed in this syndrome, among which NFTs consisting of hyperphosphorylated tau and ubiquitin-positive aggregates of transactive response DNA-binding protein of 43 kDa (TDP-43) are most common [34]. Neuropathological criteria for FTLD are based on the presence of neuronal loss and associated type of proteinopathy [35]. As a consequence of neuropathological heterogeneity, a wide range of clinical symptoms are noted in FTLD. It should be mentioned, however, that there is no exclusive one-to-one relationship between the clinical symptoms and the underlying proteinopathy, either FTLD-tau, FTLD-TDP or other, rare variants [36]. Four clinical subsyndromes can be distinguished: bvFTD is characterized by initial changes in behavior, executive function, emotion and personality [37], whereas in semantic, nonfluent and logopenic variant primary progressive aphasia (PPA), language disturbances are most prominent [38]. Clinical diagnostic criteria for these FTLD subtypes are summarized in [39]. Still other disorders belonging to the FTLD spectrum are the tauopathies corticobasal degeneration and progressive supranuclear palsy, mainly characterized by a motoric manifestation [2]. In addition, FTD with parkinsonism linked to chromosome 17 (FTDP-17) is a genetic syndrome caused by mutations in the gene coding for tau (*MAPT*) involving behavioral, cognitive and motor symptoms [40]. A subset of bvFTD subjects also progresses to amyotrophic lateral sclerosis (ALS) and vice versa, so that the presence (with differing degrees of severity) of both diseases is termed the ALS-FTD continuum [41].

Mutations in *MAPT* and *GRN*, as well as a GGGGCC hexanucleotide expansion in *C9ORF72*, account for the large majority of familial FTLD cases [36], with 10-23% of FTLD patients having an autosomal dominant inheritance pattern in the family [36]. While mutations in *MAPT* cause tauopathies, mutations in *C9ORF72* or *GRN* are most often associated with FTLD-TDP [42]. Risk factors for sporadic FTLD include head trauma [43], while single nucleotide polymorphisms in *TMEM106B* were identified as an additional risk factor in patients carrying a *GRN* mutation [44].

Finally, various molecular processes contribute to the pathogenesis of FTLD. Impaired RNA processing, RNA toxicity and disturbed protein homeostasis have been hypothesized to initiate downstream effects leading to neuronal death [45], while in a similar fashion to AD, NFT formation also contributes to neurotoxicity [46].

1.3. Dementia with Lewy bodies

A third type of dementia is DLB, showing considerable clinical and neuropathological overlap with both AD and PD(D). As such, intracellular Lewy bodies (LB) consisting of aggregated α-synuclein, are not observed exclusively in DLB, but also in PD(D). The umbrella term 'Lewy body dementia' (LBD) is thus used to denote both disorders and should not be confused with DLB [47]. Lewy bodies in DLB are first observed in the brainstem and mesencephalon, and are subsequently found throughout the cortex. These lesions are accompanied by regional atrophy in temporal and frontal lobes, but contrary to AD, the medial temporal lobe remains relatively spared [48]. Neuropathological criteria for DLB have recently been updated to assess the probability of DLB in the presence of AD-like neuropathological lesions [49, 50], as co-pathology with NFTs and Aβ plaques is often observed.

Clinical diagnosis of DLB is based on progressive cognitive decline and early deficits and fluctuations in attention, executive function and arousal. Furthermore, visual hallucinations typically occur in about 80% of patients suffering from DLB. Other core clinical features include autonomic dysfunction, rapid eye movement sleep disorder and parkinsonism, defined as the presence of bradykinesia, rigidity, resting tremor and postural instability [49]. Because of the extensive clinical and pathological overlap with AD and PD(D), DLB is thought to belong to a disease continuum comprising all of these syndromes [51].

Although a general consensus exists that genetics play a role in DLB, little information about the prevalence of a familial variant of this disease is available. In addition, a causal gene has not yet been identified [52]. Several genes, including the gene coding for α-synuclein (*SCNA*), the *APOE* ε4 allele, and glucosylceramidase β (*GBA*) do constitute risk factors for DLB, although they are also implicated in other diseases belonging to the aforementioned disease continuum [52].

With respect to the disease process underlying DLB, soluble α-synuclein monomers aggregate first to oligomers, followed by the deposition into LB. However, it remains unclear whether LB confer synaptic dysfunction and neuronal loss observed in LBD, or whether these changes should be attributed to toxic α-synuclein oligomers [53].

1.4. Parkinson's disease (dementia)

Parkinson's disease is the second most common NDD after AD [2]. Its neuropathological features resemble those of DLB, although the distribution of LB differs between these diseases, with predominant brainstem pathology in brains of PD(D) patients, less limbic and striatal AD-like pathology and a higher degree of dopaminergic neuronal loss [53, 54]. Staging of LB pathology in PD is performed according to the criteria summarized in [55, 56]. In addition, neuronal loss in the

substantia nigra pars compacta (SNpc) is regarded as a second key neuropathological feature [57]. Brain atrophy is situated in the frontal areas of PD patients, while PDD subjects show additional grey matter loss in the occipital lobe. Temporal atrophy is also observed, albeit significantly less compared to AD [58]. Clinical motor aspects of this disease have an asymmetric onset and are subsumed under parkinsonism (see section **1.3. Dementia with Lewy bodies**). These manifestations represent cardinal features for clinical diagnosis, along with a good response to L-3,4-dihydroxyphenylalanine (L-DOPA) therapy [57, 59]. Non-motor symptoms include autonomic dysfunction, anosmia, visual hallucinations, sleep disturbances and emotional dysregulation [60, 61]. With disease progression, dementia prevalence increases, amounting to >75% in PD patients with >10 years of disease duration [62]. Thus, it is clear that DLB and PD(D) are clinically alike. This prompted the use of the 'one-year rule' for differential diagnosis, which entails that a primary diagnosis of PD is proposed if motor features present more than 12 months before the start of dementia symptoms [63].

A positive family history of PD(D) occurs in about 15% of all cases [64], associated with mutations in up to 19 causal genes, including *SCNA*, and even more risk factors. In addition, exposure to environmental toxins such as pesticides represents an important non-genetic risk factor [65]. The molecular pathogenesis of PD(D) is mediated by α-synuclein oligomers and fibrils, inducing neuroinflammation and cell death. Finally, NFTs are found near α-synuclein in LB and destabilize the architecture of dopaminergic neurons, resulting in neuronal degeneration and cell death [66].

1.5. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is a NDD with affected motor neurons in the motor cortex and spinal cord [67]. Neuronal ubiquitin-positive cytoplasmic inclusions consisting of TDP-43 are commonly observed, while superoxide dismutase 1 (SOD1) and ubiquilin 2 aggregates are also noted. Staging of neuropathological changes in ALS is based on the location and spread of TDP-43 aggregates [68, 69]. Clinically, the disease manifests as initial unilateral muscle weakness in the limbs, although in about 30% of all cases, bulbar symptoms including difficulties chewing, swallowing and speaking are observed as initial signs. The disease progresses to affect most muscles, until death due to respiratory paralysis occurs [67]. Pseudobulbar affect, characterized by the inappropriate expression of emotions, such as laughing or crying uncontrollably, is present in up to 50% of ALS subjects [70, 71]. Cognitive decline in terms of language disability, impaired recognition memory and visuoperceptual disturbances, is also observed [72, 73]. The diagnosis of ALS is based on the revised El Escorial criteria, which take into account the signs of upper and lower motor neuron degeneration in conjunction with

electromyography [67, 74]. Familial ALS is present in about 10% of all cases [75] and is associated with hexanucleotide expansions in *C9ORF72*, and mutations in *SOD1* and profilin 1 (*PFN1*), among others [67]. Various other genes, such as ataxin 2 (*ATXN2*) and *TARDBP* encoding TDP-43, are hypothesized to confer a higher risk for the development of ALS [76, 77]. Furthermore, a wide variety of nongenetic risk factors have been identified, including but not limited to smoking, a high level of physical fitness, occupational exposure to chemicals, pesticides, metals and electromagnetic fields, and head trauma [78].

The molecular processes underlying ALS are complex and involve a combination of disrupted protein homeostasis, nucleocytoplasmic transport and cytoskeletal abnormalities [67], the activation of astrocytes and microglia, as well as glutamate excitoxicity [79], ultimately leading to motor neuron degeneration and neuronal loss.

It is apparent that FTLD and ALS share mutations in (predominantly) *C9ORF72*, and both show TDP-43 inclusions in the CNS, indicating a first aspect of a ALS-FTD continuum. With respect to neuroimaging, FTD-ALS is characterized by both frontotemporal cortical atrophy and white matter tract degeneration [80]. Furthermore, clinical overlap between these conditions has become increasingly appreciated in the past decades, with substantial disturbances in executive function and language, as well as emotional processing [81]. As such, ALS in association with cognitive impairment, or ALS in association with behavioral impairment are distinguished from ALS with concurrent dementia meeting the criteria for FTLD (either bvFTD, logopenic, nonfluent or semantic variant PPA) [82]. Conversely, it has been recommended to investigate signs of motoric disturbance in patients with FTLD, as similar motor deficits are observed in FTD-ALS compared to those in ALS [81].

Table 2. Overview of neurodegenerative disorders included in this thesis.

	AD	FTLD	DLB	PDD	ALS
% of dementia cases					
Early onset	29.8	13.3	2.4	1.9	N/A
Late onset	55.5	6.5	6.5	3	
Clinical features	Initial cognitive decline Behavioral disturbance	Behavioral disturbance Language disturbance (Motor disturbance)	Cognitive decline Deficit in attention and arousal Early visual hallucinations REM sleep disorder Parkinsonism Autonomic dysfunction	Parkinsonism Cognitive decline Autonomic dysfunction Visual hallucinations	Muscle weakness Pseudobulbar affect Cognitive decline
Most common genes involved	*APP* *PSEN1* *PSEN2*	*MAPT* *C9ORF72* *GRN*	No causal gene identified	*SCNA*	*SOD1* *C9ORF72* *PFN1*
Pathological protein	Aβ tau	tau TDP-43 FUS	α-synuclein	α-synuclein	TDP-43 SOD1

Prevalence data based on [83-85]. Vascular and mixed dementia account for 10-20% and 15% of dementia cases, respectively [85, 86]. Abbreviations: Aβ: amyloid-β peptide; AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; *APP*: amyloid precursor protein gene; *C9ORF72*: chromosome 9 open reading frame 72 gene; DLB: dementia with Lewy bodies; FTLD: frontotemporal lobar degeneration; FUS: fused-in sarcoma protein; *GRN*: progranulin gene; *MAPT*: microtubule-associated protein tau gene; PDD: Parkinson's disease dementia; *PFN1*: profilin 1 gene; *PSEN1/2*: presenilin 1/2 gene; *SCNA*: α-synuclein gene; TDP-43: transactive response DNA-binding protein of 43 kDa.

1.6. Therapeutics and biomarkers

It appears that, despite the clinical heterogeneity, all NDDs discussed here are characterized by pathological protein deposition in the brain (and spinal cord), followed by neuronal degeneration. However, the degree and location of neuropathological lesions in these disorders differ substantially. An overview of the neuropathological progression and spread according to the published guidelines for NFT- and amyloid pathology in AD, α-synucleinopathy in LBD and TDP-43 inclusions in ALS, can be found in **Figure 1**.
Some studies have reported disease-modifying potential of acetylcholinesterase inhibitors in AD [87, 88], while memantine in AD and riluzole in ALS diminish glutamate excitotoxicity [89-92]. Nevertheless, most therapies applied in NDDs solely target symptoms of the disease. Some of these therapies, such as L-DOPA for motor symptoms in PD and selective serotonin reuptake inhibitors or the (off-label) use of antipsychotic medication for behavioral symptoms in AD, FTLD and DLB, target monoaminergic neurotransmitter systems. Nevertheless, conflicting evidence exists about the safety and efficacy of these treatments [93, 94]. This could be due to the distinct manifestation and degree of proteinaceous aggregates in NDDs, which may differentially affect certain neurotransmitter systems and render the use of similar drugs for all of these disorders ineffective and possibly unsafe. Thus, increased knowledge of the alterations of the neurochemical systems of these NDDs, is mandatory.

In addition, information with respect to the neurochemical profile of several NDDs, offers the possibility to establish a diagnosis and to monitor the progression/treatment response of such a disease by the implementation of laboratory measurements of these neurochemicals in biological fluids. Such neurochemicals are termed biomarkers and represent a considerable field of research in NDDs. A great amount of work regarding the presence of Aβ as well as total and hyperphosphorylated tau in biological fluids has already been performed, establishing them as core diagnostic cerebrospinal fluid (CSF) biomarkers for AD [95]. Conversely, evidence for their application as biomarkers in other NDDs is unconvincing [96-98]. Alternative candidate biomarkers are CSF neurogranin for AD, neurofilament light for FTLD and ALS, or α-synuclein for DLB [99-101], but given the etiopathological overlap of some NDDs, determining a diagnostic profile based on these biomarkers often remains challenging. Since we know monoamine neurotransmitters and metabolites are affected in NDDs, combining them with previously established/proposed biomarkers for NDDs could improve the diagnostic accuracy. But then again, indications concerning altered monoaminergic content in these disorders should first be identified. In **part 2**, a short overview of

monoaminergic neurotransmitter systems will be presented, serving as a general guide for most of the work performed in this dissertation.

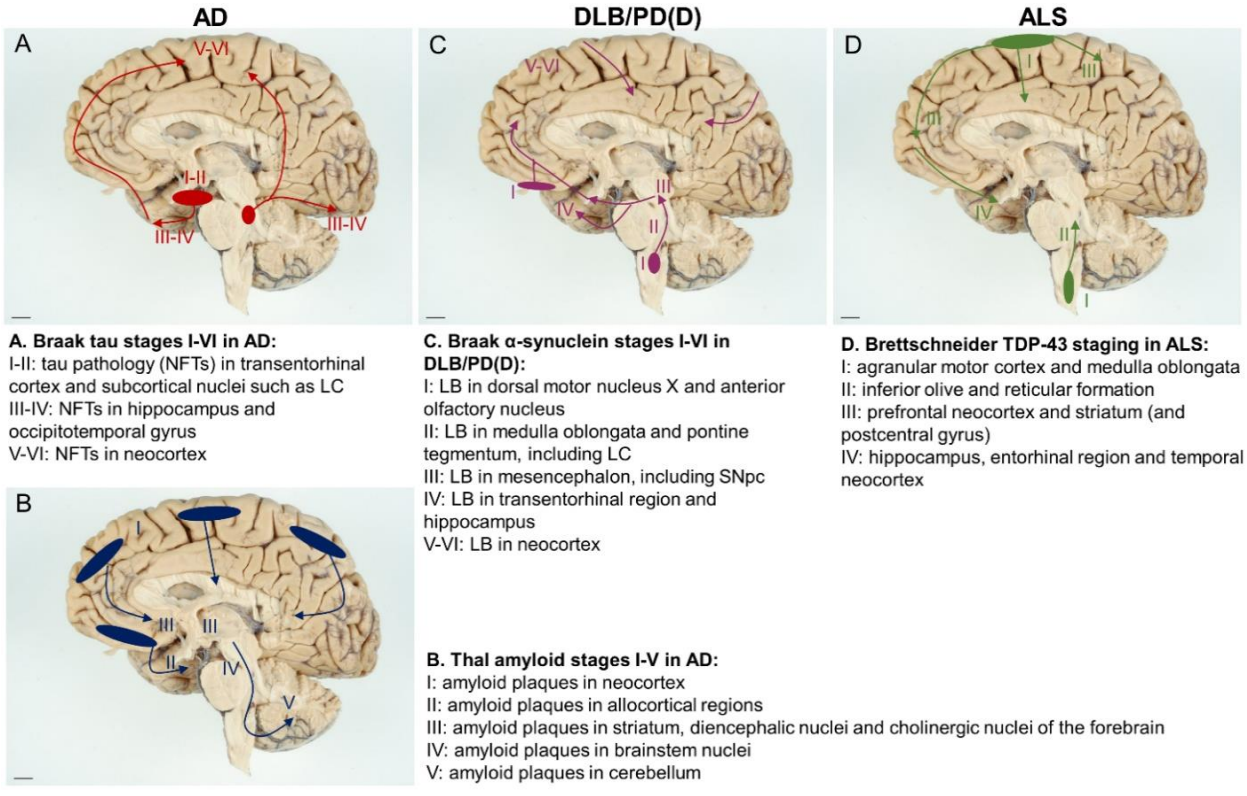

Figure 1. Neuropathological cerebral/cerebellar staging schemes for tau and amyloid-β pathology in AD [9-11], α-synuclein pathology in DLB/PD(D) [56] and TDP-43 pathology in ALS [69]. Abbreviations: AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; DLB: dementia with Lewy bodies; LB: Lewy body; NFTs: neurofibrillary tangles; LC: locus coeruleus; PD(D): Parkinson's disease (dementia); SNpc: substantia nigra pars compacta; TDP-43: transactive response DNA-binding protein of 43 kDa; Brain hemisphere depiction originates from the archives of the NeuroBioBank of the Institute Born-Bunge (NBB-IBB, n° BB 190113).

2. MONOAMINERGIC NEUROTRANSMITTER SYSTEMS

Monoamines are small molecule neurotransmitters or hormones characterized by the presence of an amino group attached to an aromatic ring via a carbon-carbon side chain [102]. This neurotransmitter class can be subdivided into catecholamines, such as dopamine (DA) and (nor)adrenaline ((N)A), and the indoleamine 5-hydroxytryptamine or serotonin (5-HT). These compounds modulate a plethora of central and peripheral functions, including cognition and behavior.

2.1. G protein-coupled receptors

Monoamines exert their effects by binding mainly to G protein-coupled receptors (GPCRs). This receptor type is characterized by seven plasma membrane-spanning domains and represents the largest family of cell surface receptors in the mammalian genome [103]. Given their implication in (monoaminergic) neurotransmission and their high prevalence in the brain, GPCRs also constitute important drug targets. Once a ligand binds to the extracellular binding pocket, a conformational change takes place facilitating coupling to intracellular guanine nucleotide-binding proteins (G proteins). Latter proteins consist of three different subunits (α, β and γ), which dissociate upon the exchange of guanine diphosphate (GDP), bound to Gα in the inactive state, for guanosine triphosphate (GTP). The free G protein subunits interact with enzymatic effectors like adenylate cyclase and phospholipase C, which subsequently regulate the formation of second messenger molecules. Finally, GTP is hydrolyzed to GDP due to the intrinsic GTPase activity of the Gα subunit, terminating the signaling process [103, 104] (**Figure 2**).

A considerable degree of G protein subunit diversity is observed, as there are 20 distinct Gα subunits, 5 Gβ subunits and 12 Gγ subunits [105]. The Gα subunits are further subdivided into four evolutionary families: (i) Gαs, upon activation stimulating and modulating adenylate cyclase, (ii) Gαi, inhibiting and modulating adenylate cyclase activity; (iii) Gαq, modulating phospholipase C activity and (iv) Gα12, regulating rho-guanine nucleotide exchange factors. The Gβγ subunits exert their effects by activating phospholipase C and GPCR kinases. Latter enzymes play a role in homologous desensitization via phosphorylation of the GPCR, which, followed by binding of β-arrestin, results in receptor – G protein uncoupling [106] (**Figure 2**). Finally, GPCRs offer unique opportunities for drug research and development, as they are known to form homo/heterodimers and higher-order oligomers, with resulting alterations in signaling properties as a target for pharmacotherapeutic tuning of various disease processes [107-109].

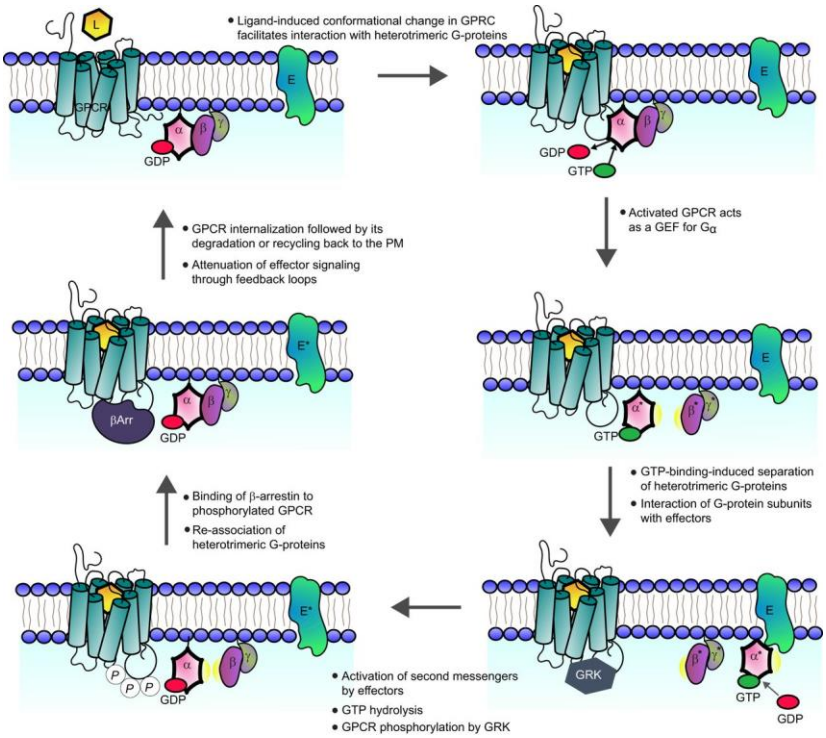

Figure 2. GPCR signaling. Binding of a ligand to the extracellular domain of the GPCR induces a conformational change, leading to the exchange of GDP for GTP. The activated Gα and Gβγ subunits subsequently interact with effectors such as AC or PLC, giving rise to a second messenger cascade. Since Gα has intrinsic GTPase activity, GTP is hydrolyzed to GDP. The GPCR is also phosphorylated by GRKs, resulting in the binding of β-arrestin and internalization of the receptor. The GPCR can then either be degraded or recycled back to the plasma membrane. Abbreviations: βArr: β-arrestin; AC: adenylate cyclase; E: effector; GDP: guanine diphosphate; GPCR: G protein-coupled receptor; GRK: G protein-coupled receptor kinase; GTP: guanine triphosphate. Reproduced with permission from the Journal of Cell Science [110].

2.2. The monoaminergic pathways

2.2.1. Dopaminergic neurotransmitter system

Although the peripheral actions of DA include the regulation of respiration, gastrointestinal motility and blood pressure, metabolic control and tumor growth [111], this monoamine has predominantly been linked to two major processes within the brain: movement and reward-related behavior [112]. Furthermore, DA plays a role in cognition, motivation, and emotion [113]. The ventral part of the mesencephalon contains two major sources of dopaminergic neurons: the SNpc and the ventral tegmental area (VTA), which send their projections to the striatum and limbic and cortical areas, respectively [114]. As such, the nigrostriatal pathway plays an important role in the control of voluntary movement, while the mesolimbic and mesocortical pathways regulate emotion-related behavior, including motivation and reward [115]. These effects are all mediated by binding of DA to five distinct GPCRs constituting the dopamine receptor (D) family. Based on the propensity to either activate or inhibit adenylate cyclase, these receptors are grouped as D1-like receptors (D1 and D5) or as D2-like receptors (D2, D3 and D4) [116]. The synthesis of DA occurs in the cytoplasm of dopaminergic neurons via the hydroxylation of the amino acid tyrosine by tyrosine hydroxylase, representing the rate-limiting step of catecholamine synthesis in general (**Figure 3**) [117]. This first step leads to the formation of L-DOPA, which is subsequently decarboxylated to DA by aromatic amino acid decarboxylase. Latter monoamine is transported into storage vesicles and can be further metabolized to NA and A. On the other hand, DA can also be catabolized by monoamine oxidase (MAO) to 3,4-dihydroxyphenylacetaldehyde (DOPAL). This unstable intermediate undergoes further metabolism by aldehyde dehydrogenase to 3,4-dihydroxyphenylacetic acid (DOPAC), representing a first stable metabolite of DA. Through the enzymatic activity of catechol-O-methyltransferase (COMT), DOPAC is finally converted to the second stable dopaminergic metabolite, homovanillic acid (HVA) [118].

2.2.2. (Nor)adrenergic neurotransmitter system

Contrary to DA, the peripheral effects of (N)A in the 'fight-or-flight response' are possibly better known than their central actions. As such, A is implicated in the regulation of autonomic activities in the brain [119], whereas NA is involved in aggressive behavior, sleep, wakefulness, attention and cognition [120, 121]. The locus coeruleus (LC) in the brainstem represents the main source of (nor)adrenergic neurons in the CNS, with projections to virtually all cortical areas [121]. However, the central source of A consists of sparse and widespread neurons in the brain [122], in conjunction with noradrenergic nerve terminals containing phenylethanolamine-N-methyltransferase (PNMT) [123]. The GPCRs belonging to

the adrenergic receptors are subdivided into the α_1, α_2 and β receptor families based on their affinity for (N)A and other ligands, and differential coupling to G proteins [121, 124]. The α_1 receptors comprise α_{1A}, α_{1B} and α_{1D}, are coupled to Gαq proteins, while α_2 receptors consisting of $\alpha_{2A\text{-}C}$, signal via Gαi proteins. The affinity of α receptors is largely equal for NA and A. Lastly, $\beta_{1\text{-}3}$ adrenoceptors are coupled to Gαs subunits and show differential affinities for NA and A, with β_1 and β_3 having equal affinity for NA and A, and β_2 having a larger affinity for A than for NA [121]. The synthesis and metabolism of (N)A is intertwined with dopaminergic metabolism (**Figure 3**). As such, NA is synthesized from DA in storage vesicles by DA β-hydroxylase. In a successive step mediated by cytoplasmic PNMT, NA is metabolized to A. Via the sequential actions of COMT and MAO, NA and A are catabolized to 3-methoxy-4-hydroxyphenylglycol (MHPG) and vanillylmandelic acid [118].

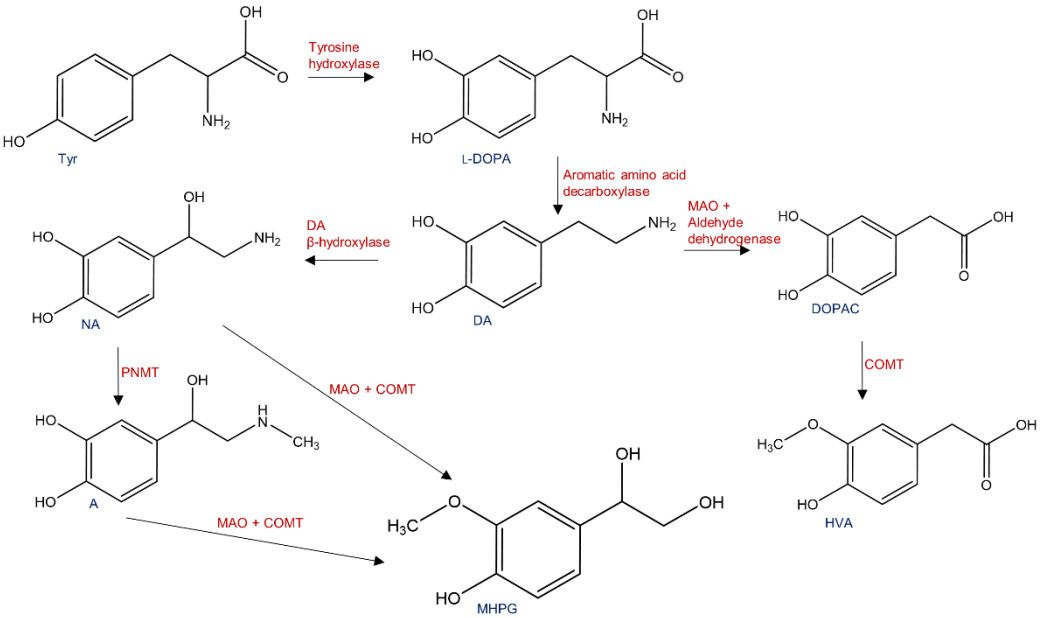

Figure 3. Catecholamine metabolism. Precursor, catecholamines, intermediate products and metabolites are indicated in blue, while enzymes are indicated in red. Abbreviations: A: adrenaline; COMT: catechol-O-methyltransferase; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; L-DOPA: L-3,4-dihydroxyphenylalanine; HVA: homovanillic acid; MAO: monoamine hydroxylase; MHPG: 3-methoxy-4-hydroxyphenylglycol; NA: noradrenaline; PNMT: phenylethanolamine-N-methyltransferase; Tyr: L-tyrosine.

2.2.3 Serotonergic neurotransmitter system

Since the initial discovery of 5-HT as a vasoconstrictor, new peripheral functions have been identified, including the regulation of hemostasis, heart rate, vascular tone, intestinal motility, vascular cell growth, immune-related functions and development of several organs [125, 126]. The large majority of peripheral 5-HT is synthesized in enterochromaffin cells in the intestine and subsequently taken up by blood platelets [126]. In the CNS, 5-HT is involved in depression, pain, circadian rhythm, aggression, feeding behavior, affective processes and anxiety [126, 127]. Here, 5-HT is produced in the raphe nuclei (RN), flanking the midline of the brainstem. The rostral cell group of the RN sends projections to the forebrain, whereas the caudal group has projections to the caudal brainstem and spinal cord [128]. As many as 14 5-HT receptors classified into seven subfamilies ($5\text{-HT}_{1\text{-}7}$) have been proposed [129]. The $5\text{-HT}_{1A,B,D,F}$ receptors preferentially couple to Gαi proteins, as well as the 5-ht_{1E} receptor, although the putative endogenous GPCR has not yet been established. In addition, $5\text{-HT}_{2A\text{-}C}$ receptors couple to Gαq/11 subunits, whereas the 5-HT_3 receptor is a ligand-gated ion channel. Serotonin receptors stimulating adenylate cyclase activity by coupling to Gαs, are 5-HT_4, 5-HT_6 and 5-HT_7. Finally, the function and coupling of $5\text{-ht}_{5A\text{-}B}$ receptors remains to be elucidated, although with little evidence of the presence of these GPCRs in native cells and tissues, coupling to Gαi/o and Gαs has previously been suggested [129].

While 95% of the essential amino acid L-tryptophan (TRP) is metabolized in the kynurenine pathway [130], the first and rate-limiting step of 5-HT synthesis is mediated by TRP hydroxylase, converting TRP to 5-hydroxytryptophan (5-HTP). This intermediate is subsequently decarboxylated by 5-HTP decarboxylase to form 5-hydroxytryptamine, 5-HT or serotonin [131], after which 5-HT is stored in synaptic vesicles. The main catabolic route for 5-HT is represented by oxidation to 5-hydroxyindoleacetic acid (5-HIAA) by MAO [132] (**Figure 4**). Alternatively, in the pineal gland and other structures, 5-HT is also metabolized to melatonin by 5-HT N-acetyltransferase and hydroxyindole-O-methyltransferase [133].

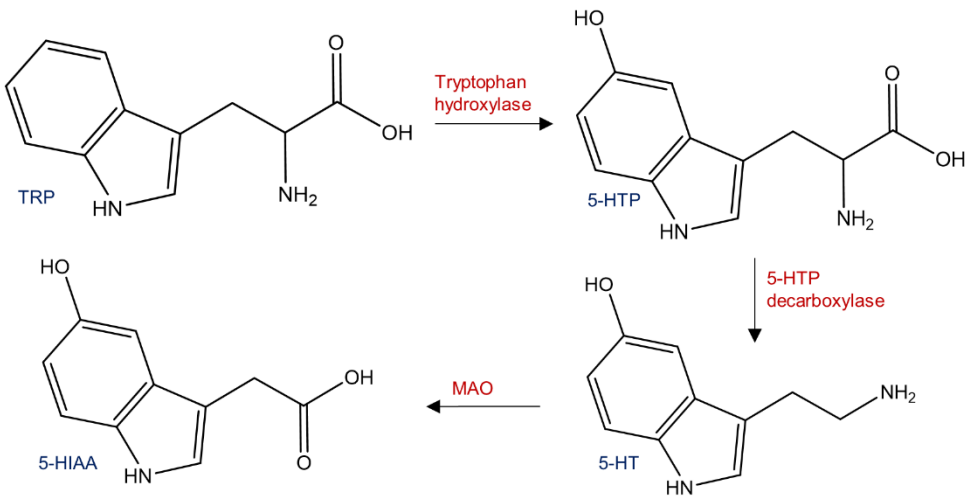

Figure 4. 5-HT metabolism. Precursor, intermediate, 5-HT and metabolites are indicated in blue, while enzymes are indicated in red. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; 5-HT: 5-hydroxytryptamine or serotonin; 5-HTP: 5-hydroxytryptophan; MAO: monoamine oxidase; TRP: L-tryptophan.

Chapter II: Aims & Outline of the dissertation

The overall aim of this thesis was to neurochemically characterize distinct neurodegenerative disorders (NDDs), with respect to monoamine and metabolite levels and their associated G protein-coupled receptors (GPCRs), in order to identify potential biomarker candidates and contribute to the fundamental insights regarding their possible role in NDDs.
While doing so, we encountered some methodological issues requiring further investigation for optimization of our (pre-)analytical methods and techniques. Despite the fact that these methodological studies were carried out after most other examinations in this dissertation, they will be presented first, followed by the results of the neurochemical characterization of NDDs in subsequent chapters.

Thus, the **first aim of this thesis** was to address the confounding effect of circadian rhythm and the rostrocaudal concentration gradient (RCG) on monoamines and metabolites in biological fluids measured by reversed-phase (ultra-) high performance liquid chromatography with electrochemical detection (RP-(U)HPLC-ECD).
Likewise, conducting reverse-transcription quantitative PCR (RT-qPCR) analyses to assess mRNA levels of several GPCRs involved in the pathophysiological neurotransmission of NDDs led us to consider an alternative normalization strategy using short interspersed nuclear elements in brain tissue of the APP23 amyloidosis mouse model for Alzheimer's disease (AD), representing the **second aim of this thesis**. Abovementioned issues and proposed solutions are presented in **Chapter III**: "*Pitfalls in RP-(U)HPLC-ECD and RT-qPCR*", as two published research papers.

Chapter IV: "*Monoaminergic disturbances in neurodegenerative disorders*" represents the work performed to accomplish the **third aim of this thesis**: distinguishing NDDs based on monoaminergic content in brain and biological fluids and identifying possible new biomarkers. Besides the monoaminergic compounds, determined via RP-(U)HPLC-ECD, we also developed an interest in the kynurenine pathway (KP) and its involvement in NDDs. Because the kynurenines were investigated by a mass spectrometry approach in only one study, the KP will be presented in the corresponding manuscript. Thus, Chapter IV comprises a research paper on monoaminergic content in brain tissue of behavioral variant frontotemporal dementia (bvFTD) versus AD and control subjects (CONTR) and its relation with behavior, as well as another paper presenting the biomarker potential of monoamines and metabolites in biological fluids of dementia with Lewy bodies (DLB), AD and FTD versus CONTR. In addition, this chapter includes a study investigating the amyotrophic lateral sclerosis (ALS)-FTD continuum with respect to monoamines and kynurenines in CSF and serum, and a viewpoint on serotonergic (dis)similarities between ALS and Parkinson's disease (PD).

Lastly, we also investigated the occurrence of neurotransmission-related GPCRs (serotonin (5-hydroxytryptamine; 5-HT) receptor 1A, 7 (5-HT_{1A}, 5-HT_7) and β_3 adrenoceptor) in brain tissue of AD versus CONTR using RT-qPCR, and their correlation with monoamine content and behavior. These data are grouped into one manuscript with RT-qPCR results of cholinergic and glutamatergic GPCRs, and protein data of 5-HT_6 expression obtained by western blot and double-labelling immunofluorescence in the hippocampus of the APP23 model. With **Chapter V**, we thus tried to accomplish the **fourth aim of this thesis**: providing evidence regarding pathophysiological expression of GPCRs in AD, and how these expression levels relate to concentrations of monoaminergic compounds.

The aims and outline are graphically represented in **Figure 1**.

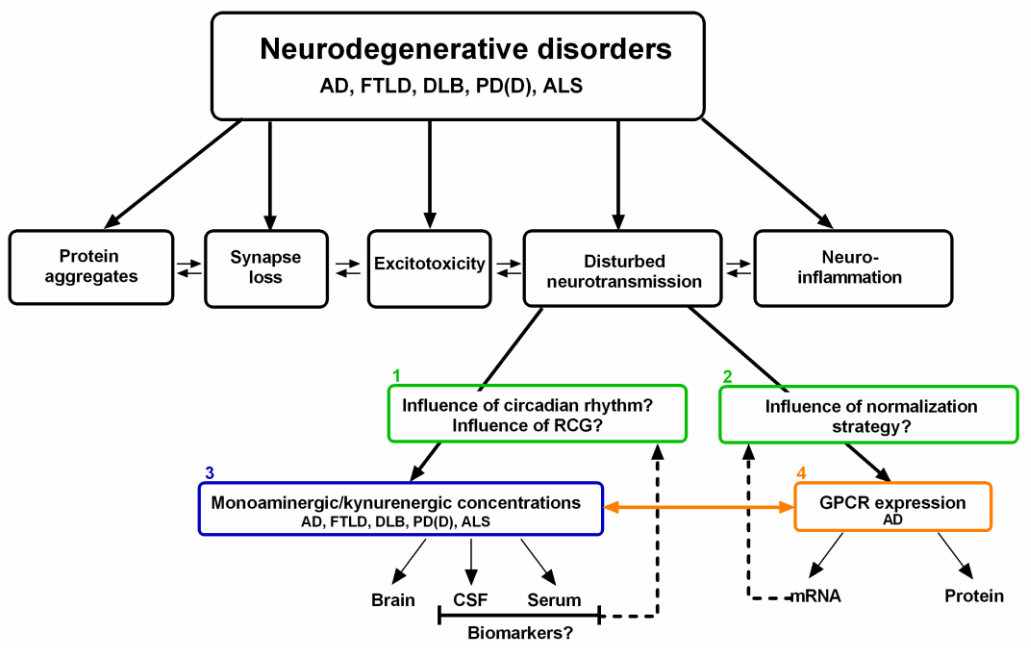

Figure 1. Graphical representation of the aims and outline of the thesis. Numerals represent the four aims of the project, while green, blue and orange text boxes indicate the second, third and fourth chapter, respectively, containing the corresponding aims. Dotted black lines represent methodological issues encountered while conducting (ultra)-high performance liquid chromatography with electrochemical detection and reverse-transcription quantitative PCR, prompting investigation of these issues as separate aims. Abbreviations: AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; DLB: dementia with Lewy bodies; FTLD: frontotemporal lobar degeneration; GPCR: G protein-coupled receptor, PD(D): Parkinson's disease (dementia); RCG: rostrocaudal concentration gradient in cerebrospinal fluid.

Chapter III

Pitfalls in the setup of RP-(U)HPLC-ECD and RT-qPCR analyses

Chapter III.1. Sampling issues of cerebrospinal fluid and plasma monoamines: investigation of the circadian rhythm and rostrocaudal concentration gradient

Jana Janssens[a,1], Sawal D. Atmosoerodjo[b,1], Yannick Vermeiren[a,d], Anthony R. Absalom[c], Izaak den Daas[b], Peter P. De Deyn[a,d,e,f,*]

[a]*Laboratory of Neurochemistry and Behavior, Institute Born-Bunge, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium*

[b]*QPS Netherlands BV, Groningen, The Netherlands*

[c]*Department of Anaesthesiology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands*

[d]*Department of Neurology and Alzheimer Center, University of Groningen and University Medical Center Groningen (UMCG), Hanzeplein 1, 9713 GZ Groningen, The Netherlands*

[e]*Department of Neurology and Memory Clinic, Hospital Network Antwerp (ZNA) Middelheim and Hoge Beuken, Lindendreef 1, 2020 Antwerp, Belgium*

[f]*Biobank, Institute Born-Bunge, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium*

[1]These authors contributed equally to this work.

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ABSTRACT

Biomarkers for neurodegenerative dementias offer interesting prospects regarding diagnosis and disease monitoring. Monoamines such as dopamine, (nor)adrenaline, serotonin (5-hydroxytryptamine or 5-HT), and their respective metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylglycol (MHPG), and 5-hydroxyindoleacetic acid (5-HIAA), were shown to be altered in dementia, including Alzheimer's disease (AD). Biomarker research is hampered by potential confounds including the influence of time of day and volume of cerebrospinal fluid (CSF) collected. Therefore, the possibility of a circadian rhythm in CSF and plasma, and the presence of a rostrocaudal concentration gradient (RCG) in CSF for aforementioned monoamines/metabolites, were investigated.

Circadian rhythmicity was assessed using reversed-phase ultra-high performance liquid chromatography with electrochemical detection (RP-UHPLC-ECD) to measure monoamine/metabolite concentrations in 271 paired CSF and plasma samples, successively collected over a period of 30 hours and derived from eight healthy subjects. Plasma samples were also analyzed for melatonin, serving as positive control analyte, using ELISA. The RCG examination entailed RP-UHPLC-ECD analyses on five consecutive CSF samples derived from 10 patients with AD and 10 non-AD/control subjects.

Besides a diurnal rhythm for melatonin, we found a similar rhythmicity for plasma HVA, with acrophases occurring between 02:00 and 06:00 hours, in four out of seven subjects. Three and two subjects showed a circadian rhythm for CSF HVA and 5-HIAA, respectively. No rhythmicity was observed in any other compound. We found that only CSF MHPG, HVA and 5-HIAA levels differed across CSF fractions, and that these changes in 5-HIAA levels varied in the AD versus non-AD/control group. Positive correlations between CSF volume and HVA and 5-HIAA levels, indicative of a RCG, were also observed. Such a RCG could not be detected for the other monoamines/metabolites. Our results stress the importance of standardizing sampling procedures of biological fluids with respect to time of day, volume and number of samples.

1. INTRODUCTION

1.1. Monoamines as biomarkers for neurodegenerative dementias

Cerebrospinal fluid (CSF)- and blood-based biomarkers represent interesting opportunities for the differential diagnosis of neurodegenerative dementias [134]. For example, amyloid-β peptide of 42 amino acids ($A\beta_{1-42}$), total tau (T-tau) and tau phosphorylated at threonine 181 ($P\text{-}Tau_{181P}$) are widely known as the standard CSF biomarker panel to aid in the differential diagnosis of Alzheimer's disease (AD) [135, 136]. Besides their application in diagnostic evaluation, biomarkers are implemented in other contexts of use, such as disease- and treatment response monitoring [137]. In this aspect, blood-based biomarkers are preferred over CSF markers given the relative invasiveness and practical disadvantages inherent to a lumbar puncture (LP) [137, 138]. Nevertheless, a suitable blood biomarker for AD diagnosis or disease monitoring, is still lacking. Some promising candidate molecules (plasma T-tau and serum neurofilament light chain) have already been identified (reviewed by [139]), but still face several challenges, among which are a lack of reproducibility [140, 141].

In addition, despite their usefulness for the diagnosis of AD, CSF $A\beta_{1-42}$, T-tau and $P\text{-}Tau_{181P}$ were proven to have little efficacy in diagnosing dementias other than AD [96-98, 142]. However, distinct dementia types were previously hypothesized to have dissimilar neurochemical underpinnings with regard to monoamine content in the CNS, including dopamine (DA), (nor)adrenaline ((N)A), and serotonin or 5-hydroxytryptamine (5-HT), and their respective metabolites, i.e. 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycol (MHPG), and, 5-hydroxyindoleacetic acid (5-HIAA) [143]. Studies investigating brain areas of patients suffering from AD, frontotemporal dementia (FTD) and dementia with Lewy bodies (DLB) support this theory [143, 144]. These dissimilarities in monoaminergic compounds between AD and other dementias, have also been identified in CSF and serum [145-149]. For instance, the addition of CSF (and serum) MHPG - the major metabolite of (N)A indicating noradrenergic turnover [150] - to the standard CSF biomarker panel for AD, improved the ability of the panel to differentiate AD and DLB [146, 147]. Taken together, monoamines and/or their metabolites in CSF and blood are potentially of added value as diagnostic biomarkers for dementia, whether they are combined with a previously validated biomarker panel, or implemented as stand-alone compounds [147].

1.2. Difficulties associated with sampling of CSF and plasma biomarkers

One of the difficulties concerning biomarker research, is the lack of reproducibility across laboratories [134]. The pre-analytical phase, comprising sample collection, -handling, -transport, and -storage, is reported to be specifically prone to errors, accounting for up to 70% of all mistakes in laboratory diagnostics [151, 152], and possibly impacting the quality of biomarker assessment [134]. Efforts should be made to develop a standardized and holistic approach covering the use of correct laboratory equipment, sampling and storage volume, appropriate temperature for transport and storage, centrifugation settings and fasting status of study participants [134, 141, 151, 153].

Another possible source of variability between the findings of different groups is the potential influence of time of day and volume of CSF aspirated. This work will primarily focus on sampling time, with respect to the circadian rhythm of monoamines and/or their metabolites in CSF and plasma, and the presence of a possible rostrocaudal concentration gradient (RCG) of these compounds in CSF.

1.2.1. Circadian rhythm of monoaminergic compounds in CSF and plasma

While some studies suggest the existence of a physiological circadian rhythm of CSF Aβ levels [154-156], little evidence exists concerning possible circadian fluctuations of monoaminergic compounds in human (patho)physiology. Dopaminergic compounds, including DA and HVA, were found to fluctuate diurnally in the CSF of patients suffering from Parkinson's disease, restless legs syndrome and control subjects [157]. Alternatively, a circadian rhythm for 5-HT was reported in serum derived from healthy and schizophrenic subjects [158], as well as in whole blood samples of controls and depressed patients [159]. The main metabolite of 5-HT, 5-HIAA, was also shown to have a circadian rhythm contrary to that of its precursor in whole blood [159]. Diurnal fluctuations of plasma and serum (N)A have also been reported [160, 161], although other studies found an ultradian rhythm in the noradrenergic neurotransmitter system, consisting of 20-30 pulses of (N)A per 24 hours (hrs), depending on the analysis software [162]. Finally, concentrations of plasma MHPG were also shown to vary diurnally in six control subjects [163]. These findings could also be corroborated by a later study investigating plasma MHPG levels in healthy volunteers as well as in patients suffering from depression [164].

1.2.2. Rostrocaudal concentration gradient

Whereas limited evidence exists concerning diurnal rhythms of monoamines/metabolites in biological fluids, state of the art evidence generally agrees on the existence of a RCG for CSF HVA and 5-HIAA. A RCG of these compounds was indeed observed in healthy adult control subjects as well as in patients suffering from neuropsychiatric diseases, such as vascular dementia, mixed dementia, organic depression and psychosis, which did not affect CSF circulation. In contrast, MHPG levels did not show any variation in consecutive CSF samples [165]. Similar findings were reported in a study analyzing monoaminergic metabolites in CSF derived from children and adolescents aged 6.50–17.25 years with obsessive-compulsive disorder and/or attention deficit disorder [166]. In addition, levels of HVA and 5-HIAA in consecutive CSF fractions derived from patients with (possible) hydrocephalus also rose with increasing sampling volume in two separate studies [167, 168]. Concentrations of MHPG were previously reported to be comparable across CSF fractions [166, 168], although another study reported increased MHPG levels with increasing CSF sampling volume [169]. However, current literature provides little support for a RCG in levels of monoamine neurotransmitters. Most of the aforementioned studies also date from two decades ago, and analytical research methods have improved ever since.

1.3. Aims and hypothesis

The aim of this work is to elaborate on the presence of a possible circadian rhythm in plasma and CSF monoamines and their metabolites to establish an optimal sampling time of these biological fluids. We included plasma melatonin as a positive control analyte, given the extensive evidence of its circadian rhythm [170-173]. Based on the available literature, we expected to find a circadian rhythm in plasma and CSF for 5-HT and 5-HIAA in particular, since these compounds are closely related to melatonin. In addition, we intended to provide evidence for a RCG of monoamine neurotransmitters and their metabolites, with special regard to HVA and 5-HIAA.

2. MATERIALS & METHODS

2.1. Study population

The inclusion procedure for the study of circadian rhythm has been described in detail in previous work [174, 175]. In short, eight healthy males aged 50-75 were enrolled, of whom seven completed the entire protocol. The exclusion parameters for this study entailed a body mass index >27 $kg.m^{-2}$, body weight >100 kg, acute

cellulitis, onychomycosis of the feet, increased white blood cell count, poor venous access, history of lower back pain, significant kyphosis and/or scoliosis or other spinal column deformities, persisting fibromyalgia, a history of difficult LP, a history of and/or any sign or symptom indicating abnormal hemostasis or blood dyscrasia and any disorder which could lead to increased intracranial pressure, such as glaucoma or hydrocephalus-related pathology. Elderly men were chosen since they have lower intradural cavity pressure and thus have a reduced likelihood of CSF leakage and post dural puncture headache. After a screening period, the subjects arrived at the clinic on day -2. During the next day (day -1), the volunteers received 2 L of 0.9% saline solution by intravenous infusion. On day 1, intradural catheterization was performed under strict aseptic conditions by an experienced anesthesiologist. At 12 hrs and 36 hrs after intradural catheterization, the subjects received fraxiparine for antithrombotic prophylaxis. During this period, subjects were allowed to move around freely and samples of 2 mL each were collected in 5 mL polypropylene tubes by interval sampling over a 30-hour period starting at 10:00 hrs (t=1) of day 1 (Figure 1). Samples were withdrawn at hourly intervals by aspiration with a syringe during the first 12 hrs, at four-hour intervals between sampling times 13 and 16, and, finally, at two-hour intervals from sampling time 16 until 19 (Figure 1).

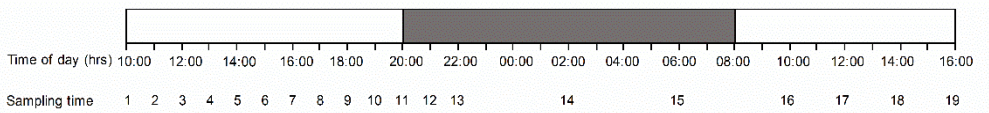

Figure 1. Time schedule of CSF and plasma sampling. Sampling started at 10:00 hrs on day 1, and lasted until 16:00 hrs on day 2. During the first 12 hrs, CSF and plasma were collected hourly, followed by three four-hour sampling intervals. For the last six hours of the protocol, samples were taken every two hrs. Abbreviations: CSF: cerebrospinal fluid; hrs: hours.

This led to a total of 19 paired CSF and plasma serial samples per subject, resulting in a total of 271 CSF and plasma samples. This study was approved by the local medical ethics committee (*Stichting Beoordeling Ethiek Bio-Medisch Onderzoek*, Assen, The Netherlands) and conducted in compliance with the Helsinki declaration and the Guideline for Good Clinical practice.

For investigation of the RCG, 10 probable AD patients and 10 probable non-AD/control subjects were included via a study protocol concerning CSF biomarkers of dementia [176]. The diagnosis of probable AD was routinely made according to the revised criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [16]. Additionally, all AD patients fulfilled the criteria of the

Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV-TR) [177]. The clinically diagnosed AD patients underwent a LP and neurocognitive assessment including the Mini-Mental State Examination [178] as part of their diagnostic work-up of probable dementia. In addition, an AD-specific, pathological CSF biomarker profile was determined in these patients shortly thereafter (i.e. CSF levels of $A\beta_{1\text{-}42}$<638.5 pg/ml, T-tau>296.5 pg/ml, and P-tau_{181P}>56.5 pg/ml [96]. Similarly, age- and gender-matched individuals who had subjective memory complaints and/or were referred to a neurologist on the suspicion of dementia, underwent LP and neurocognitive assessment. Although these 10 subjects had normal, physiological biomarker profiles, a non-AD dementia diagnosis could not be fully excluded solely based upon the measured $A\beta_{1\text{-}42}$, T-tau and P-tau_{181P} levels. All included subjects were recruited at the Memory Clinic of the Hospital Network Antwerp Middelheim (ZNA) and Hoge Beuken (Antwerp, Belgium). According to the aforementioned protocol, five consecutive fractions of CSF were collected from both the non-AD/control and AD groups [176]. Lumbar puncture was performed at the L3/L4 or L4/L5 interspace between 08:00 and 10:00 hrs, after fasting and abstention from smoking for at least 12 hrs. Approximately 16.5 mL of CSF was collected in an ordered manner: C1 (4.5 mL), C2 (1.5 mL), C3 (1.5 mL), C4 (4.5 mL) and C5 (4.5 mL) fractions were sampled in polypropylene vials (Nalgene; VWR, Leuven, Belgium), making up a total of 100 samples for the RCG analysis of CFS monoamines. Samples were placed in liquid nitrogen immediately after LP, and stored at -80°C until neurochemical analysis. For routine AD CSF biomarker analyses, the C2 fraction was used. Biomarkers were measured via single analyte ELISA-kits (INNOTEST, Fujirebio, Ghent, Belgium).

2.2. RP-UHPLC-ECD

The quantification of CSF and plasma monoamines and their corresponding metabolites was performed using an optimized Alexys Neurotransmitter Analyzer with electrochemical detection (ECD) (Antec Leyden BV, Zoeterwoude, The Netherlands). This reversed-phase ultra-high performance liquid chromatography (RP-UHPLC) system operated at an isocratic flow rate of 75 µL/min. The Decade II electrochemical detector was equipped with a thin layered electrochemical VT03 flow cell fitted with a glassy carbon 0.7 mm working electrode and an in situ Ag/AgCl (ISAAC) reference electrode. Integration of chromatograms was performed with channel integration M018/EN25B Clarity software (DataApex Ltd., Prague, The Czech Republic, version 6.2). The mobile phase consisted of 11% methanol combined with citric acid (100 mM), phosphoric acid (100 mM), octane-1-sulfonic acid sodium salt (2.8 mM), KCl (8 mM), and, ethylenediaminetetraacetic

acid (0.1 mM). The pH of the mobile phase was set at 3.0. Samples of 5 µL were loaded with an Alexys AS 110 Autosampler. Separation was achieved using a short 15 cm Waters Acquity Column (BEH C18, 1 mm diameter, particle size 1.7 µm), delivering optimal performances for monoamine analysis. Total runtime for each sample was under 15 min, after which all eight monoamines and metabolites were detected. With respect to the sample preparation protocol, Amicon Ultracentrifugal filters (3,000 Da; Millipore, Ireland) were washed twice by centrifugation of 450 µL of sample preparation buffer at 14,000 x g for 25 min at 4°C. Next, 400 µL of plasma or CSF was directly transferred to the washed Amicon filters and centrifuged at 14,000 x g for 40 min, still at 4°C. After centrifugation, one fraction of the eluate was diluted 1:4 and injected in the RP-UHPLC-ECD system in the case of both CSF and plasma, while a second 1:20 plasma dilution was also analyzed.

2.3. ELISA

Enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturer's instructions (RE54021; IBL. International GMBH, Hamburg, Germany) to determine plasma melatonin levels.

2.4. Statistics

For the analysis of circadian rhythm, mixed linear model analysis implementing compound symmetry covariance structure with heterogeneous variances, was performed on log-transformed plasma melatonin and plasma/CSF monoamine concentrations. Parameter estimation for these models was obtained implementing the restricted maximum likelihood method. In case a statistically significant effect of time was detected, post-hoc analysis consisted of pairwise comparisons based on the least-squares means, with Bonferroni-adjusted p-values (statistically significant if $P<0.05$). In addition, single cosinor analysis was applied to estimate the acrophase, amplitude and midline estimating statistic of rhythm (MESOR) of circadian rhythms displayed by melatonin and monoaminergic compounds. For the investigation of the RCG in CSF monoamines and/or metabolites, the independent-samples T-test and chi-square statistics were applied to verify if AD- and non-AD/CONTR groups were age- and gender-matched, respectively. In addition, mixed factorial ANOVA with CSF fraction as within-subjects variable and diagnostic class as between-subjects factor, was used to analyze the effects of the possible RCG and/or disease status on CSF monoamine/metabolite levels. In case the assumption of sphericity was violated, the Greenhouse-Geisser correction was applied. Post-hoc pairwise comparisons based on the estimated marginal means were performed to detect differences in

monoamine/metabolite levels between CSF fractions. Again, p-values were adjusted according to the Bonferroni correction for multiple comparisons. Finally, the correlation between withdrawn CSF volume and concentrations of monoaminergic compounds was tested using Spearman's correlation statistics. All statistical analyses, except for cosinor analysis, were performed using SPSS 24.0 for Windows (IBM SPSS Software, Armonk, NY, IBM Corp.). Cosinor models were fitted using the cosinor package in R [179], version 3.4.0 for Windows, while the zero-amplitude test for the detection of a statistically significant rhythm was performed using the cosinor2 package [180]. Figures were produced using GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

3. RESULTS

3.1. Demography – circadian rhythm

The demographic characteristics of subjects included for the study of circadian rhythm, are summarized in Table 1. Plasma and CSF samples were stored at -70°C within 30 min after sampling, and were thawed twice beforehand to facilitate assays for other substances [174].

Table 1. Demographics of the subjects included for the study of circadian rhythm.

Parameter	
Age (years)	62.5 (8.3)
Gender (male/female)	8/0
Weight (kg)	79.4 (9.5)
Height (cm)	179.0 (16.3)
BMI (kg/m^2)	24.3 (2.6)

Data are represented as median, with the IQR denoted between brackets. Abbreviations: BMI: body mass index; IQR: interquartile range.

3.2. Mixed linear model analysis to detect concentration changes over time

The concentration changes of plasma melatonin and CSF and plasma HVA over time, are depicted in Figure 2. Mixed linear model analysis on log-transformed melatonin values indicated a statistically significant effect of sampling time ($F(18, 24.79)=3.61$; $P<0.05$), with pronounced post-hoc differences between time point 14 of sampling, corresponding to 02:00 hrs, and almost all other time points (Table 2). In addition, log-transformed HVA concentrations in CSF ($F(18, 23.39)=5.16$; $P\leq0.001$), as well as in plasma ($F(18, 17.34)=2.58$; $P<0.05$), varied significantly

across time points. No differences could be detected in plasma HVA levels after correcting for multiple comparisons. As for log-transformed 5-HIAA levels in CSF, a significant effect of sampling time was also found ($F(18, 21.61)=2.15$; $P<0.05$), although only differences between the second and 17th, as well as between the second and 19th time points remained significant after Bonferroni correction (Table 2). Finally, a statistically significant difference across sampling times was detected for log-transformed plasma DOPAC levels ($F(18, 14.30)=2.48$; $P<0.05$), although only the pairwise comparison between the 17th and 18th time point remained significant after the post-hoc analysis. Statistically significant post-hoc differences in monoaminergic metabolite concentrations are summarized in Table 2, and raw data representing the mean concentrations at each significant time point, are represented in Supplementary Table 1.

3.3. Circadian rhythm analysis

A statistically significant diurnal rhythm could be corroborated for plasma melatonin in five out of seven subjects, while four out of seven subjects showed such a rhythmicity for plasma HVA. All estimated parameters and statistics for plasma melatonin and HVA in each subject are represented in Supplementary Table 2. The acrophase of plasma melatonin, indicating the time at which its concentrations were highest, was at night, ranging from about 02:00 to 05:30 hrs and corresponding to sampling point 14, for which most of the pairwise differences were found (Figure 2, panel A; Table 2). A clear surge of plasma melatonin levels can indeed be observed at sampling time 14, followed by a rapid decrease of melatonin concentration. For two out of seven subjects, the assumption of normality of residuals was violated. In case of plasma HVA, four out of seven subjects showed a statistically significant diurnal rhythm, of which the acrophase ranged from about 01:00 to 04:30 hrs. Here, a more steady rise and decrease in analyte concentrations was observed (Figure 2, panel B). However, the assumption of normality was not met for three individuals. No circadian rhythm could be observed in plasma DOPAC concentrations. Remarkably, a larger variability in CSF analyte levels was generally observed compared to plasma. Two out of seven subjects also showed a significant rhythm for CSF HVA, with peak levels at 04:53 hrs, while the acrophase of the third individual with a significant rhythmicity, was at around 20:00 hrs (Figure 2, panel C). In addition, CSF HVA data of two subjects did not comply with the assumption of independence of residuals. Our results also indicated a 24-hour rhythm in CSF 5-HIAA levels in two out of seven subjects, with acrophases ranging from 20:36–04:09 hrs (Figure 2, panel D). Lastly, data of four subjects did not comply with the assumption of independent observations, and an

additional non-normal distribution of CSF 5-HIAA levels was found in two of these four participants.

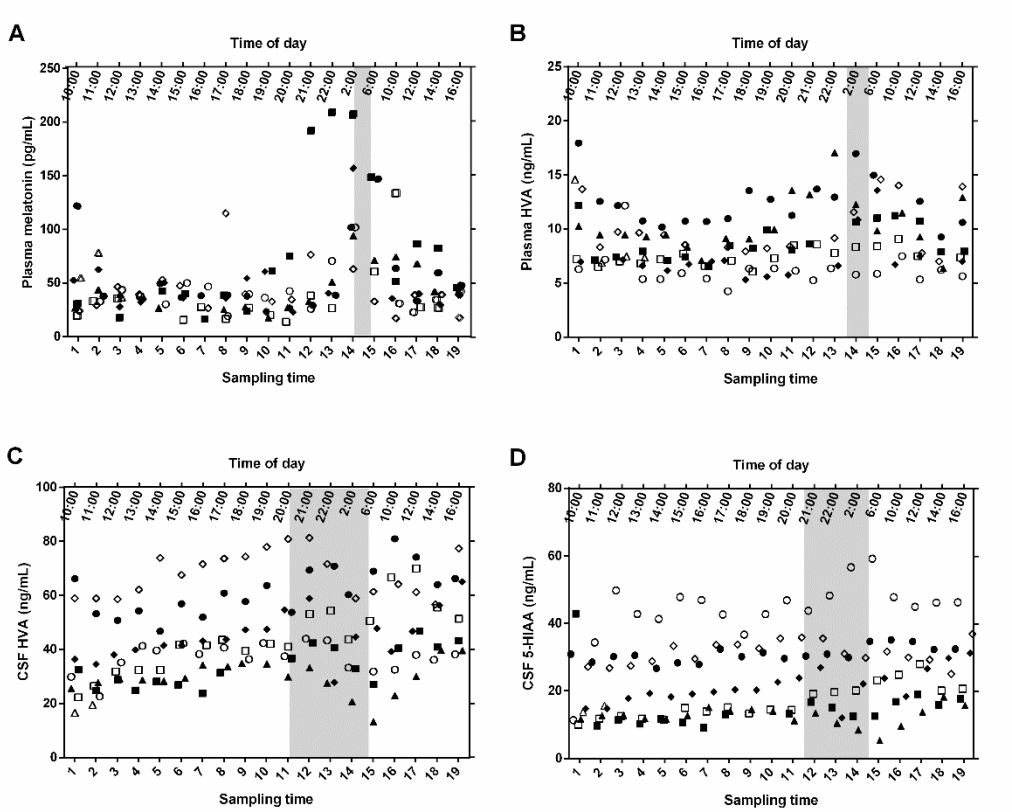

Figure 2. Plasma melatonin and HVA concentrations, as well as CSF HVA and 5-HIAA levels, as a function of sampling time (panels A, B, C, and D respectively). The range of statistically significant acrophases for each compound, is indicated in grey. A: The plasma melatonin concentration at time point 14 differed significantly compared to almost all other time points. B: The acrophase of plasma HVA is situated in a similar time frame compared to melatonin. C, D: CSF HVA and 5-HIAA levels did not show a clear diurnal rhythm, and were characterized by a rather high variability. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; CSF: cerebrospinal fluid; HVA: homovanillic acid.

Table 2. Pairwise comparisons between sampling times of melatonin or monoaminergic compounds in CSF or plasma.

Parameter	Pairwise comparison (Sampling time)	Mean difference ± SE	p-value
Plasma Melatonin (Log(pg/mL))	2-14	-0.404 ± 0.089	0.036
	3-14	-0.508 ± 0.090	0.004
	4-14	-0.486 ± 0.072	0.001
	5-14	-0.438 ± 0.085	0.006
	6-14	-0.491 ± 0.096	0.005
	7-14	-0.569 ± 0.107	0.002
	8-14	-0.504 ± 0.121	0.031
	9-14	-0.500 ± 0.083	0.001
	10-14	-0.524 ± 0.101	0.003
	11-14	-0.548 ± 0.103	0.002
	14-17	0.423 ± 0.100	0.036
	14-18	0.415 ± 0.090	0.017
	14-19	0.479 ± 0.093	0.004
CSF HVA (Log(ng/mL))	2-10	-0.164 ± 0.036	0.044
	2-12	-0.205 ± 0.035	0.003
	2-17	-0.179 ± 0.037	0.006
	2-19	-0.208 ± 0.035	0.004
	3-12	-0.143 ± 0.029	0.005
	3-18	-0.116 ± 0.026	0.024
	3-19	-0.146 ±0.028	0.002
CSF 5-HIAA (Log(ng/mL))	2-17	-0.172 ± 0.040	0.044
	2-19	-0.179 ± 0.040	0.030
Plasma DOPAC (Log(ng/mL))	17-18	0.129 ± 0.026	0.027

Melatonin, HVA, 5-HIAA and DOPAC concentrations were log-transformed to fulfill the assumption of normality of residuals. Only statistically significant differences after post-hoc Bonferroni correction ($P < 0.05$) are displayed. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; CSF: cerebrospinal fluid; HVA: homovanillic acid; SE: standard error.

3.4. Demography – rostrocaudal concentration gradient

The study population consisted of age- and gender matched individuals in both non-AD/CONTR (n=10) and AD (n=10) groups. Demographic parameters are summarized in Table 3.

Table 3. Demographic and biochemical parameters of subjects included in the RCG study.

Parameter		Non-AD/CONTR	AD	Test statistic
Age (years)		68.8 ± 12.0	73.3 ± 4.7	t(11.70)=-1.11 *P*>0.05
Gender (male/female)		5/5	5/5	χ^2=0 *P*>0.05
Storage time (months)		47.3 ± 3.0	47.1 ± 3.6	t(18.00)=0.13 *P*>0.05
CSF MHPG (ng/mL)	C1	23.8 ± 11.5[a]		F(2.66, 47.88)=8.67 ***P*<0.001**
	C2	19.1 ± 9.3[a,e,g]		
	C3	24.8 ± 12.0[e]		
	C4	22.0 ± 10.5		
	C5	23.9 ± 11.2[g]		
CSF HVA (ng/mL)	C1	30.8 ± 13.9[a,b,c,d]		F(2.32, 41.71)=29.85 ***P*<0.001**
	C2	37.5 ± 16.4[a,g]		
	C3	38.0 ± 15.9[b,i]		
	C4	35.2 ± 15.0[c,j]		
	C5	44.3 ± 18.9[d,g,i,j]		
CSF 5-HIAA (ng/mL)	C1	12.6 ± 5.1	19.2 ± 8.2[a,b,c,d]	F(2.56, 45.99)=24.97 ***P*<0.001** F(2.56, 45.99)=3.81 ***P*<0.05**
	C2	15.0 ± 5.8	23.9 ± 11.3[a,g]	
	C3	15.3 ± 5.1	24.5 ± 11.4[b,i]	
	C4	13.9 ± 4.7[j]	23.0 ± 9.0[c,j]	
	C5	16.8 ± 4.7[j]	28.8 ± 10.2[d,e,f,j]	

All data are presented as mean ± SD. Significant differences after post-hoc correction for multiple comparisons (*P*<0.05) are indicated by superscript letters a, b, c, d, e, f, g, h, i, and j for differences between C1 and C2, C1 and C3, C1 and C4, C1 and C5, C2 and C3, C2 and C4, C2 and C5, C3 and C4, C3 and C5, and, C4 and C5, respectively. Statistically significant differences after independent samples T-tests between the non-AD/CONTR and the AD group, or mixed model ANOVA, are depicted in the rightmost column. In case of 5-HIAA, the effect of CSF subsample is first represented, followed by the statistical significance of the interaction between CSF fraction and diagnostic class. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; AD: Alzheimer's disease; C1-5: five consecutive CSF fractions containing 4.5 mL, 1.5 mL, 1.5 mL, 4.5 mL and 4.5 mL, respectively; CONTR: control; CSF: cerebrospinal fluid; HVA: homovanillic acid; MHPG: 3-methoxy-4-hydroxyphenylglycol; RCG: rostrocaudal concentration gradient.

3.5. Mixed model ANOVA

Mixed model ANOVA was performed to verify effects of a possible RCG and diagnostic category on a subset of metabolite levels, as (N)A, DA, 5-HT and DOPAC could not be reliably detected in these CSF samples. A significant effect of CSF

fraction was found for MHPG ($F(2.66, 47.88)=8.67$; $P<0.001$), while the effect of CSF fraction was not different between diagnostic classes ($F(2.66, 47.88) = 1.52$; $P>0.05$). Likewise, HVA levels were found to differ across CSF fractions ($F(2.32,41.71) = 29.85$; $P<0.001$), without a significant interaction effect between diagnostic class and CSF subsample ($F(2.32, 41.71)=2.96$; $P>0.05$). A statistically significant effect of CSF fraction on 5-HIAA levels was also detected ($F(2.56,45.99)=24.97$; $P<0.001$). Moreover, the behavior of the RCG was different in each diagnostic class, indicated by a significant interaction effect ($F(2.56, 45.99)=3.81$; $P<0.05$). The levels of monoamine metabolites in each subfraction and post-hoc differences, are displayed in Table 3. No global upward trend could be observed for CSF MHPG levels (Figure 3, panel A), while diminished levels were noted in the C2 and C4 fraction. In addition, a similar pattern was detected for CSF HVA and 5-HIAA levels (Figure 3, panel B and C, respectively), characterized by a general increase with collected CSF volume. A drop in both analyte levels could also be recognized in the C4 subsample. Lastly, markedly higher CSF 5-HIAA concentrations were observed in the AD group compared to non-AD/control individuals.

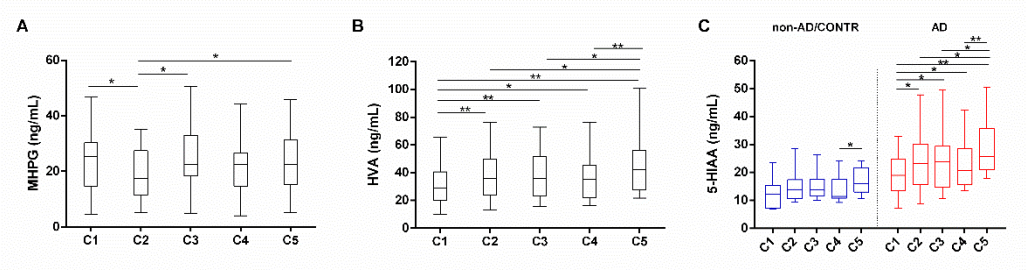

Figure 3. Concentrations of MHPG, HVA and 5-HIAA in each CSF fraction (panels A, B, and C, respectively). In A and B, the combined results of the non-AD/CONTR group and the AD group are displayed, since no interaction effect was found for either MHPG or HVA. In case of 5-HIAA, the results are depicted separately for each diagnostic category. Statistically significant post-hoc differences between CSF fractions after Bonferroni correction, are indicated by one ($P<0.05$) or two ($P<0.001$) asterisks. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; AD: Alzheimer's disease; CONTR: control; CSF: cerebrospinal fluid; HVA: homovanillic acid; MHPG: 3-methoxy-4-hydroxyphenylglycol.

3.6. Correlation between metabolite concentrations and CSF fraction

Spearman's correlation statistics showed that both CSF HVA ($rs(18)=0.221$; $P<0.05$) and 5-HIAA ($rs(18)=0.213$; $P<0.05$) levels increased with collected CSF volume, whereas no association was found between MHPG levels and CSF fraction ($rs(18)=0.043$; $P>0.05$). As CSF fraction impacted CSF 5-HIAA levels differently in the non-AD/CONTR group compared to the AD group, we also investigated whether

different correlations could be found in those two diagnostic categories. However, no statistically significant association was found between CSF 5-HIAA levels and CSF fraction when non-AD/CONTR ($rs(8)=0.211$; $P>0.05$) and AD ($rs(8)=0.254$; $P>0.05$) groups were analyzed separately.

4. DISCUSSION

4.1. Circadian rhythm of melatonin and monoamines

Regarding circadian rhythm analyses in CSF, mainly negative results were found. Indeed, previous reports indicate the absence of diurnal rhythmicity in CSF DOPAC [157] and MHPG, albeit in non-human primates [181]. Concentrations of CSF HVA and 5-HIAA did show a 24-hour rhythm, although only in three and two subjects, respectively. Moreover, the acrophases of these rhythms showed a large variability, causing these results to remain inconclusive. Five and four out of the seven subjects who completed the protocol, showed a statistically significant circadian rhythm for plasma melatonin and HVA, respectively, which might be due to the non-normal distribution of these analyte concentrations. In addition, it is possible that the sleep-wake cycle of those two and three subjects was disturbed as a consequence of the hospital setting. Interestingly, the acrophases of melatonin and HVA, either in plasma or CSF, were situated in a similar time frame (01:00-06:00 hrs). This might be explained by the fact that both melatonin and DA are regulated by the major circadian pacemaker in the hypothalamus, the suprachiasmatic nucleus (SCN) [173, 182-184]. Briefly, the SCN regulates melatonin synthesis in the pineal gland via stimulatory and inhibitory stimuli, highly dependent on the light signal received from the retina [185, 186]. Dopaminergic activity is also hypothesized to be organized by the SCN. As an example, behavioral and neurochemical disturbances were observed in mice lacking Rev-erbα, a nuclear receptor involved in the feedforward loop of the molecular clockwork of circadian rhythm [187]. These mice exhibited increased production of tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis [188], and increased DA turnover, indicated by a surge in HVA/DA ratios [189]. In addition, protein levels of TH and DA transporters have been reported to be higher at night in the nucleus accumbens of male Sprague-Dawley rats [184], coinciding with our findings of peak HVA levels in both plasma and CSF at night. However, it should be noted that the evidence for dopaminergic regulation by the SCN was mainly raised in a preclinical setting, which might complicate a straightforward comparison with the results of this work. Our hypothesis stated that a circadian rhythm would be expected in the serotonergic system, as this is closely related to melatonin. However, no such diurnal variation

was observed in these compounds, except for CSF 5-HIAA in only two out of seven participants. The relatively high variability of our measurements in combination with a small sample size, might account for these negative results.
We are also aware of the possible confounding influence of diet on monoaminergic content [190], with high-carbohydrate meals causing increased tryptophan and 5-HT contents in the brain. In addition, levels of DA and (N)A increase in response to meals consisting of 40% protein. Since we did not measure brain monoamine levels and since we only found evidence of a circadian rhythm in metabolites of these compounds, it is difficult to determine whether food consumption affected our findings. Nevertheless, the observed similarities in circadian rhythm of melatonin, a metabolite of 5-HT, and HVA, are not entirely consistent with a dietary influence on these molecules. Since subjects participating in this study received standardized meals mainly consisting of carbohydrates, the increase in melatonin could be foreseen. The surge of HVA at the same time point, however, is contradictory with the effect of high-carbohydrate meals. In addition, three surges in melatonin, one after each meal of the day, would also be expected if diet had truly influenced our observations.
Altogether, we propose to sample plasma and CSF at standardized times when the variation in monoamine/metabolite levels is lowest. According to our results, this would be in the early afternoon (12:00-15:00 hrs).

4.2. Rostrocaudal concentration gradient

As expected from previous reports, no RCG was observed for CSF MHPG, while HVA and 5-HIAA did show increasing levels in consecutive CSF fractions. Since MHPG readily passes the blood-brain [191], as well as the blood-CSF barrier [192], CSF MHPG levels might not correlate with central noradrenergic metabolism [191], possibly because of constant inflow from the blood compartment [193, 194]. Moreover, HVA and 5-HIAA are removed from the CSF by diffusion, bulk flow of CSF and active transport mechanisms (reviewed in [195]). This difference in clearance mechanisms between CSF MHPG, HVA and 5-HIAA, might additionally explain the observed differences in concentration gradient of these compounds. Furthermore, a decrease in the C4 fraction was observed for HVA, 5-HIAA and MHPG, as well as a reduction of MHPG levels in the C2 fraction. It should be noted that the C2 fraction of each study subject had already been used for determination of the standard AD biomarker panel, and that CSF MHPG levels, contrary to HVA and 5-HIAA concentrations, were found to decrease even after a single freeze-thaw cycle [196]. Nevertheless, it remains unclear why CSF MHPG, HVA and 5-HIAA levels were reduced in the C4 fraction. In most other studies reporting the existence of a RCG

in CSF HVA and 5-HIAA concentrations, a larger total volume of CSF was collected [165, 167, 168]. Thus, as the decrease in CSF analyte levels occurs after the first 7.5 mL in our study, it is possible that this reflects normal biological variation, of which the effect could have been less pronounced if larger, and most importantly, more fractions had been collected. This might also account for the shallow slope observed for all analyzed compounds in Figure 3. Generally, our results suggest that the presence of a RCG of monoaminergic metabolites such as HVA and 5-HIAA, might indeed influence the comparison of these compounds between distinct factorial groups. We therefore suggest that investigators analyze the first CSF fraction from all study participants, so that the possible effect of the RCG is avoided.

Lastly, we observed consistently higher CSF 5-HIAA levels in the AD group versus non-AD/control participants. Different factors might account for this apparent discrepancy with the existing literature, in which predominantly lower or unchanged CSF 5-HIAA levels were noted in AD patients versus controls [147, 149, 197], and in which a negative correlation was found between AD severity and brain 5-HT and 5-HIAA levels [198, 199]. The most important factor is probably the presence of non-AD dementia types, which might also display disturbances in monoamine content, in the 'control' group. In addition, the individual dementia stage of the patients at the moment of CSF collection, might also have influenced our findings.

4.3. Strengths, limitations and relevance

All analyses for the study of circadian rhythm were performed on a valuable and extensive dataset incorporating paired CSF and plasma samples derived from eight healthy volunteers, during a 30-hour period. It should be noted, however, that only seven subjects completed the whole protocol, possibly decreasing statistical power. Samples were thawed twice beforehand, which might have impacted our results as freeze/thaw cycles could affect the stability of monoaminergic compounds. However, it has been demonstrated that CSF HVA and 5-HIAA remain relatively stable even after six freeze-thaw cycles of variable duration [196, 200]. In addition, plasma melatonin levels were previously found to be unchanged after three freeze/thaw cycles [201]. The results of the linear mixed model analysis were concordant with the circadian rhythm findings, illustrated by the fact that the individual acrophases corresponded with sampling time 14, which was the only time point at which plasma melatonin levels differed significantly compared to almost all other time points. Therefore, another strength of this study can be found in the relatively accurate estimation of diurnal melatonin rhythm. On the one hand,

the long sampling intervals during the night might have hampered an even more meticulous evaluation of the circadian rhythm of all compounds, as the acrophases of all diurnal compounds occurred during these four-hour intervals. On the other hand, the circadian rhythm might have been disturbed if the subjects were woken up more frequently during the night for sampling purposes. Furthermore, as the circadian rhythm of melatonin is widely accepted to occur around 02:00–04:00 hrs [170-173], our results corroborated these previous findings. Thus, melatonin could be considered a positive control compound in this circadian rhythm study.

Concerning the investigation of the RCG, the use of CSF samples derived from subjects who underwent neuropsychological assessment and CSF biomarker analysis, increased the reliability of our results. Moreover, storage- and handling conditions were similar across all samples, thereby minimally affecting differences between groups or CSF fractions. However, the disease state of our study subjects has not been confirmed neuropathologically, and the sample size used for this investigation was rather small, so that the effect of a possible RCG on monoaminergic metabolites might have been more pronounced if more subjects were included in the analysis.

This work focuses on the methodological aspects of neurochemical (biomarker) studies investigating monoamines and/or their metabolites. These compounds are especially of interest since they were previously found to be altered in several forms of dementia [143, 145-149] and since currently, most of the available treatment options are associated with a lack of efficiency and/or important side effects. Therefore, the assessment of alterations in these compounds should be free of misinterpretation and/or bias due to the circadian rhythm and the RCG, so that monoamines/metabolites can be identified as reliable biomarkers and/or therapeutic targets in dementia.

5. CONCLUSIONS

In general, pre-analytical factors should be carefully considered before the onset of studies investigating (monoaminergic) compounds in biological fluids. Despite relatively small study populations in this work, we found that plasma HVA levels vary according to a circadian rhythm similar to that of melatonin, which could be explained by a shared control mechanism in the SCN. Our results indicated that plasma and CSF should ideally be sampled in the early afternoon for the analysis of monoamines and their metabolites. Lastly, since a RCG was observed for CSF HVA and 5-HIAA, it is advisable to standardize the number and volume of CSF samples, so that comparable biological specimens can be analyzed in future studies. To avoid confounding effects of the RCG, we propose to analyze the first 1.5 mL of CSF collected in all study participants.

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7. SUPPLEMENTARY MATERIAL

Supplementary Table 1. Pairwise comparisons between sampling times.

Parameter	Pairwise comparison (sampling time)	Mean concentration at denoted sampling times
Plasma melatonin (pg/mL)	2-14	42.5 – 120.6
	3-14	35.4 – 120.6
	4-14	36.2 – 120.6
	5-14	42.1 – 120.6
	6-14	37.6 – 120.6
	7-14	32.3 – 120.6
	8-14	41.3 – 120.6
	9-14	35.7 – 120.6
	10-14	36.1 – 120.6
	11-14	34.7 – 120.6
	14-17	120.6 – 45.3
	14-18	120.6 – 51.4
	14-19	120.6 – 38.9
CSF HVA (ng/mL)	2-10	33.4 – 51.3
	2-12	33.4 – 54.6
	2-17	33.4 – 52.4
	2-19	33.4 – 54.4
	3-12	38.8 – 54.6
	3-18	38.8 – 49.9
	3-19	38.8 – 54.4
CSF 5-HIAA (ng/mL)	2-17	19.3 – 28.1
	2-19	19.3 – 28.7
Plasma DOPAC (ng/mL)	17-18	4.2 – 3.2

Mean concentrations of plasma melatonin and DOPAC, as well as CSF HVA and 5-HIAA, at sampling times which were significantly different after post-hoc pairwise comparisons. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid.

Supplementary Table 2. Circadian rhythm parameter estimates of plasma melatonin and HVA, as well as CSF HVA and 5-HIAA.

Subject ID	Amplitude (ng-pg/mL)	CI (ng-pg/mL)	Acrophase (radians)	CI (radians)	Acrophase (hrs)	CI (hrs)	MESOR (ng-pg/mL)	CI (ng-pg/mL)	p-value
Plasma melatonin (pg/mL)									
1	**45.35**	**28.32 - 62.38**	**-1.40**	**-1.74 - (-1.07)**	**05:21**	**04:05 - 06:39**	**70.52**	**57.89 - 83.14**	**< 0.001**
2	**24.85**	**10.48 - 39.21**	**-0.76**	**-1.22 - (-0.30)**	**02:54**	**01:09 - 4:39**	**54.72**	**43.97 - 65.47**	**< 0.05**
3	**90.43**	**61.31 - 119.54**	**-0.50**	**-0.79 - (-0.21)**	**01:55**	**00:48 - 03:01**	**115.37**	**93.63 - 137.11**	**< 0.001**
4*	26.80	2.02 - 51.57	-1.47	-2.31 - (-0.64)	05:37	02:26 - 08:49	44.26	25.80 - 62.72	> 0.05
5	**27.26**	**14.32 - 40.20**	**-1.24**	**-1.62 - (-0.86)**	**04:44**	**03:17 - 06:11**	**53.19**	**44.39 - 61.99**	**< 0.01**
6	**43.33**	**20.69 - 65.98**	**-0.84**	**-1.22 - (-0.46)**	**03:13**	**01:46 - 04:39**	**70.90**	**54.78 - 87.01**	**< 0.01**
7*	13.41	-2.46 - (-29.28)	0.63	-0.78 - 2.04	21:35	16:13 - 02:59	44.81	30.70 - 58.93	> 0.05
Plasma HVA (ng/mL)									
1*	3.16	1.92 - 4.40	-1.04	-1.36 - (-0.71)	03:58	02:43 - 05:11	13.92	13.02 - 14.82	< 0.001
2*	0.78	-0.28 - 1.84	0.89	-0.74 - 2.54	20:36	14:18 - 02:50	6.27	5.35 - 7.19	> 0.05
3	**1.99**	**0.93 - 3.05**	**-1.15**	**-1.62 - (-0.69)**	**04:23**	**02:38 - 06:11**	**9.66**	**8.86 - 10.47**	**< 0.01**
4	**0.85**	**0.29 - 1.42**	**-0.77**	**-1.30 - (-0.24)**	**02:56**	**00:55 - 04:58**	**7.86**	**7.46 - 8.25**	**< 0.05**
5	**2.67**	**1.25 - 4.10**	**-0.24**	**-0.76 - 0.29**	**00:55**	**22:54 - 02:54**	**11.43**	**10.31 - 12.55**	**< 0.01**
6	**2.64**	**1.52 - 3.75**	**-1.19**	**-1.57 - (-0.80)**	**04:33**	**03:04 - 06:00**	**8.35**	**7.52 - 9.19**	**< 0.01**
7*	2.54	0.84 - 4.24	-1.42	-2.03 - (-0.82)	05:25	03:08 - 07:45	10.80	9.52 - 12.08	< 0.05

Supplementary Table 2. Continued.

Subject ID	Amplitude (ng-pg/mL)	CI (ng-pg/mL)	Acrophase (radians)	CI (radians)	Acrophase (hrs)	CI (hrs)	MESOR (ng-pg/mL)	CI (ng-pg/mL)	p-value
CSF HVA (ng/mL)									
1	6.20	-0.81 - 13.2	-1.03	-1.93 - (-0.12)	03:56	00:28 - 07:22	64.28	59.27 - 69.29	> 0.05
2	**5.84**	**3.03 - 8.65**	**-1.28**	**-1.78 - (-0.78)**	**04:53**	**02:59 - 06:48**	**35.76**	**33.50 - 38.01**	**< 0.01**
3*	2.91	-2.44 - 8.25	0.50	-1.70 - 2.70	22:05	13:41 - 06:29	34.26	29.72 - 38.80	> 0.05
4	3.64	-6.10 - 13.38	-0.29	-2.88 - 2.30	01:06	15:13 - 11:00	45.64	38.08 - 53.20	> 0.05
5	**9.58**	**7.05 - 12.11**	**-1.28**	**-1.50 - (-1.06)**	**04:53**	**04:03 - 05:44**	**25.80**	**23.97 - 27.64**	**< 0.001**
6*	5.17	-1.17 - 11.51	1.28	0.02 - 2.55	19:07	14:16 - 23:55	43.84	38.76 - 48.93	> 0.05
7	**10.37**	**7.06 - 13.69**	**1.10**	**0.75 - 1.46**	**19:48**	**18:25 - 21:08**	**66.53**	**63.76 - 69.29**	**< 0.001**
CSF 5-HIAA (ng/mL)									
1*	1.44	-0.31 - 3.19	-1.50	-2.58 - (-0.42)	05:43	01:36 – 09:51	31.44	30.13 - 32.74	> 0.05
2*	5.37	-1.86 - 12.60	-0.30	-1.60 - 0.99	01:09	20:14 - 06:06	46.23	40.64 - 51.83	> 0.05
3*	3.57	-2.65 - 9.78	1.32	-0.31 - 2.96	18:56	12:42 – 01:10	15.75	11.11 - 20.40	> 0.05
4	3.23	-0.79 - 7.25	-1.02	-2.02 - (-0.02)	03:54	00:04 - 07:43	18.28	15.41 - 21.16	> 0.05
5	**4.11**	**2.75 - 5.46**	**-1.09**	**-1.35 - (-0.82)**	**04:09**	**03:07 - 05:09**	**10.82**	**9.85 - 11.79**	**< 0.001**
6*	1.90	-1.95 - 5.75	1.43	-0.53 - 3.39	05:27	11:03 - 02:01	20.68	17.69 - 23.68	> 0.05
7	**3.52**	**1.81 - 5.22**	**0.89**	**0.32 - 1.47**	**20:36**	**18:24 - 22:47**	**31.16**	**29.68 - 32.64**	**< 0.05**

Parameters are depicted in bold in case of a statistically significant circadian rhythm, while an asterisk indicates subjects with data violating the assumptions for the circadian rhythm analysis. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; CI: 95 % confidence interval; CSF: cerebrospinal fluid; hrs: hours; HVA: homovanillic acid; MESOR: midline estimating statistic of rhythm.

Chapter III.2. Evaluating the applicability of mouse SINEs as an alternative normalization approach for RT-qPCR in brain tissue of the APP23 model for Alzheimer's disease

Jana Janssens[a,1], René A. J. Crans[b,c,1], Kathleen Van Craenenbroeck[b], Jo Vandesompele[d,e], Christophe P. Stove[b], Debby Van Dam[a,f,*], Peter P. De Deyn[a,f,g,h]

[a] *Laboratory of Neurochemistry and Behavior, Institute Born-Bunge, University of Antwerp, Universiteitsplein 1, BE-2610 Wilrijk, Belgium*

[b] *Laboratory for GPCR Expression and Signal Transduction (L-GEST) – Laboratory of Toxicology, Ghent University, Ottergemsesteenweg 460, BE-9000 Ghent, Belgium*

[c] *Unitat de Farmacologia, Departament Patologia i Terapèutica Experimental, Facultat de Medicina, IDIBELL, Universitat de Barcelona, 08907 L'Hospitalet de Llobregat, Spain*

[d] *Center for Medical Genetics, Ghent University Hospital, C. Heymanslaan 10, BE-9000 Ghent, Belgium*

[e] *Cancer Research Institute Ghent (CRIG), Ghent University, BE-9000 Ghent, Belgium.*

[f] *Department of Neurology and Alzheimer Research Center, University Medical Center Groningen (UMCG), Hanzeplein 1, 9713 GZ Groningen, The Netherlands*

[g] *Department of Neurology and Memory Clinic, Hospital Network Antwerp (ZNA) Middelheim and Hoge Beuken, Lindendreef 1, BE-2020 Antwerp, Belgium*

[h] *Biobank, Institute Born-Bunge, University of Antwerp, Universiteitsplein 1, BE-2610 Antwerp, Belgium*

[1]These authors contributed equally to this work.

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ABSTRACT

Background: The choice of appropriate reference genes (RGs) for use in reverse transcription quantitative polymerase chain reaction (RT-qPCR) has been thoroughly investigated, since the inclusion of unstable RGs might cause inaccurate gene expression results.

New method: Short interspersed nuclear elements (SINEs) such as B elements, might represent an alternative solution given the high occurrence of these repetitive elements in the rodent genome and transcriptome. We performed RT-qPCR to investigate the stability of nine commonly used RGs and two B elements, B1 and B2, across different age- and genotype-related experimental conditions in the hippocampus and cortex of the APP23 amyloidosis mouse model for Alzheimer's disease. Gene stability was assessed using geNorm, NormFinder and BestKeeper. Human amyloid precursor protein (*APP*) levels in transgenic versus wild-type animals were also determined to validate the use of B elements as an alternative normalization approach.

Results: Whereas B elements were stably expressed in the hippocampus, they were ranked as least stable in the cortex. The optimal normalization factor (NF) in hippocampus was a combination of *Gapdh* and *Rpl13a*, whereas in cortex, *Actb* and *Tbp* constituted the ideal NF.

Comparison with Existing Method: When comparing B1 and B2 as NFs for *APP* with the optimal panel of RGs in hippocampus, we found that B1 and B2 performed similarly to the optimal NF, while these SINEs performed less well in cortex.

Conclusions: Although B elements are suitable as an alternative normalization strategy in the hippocampus, they do not represent a universal normalization approach in the APP23 model.

1. INTRODUCTION

The reverse transcription quantitative polymerase chain reaction (RT-qPCR) is one of the most commonly used techniques in the field of molecular biology [202], and has become the gold standard for quantification of mRNA in biological samples [203, 204] given its flexibility, sensitivity, specificity and the possibility for high-throughput analyses. Quantitative determination of gene expression is either relative or absolute, however, both methods are associated with a number of pitfalls. Since absolute quantification requires the inclusion of a standard curve [203, 205, 206], a major downside of this method is the need for such a serial dilution on each plate, thus hampering large RT-qPCR studies incorporating many samples and/or genes. In contrast, the delta-delta Cq method [207] for relative quantification normalizes expression of the gene of interest to one or more endogenous reference genes (RGs), serving as internal controls. The internal control genes should ideally be constitutively and stably expressed across cell/tissue types, developmental stages, age and treatment conditions.

The selection and validation of appropriate RGs received a lot of attention in the previous decade [208-211], as the inclusion of unstably expressed RGs might give rise to an important source of bias. As such, commonly used RGs including glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta-actin (*ACTB*), and 18S rRNA have repeatedly been shown to be variably expressed across experimental parameters and tissues [202, 211, 212]. Another topic of discussion was the ideal number of RGs to be included in the experimental design. Following the publication of the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines for studies reporting RT-qPCR results, which recommended the inclusion of at least two RGs [213], a restricted literature review investigated the number of RGs used in gene expression studies in the period from 2010 until 2015, showing that merely 13% of all gene expression studies used more than one RG [214]. Another survey investigating papers published from 2009 until 2011 in journals with a wide range of impact factors (IF), noted that almost 30% of journals with an IF < 5 and 73% of high-impact journals failed to publish a single paper including validated RGs [215]. The notion that (the number of) appropriate RGs should be validated for each experimental design, is currently becoming increasingly accepted in the field. However, the repeated validation of RGs for each new study entails important disadvantages, as it renders genetic research more expensive and labor-intensive. Limited RNA sample availability also hinders an extensive investigation of appropriate RGs. These drawbacks, in addition to the unstable expression of historical RGs (e.g. *GAPDH*, *ACTB* and 18S rRNA), motivate

the search for alternative normalization approaches. Short interspersed nuclear elements (SINEs) are a class of highly occurring retrotransposons, which are generally 100-500 base pairs (bp) in length [216, 217]. Since SINEs make up approximately 10% of the total mammalian genome [218-222] and since they are mainly located in intronic and untranslated regions of many genes [223], it is hypothesized that temporary or event-related changes in a certain number of genes will not have a large impact on the overall SINE content in the transcriptome [224, 225]. Therefore, SINEs might represent a valuable alternative to the regularly used RGs. As an example, expressed Alu repeats were previously found to be an accurate normalization tool for RT-qPCR studies in human blood [226], human embryonic stem cells [227], and cancer cells [228]. This family of retrotransposons, derived from 7SL RNA [229, 230], is ubiquitously present in the primate genome [231], and contains a common restriction site for AluI [232]. Due to the ability to integrate in various regions of the genome, Alu repeats can regulate gene expression by acting as transcriptional enhancers [233], by influencing pre-mRNA splicing [234, 235] or other documented mechanisms (for review, see [236-238]).

The identification of a second family of SINEs, present in the rodent genome [239-241], arose from the analysis of the structural features of pre-mRNA [242]. Its nomenclature is based on the homology to double-stranded (ds) regions in pre-mRNA, termed as dsRNA-B molecules [239, 242, 243]. Further analysis of the DNA sequences complementary to dsRNA-B showed two abundant categories, B1 and B2 [239], each with a distinct degree of sequence variation [239], and derived from distinct RNA ancestor molecules. The first category, B1, consists of sequences with a total length of 130 bp [240]. Similar to Alu repeats, B1 elements are derived from 7SL RNA and were termed quasidimers because of an internal duplication of 29 bp [244, 245]. In contrast, B2 sequences, which are generally 190 bp in length, originate from tRNA [246, 247]. Analogous to the Alu repeats, B elements are hypothesized to be involved in splicing, processing of RNA polymerase II transcripts, and the regulation of gene expression [241]. Thus, since B1 and B2 elements are well characterized and because of their ubiquitous presence in the rodent genome and transcriptome, these SINEs might represent an interesting alternative to classical RGs in RT-qPCR experiments. In this paper, we investigated the applicability of nine commonly used mouse RGs: *Gapdh*, *Actb*, beta-2-microglobulin (*B2m*), phosphoglycerate kinase 1 (*Pgk1*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*Ywhaz*), beta-glucuronidase (*Gusb*), TATA-box binding protein (*Tbp*) and ribosomal protein L13a (*Rpl13a*), as well as B1 and

B2 elements as a normalization strategy for gene expression analysis in hippocampal and cortical tissue of the APP23 amyloidosis mouse model for Alzheimer's disease (AD). Although various work reported about methylation of B elements [248], posttranscriptional gene regulation [249], and their transcriptional response to viral cell infection [250], few previous studies used these SINEs as a normalization approach for RT-qPCR experiments. Only one recent study performed a similar experimental analysis concerning the applicability of B elements for normalization of gene expression in hippocampus and cortex of a rat model of temporal lobe epilepsy [225], while, to the best of our knowledge, these SINEs have not previously been used for analogous purposes in mice.

2. MATERIALS & METHODS

2.1. Animal model and tissue collection

The APP23 model is a transgenic mouse model, containing human cDNA of *APP* with the Swedish double mutation (K670N/M671L), resulting in overexpression of *APP* and causing a familial form of early-onset AD in humans. From the age of 6 months onwards, APP23 mice display neuropathological lesions, i.e. Aβ plaques and tau-related pathology [251-253]. Further characterization with respect to cognitive and behavioral abnormalities, and validation of this model, have been described previously [253, 254]. Male mice were group-housed in standard mouse cages in the animal facility of the University of Antwerp, in a 12:12 h light-dark cycle (lights on at 08:00 hrs, lights off at 20:00 hrs). Room temperature was maintained constant at 22 ± 2°C, while the humidity was 55 ± 5%. Food and water were supplied *ad libitum*. Genotyping was performed on tissue derived from ear punches using custom primers (Biolegio, Nijmegen, The Netherlands). Hemizygous (HEM) (n=5) and wild-type (WT) (n=5) mice belonging to each age group (6-8 weeks, 6, and 24 months) were euthanized by cervical dislocation, followed by brain collection and regional dissection of each hemisphere using binoculars. First, both bulbi were dissected, followed by the cerebellum and pons/brain stem. Cortex tissue was sliced off using a scalpel, and as such removed from the underlying white matter. In addition, cortical tissue was visually checked for remaining pieces of white matter. A small sagittal midline incision was made to detach the left and right hippocampus from the fornix, after which a forceps was used to remove both hippocampi in the caudolateral direction. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until further use. All procedures involving animals were approved by the local ethics committee for laboratory

animal experiments (file number 2016-06) and complied with the European Communities Council Directive (2010/63/EU) and ARRIVE guidelines.

2.2. RNA extraction and cDNA synthesis

All analyses were performed on left hemispheric cortices (n=30) and hippocampi (n=29), derived from HEM and WT animals aged 6-8 weeks, 6 and 24 months, except for one right hemispheric hippocampus implemented in the 24-month-old group. RNA extraction was performed using the RNeasy® Plus Universal Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. In addition, on-column DNase treatment was performed using the RNase-free DNase set (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA eluate volume varied between 22 and 25 µl. RNA concentration and purity were determined by UV-VIS spectroscopy measurements at 230, 260 and 280 nm with the Nanodrop 1000 instrument (ThermoFisher Scientific, Waltham, MA, USA). Individual tissue weights, RNA yield and purity are comprised in Supplementary Table 1. Reverse transcription was performed on 800 ng RNA, using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) in 20 µL reactions. To analyze expression stability of B elements and *Hprt* in hippocampus, cDNA samples were diluted 1:1000, while a dilution of 1:10 was adopted for *Actb*, *B2m*, *Gapdh*, *Gusb*, *Pgk1*, *Tbp*, *Rpl13a* and *Ywhaz*. In addition, cDNA was diluted 1:10 for *Actb*, *B2m*, *Gusb*, *Hprt*, *Pgk1*, *Tbp*, *Rpl13a* and *Ywhaz*, and 1:1000 for *Gapdh* and B elements in the case of cortical samples. Expression levels of the human *APP* construct as well as mouse *App* were also analyzed, and normalized to the best combination of RGs according to gene stability software, or to B elements. For this analysis, cDNA was diluted 1:10 and 1:1000 for hippocampus and cortex, respectively. All cDNA samples were stored at -80°C until further use.

2.3. Primer design

Primers for all RGs were developed and checked for specificity using the NCBI Primer-BLAST software (Table 1) and RefSeq mRNA sequences of *Mus musculus*, while sequences for B1 and B2 elements were designed using Primer3 software, and checked for specificity using NCBI Primer-BLAST (J.V., UGent). All primers were synthesized by Biolegio (Nijmegen, The Netherlands).

2.4. Reverse transcription quantitative PCR

Reverse transcription quantitative PCR analyses were performed on the Applied Biosystems StepOnePlus instrument (Foster City, CA, USA), with automatic

threshold settings. All analyses were carried out in 10 µL reactions containing 5 µL of the Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 1.5 µL of each primer (250 nM final concentration) and 2 µL cDNA (8 ng and 0.08 ng cDNA in case of a 1:10 dilution and 1:1000 dilution, respectively). Cycling conditions for all analyses consisted of initial denaturation at 95°C during 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 60 seconds. Finally, melting curve analysis was performed by heating from 60°C to 95°C in increments of 0.3°C/s. cDNA samples and corresponding negative control reactions to which no reverse transcriptase was added during cDNA synthesis (-RT reactions), were analyzed in triplicate using StepOne Software v2.3 (Applied Biosystems, Foster City, CA, USA). All corresponding -RT reactions were negative (defined as reactions with an undetermined Cq or if the difference between the -RT control and the corresponding cDNA sample was ≥10 Cq values). PCR efficiencies for each assay were determined using technical triplicates of 1:2 or 1:10 serial dilutions, consisting of up to six dilution points, of a representative cDNA sample and are displayed in Table 1. NRQs of B elements and *APP* were calculated using the qbase+ software (version 3.1, Biogazelle), implementing an efficiency-corrected delta-delta Cq method (Hellemans et al., 2007). Melt curves of each RT-qPCR product are shown in Appendix B.

Table 1. Primer sequences, amplicon lengths and PCR efficiencies of every assay.

Symbol	Sequence	Accession number	Amplicon length (bp)	PCR efficiency (%)
Actb	5'-GGCTGTATTCCCCTCCATCG-3' 5'-CCAGTTGGTAACAATGCCATGT-3'	NM_007393.5	154	97.3
APP	5'-AGAAGGACAGACAGCACACC-3' 5'-TCATAACCTGGGACCGGATCT-3'	NM_001136130.2	90	94.5
B1 element	5'-GTGGCGCACGCCTTTAAT-3' 5'-GCTGGCCTCGAACTCAGAAA-3'	NC_000067.6	68	95.3
B2 element	5'-CAATTCCCAGCAACCACATG-3' 5'-ACACACCAGAAGAGGGCATCA-3'	NC_000067.6	69	98.8
B2m	5'-GTATACTCACGCCACCCACC-3' 5'-TGGGGGTGAATTCAGTGTGAG-3'	NM_009735.3	193	108.6
Gapdh	5'-AGGTCGGTGTGAACGGATTTG-3' 5'-GGGGTCGTTGATGGCAACA-3'	NM_001289726.1	95	98.5
Gusb	5'-GGCGATGGACCCAAGATACC-3' 5'-TGAATCCCATTCACCCACACA-3'	NM_010368.1	88	106.0
Hprt	5'-CTTCCTCCTCAGACCGCTTT-3' 5'-CATCATCGCTAATCACGACGC-3'	NM_013556.2	85	99.1
Pgk1	5'-CTCCGCTTTCATGTAGAGGAAG-3' 5'-GACATCTCCTAGTTTGGACAGTG-3'	NM_008828.3	117	109.1

Table 1. Continued.

Symbol	Sequence	Accession number	Amplicon length (bp)	PCR efficiency (%)
Rpl13a	5'-CCCTCCACCCTATGACAAGAAAA-3' 5'-TAGGCTTCAGCCGAACAACC-3'	NM_009438.5	71	96.4
Tbp	5'-GGTATCTGCTGGCGGTTTGG-3' 5'-GAAATAGTGATGCTGGGCACTG-3'	NM_013684.3	73	99.7
Ywhaz	5'-TGTCACGGTGTGGACGC-3' 5'-ATGACGTCAAACGCTTCTGG-3'	NM_011740.3	119	100.8

Abbreviations: *Actb*: beta-actin; *B2m*: beta-2-microglobulin; bp: base pairs; *Gapdh*: glyceraldehyde-3-phopshate dehydrogenase; *Gusb*: beta-glucuronidase; *Hprt*: hypoxanthine guanine phosphoribosyl transferase; *Pgk1*: phosphoglycerate kinase 1; *Rpl13a*: ribosomal protein L13A; *Tbp*: TATA-box binding protein; *Ywhaz*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

2.5. Software and statistical analysis

Gene stability analysis was carried out on previously mentioned nine RGs, in conjunction with the two B elements separately and combined. We analyzed all RGs using qbase+ software, version 3.1, incorporating the geNorm algorithm [255]. This algorithm ranks distinct RGs based on the geNorm M value, which represents the average pairwise variation of remaining RGs after stepwise exclusion of the least stable genes. In addition, the geNorm V value indicates the pairwise variation of two sequential NFs upon inclusion of additional genes. Respective cutoff values for geNorm M and V are 0.50 and 0.15, so that all RGs with a geNorm M value <0.50 are generally classified as stable RGs. A geNorm V value <0.15 indicates that there is no benefit in including additional RGs for normalization purposes. Moreover, the NormFinder algorithm, v0.953, was used as an add-on in Microsoft Excel and ranks RGs taking into account a systematic error measure based on the inter- and intragroup variation. Similar to geNorm, the RG with the lowest stability value, is ranked as most stable [256]. Raw Cq values were transformed into efficiency-adjusted relative quantities before performing the NormFinder analysis, as described previously [208]. Lastly, the BestKeeper algorithm, version 1, was implemented in Microsoft Excel, calculating the geometric mean of all RGs included in the study as the BestKeeper Index, as well as the correlation coefficient of each individual gene with aforementioned index. Consequently, the gene with the highest correlation factor, is characterized by the most stable expression.

Since these three algorithms use distinct approaches to rank RGs, we compared the stability scores from geNorm, NormFinder and BestKeeper, and finally, compiled them into one final rank using the RankAggreg package [257] in R, version 3.4.0 for

Windows, using the cross-entropy Monte Carlo algorithm with Spearman's footrule distance (Supplementary File 1).

Relative quantities of B elements and *APP*, either normalized to the optimal NF as determined by rank aggregation analysis, or to a NF containing B elements in the case of *APP*, were log-transformed for all statistical analyses. Test statistics were considered statistically significant if $P<0.05$. To detect the effects of age and genotype on NRQs of *APP* and B elements, two-way ANOVA was applied, with Tukey's post-hoc tests for age, if applicable. In case the assumption of equality of variances was violated, separate one-way Welch's ANOVA and independent samples T-tests were performed. All statistical analyses were performed in IBM SPSS Statistics for Windows, version 24.0.

3. RESULTS

3.1. Sample size

In total, 30 mice were included in the RT-qPCR experiments. Age groups of 6-8 weeks, 6, and 24 months, consisted of 10 mice each, with 5 HEM APP23 animals and 5 WT littermates in all age groups.

3.2. Expression profiles based on raw Cq values

Descriptive statistics of Cq values for each B element and RG in hippocampus and cortex are depicted in Supplementary Figures 1 and 2. In hippocampus, almost all genes and B elements show a similar expression pattern across age and genotype groups. In animals aged 24 months, however, higher levels of *B2m* can be discerned relative to animals aged 6-8 weeks and 6 months. Overall, the variation in Cq values represented by the interquartile range is larger in cortical tissue compared to hippocampal samples for all tested genes. Surprisingly, the expression profiles of the B elements in the cortex showed the highest variability.

3.3. Stability ranking of reference genes according to geNorm, NormFinder and BestKeeper

For both tissue types, the ranking of RGs was largely similar between the three algorithms. The ranking of all RGs and separate B elements, as well as their gene stability scores, are displayed in Table 2. In hippocampal tissue, B1 and B2 performed relatively well, with a top 5 ranking in the gene list generated by each algorithm (Figure 1A-C). Furthermore, almost all genes included in the analysis proved to be acceptable RGs, indicated by the finding that all RGs had a geNorm M value <0.50. Nevertheless, caution should be exercised when choosing *B2m* as RG

in hippocampus, since this RG shows considerably higher stability indices compared to the other RGs in both NormFinder and BestKeeper algorithms. Furthermore, B elements and *B2m* were amongst the least favorably ranked genes in cortical tissue, crossing the geNorm M threshold value. NormFinder and BestKeeper algorithms both appear to confirm this finding, with distinctly higher stability indices for B elements as well as *B2m*. In addition, when both B1 and B2 were included, B1 had a higher ranking compared to B2 in hippocampus, while both SINEs were ranked as the two least stably expressed transcripts in cortex (Figure 1D-F). As the BestKeeper algorithm is limited to 10 genes, *B2m* was excluded from the analysis since it was the least stably expressed transcript according to both geNorm and NormFinder.

Table 2. Stability ranking of single B elements and RGs according to geNorm, NormFinder and BestKeeper in hippocampus and cortex.

Hippocampus						Cortex					
geNorm		NormFinder		BestKeeper		geNorm		NormFinder		BestKeeper	
Rank - Gene	geNorm M	Rank- Gene	Stability value	Rank- Gene	1- Correlation Coefficient	Rank- Gene	geNorm M	Rank- Gene	Stability value	Rank- Gene	1- Correlation Coefficient
B1 compared to RGs											
1-*Gapdh*	0.161	1-*Rpl13a*	0.173	1-*Rpl13a*	0.045	1-*Actb*	0.253	1-*Hprt*	0.087	1-*Actb*	0.079
2-*Actb*	0.168	2-*Hprt*	0.279	2-*Gapdh*	0.070	2-*Ywhaz*	0.261	2-*Tbp*	0.160	2-*Tbp*	0.083
3-*Ywhaz*	0.171	3-B1	0.296	3-*Actb*	0.072	3-*Tbp*	0.269	3-*Gapdh*	0.183	3-*Gapdh*	0.085
4-*Rpl13a*	0.201	4-*Gapdh*	0.297	4-*Pgk1*	0.074	4-*Gapdh*	0.305	4-*Ywhaz*	0.220	4-*Hprt*	0.111
5-B1	0.219	5-*Actb*	0.341	5-B1	0.084	5-*Hprt*	0.348	5-*Actb*	0.313	5-*Ywhaz*	0.113
6-*Pgk1*	0.232	6-*Gusb*	0.412	6-*Hprt*	0.131	6-*Pgk1*	0.388	6-*Pgk1*	0.346	6-*Pgk1*	0.115
7-*Tbp*	0.260	7-*Pgk1*	0.425	7-*Ywhaz*	0.147	7-*Rpl13a*	0.416	7-*Rpl13a*	0.440	7-*Rpl13a*	0.129
8-*Hprt*	0.288	8-*Tbp*	0.428	8-*Tbp*	0.165	8-*Gusb*	0.444	8-*Gusb*	0.452	8-*Gusb*	0.167
9-*Gusb*	0.324	9-*Ywhaz*	0.435	9-*Gusb*	0.182	9-*B2m*	0.517	9-*B2m*	0.628	9-B1	0.341
10-*B2m*	0.423	10-*B2m*	1.199	10-*B2m*	0.537	10-B1	0.653	10-B1	8.012	10-*B2m*	0.530
B2 compared to RGs											
1-*Gapdh*	0.161	1-*Rpl13a*	0.172	1-*Rpl13a*	0.042	1-*Actb*	0.253	1-*Hprt*	0.059	1-*Actb*	0.080
2-*Actb*	0.168	2-*Hprt*	0.281	2-*Gapdh*	0.065	2-*Ywhaz*	0.261	2-*Tbp*	0.148	2-*Tbp*	0.082
3-*Ywhaz*	0.171	3-*Gapdh*	0.288	3-*Actb*	0.069	3-*Tbp*	0.269	3-*Gapdh*	0.152	3-*Gapdh*	0.091
4-*Rpl13a*	0.201	4-*Actb*	0.337	4-*Pgk1*	0.073	4-*Gapdh*	0.305	4-*Ywhaz*	0.199	4-*Ywhaz*	0.110
5-*Pgk1*	0.220	5-B2	0.367	5-*Hprt*	0.131	5-*Hprt*	0.348	5-*Actb*	0.232	5-*Hprt*	0.129
6-B2	0.253	6-*Gusb*	0.418	6-*Ywhaz*	0.141	6-*Pgk1*	0.388	6-*Rpl13a*	0.312	6-*Pgk1*	0.142
7-*Tbp*	0.277	7-*Tbp*	0.423	7-*Tbp*	0.160	7-*Rpl13a*	0.416	7-*Gusb*	0.333	7-*Rpl13a*	0.151
8-*Hprt*	0.302	8-*Ywhaz*	0.425	8-B2	0.176	8-*Gusb*	0.444	8-*Pgk1*	0.450	8-*Gusb*	0.154
9-*Gusb*	0.337	9-*Pgk1*	0.429	9-*Gusb*	0.196	9-*B2m*	0.517	9-*B2m*	0.571	9-B2	0.474
10-*B2m*	0.435	10-*B2m*	1.203	10-*B2m*	0.550	10-B2	0.684	10-B2	3.308	10-*B2m*	0.515

Gene ranks indicate the stability of gene expression, with 1 corresponding to the most stable gene, and 10 to the least stable. Abbreviations: *Actb*: beta-actin; *B2m*: beta-2-microglobulin; *Gapdh*: glyceraldehyde-3-phopshate dehydrogenase; geNorm M: average expression stability; *Gusb*: beta-glucuronidase; *Hprt*: hypoxanthine guanine phosphoribosyl transferase; *Pgk1*: phosphoglycerate kinase 1; RGs: reference genes; *Rpl13a*: ribosomal protein L13A; *Tbp*: TATA-box binding protein; *Ywhaz*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

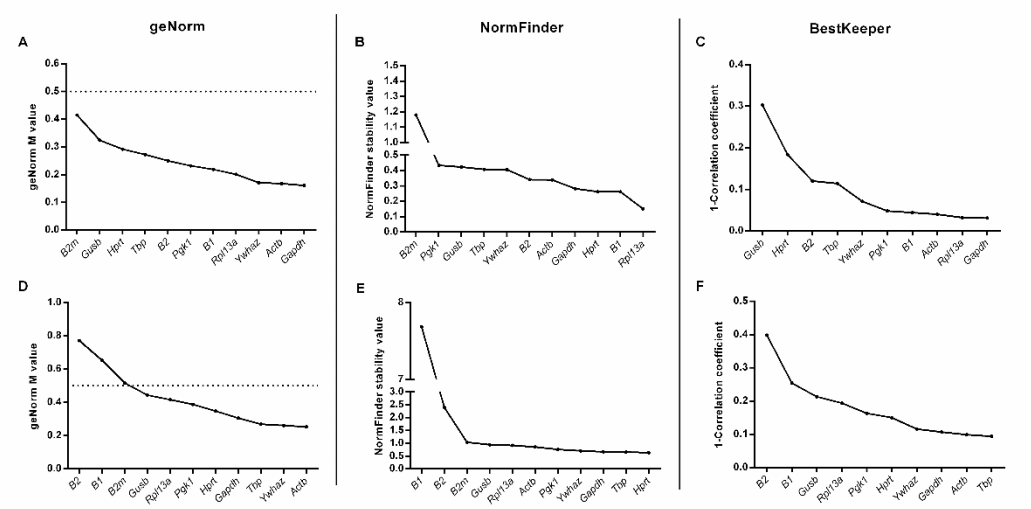

Figure 1. Stability ranking of both B elements and RGs according to geNorm, NormFinder and BestKeeper. A, B, C depict findings in hippocampus, while D, E, F, illustrate results in cortex. The higher the stability value on the y-axis, the less stably the corresponding gene transcript is expressed. For geNorm analyses, the proposed geNorm M value cutoff of 0.50 is indicated by the dotted line. In case of BestKeeper analysis, *B2m* was not included as the algorithm is limited to a maximum of 10 genes and *B2m* was the least stably expressed RG as reported by geNorm and NormFinder. Abbreviations: *Actb*: beta-actin; *B2m*: beta-2-microglobulin; *Gapdh*: glyceraldehyde-3-phopshate dehydrogenase; geNorm M: average expression stability; *Gusb*: beta-glucuronidase; *Hprt*: hypoxanthine guanine phosphoribosyl transferase; *Pgk1*: phosphoglycerate kinase 1; RGs: reference genes; *Rpl13a*: ribosomal protein L13A; *Tbp*: TATA-box binding protein; *Ywhaz*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

3.4. Rank aggregation analysis

Although the ranking of all analyzed genes was fairly similar within tissue type, some genes performed differently when ranks were compared across algorithms. A final overall ranking including either B1 or B2, indicated that in hippocampus of the APP23 model, *Rpl13a* showed the most stable expression, followed by *Gapdh* and *Actb*. When a combination of both B elements was included, B1 was ranked as the fourth most stably expressed gene transcript, while B2 was ranked sixth. In cortex, *Actb*, *Tbp* and *Ywhaz*, showed the most stable transcript levels overall, whereas B1 and B2 were ranked last.

Since B1 and B2 had a very distinct stability in hippocampus compared to cortex, they were excluded from analysis when an overall ranking across tissues and across algorithms was performed, to obtain an indication of the most stable gene transcripts in experiments investigating gene expression levels in cortex and

hippocampus. This rank aggregation returned *Actb* as the most stably expressed transcript, followed by *Gapdh* and *Ywhaz* (Table 3).

Table 3. Final stability ranking of RGs.

Rank	RG
1	*Actb*
2	*Gapdh*
3	*Ywhaz*
4	*Hprt*
5	*Tbp*
6	*Rpl13a*
7	*Pgk1*
8	*Gusb*
9	*B2m*

Final stability ranking of RGs was obtained after rank aggregation analysis implementing the cross-entropy Monte-Carlo algorithm with Spearman's footrule distance across algorithms and tissue types. Rank 1 corresponds to the most stably expressed RG, while rank 9 represents the least stable RG. Abbreviations: *Actb*: beta-actin; *B2m*: beta-2-microglobulin; *Gapdh*: glyceraldehyde-3-phopshate dehydrogenase; *Gusb*: beta-glucuronidase; *Hprt*: hypoxanthine guanine phosphoribosyl transferase; *Pgk1*: phosphoglycerate kinase 1; RG: reference gene; *Rpl13a*: ribosomal protein L13A; *Tbp*: TATA-box binding protein; *Ywhaz*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

3.5. Number of reference genes to be included

The pairwise variation between normalization factors (NFs) incorporating n and n+1 RGs, was lower than the proposed cut-off value of 0.15 for all n (Figure 2). For hippocampal as well as cortical tissue, the pairwise variation between NFs containing two and three RGs was smaller than 0.15, indicating that the addition of a third gene was not required. However, the geNorm V values were consistently higher in cortex (Figure 2B, Supplementary Figure 3C, D) compared to hippocampus (Figure 2A, Supplementary Figure 3A, B), regardless of the number of candidate RGs.

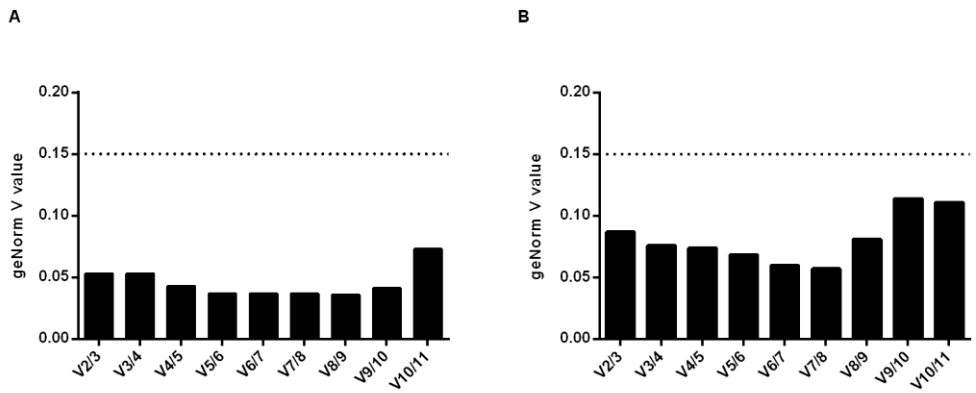

Figure 2. Pairwise variation of NFs upon inclusion of an additional RG. geNorm analyses were performed in hippocampal tissue (A) and cortex (B), incorporating both B elements. The optimal number of RGs is indicated by the cutoff value of 0.15, below which the benefit of including an $(n+1)^{th}$ RG is limited. Abbreviations: NF: normalization factor; $V_{(n/n+1)}$: pairwise variation between normalization factors incorporating a consecutive number of reference genes; RG: reference gene.

3.6. Normalized expression of B elements and *APP*

Since the results of the geNorm V analysis indicated that two RGs should be included in the normalization panel, and as rank aggregation indicated the best RGs in either tissue, we normalized expression levels of B1 and B2 to the optimal NF in hippocampus (*Gapdh* and *Rpl13a*) and cortex (*Actb* and *Tbp*). Both SINEs are characterized by a stable expression in hippocampus across age and genotype, whereas in cortex, more variability across experimental conditions could be observed (Figure 3). These observations were corroborated by the results of a two-way ANOVA, with non-significant effects of age [($F(2, 24)=0.703$; $P>0.05$) and ($F(2, 24)=0.071$; $P>0.05$)] and genotype [($F(1, 24)=0.163$; $P>0.05$) and ($F(1, 24)=0.158$; $P>0.05$)] on log-transformed normalized relative quantities (NRQ) of B1 and B2, respectively. Furthermore, no significant interaction effects between age and genotype were found for normalized expression of B1 ($F(2, 24)=0.104$; $P>0.05$) and B2 ($F(2, 24)=0.128$; $P>0.05$) in hippocampal tissue. Welch's ANOVA indicated a non-significant age effect on B1 levels in the cortex (Welch's $F(2, 15.707)=1.12$; $P>0.05$). An independent samples T-test indicated no significant effect of genotype on normalized B1 expression levels ($t(28)=-1.182$; $P>0.05$). Similar results were found for B2, without significant effects of age ($F(2, 24)=1.389$; $P>0.05$) nor genotype ($F(1, 24)=0.009$; $P>0.05$), as well as a non-significant interaction term ($F(2, 24)=1.218$; $P>0.05$).

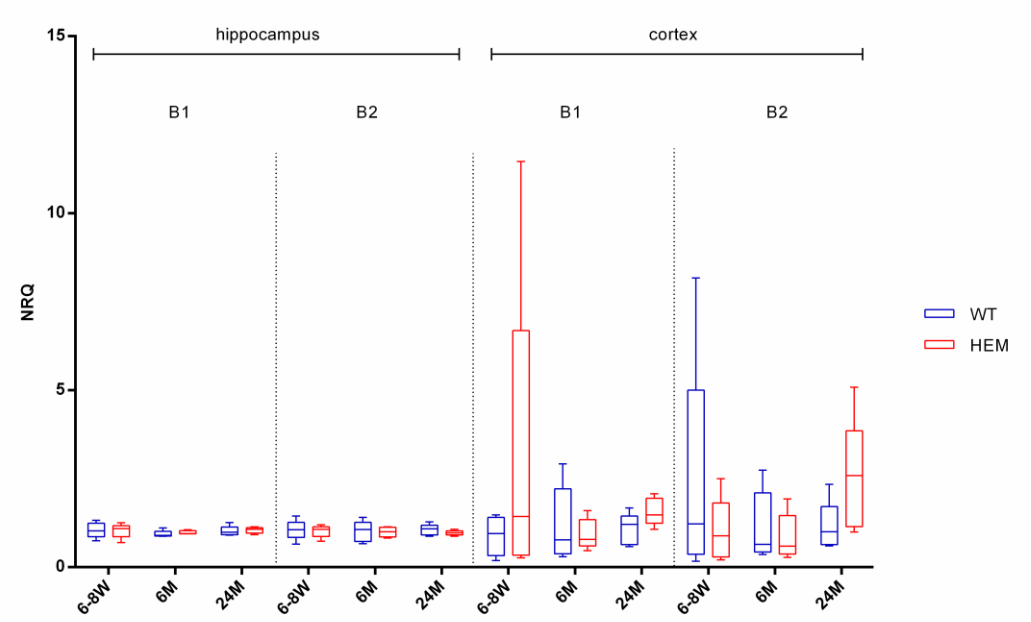

Figure 3. Normalized expression levels of B1 and B2 in WT (n=5) and HEM (n=5) mice by age group, to a normalization factor including *Gapdh* and *Rpl13a* in hippocampus, and *Actb* and *Tbp* in cortex. Data are represented as box and whisker plots with minimum and maximum values. Abbreviations: *Actb*: beta-actin; *Gapdh*: glyceraldehyde-3-phosphate dehydrogenase; HEM: hemizygous; M: months; NRQ: normalized relative quantities; *Rpl13a*: ribosomal protein L13A; SD: standard deviation; *Tbp*: TATA-box binding protein; W: weeks; WT: wild-type.

In addition, normalized expression of amyloid precursor protein (*APP*) in hippocampus and cortex was also determined using previously determined optimal NF for each tissue (Supplementary Figure 4). Transgenic APP23 animals were characterized by a (8.5 ± 0.9)-fold, (11.0 ± 3.0)-fold, and (10.2 ± 2.2)-fold overexpression of *APP* in hippocampal tissue of HEM mice aged 6-8 weeks, 6 months and 24 months, respectively. Conversely, in cortical tissue, *APP* levels were expressed (2.1 ± 1.0)-fold in HEM compared to WT animals aged 6-8 weeks, while a (3.8 ± 4.1)-fold and (19.4 ± 15.0)-fold overexpression were found in HEM mice aged respectively 6 and 24 months. The results of a two-way ANOVA showed a significant effect of age ($F(2, 24)=9.162$; $P\leq0.001$) and genotype ($F(1, 24)=1849.160$; $P<0.001$) on normalized *APP* levels in hippocampus, while the difference between WT and HEM animals was not significantly influenced by age ($F(2, 24)=2.173$; $P>0.05$). Regardless of genotype, differences in *APP* expression were found between the youngest age group (6-8 weeks) and the 6- ($P\leq0.001$) and 24-month-old ($P<0.05$) groups after Tukey's post-hoc tests. In contrast, NRQs of *APP* in WT versus HEM animals were differently influenced by age in cortical tissue

(F(2, 22)=6.291; *P*<0.05), despite a non-significant effect of age on *APP* levels (F(2, 22)=1.676; *P*>0.05) regardless of genotype. Genotype, however, did have a highly significant effect on *APP* expression in the cortex (F(2, 22)=47.827; *P*<0.001).

To investigate the use of B elements as a novel normalization approach, we also compared the *APP* expression profile across genotypes when normalized to B1, B2, and a combination of these B elements (Figure 4). We found that the overexpression levels of *APP* in hippocampus were (9.8 ± 3.3)-fold, (10.3 ± 3.6)-fold, (10.1 ± 3.3)-fold, and (10.0 ± 2.7)-fold, when respective NFs containing B1, B2, B1 and B2, or *Rpl13a* and *Gapdh*, were implemented. In cortex, overexpression of *APP* in HEM compared to WT animals was (6.1 ± 5.2)-fold, (6.9 ± 4.9)-fold, (6.5 ± 4.7)-fold, and (6.7 ± 8.1)-fold when B1, B2, a combination of these SINEs, or the optimal combination of *Actb* and *Tbp* were used as NF, respectively.

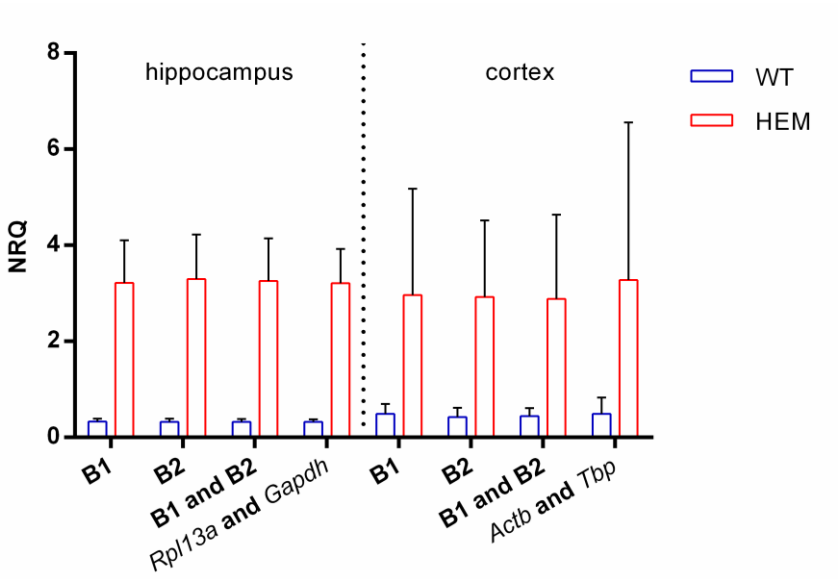

Figure 4. *APP* overexpression levels in hippocampus and cortex. For hippocampus, NRQs of WT (n=15) versus HEM (n=15) animals were obtained using four NFs, including either B1, B2, a combination of B1 and B2, and the optimal combination of *Rpl13a* and *Gapdh*. In case of cortical tissue, 13 WT and 15 HEM animals were included for the analysis of APP levels normalized to B1, B2, a combination of these B elements, and the optimal NF comprising *Actb* and *Tbp*. Data are represented as mean ± SD. Abbreviations: *APP*: amyloid precursor protein; *Gapdh*: glyceraldehyde-3-phosphate dehydrogenase; HEM: hemizygous; NFs: normalization factors; NRQ: normalized relative quantities; *Rpl13a*: ribosomal protein L13A; SD: standard deviation; WT: wild-type.

4. DISCUSSION

4.1. Strengths and limitations

Our study comprised three equally sized age groups, from juvenile to old age, comprising WT and HEM animals of the well characterized APP23 amyloidosis mouse model [251, 252], thus introducing a considerable amount of biological variation and facilitating statistical analysis. In addition, nine commonly used RGs were analyzed in conjunction with two SINEs, using three independent gene ranking algorithms. Since those three algorithms indicated slightly different rankings, rank aggregation was performed to retrieve a final ranking of all RGs and B elements combined, adding further reliability to our study. Apart from gene stability analysis, we also conducted a normalization approach implementing B elements to investigate the expression of *APP*, which is known to be overexpressed in HEM relative to WT mice. Despite the fact that no protein-level analyses were conducted, our study adds relevant information to the characterization of the APP23 model, unambiguously reporting *APP* transgene mRNA levels in transgenic versus WT mice.

A study aspect conceived as both a strength and a limitation, is that regional brain dissection was applied to collect brain samples. Although we have ample expertise in doing so and although this technique was performed with great precision, it gives rise to a larger amount of unwanted variability compared to other techniques such as laser capture microdissection. Moreover, the BestKeeper algorithm does not produce a gene ranking by itself. Other rankings, e.g. based on coefficient of variation and standard deviation, might give rise to alternative results. However, we believe that a gene ranking analysis should preferentially be based on the relation between genes or a measure taking into account distinct sources of variation, and, therefore, we chose to implement the correlation coefficient between each candidate RG and the BestKeeper Index.

4.2. Stability of B elements and commonly used reference genes in hippocampus and cortex

In both hippocampus and cortex, the results of the gene stability analysis were comparable between the three distinct algorithms, with B elements having stability values lower than the geNorm threshold value of 0.5 in hippocampus, while in cortex, their expression levels were less stable compared to the RGs, with stability values near or above the threshold. Two of the most implemented RGs in literature, *Actb* and *Gapdh* [258, 259], were in the top 5 of most stably expressed transcripts,

with geNorm M values far below the threshold value, regardless of tissue type. Moreover, in the final rank merging all analysis methods and tissues, and including only commonly applied RGs, *Actb* and *Gapdh* were classified as the most stable candidate genes. These results do not entirely comply with previous reports on RG stability in the literature. A study investigating the expression stability of six commonly reported RGs in brain regions of mice at postnatal day 7 and at 6 months of age, found that *Gapdh* was ranked sixth of all analyzed genes in the latter age group, with a geNorm M value of 13.396 [260]. However, it should be noted that 6-month-old CD1 mice were first anesthetized by inhalation of isoflurane, followed by decapitation and brain collection, while our results were obtained in transgenic mice with a C57BL/6J background and in the absence of potentially interfering anesthetics. Indeed, inhalation anesthesia with isoflurane has been reported to induce changes in mRNA, even after a short time span [261]. In contrast, another report, focusing on the stability of RGs in the developing mouse brain, indicated that *Actb* was the second most stably expressed RG in the male developing C57BL/6 mouse brain across time points ranging from embryonic day 11.5 until 15.5, with a GeoMean score of 2.73 [262]. As our study pinpointed aging and disease progression rather than development, and since we used a distinct method of euthanasia along with a different mouse model, it is difficult to compare our results with the findings of these previous studies.

4.3. Tissue-specific differences in stability of B elements

When B1 and B2 were normalized to the ideal normalization panel in hippocampus and cortex, both SINEs showed stable expression across hippocampal samples. Although still moderately stable, these SINEs were characterized by a lesser stability in cortex compared to hippocampus. Surprisingly, no significant effects of age or genotype were found in normalized B1 nor B2 levels in the cortex, while their mean values vary considerably (Figure 3). Possibly, the variation in normalized levels of the B elements was too high to detect a statistically significant difference. Although the macroscopic dissection method used for this work is not as accurate as laser capture microdissection, it is unlikely this influenced our findings. Despite the fact that all samples were visually checked for the presence of pieces of white matter, we cannot exclude the possibility that some traces were still present in the collected samples, thereby minimally altering the relative amount of cortex tissue. However, applying laser capture microdissection, would have made the tissue collection process more labor-intensive and time-consuming. Secondly, epigenetic changes such as DNA methylation or histone modification are hallmarks of genomic instability [263] and might underlie the disparity with regard to the

stability of B elements in hippocampus versus cortex. As such, B1 elements were found to be methylated in somatic [216] and embryonic cells [264], with a higher degree of methylation and subsequent transcriptional repression, compared to B2 [264].

Besides B elements, human Alu sequences are also known methylation targets [265-267]. In addition, these SINEs were previously shown to exhibit age- and tissue-specific differential methylation [268-271]. One study even showed that methylation differences between brain areas were more apparent than differences in methylation profile with respect to age, gender, postmortem delay, race, diagnosis, or cause of death [272].

Since we did not generate proof of the nature, nor the direction of epigenetic changes, we can only speculate that a tissue-specific differential degree of epigenetic signaling across age groups and, possibly, genotypes, underlies the differences in the stability of B elements in cortex versus hippocampus. However, to the best of our knowledge, no reports exist of distinct age-or genotype-related epigenetic regulation of SINE expression in brain areas of the APP23 model. Since the first amyloid-β (Aβ)-plaques appear in the frontal cortex of HEM APP23 mice aged 6 months [252], preceded by an increase in soluble Aβ oligomers from the age of 6-8 weeks onwards [273], one could hypothesize that this brain region displays early epigenetic changes as a consequence of initial Aβ exposure, as was previously suggested in female mice of the 5xFAD model [274, 275] and in murine cerebral endothelial cells exposed to synthetic $A\beta_{1\text{-}40}$ peptides [276]. However, one would also expect similar alterations in hippocampal samples of older age groups of the APP23 model, as Aβ plaques are present in virtually all brain regions in HEM APP23 mice aged 24 months [252]. Thus, it remains unclear why B elements are less stably expressed in cortex compared to hippocampus.

4.4. *APP* overexpression to validate the use of B elements as a novel normalization approach

Previously, the transgene in APP23 HEM animals was reported to be sevenfold overexpressed relative to endogenous *App* mRNA, as determined by semi-quantitative PCR and confirmed by western blot experiments [251, 252]. However, it is unclear if these expression levels were determined across several ages or in a single age group. The use of a semi-quantitative technique, along with the number of tissue samples included in these analyses, further hamper a straightforward comparison of our *APP* overexpression data with these initially reported results. However, regardless of age, *APP* was at least twofold overexpressed in HEM relative

to WT animals in the cortex of the youngest age group, while an increasing trend was observed in the older groups (Supplementary Figure 4). Conversely, tissue samples derived from hippocampus showed a relatively constant expression of *APP* in all age groups. Thus, as suggested in the original paper describing the APP23 model, crossing a certain threshold expression of *APP* bearing human disease-associated mutations, might represent an initial incentive for neuropathology [251]. In addition, a specific threshold of two- to threefold *APP* overexpression was previously proposed for successful reproduction of pathological AD-like features [277]. In the youngest age group of this study, *APP* is also overexpressed in HEM versus WT animals, although no plaque-related neuropathology is present at this age. However, as stated previously, high levels of soluble Aβ oligomers were found at this early stage [273] as a possible result of initial *APP* overexpression. Lastly, when hippocampal *APP* levels were not only normalized to the ideal panel of RGs, but also to B1, B2, or a combination of both (Figure 4), the overexpression of human *APP* was almost identical across all normalization strategies, indicating that these SINEs can indeed be adopted as a novel normalization approach in hippocampus. In cortex, the results of Figure 4 appear to indicate that B1, B2 and a combination of these SINEs are superior to the previously determined optimal NF consisting of *Actb* and *Tbp*. However, one should bear in mind that this figure depicts normalized *APP* levels and that the large standard deviation of *APP* NRQs, when the optimal NF was used for normalization purposes, might just reflect a large biological variation of *APP* levels in the cortex of HEM mice. In this aspect, B elements as NF in cortex might fail to indicate the actual results, leading researchers to draw incorrect conclusions. Conversely, a recent paper showed that 11 expressed repeat elements in mice, other than B1 and B2 (amongst others lower in copy number), were superior to commonly used RGs in the literature with respect to normalization purposes in a wide range of experimental setups [224]. Thus, as contemporary research is providing growing evidence concerning the applicability of repeat elements with respect to normalization of mRNA expression levels in various conditions [224, 226, 228, 278], they might become increasingly popular as reference targets.

5. CONCLUSIONS

In conclusion, this study emphasizes the importance of validating RGs in distinct tissue types for optimal normalization results. The expression levels of B elements and *APP* varied to a much larger extent in cortex compared to hippocampus, which might be due to epigenetic alterations. In addition, we provide first evidence for the

use of mouse B elements as an alternative normalization approach in hippocampus, but not cortex, of the APP23 amyloidosis mouse model.

6. ACKNOWLEDGEMENTS

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7. SUPPLEMENTARY MATERIAL

Supplementary File 1. R script – Rank aggregation analysis

```
library(RankAggreg)

library(gtools)

#geNorm, Normfinder, BestKeeper for HC_B1

x <- matrix (c("Gapdh", "Actb", "Ywhaz", "Rpl13a", "B1", "Pgk1", "Tbp", "Hprt", "Gusb", "B2m",
"Rpl13a", "Hprt", "B1", "Gapdh", "Actb", "Gusb", "Pgk1", "Tbp", "Ywhaz", "B2m", "Rpl13a", "Gapdh",
"Actb", "Pgk1", "B1", "Hprt", "Ywhaz", "Tbp", "Gusb", "B2m"),nrow=3, ncol=10, byrow=TRUE)

w <- matrix (c(0.161, 0.168, 0.171, 0.201, 0.219, 0.232, 0.26, 0.288, 0.324, 0.423,
0.172503040293069, 0.278569361760678, 0.295753365118314, 0.296697793885603,
0.341434726963637, 0.411987892506757, 0.425380917048134, 0.428335010025596,
0.434524407987751, 1.19883716315127, 0.045, 0.07, 0.072, 0.074, 0.084, 0.131, 0.147, 0.165,
0.182, 0.537),nrow=3, ncol=10, byrow=TRUE)

List_B1 <- RankAggreg (x, 10, w, method="CE", distance="Spearman", maxIter=1000, conIvn=7,
rho=0.1)

#geNorm, Normfinder and BestKeeper for HC_B2

x <- matrix (c("Gapdh", "Actb", "Ywhaz", "Rpl13a", "Pgk1", "B2", "Tbp", "Hprt", "Gusb", "B2m",
"Rpl13a", "Hprt", "Gapdh", "Actb", "B2", "Gusb", "Tbp", "Ywhaz", "Pgk1", "B2m", "Rpl13a", "Gapdh",
"Actb", "Pgk1", "Hprt", "Ywhaz", "Tbp", "B2", "Gusb", "B2m"),nrow=3, ncol=10, byrow=TRUE)

w <- matrix (c(0.161, 0.168, 0.171, 0.201, 0.22, 0.253, 0.277, 0.302, 0.337, 0.435,
0.171804117131319, 0.280836395227674, 0.288028782748222, 0.337211461393368,
0.36667626083978, 0.417852995687761, 0.423031917378859, 0.424806392681345,
0.429277677377594, 1.2034910521629, 0.042, 0.0649999999999999, 0.069, 0.073, 0.131, 0.141,
0.16, 0.176, 0.196, 0.55), nrow=3, ncol=10, byrow=TRUE)

List_B2 <- RankAggreg (x, 10, w, method="CE", distance="Spearman", maxIter=1000, conIvn=7,
rho=0.1)

#geNorm, Normfinder, BestKeeper for CX_B1

x <- matrix (c("Actb", "Ywhaz", "Tbp", "Gapdh", "Hprt", "Pgk1", "Rpl13a", "Gusb", "B2m", "B1",
"Hprt", "Tbp", "Gapdh", "Ywhaz", "Actb", "Pgk1", "Rpl13a", "Gusb", "B2m", "B1", "Actb", "Tbp",
"Gapdh", "Hprt", "Ywhaz", "Pgk1", "Rpl13a", "Gusb", "B1", "B2m"),nrow=3, ncol=10, byrow=TRUE)

w <- matrix (c(0.253, 0.261, 0.269, 0.305, 0.348, 0.388, 0.416, 0.444, 0.517, 0.653,
0.0871525709263926, 0.160427595012405, 0.183309508692961, 0.220185477007951,
0.312844296118144, 0.346100922578084, 0.440322540305686, 0.451972203626736,
0.627624306433722, 8.01213787586458, 0.079, 0.083, 0.085, 0.111, 0.113, 0.115, 0.129, 0.167,
0.341, 0.53),nrow=3, ncol=10, byrow=TRUE)

List_B1 <- RankAggreg (x, 10, w, method="CE", distance="Spearman", maxIter=1000, conIvn=7,
rho=0.1)
```

```
plot(List_B1)

#geNorm, Normfinder, BestKeeper for CX_B2

x <- matrix (c("Actb", "Ywhaz", "Tbp", "Gapdh", "Hprt", "Pgk1", "Rpl13a", "Gusb", "B2m", "B2",
"Hprt", "Tbp", "Gapdh", "Ywhaz", "Actb", "Rpl13a", "Gusb", "Pgk1", "B2m", "B2", "Actb", "Tbp",
"Gapdh", "Ywhaz", "Hprt", "Pgk1", "Rpl13a", "Gusb", "B2", "B2m"),nrow=3, ncol=10, byrow=TRUE)

w <- matrix (c(0.253, 0.261, 0.269, 0.305, 0.348, 0.388, 0.416, 0.444, 0.517, 0.684,
0.0592977517489527, 0.148448205093243, 0.151554603819292, 0.198810923621684,
0.231585741309127, 0.311947524696954, 0.332931413338353, 0.449928866585664,
0.571097740114864, 3.30761409901505, 0.08, 0.082, 0.091, 0.11, 0.129, 0.142, 0.151, 0.154, 0.474,
0.515),nrow=3, ncol=10, byrow=TRUE)

List_B2 <- RankAggreg (x, 10, w, method="CE", distance="Spearman", maxIter=1000, conIvn=7,
rho=0.1)

#geNorm, NormFinder and BestKeeper correlation coefficient for HKG in both HC and CX

x <- matrix (c("Gapdh", "Actb", "Ywhaz", "Rpl13a", "Pgk1", "Tbp", "Hprt", "Gusb", "B2m", "Rpl13a",
"Hprt", "Gapdh", "Actb", "Gusb", "Pgk1", "Tbp", "Ywhaz", "B2m", "Rpl13a", "Gapdh", "Actb", "Pgk1",
"Hprt", "Ywhaz", "Gusb", "Tbp", "B2m", "Actb", "Ywhaz", "Tbp", "Gapdh", "Hprt", "Pgk1", "Rpl13a",
"Gusb", "B2m", "Actb", "Hprt", "Gusb", "Tbp", "Gapdh", "Rpl13a", "Ywhaz", "Pgk1", "B2m", "Actb",
"Gapdh", "Tbp", "Hprt", "Ywhaz", "Pgk1", "Rpl13a", "Gusb", "B2m"),nrow=6, ncol=9, byrow=TRUE)

w <- matrix (c(0.161, 0.168, 0.171, 0.201, 0.22, 0.256, 0.296, 0.335, 0.443, 0.198883828584835,
0.302745411365983, 0.307835280877475, 0.337969579797726, 0.404473684629807,
0.433874695591076, 0.447394594937274, 0.464781254680886, 1.22225120606555, 0.051,
0.074, 0.077, 0.08, 0.135, 0.156, 0.169, 0.174, 0.515, 0.253, 0.261, 0.269, 0.305, 0.348, 0.388, 0.416,
0.444, 0.517, 0.47818327736617, 0.509644561191983, 0.56304601473059, 0.578738005187571,
0.626568712545538, 0.718513244277592, 0.782401655726401, 0.816541077802296,
1.1739061535887, 0.0600000000000001, 0.0669999999999999, 0.069, 0.091, 0.097, 0.101, 0.116,
0.165, 0.534), nrow=6, ncol=9, byrow=TRUE)

List_HKG <- RankAggreg (x, 9, w, method="CE", distance="Spearman", maxIter=1000, conIvn=7,
rho=0.1)
```

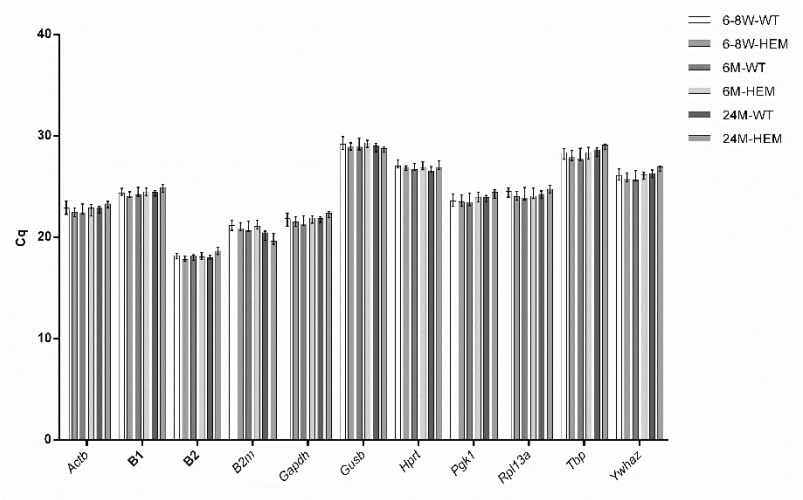
Supplementary Table 1. Individual weights, RNA concentrations and purity values of hippocampal and cortical tissue samples.

	Hippocampus				Cortex			
Age	**weight (mg)**	**RNA concentration (ng/µL)**	**A260/280**	**A260/230**	**weight (mg)**	**RNA concentration (ng/µL)**	**A260/280**	**A260/230**
6-8 weeks	10.8	336.7	2.10	2.20	21.3	958.7	2.12	2.06
	16.5	550.5	2.14	2.12	24.6	1070.4	2.12	2.11
	15.7	396.9	2.08	1.86	30.7	1491.7	2.13	2.28
	18.8	739.9	2.13	2.02	28.5	1401.8	2.12	2.20
	15.3	554.8	2.13	2.03	25.7	1117.2	2.12	2.15
	17.2	567.4	2.13	1.88	24.2	1044.9	2.12	2.10
	13.7	467.4	2.04	2.07	27.5	1612.4	2.11	2.25
	13.8	414.7	2.06	1.79	26.4	1543.7	2.14	2.27
	13.8	554.1	2.12	2.06	28.9	1586.9	2.13	2.22
	13.5	403.6	2.06	2.11	32.5	1737.0	2.13	2.03
6 months	21.1	373.9	2.08	1.78	22.6	827.1	2.12	2.11
	15.0	466.9	2.04	2.06	32.1	1731.6	2.10	2.27
	15.6	528.8	2.11	1.86	25.0	1225.9	2.14	2.13
	14.5	424.0	2.09	1.87	32.2	1709.9	2.13	2.23
	18.3	553.7	2.14	1.94	29	1320.7	2.12	2.17
	12.2	353.8	2.10	1.69	30.9	1216.9	2.11	2.18
	14.5	396.5	2.08	1.96	26.5	1183.4	2.11	2.29
	16.8	579.5	2.16	1.97	27.6	1434.2	2.11	2.27
	11.5	343.3	2.09	1.47	24.3	1048.4	2.13	2.07
	13.0	435.4	2.07	1.94	30.1	1551.9	2.12	2.28

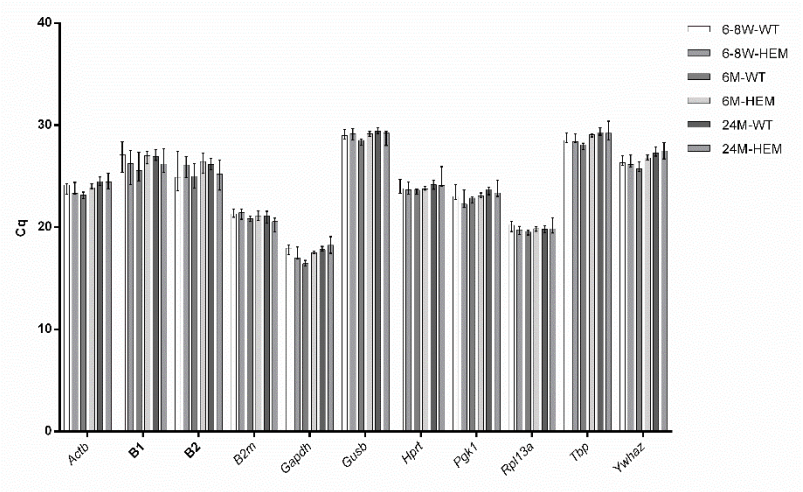
Supplementary Table 1. Continued.

	Hippocampus				Cortex			
Age	**weight (mg)**	**RNA concentration (ng/µL)**	**A260/280**	**A260/230**	**weight (mg)**	**RNA concentration (ng/µL)**	**A260/280**	**A260/230**
24 months	22.3	774.3	2.13	1.98	27.1	1065.6	2.11	2.22
	19.3	730.7	2.12	1.91	31.4	1460.1	2.11	2.26
	20.7	674.9	2.14	2.01	24.7	1145.5	2.10	2.09
	19.2	624.2	2.12	2.08	26.7	1436.0	2.13	2.19
	16.0	422.6	2.06	2.06	26.6	1177.6	2.12	2.14
	22.0	832.4	2.13	2.12	18.3	700.8	2.09	2.07
	24.3	805.4	2.12	1.99	20.2	950.7	2.12	2.18
	18.2	611.1	2.12	2.04	27.1	1278.7	2.08	1.97
	13.5	536.6	2.11	2.16	25.1	1252.0	2.13	2.16
	19.7	737.1	2.13	2.14	22.8	1168.3	2.13	2.19

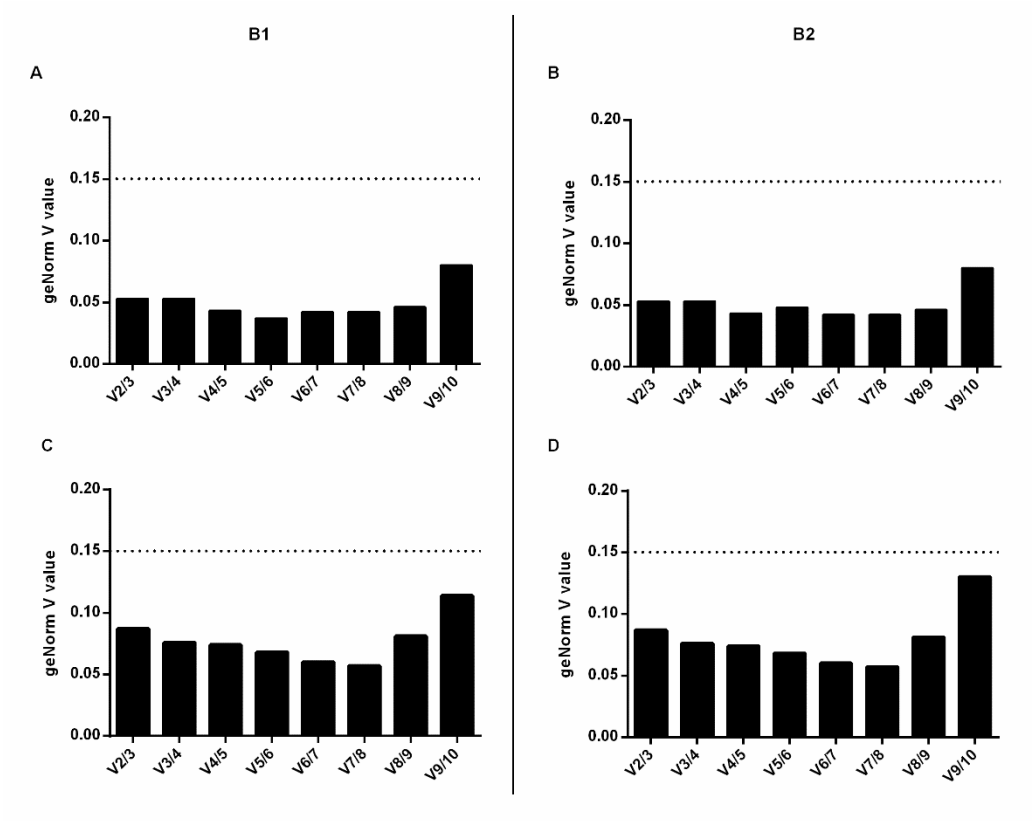
Individual data for each animal are presented.



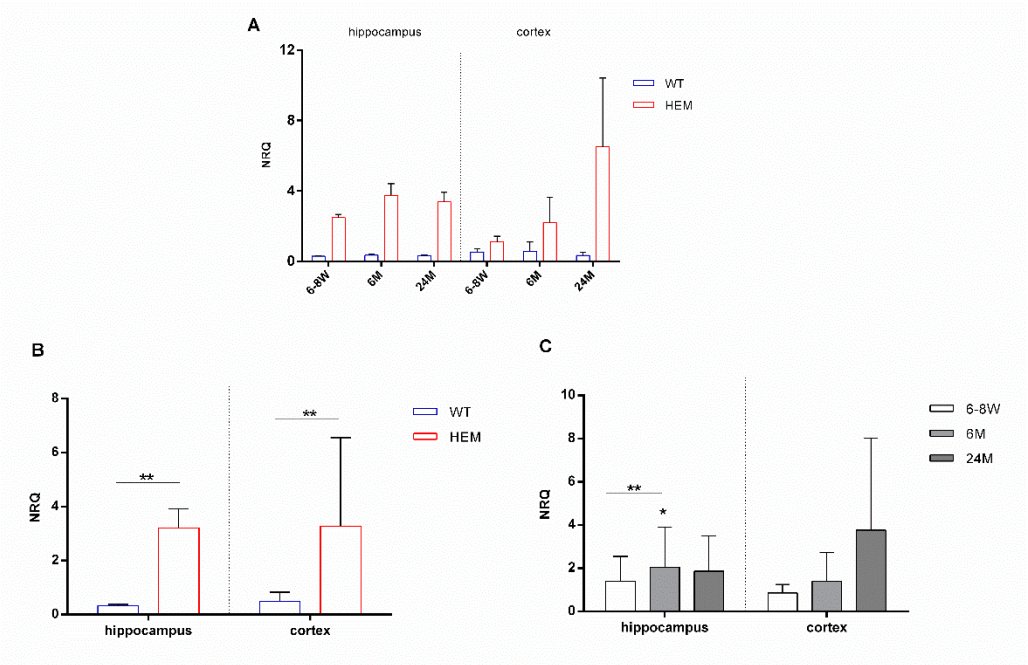
Supplementary Figure 1. Cq values of all RGs and B elements in the hippocampus of APP23 WT (n=5) and HEM (n=5) mice aged 6-8 weeks, 6, and 24 months. Data are represented as median with interquartile range. Abbreviations: Cq: quantitation cycle; HEM: hemizygous; M: months; RGs: reference genes; W: weeks; WT: wild-type.



Supplementary Figure 2. Cq values of all RGs and B elements in the cortex of APP23 WT (n=5) and HEM (n=5) mice aged 6-8 weeks, 6, and 24 months. Data are represented as median with interquartile range. Abbreviations: Cq: quantitation cycle; HEM: hemizygous; RGs: reference genes; SD: standard deviation; WT: wild-type.



Supplementary Figure 3. Pairwise variation of NFs upon inclusion of an additional RG. geNorm analyses were performed in hippocampal tissue (A, B) and cortex (C, D), with either B1 or B2. The optimal number of RGs is indicated by the cutoff value of 0.15, below which the benefit of including an $(n+1)^{th}$ RG is limited. Abbreviations: NF: normalization factor; $V_{(n/n+1)}$: pairwise variation between normalization factors incorporating a consecutive number of reference genes; RG: reference gene.



Supplementary Figure 4. A. Normalized expression levels of *APP* in WT and HEM mice aged 6-8 weeks, 6 months and 24 months. B. Normalized expression of *APP* in pooled age groups. C. Normalized expression of *APP* levels in pooled genotype groups. In all cases, *APP* was normalized to the optimal normalization panel in each tissue. For analyses in hippocampus, five WT and HEM animals were included in all age groups, whereas for cortex, the 6-8W and 24M WT groups contained four mice. Data are represented as mean ± SD, whereas statistical analyses were performed on log-transformed data. Statistical differences with $P<0.05$ and $P\leq0.001$, are indicated by respectively one and two asterisks. Abbreviations: APP: amyloid precursor protein; HEM: hemizygous; M: months; NRQ: normalized relative quantities; W: weeks; WT: wild-type.

Chapter IV

Monoaminergic disturbances in neurodegenerative disorders

Chapter IV.1. Brain serotonergic and noradrenergic deficiencies in behavioral variant frontotemporal dementia compared to early-onset Alzheimer's disease

Yannick Vermeiren[a,b,1], Jana Janssens[a,1], Tony Aerts[a], Jean-Jacques Martin[d], Anne Sieben[d,e], Debby Van Dam[a,b], Peter P. De Deyn[a,b,c,d]

[a]*Department of Biomedical Sciences, Laboratory of Neurochemistry and Behavior, Institute Born-Bunge, University of Antwerp, Wilrijk (Antwerp), Belgium*
[b]*Department of Neurology and Alzheimer Research Center, University of Groningen and University Medical Center Groningen (UMCG), Groningen, The Netherlands*
[c]*Department of Neurology and Memory Clinic, Hospital Network Antwerp (ZNA) Middelheim and Hoge Beuken, Antwerp, Belgium*
[d]*Biobank, Institute Born-Bunge, University of Antwerp, Wilrijk (Antwerp), Belgium*
[e]*Department of Neurology, University Hospital Ghent and University of Ghent, Ghent, Belgium*

[1]These authors contributed equally to this work

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ABSTRACT

Routinely prescribed psychoactive drugs in behavioral variant frontotemporal dementia (FTD) for improvement of (non)cognitive symptoms are primarily based on monoamine replacement or augmentation strategies. These were, however, initially intended to symptomatically treat other degenerative, behavioral or personality disorders, and thus lack disease specificity. Moreover, current knowledge on brain monoaminergic neurotransmitter deficiencies in this presenile disorder is scarce, particularly with reference to changes in Alzheimer's disease (AD). The latter hence favors neurochemical comparison studies in order to elucidate the monoaminergic underpinnings of FTD compared to early-onset AD, which may contribute to better pharmacotherapy. Therefore, frozen brain samples, i.e. Brodmann area (BA) 6/8/9/10/11/12/22/24/46, amygdala and hippocampus, of 10 neuropathologically confirmed FTD, AD and control subjects were analyzed by means of reversed-phase high-performance liquid chromatography. Levels of serotonergic, dopaminergic and noradrenergic compounds were measured. In nine brain areas, serotonin (5-HT) concentrations were significantly increased in FTD compared to AD patients, while 5-hydroxyindoleacetic acid/5-HT ratios were decreased in eight regions, also compared to controls. Furthermore, in all regions, noradrenaline (NA) levels were significantly higher, and, 3-methoxy-4-hydroxyphenylglycol/NA ratios significantly lower in FTD than in AD and controls. Contrarily, significantly higher dopamine (DA) levels and reduced homovanillic acid/DA ratios were only found in BA12 and BA46. Results indicate that FTD is defined by distinct serotonergic and noradrenergic deficiencies. Additional research regarding the interactions between both monoaminergic networks is required. Similarly, clinical trials investigating the effects of 5-HT_{1A} receptor antagonists or NA-modulating agents, such as $\alpha_{1/2}$/β_1-blockers, seem to have a rationale and should be considered.

1. INTRODUCTION

Inasmuch as remarkable progress in diagnostic, molecular, genetic and neuropathological aspects of frontotemporal dementia (FTD) has been made in recent years, efficient neurotransmitter-based pharmacotherapies for substantial improvement of cognitive and noncognitive symptoms, however, are still lacking, with no Food and Drug Administration (FDA)-approved treatment at the moment [279]. In addition, routinely prescribed psychoactive drugs in FTD are primarily based on monoamine replacement or augmentation strategies, which, conversely, have been developed to symptomatically treat other neurodegenerative, behavioral or personality disorders, such as Alzheimer's disease (AD), Parkinson's disease, major depressive disorder, obsessive-compulsive disorder, and, schizophrenia [280, 281]. Moreover, current knowledge on brain monoaminergic neurotransmitter deficiencies in this devastating presenile disorder is scarce, particularly with reference to changes in AD [282], still the most common form of primary early-onset dementia [36]. The latter thus favors neurochemical comparison studies in order to fully clarify the monoaminergic underpinnings of FTD as opposed to early-onset AD, which could contribute to the development of more dementia subtype-specific pharmacological treatments in the long term, and, provide more insight into which combinations of currently administered psychotropic drugs may be more effective.

In this context, Bowen et al. [282] evidenced that there might be a severely imbalanced serotonergic system in frontal and temporal cortex of FTD compared to AD patients, whereas there are no similar reports in literature of studies that examined noradrenergic and dopaminergic brain changes. On the other hand, several cerebrospinal fluid (CSF) studies have been performed previously, even though results were found to be inconsistent. More specifically, some studies observed no alterations in CSF 5-hydroxyindoleacetic acid (5-HIAA; metabolite of serotonin (5-HT; 5-hydroxytryptamine)) content between FTD and AD [149, 176, 280], whereas Engelborghs et al. [283] demonstrated higher CSF homovanillic acid (HVA; metabolite of dopamine (DA)) to 5-HIAA ratios in FTD, which was interpreted as a reflection of the inhibitory modulation of the serotonergic system on dopaminergic functioning [284, 285]. As for the dopaminergic neurotransmitter system, Sjögren et al. [149] concluded that only CSF HVA levels in FTD compared to healthy controls were reduced, while the study of Francis et al. [286] did not report such alterations. Interestingly, the same holds true for the comparison with AD patients [176, 283]. Additionally, Engelborghs et al. [283] also revealed a strong association between physically nonaggressive behavior and CSF levels of 3,4-dihydroxyphenylacetic acid (DOPAC; metabolite of DA) in a subgroup of FTD subjects, which indicates that monoaminergic neurotransmitter alterations might

certainly underlie dementia-specific neuropsychiatric symptoms (NPS) [144, 287]. Finally, there is little evidence on noradrenergic deficiencies in FTD compared to AD, with only one CSF study that showed significantly higher 3-methoxy-4-hydroxyphenylglycol (MHPG; metabolite of (nor)adrenaline ((N)A)) levels in FTD [149]. Engelborghs et al. [283] and Vermeiren et al. [176], however, could not corroborate these findings. Sjögren et al. [149] also suggested that CSF MHPG might be useful to differentiate between both neurodegenerative conditions. Correspondingly, the addition of CSF MHPG to the traditional set of AD biomarkers (amyloid-$\beta_{1\text{-}42}$ peptide, total- and phosphorylated tau protein) has shown to improve the discrimination of dementia with Lewy bodies (DLB) from AD, but not FTD, whereas the differentiation of AD from FTD only improved marginally [146].

Besides neurochemical studies quantitatively measuring CSF or brain tissue monoamine levels, neuroimaging, receptor, and, transporter binding studies also found strongly decreased 5-HT_{1A} and 5-$HT_{2(A)}$ receptor bindings, a decreased presynaptic striatal DA transporter binding, and, an unchanged to decreased postsynaptic striatal D2 receptor binding in FTD patients compared to healthy elderly [280, 288, 289]. The authors further implied that selective serotonin reuptake inhibitors (SSRIs) as antidepressants may be used as a first-line treatment to reduce NPS in FTD, even though most related clinical trials were small and uncontrolled [280]. As a result, future neurochemical FTD studies have become essential.

Altogether, more insight into the distribution of brain monoamines and metabolites in FTD compared to AD could subsequently not only contribute to a better monoaminergic-based therapeutic approach and understanding of disease-related pathophysiological mechanisms but might even enable the appraisal of their added biomarker value. Therefore, the present study determined the levels of eight monoamines and metabolites in various postmortem brain regions of age- and gender-matched neuropathologically defined patients with FTD, AD, and, a healthy control group. Except for the intergroup comparisons, the relationship between antemortem NPS and the analyzed neurochemical compounds were examined in both dementia subtypes as well. On the whole, and, based on aforementioned studies, we mainly expect to find a severely impaired serotonergic and dopaminergic neurotransmission in FTD compared to AD brain with significantly altered 5-HT, DA, DOPAC and HVA levels, and, to a lesser extent 5-HIAA levels, whereas the noradrenergic system might have remained relatively preserved (except for MHPG levels).

2. MATERIALS AND METHODS

2.1. Study population and protocol

Frontotemporal lobar degeneration (FTLD) is an umbrella term encompassing a group of heterogeneous pathological disorders which are characterized by relatively selective frontotemporal atrophy and disease onset before the age of 65 in 75-80% of patients. Two main clinical phenotypes exist, including the behavioral variant FTD, which accounts for more than 50% of patients, and, in the current study referred to as 'FTD', in addition to primary progressive aphasia, which can be further categorized into primary nonfluent aphasia and semantic dementia [36, 290]. Primary characteristics of FTD are impairment of executive functions and severe behavioral changes, while at least in the initial stages of the disease, memory and perceptuospatial skills remain intact [291].

Besides neuropathologically confirmed FTD patients (n=10), ten neuropathologically confirmed AD patients and ten age-matched control subjects were included in our study population as well. All samples were retrospectively selected from the Biobank of the Institute Born-Bunge (University of Antwerp, Antwerp, Belgium). Initially, patients with probable AD according to the NINCDS-ADRDA criteria of McKhann et al. [16, 292] were recruited at the Memory Clinic of the Hospital Network Antwerp (ZNA-Middelheim and ZNA-Hoge Beuken, Antwerp, Belgium) for inclusion in a prospective, longitudinal study on NPS [293]. The latter also applies to all included FTD and control subjects. Probable FTD was diagnosed using the criteria of Neary et al. [39]. All patients also fulfilled the DSM-IV-TR criteria for dementia [177]. The ten age- and gender-matched included AD subjects were part of a larger group of 40 on which neurochemical NPS-related brain research has been conducted previously [199, 294].

Apart from general physical and neurological examinations, blood screening tests, structural neuroimaging by CT, MRI or SPECT, neuropsychological examination (Mini-Mental State Examination scores (MMSE)) and optional CSF/blood sampling for biomarker and/or DNA analyses, a baseline behavioral assessment was routinely performed as well. Follow-up behavioral ratings of AD and FTD patients were performed, if possible. Age-matched control subjects were hospitalized in the Middelheim General Hospital (Antwerp, Belgium), and gave brain donation autopsy consent shortly before death. Moreover, clinical neurological examination and a retrospective review of the clinical history, neuropsychological examination and hospital records, did not demonstrate any evidence of dementia, psychiatric antecedents or cognitive decline. Furthermore, none of the control subjects suffered from central nervous system pathology which was neuropathologically confirmed. Death causes were carcinoma (esophageal (n=1); cervical (n=1); lung

(n=2); neuroendocrine (n=2)), multiple myeloma (n=1)), liver cirrhosis (n=1), cardiovascular disease/metabolic syndrome (n=1) and Burkitt's lymphoma (n=1). Written informed consents concerning autopsy and subsequent use of brain tissue, clinical documentation and behavioral information for research purposes were obtained from all participants. The study was approved by the Medical Ethical Committee of the Middelheim General Hospital (Antwerp, Belgium), and conducted in compliance with the Helsinki Declaration.
In case AD, FTD or control subjects who gave brain donation consent, died, brain autopsy was performed within eight hours postmortem, followed by freezing of the left hemisphere at -80°C for neurochemical analysis, and fixation of the right hemisphere in formalin (12%) for neuropathological examination.

2.2. Behavioral assessment

Behavior of AD and FTD patients was assessed together with relatives and/or nursing staff using a battery of behavioral assessment scales, including: the Behavioral Pathology in Alzheimer's Disease Rating Scale (Behave-AD) [295]; Middelheim Frontality Score (MFS) [291]; Cohen-Mansfield Agitation Inventory (CMAI) [296]; and Cornell Scale for Depression in Dementia (CSDD) [297]. Dementia staging was based on the Global Deterioration Scale (GDetS) with a range varying from 1 (nondemented) to 7 (terminal stage of dementia) [298]. During each NPS rating, only the behavioral phenomena covering the last two weeks prior to the assessment were included and rated. Behavioral assessments were repeated during each neurological follow-up examination in the hospital, if possible (n=2 for AD with one (n=1) and two (n=1) follow-up ratings; n=4 for FTD with one (n=1), three (n=2) and four (n=1) follow-up ratings). A final retrospective behavioral scoring was performed in case patients died approximately more than two weeks after the last follow-up visit. However, in total, eight AD and six FTD patients underwent only one rating close to death, given the short amount of time which was left since they entered our study protocol. No behavioral scores were available for the control group. For this study, behavioral scores of the final assessment as close as possible to date of death were used.

2.3. Neuropathological evaluation

Neuropathological diagnosis was performed on the formaldehyde-fixated right hemisphere. A standard selection of 10 to 13 regionally dissected brain regions, including frontal, temporal and occipital blocks of the neocortex, amygdala, hippocampus (at the level of the posterior part of the amygdala and the lateral geniculate body), basal ganglia, thalamus, brain stem, substantia nigra (SN), pons at the level of the locus coeruleus (LC) and cerebellum, was embedded in paraffin and routinely stained with haematoxylin-eosin, cresyl violet and Bodian's

technique. Furthermore, routinely applied immunostains were 4G8 (amyloid) and AT8 (P-tau$_{181\text{-}P}$), as well as staining against hyperphosphorylated transactive response DNA-binding protein (TDP)-43 and ubiquitin. When the presence of Lewy bodies was suspected on haematoxylin-eosin and ubiquitin immunoreactivity, an anti-α-synuclein staining was applied.
AD patients were neuropathologically diagnosed according to the criteria of Braak and Braak [10], Braak et al. [11], and, Jellinger and Bancher [299] to decide on definite AD. Additionally, the 'ABC' scoring method of Montine et al. [8] was applied to all AD brains collected after May 2011 (n=2). FTD patients on the other hand, were diagnosed using the criteria by Cairns et al. [35] and Mackenzie et al. [42, 300, 301], which propose a new terminology for FTLD-subtypes and a classification of TDP-43 proteinopathies into types A-D [301]. The overall histopathological diagnoses of the included FTD subjects were FTLD-tau/Pick's disease (n=3) and FTLD with ubiquitin-positive inclusions (FTLD-U) (n=7), of which the FTLD-U patients could be further categorized into FTLD-TDP-43 type A (n=3), FTLD-TDP-43 type B (n=3) and FTLD-ubiquitin proteasome system (UPS) (n=1).

2.4. Regional brain dissection

Regional brain dissection of the left frozen hemisphere was performed according to a standard procedure [199, 294] during which 21 brain regions are dissected. With regard to this study design, a total of 11 behaviorally and neurochemically relevant brain areas were ultimately analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) with electrochemical detection (ECD), i.e. Brodmann area (BA) 6, 8, 9 and 10 (medial and prefrontal cortex), BA11 and 12 (orbitofrontal cortex), BA22 (temporal cortex), BA24 (cingulate gyrus), BA46 (dorsolateral prefrontal cortex), amygdala and hippocampus.

2.5. Neurochemical analysis, sample preparation procedure and pH measurement

A recently optimized and validated RP-HPLC-ECD system for the fast and simultaneous detection of monoaminergic compounds in human brain tissue was used to simultaneously measure the concentrations of 5-HT, (N)A, DA, and their respective metabolites, i.e. 5-HIAA, MHPG and DOPAC/HVA [39]. In short, sample analysis was performed using an Alexys™ Dual Monoamines Analyzer (Antec Leyden BV, Zoeterwoude, The Netherlands) by which each brain tissue sample was directly analyzed in duplicate. Output ranges were 500 pA and 1 nA with two electrochemical VT03 flow cells each containing a glassy carbon working electrode of 0.7 mm and an in situ Ag/AgCl reference electrode at 670 mV placed in a Decade II electrochemical detector (Antec Leyden BV, Zoeterwoude, The Netherlands). An isocratic flow rate of 40 µL of mobile phase per minute was set for both LC 110

pumps. The optimal conditions for separation of the monoaminergic compounds were obtained using a mobile phase comprising 13% methanol combined with a mixture of phosphoric (50 mM) and citric acid (50 mM), octane-1-sulfonic acid sodium salt (1.8 mM), KCl (8 mM) and ethylenediaminetetraacetic acid (EDTA) (0.1 mM) (pH 3.6). Samples were loaded onto two microbore ALF-125 columns (250 mm x 1.0 mm, 3 μm particle size) filled with a porous C18 silica stationary phase. Separation of the monoamines and metabolites was achieved at a stable column and VT03 flow cell temperature of 36°C with a total runtime of approximately 45 min per sample. Levels of the monoaminergic compounds were calculated using Clarity™ Software (DataApex Ltd, 2008, Prague, The Czech Republic). All purchased chemicals were of analytical grade. The brain sample preparation procedure prior to RP-HPLC-ECD analysis was fast and simple, and, performed as described in Van Dam et al. [302]. Samples need to be nonacidotic (i.e. pH>6.1) [303, 304] in order to guarantee high-quality brain tissue since acidosis may induce alterations in neurotransmitter concentrations and enzyme activities. Several factors such as a prolonged death struggle, antemortem stroke and a long postmortem delay could acidify brain tissue [305]. For this study, pH values of the cerebellar cortex were measured since the cerebellar pH has previously been shown to be most representative for the entire brain hemisphere [306]. The accompanying analytical procedure was performed as described by Stan et al. [306].

2.6. Statistical analysis

Nonparametric statistics were applied due to the limited number of patients and ordinal variables (behavioral scores). A Shapiro Wilk test of normality was first performed to test whether our obtained data complied with a normally distributed study population. Fisher's Exact test was applied to compare male/female ratios and patients taking/not taking psychotropic medication across groups. Kruskal-Wallis analyses with post-hoc Mann-Whitney U tests were used for comparison of all demographic, clinical, behavioral, pH and monoaminergic data between AD, FTD and control subjects. In all cases, only data remaining statistically significant following a Bonferroni correction for multiple comparisons (P<0.017 for three group comparisons) were considered significant. Mann-Whitney U tests were also applied to look at potential confounding effects of psychotropic medication within each group. Finally, in order to calculate neurochemical correlations of MFS-, CMAI-, CSDD- and Behave-AD cluster scores in the total group of AD (n=10) and FTD (n=10) patients, nonparametric Spearman's Rank Order correlation statistics were applied. Again, a Bonferroni correction was performed and only those significant data were taken into account (P<0.000022).
All analyses were performed using SPSS 22.0 for Windows (IBM SPSS Software, Armonk, NY, IBM Corp).

3. RESULTS

3.1. Demographics, clinical data, behavioral assessment scores, dementia staging and pH values

Corresponding data are summarized in Table 1 and the electronic Supplementary Material. The AD, FTD and control groups were age- and gender-matched. Moreover, the number of patients taking/not taking psychotropic medication was comparable between all groups (P>0.05). In the AD group, administered subtypes of psychotropic medication were antidepressants (n=1), antipsychotics (n=2) and cholinesterase inhibitors (n=1). In the FTD group, patients were on antidepressants (n=4), antipsychotics (n=2), cholinesterase inhibitors (n=1) and benzodiazepines (n=2). Lastly, some control subjects were on antidepressants (n=1) and benzodiazepines (n=1). There were statistically significant differences regarding storage times of the frozen brain material between the AD and control group, and, the FTD and control group (P<0.001 and P<0.05, respectively). The average interval between the last behavioral rating and time of death was 0 and 2.9 days for the AD and FTD groups, respectively. Postmortem delay, GDetS scores and pH-values were comparable between groups. Additionally, all FTD and control subjects had cerebellar pH values >6.1. In contrast, two AD patients had low cerebellar pH-values (<6.1), for which supplementary pH analyses on the remaining 11 brain regions were performed. Eventually, brain samples with acidotic pH values were excluded from statistical analysis, i.e.: BA8 (n=1), BA9 (n=1), BA22 (n=1), and BA46 (n=1).

Table 1. Demographics, clinical data, dementia staging and pH values.

Parameter	AD (n=10)	FTD (n=10)	Controls (n=10)	Kruskal-Wallis/Mann-Whitney U
Demographics and clinical data				
Age at onset dementia (years)	60.0 ± 7.8 (49-71)	56.5 ± 5.7 (47-65)	N/A	P>0.05
Age at death (years)	66.4 ± 6.4 (58-75)	63.4 ± 6.3 (51-72)	65.7 ± 5.3 (57-73)	P>0.05
Gender, male/female (n)	7/3	7/3	6/4	Fisher's Exact test = 0.418 P>0.05
Storage time tissue at -80°C (years)	3.0 ± 1.2[aa] (0.3-9.9)	4.9 ± 1.4[b] (0.7-15.0)	9.9 ± 0.7[aa,b] (4.3-11.6)	***P*≤0.001**
Taking/not taking psychotropic medication (n)	3/7	5/5	2/8	Fisher's Exact test = 2.015 P>0.05

Table 1. Continued.

Parameter	AD (n=10)	FTD (n=10)	Controls (n=10)	Kruskal-Wallis/Mann-Whitney U
Dementia staging and pH data				
GDetS score (/7): dementia staging	6.6 ± 0.7 (5-7)	6.7 ± 0.7 (5-7)	N/A	*P*>0.05
pH values cerebellar brain tissue	6.3 ± 0.4 (5.9-7.1)	6.4 ± 0.3 (6.1-6.8)	6.3 ± 0.2 (6.1-6.6)	*P*>0.05

Mean ± SD; minimum-maximum range is displayed between parentheses. The results of Kruskal-Wallis and Mann-Whitney U tests for 3 and 2 group comparisons, respectively, are displayed in the rightmost column. Significant *P* values (<0.05) are depicted in bold. One and two superscript letters are used to indicate significant group differences after post-hoc Mann-Whitney U analyses with *P*<0.017 and *P*<0.001, respectively. Letters a and b signify differences between AD and controls[a] and FTD and controls[b]; *Postmortem delay indicates the number of hours between time of death and storage of the brain at -80°C. Abbreviations: AD: Alzheimer's disease; FTD: frontotemporal dementia; GDetS: Global Deterioration Scale; N/A: not applicable.

Brain MRI data (not shown) revealed that 7 out of 10 FTD subjects had no asymmetric brain degeneration on average two years before brain autopsy. There was a maximum of 39 and minimum of 3 months between MRI scans and death. Of the three individuals with asymmetric brain atrophy, one had predominant left atrophy of temporal and frontal lobes, and the two others had a slightly more pronounced right atrophy of the temporal horn (but not frontal lobe).

As for the behavioral data, Behave-AD cluster B (*P*<0.05), AB (*P*<0.05), D (*P*<0.05) and total (*P*<0.05) scores, as well as the CMAI cluster 1 (*P*<0.05), 3 (*P*<0.05), and, CSDD total scores (*P*<0.05) were all significantly higher in the AD group as compared to their FTD counterparts (Supplementary Table 1).

3.2. Neurochemical results

Monoaminergic data of the intergroup comparisons are summarized in Table 2. Nonsignificant data were omitted. Likewise, the most significant group differences regarding 5-HIAA/5-HT ratios, 5-HT levels, MHPG/NA ratios and NA levels are represented in Figures 1-4.

3.2.1. Serotonergic findings

Firstly, 5-HIAA/5-HT ratios, indicative of the catabolic serotonergic turnover, were significantly lower in FTD compared to AD subjects in eight brain regions (BA8, *P*<0.05; BA9, *P*<0.05; BA10, *P*<0.05; BA11, *P*<0.05; BA12, *P*<0.05; BA22, *P*<0.0001; BA46, *P*<0.05; and hippocampus, *P*<0.05). The same applied to five brain regions for the FTD versus control group comparison (BA8, BA9, BA11, BA12, and BA22; for all: *P*<0.05). Remarkably, only in BA22, a significant difference in 5-HIAA/5-HT

ratios could be detected between AD and control subjects, with higher values in the AD group (P<0.05) (Figure 1).

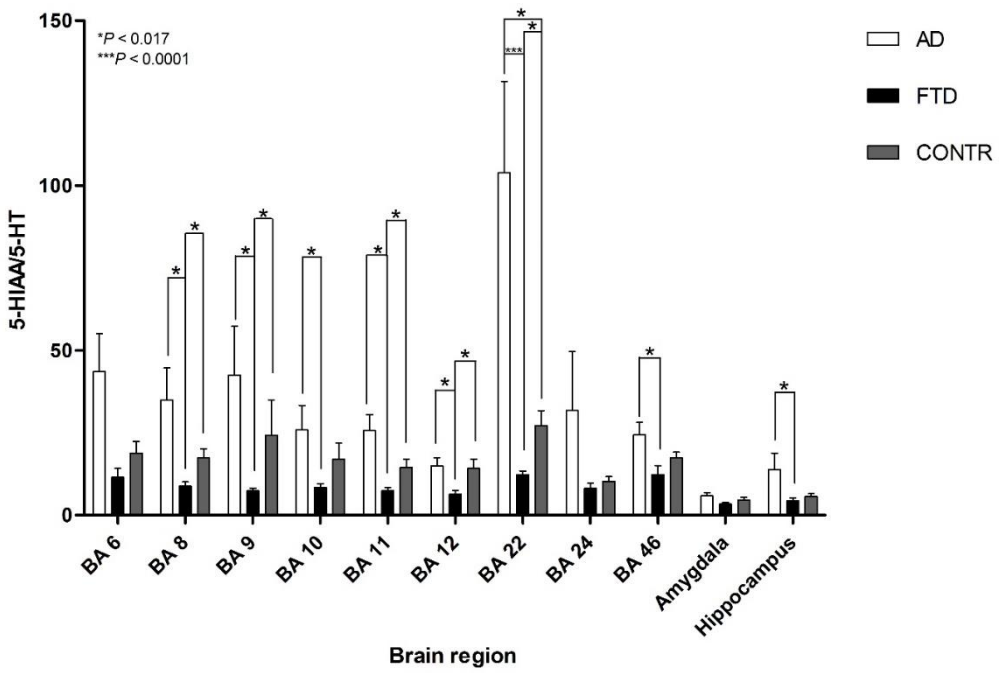

Figure 1. 5-HIAA/5-HT ratios, indicative of the catabolic serotonergic turnover, across eleven brain regions in AD compared to FTD and control subjects. Data are presented as mean with SD. Only differences (Kruskal Wallis, P<0.05) remaining statistically significant after post-hoc Mann-Whitney U tests with Bonferroni correction are indicated by star symbols (*P<0.017; ***P<0.0001). Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; 5-HT: 5-hydroxytryptamine; AD: Alzheimer's disease; BA: Brodmann area; CONTR: controls; FTD: frontotemporal dementia.

Furthermore, 5-HT concentrations were higher in FTD than in AD patients in nine out of 11 brain regions (BA6, P<0.05; BA8, P<0.05; BA9, P<0.05; BA11, P<0.05; BA12, P≤0.001; BA22, P≤0.001; BA46, P<0.05; amygdala, P<0.05; and hippocampus, P<0.05). In the amygdala and hippocampus, 5-HT levels were significantly higher in control subjects compared to AD patients as well (P<0.0001 and P≤0.001, respectively). No significant differences, however, were identified between the FTD and control group. Notably, in BA10 and BA24, no significant group differences were found (Figure 2).

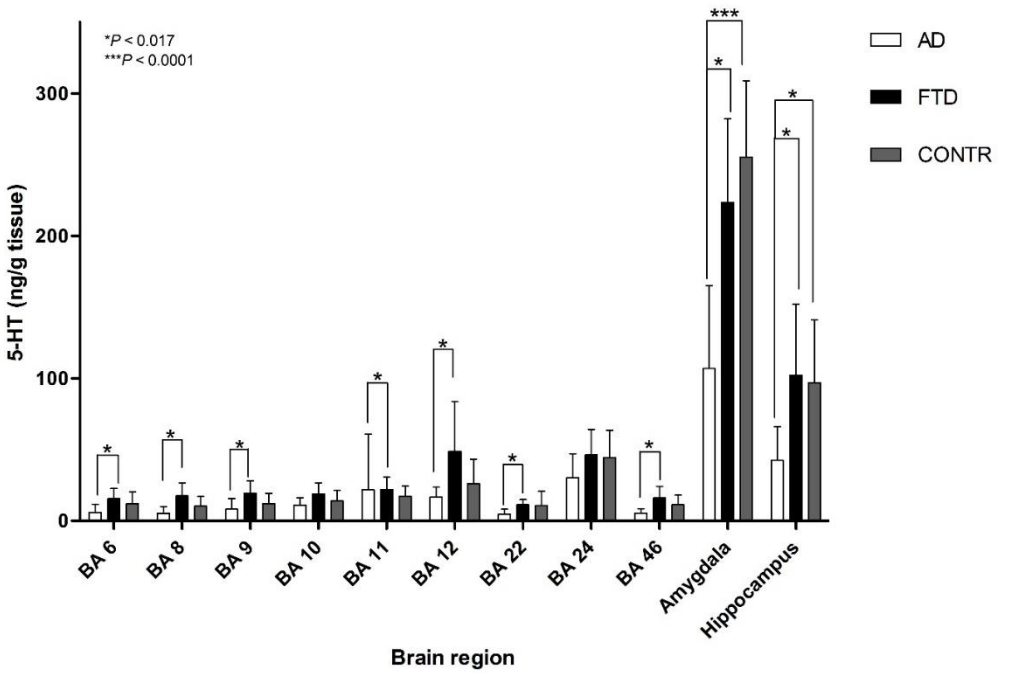

Figure 2. 5-HT levels across eleven brain regions in AD compared to FTD and control subjects. Data are presented as mean with SD. Only differences (Kruskal Wallis, $P<0.05$) remaining statistically significant after post-hoc Mann-Whitney U tests with Bonferroni correction are indicated by star symbols (*$P<0.017$; ***$P<0.0001$). Abbreviations: 5-HT: 5-hydroxytryptamine; AD: Alzheimer's disease; BA: Brodmann area; CONTR: controls; FTD: frontotemporal dementia.

Lastly, in BA22, 5-HIAA concentrations were significantly higher in AD compared to FTD patients ($P<0.05$) whereas in the amygdala, 5-HIAA levels were significantly lower in AD compared to control subjects ($P\leq0.001$) (Table 2).

3.2.2. Noradrenergic findings

For all brain regions analyzed, FTD patients had the lowest MHPG/NA ratios, indicative of the catabolic noradrenergic turnover, compared to their AD counterparts, with the most pronounced differences in BA6, BA9, BA10, BA11, BA24, amygdala and hippocampus (for all: $P<0.0001$; Figure 3). Additionally, in BA9, BA10, BA11, BA22, BA24 and BA46 (for all: $P<0.05$), MHPG/NA ratios were significantly lower in FTD as opposed to control subjects as well. Moreover, in the prefrontal cortex, ratios were higher in the AD compared to the control group (BA6, $P<0.05$; BA8, $P<0.05$; BA9, $P\leq0.001$; and BA10, $P<0.05$).

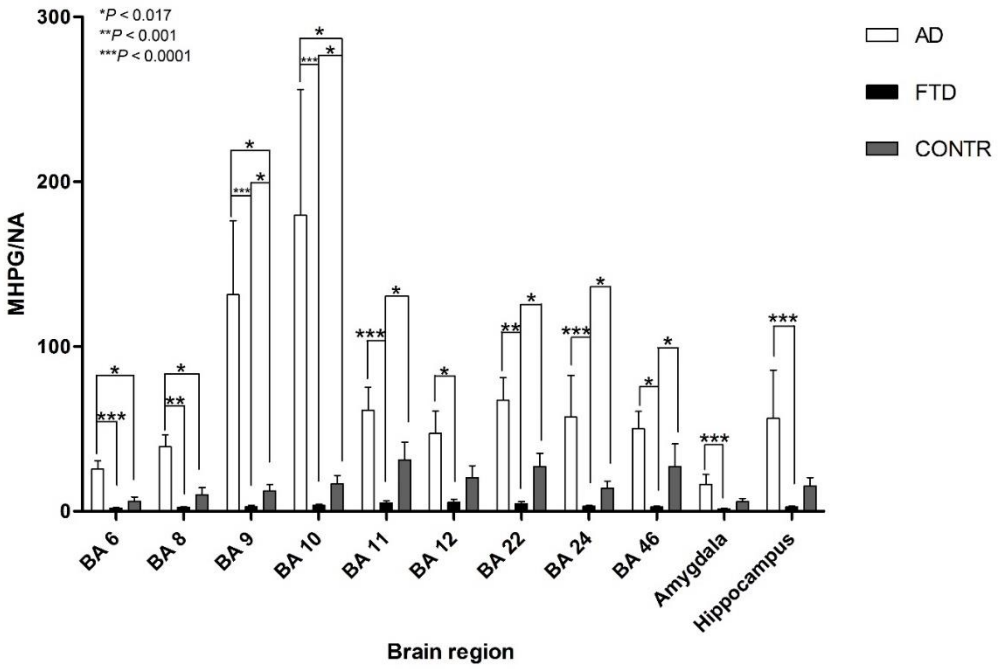

Figure 3. MHPG/NA ratios, indicative of the catabolic noradrenergic turnover, across eleven brain regions in AD compared to FTD and control subjects. Data are presented as mean with SD. Only differences (Kruskal Wallis, $P<0.05$) remaining statistically significant after post-hoc Mann-Whitney U tests with Bonferroni correction are indicated by star symbols (*$P<0.017$; **$P<0.001$; ***$P<0.0001$). Abbreviations: AD: Alzheimer's disease; BA: Brodmann area; CONTR: controls; FTD: frontotemporal dementia; MHPG: 3-methoxy-4-hydroxyphenylglycol; NA: noradrenaline.

In contrast, NA levels were significantly higher in the FTD than in the AD group for all 11 brain regions, while in BA6 ($P<0.001$), BA8 ($P\leq0.001$), BA9 ($P<0.001$), BA10 ($P<0.05$), BA11 ($P<0.05$), BA24 ($P<0.05$) and amygdala ($P<0.05$), NA levels were significantly lower in AD as opposed to controls. Furthermore, in BA8 ($P<0.05$) and BA46 ($P<0.05$), NA levels were significantly higher in FTD patients in comparison with control subjects (Figure 4).

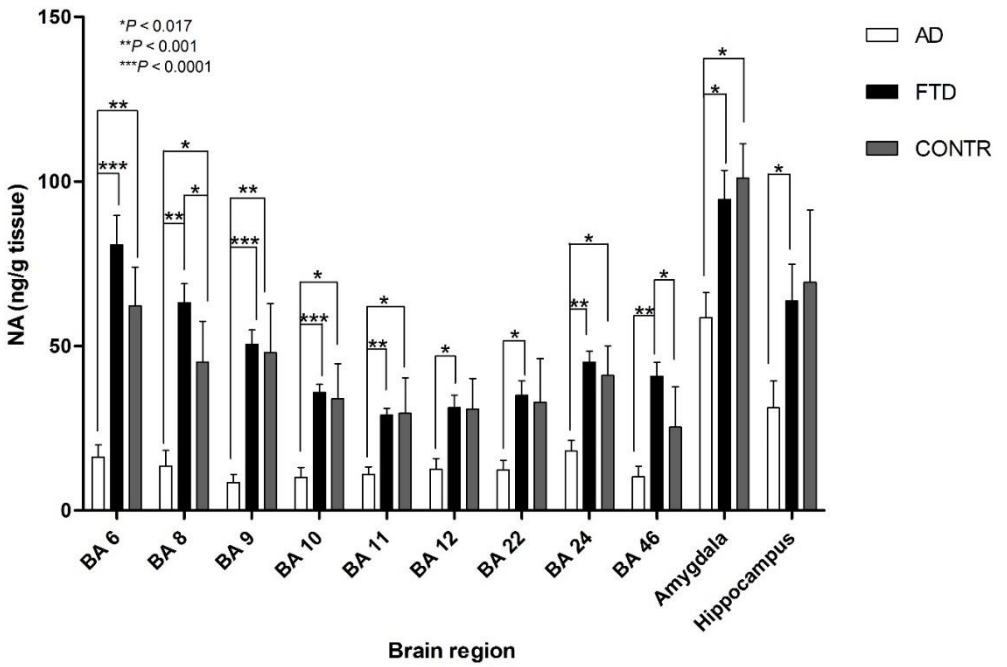

Figure 4. NA levels across eleven brain regions in AD compared to FTD and control subjects. Data are presented as mean with SD. Only differences (Kruskal Wallis, $P<0.05$) remaining statistically significant after post-hoc Mann-Whitney U tests with Bonferroni correction are indicated by star symbols (*$P<0.017$; **$P<0.001$; ***$P<0.0001$). Abbreviations: AD: Alzheimer's disease; BA: Brodmann area; CONTR: controls; FTD: frontotemporal dementia; NA: noradrenaline.

Finally, MHPG concentrations were significantly lower in FTD than in AD patients in eight regions (BA9, $P\leq0.001$; BA10, $P<0.001$; BA11, $P<0.001$; BA22, $P<0.001$; BA24, $P<0.05$; BA46, $P<0.05$; amygdala, $P<0.001$; and hippocampus, $P\leq0.001$). There were no significant differences in MHPG levels between the AD and control group. Contrarily, MHPG concentrations were higher in control subjects than in FTD patients in BA9, BA11, BA22, BA24, amygdala and hippocampus (for all: $P<0.05$).

3.2.3. Dopaminergic findings

Overall, significant dopaminergic group differences were scarce, with only higher DA levels and lower HVA/DA ratios, indicative of the catabolic dopaminergic turnover, in BA12 (for both: $P<0.05$), and higher DA levels in BA46 ($P<0.05$) in FTD compared to AD patients. In BA6 and BA46, higher DA levels (both $P<0.05$) and lower HVA/DA ratios (for both: $P<0.05$) in FTD compared to control subjects were observed accordingly. No significant differences concerning HVA/DA ratios were

found between AD and control groups, apart from higher DOPAC/DA ratios in BA22 of AD subjects (P<0.05) (Table 2).

Table 2. Brain region-specific monoaminergic data of AD, FTD and control subjects.

Brain region	MA, MT or ratio	AD (n=10)	FTD (n=10)	Controls (n=10)	Kruskal Wallis
BA6	NA (ng/g)	13.7 (8.7-18.7) n=10^{aa,ccc}	83.9 (65.9-101.5) n=10^{ccc}	54.9 (30.8-87.7) n=10^{aa}	P<0.001
	DA (ng/g)	6.5 (4.0-10.6) n=10	10.0 (6.2-15.7) n=10^{b}	4.5 (2.7-7.0) n=10^{b}	P<0.05
	5-HT (ng/g)	3.9 (1.4-10.7) n=10^{c}	16.8 (11.0-22.1) n=10^{c}	9.4 (6.5-15.4) n=10	P<0.05
	HVA/DA	20.2 (16.5-38.2) n=10	15.3 (8.5-29.1) n=10^{b}	41.3 (17.6-68.0) n=10^{b}	P<0.05
	MHPG/NA	23.5 (13.7-40.7) n=10^{a,ccc}	1.9 (0.9-2.6) n=10^{ccc}	3.1 (1.2-8.8) n=10^{a}	P<0.001
BA8	NA (ng/g)	7.9 (6.1-15.1) n=9^{a,cc}	63.8 (46.2-82.6) n=10^{b,cc}	33.8 (19.5-54.4) n=10^{a,b}	P<0.001
	5-HT (ng/g)	2.8 (1.8-9.1) n=9^{c}	19.1 (9.5-23.4) n=10^{c}	9.4 (5.3-12.6) n=10	P<0.05
	5-HIAA/5-HT	24.6 (12.8-45.1) n=9^{c}	7.2 (5.9-10.8) n=10^{b,c}	15.2 (11.7-23.5) n=10^{b}	P<0.05
	MHPG/NA	48.9 (16.6-54.6) n=9^{a,cc}	2.1 (1.4-3.5) n=10^{cc}	4.6 (1.5-16.2) n=10^{a}	P≤0.001
BA9	MHPG (ng/g)	465.7 (311.8-539.4) n=9^{c}	90.2 (57.9-175.4) n=10^{b,c}	291.4 (156.9-553.2) n=10^{b}	P<0.05
	NA (ng/g)	4.0 (2.7-16.0) n=9^{aa,ccc}	55.0 (34.1-59.5) n=10^{ccc}	33.5 (26.0-46.3) n=10^{aa}	P<0.0001
	5-HT (ng/g)	6.0 (2.9-11.9) n=9^{c}	19.0 (10.1-25.8) n=10^{c}	11.6 (6.1-15.4) n=10	P<0.05
	5-HIAA/5-HT	28.0 (10.4-72.5) n=9^{c}	6.4 (5.5-9.9) n=10^{b,c}	15.2 (8.9-20.3) n=10^{b}	P<0.05
	MHPG/NA	59.3 (23.5-237.3) n=9^{a,ccc}	2.3 (1.2-4.4) n=10^{b,ccc}	6.5 (4.7-23.4) n=10^{a,b}	P<0.0001
BA10	MHPG (ng/g)	508.9 (246.1-1080.1) n=10^{cc}	113.7 (65.3-174.1) n=10^{cc}	245.9 (146.6-567.7) n=10	P<0.05
	NA (ng/g)	5.1 (3.8-16.1) n=10^{a,ccc}	36.5 (31.1-40.7) n=10^{ccc}	24.4 (16.1-36.11) n=10^{a}	P<0.001
	5-HIAA/5-HT	19.7 (10.1-33.6) n=10^{c}	7.9 (5.9-10.2) n=10^{c}	13.0 (7.4-19.0) n=10	P<0.05
	MHPG/NA	97.4 (21.1-236.0) n=10^{a,ccc}	3.0 (2.2-4.7) n=10^{b,ccc}	9.7 (4.6-31.2) n=10^{a,b}	P≤0.0001

Table 2. Continued.

Brain region	MA, MT or ratio	AD (n=10)	FTD (n=10)	Controls (n=10)	Kruskal Wallis
BA11	MHPG (ng/g)	473.6 (257.7-728.0) n=10^{cc}	113.3 (50.7-182.5) n=10^{b,cc}	432.8 (287.7-671.9) n=10^{b}	$P \leq 0.001$
	NA (ng/g)	8.0 (6.2-17.3) n=9^{a,cc}	29.8 (26.3-31.7) n=10^{cc}	19.7 (13.2-25.9) n=10^{a}	$P \leq 0.001$
	5-HT (ng/g)	11.2 (6.5-13.8) n=10^{c}	21.8 (14.0-27.5) n=10^{c}	18.0 (11.0-21.7) n=10	$P<0.05$
	5-HIAA/5-HT	27.9 (12.2-37.4) n=10^{c}	6.4 (4.2-10.2) n=10^{b,c}	11.7 (9.0-18.8) n=10^{b}	$P<0.05$
	MHPG/NA	56.0 (29.5-99.7) n=9^{ccc}	3.8 (2.5-6.7) n=10^{b,ccc}	21.6 (9.0-39.9) n=10^{b}	$P<0.001$
BA12	NA (ng/g)	8.1 (5.7-19.3) n=10^{c}	30.2 (19.4-42.8) n=10^{c}	20.7 (13.6-41.7) n=10	$P<0.05$
	A (ng/g)	4.2 (2.4-6.0) n=5^{c}	12.4 (9.1-12.9) n=7^{c}	8.0 (4.9-14.5) n=4	$P<0.05$
	DA (ng/g)	2.9 (2.5-5.7) n=10^{c}	8.9 (5.0-17.2) n=10^{c}	4.3 (2.7-6.6) n=10	$P<0.05$
	5-HT (ng/g)	16.1 (9.3-22.8) n=10^{c}	31.1 (23.9-85.5) n=10^{c}	23.5 (15.1-33.0) n=10	$P<0.05$
	5-HIAA/5-HT	13.6 (9.2-20.7) n=10^{c}	5.3 (3.7-9.0) n=10^{b,c}	12.5 (6.5-20.3) n=10^{b}	$P<0.05$
	HVA/DA	54.4 (31.0-73.5) n=10^{c}	17.2 (13.4-22.6) n=10^{c}	51.4 (21.0-71.1) n=10	$P<0.05$
	MHPG/NA	40.8 (16.5-69.5) n=10^{c}	3.2 (2.7-5.1) n=10^{c}	11.3 (1.7-39.1) n=10	$P<0.05$
BA22	MHPG (ng/g)	554.9 (471.5-779.9) n=9^{cc}	103.1 (60.3-198.0) n=10^{b,cc}	360.5 (260.4-360.7) n=10^{b}	$P \leq 0.001$
	NA (ng/g)	10.0 (6.23-19.9) n=7^{c}	33.6 (21.8-50.6) n=10^{c}	18.7 (13.6-28.5) n=10	$P<0.05$
	5-HIAA (ng/g)	317.7 (144.8-485.8) n=9^{c}	117.7 (97.4-197.0) n=10^{c}	171.5 (121.7-320.6) n=10	$P<0.05$
	5-HT (ng/g)	4.2 (1.9-7.3) n=9^{c}	12.8 (8.3-13.5) n=10^{c}	7.4 (3.8-11.9) n=10	$P<0.05$
	5-HIAA/5-HT	67.8 (33.8-175.4) n=9^{a,ccc}	11.9 (9.0-14.9) n=10^{b,ccc}	23.5 (18.0-36.7) n=10^{a,b}	$P<0.0001$
	DOPAC/DA	2.4 (1.5-4.7) n=8^{a}	1.1 (0.8-2.6) n=10	1.0 (0.5-1.5) n=10^{a}	$P<0.05$
	MHPG/NA	83.5 (27.1-91.4) n=7^{cc}	3.6 (2.0-5.7) n=10^{b,cc}	20.0 (7.8-46.9) n=10^{b}	$P \leq 0.001$
BA24	MHPG (ng/g)	552.0 (252.0-856.3) n=10^{cc}	101.4 (65.7-164.6) n=10^{b,cc}	336.9 (158.1-587.5) n=10^{b}	$P \leq 0.001$
	NA (ng/g)	17.6 (10.5-22.7) n=9^{a,cc}	45.3 (36.8-53.0) n=10^{cc}	33.5 (26.4-43.4) n=10^{a}	$P \leq 0.001$
	MHPG/NA	36.9 (14.9-67.0) n=9^{ccc}	2.3 (1.8-3.5) n=10^{b,ccc}	9.3 (3.4-21.9) n=10^{b}	$P<0.001$

Table 2. Continued.

Brain region	MA, MT or ratio	AD (n=10)	FTD (n=10)	Controls (n=10)	Kruskal Wallis
BA46	MHPG (ng/g)	205.6 (145.4-514.3) n=9^{c}	82.6 (47.4-156.1) n=10^{c}	105.8 (58.6-462.4) n=10	*P*<0.05
	NA (ng/g)	5.5 (4.4-14.8) n=9^{cc}	41.7 (30.8-52.1) n=10^{b,cc}	12.7 (6.8-22.1) n=10^{b}	*P*≤0.001
	DA (ng/g)	3.0 (2.1-8.4) n=9^{c}	12.2 (6.9-17.7) n=10^{b,c}	4.2 (3.4-5.5) n=10^{b}	*P*<0.05
	5-HT (ng/g)	6.0 (3.0-7.6) n=9^{c}	18.1 (9.4-22.4) n=10^{c}	9.9 (6.6-14.6) n=10	*P*<0.05
	5-HIAA/5-HT	27.8 (11.6-33.4) n=9^{c}	9.4 (7.1-14.2) n=10^{c}	17.8 (15.3-20.0) n=10	*P*<0.05
	HVA/DA	29.1 (15.1-52.4) n=9	11.1 (7.8-18.5) n=10^{b}	32.8 (16.7-48.0) n=10^{b}	*P*<0.05
	MHPG/NA	57.8 (20.5-75.8) n=9^{c}	2.7 (1.1-3.0) n=10^{b,c}	11.4 (4.2-37.9) n=10^{b}	*P*≤0.001
Amygdala	MHPG (ng/g)	651.3 (327.3-1009.7) n=9^{cc}	124.9 (70.8-205.1) n=9^{b,cc}	304.8 (193.5-759.3) n=10^{b}	*P*<0.05
	NA (ng/g)	59.8 (40.3-78.7) n=9^{a,c}	104.3 (66.2-114.5) n=9^{c}	84.5 (77.5-121.8) n=10^{a}	*P*<0.05
	5-HIAA (ng/g)	481.2 (317.4-668.1) n=9^{a}	765.6 (471.9-878.4) n=9	999.8 (754.5-1270.4) n=10^{a}	*P*<0.05
	HVA (ng/g)	558.6 (378.5-706.9) n=9^{a}	562.5 (395.3-779.1) n=9^{b}	1132.5 (751.0-1421.4) n=10^{a,b}	*P*<0.05
	5-HT (ng/g)	109.6 (49.4-139.9) n=9^{aaa,c}	225.2 (173.3-274.5) n=9^{c}	244.9 (221.7-297.2) n=10^{aaa}	*P*=0.001
	MHPG/NA	7.9 (4.8-26.5) n=9^{ccc}	1.7 (0.8-2.1) n=9^{ccc}	3.0 (1.4-9.9) n=10	*P*=0.001
Hippo-campus	MHPG (ng/g)	716.8 (290.0-1099.3) n=10^{c}	140.0 (71.9-193.7) n=9^{b,c}	416.3 (232.9-713.5) n=10^{b}	*P*<0.05
	NA (ng/g)	23.0 (17.2-36.7) n=9^{c}	50.4 (40.6-82.3) n=9^{c}	43.0 (25.8-81.9) n=10	*P*<0.05
	5-HT (ng/g)	41.4 (20.2-61.1) n=10^{a,c}	85.9 (59.0-144.6) n=9^{c}	87.8 (69.3-111.2) n=10^{a}	*P*<0.05
	5-HIAA/5-HT	9.3 (6.5-12.7) n=10^{c}	3.3 (2.6-5.9) n=9^{c}	5.3 (3.4-7.3) n=10	*P*<0.05
	MHPG/NA	20.1 (9.2-70.7) n=9^{ccc}	2.8 (1.1-3.5) n=9^{ccc}	8.2 (2.3-31.4) n=10	*P*≤0.001

Median with IQR denoted between parentheses and *P* values after Kruskal Wallis analysis in the rightmost column (*P*<0.05). Only data that remained statistically significant after post-hoc Mann-Whitney U tests with Bonferroni correction (*P*<0.017 for three group comparisons, one superscript letter) are displayed. Two and three superscript letters are used to indicate significant differences with *P*<0.001 and *P*<0.0001, respectively. Letters a, b and c signify differences between AD and controls^{a}, FTD and controls^{b}, and, AD and FTD^{c}. Abbrevations: 5-HIAA: 5-hydroxyindoleacetic acid; 5-HT: 5-hydroxytryptamine (serotonin); A: adrenaline; AD: Alzheimer's disease; BA: Brodmann area; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; FTD: frontotemporal dementia; HVA: homovanillic acid; MA/MT: monoamine and metabolite; MHPG: 3-methoxy-4-hydroxyphenylglycol; NA: noradrenaline.

3.3. Neurochemical correlates of NPS in AD and FTD

Neurochemical correlates of NPS in the AD and FTD groups are presented in Supplementary Table 2. Due to the extensive amount of data with $P<0.05$, only the most significant correlations were finally retained ($P<0.005$). None of these correlations, however, remained statistically significant following a total Bonferroni correction for multiple comparisons (i.e. $P<0.00002$).

As for the AD group, NA levels in BA12 correlated with CMAI cluster 1 scores (aggressive behavior) ($P<0.001$, r(s)=+0.914, n=10), whereas in BA22, HVA/5-HIAA ratios correlated with Behave-AD cluster G scores (anxieties and phobias) ($P\leq0.001$, r(s)=+0.850, n=9). Furthermore, hippocampal 5-HIAA concentrations inversely correlated with Behave-AD total scores, CMAI cluster 3 scores (verbally agitated behavior) and CMAI total scores ($P\leq0.0001$, r(s)=-0.927, n=10; $P<0.0001$, r(s)=-0.939, n=10; and $P<0.001$, r(s)=-0.903, n=10). Hippocampal HVA/5-HIAA ratios also correlated with Behave-AD cluster G scores, CMAI cluster 2 (physically nonaggressive behavior) and cluster 3 scores ($P<0.05$, r(s)=+0.816, n=10; $P\leq0.001$, r(s)=+0.872, n=10; and $P\leq0.001$, r(s)=+0.865, n=10), as well as Behave-AD and CMAI total scores ($P<0.001$, r(s)=+0.902, n=10; and $P<0.001$, r(s)=+0.903, n=10).

In the FTD group, NA levels in BA9 inversely correlated with Behave-AD cluster D scores (aggressiveness) ($P<0.05$, r(s)=-0.813, n=10). Additionally, 5-HT concentrations in BA12 were inversely associated with Behave-AD global scores ($P<0.05$, r(s)=-0.834, n=10). Lastly, in BA46, HVA/DA ratios correlated with CMAI total scores ($P<0.05$, r(s)=+0.812, n=10).

3.4. Possible confounding effects of psychotropic medication

In the AD group, 5-HT levels in BA10, DOPAC/DA ratios in BA22 and MHPG/NA ratios in the hippocampus (for all: $P<0.05$) were significantly higher in patients who were on psychotropic medication before death (n=3) compared to patients free of such medication (n=7).
Concerning the FTD group, MHPG/NA ratios in both BA6 and BA8 (both $P<0.05$) were significantly lower in pharmacologically-treated patients (n=5) compared to those who were free of psychotropic medication (n=5). The same applies to MHPG levels in BA8, amygdala and hippocampus and, finally, DA levels in BA9, and DOPAC and 5-HT levels in the amygdala (for all: $P<0.05$).
As for the control subjects, 5-HIAA levels in BA6, BA8, BA12, BA22, BA46 and amygdala, as well as NA levels in BA10 and BA22 were significantly lower in patients on psychotropic agents (n=2) compared to their medication-free counterparts (n=8) (for all: $P<0.05$).

Because four times as many FTD patients where on antidepressants (n=4; i.e. trazodone (n=2), amitriptyline (n=1) and sertralin (n=1) compared to AD and control subjects (n=1 for both groups), these drugs may certainly have influenced our serotonergic and/or noradrenergic results. However, only 5-HIAA/5-HT ratios in BA6 and BA9, and, MHPG/NA ratios in BA6, BA8 and hippocampus, were significantly lower in the subgroup of FTD subjects on antidepressants (n=4) compared to their antidepressant-free counterparts (n=6). There were no significant alterations regarding 5-HT or NA levels.

4. DISCUSSION

4.1. Monoaminergic findings

The key findings of the present study are that FTD patients have a remarkably distinct monoaminergic profile compared to AD patients, with predominantly imbalanced levels of brain serotonergic and noradrenergic compounds, and, an apparently unaltered dopaminergic neurotransmitter system. The latter is in sharp contrast with some of the aforementioned studies [280, 283, 288] and our initial expectations.

Increased 5-HT levels, corresponding with the findings of Bowen et al. [282], combined with decreased 5-HIAA/5-HT ratios in practically every analyzed brain region of FTD compared to AD and/or control subjects, could possibly be indicative of a serotonergic neurotransmitter imbalance in patients suffering from FTD. In general, this presenile dementia subtype is characterized by a more extensive loss of pyramidal neurons in the supragranular layers of the frontotemporal cortex than AD [307, 308]. In addition, these layers are enriched with 5-HT_{1A} receptors that have been reported to index the soma of corticocortical glutamatergic pyramidal neurons in the neocortex [286, 309], and, 5-HT concentrations have shown to inhibit glutamate release via these receptors [310]. Our observed serotonergic alterations could therefore reflect a relative excess of extraneuronal 5-HT in relation to the number of surviving glutamatergic pyramidal neurons. As a consequence, cognitive and behavioral symptoms in FTD might arise as a result of deficient glutamatergic neurotransmission. This excess of 5-HT may be due to preservation of 5-HT afferents, as was claimed by Bowen et al. [282], based on increased 5-HT reuptake site measures in FTD compared to AD. The mean reuptake value in temporal, parietal and frontal cortex for their FTD group was no less than 153% and 120% of AD and control values. Such preservation of serotonergic indices could be due to collateral sprouting, as has been demonstrated in animal models [311]. The authors further suggest that a 5-HT_{1A} receptor antagonist may be indicated to counteract the overstimulation of 5-HT on these receptors [282].

Interestingly, 5-HT_{1A} receptors or even 5-HT itself could represent neurochemical markers for glutamatergic neurotransmission in FTD accordingly [312]. Clinical trials with SSRIs (e.g. paroxetine, fluvoxamine, sertraline, citalopram) or other serotonergic antidepressants (e.g. trazodone) also led to conflicting results, with mixed treatment effects on both cognition and behavior, and, even a worsening of cognitive functioning in one trial (paroxetine; for review, see [280] and [281]), which fits our aforementioned postulation.

As for the serotonergic metabolite 5-HIAA, levels were only significantly decreased in the temporal cortex (BA22) of FTD compared to AD subjects. Such preservation of 5-HIAA is largely consistent with previous comparative studies that not only examined frontal, temporal and parietal brain tissue [282], but CSF as well [149, 176, 283].

Apart from neocortical brain areas, the present study also analyzed amygdala and hippocampus, which yielded similar results, and therefore our findings point in the direction of a more diffuse serotonergic brain deficiency in FTD. In this context, a 40% reduction in the number of serotonergic raphe nuclei (RN) neurons has previously been observed [313], even though the accompanying postsynaptic serotonergic dysfunction via their ascending projections to the forebrain, including the amygdala, hippocampus and other subcortical nuclei, certainly necessitates further neurochemical investigation, given that a neuronal loss in RN is rather suggestive for an overall decrease (and not increase) in 5-HT levels. Unfortunately, glutamate levels, 5-HT reuptake values or RN atrophy measures are lacking in our study.

Similar to the serotonergic data, we found even more significantly increased NA levels and decreased MHPG/NA ratios in all 11 regions in FTD brain. Additionally, MHPG levels were strongly decreased in eight out of 11 brain regions. These noradrenergic abnormalities suggest that the LC, the principal site for brain synthesis of NA, may be severely damaged in FTD. Yang and Schmitt [313], however, found no neuronal loss in the LC of 12 FTD compared to 30 AD patients, whereas in latter AD group, both the RN and LC were severely affected. Nevertheless, the LC also receives serotonergic innervation from the upper RN [314] with predominantly inhibitory effects on the firing of these LC neurons, which is mediated by receptors of the 5-HT_1 family [315, 316]. Hence, even though the LC appears to be structurally intact, it cannot be excluded that serotonergic deafferentation might eventually lead to secondary, postsynaptic noradrenergic changes in the frontotemporal cortex, hippocampus and amygdala among others. This remains purely hypothetical. Contrarily, previous studies that examined NA levels and its main metabolite, MHPG, in CSF, did not find such noradrenergic alterations [176, 283], except for Sjögren et al. [149], who found higher CSF MHPG

levels in FTD compared to both early- and late-onset AD. Lastly, it is of interest to mention that Sparks et al. [317] observed a decreased monoamine oxidase A (MAO-A) enzyme activity in the temporal lobe of patients with Pick's disease, which might – at least partly – explain the increased NA levels and decreased MHPG/NA ratios in our FTD group. This particular enzyme plays a strategic role in inactivating catecholamines that are free within the nerve terminal endings, of which MAO-A preferentially deaminates NA and 5-HT [318]. On the other hand, the authors found increased MAO-A activity in the frontal lobe of AD subjects, which corresponds to the decreased NA levels and increased ratios of our AD group as well.

Similarly, based on our results, it can be concluded that the dopaminergic neurotransmitter system remained largely unaffected in FTD compared to AD patients and healthy controls. In general, the handful of significant differences that were found, may partly be attributed to psychotropic drug therapy shortly before death (see section 3.4. in Results). The latter is in stark contrast to previous findings of Engelborghs et al. [283], who reported an increased activity of dopaminergic neurotransmission due to a potentially altered inhibitory control of the serotonergic system, represented by increased HVA/5-HIAA ratios, in CSF of 25 FTD compared to 181 AD patients. No such differences in HVA/5-HIAA ratios were found in any of the analyzed brain regions in our study, suggesting that the serotonergic inhibitory control on dopaminergic neurotransmitter release was supposedly not affected in AD nor FTD. We have to bear in mind, however, that the hypothesis that monoamine levels in peripheral body fluids reflect central monoaminergic metabolism is based on assumptions (e.g. the assumption that a change in monoaminergic metabolism in a discrete brain area is measurable in large compartments such as CSF) [319]. Conversely, there are other CSF reports that agree with the theory of a relatively spared dopaminergic system in FTD [149, 176]. With regard to studies that analyzed brain tissue samples, one case study found unchanged DA levels in different regions of the neo- and allocortex [320], whereas another case study found dramatically reduced levels of DA and HVA in several neocortical brain areas, as well as thalamus, SN, and striatum [321], both in comparison with identical regions of seven control brains. Lastly, in a small study of only three FTD patients, CSF and brain tissue measurements also suggested that DA release remained relatively intact [286]. Overall, these contradicting results certainly necessitate further investigation since the impairment of the serotonergic system may have a profound impact on the dopaminergic system [313].

Finally, it is of note that serotonergic pathways – apart from strong interactions with dopaminergic, noradrenergic and glutamatergic systems – are also known to intensively interact with the cholinergic system, and, that the 5-HT_{1A} receptor can facilitate various types of memory by enhancing cholinergic as well as

glutamatergic neurotransmission if antagonized [322], making it a valuable and strategic therapeutic target.

4.2. Neurochemical correlates of NPS

Strikingly, most of the correlations pertained to agitated and/or aggressive behavior in AD as well as FTD patients. In the AD group, considering the ten patients were part of a larger group of 40 in total on which similar research was conducted (See section 2.1. in Materials & methods), results therefore correspond well with those of our previous studies [199, 294].

As for FTD patients, an increased enzymatic turnover of DA to DOPAC and then HVA, as represented by HVA/DA ratios, in the middle frontal gyrus (BA46) might be related to agitated behavior (CMAI total score). To some degree, this finding is consistent with Engelborghs et al. [283] who mentioned that CSF DOPAC levels of FTD patients correlated with physically nonaggressive behavior (CMAI cluster 2 score) and agitation in general (CMAI cluster 3 score). Moreover, CSF DOPAC levels were able to predict future aggressive and agitated behavior in FTD patients.
In both study groups, NA levels of the prefrontal cortex (BA12 and BA9) correlated with aggressive behavior, albeit inversely in FTD patients. The correlation in AD partially corresponds with the results of Matthews et al. [323], who observed a similar correlation between aggressive behavior and severity of noradrenergic cell loss in the LC of AD patients before. The same monoamine was thus associated with more and less apparent aggressive behavior in comparable prefrontal regions in AD and FTD brain, respectively.
Results need to be interpreted with caution, however, since none of these correlations described above remained statistically significant following Bonferroni correction for multiple comparisons.

4.3. Study strengths and weaknesses

All AD and FTD patients were clinically and behaviorally well-characterized by combining clinical, neuropsychological and neuroimaging data, as well as behavioral assessment scores obtained during baseline and follow-up investigations. These data gave rise to the clinical diagnosis of probable AD or FTD. In addition, postmortem neuropathological examination of the formalin-fixated right hemisphere established the diagnosis of definite AD or FTD, and all groups were age- and gender-matched. Moreover, all brain dissections of the frozen left hemispheres, as well as the neuropathological assessments of the right hemispheres were always performed by the same neuropathologist(s)/scientist, thus minimizing variability. Postmortem degradation of the neurochemical compounds was minimized by the inclusion of two quality control measures. Firstly, only nonacidotic brain tissue samples were included. Secondly, the average

postmortem delay in all three groups remained more than sufficient within the six to eight hour range (4-5 hours) (e.g. if compared to the study of Bowen et al. [282] (30-48 hours)).

As opposed to aforementioned strengths, this study also comprised a number of limitations. As such, behavioral assessment scores of the control group were not available and those of the FTD group lacked sufficient variation (Supplementary Table 1) so that, eventually, very little significant neurochemical correlates of NPS were found in this group. Nonparametric tests were also applied due to the limited number of subjects in each study group (n=10) and ordinal variables (behavioral scores), and, because our data did not comply with those of a normally distributed population (data not shown), statistical power might potentially have been lost. Unfortunately, MMSE scores were unsuitable or absent for data analysis owing to the severe disease progression in some patients (e.g. mutism) or because obtained MMSE scores were not recent enough. Furthermore, the existing information on monoamine deficits in particular with reference to distinct histological FTLD subgroups, such as FTLD with (Pick's disease) and without (FTLD-U) tau pathology [36], is sparse [282], and might have introduced some bias in our neurochemical dataset not only due to heterogeneity in spreading of TDP-43 positive inclusions across the different cortical layers depending on the FTLD-TDP-43 subtype [324], but also due to interindividual variation in topographic distribution of pathology. We also have to acknowledge that most of the included AD and FTD subjects had advanced disease stages, which may lead to dissimilar findings in patients with early disease stages, still the main target group for pharmaceutical intervention and biomarker discovery and verification. The variable degree of cortical atrophy between and within patients groups may also have introduced a neurochemical bias, given that there were three out of ten FTD subjects with asymmetric brain degeneration (visualized on MRI). The latter is in reference with Whitwell et al. [325], who found a minority of 35% of behavioral variant FTD patients with asymmetric frontal lobes (15% asymmetric right and 20% asymmetric left).

With regard to the use of antidepressants, our serotonergic and noradrenergic results may have been influenced by confounding medication effects to some degree, particularly in BA6, BA8, BA9 and hippocampus (See 3.4. Results). On the other hand, possible confounding psychotropic drug effects in general may have introduced type I errors since several noradrenergic alterations were observed as well, although restricted to MHPG/NA ratios and/or MHPG levels of BA6, BA8, amygdala, and hippocampus (See section 3.4. in Results). However, neurochemical effects of psychotropic drugs may last for up to several weeks after cessation of treatment. Therefore, neurochemical data of patients who did not take

psychotropic medication at the date of death may also be influenced by these effects.

5. CONCLUSIONS

By and large, our findings support the premise that FTD and AD are distinguishable by their monoaminergic profiles not only in frontotemporal brain regions, but also in amygdala and hippocampus. More specifically, FTD seems to be predominantly characterized by imbalanced levels of brain serotonergic and noradrenergic compounds, and, by an apparently unaltered dopaminergic neurotransmitter system. We speculate that the observed serotonergic alterations might be caused by preservation of 5-HT afferents (and thus 5-HT levels), consequently leading to an underactivity of prefrontal glutamatergic neurotransmission, as was previously concluded by Bowen et al. [282]. On the other hand, we are the first, to our knowledge, to report on severe brain noradrenergic neurotransmitter deficiencies in FTD compared to AD, hypothetically resulting from an impaired connection between the RN and LC. Tackling both of these monoaminergic disturbances might, therefore, improve cognitive and/or behavioral deficits in patients suffering from this presenile disorder. Additionally, clinical trials that investigate the effects of 5-HT_{1A} receptor antagonists and/or NA-modulating agents, such as $\alpha_{1/2}$- or β_1-blockers [326, 327], may be considered. For instance, the efficacy of α_2-adrenoreceptor antagonists has been demonstrated before in three FTD subjects [328], and a novel generation of promising α_{2C}-antagonists, such as ORM-10921/ORM-12741, is currently being tested (phase 2 clinical trial NCT02471196), albeit in AD [327, 329].

Our study would of course have been more informative if some of the alternative indicators of neurotransmission, such as binding potential measures of 5-$HT_{1A/2A}$-, glutamate- and α- or β-noradrenergic receptors, and, MAO and cholinergic enzyme activities had been determined simultaneously. Moreover, given the discriminative features of both serotonergic (5-HT levels and 5-HIAA/5-HT ratios) and noradrenergic (MHPG, NA levels and MHPG/NA ratios) compounds, future studies should examine their added potential in CSF in combination with the traditional set of AD biomarkers, as was previously attempted by Herbert et al. [146], and of which a good first indication came from CSF MHPG levels.
Finally, the strong and reciprocal connections between the RN and LC, as well as their postsynaptic efferents to the neo- and allocortex, certainly necessitate further investigation, accompanied with a complete topographic mapping of monoaminergic alterations in FTD and AD brain, including not only the RN and LC, but also the SN and striatum.

6. ACKNOWLEDGEMENTS

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7. SUPPLEMENTARY MATERIAL

Supplementary Table 1. Behavioral data of AD and FTD patients.

Behavioral assessment scores	AD (n=10)	FTD (n=10)	Mann-Whitney U
MFS total score (/10)	5.3 ± 1.5 (3-8)	5.7 ± 1.8 (3-9)	*P*>0.05
Behave-AD cluster A (/21) *Paranoid and delusional ideation*	1.7 ± 3.5 (0-11)	0.0 ± 0.0 (0-0)	*P*>0.05
Behave-AD cluster B (/15) *Hallucinations*	1.1 ± 1.4 (0-3)	0.0 ± 0.0 (0-0)	***P*<0.05**
Behave-AD cluster AB (/36) *Psychosis*	2.8 ± 3.9 (0-12)	0.0 ± 0.0 (0-0)	***P*<0.05**
Behave-AD cluster C (/9) *Activity disturbances*	3.4 ± 3.4 (0-9)	1.5 ± 1.7 (0-4)	*P*>0.05
Behave-AD cluster D (/9) *Aggressiveness*	4.5 ± 2.7 (0-9)	1.1 ± 2.0 (0-6)	***P*<0.05**
Behave-AD cluster E (/3) *Diurnal rhythm disturbances*	0.5 ± 0.7 (0-2)	0.3 ± 0.7 (0-2)	*P*>0.05
Behave-AD cluster F (/6) *Affective disturbances*	1.6 ± 1.6 (0-4)	0.6 ± 1.1 (0-3)	*P*>0.05
Behave-AD cluster G (/12) *Anxieties and phobias*	1.5 ± 2.1 (0-5)	0.1 ± 0.3 (0-1)	*P*>0.05
Behave-AD total score (/75)	14.3 ± 11.4 (1-36)	3.6 ± 4.4 (0-13)	***P*<0.05**
Behave-AD global score (/3) *Caregiver burden*	1.8 ± 1.2 (0-3)	0.9 ± 1.0 (0-3)	*P*>0.05
CMAI cluster 1 (/70) *Aggressive behavior*	17.8 ± 9.9 (10-39)	10.2 ± 0.6 (10-12)	***P*<0.05**
CMAI cluster 2 (/77) *Physically nonaggressive behavior*	25.2 ± 11.2 (11-39)	20.9 ± 10.7 (11-43)	*P*>0.05
CMAI cluster 3 (/56) *Verbally agitated behavior*	16.0 ± 8.0 (8-33)	9.4 ± 3.0 (8-16)	***P*<0.05**
CMAI total score (/203)	59.0 ± 22.3 (29-92)	40.5 ± 13.0 (29-71)	*P*>0.05

Supplementary Table 1. Continued.

Behavioral assessment scores	AD (n=10)	FTD (n=10)	Mann-Whitney U
CSDD total score (/38)	12.5 ± 4.2	8.6 ± 3.7	***P*<0.05**
Depression	(5-19)	(4-17)	

Mean ± SD; minimum-maximum range is displayed between parentheses. Significant *P* values (<0.05) after Mann-Whitney U statistical analyses are depicted in **bold**. Abbreviations: AD: Alzheimer's disease; Behave-AD: Behavioral Pathology in Alzheimer's Disease Rating Scale; CMAI: Cohen-Mansfield Agitation Inventory; CSDD: Cornell Scale for Depression in Dementia; FTD: frontotemporal dementia; MFS: Middelheim Frontality Score.

Supplementary Table 2. Neurochemical correlates of NPS in AD and FTD.

Study group	Brain region	MA, MT or ratio	NPS feature	Spearman's Rank Order statistics
AD n=10	BA6	HVA (ng/g)	Behave-AD cluster D *Aggressiveness*	r(s)=+0.862, *P*≤0.001, n=10
	BA8	MHPG (ng/g)	CSDD	r(s)=+0.840, *P*<0.05, n=9
	BA9	5-HT (ng/g)	MFS Total score	r(s)=-0.886, *P*≤0.001, n=9
	BA10	5-HIAA/5-HT	Behave-AD cluster G *Anxieties and phobias*	r(s)=+0.876, *P*<0.05, n=10
	BA12	NA (ng/g)	CMAI cluster 1 *Aggressive behavior*	**r(s)=+0.914, *P*<0.001, n=10**
	BA22	HVA/5-HIAA	Behave-AD cluster G *Anxieties and phobias*	r(s) =+0.890, *P*≤0.001, n=9
	BA24	5-HT (ng/g)	MFS Total score	r(s)=-0.883, *P*≤0.001, n=10
		5-HIAA/5-HT	MFS Total score	r(s)=+0.827, *P*<0.05, n=10
	Hippocampus	MHPG (ng/g)	Behave-AD cluster B *Hallucinations*	r(s)=-0.822, *P*<0.05, n=10
			Behave-AD Total score	r(s)=-0.829, *P*<0.05, n=10
		5-HIAA (ng/g)	Behave-AD cluster AB *Psychosis*	r(s) = -0.830, *P*<0.05, n=10
			Behave-AD cluster C *Activity disturbances*	r(s)=-0.853, *P*<0.05, n=10
			Behave-AD Total score	**r(s)=-0.927, *P*≤0.0001, n=10**
			CMAI cluster 3 *Verbally agitated behavior*	**r(s)=-0.939, *P*<0.0001, n=10**
			CMAI Total score	**r(s)=-0.903, *P*<0.001, n=10**
		HVA/5-HIAA	Behave-AD cluster C *Activity disturbances*	r(s)=+0.853, *P*<0.05, n=10
			Behave-AD cluster G *Anxieties and phobias*	r(s)=+0.816, *P*<0.05, n=10
			Behave-AD Total score	**r(s)=+0.902, *P*<0.001, n=10**
			Behave-AD Global score	r(s)=+ 0.821, *P*<0.05, n=10
			CMAI cluster 2 *Physically nonaggressive behavior*	r(s)=+0.872, *P*≤0.001, n=10 r(s)=+0.865, *P*≤0.001, n=10
			CMAI cluster 3 *Verbally agitated behavior*	**r(s)=+0.903, *P*<0.001, n=10**
			CMAI Total score	
		5-HIAA/5-HT	Behave-AD cluster D *Aggressiveness*	r(s)=-0.832, *P*<0.05, n=10
FTD n=10	BA9	NA (ng/g)	Behave-AD cluster D *Aggressiveness*	r(s)=-0.813, *P*<0.05, n=10
	BA12	5-HT (ng/g)	Behave-AD Global score	r(s)=-0.834, *P*<0.05, n=10
	BA46	HVA/DA	CMAI Total score	r(s)=+0.812, *P*<0.05, n=10

Supplementary Table 2 only presents data with *P* values <0.005. Most significant correlations are depicted in **bold**. None of these correlations, however, remained statistically significant following a

total Bonferroni correction for multiple comparisons (P<0.000022). Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; 5-HT: 5-hydroxytryptamine (serotonin); AD: Alzheimer's disease; BA: Brodmann area; Behave-AD: Behavioral Pathology in Alzheimer's Disease Rating Scale; CMAI: Cohen-Mansfield Agitation Inventory; CSDD: Cornell Scale for Depression in Dementia; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; FTD: frontotemporal dementia; HVA: homovanillic acid; MA/MT: monoamine and metabolite; MFS: Middelheim Frontality Score; MHPG: 3-methoxy-4-hydroxyphenylglycol; NA: noradrenaline.

Chapter IV.2. CSF and serum MHPG improve Alzheimer's disease versus dementia with Lewy bodies differential diagnosis

Jana Janssens[a,b,1], Yannick Vermeiren[a,b,1], Erik Fransen[c], Tony Aerts[a], Debby Van Dam[a,b], Sebastiaan Engelborghs[a,d], Peter P. De Deyn[a,b,d,e]

[a]*Laboratory of Neurochemistry and Behavior, Institute Born-Bunge, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk (Antwerp), Belgium.*
[b]*Department of Neurology and Alzheimer Research Center, University of Groningen and University Medical Center Groningen (UMCG), Hanzeplein 1, 9713 GZ Groningen, The Netherlands*
[c]*StatUa Center for Statistics, University of Antwerp, Prins Boudewijnlaan 43, 2650 Edegem, Belgium*
[d]*Department of Neurology, Memory Clinic of Hospital Network Antwerp (ZNA) Middelheim and Hoge Beuken, Lindendreef 1, 2020 Antwerp, Belgium*
[e]*Biobank, Institute Born-Bunge, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk (Antwerp), Belgium*

[1]These authors contributed equally to this work.

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ABSTRACT

Introduction: Given the challenges concerning the differential diagnosis of dementia, we investigated the possible added value of monoaminergic compounds to the standard cerebrospinal fluid (CSF) Alzheimer's disease (AD) biomarkers. Particularly regarding the AD versus dementia with Lewy bodies (DLB) comparison, monoamines or their metabolites might have added discriminative value as there is a more severe neuropathological burden in the locus coeruleus (LC) of DLB patients, the principal site of noradrenaline synthesis.

Methods: We applied ELISA to analyze CSF amyloid-β peptide of 42 amino acids, total tau and tau phosphorylated at threonine 181, in patients with AD, frontotemporal dementia, DLB/Parkinson's disease dementia (PDD), and controls. RP-HPLC-ECD was implemented to study monoamine and metabolite levels in CSF and serum. Stepwise forward conditional logistic regression and ROC curve analyses were performed to assess the diagnostic accuracy of these newly fitted models containing the most discriminative indicators of disease status.

Results: Most significant differences in CSF and serum were confined to the noradrenergic system. More specifically, CSF 3-methoxy-4-hydroxyphenylglycol (MHPG) levels were higher, and, serum MHPG levels lower, in DLB patients compared to all other groups. Addition of CSF and serum MHPG levels to the CSF AD biomarker panel significantly increased diagnostic accuracy between DLB/PDD and AD. Interestingly, a model only including CSF and serum MHPG without the classic AD biomarker panel reached similar AUC values.

Conclusion: We hypothesize that varying degrees of neuronal loss in the LC of DLB/PDD versus AD patients result in differentially altered MHPG levels, making this metabolite a valuable biomarker.

1. INTRODUCTION

Alzheimer's disease (AD) and associated neurodegenerative brain disorders remain an important health care burden [330]. Recent findings from the Alzheimer's Association indicate that in 2015, 46.8 million people suffered from dementia worldwide, while this number is expected to increase in the next couple of decades [331], giving rise to even higher health strategies. Early detection of this neurocognitive disorder, combined with treatment strategies in the initial stages of the disease could aid in its reduction [332, 333].

Although current cerebrospinal fluid (CSF) biomarkers for AD diagnosis (amyloid-β ($A\beta_{1\text{-}42}$), total (T)-tau and tau phosphorylated at threonine 181 ($P\text{-}tau_{181P}$)), as comprised in the International Working Group-2 criteria [135], are widely used in clinical research, they still bring about several challenges. As such, they lack specificity to accurately discriminate between AD and dementia with Lewy bodies (DLB), which is especially complicated by the presence of AD co-pathology in DLB patients [334]. Thus, apart from shared clinical symptoms, some dementia types can also share a common etiology. Since overlapping concentrations exist in CSF T-tau and $P\text{-}tau_{181P}$ between AD, frontotemporal dementia (FTD), DLB and vascular AD [98], current diagnostics often require additional imaging investigations for differential dementia diagnosis. Another pitfall associated with CSF biomarkers is that their diagnostic performance decreases with age [335]. Given aforementioned complications and the practical difficulties associated with CSF sampling, the search for efficient blood biomarkers is imperative [138, 336]. Nevertheless, variability of the distinct blood constituents involves supplementary challenges concerning reproducibility of biomarker analysis. Additionally, questions arise about the applicability of blood biomarkers as the blood compartment is not in direct contact with the central nervous system (CNS), and might therefore inaccurately reflect changes in disease progression [336]. Moreover, it was shown that plasma and CSF $A\beta_{1\text{-}42}$ levels did not correlate in either patients with AD, non-AD, mild cognitive impairment (MCI) and control subjects [337]. Although a recently published paper provided first evidence for plasma neurofilament light as a potential blood biomarker for AD [338], such a blood biomarker for discrimination between AD and non-AD cases, to the best of our knowledge, has not been identified nor validated yet.

Recent studies, however, indicate that monoaminergic neurotransmitter profiles could represent an added value in improving etiological dementia diagnosis [144]. One of the first indications of this hypothesis was provided by Aerts and colleagues, who proved that addition of CSF 3-methoxy-4-hydroxyphenylglycol (MHPG), main metabolite of the monoamine (nor)adrenaline ((N)A) and indicative of central noradrenergic activity [150], to the classical biomarker profile of AD, could

increase both sensitivity and specificity for the discrimination between AD and DLB [145, 146]. This hypothesis might be further strengthened by the notion that distinct MHPG levels between AD and DLB patients were observed in 8 out of 11 brain regions, with DLB patients demonstrating significantly reduced MHPG levels [144]. Other studies investigating monoamine neurotransmitter levels in brain tissue equally gave rise to the awareness that AD and FTD differ in serotonergic and noradrenergic neurotransmitter content [143, 282], while an earlier study reported that CSF MHPG levels were considerably higher in FTD patients as compared to AD patients [149]. It was also noted that CSF NA and MHPG levels were increased in patients with advanced AD as compared to subjects suffering from moderate AD or controls, suggesting hyperactivity of the noradrenergic system in the end stage of the disease [339]. Furthermore, extensive evidence demonstrates that the locus coeruleus, the main NA-producing nucleus in the brain, is severely affected by Lewy pathology in Parkinson's disease dementia (PDD) [340], as well as associated with severe cell death which might affect the dopaminergic nigrostriatal pathway through loss of noradrenergic innervation [341-344]. This Lewy pathology in PDD has even been shown to precede the appearance of α-synuclein inclusions and neuronal loss in the dopaminergic substantia nigra [340, 345-350], indicating an undeniable role of noradrenergic deficits in PDD. Interestingly, MHPG easily passes the blood-brain [191] and blood-CSF [192] barrier. Taking into account all of the above, it appears that monoaminergic systems are indeed differentially implicated in distinct dementia subtypes, and could potentially serve as predictive markers.

Accordingly, this study aimed at identifying predictive monoamine biomarkers in both CSF and serum derived from patients suffering from AD, FTD, DLB/PDD, age-matched controls (CONTR) and young control (Y-CONTR) subjects. We hypothesized that these fluid monoamine markers, especially with regard to MHPG, could add significantly to the classical CSF AD biomarker panel, thus increasing diagnostic accuracy.

2. MATERIALS & METHODS

2.1. Study population

Paired CSF-serum samples derived from patients with probable AD (n=52), FTD (n=59), DLB (n=39), PDD (n=14), as well as CONTR (n=88) and Y-CONTR (n=32), were selected from the Biobank of the Institute Born-Bunge. All patients included in the AD, FTD, DLB, PDD, and Y-CONTR groups were included in a prospective, longitudinal study on neuropsychiatric symptoms (NPS) [293] between 2001-2011, and originally recruited at the Memory Clinic of the Hospital Network Antwerp (ZNA) Middelheim and Hoge Beuken as part of their diagnostic clinical

work-up. At inclusion, subjects underwent neuropsychological assessment and behavioral analysis as described earlier [293]. In case consented patients died, brain autopsy was performed within 6 h postmortem. The left hemisphere was frozen at -80°C, while the right hemisphere was fixated in formalin (12%) for neuropathological examination, which was performed as described earlier [143, 144]. None of the age-matched control subjects nor young controls suffered from neurological disease. Additionally, control subjects were excluded in case of psychiatric antecedents or suspicion of CNS pathology. Thus, the control group consisted of patients requiring lumbar radiculography as they suffered from mechanical low back pain, subjects with peripheral nervous system disorders and patients with subjective complaints which were not due to disorders of the central, nor the peripheral nervous system [96]. Lastly, the study was approved by the Medical Ethical Committee of the Middelheim General Hospital (Antwerp, Belgium; approval numbers 2805 and 2806) and conducted in compliance with the Helsinki Declaration.

2.2. CSF and serum sampling

Sampling of CSF was performed according to Vermeiren et al [176]. In short, a lumbar puncture was performed at the L3/L4 or L4/L5 intervertebral space between 08.00 and 10.00 hrs. Patients and controls fasted overnight and abstained from smoking for at least 12 hours. In total, 16.5 mL CSF was collected in 5 fractions using polypropylene vials as described by Engelborghs et al. [96].
Total blood was sampled into two serum gel tubes coated with clotting activator (S-Monovette® 7.5 mL Z-gel (Sarstedt, Nümbrecht, Germany)) and centrifuged at 3,000 rpm for 10 minutes. Serum aliquots were subsequently distributed to marked polypropylene vials and frozen in liquid nitrogen. Of note, part of the CONTR group (n=43) followed the protocol specified above (i.e. matched CSF-serum samples after overnight fasting) [293], whereas serum-only samples of the remaining 45 control subjects were obtained at different time points and under a distinct clinical setting. All samples were stored in the Biobank of the Institute Born-Bunge at -80°C.

2.3. CSF $A\beta_{1\text{-}42}$, T-tau, P-tau_{181P} analysis using ELISA

CSF analyses of $A\beta_{1\text{-}42}$, T-tau, P-tau_{181P} were performed by means of ELISA, as part of a clinical diagnostic work-up. Cut-off values were derived from the lower and upper detection limits inherent to the ELISA-kits (INNOTEST® amyloid-$\beta_{(1\text{-}42)}$, INNOTEST® hTAU-Ag, and INNOTEST® PHOSPHO-$TAU_{(181P)}$, for CSF $A\beta_{1\text{-}42}$, T-tau and P-tau_{181P}, respectively (Fujirebio, Ghent, Belgium)), i.e. 125 pg/mL and 2,000 pg/mL for $A\beta_{1\text{-}42}$, 75 pg/mL and 1,200 pg/mL for T-tau, and , lastly, 15.6 pg/mL and

500 pg/mL for P-tau$_{181P}$. The detailed CSF analysis protocol has already been published by Le Bastard et al. [351].

2.4. RP-HPLC-ECD

The monoamines dopamine (DA), tryptophan (TRP), serotonin (5-HT) and (N)A, as well as their respective metabolites (homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA), and MHPG) were analyzed in paired CSF-serum samples by means of an optimized and validated reversed-phase high-performance liquid chromatography (RP-HPLC) system with electrochemical detection (ECD) (ALEXYS™ Monoamine Analyzer, Antec Leyden, Zoeterwoude, Netherlands) [302]. The sample preparation protocol was standardized and consisted of a precolumn purification using Amicon® Ultra 0.5 Centrifugal Filters (cutoff 3,000 Da; Millipore, Ireland), washed twice with 450 µL sample buffer during centrifugation (14,000 x g, 25 minutes, 4°C). Subsequently, CSF and serum samples were loaded onto the prewetted columns and centrifuged at 4°C for 40 minutes at 14,000 x g. The filtrate was diluted 1:2 and 1:7 for CSF, while 1:4 and 1:15 dilutions were implemented for serum. Finally, diluted samples were injected automatically onto an ALF-125 column (C18; 250 mm x 1.0 mm, 3 µm particle size). Further specifications of the RP-HPLC-ECD procedure have been described by Van Dam et al. [302].

2.5. Statistics

As our dataset was characterized by non-normally distributed variables, nonparametric statistical tests were performed. All continuous variables were tested for differences between diagnostic classes using the Kruskal-Wallis test, followed by a post-hoc analysis using the Mann-Whitney U tests with a Bonferroni correction for multiple comparisons (P<0.005). Additionally, the association between the diagnostic classes and categorical variables such as gender, was tested using the chi-square test. In case the expected cell count was less than 5, Fisher's Exact test was used. To identify the most discriminative indicators of disease status, stepwise forward conditional logistic regression analysis was applied, with disease status as dependent variable and a combination of standard AD biomarkers and monoamines or metabolites as explanatory variables. Age was included in every regression model. Subsequently, area under the curve (AUC) values belonging to the respective ROC curves of models fitted with and without the addition of monoamines and/or metabolites, were compared by performing DeLong tests. Finally, optimal cut-off values were determined by maximization of the Youden's index [352]. All statistical analyses, except for DeLong tests, were performed using SPSS 24.0 for Windows (IBM SPSS Software, Armonk, NY, IBM Corp). The DeLong

test was carried out as implemented in the pROC package in R, version 3.4.0 for Windows, specifically designed to investigate partial AUC values (pROC) [353].

3. RESULTS

3.1. Demographical and clinical data

Table 1 contains corresponding demographical and clinical information.
Whereas all patient groups were gender-matched ($P>0.05$), age of CSF and serum sampling was only comparable between the AD and DLB/PDD groups ($P>0.05$), as well as between the AD and CONTR groups ($P>0.05$) and DLB/PDD and CONTR groups ($P>0.05$). Furthermore, significant differences were detected for cognitive test scores ($P<0.001$) between all study groups. For instance, Mini-Mental State Examination scores were much lower in the AD group compared to DLB/PDD, FTD and CONTR groups. Indication of disease stage by global deterioration scale scores indicated that AD patients were somewhat more advanced than DLB/PDD and FTD counterparts. Scores of the neuropsychological assessment were only included if there were no more than four months between the moment of testing and sampling. In total, 42 out of 52 AD, 15 out of 59 FTD, and, 9 out of 53 DLB/PDD patients, respectively, had neuropathological confirmation of their clinical diagnoses. Additionally, out of the 9 neuropathologically-defined DLB patients, 7 had concomitant AD pathology.
Administered classes of psychotropic medication can be found in Table 1 for each diagnostic category.

3.2. Neurochemical comparisons of biomarkers and monoamines

We found statistically significant differences in CSF levels of $A\beta_{1\text{-}42}$ ($P<0.0001$), T-tau ($P<0.0001$) and P-Tau_{181P} ($P\leq0.001$) for the AD versus FTD comparison, as well as between AD and DLB/PDD ($P\leq0.001$, $P<0.0001$, and $P<0.0001$ for $A\beta_{1\text{-}42}$, T-tau, and P-Tau_{181P}, respectively), as well as between the AD and Y-CONTR groups ($P<0.0001$ for all three biomarkers). Differences in concentrations of $A\beta_{1\text{-}42}$ ($P<0.0001$) and T-tau ($P<0.0001$), but not P-Tau_{181P} ($P<0.05$), in AD versus CONTR subjects were also deemed significant. A complete description of biomarker levels between diagnostic categories can be found in Table 2.

Table 1. Demographic and clinical data of the study population.

Parameter	AD (n=52)	FTD (n=59)	DLB/PDD (n=53)	CONTR (n=88)	Y-CONTR (n=32)	Test statistics
Age at sampling (y)	75.5 ± 8.2[aa,ddd] (56.0-89.1) (n=52)	68.8 ± 9.5[aa,eee,f,ggg] (40.8-83.3) (n=59)	76.6 ± 6.0[eee,iii] (61.5-88.5) (n=53)	73.8 ± 10.7[f,jjj] (50.4-92.8) (n=88)	38.2±8.8[ddd,ggg,iii,jjj] (17.1-50.0) (n=32)	X^2=100.2 *P*<0.00001
MMSE (/30)	13.7 ± 5.8[aa,ccc] (3.0-25.0) (n=39)	19.1 ± 7.3[aa,fff] (1.0-30.0) (n=43)	17.2 ± 6.6[hhh] (3.0-28.0) (n=45)	28.2 ± 1.6[ccc,fff,hhh] (24.0-30.0) (n=39)	N/A	X^2=84.2 *P*<0.00001
HDS (/10)	6.9 ± 1.3[ccc] (4.2-9.1) (n=28)	7.3 ± 1.7[fff] (2.9-9.8) (n=31)	8.0 ± 5.0[hhh] (2.4-34.0) (n=32)	9.7 ± 0.3[ccc,fff,hhh] (9.1-10.0) (n=17)	N/A	X^2=40.1 *P*<0.00001
BNT (/60)	24.2 ± 12.5[a,bbb,ccc] (4.0-48.0) (n=28)	34.9 ± 13.4[a,fff] (1.0-57.0) (n=35)	38.3 ± 10.3[bbb,hhh] (13.0-54.0) (n=38)	51.2 ± 4.7[ccc,fff,hhh] (36.0-57.0) (n=23)	N/A	X^2=53.9 *P*<0.00001
VFT	20.2 ± 10.6[ccc] (2.0-45.0) (n=25)	25.2 ± 15.2[fff] (0.0-56.0) (n=31)	24.1 ± 9.6[hhh] (6.0-49.0) (n=34)	51.0 ± 13.3[ccc,fff,hhh] (34.0-90.0) (n=24)	N/A	X^2=48.2 *P*<0.00001
GDetS (/7)	5.7 ± 0.7[aaa,ccc] (4.0-7.0) (n=38)	4.9 ± 1.0[aaa,fff] (3.0-7.0) (n=33)	5.2 ± 1.0[hhh] (3.0-7.0) (n=38)	1.5 ± 0.6[ccc,fff,hhh] (1.0-3.0) (n=39)	N/A	X^2=97.5 *P*<0.00001
Gender (Male/Female)	30/22	30/29	37/16	46/42	15/17	Pearson X^2=6.4 *P*>0.05
Psychotropic medication (n/y)	13/39	16/43	10/43	52/36	25/7	Pearson X^2=51.1 *P*<0.00001
Anti-Alzheimer's medication (n/y)	33/19	46/13	38/15	88/0	32/0	Pearson X^2=45.4 *P*<0.00001
Anti-Parkinson's medication (n/y)	50/2	55/4	28/25	88/0	32/0	Pearson X^2=90.1 *P*<0.00001
Hypnotic, Sedative or Anxiolytic medication (n/y)	42/10	46/13	41/12	69/19	29/3	Pearson X^2=2.8 *P*>0.05
Antidepressant medication (n/y)	34/18	41/18	34/19	67/21	27/5	Pearson X^2=6.0 *P*>0.05

Table 1. Continued.

Parameter	AD (n=52)	FTD (n=59)	DLB/PDD (n=53)	CONTR (n=88)	Y-CONTR (n=32)	Test statistics
Antipsychotic medication (n/y)	29/23	34/25	40/13	86/2	32/0	Pearson X^2=58.1 *P*<0.00001
Antiepileptic medication (n/y)	52/0	57/2	53/0	82/6	32/0	Fisher's Exact Test=6.6 *P*>0.05

Data are represented as mean ± SD with minimum-maximum ranges between brackets. Test statistics of the Kruskal-Wallis and Chi-Square (or Fisher's Exact) analysis can be found in the rightmost column, while statistically significant differences with $P \leq 0.005$, $P \leq 0.001$, and $P \leq 0.0001$ after M-W U analysis with post-hoc Bonferroni corrections are depicted by one, two or three superscript letters, respectively. Superscript letters denote differences between following groups, a: AD and FTD, b: AD and DLB/PDD, c: AD and CONTR, d: AD and Y-CONTR, e: FTD and DLB/PDD, f: FTD and CONTR, g: FTD and Y-CONTR, h: DLB/PDD and CONTR, i: DLB/PDD and Y-CONTR, and j: CONTR and Y-CONTR, respectively. Only cognitive test scores of no more than four months before date of sampling were included in the analyses. Abbreviations: AD: Alzheimer's disease; BNT: Boston Naming Test; CONTR: controls; DLB/PDD: dementia with Lewy bodies/Parkinson's disease dementia; FTD: frontotemporal dementia; GDetS: Global Deterioration Scale; HDS: Hierarchic Dementia Scale; MMSE: Mini-Mental State Examination; N/A: not applicable; VFT: Verbal Fluency Test; Y-CONTR: young controls.

Table 2. Concentrations of classic CSF AD biomarkers.

Parameter	AD (n=52)	FTD (n=59)	DLB/PDD (n=53)	CONTR (n=88)	Y-CONTR (n=32)	Test statistics
$A\beta_{1\text{-}42}$ (pg/mL)	432.3 ± 172.2^{aaa,bb,ccc,ddd} (125.0-1159.0) (n=47)	680.1 ± 245.2^{aaa} (282.0-1200.0) (n=58)	584.9 ± 235.4^{bb} (274.0-1327.0) (n=51)	803.7 ± 275.9^{ccc} (302.0-1212.0) (n=19)	1014.6 ± 162.2^{ddd} (725.0-1265.0) (n=16)	X^2=62.8 *P*<0.00001
T-tau (pg/mL)	581.5 ± 285.2^{aaa,bbb,ccc,ddd} (108.0-1200.0) (n=47)	362.8 ± 190.9^{aaa} (97.0-956.0) (n=58)	304.2 ± 189.7^{bbb} (17.0-1200.0) (n=50)	295.7 ± 144.7^{ccc} (126.0-680.0) (n=19)	185.6 ± 57.0^{ddd} (96.0-320.0) (n=16)	X^2=49.3 *P*<0.00001
P-tau_{181P} (pg/mL)	71.0 ± 31.7^{aa,bbb,ddd} (16.0-152.0) (n=47)	52.7 ± 24.6^{aa} (19.0-116.0) (n=58)	50.1 ± 24.9^{bbb} (19.0-151.0) (n=50)	53.4 ± 21.7 (23.0-110.0) (n=19)	37.1 ± 9.7^{ddd} (21.0-52.0) (n=16)	X^2=24.1 *P*<0.0001

Data represented as mean ± SD with minimum-maximum ranges between brackets. Test statistics of the Kruskal-Wallis analysis can be found in the rightmost column, while statistically significant differences with $P \leq 0.005$, $P \leq 0.001$, and $P \leq 0.0001$ after M-W U analysis with Bonferroni post-hoc corrections ($P \leq 0.0125$) are depicted by one, two and three superscript letters, respectively. Superscript letters denote differences between following groups, a: AD and FTD, b: AD and DLB/PDD, c: AD and CONTR, and, d: AD and Y-CONTR, respectively. Abbreviations: $A\beta_{1\text{-}42}$: amyloid-β peptide of 42 amino acids; AD: Alzheimer's disease; CONTR: controls; DLB/PDD: dementia with Lewy bodies/Parkinson's disease dementia; FTD: frontotemporal dementia; P-tau_{181P}: tau phosphorylated at threonine 181; T-tau: total tau; Y-CONTR: young controls.

Similar analyses were performed in paired CSF and serum samples, taking into account all monoamines, their metabolites and calculated ratios. In CSF, we found 48 significant differences between diagnostic categories, while 59 statistically significant distinctions could be identified in serum. Most of these differences were identified in the noradrenergic system and, therefore, we will only focus on MHPG in CSF and serum (Figure 1). Significant differences in CSF MHPG were found when we compared all diagnostic categories (for all: $P<0.0001$), except for the comparisons between AD and FTD, AD and CONTR, and, lastly, FTD and CONTR. Likewise, distinct serum MHPG levels could be observed for all group comparisons ($P\leq0.001$), other than those between AD and FTD, and AD and CONTR.
We refer to the Supplementary Table 1 for the complete dataset of CSF and serum monoamines for each diagnostic category.

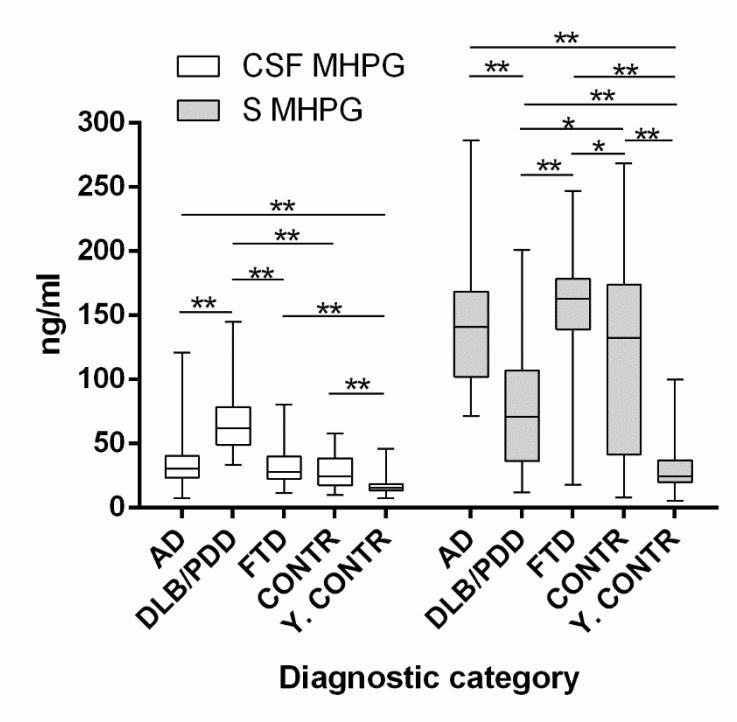

Figure 1. CSF and serum MHPG levels in all diagnostic categories. Data are represented as median ± IQR. Significant differences between groups after M-W U tests with post-hoc Bonferroni correction ($P<0.005$) are indicated by one ($P=0.001$) or two asterisks ($P<0.001$).
Abbreviations: AD: Alzheimer's disease; CONTR: controls; CSF: cerebrospinal fluid; DLB/PDD: dementia with Lewy bodies/Parkinson's disease dementia; FTD: frontotemporal dementia; IQR: interquartile range; MHPG: 3-methoxy-4-hydroxyphenylglycol; M-W U: Mann-Whitney U; S: serum; Y-CONTR: young controls.

3.3. Added value of monoamines on standard biomarkers

Table 3 contains diagnostic accuracy values corresponding to each fitted model. Besides the classic CSF biomarker set for AD, i.e. $A\beta_{1\text{-}42}$, T-tau and P-Tau_{181P}, as well as age of sampling, CSF and serum NA and its metabolite MHPG were among the most predictive markers. In case of distinctions between AD and DLB/PDD, we found that the AUC values differed significantly (P<0.001) between the fitted models with and without addition of CSF (P<0.001) and serum (P=0.001) MHPG. When concentrations of only CSF and serum MHPG were included in a model without the classic AD biomarker panel, this difference could even be maintained (P<0.05).

Table 3. Results of DeLong tests indicating discriminative power between distinct diagnostic categories.

BM-based discrimination between	Selected CSF BM	Selected CSF and/or serum MA or MT	AUC	95% CI	S (%)	Sp (%)
Without MA-MT						
AD and FTD	$A\beta_{1\text{-}42}$/ P-tau_{181P} ratio*	N/A	0.81	0.73-0.90	66	85
AD and DLB/PDD	$A\beta_{1\text{-}42}$, T-tau & P-tau_{181P}	N/A	0.82[a(a)]	0.73-0.91	88	73
AD and CONTR	$A\beta_{1\text{-}42}$/T-tau ratio*	N/A	0.88	0.75-1.00	89	82
AD versus non-AD	$A\beta_{1\text{-}42}$, T-tau & P-tau_{181P}	N/A	0.87[a]	0.80-0.94	90	76
With MA-MT						
AD and FTD	$A\beta_{1\text{-}42}$/ P-tau_{181P} ratio*	CSF NA	0.85	0.78-0.93	69	90
AD and DLB/PDD	$A\beta_{1\text{-}42}$, T-tau & P-tau_{181P}	CSF & serum MHPG	0.99[aa]	0.97-1.00	98	95
	N/A (without BM)	CSF & serum MHPG	0.98[a]	0.95-1.00	98	95
AD and CONTR	$A\beta_{1\text{-}42}$/T-tau ratio*	CSF NA	0.94	0.88-1.00	89	92
AD versus non-AD	$A\beta_{1\text{-}42}$, T-tau & P-tau_{181P}	CSF NA & TRP and serum 5-HIAA	0.95[a]	0.91-0.99	97	82

Optimal cutoff values were determined by maximizing the Youden's index. Superscript letters "a" added to the AUC values indicate statistical significance: [a]P<0.005; [aa]P<0.001. An asterisk indicates the inclusion of biomarker ratios rather than separate biomarker levels for comparisons between AD and both FTD and CONTR, as described earlier in Struyfs et al., 2015 [354]. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; $A\beta_{1\text{-}42}$: amyloid-β peptide of 42 amino acids; AD: Alzheimer's disease; AUC: area under the curve; BM: biomarkers; CI: confidence interval; CONTR: controls; CSF: cerebrospinal fluid; DLB/PDD: dementia with Lewy bodies/Parkinson's disease dementia; FTD: frontotemporal dementia; MHPG: 3-methoxy-4-hydroxyphenylglycol; MA & MT: monoamines and metabolites; N/A: not applicable; NA: noradrenaline; P-tau_{181P}: tau phosphorylated at threonine 181; S: sensitivity; Sp: specificity; TRP: tryptophan; T-tau: total tau.

Sub-analysis in the group of neuropathologically-characterized AD (n=42) and DLB (n=9) subjects confirmed previous finding, with the AUC value increasing from 0.70 to 0.99 (P<0.001) if solely CSF and serum MHPG were included in the model instead of the core CSF AD biomarkers. Models fitted for comparisons between the AD group and both the FTD and CONTR groups, could not be significantly improved by addition of CSF or serum monoamines. When an overall distinction between AD and non-AD was made, the model with addition of CSF NA, serum 5-HIAA and CSF TRP (for all: P<0.05) was characterized by a significantly raised AUC (P<0.05). Lastly, none of the distinctions including Y-CONTR could be improved by addition of monoamines and/or metabolites, as sensitivity and specificity values already reached 100%.

3.4. Psychotropic medication comparisons: effect on monoamines

Concerning the influence of medication on monoaminergic compounds, we found an expected effect of antidepressants on serotonergic compounds and ratios in all diagnostic categories except Y-CONTR. Significant differences could also be detected in DA levels in FTD (serum DA; P<0.05) and DLB/PDD (CSF DA; P<0.05) between patients taking and not taking anti-Alzheimer's medication. Overall, concentrations of DA were altered between patients taking and not taking psychotropic medication in CSF samples of the FTD group (P<0.05), and, serum samples of the DLB/PDD group (P<0.05). In addition, noradrenergic alterations could be observed between patients on anti-Parkinson's medication and patients free of such medication, with CSF MHPG/NA ratios being significantly higher in patients not taking anti-Parkinson's drugs in AD (P<0.05) and DLB/PDD groups (P<0.05). Likewise, serum MHPG/NA ratios in DLB/PDD subjects were higher in patients not taking anti-Parkinson's drugs (P<0.0001). In the same study group, CSF and serum NA levels were higher (P<0.05 and P<0.0001, respectively), in patients free of anti-Parkinson's medication. Lastly, use of antipsychotic medication significantly influenced CSF and serum NA levels in the DLB/PDD group, as well as CSF and serum MHPG/NA ratios (for all: P<0.05).

4. DISCUSSION

Similar to our results, CSF MHPG was reported to increase discriminative power between AD and DLB, although this effect was not apparent in comparisons with other dementia types [145, 146]. Besides monoamines in CSF, we investigated whether such compounds in serum could equally serve as valuable blood biomarkers. Indeed, addition of serum MHPG and 5-HIAA proved to be useful in discriminating AD versus non-AD, increasing sensitivity and specificity values. Several studies have already indicated that noradrenergic cell loss in the LC is apparent in both AD [355-357] and DLB/PDD [358, 359]. Although both dementias are characterized by LC degeneration, cell loss in the brainstem has been reported to be more prominent in DLB/PDD [360]. The authors found a significantly lower number of tyrosine-hydroxylase labeled neurons at the 70% level of the LC in DLB patients compared to their AD counterparts, with the most rostral (0%) section defined as the beginning of the trochlear nucleus, and the most caudal end defined as the rostral edge of the trigeminal motor nucleus [361]. Moreover, two other studies also reported more severe, albeit not significant, cell loss in DLB/PDD compared to AD [358, 359]. Further evidence supporting these findings can be found in the fact that the LC is initially involved DLB/PDD neuropathology, while AD patients at first show Aβ plaques in neo- and allocortical brain regions, with amyloid pathology only reaching the brainstem nuclei in more advanced stages, i.e. Thal stages 4 and 5 [8, 9]. Tau pathology affects brainstem nuclei only in mid-to-late AD stages, coinciding with Braak stages IV-VI [10, 362]. The majority of patients suffering from DLB, as well as about half of PDD subjects, also show AD pathology [363], possibly exacerbated by the notion that the A53T mutant of alpha-synuclein, although rare [364], is able to promote the association of tau-fibrils [365]. Taken together, these factors might result in a more severe noradrenergic cell loss in the LC due to a heavier neuropathological load. It was shown that in patients suffering from AD, this cell loss corresponded with an increased noradrenergic turnover (as a compensatory mechanism) in brain areas receiving efferent projections from the LC [339, 366], such as the hippocampus and amygdala [367]. Our results support this finding in CSF of AD and DLB patients, with increased MHPG/NA ratios, indicating increased noradrenergic turnover, in both conditions. Furthermore, we observed that these ratios were higher in DLB compared to AD patients (Supplementary Table 1). Latter event might be explained by a more severe neuropathological load in DLB patients, resulting in a more extensive compensatory mechanism.

Although a preceding study questioned the use of CSF MHPG as a reliable indicator of central noradrenergic activity given the diffusion of MHPG into spinal cord tissue [191], it was suggested that both CSF NA and MHPG levels reflected NA metabolism

in the brain [192, 339, 368, 369], while plasma NA was hypothesized to mirror noradrenergic turnover of peripheral sympathetic neurons [169, 339]. A more recent study confirmed that MHPG, unlike NA [20], passes the blood-brain, as well as the blood-CSF barrier [194], thus strengthening latter theory. Still others suggested that degeneration of peripheral noradrenergic neurons may occur as a prodromal state of DLB and Parkinson's disease (PD) [370, 371], preceding neuropathological lesions in the LC. Another study reported that patients originally diagnosed with pure autonomic failure (PAF), may later on convert to multiple system atrophy, or DLB/PDD [371, 372]. Our results indeed indicated lowered NA and increased MHPG levels in the CSF of the DLB/PDD group, possibly reflecting the hypothesis that extensive damage to the LC results in lowered and increased brain NA and MHPG levels, respectively, which are mirrored in the CSF of these patients. In AD, we found a trend towards increased CSF MHPG levels, in addition to significantly decreased NA levels compared to controls.

The direction of change of NA and MHPG levels has been the subject of previous debate. For instance, levels of CSF NA and MHPG were found to be unchanged or enhanced in patients suffering from (severe) AD [146, 339, 373]. It has also been reported that CSF MHPG levels were unchanged or decreased in PDD [374], and, decreased in DLB patients [146]. However, CSF NA levels between AD and DLB/PDD patients did not differ, which was in concordance with previous findings [176]. We hypothesize that we could not corroborate previous findings regarding CSF NA because this compound was influenced by several types of psychotropic medication in the DLB/PDD group, while little information pertaining to medication effects is provided in the study conducted by Herbert et al. (2014). Lastly, next to the proposed hypothesis, differences in study population characteristics and a considerable variation in CSF MHPG levels in the DLB group reported by Herbert et al. (2014), might also have caused this discrepancy compared to preceding studies. In serum, both diagnostic categories showed increased MHPG and NA concentrations, which may be explained by additional NA release by peripheral noradrenergic neurons, as well as peripheral NA metabolism. Conversely, patients suffering from PAF are characterized by lowered plasma NA levels [371], as a result of autonomic dysfunction. This finding could not be corroborated in our study, possibly since serum is a large compartment in which several confounding biological processes occur, possibly masking previously mentioned effects.

One of the limitations of this study is the variation of sampling procedures in the control group, which could give rise to a considerable amount of variation in serum MHPG levels in this population (Figure 1). In addition, the biochemical analysis of MHPG and NA, which are sensitive to pre-analytical variability effects of

temperature and oxidation, still remains challenging. We also found that medication use was not comparable between diagnostic categories, and, moreover, that psychotropic drug use might have influenced various monoaminergic compounds, such as the serotonergic and noradrenergic ones (see section 3.4). However, it should be taken into account that none of the serotonergic parameters influenced by antidepressant medication, were included in the logistic regression models, with the sole exception of serum 5-HIAA for the distinction between AD and non-AD. Lastly, none of the drug classes influenced concentrations of serum nor CSF MHPG, a crucial compound included in the newly fitted models. This is a strength of the study in addition to the considerable amount of available clinical and neuropsychological data, as well as the additional neuropathological confirmation.

5. CONCLUSION

We observed that CSF and serum monoamines have an added value in the differential diagnosis of dementia, next to the classical CSF AD biomarker panel. In particular, we found that CSF and serum MHPG were the most valuable markers to discriminate DLB/PDD from AD. To verify these findings, future studies should focus on age-matched, and, preferably, medication-free patient populations. Beforehand, various methodological and confounding aspects that might interfere with the analysis of CSF/serum MHPG, such as sample handling, lumbar puncture, and dietary effects, should be vigorously investigated.

6. ACKNOWLEDGEMENTS

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7. SUPPLEMENTARY MATERIAL

Supplementary Table 1. CSF and serum levels of monoamines and metabolites.

Parameter	AD (n=52)	FTD (n=59)	DLB/PDD (n=53)	CONTR (n=88)	Y-CONTR (n=32)	Test statistic
CSF						
MHPG (ng/mL)	36.4 ± 22.3[bbb,ddd] (7.3-120.8) (n=47)	32.2 ± 15.1[eee,ggg] (11.4-80.7) (n=59)	66.9 ± 24.0[bbb,eee,hhh,iii] (33.3-145.0) (n=53)	27.9 ± 11.4[hhh,jjj] (10.0-57.9) (n=43)	16.5 ± 6.9[ddd,ggg,iii,jjj] (7.3-46.0) (n=32)	X^2=119.9 *P*<0.00001
NA (ng/mL)	0.6 ± 0.7[aa,ccc,ddd] (0.8-3.5) (n=41)	1.0 ± 0.7[aa,ggg] (0.1-3.3) (n=59)	1.1 ± 1.3[h,iii] (0.0-8.5) (n=50)	1.4 ± 0.8[ccc,h] (0.1-5.0) (n=43)	1.5 ± 0.4[ddd,ggg,iii] (0.7-2.3) (n=32)	X^2=47.2 *P*<0.00001
A (ng/mL)	0.7 ± 0.9[aaa] (0.3-5.5) (n=44)	0.9 ± 2.4[aaa,eee,ggg] (0.1-14.4) (n=56)	0.8 ± 1.2[eee] (0.1-8.9) (n=59)	0.6 ± 0.5 (0.1-2.2) (n=38)	0.9 ± 1.2[ggg] (0.3-5.9) (n=20)	X^2=32.1 *P*<0.0001
DOPAC (ng/mL)	2.6 ± 1.9[bbb] (0.1-7.6) (n=47)	2.3 ± 2.6 (0.1-12.2) (n=59)	1.2 ± 1.2[bbb,hh] (0.1-6.8) (n=46)	2.9 ± 3.5[hh] (0.2-13.2) (n=43)	1.8 ± 1.8 (0.2-9.6) (n=32)	X^2=20.6 *P*<0.00001
5-HIAA (ng/mL)	21.9 ± 15.1 (4.3-80.2) (n=47)	22.2 ± 9.4[ee] (3.1-52.8) (n=59)	16.5 ± 9.2[ee,hhh] (4.4-38.8) (n=53)	26.5 ± 11.2[hhh,jjj] (6.8-60.2) (n=43)	17.2 ± 6.9[jjj] (7.1-36.2) (n=32)	X^2=29.1 *P*<0.00001
DA (ng/mL)	3.3 ± 4.6[ccc,ddd] (0.5-28.7) (n=47)	2.2 ± 1.8[f,ggg] (0.5-9.9) (n=59)	1.8 ± 1.1[iii] (0.3-6.8) (n=53)	1.6 ± 1.4[ccc,f,jjj] (0.4-7.0) (n=43)	0.7 ± 0.2[ddd,ggg,iii,jjj] (0.4-1.4) (n=32)	X^2=62.6 *P*<0.00001
HVA (ng/mL)	45.4 ± 32.7 (7.7-174.4) (n=47)	47.2 ± 24.3 (6.7-147.0) (n=59)	49.9 ± 62.5 (5.9-470.0) (n=53)	54.6 ± 23.5[jj] (15.8-106.3) (n=43)	37.0 ± 14.7[jj] (7.6-66.2) (n=32)	X^2=11.7 *P*=0.020
TRP (ng/mL)	337.8 ± 122.1[a,c] (161.4-883.3) (n=47)	385.3 ± 99.6[a] (227.8-896.4) (n=59)	n.d.	412.8 ± 160.4[c,j] (134.9-1208.9) (n=43)	332.8 ± 52.4[j] (220.5-463.6) (n=32)	X^2=18.0 *P*<0.00001

Supplementary Table 1. Continued.

Parameter	AD (n=52)	FTD (n=59)	DLB/PDD (n=53)	CONTR (n=88)	Y-CONTR (n=32)	Test statistic
CSF						
5-HT (ng/mL)	n.d.	n.d.	n.d.	0.9 ± 0.6 (0.3-2.1) (n=7)	0.7 ± 0.3 (0.4-1.5) (n=9)	X^2=0.7 *P*=0.864
MHPG/NA	114.8 ± 155.6^{aaa,ccc,ddd} (8.1-972.1) (n=41)	62.0 ± 97.5^{aaa,eee,f} (5.7-695.8) (n=59)	273.9 ± 526.9^{eee,hhh,iii} (6.9-2949.8) (n=50)	42.1 ± 74.0^{ccc,f,hhh,jjj} (3.2-440.1) (n=43)	12.2 ± 7.4^{ddd,iii,jjj} (4.3-37.1) (n=32)	X^2=94.3 *P*<0.00001
DOPAC/DA	1.2 ± 1.9^{ccc,ddd} (0.0-10.3) (n=47)	1.6 ± 2.1 (0.0-10.4) (n=59)	0.9 ± 1.2^{hhh,iii} (0.1-6.2) (n=46)	2.5 ± 3.4^{ccc,hhh} (0.1-16.5) (n=43)	2.5 ± 2.3^{ddd,iii} (0.3-11.2) (n=32)	X^2=34.0 *P*<0.00001
HVA/DA	20.7 ± 12.5^{ccc,ddd} (3.2-44.0) (n=47)	29.1 ± 16.4^{fff} (3.8-65.4) (n=59)	35.9 ± 39.6^{h,iii} (4.9-233.3) (n=53)	51.2 ± 30.7^{ccc,fff,h} (6.2-114.7) (n=43)	54.0 ± 25.1^{ddd,iii} (12.9-126.2) (n=32)	X^2=53.3 *P*<0.00001
5-HIAA/5-HT	n.d.	n.d.	n.d.	38.8 ± 20.0 (17.1-71.4) (n=7)	22.4 ± 11.2 (9.4-74.1) (n=9)	X^2=3.4 *P*=0.330
HVA/5-HIAA	2.2 ± 1.0 (0.6-4.5) (n=47)	2.3 ± 1.1 (0.6-7.5) (n=59)	3.5 ± 3.2 (0.5-17.9) (n=53)	2.2 ± 0.8 (0.9-4.5) (n=43)	2.2 ± 0.7 (0.7-3.9) (n=32)	X^2=4.2 *P*=0.381
Serum						
MHPG (ng/mL)	142.7 ± 47.0^{bbb,ddd} (71.7-286.5) (n=50)	160.7 ± 36.9^{eee,ff,ggg} (18.1-246.9) (n=56)	78.0 ± 45.7^{bbb,eee,hh,iii} (12.2-201.0) (n=52)	119.6 ± 70.6^{ff,hh,jjj} (8.0-268.7) (n=81)	29.0 ± 16.7^{ddd,ggg,iii,jjj} (5.3-100.2) (n=32)	X^2=105.6 *P*<0.00001
NA (ng/mL)	3.8 ± 9.8^{d} (0.1-65.9) (n=49)	4.2 ± 17.9^{e} (0.2-129.7) (n=54)	7.5 ± 14.8^{e,h,iii} (0.2-74.7) (n=40)	1.6 ± 3.3^{h,j} (0.1-29.6) (n=78)	0.9 ± 0.6^{d,iii,j} (0.3-3.0) (n=24)	X^2=23.7 *P*<0.00001
A (ng/mL)	2.1 ± 9.3 (0.2-63.9) (n=46)	0.7 ± 0.3^{gg} (0.3-1.7) (n=50)	1.2 ± 1.1 (0.0-5.9) (n=44)	1.3 ± 3.5 (0.2-28.9) (n=67)	1.0 ± 0.5^{gg} (0.5-2.9) (n=20)	X^2=34.0 *P*<0.00001

Supplementary Table 1. Continued.

Parameter	AD (n=52)	FTD (n=59)	DLB/PDD (n=53)	CONTR (n=88)	Y-CONTR (n=32)	Test statistic
Serum						
DOPAC (ng/mL)	5.0 ± 11.2[aa,bb,d] (1.3-78.0) (n=50)	2.6 ± 2.9[aa,eee] (0.9-17.9) (n=54)	9.5 ± 12.1[bb,eee,hhh,iii] (0.6-62.7) (n=51)	3.8 ± 4.5[hhh] (0.8-31.1) (n=80)	2.2 ± 1.2[d,iii] (0.2-6.1) (n=31)	X^2=14.0 *P*=0.007
5-HIAA (ng/mL)	11.1 ± 13.8[d] (3.0-72.5) (n=50)	6.1 ± 2.9[ff,ggg] (1.9-16.2) (n=56)	7.1 ± 3.6[iii] (1.4-19.0) (n=52)	8.1 ± 3.9 [ff,jjj] (3.1-20.5) (n=81)	6.0 ± 8.4[d,ggg,iii,jjj] (2.1-45.6) (n=32)	X^2=42.2 *P*<0.00001
DA (ng/mL)	8.3 ± 29.8 (0.8-201.3) (n=50)	1.9 ± 1.1[f] (0.7-8.1) (n=56)	3.6 ± 3.4 (0.2-21.1) (n=52)	3.5 ± 4.9[f] (0.6-30.0) (n=81)	1.8 ± 0.5 (1.1-3.7) (n=32)	X^2=14.8 *P*<0.005
HVA (ng/mL)	22.7 ± 32.3[aa,ddd] (3.2-141.3) (n=50)	10.6 ± 7.8[aa,eee,ggg] (4.3-51.3) (n=56)	38.1 ± 60.2[eee,hh,iii] (4.2-250.7) (n=52)	11.1 ± 6.0[hh,jjj] (4.0-42.1) (n=81)	5.6 ± 1.4[ddd,ggg,iii,jjj] (3.5-10.0) (n=32)	X^2=70.4 *P*<0.00001
TRP (ng/mL)	n.d.	2376.7 ± 663.4[ggg] (1057.2-3590.7) (n=26)	2566.3 ± 607.4[iii] (1379.8-4284.6) (n=52)	2615.7 ± 849.7[jjj] (1238.8-4683.1) (n=34)	1626.2 ± 580.5[ggg,iii,jjj] (971.0-3895.7) (n=32)	X^2=42.3 *P*<0.00001
5-HT (ng/mL)	77.5 ± 66.1 (1.6-336.9) (n=50)	65.2 ± 52.4 (1.8-204.7) (n=56)	56.1 ± 49.9 (2.0-203.4) (n=52)	63.1 ± 44.3 (1.2-169.9) (n=81)	69.6 ± 39.5 (10.9-134.7) (n=32)	X^2=4.9 *P*=0.296
MHPG/NA	150.0 ± 142.6[bbb,ddd] (2.2-592.9) (n=49)	211.0 ± 184.6[eee,f,ggg] (1.1-1004.9) (n=54)	66.1 ± 103.2[bbb,eee,hhh] (1.3-541.3) (n=40)	148.3 ± 191.0[f,hhh,jj] (5.9-1257.4) (n=78)	43.6 ± 31.6[ddd,ggg,jj] (8.6-133.7) (n=24)	X^2=50.7 *P*<0.00001
DOPAC/DA	1.6 ± 1.1 (0.1-6.4) (n=50)	1.5 ± 1.3[ee] (0.1-8.1) (n=54)	3.3 ± 3.0[ee,hhh,ii] (0.4-12.8) (n=51)	1.7 ± 2.0[hhh] (0.1-13.4) (n=80)	1.3 ± 0.8[ii] (0.1-3.8) (n=31)	X^2=19.8 *P*=0.001
HVA/DA	8.1 ± 8.8[c,ddd] (0.7-53.9) (n=50)	6.0 ± 3.7[ggg] (1.3-23.1) (n=56)	15.7 ± 22.4[h,iii] (0.6-126.3) (n=52)	4.9 ± 3.0[c,h,j] (0.6-21.7) (n=81)	3.3 ± 1.1[ddd,ggg,iii,j] (1.2-6.1) (n=32)	X^2=34.4 *P*<0.00001

Supplementary Table 1. Continued.

Parameter	AD (n=52)	FTD (n=59)	DLB/PDD (n=53)	CONTR (n=88)	Y-CONTR (n=32)	Test statistic
Serum						
5-HIAA/5-HT	0.4 ± 0.6[dd] (0.0-2.4) (n=50)	0.3 ± 0.5 (0.0-2.2) (n=56)	0.5 ± 0.8[ii] (0.0-3.3) (n=52)	0.6 ± 1.4[jjj] (0.0-9.1) (n=81)	0.2 ± 0.4[dd,ii,jjj] (0.0-2.2) (n=32)	X^2=18.0 *P*=0.001
HVA/5-HIAA	2.3 ± 2.9 (0.5-19.9) (n=50)	2.2 ± 3.4[e] (0.4-27.6) (n=56)	7.5 ± 15.6[e,hhh,iii] (0.4-81.6) (n=52)	1.5 ± 0.5[hhh] (0.4-3.1) (n=81)	1.4 ± 0.5[iii] (0.2-3.0) (n=32)	X^2=22.6 *P*<0.00001

Data are represented as mean ± SD with minimum-maximum ranges between brackets. Test statistics of the Kruskal-Wallis analysis can be found in the rightmost column, while statistically significant differences with $P \leq 0.005$, $P \leq 0.001$, and $P \leq 0.0001$ after M-W U analysis with Bonferroni post-hoc corrections are depicted by one, two and three superscript letters, respectively. Superscript letters denote differences between following groups a: AD and FTD, b: AD and DLB/PDD, c: AD and CONTR, d: AD and Y-CONTR, e: FTD and DLB/PDD, f: FTD and CONTR, g: FTD and Y-CONTR, h: DLB/PDD and CONTR, i: DLB/PDD and Y-CONTR, and j: CONTR and Y-CONTR, respectively. Abbreviations: 5-HIAA: 5-hydroxyindoleacetic acid; 5-HT: 5-hydroxytryptamine/serotonin; A: adrenaline; AD: Alzheimer's disease; CONTR: controls; DA: dopamine; DLB/PDD: dementia with Lewy bodies/Parkinson's disease dementia; DOPAC: 3,4-dihydroxyphenylacetic acid; FTD: frontotemporal dementia; HVA: homovanillic acid; NA: noradrenaline; MHPG: 3-methoxy-4-hydroxyphenylglycol; TRP: tryptophan; Y-CONTR: young controls.

Chapter IV.3. Monoaminergic and kynurenergic characterization of frontotemporal dementia and amyotrophic lateral sclerosis in cerebrospinal fluid and serum

Jana Janssens[1,2], Yannick Vermeiren[1,2], Martijn van Faassen[3], Claude van der Ley[3], Ido P. Kema[3], Peter P. De Deyn[1,2,4].

[1]Department of Biomedical Sciences, Neurochemistry and Behavior, Institute Born-Bunge (IBB), University of Antwerp, Wilrijk, Belgium.
[2]Department of Neurology, Alzheimer Center Groningen, University Medical Center Groningen (UMCG) and University of Groningen, Groningen, The Netherlands.
[3]Department of Laboratory Medicine, University Medical Center Groningen (UMCG) and University of Groningen, Groningen, The Netherlands.
[4]Faculty of Medicine and Health Sciences, University of Antwerp, Wilrijk, Belgium.

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ABSTRACT

Exploring the neurochemical continuum between frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) with respect to monoamines and kynurenines in cerebrospinal fluid (CSF) and serum, may be useful to identify possible new research/therapeutic targets. Hence, we analysed monoamines and kynurenines in CSF and serum derived from patients with FTD (n=39), ALS (n=23), FTD-ALS (n=4) and age-matched control subjects (n=26), using reversed-phase ultra-high performance liquid chromatography (RP-UHPLC) with electrochemical detection (ECD) and liquid chromatography tandem mass spectrometry, respectively.

We noted a shared dopaminergic disturbance in FTD and ALS when compared to CONTR, with significantly increased serum DA levels and decreased DOPAC concentrations, as well as decreased DOPAC/DA ratios in both disease groups. In CSF, significantly reduced DOPAC concentrations in FTD and ALS were observed as well. Here, a significant increase in DA levels and decrease in DOPAC/DA ratios was only found in FTD relative to CONTR. With respect to the kynurenine pathway (KP), we only found decreased HK/XA ratios, indicative for vitamin B6 status, in serum of ALS subjects compared to FTD.

The dopaminergic commonalities observed in FTD and ALS might relate to a disturbance of dopaminergic nerve terminals in projection areas of the substantia nigra and/or ventral tegmental area, although these findings should first be confirmed in brain tissue. Lastly, based on the results of this work, the KP does not hold promise as a research/therapeutic target in FTD and ALS.

1. INTRODUCTION

Frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) are devastating neurodegenerative disorders demonstrating genetic and neuropathologic overlap. The most common shared genetic mutation is a GGGGCC expansion in *C9ORF72*, present in approximately 30-47% [375, 376] and 25-34% [375, 377] of familial ALS and FTD cases, respectively. This hexanucleotide expansion is most often associated with the presence of cytoplasmic inclusions containing transactive response DNA-binding protein of 43 Da (TDP-43) in both diseases [378, 379]. Consequently, these inclusions are found in up to 97% of ALS and 45% of FTD cases [380]. Next to the shared genetic and neuropathologic characteristics, similarities in clinical presentation are also observed. Behavioral abnormalities and/or signs of cognitive impairment including executive dysfunction, are observed in 50% of patients with ALS, while 15% of these subjects reach the diagnostic criteria for FTD [381-384]. Likewise, similar frequencies of motor disturbances are observed in FTD [377, 385].

Although this clinicopathological overlap has been well covered in the scientific literature, less is known about (monoaminergic) neurotransmitter disturbances in ALS. As this condition is currently treated with strategies primarily targeting glutamatergic neurotransmission and oxidative stress using riluzole and edaravone, respectively [386-388], offering limited benefit, other options based on alternative neurotransmitter systems could be explored. The relative success of selective serotonin reuptake inhibitors (SSRIs) in FTD might point to monoamines as interesting research targets in this disorder, and possibly also in ALS. Furthermore, as previous research demonstrated monoaminergic disturbance in brain tissue (reviewed in [389])and cerebrospinal fluid (CSF) of FTD patients [147, 149, 283], investigating the occurrence of these substances in easily accessible body fluids such as blood, plasma or serum in ALS might be of interest to determine whether FTD and ALS also share neurochemical characteristics.

Due to its implication in (neuro-)inflammation, the kynurenine pathway (KP) has received increasing attention in immune-related diseases such as cancer [390, 391], HIV [392], and several neurodegenerative disorders including ALS [393-395]. The KP starts with the oxidative metabolism of the essential amino acid L-tryptophan (TRP), by the rate-limiting enzymes indolamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), accounting for 95 % of L-tryptophan degradation, [130] (Figure 1). The most potent activator of IDO is the cytokine interferon gamma (IFN-γ) [396], while TDO is primarily induced by glucocorticoids

and substrate activation [397, 398]. The activation of both enzymes leads to an unstable intermediate, N-formylkynurenine, which is subsequently metabolized to L-kynurenine (KYN). In turn, this compound is converted to the end-product nicotinamide adenine dinucleotide (NAD^+) in sequential steps producing the neuroprotective NMDA-receptor antagonist kynurenic acid (KA) [399, 400], the oxidative stress generator 3-hydroxykynurenine (3-HK) [401], the immunomodulatory (3-hydroxy)anthranilic acid ((3-H)AA) [402], the neuroprotective picolinic acid (PA) [403] and the NMDA-receptor agonist quinolinic acid (QA) [404]. Next to these well-known metabolites, the KP also results in the formation of xanthurenic acid (XA), which is hypothesized to act as a neuroprotectant [405], the possibly anti-inflammatory cinnabarinic acid [406] and nicotinic acid or vitamin B3 [130]. Although few researchers have previously focused on determining the KP in samples derived from patients with ALS, it appears that concentrations of some neuroprotective and neurotoxic metabolites are altered in CSF and serum [393, 407]. In FTD, however, little information regarding a possible dysregulation of this metabolic pathway can be found. Given the involvement of the KP in neuroinflammation and since this pathway is linked to the production of 5-HT via their common precursor TRP, not only 5-HT and other related monoamines, but also compounds of the KP could possibly represent an interesting means to investigate the neurochemical continuum of ALS-FTD.

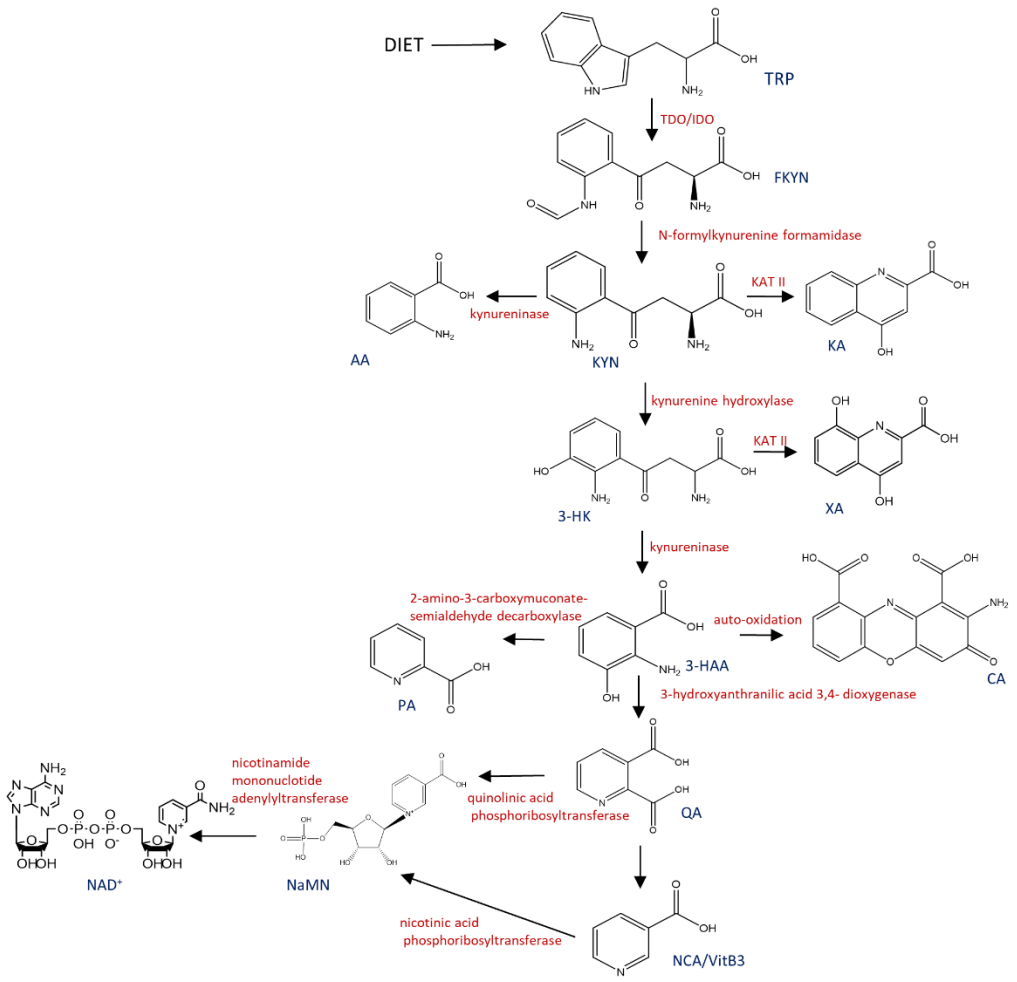

Figure 1. Schematic representation of the kynurenine pathway. Abbreviations: 3-HAA: 3-hydroxyanthranilic acid; 3-HK: 3-hydroxykynurenine; AA: anthranilic acid; CA: cinnabarinic acid; FKYN: N-formylkynurenine; IDO: indoleamine 2,3-dioxygenase; KA: kynurenic acid; KAT: kynurenine aminotransferase; KYN: l-kynurenine; NAD^+: nicotinamide adenine dinucleotide; NaMN: nicotinic acid mononucleotide; NCA/VitB3: nicotinic acid or Vitamin B3; PA: picolinic acid; QA: quinolinic acid; TDO: tryptophan 2,3-dioxygenase; TRP: tryptophan; XA: xanthurenic acid.

2. MATERIALS & METHODS

2.1. Study population

This retrospective study used CSF and serum samples derived from the NeuroBioBank of the Institute Born-Bunge (NBB-IBB (n°BB190113), Wilrijk). Monoamines and metabolites were analyzed in CSF samples of patients suffering from probable FTD (n=39) [39], FTD-ALS (n=4), ALS (n=23), and age-matched control subjects (CONTR) (n=26). Serum samples were derived from the same patients, although for 3 ALS subjects, no serum samples were available. Patients and control subjects were recruited at the Memory Clinic of the Hospital Network Antwerp (ZNA) Middelheim and Hoge Beuken, as CSF and serum sampling was part of their clinical diagnostic workup. Psychiatric antecedents and central nervous system pathology other than FTD and ALS were regarded as exclusion criteria. Control subjects came to the clinic because of (tension) headache, lumbar canal stenosis, cervicalgia/cervical myelopathy, carpal tunnel syndrome, nausea, chronic gait disorders, periodic fever syndrome, leucopenia, struma simplex, facial arteriovenous malformations, and sinusitis. In patients who gave brain donation consent, brain autopsy was generally performed within 6-8 hours postmortem. The right hemisphere was immersion-fixated in a 12% formalin solution for neuropathological examination, while the left hemisphere was frozen at –80°C [143, 144]. In the FTD-ALS group, only one subject had neuropathological confirmation of the initial clinical diagnosis, with molecular lesions classified as TDP-43 Type B. Similarly, only one subject was neuropathologically confirmed as definite ALS. Genetically, 11 FTD subjects had a hexanucleotide expansion in *C9ORF72*, three patients had a *GRN* mutation, two subjects had a *MAPT* mutation and two other patients had a mutation in either *TBK1* or *VCP*. A summary of neuropathologically confirmed cases and related genetics in the FTD group can be found in Figure 2. This study was approved by the Medical Ethical Committee of the Middelheim General Hospital (Antwerp, Belgium, file numbers 2805 and 2806), and was conducted in line with the Helsinki Declaration.

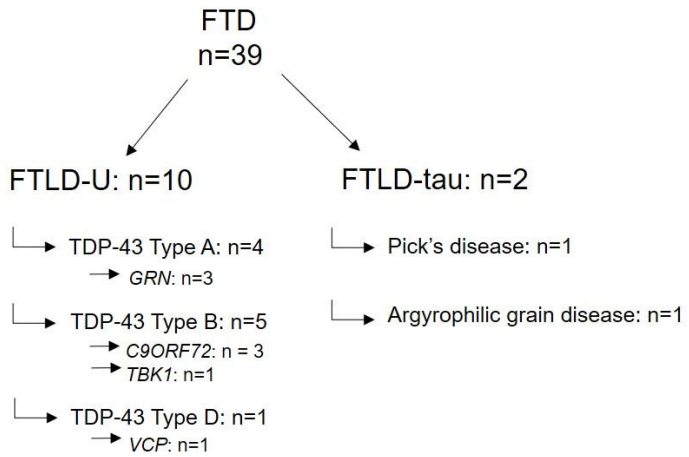

Figure 2. Overview of neuropathological diagnoses in the FTD group. Abbreviations: FTLD-U: frontotemporal lobar degeneration with ubiquitin-positive inclusions; FTD: frontotemporal dementia; TDP-43: transactive response DNA-binding protein of 43 kDa.

2.2. Sampling of cerebrospinal fluid and serum

Lumbar puncture was performed at the L3/L4 or L4/L5 intervertebral space to collect a total volume of 16.5 mL, which was divided across five fractions of 4.5 mL, 1.5 mL, 1.5 mL, 4.5 mL and 4.5 mL, respectively, in polypropylene vials (Nalgene; VWR, Leuven, Belgium) [96]. Serum was obtained after total blood sampling into two serum gel tubes with clotting activator (S-Monovette 7.5 mL Z-gel (Sarstedt, Nümbrecht, Germany)), which were centrifuged during 10 min at 3,000 rpm. Afterwards, serum was distributed to polypropylene vials. Both CSF and serum samples were frozen and stored at -80°C until analysis.

2.3. RP-UHPLC-ECD

An optimized and validated reversed-phase ultra-high-performance liquid chromatography (RP-UHPLC) system with electrochemical detection (ECD) was used to determine (nor)adrenaline ((N)A) and its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG), dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), as well as 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). The sample preparation consisted of a purification on Amicon® Ultra 0.5 Centrifugal Filters (cutoff 3,000 Da; Millipore, Ireland), which were washed twice beforehand with 450 µL buffer while centrifuging (14,000 x g, 25 minutes, 4°C). Serum and CSF were brought onto these columns, followed by centrifugation at 4°C for 40 min at 14,000 x g. The resulting CSF filtrate was diluted 1:2, and either 1:7 or 1:10, while the filtered

serum was diluted 1:5 and 1:25 or 1:30. This RP-UHPLC system operated at an isocratic flow rate of 75 µL/min. The Decade II electrochemical detector was equipped with a thin layered electrochemical VT03 flow cell fitted with a glassy carbon 0.7 mm working electrode and an in situ Ag/AgCl (ISAAC) reference electrode. Samples of 5 µL were loaded with an Alexys AS 110 Autosampler. Separation was achieved using a short 15 cm Waters Acquity Column (BEH C18, 1 mm diameter, particle size 1.7 µm; Waters; Etten-Leur; the Netherlands) [302].

2.4. LC-MS/MS

Concentrations of l-tryptophan (TRP), l-kynurenine (KYN), 3-hydroxykynurenine (3-HK), anthranilic acid (AA), kynurenic acid (KA), xanthurenic acid (XA), quinolinic acid (QA), picolinic acid (PA) and nicotinic acid (NA) were determined at the department of Laboratory Medicine of the University Medical Center Groningen by liquid chromatography in combination with isotope dilution tandem mass spectrometry (LC-MS/MS) essentially as described by [408]. In short, 100 µL of CSF or 50 µL of serum was mixed with deuterated or 13C labeled internal standards of the KP compounds under investigation. These mixtures were extracted with Strata X-A 96-well plates with a pore size of 33 µm and sorbent mass of 30 mg/well (Cat. n°: 8E-S123-TGB; Strata-X, Phenomenex, Utrecht, the Netherlands) and eluted with 3 M HCl in 1-butanol. Finally, 1 µL was injected into an Acquity UHPLC (Waters) equipped with a Phenomenex Luna column (Omega C18, 100 x 2.1 mm, particle size 1.6 µm), and coupled to a XEVO TQ-S MS/MS system (Waters; Etten-Leur, the Netherlands). KYN/TRP ratios were calculated as a measure for TDO and IDO activity [409] and HK/XA ratios as a measure for vitamin B6 function since the enzyme converting HK to XA, kynurenine aminotransferase II, is pyridoxal phosphate-dependent [410].

2.5. Statistics

One-way ANOVA was applied for continuous demographic parameters such as age and storage time. In case the assumption of homogeneity of variances was violated, Welch's ANOVA was applied. Fisher's Exact tests were applied to investigate the association between categorical variables such as disease category, gender and medication use.

Nonparametric statistics were applied to test inter-group differences of monoamines and kynurenines due to the non-normal distribution of these parameters in CSF and serum. Kruskal-Wallis analyses were applied as omnibus tests, while post-hoc analyses were performed with Mann-Whitney U statistics. Correction for multiple testing was applied using the Benjamini-Hochberg

procedure. The results for the FTD-ALS group are presented solely as median concentrations, as the size of this group was very small and often even reduced to n=2 or n=3. Therefore, we opted not to include monoaminergic and kynurenergic concentrations of this group in statistical tests for group comparisons. Lastly, Spearman's rank correlation analysis was used to assess the relationship between storage time and concentrations of monoamines and kynurenines. Again, Benjamini-Hochberg corrections were applied to account for multiple testing. All statistical analyses were performed using SPSS version 25.0 for Windows.

3. RESULTS

3.1. Demographics

Table 1 summarizes the demographic details of the study population. Additional information about the types of medication across the different disease groups can be found in Supplementary Table 1.

Table 1. Demographics of the study populations for analysis of monoamines and kynurenines.

Parameter	Data-set	Sample type	CONTR	FTD	FTD-ALS	ALS	Test statistic
Age at sampling (years)	M	CSF	67.0 ± 8.0 n=26	67.4 ± 11.6 n=39	64.7 ± 5.7 n=3	67.2 ± 10.3 n=23	F(3,87)=0.069 *P*>0.05
		Serum	67.2 ± 8.3 n=26	67.4 ± 11.6 n=39	64.7 ± 5.7 n=3	66.9 ± 10.4 n=20	F(3,84)=0.071 *P*>0.05
	K	CSF	67.3 ± 8.1 n=25	67.4 ± 11.6 n=39	64.7 ± 5.7 n=3	67.6 ± 10.5 n=22	F(3,85)=0.071 *P*>0.05
		Serum	67.0 ± 8.0 n=26	67.4 ±11.6 n=39	64.7 ± 5.7 n=3	66.9±10.4 n=20	F(3,84)=0.072 *P*>0.05
Male/Female	M	CSF	14/12	20/19	1/3	21/2	FE=14.2 *P*<0.05
		Serum	14/12	20/19	1/2	18/2	FE=10.9 *P*<0.05
	K	CSF	14/11	20/19	1/3	20/2	FE=13.1 *P*<0.05
		Serum	14/12	20/19	1/2	18/2	FE=10.9 *P*<0.05
Taking/not taking medication	M	CSF	9/7	27/7	0/1	11/6	FE=5.2 *P*>0.05
		Serum	10/6	27/7	0/1	11/5	FE=4.1 *P*>0.05
	K	CSF	9/7	27/7	0/1	11/6	FE=5.2 *P*>0.05
		Serum	9/7	27/7	0/1	11/5	FE=5.1 *P*>0.05
Storage time (months)	M	CSF	202.5 ± 30.6 n=26	132.8 ± 62.1 n=39	158.3 ± 44.4 n=3	171.9 ± 76.2 n=23	Welch=10.930 *P*<0.05
		Serum	203.0 ± 30.6 n=26	133.2 ± 62.2 n=39	158.3 ± 44.4 n=3	187.3 ± 68.2 n=20	Welch = 10.848 *P*<0.05
	K	CSF	213.0 ± 30.8 n=25	144.6 ± 62.2 n=39	169.7 ± 44.2 n=3	185.9 ± 77.3 n=22	Welch=10.285 *P*<0.05
		Serum	214.2 ± 30.7 n=26	144.6 ± 62.1 n=39	169.7 ± 44.2 n=3	198.8 ± 68.0 n=20	Welch=10.760 *P*<0.05

Data regarding age at sampling and sampling time are represented as mean ± standard deviation. For the numbers of subjects taking/not taking medication, psychotropic substances, riluzole and dietary vitamin B supplements were taken into account. Abbreviations: ALS: amyotrophic lateral sclerosis; CONTR: control; CSF: cerebrospinal fluid; FE: Fisher's Exact; FTD: frontotemporal dementia; FTD-ALS: frontotemporal dementia – amyotrophic lateral sclerosis; K: kynurenines; M: monoamines.

3.2. Monoamines

Alterations in the dopaminergic system across disease groups were noted in both CSF ($H(2)=9.017$, $P<0.05$ for DOPAC; $H(2)=17.963$, $P<0.001$ for DA; $H(2)=12.392$, $P<0.05$ for DOPAC/DA and $H(2)=7.252$, $P<0.05$ for HVA/DA) and serum ($H(2)=12.116$, $P<0.05$ for DOPAC, $H(2)=16.850$, $P<0.001$ for DA; and $H(2)=19.438$, $P<0.0001$ for DOPAC/DA). A general decrease in DOPAC values was observed in FTD and ALS subjects compared to the CONTR group. Increased DA concentrations in CSF were found in the FTD versus the CONTR and ALS groups. In serum, DA levels were also highly significantly increased in the FTD subjects compared to CONTR individuals, while a similar result was observed between the CONTR and ALS group. Analysis of the DOPAC/DA ratio revealed a disturbed dopaminergic catabolism in FTD versus the CONTR group, evidenced by significantly lower CSF and serum DOPAC/DA ratios in the former category. Furthermore, serum DOPAC/DA ratios were severely decreased in ALS subjects compared to healthy CONTR subjects (Figure 2). Another index of DA catabolism, HVA/DA, was also significantly decreased in FTD compared to CONTR subjects. Finally, CSF MHPG differed across diagnostic categories ($H(2)=8.833$, $P<0.05$), with the only significant difference after post-hoc correction indicating higher MHPG levels in the FTD compared to the CONTR group. All results of post-hoc comparisons are depicted in Supplementary Table 2.

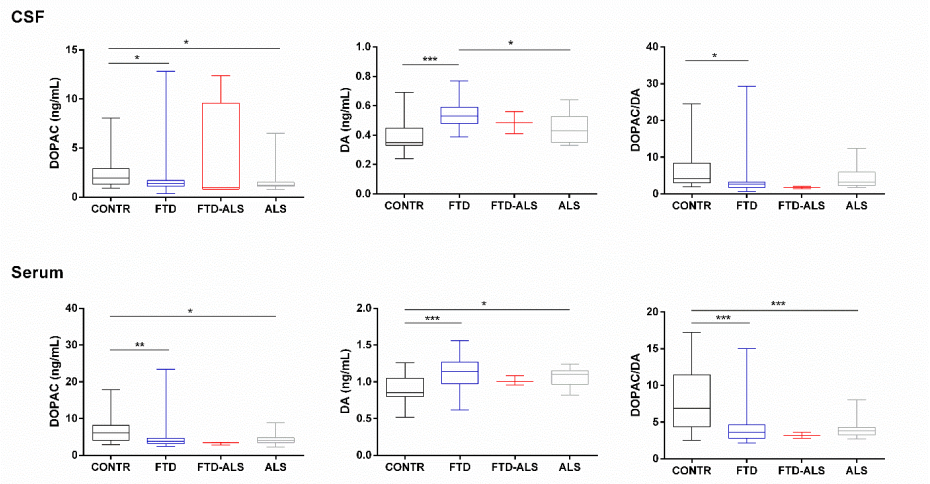

Figure 3. Dopaminergic findings across diagnostic categories. Data are represented as box- and whisker plots with minimum-maximum ranges. Statistically significant differences after Mann-Whitney U tests with Benjamini-Hochberg post-hoc corrections are depicted by one, two or three asterisks if $P \leq 0.05$, 0.001, or 0.0001, respectively. Sample sizes for CSF DOPAC are: CONTR: n=26, FTD: n=39, FTD-ALS: n=4, ALS: n=23, sample sizes for CSF DA and DOPAC/DA are CONTR: n=21, FTD: n=35, FTD-ALS: n=2, ALS: n=17.

Sample sizes for serum DOPAC are: CONTR: n=26, FTD: n=39, FTD-ALS: n=3, ALS: n=20, sample sizes for serum DA and DOPAC/DA are: CONTR: n=26, FTD: n=35, FTD-ALS: n=3, ALS: n=20. The FTD-ALS group was not included in the statistical analysis. Abbreviations: ALS: amyotrophic lateral sclerosis; CONTR: control; CSF: cerebrospinal fluid; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; FTD: frontotemporal dementia; FTD-ALS: frontotemporal dementia – amyotrophic lateral sclerosis.

3.3. Kynurenines

No statistically significant differences were found after Kruskal-Wallis analysis for individual compounds belonging to the KP. We did find a difference in serum HK/XA ratios across diagnostic categories (H(2)=7.007, $P<0.05$), with lower serum HK/XA ratios in the ALS versus the FTD group (Figure 3). Post-hoc statistics, in addition to median values for HK/XA in each group, are summarized in Supplementary Table 2.

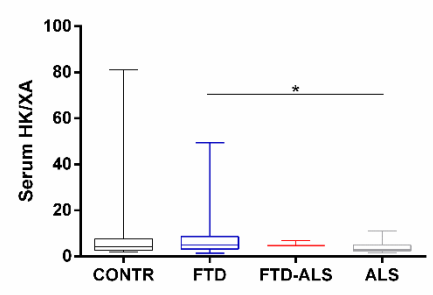

Figure 4. Serum HK/XA ratios across diagnostic categories. Data are represented as box- and whisker plots with minimum-maximum ranges. Statistically significant differences after Mann-Whitney U analyses with Benjamini-Hochberg corrections are indicated by an asterisk ($P<0.05$). Sample sizes for serum HK/XA are: CONTR: n=25, FTD: n=39, FTD-ALS: n=3, ALS: n=19. The FTD-ALS group was not included in the statistical analysis. Abbreviations: ALS: amyotrophic lateral sclerosis; CONTR: control; FTD: frontotemporal dementia; FTD-ALS: frontotemporal dementia-amyotrophic lateral sclerosis; HK: 3-hydroxykynurenine; XA: xanthurenic acid.

3.4. Influence of psychotropic medication and storage time

When equivalent analyses were performed to detect differences in monoamine/kynurenine content only in subjects who were free of psychotropic medication and who did not take riluzole nor supplements containing vitamin B, a trend for increased CSF DA levels in FTD and ALS compared to CONTR could be observed (H(2)=5.751; $P=0.056$), but none of the monoaminergic differences remained statistically significant. In contrast, a significant difference was found for CSF TRP across groups (H(2)=7.558; $P<0.05$), with higher TRP levels in the FTD and ALS groups compared to the CONTR group (U=6.000; $P<0.05$ and U=5.000; $P<0.05$, respectively).

We found that CSF MHPG and DA were negatively correlated with storage time (rs(89)=-0.409; $P<0.0001$ and rs(73)=-0.330; $P<0.05$), whereas a positive

association between storage time and 5-HIAA (rs(89)=0.286; $P<0.05$) as well as 5-HIAA/5-HT ratios (rs(87)=0.346; $P\leq0.001$) was noted. In serum, no such associations could be detected. As for kynurenines, only AA in CSF decreased with increasing storage time (rs(87)=-0.288; $P<0.05$).

4. DISCUSSION

4.1. Monoamines

A general dopaminergic disturbance was noted in FTD and ALS compared to CONTR subjects, largely corresponding to previous reports. Single photon emission computed tomography has shown decreased frontal uptake of a dopamine 2 receptor (D2) postsynaptic ligand, as well as a relative frontal hypoperfusion in FTD versus CONTR and subjects with Alzheimer's disease [411]. Reduced uptake of ^{11}C-2β-carbomethoxy-3β-(4-fluorophenyl) tropane (^{11}C-CFT), a cocaine analogue targeting DA transporters (DAT), in the putamen of FTD subjects was also observed in another study [412]. In addition, ALS subjects showed decreased striatal binding of ^{123}I-N-(3-iodopropen-2-yl)-2β-carbomethoxy-3β-(4-chlorophenyl)-tropane (^{123}IPT), a cocaine analogue binding specifically to DATs [413]. Furthermore, increased DA levels and decreased HVA/DA ratios were observed in Brodmann area (BA)46 and BA6 of FTD subjects compared to CONTR [143]. Taken together, these findings may all indicate disrupted dopaminergic nerve terminals in projection areas of the substantia nigra (SN) and ventral tegmental area (VTA). Reduced presynaptic uptake of ^{123}IPT and decreased binding of ^{11}C-CFT in ALS and FTD, might reflect a dysfunction of dopaminergic nerve terminals, possibly related to presynaptic reuptake mechanisms mediated by DAT. This might explain our observation of increased CSF DA levels in these disease groups. The decreased DOPAC concentrations and DOPAC/DA ratios may then be a result of impaired DOPAC synthesis due to dopaminergic neuron dysfunction. Exploring the observed dopaminergic alterations in brain tissue of FTD and ALS subjects is mandatory to assess the validity of this hypothesis.

It would also be interesting to compare the dopaminergic disturbance in the FTD-ALS continuum with that observed in Parkinson's disease (PD). In a previous study on dopaminergic alterations in CSF of synucleinopathy patients [414], among which PD, dopaminergic data of the PD group were reduced to eliminate influences of dopaminergic medication, resulting in a PD group (n=17) in which only PD patients with CSF and plasma L-DOPA values ≤ 5 nmol/L and 10 nmol/L, were included. We calculated DA ratios and DOPAC ratios in CSF for the disease groups included in our study versus the control group (CONTR), as well as indentical ratios the PD group versus CONTR in the previous report [414]. We observed that the

ratios of DOPAC in the FTD, FTD-ALS and ALS groups versus CONTR were 0.7, 0.5 and 0.6, respectively, while this ratio was 0.4 if the DOPAC concentrations of the PD group were normalized to CONTR. For DA, we noted ratios of 1.3 in both FTD as well as FTD-ALS groups, and 1.0 in ALS compared to CONTR. In the study on catecholaminergic alterations in synucleinopathy subjects [414], the PD/CONTR ratio for DA was 0.8.

In this respect, it is clear that the extent of the dopaminergic disturbance is most evident in PD, with severely decreased CSF DOPAC and DA concentrations compared to CONTR. This finding of lowered CSF DA and DOPAC concentrations in PD are supported by several other studies [415, 416], even though the sample sizes of these studies were rather small (n=4 and n=7, respectively). It is interesting to note that we did not find decreased DA concentrations in FTD or ALS, possibly reflecting a distinct disease mechanism from PD.

The dopaminergic alterations in serum of FTD and ALS versus CONTR subjects resembled those in CSF, which is surprising since DA nor DOPAC readily cross the blood-brain barrier (BBB) [417, 418]. However, several studies have demonstrated impaired BBB integrity in FTD and ALS [419, 420], which might account for the similarities observed in CSF and serum.
The use of psychotropic medication may have influenced our findings since the dopaminergic alterations could not be detected between FTD, ALS or CONTR in a medication-free subgroup. However, given the low number of observations in this analysis (n=3-9 per group), type II errors cannot be ruled out.
The longer storage time in the CONTR group might have led to a false positive result regarding the increased MHPG and DA levels in FTD compared to the CONTR group. Thus, the absolute DA levels in CSF should be considered carefully, while the DOPAC concentrations and the dopaminergic turnover in CSF remain unaffected by storage time.
Based on the therapeutic strategies applied in FTD, we expected to find serotonergic (dis)similarities, in the ALS-FTD continuum. However, our results suggest a shared dopaminergic disturbance in CSF and serum, except for CSF DA levels. This finding may shift the research in these neurodegenerative conditions to DA-based approaches.

4.2. Kynurenines

In previous studies investigating the KP in ALS, KYN levels, neuroprotective and neurotoxic KP compounds appeared to be altered in this condition [393, 407]. It is possible we could not reproduce these findings because of differences in CSF handling, alternative analytical conditions, or distinct demographics. Since the study of Chen et al. [393] included non-age-matched CONTR and ALS groups

(average age of 36 and 58 years, respectively) and since the KP is involved in aging, these results, particularly with respect to CSF KYN [421], KYN/TRP ratios as a measure for IDO-activity, and concentrations of QA [422] could also be explained by the higher average age in the ALS group.
The results of subjects who did not take psychotropic medication, riluzole or vitamin B supplements did indicate increased CSF TRP levels between FTD and ALS versus CONTR. It is difficult to speculate about the (patho)physiological meaning of this result, as lowered KYN/TRP ratios would indicate a downregulation of IDO/TDO activity, but no such results could be observed. In addition, no serotonergic differences were observed in FTD and ALS versus the CONTR group, indicating that TRP metabolism was not shifted towards the synthesis of serotonergic compounds in these groups. Lastly, our observation of decreased serum HK/XA ratios in ALS compared to FTD could have been influenced by storage time. Based on the results of this study, we suggest that the KP offers limited therapeutic value/research potential for the ALS-FTD continuum.

4.3. Future perspectives

Although the SN was shown to be affected in FTD and ALS [423, 424], the differential pathology in the SN and/or VTA of FTD and ALS patients does not represent a well-studied topic. It would be interesting to investigate the correlation between neuropathological observations in the SN and VTA, and dopaminergic disturbance in their projection areas in postmortem brain tissue. Alternatively, resting state functional MRI may be applied to assess the functional connectivity between midbrain dopaminergic centers and (pre)frontal cortical regions, combined with CSF sampling in FTD and ALS subjects. Follow-up of these patients may then result in a better understanding of dopaminergic disturbance, and how this is reflected in bodily fluids. In addition, we recommend the analysis of CSF and serum on a clinically better characterized population, certainly with respect to the ALS group.

In conclusion, we found that ALS and FTD share a dopaminergic disturbance in serum, while these changes are also partly present in CSF. This could possibly be caused by dopaminergic terminal dysfunction in (cortical) projection areas of the SN and VTA. The compounds of the KP did not appear to be altered in FTD or ALS.

5. ACKNOWLEDGEMENTS

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6. SUPPLEMENTARY MATERIAL

Supplementary Table 1. Types of medication across diagnostic categories.

Parameter	Compounds of interest	Sample type	CONTR	FTD	FTD-ALS	ALS	Test statistic
Taking/not taking psychotropic medication	Monoamines	CSF	9/7	25/9	0/1	4/13	Fisher's Exact=12.6; *P*<0.05
		Serum	10/6	25/9	0/1	4/12	Fisher's Exact=11.8; *P*<0.05
	Kynurenines	CSF	9/7	25/9	0/1	4/13	Fisher's Exact=12.6; *P*<0.05
		Serum	9/7	25/9	0/1	4/12	Fisher's Exact=11.6; *P*<0.05
Taking/not taking riluzole	Monoamines	CSF	0/16	0/34	0/1	7/10	Fisher's Exact=18.4; *P*<0.05
		Serum	0/16	0/34	0/1	7/9	Fisher's Exact=19.2; *P*<0.05
	Kynurenines	CSF	0/16	0/34	0/1	7/10	Fisher's Exact=18.4; *P*<0.05
		Serum	0/16	0/34	0/1	7/9	Fisher's Exact=19.2; *P*<0.05
Taking/not taking VitB	Monoamines	CSF	0/16	7/27	0/1	5/12	Fisher's Exact=6.1; *P*>0.05
		Serum	0/16	7/27	0/1	5/11	Fisher's Exact=6.5; *P*>0.05
	Kynurenines	CSF	0/16	7/27	0/1	5/12	Fisher's Exact=6.1; *P*>0.05
		Serum	0/16	7/27	0/1	5/11	Fisher's Exact=6.5; *P*>0.05

Abbreviations: ALS: amyotrophic lateral sclerosis; CONTR: control; CSF: cerebrospinal fluid; FTD: frontotemporal dementia; FTD-ALS: frontotemporal dementia – amyotrophic lateral sclerosis; VitB: vitamin B.

Supplementary Table 2. Concentrations of statistically significant monoamines/ kynurenines, metabolites and ratios.

Parameter	CONTR	FTD	FTD-ALS	ALS	Test statistic
CSF MHPG (ng/mL)	14.2 (7.7)[a,b] n=26	21.0 (13.2)[a] n=39	25.3 (26.5) n=4	19.5 (7.8)[b] n=23	[a]U=298.0 *P*<0.05 [b]U=190.0 *P*<0.05
CSF DOPAC (ng/mL)	1.9 (1.6)[a,b] n=26	1.4 (0.6)[a] n=39	1.0 (8.8) n=4	1.2 (0.4)[b] n=23	[a]U=307.5 *P*<0.05 [b]U=173.0 *P*<0.05
CSF DA (ng/mL)	0.4 (0.1)[a] n=21	0.5 (0.1)[a,c] n=35	0.5 n=2	0.4 (0.2)[c] n=17	[a]U=136.0 *P*<0.0001 [c]U=161.5 *P*<0.05
CSF DOPAC/DA	4.2 (5.4)[a] n=21	2.8 (1.6)[a] n=35	1.7 n=2	3.2 (3.7) n=17	[a]U = 167.0 *P*≤0.001
CSF HVA/DA	101.2 (75.6)[a] n=21	72.1 (93.3)[a] n=35	44.4 n=2	106.8 (59.5) n=17	[a]U=221.0 *P*<0.05
Serum DOPAC (ng/mL)	6.9 (4.2)[a,b] n=26	3.8 (1.5)[a] n=39	3.4 n=3	4.0 (1.3)[b] n=20	[a]U=265.0 *P*<0.05 [b]U=140.0 *P*≤0.001
Serum DA (ng/mL)	0.9 (0.3)[a,b] n=26	1.1 (0.3)[a] n=35	1.0 n=3	1.1 (0.2)[a,b] n=20	[a]U=200.5 *P*<0.001 [b]U=115.5 *P*≤0.001
Serum DOPAC/DA	6.9 (7.1)[a,b] n=26	3.6 (1.8)[a] n=35	3.2 n=3	3.8 (1.0)[b] n=20	[a]U=181.0 *P*<0.001 [b]U=100.0 *P*<0.001
Serum HK/XA	4.1 (4.8) n=25	5.0 (5.4)[c] n=39	4.3 n=3	4.7 (2.5)[c] n=19	[c]U=207.0 *P*<0.05

Data are represented as median with interquartile range between brackets. Differences in monoamines, metabolites or ratios between disease groups remaining statistically significant after Benjamini-Hochberg correction, are depicted by superscript letters a, b and c for CONTR versus FTD, CONTR versus ALS and FTD versus ALS, respectively. Abbreviations: ALS: amyotrophic lateral sclerosis; CONTR: control; CSF: cerebrospinal fluid; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; FTD: frontotemporal dementia; FTD-ALS: frontotemporal dementia – amyotrophic lateral sclerosis; HK: 3-hydroxykynurenine; HVA: homovanillic acid; XA: xanthurenic acid.

Chapter IV.4. Serotonergic dysfunction in amyotrophic lateral sclerosis and Parkinson's disease: similar mechanisms, dissimilar outcomes

Yannick Vermeiren[a,b], Jana Janssens[a,b], Debby Van Dam[a,b], Peter P. De Deyn[a,b,c]

[a]*Department of Biomedical Sciences, Laboratory of Neurochemistry and Behavior, Institute Born-Bunge, University of Antwerp, Wilrijk (Antwerp), Belgium*
[b]*Department of Neurology and Alzheimer Center, University of Groningen and University Medical Center Groningen (UMCG), Groningen, Netherlands*
[c]*Department of Neurology, Memory Clinic of Hospital Network Antwerp (ZNA) Middelheim and Hoge Beuken, Antwerp, Belgium*

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) share similar pathophysiological mechanisms. From a neurochemical point of view, the serotonin (5-hydroxytryptamine; 5-HT) dysfunction in both movement disorders – related to probable lesioning of the raphe nuclei – is profound, and, therefore, may be partially responsible for motor as well as non-motor disturbances. More specifically, in ALS, it has been hypothesized that serotonergic denervation leads to loss of its inhibitory control on glutamate release, resulting into glutamate-induced neurotoxicity in lower and/or upper motor neurons, combined with a detrimental decrease of its facilitatory effects on glutamatergic motor neuron excitation. Both events then may eventually give rise to the well-known clinical motor phenotype. Similarly, disruption of the organized serotonergic control on complex mesencephalic dopaminergic connections between basal ganglia (BG) nuclei and across the BG-cortico-thalamic circuits, has shown to be closely involved in the onset of parkinsonian symptoms. Levodopa (L-DOPA) therapy in PD largely seems to confirm the influential role of 5-HT, since serotonergic rather than dopaminergic projections release L-DOPA-derived dopamine, particularly in extrastriatal regions, emphasizing the strongly interwoven interactions between both monoamine systems.

Apart from its orchestrating function, the 5-HT system also exerts neuroprotective and anti-inflammatory effects. In line with this observation, emerging therapies have recently focused on boosting the serotonergic system in ALS and PD, which may provide novel rationale for treating these devastating conditions both on the disease-modifying, as well as symptomatic level.

1. BACKGROUND

The neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) is produced in the raphe nuclei (RN), a moderately sized cluster of caudal and rostral neurons (B1-B9) found in the brainstem [425]. Axons arising from the caudal group (B1-B4) form a descending system projecting to the spinal cord, cerebellum, pontine and midbrain structures, whereas ascending fibers emanating from the more rostral clusters (B5-B9) connect with the cerebral cortex, (hypo)thalamus, basal ganglia and hippocampus among others. About roughly 300,000 5-HT-containing neurons in the human brain bear a tremendous number of collateral branches so that the serotonergic system densely innervates nearly all brain regions [426]. It is, therefore, not surprising that this extensive neuronal network is implicated in the regulation of numerous physiological events, such as hormone secretion, sleep-wake cycle, motor control, immune system functioning, nociception, food intake, energy balance/metabolism, cardiovascular/respiratory functioning, body temperature, affect/aggression, consciousness, learning and memory [427, 428]. Serotonin receptors are subdivided into seven families (5-HT_{1-7}), based on structural, biochemical and pharmacological characteristics, resulting into 14 subtypes (5-$HT_{1A/1B/1D/1e/1F}$, 5-$HT_{2A/2B/2C}$, 5-HT_3, 5-HT_4, 5-$HT_{5A/5b}$, 5-HT_6 and 5-HT_7). With the sole exception of 5-HT_3, which belongs to the ligand gated ion channels, all 5-HT receptors are G protein-coupled receptors, mediating a variety of physiological and behavioral functions [429]. Regarding amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) pathophysiology, especially 5-$HT_{1A/1B}$ and 5-$HT_{2A/2B/2C}$ seem crucial [430-433]. In short, the 5-HT_{1A} receptor is expressed in the RN as a presynaptic autoreceptor, while it also functions as a postsynaptic heteroreceptor in areas of the limbic system, such as the prefrontal cortex, hippocampus, lateral septum, and amygdala, as well as in (hypo)thalamus, and, basal ganglia [434]. Activation of 5-HT_{1A} autoreceptors on the cell bodies or dendrites of the RN neurons exerts inhibitory feedback in response to local 5-HT release. The 5-HT_{1B} receptors are centered on axonal terminals of (non)serotonergic neurons, mainly found in the basal ganglia and substantia nigra (SN) [435]. Interestingly, it has been indicated that 5-HT_{1B} receptors are preferentially located on presynaptic terminals of ɣ-amino-butyric acid (GABA)ergic neurons, and, it has also been suggested that thalamostriatal and/or corticostriatal glutamatergic neurons express presynaptic 5-HT_{1B} receptors [436]. In contrast, the 5-HT_{2A} receptor is found mainly in the periphery and neocortical areas, where they are implicated in the pathogenesis of schizophrenia and hallucinations [129, 437]. These receptors are highly expressed in both pyramidal cells and GABAergic interneurons. Moreover, cerebral 5-HT_{2B} receptors are present in the cerebellum, cerebral cortex, hypothalamus, corpus callosum and amygdala, causing anxiolytic effects among others [438]. Noteworthy, this receptor subtype is likely to be expressed by RN neurons, where this autoreceptor might play a role in the regulation of the serotonin transporter (SERT) [439]. Next, the 5-HT_{2C} subtype

is widely distributed throughout the brain and has been proposed as the main mediator of the different actions of 5-HT in the central nervous system (CNS) [129]. Additionally, 5-HT_{2C} receptors are commonly found in the choroid plexus, where they modulate cerebrospinal fluid (CSF) production [440].
The serotonergic system is organized in such a way that it exerts widespread effects on targeted neurons, such as motor neuron excitability threshold control, and interacts with many other neurotransmitters, including dopamine (DA), noradrenaline (NA), glutamate, GABA and various peptides [427]. Remarkably, 5-HT also plays an important part in the development of the embryonic nervous system, which relates to neurite outgrowth and other aspects of neuronal differentiation, including synaptogenesis [441]. Given its complex but critical modulating characteristics, 5-HT can be regarded as one of the principal orchestrators of the CNS, with a very significant role in motor activity. In PD and ALS, two invariably fatal neurodegenerative conditions, the motor and non-motor features have been partially attributed to disease-related malfunctioning of this overseeing neurotransmitter system.

2. SEROTONERGIC DEGENERATION IN PD AND ALS

Staging of brain pathology in PD demonstrated an early involvement of Lewy body depositions within the RN. In more detail, Halliday et al. (1990) [442] firstly described a 56% loss of serotonergic neurons in the median RN of PD compared to control brain. Afterwards, Braak et al. (2003) [56] determined six stages in the evolution of PD-related pathology, with lesions being present in the median RN in the caudal brainstem already from stage two onwards. Furthermore, 5-HT depletion was observed in various target areas of the RN, such as in the basal ganglia, hypothalamus, hippocampus and prefrontal cortex [443, 444]. This was later confirmed by in vivo imaging studies, revealing new insights. For instance, Politis et al. (2010) [445] applied ^{11}C-DASB-PET to early-stage PD patients, and demonstrated reduced SERT binding in the caudate nucleus, (hypo)thalamus, and anterior cingulate cortex, whereas PD subjects with established disease showed additional ^{11}C-DASB binding reductions in the putamen, insula, posterior cingulate cortex, and, prefrontal cortex. Further binding reductions were noticed in the ventral striatum, RN, and amygdala of advanced PD patients. Interestingly, the loss of SERT binding in the RN occurred in later stages, pointing to an earlier loss of serotonergic projections instead of the neurones themselves.
In ALS, distribution patterns of transactive response DNA-binding protein (TDP)-43 intraneuronal inclusions have only recently been investigated, summing up into a total of four discriminative neuropathological stages [69]. Notably, it has been theorized that sites with projections to the cortex remain intact in ALS, unlike those receiving corticofugal axonal projections, supporting the hypothesis of prion-like propagation of TDP-43, potentially from the motor cortex downwards (dying forward/back hypotheses, Figure 1). In agreement, the upper RN with diffuse cortical projections barely become affected by TDP-43 pathology in ALS, which is

in great contrast with PD or Alzheimer's disease (AD) [69]. Nevertheless, a marked reduction in both cortical and RN 5-HT_{1A} receptor binding (21%) has been observed [446], and, several studies previously evidenced decreased levels of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA; main metabolite of 5-HT) or tryptophan (precursor of 5-HT) in CSF, plasma and/or spinal cord [447-450]. Platelet 5-HT levels also positively correlated with survival in ALS subjects [451]. Consequently, it has been postulated that 5-$HT_{1A/2}$ receptor (anta)agonists, 5-HT precursors (e.g. 5-hydroxytryptophan (5-HTP)) [452] or 5-$HT_{2B/C}$ receptor inverse agonists [453] might improve locomotor function and even strategically interfere with ALS disease course. On the whole, the serotonergic theory in ALS has gained renewed interest especially due to several recent publications [433, 453, 454].

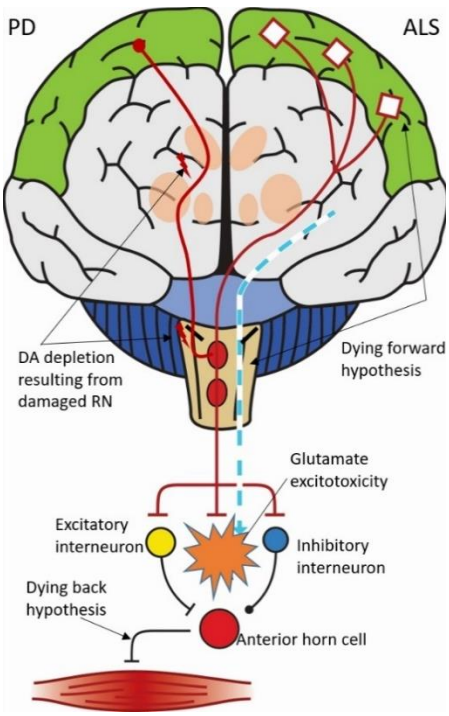

Figure 1. Schematic representation of dysfunctional serotonergic pathway interactions in ALS and PD, mediated by lesioned raphe nuclei (RN) centered in the brainstem. In summary, serotonergic loss in amyotrophic lateral sclerosis (ALS) brain and subsequent loss of its inhibitory control on glutamate release cause glutamate-induced excitotoxicity leading to upper/lower motor neuron damage. In this regard, the dying forward hypothesis proposes that ALS is a disorder primarily of the corticomotoneurons, with anterior horn cell degeneration propagated via an anterograde glutamate-dependent excitotoxic process. In contrast, the dying back hypothesis proposes that ALS begins within the muscle or neuromuscular junction, with pathogens being retrogradely transported from the neuromuscular junction to the cell body where they may exert their deleterious effects. Simultaneously, this figure illustrates the pathophysiological serotonergic-dopaminergic interactions on the striatal level in Parkinson's disease (PD), where lesioning of the RN (red spheres) in addition to dopamine (DA) depletion in the striatum and substantia nigra (black-bolded dashes) give rise to a net decreased activity of the motor cortex. Adapted from Vucic et al. (2013), ©2013 with permission from BMJ Publishing Group Ltd.

3. 5-HT AND THE CONTROL OF MOTOR NEURON EXCITABILITY: POSSIBLE IMPLICATIONS

The indolamine 5-HT has facilitatory effects on glutamatergic motor neuron excitation by augmenting weak or polysynaptic inputs, bringing motor neurons to threshold. This effect on spinal motor neurons is exerted through 5-$HT_{1/2}$ receptors (reviewed in [428]). In ALS, serotonergic denervation has been hypothesized to lead to significant loss of inhibitory control on glutamate release, via decreased binding on presynaptic 5-HT_{1B} receptors, triggering glutamate-induced

neurotoxicity, and, eventually, rapid-onset loss of upper and lower motor neurons [455]. Upper motor neurons are glutamatergic neurons located in layer V of the motor cortex, project to spinal motor neurons through the corticospinal tract, and are the major source of descending motor commands for voluntary movement [456]. Meanwhile, progressive degeneration of 5-HT neurons in the motor cortex, RN and their projections may lead to a compensatory increase in glutamate excitation [449], adding up to the clinical motor phenotype (Figure 1). Conversely, motor neuron groups such as the oculomotor, trochlear and abducens nuclei, and the cerebellum, which only receive sparse serotonergic innervation, appear more resistant to the process of neurodegeneration in ALS. Moreover, it is possible that differences between bulbar and spinal ALS in the course of the disease may be related to the degree of cerebral 5-HT depletion, which seems more extensive in the bulbar subtype [446]. Additionally, 5-HT is a precursor of melatonin, which inhibits glutamate release and glutamate-induced neurotoxicity [457].

One of the possible implications of serotonergic degeneration with regard to motor symptoms in ALS, is spasticity [453, 454]. For instance, El Oussini and colleagues (2017) [454] recently demonstrated that degeneration of brainstem 5-HT neurons in transgenic SOD1 (G37R) mice, more particularly the dorsal and median RN, induced spasticity. This hyperreflexia is able to compensate for motor deficits, allowing the maintenance of motor function after disease onset. Spasticity is a painful symptom which can severely restrict quality of life on a daily basis. Remarkably, SB206553 administration, a 5-$HT_{2B/C}$ receptor inverse agonist, completely abolished spasticity symptoms [454, 458]. The authors further stress that selective degeneration of the RN might directly lead to motor neuron hyperexcitability and spasticity – rather than degeneration of upper motor neurons in the cerebral motor cortex.

4. RELATED TREATMENT OPTIONS

So far, riluzole and edaravone – of which latter drug has recently been FDA-licensed in the US and Japan [459] – only modestly improve motor symptoms and daily functioning in ALS patients, but with a reasonable safety profile of riluzole 100 mg daily [387]. However, the treatment regimen for edaravone is inconvenient and costly [459]. Riluzole acts as an N-methyl-D-aspartate (NMDA)-receptor antagonist, whereas edaravone is a free radical scavenger. Both mechanisms of action thus support the glutamate excitotoxicity-driven hypothesis.

As for clinical trials with serotonergic therapies, Meiniger et al. (2004) [460] carried out two randomized, double-blind, placebo-controlled multicenter studies (phase III) with xaliproden, a 5-HT_{1A} receptor agonist which has neurotrophic and neuroprotective effects, to assess its safety and efficacy at two doses. ALS patients were randomly assigned to placebo, 1 mg or 2 mg xaliproden orally once daily as a monotherapy (867 patients), or, to the same regimen with addition of riluzole 50 mg (1210 patients). In the end, however, the primary outcome measures (time to

death, tracheostomy, or permanent assisted ventilation) did not reach statistical significance. There only was a therapeutic benefit on the second outcome measure, i.e. vital capacity (maximum volume of air exhaled at a steady state after maximum inhalation of a single breath) at the 1 mg and 2 mg dose without riluzole, which let the authors conclude that xaliproden does not effectively slow down disease progression. In short, strong evidence is currently lacking and insufficient regarding the potential benefits of serotonergic therapies in ALS, despite of its remarkable 5-HT-related pathophysiological characteristics described above.
Finally, monoamine oxidase-B (MAO-B) inhibitors such as deprenyl, rasagiline or selegiline affect the release and increase the content of not only DA and NA, but also of 5-HT (reviewed in [461]). MAO-B inhibitors also have neuroprotective properties. Following the introduction of rasagiline to the therapeutic armamentarium for PD, various successes have been reported [462, 463]. In ALS, results are less consistent, with selegiline treatment having no significant effect on the rate of clinical progression or outcome in ALS as evidenced by Lange et al. (1998) [464], whereas deprenyl and rasagiline seem more promising, but necessitate further scrutiny [465, 466].

5. SEROTONERGIC MODULATION OF BASAL GANGLIA AND MESENCEPHALIC DOPAMINERGIC ACTIVITY IN PD

The basal ganglia (BG) are composed of the striatum (caudate nucleus and putamen), subthalamic nucleus (STN), internal and external globus pallidus (GPi/e) and SN, and are part of the BG-cortico-thalamic circuits. This highly organized network is important for motor control, emotion and cognition. It has been firmly established that BG nuclei receive vast serotonergic input mainly coming from the rostral RN clusters (B7), with effects on mesencephalic dopaminergic activity depending on the specific nucleus and its receptor distribution (excitatory via $5\text{-HT}_{1A/1B/2A/3/4/7}$ and inhibitory via $5\text{-HT}_{2C/6}$ receptors [432, 467, 468]. In PD, lesioning of the RN in addition to DA depletion in the striatum and SN – particularly of the pars compacta (SNpc) – are hallmarks of the disease, leading to overactivation of the output regions of the BG, i.e. GPi and SN pars reticulata (SNpr), which contain large GABAergic neurons. This cascade results in a net decreased activity of the supplementary motor areas, premotor and primary motor cortices, triggering parkinsonian symptoms [469] (Figure 1). Overall, the loss of 5-HT neurons is not as profound as the loss of DA neurons, and may not be sufficient to cause motor or non-motor symptoms per se, however, both systems closely interact, and combined depletion certainly seems to aggravate the situation, as was shown in a parkinsonian rat model [470]. Moreover, 5-HT and 5-HIAA levels, as well as SERT expression, are reduced in various BG nuclei [471-473], and the serotonergic system is strongly involved in the mechanism of action of antiparkinsonian therapeutics, such as levodopa (L-DOPA), and high frequency stimulation of the STN [474].

6. L-DOPA ACTIONS VIA SEROTONERGIC NERVE TERMINALS IN PD: THE INFLUENTIAL EFFECT OF 5-HT

Levodopa, the metabolic precursor of DA, is a well-established symptomatic treatment for the motor deficits in PD. Paradoxically, L-DOPA-induced dyskinesia (LID), as well as hallucinations, are unfortunate but more or less inevitable corollaries of its long-term administration [475]. Despite the traditional assumption that L-DOPA is transformed in residual striatal dopaminergic neurons into DA, interestingly, a more important role for serotonergic than dopaminergic projections has been identified for the increase of extracellular DA, predominantly in prefrontal cortex, nucleus accumbens, STN, hippocampus and additional extrastriatal regions [475]. Briefly, 5-HT neurons convert exogenous L-DOPA into DA and store neo-synthesized DA into vesicles for exocytosis via vesicular monoamine transporter 2, as was originally shown in rats [476]. Since the distribution of 5-HT terminals in the brain is very different from dopaminergic innervation, the magnitude of effect in extrastriatal regions is tremendous compared to physiological conditions, especially at low L-DOPA doses, so that 5-HT in fact controls the dopaminergic output in a state and region-dependent manner [474].

Latter phenomenon has even led to the assumption that future envisaged pharmacotherapeutic strategies to treat LID should specifically aim at controlling L-DOPA-stimulated DA release from extrastriatal 5-HT neurons [468, 475]. Recently, the use of 5-HTP [477] or 5-$HT_{1A/B}$ receptor agonists (e.g. eltoprazine or buspirone) [475, 478] – influencing DA release indirectly via action on the overall 5-HT tone – has been proposed. As for exacerbation of psychosis by L-DOPA treatment – attributed to excessive DA release in the mesolimbic areas rather than the motor striatum, mediated by hypersensitive 5-HT signalling – a favourable role for 5-HT_{2A} receptor inverse agonists (e.g. pimavanserin) or 5-HT_{2A} antagonists (e.g. low doses of clozapine) has likewise been demonstrated [430]. These findings suggest that the serotonergic system may even adapt to the lack of DA by adopting anatomical and functional transformations in PD.

7. ALTERATIONS IN OTHER MONOAMINE NEUROTRANSMITTER SYSTEMS

NA levels have been previously reported to be significantly increased in the cervical, thoracic and lumbar spinal cord of ALS patients compared to controls [449], with highest concentrations measured in ventral and intermediate grey matter. In CSF, a similar increase has been noted [479]. Independently of 5-HT, NA increases the excitability of motor neurons to glutamate [480]. Bertel et al. (1991) [449] further discussed that in all probability, it is unlikely that the noradrenergic changes were due to the effect of tissue shrinkage – since concentrations were expressed in ng/g wet weighed tissue – but rather a consequence of denser

noradrenergic (neosympathetic) innervation, such as from sprouting of noradrenergic fibers into affected areas. In PD, the noradrenergic dysfunction has been investigated in more detail. In summary, α-synuclein depositions in the locus coeruleus (stage 2), the brain's main source of NA, have been evidenced to precede that of the SN (stage 3) [56]. Consequently, neuronal loss in this noradrenergic nucleus and the accompanying noradrenergic deficiency both on the central and peripheral level have been related to various motor and non-motor (cognitive) symptoms, including the progression to (prodromal) dementia [481].
A potential dopaminergic deficit in ALS has only been scarcely investigated, with significantly reduced striatal DA transporter expression in patients with bulbar- or limb-onset compared to controls (^{123}I-IPT-SPECT) [413], while in drug-naïve, sporadic ALS patients, decreased striatal D2-receptor binding could be partially reversed by riluzole [482]. On the contrary, no differences in spinal DA concentrations were found between ALS and control subjects [449]. No research has been performed yet with regard to 5-HT-DA interactions in mesencephalic brain areas or BG nuclei, but a study by Xu and colleagues [483] observed an abnormal cortical-BG network in ALS after applying resting state fMRI and voxel-wise network analysis.

8. THE NON-MOTOR OUTCOMES OF SEROTONERGIC DYSFUNCTION IN ALS AND PD

New findings point at an important link between non-linear progressive degeneration of serotonergic terminals and non-motor disturbances in PD, such as depression, fatigue, weight loss, and anxiety [484, 485]. Similarly, cognitive impairment and dementia are major issues in PD, and might be ascribed to serotonergic dysfunction too [485]. In this respect, a phase 2 trial is currently evaluating the safety, tolerability and efficacy of SYN120, a dual 5-HT_6/5-HT_{2A} antagonist, in 80 PD dementia patients over a 16-week period (SYNAPSE; NCT02258152 (clinicaltrials.gov)). As for ALS, fatigue and abnormal peripheral glucose metabolism have been suggested [486]. Major depressive disorder, in which an approximate 12% reduction of cortical 5-HT_{1A} binding is seen in non-ALS cases [487], is relatively rare in ALS patients, even in later stages [488]. More recently, Vercruysse and colleagues (2016) [489] indicated that serotonergic axonal loss in the arcuate nucleus of the hypothalamus in combination with decreased hypothalamic 5-HT levels primarily caused a melanocortin deficit in mutant SOD1 (G86R) mice, which contributed to dysregulated food intake/weight loss.
Furthermore, self-referential thinking (i.e. reflecting one's mental self) is a key cognitive process which seems to be regulated by 5-HT_{1A} receptors within the default mode network, which comprises the precuneus, posterior cingulate cortex, medial prefrontal cortex, and, the temporoparietal junction [490]. In this regard, Fomina et al. (2017) [491] observed electroencephalography correlates

(bandpower) of self-referential thinking in the medial prefrontal cortex of healthy individuals, but not ALS patients. The authors concluded that these cognitive abnormalities, such as anosognosia, may well be in compliance with the proposed serotonergic theory in ALS.

9. (DIS)SIMILARITIES: 5-HT AS A CRUCIAL DISEASE MODIFIER

The process of normal, healthy ageing has complex effects on central and peripheral serotonergic transmission. Accumulating (pre)clinical evidence suggests a linear and gradual decline of 5-HT connections from the RN, as well as altered SERT and 5-$HT_{1A/2A}$ receptor expressions in multiple brain regions [198]. However, in ALS and PD, RN neuronal loss and/or loss of serotonergic projections due to marked and early TDP-43 and α-synuclein depositions in target areas might cause major imbalance in monoaminergic neurotransmission across the brain [446, 453, 484], accounting for numerous motor, behavioral and cognitive dysfunctions.

The neurochemical similarity between ALS and PD, is that in both conditions, the supervising but damaged serotonergic system has lost pre- and postsynaptic regulatory functions on neighboring systems, leading to loss of inhibitory control of glutamate release and loss of facilitatory effects of glutamatergic motor neuron excitation in ALS, whereas in PD, this results in alterations of the complex serotonergic modulation of mesencephalic dopaminergic systems. Maybe unexpectedly, this largely and selectively affects upper and lower motor neurons in ALS brain and spinal cord, causing neuromuscular disease, while in PD, the effects rather remain central, i.e. at the level of the SNpc/pr, BG nuclei and (extra)striatal regions. Serotonergic alterations in ALS brain and RN have been found before [446], but the overall outcome of the serotonergic shortage on the corticocerebral level remains to be elucidated. In addition, autonomic and olfactory dysfunction in PD have been ascribed to peripheral noradrenergic alterations, potentially resulting from LC lesioning, and, likely, far preceding the motor deficits [481]. In contrast, there is a fairly dissimilar clinical outcome for both neurodegenerative diseases, with more 5-HT-associated non-motor disturbances in PD versus a very typical motor – but less non-motor – region-dependent degenerative pattern in ALS, causing the well-characterized limb- or bulbar-onset phenotype (Figure 2).

Another very important peculiarity of 5-HT, which underscores its disease-influencing potential in ALS and PD, is its neuroprotective role through controlling energy homeostasis via still incompletely characterized mechanisms [492]. As such, new preclinical studies are emerging, which have already shown that 5-$HT_{1A/2B}$ receptor stimulation on astrocytes and microglia promotes proliferation and upregulation of antioxidative molecules, slowing down or even reversing the disease process in ALS [433], and protecting dopaminergic neurons in PD [431].

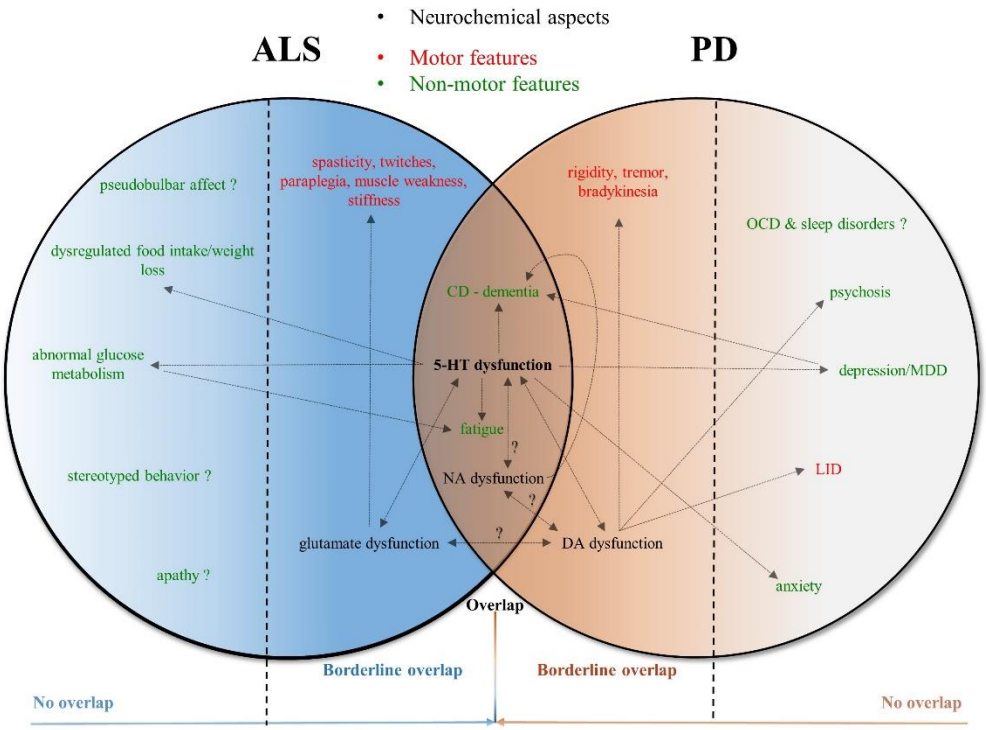

Figure 2. Venn diagram—visualization of the complex interplay of neurochemical and clinical keystones in ALS and PD. This figure depicts the complex interplay between neurochemical characteristics in ALS and PD related to their disease-specific clinical (i.e., motor and non-motor) outcome. Mutually influential and/or synergistic interactions are indicated with arrowheads at both ends. Question marks over the arrows refer to partially-proven or suggestive mechanisms of neurochemical features. The Venn diagram clearly shows that serotonergic dysfunction is the central overlap in the overall pathophysiology of ALS and PD, whereas the neurochemical causes of the clinical non-motor (i.e., behavioral) disturbances, particularly in ALS, necessitate further scrutiny. Similarly, the reciprocal interaction between the noradrenergic and serotonergic/dopaminergic disturbances in ALS and PD remains to be elucidated, even though in PD, NA dysfunction has been previously linked with CD and dementia progression. Abbreviations: 5-HT: serotonin (5-hydroxytryptamine); ALS: amyotrophic lateral sclerosis; CD: cognitive deterioration; DA: dopamine; LID: levodopa-induced dyskinesia; MDD: major depressive disorder; NA: noradrenaline; OCD: obsessive-compulsive disorder; PD: Parkinson's disease.

10. WHY IT MATTERS?

So far, there is total absence of easily-accessible biological markers in CSF or blood for ALS or PD, rendering the diagnosis of both disease entities sometimes fairly complex, laborious and challenging. Accordingly, the differential diagnosis among similar syndromes, including progressive supranuclear palsy, multiple system atrophy or corticobasal degeneration, may be quite difficult. Future studies should, therefore, focus on the serotonergic dysfunction in ALS and PD, and reveal if serotonergic markers alone or in combination with other biological factors, such as

the LDL/HDL ratio, plasma ApoE, or various neuroinflammatory compounds [451], could be useful for routine diagnostic work-up of patients in clinics.
Additionally, serotonergic approaches in ALS and PD may alleviate disease burden on both the motor and non-motor level, and may hold great potential to influence the disease course, even though clinical trials with 5-HT modulating agents are currently scarce. Hypothetically, other neurodegenerative disorders, such as AD, dementia with Lewy bodies or PD plus syndromes, could – at least in part – share a fundamentally-alike monoaminergic pathophysiology, promoted by very early protein depositions in strategic brainstem nuclei. One might, therefore, wonder whether the universal quest for efficient symptomatic and disease-modifying therapies might, perhaps, be narrowed down to a monoaminergic-based derivative.

Chapter V

Expression of G protein-coupled receptors in Alzheimer's disease

Chapter V.1. Examining neurotransmission in Alzheimer's disease: disturbances in G protein-coupled receptor expression and their correlation with monoaminergic compounds

Compiled results of several research projects within the Laboratory of Neurochemistry and Behavior, and in collaboration with prof dr. María Ramírez (Department of Pharmacology and Toxicology, University of Navarra, Pamplona, Spain).

ABSTRACT

We mapped neurochemical disturbances with respect to G protein-coupled receptors (GPCRs) and neurotransmitter concentrations in postmortem brain material of patients suffering from Alzheimer's disease (AD) and the APP23 amyloidosis mouse model for this condition. With this work, we aimed to contribute to the knowledge of the involvement of certain GPCRs in AD, and aid in the identification of a novel research target.

The human study population consisted of clinically well-characterized and neuropathologically confirmed AD patients (n = 42) and age-matched control subjects (CONTR) (n = 18). Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and high-performance liquid chromatography were performed on Brodmann area (BA) 7, BA10, BA22, BA24, hippocampus, amygdala, thalamus and cerebellum to measure mRNA levels of serotonin (5-hydroxytryptamine; 5-HT) receptor 1A (*HTR1A*), serotonin receptor 7 (*HTR7*) and β-adrenergic receptor 3 (*ADRB3*), as well as the concentrations of various monoamine neurotransmitters and their metabolites. RT-qPCR was also performed on hippocampal tissue of wild-type (WT) and APP23 hemizygous (HEM) mice aged 6-8 weeks, 3-, 6-, 12-, 18-, and 24-months to investigate expression levels of muscarinic acetylcholine receptor 1 (*Chrm1*) and metabotropic glutamate receptor 5 (*Grm5*). Western blot was performed to assess protein expression levels of serotonin receptor 6 (5-HT_6) in hippocampus of the APP23 model, and qualitative double-labelling immunofluorescence was attempted to investigate the co-occurrence of this receptor with neuronal, astrocytic and microglial markers.

We found a general serotonergic disturbance in AD, characterized by upregulated *HTR1A* expression levels in hippocampus and amygdala and decreased hippocampal 5-HT concentrations. In addition, decreased levels of *HTR7* mRNA were observed in BA10, whereas hippocampal *ADRB3* expression was increased in AD versus CONTR. An inverse correlation was found between hippocampal *HTR1A* levels and the presence of anxieties and phobias in AD. These findings might indicate an increase in presynaptic autoreceptors, resulting in lowered 5-HT levels, and a protective role of 5-HT_{1A} against anxiety. *Chrm1* and *Grm5* showed age-dependent mRNA expression levels, and only differed between WT and HEM animals at the age of 6 months, while no age- or genotype effects could be identified for 5-HT_6 protein expression. Qualitative immunofluorescence analyses indicated the location of 5-HT_6 on neuronal hippocampal cells.
In general, we recommend future research to focus on techniques investigating receptor-ligand functionality, and its implication in cognition and behavior.

1. INTRODUCTION

Previous chapters in this work have focused predominantly on neurotransmitter levels in brain tissue and biological fluids, although expression levels of the corresponding neurotransmitter receptors, are equally (or even more) important. We therefore investigated mRNA expression levels of the metabotropic glutamate receptor 5 (*Grm5*) and muscarinergic acetylcholine (ACh) receptor 1 (*Chrm1*) in hippocampal tissue of the APP23 amyloidosis mouse model for Alzheimer's disease (AD). In frontal, temporal, parietal and limbic structures, as well as thalamus and cerebellum of patients suffering from AD and healthy control subjects (CONTR), mRNA levels of the serotonin (5-hydroxytryptamine, 5-HT) receptor 1A (*HTR1A*), 5-HT receptor 7 (*HTR7*) and the β_3-adrenoceptor (*ADRB3*) were analyzed. Lastly, protein expression of the 5-HT receptor 6 (5-HT_6) was analyzed in hippocampus of the APP23 model using western blot and double-labelling immunofluorescence. In this introductory section, the implication of abovementioned G protein-coupled receptors (GPCRs) in AD will be discussed.

Firstly, considerable evidence has been collected on the involvement of disturbed cholinergic neurotransmission, including muscarinic ACh receptors (M receptors), in the pathological mechanisms underlying AD. Activation of the predominantly postsynaptic M1, which signals via Gαq/11 proteins and subsequent activation of phospholipase C [493-496], restores cognition and attenuates AD-like pathology in distinct animal models [497]. Xanomeline, an agonist of the M1/M4 receptors, was reported to exert positive effects on cognition and various neuropsychiatric symptoms in AD, although its limited tolerability precluded further clinical use [498]. Tau phosphorylation in transfected PC12 cells was also decreased by activation of M1 [499], which would comply with the amelioration of cognitive functioning [500, 501] upon stimulation of this GPCR. In addition, activation of M1 promotes the production of the neuroprotective soluble APPα (sAPPα), while amyloid-β (Aβ) formation is inhibited [502, 503]. Conversely, Aβ is hypothesized to disrupt intracellular signaling of the M1, resulting in a positive feedback loop promoting the production of Aβ [504, 505], while the levels of *Chrm1* have been reported to remain largely unaltered [504, 506, 507], although some authors also report increased [508] or decreased [509] levels of M1 in AD compared to CONTR.

Metabotropic glutamate (Glu) receptors (mGluRs) are known for their role in learning and memory, including synaptic plasticity. In AD brain, pyramidal glutamatergic neurotransmission is severely disrupted in the hippocampus and several areas of the neocortex implicated in cognition-related processes [510]. Since targeting N-methyl-D-aspartate (NMDA) receptors in an attempt to mediate glutamate excitotoxicity results in an overall impaired fast excitatory

neurotransmission, sedation and ataxia [511-513], these ionotropic receptors do not represent an interesting target for long-term therapy. Conversely, the metabotropic glutamate receptor 5 (mGluR5) is coupled to Gαq/11 proteins [514] and is mainly located in the postsynaptic membrane [515, 516], where it is postulated to interact with and modulate NMDA receptors [517-519]. Furthermore, soluble Aβ oligomers were found to interact with cellular prion protein, which led to synapse loss via the physical interaction with mGluR5 and the activation of Fyn kinase [520]. In this study, antagonism of mGluR5 resulted in restored synapse density, and associated improvement in learning and memory in familial AD (FAD) transgenic mice. Genetic ablation of *Grm5* in the APPswe/PS1ΔE9 mouse model was found to rescue spatial learning deficits in the Morris water maze [521]. In autoradiography experiments, a higher binding of [^{18}F]PSS232 to mGluR5 was observed in frontal cortex and hippocampus of AD subjects compared to CONTR [522]. In other studies, increased expression of mGluR5 was confirmed in postmortem brain of aged subjects, as well as patients suffering from AD [523], and in Tg-ArcSwe mice [524]. These findings appear to fit within the hypothesis that pathological overexpression or -stimulation of mGluR5 leads to increased neurotoxicity mediated by NMDA receptors.

Although ACh and Glu are perhaps the most widely reported neurotransmitters to be disturbed in AD, 5-HT also plays an important role in behavior and cognition, possibly via modulation of cholinergic, glutamatergic, dopaminergic and GABA (γ-aminobutyric acid)-ergic neurotransmission [198, 525]. Previous reports have observed decreased 5-HT levels in AD brain [143, 526], correlating with cognitive performance [526]. Further (pre)clinical evidence also points to the relation between reduced serotonergic neurotransmission and cognitive deficits (reviewed by [198])

The first serotonergic receptor discussed here, is the 5-HT$_{1A}$ receptor, which transduces intracellular signals via Gi/o [527, 528], and thus inhibits the formation of cAMP. Both postsynaptic and presynaptic 5-HT$_{1A}$ receptors are present in the brain, although presynaptic autoreceptors are predominantly expressed in raphe nuclei neurons [529, 530]. Since 5-HT$_{1A}$ is expressed on cholinergic neurons in the medial septal regions and diagonal band nuclei [531], the brain regions representing an important source of cholinergic afferents to the hippocampus [532] and cortex [533], stimulation of this receptor results in reduced ACh release and impaired memory [534]. Alternatively, glutamatergic pyramidal cells in the hippocampus and cortex also express 5-HT$_{1A}$ [535] and stimulation of this GPCR is thus hypothesized to interfere with excitatory events upon NMDA receptor activation [134, 534, 536]. Antagonism of 5-HT$_{1A}$ was associated with improved performance in the Morris water maze in rats treated with 8-OH-DPAT, a 5-HT$_{1A}$ agonist [537, 538]. Several other studies corroborated this finding [539-541]. A

vast amount of studies have investigated the expression of 5-HT_{1A} (reviewed in [198, 542]), but overall, a reduced density of 5-HT_{1A} (binding sites) is observed in various brain regions in AD versus CONTR brain [543, 544]. This marked reduction has also been associated with neuronal loss in the CA1 region of the hippocampus [543]. In this respect, 5-HT_{1A} might function as a marker of neuronal loss in AD.
Concerning 5-HT_6, a similar pattern of impaired learning and memory upon activation and improved spatial learning upon inhibition of this receptor is observed [545-547]. This GPCR is solely expressed postsynaptically in neuronal cilia [548], and leads to increased intracellular cAMP levels via coupling to adenylate cyclase through Gαs. An alternative signaling mechanism with coupling to Gαq/11 has also been described [549]. Furthermore, 5-HT_6 has been reported to interact with Fyn kinase, with activation of this receptor resulting in increased phosphorylation of tau [550, 551]. Since agonism and antagonism of 5-HT_6 both have been reported to modulate cholinergic and glutamatergic neurotransmission [551, 552], reduced 5-HT_6 levels observed in AD [553, 554], might represent a compensatory mechanism to increase ACh and Glu to normal concentrations.
Lastly, 5-HT_7 signals postsynaptically via Gαs or Gα12 [555-557]. Mainly preclinical evidence has suggested that this GPCR is involved in the regulation of thermoregulation, cognition and various behaviors including stress, circadian rhythmicity and depression (reviewed in [558, 559]). Possibly due to the differential transduction mechanisms of 5-HT_7, agonism and antagonism of this receptor can produce both deficits or improvement in cognition [558, 560]. This is even further complicated by the reported heterodimerization with 5-HT_{1A} [561], which may considerably alter signaling mechanisms under certain conditions [562]. Finally, to the best of our knowledge, no studies have reported AD-related changes in 5-HT_7 levels at the moment of writing.

The last GPCR discussed here, the adrenergic β_3 receptor, is positively coupled to adenylate cyclase via Gαs [563-565] and belongs to the noradrenergic neurotransmitter system, which is disturbed in AD as well (reviewed in [566]). Besides loss of noradrenergic neurons in the locus coeruleus [323, 356, 567-569], generally lowered noradrenaline (NA) levels and higher 3-methoxy-4-hydroxyhenylglycol (MHPG) concentrations were observed in various frontal, temporal and limbic structures in AD versus CONTR [143]. These results corroborated previous findings obtained by high-performance liquid chromatography experiments [323, 570, 571], but contradicted reports of unchanged or increased NA levels in AD [572-574]. However, β_3 has a prominent role in the regulation of metabolism in adipose tissues [575, 576] and cardiac function [577, 578]. In the CNS, this receptor controls appetite [579] and is involved in memory processes [93]. It was shown that a knock-out mouse model exhibited impairment in the novel object recognition and social discrimination tasks [580].

Conversely, agonism of β_3 in chicks infused with Aβ peptides, was able to rescue memory impairment in an avoidance discrimination task [581]. These findings seem to indicate that β_3 confers procognitive effects, although reports about β_3 levels in AD brain are still lacking.

Although an extensive amount of information is present for some of the GPCRs introduced in the paragraphs above, investigation of alterations of all abovementioned GPCRs and their correlation with neurotransmitter levels and behavior in AD, could possibly improve insights into the (patho)physiological occurrence and role of these receptors.

2. MATERIALS & METHODS

2.1. Study population and sample size

The study population for the investigation of mRNA levels of *HTR1A*, *HTR7* and *ADRB3* (gene names for 5-HT_{1A}, 5-HT_7 and β_3 adrenergic receptor, respectively), consisted of neuropathologically confirmed AD subjects (n=42) and CONTR (n=18), which were selected from the NeuroBioBank of the Institute Born-Bunge (University of Antwerp, Antwerp, Belgium). Clinical, neuropsychological and neuropathological characterization of study participants was performed as described previously [143].
Analyses of mRNA of *Chrm1* and *Grm5* (gene names for M1 and mGluR5) and protein levels of 5-HT_6, were performed in the APP23 mouse model. In short, the APP23 model is a transgenic mouse model, containing human cDNA of APP with the Swedish double mutation (K670N/M671L) under control of the murine neuronal Thy-1 promotor, resulting in age-dependent overexpression of APP and causing a familial form of early-onset AD in humans [251, 252]. Further neuropathological, cognitive, and behavioral aspects of this model, as well as housing conditions and genotyping are described in [253, 582]. Male hemizygous (HEM) (n=5) and wild-type (WT) (n=5) mice belonging to each age group (6-8 weeks, 3, 6, 12, 18 and 24 months) were euthanized by cervical dislocation, followed by brain collection and regional dissection of each hemisphere.

2.2. Brain dissection

Human brain samples were dissected according to a standard procedure as described in [143, 199, 294]. For this study, frontal (Brodmann area (BA) 10), temporal (BA22) and limbic regions (BA24, hippocampus, amygdala) relevant to cognition and behavior in AD, as well as BA7, thalamus and cerebellum were subsequently analyzed using reversed transcription quantitative polymerase chain reaction (RT-qPCR) and correlated with monoaminergic HPLC-data from previous

studies with the same study population. Methodology regarding latter experiments, can be found in the respective papers [199, 294].
Mouse brain samples were collected according to [582]. Following cervical dislocation, regional brain dissection was performed to obtain and weigh the olfactory bulbs, cerebellum, brain stem, cortex, which consisted of frontal, temporal, parietal and occipital areas, hippocampus, hypothalamus and striatum. After dissection, these tissues were frozen at -80°C until further use. Due to time constraints and the known neuronal loss in the CA1 area of the hippocampus in APP23 mice, only hippocampal samples were analyzed in subsequent experiments. Left and right hippocampus was used for RT-qPCR and western blot, respectively, except for one animal in the 6-month-old and 24-month-old groups. For these animals, left hippocampus was used for western blot, and right hippocampus was used for RT-qPCR.

2.3. RNA extraction and cDNA synthesis

Since RT-qPCR analyses on human and mouse samples were performed at different research institutes, being the Center for Applied Medical Research (University of Navarra, Pamplona, Spain) and the Laboratory of Neurochemistry and Behavior (University of Antwerp, Antwerp, Belgium), respectively, the methods and used materials differ.
Ribonucleic acid extraction from 20 mg of human brain tissue was performed using the Nucleospin RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Afterwards, RNA concentration and purity were determined with UV-VIS spectrophotometry using the Nanodrop 1000 instrument (Thermo Fisher Scientific, Waltham, MA, USA), and samples were stored at -80°C until further use. Reverse transcription was conducted on 300 ng RNA, using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA). All resulting cDNA samples were diluted 1:10 and stored at -80°C until qPCR analyses.
Regarding mouse brain samples, RNA extraction and cDNA synthesis were performed as described previously [582]. All cDNA samples were diluted 1:10 and stored at -80°C until further use.

2.4. Primer design

The qPCR assay performed on human brain samples, employed specific Taqman probes which were pre-designed by Applied Biosystems (Applied Biosystems, Foster City, CA, USA) with assay-IDs Hs-04194798_s1 (*HTR7*), Hs-00265014_s1 (*HTR1A*), Hs-00609046_m1 (*ADRB3*). Efficiency of these assays was not determined experimentally, but assumed to be 100%.

Primers for the qPCR assays on mouse samples, comprising two target genes and four reference genes, were developed and checked for specificity using the NCBI Primer-BLAST software (Table 1) and RefSeq mRNA sequences of *Mus musculus*. These primers were synthesized by Biolegio (Nijmegen, The Netherlands).

Table 1. Primer sequences, amplicon lengths and efficiencies of qPCR assays in murine hippocampus.

Symbol	Sequence	Accession number	Amplicon length	PCR efficiency (%)
Actb	5'-GGCTGTATTCCCCTCCATCG-3' 5'-CCAGTTGGTAACAATGCCATGT-3'	NM_007393.5	154	105.6
B2m	5'-GTATACTCACGCCACCCACC-3' 5'-TGGGGGTGAATTCAGTGTGAG-3'	NM_009735.3	193	106.7
Chrm1	5'-AGCTACAGTGACAGGCAACC-3' 5'-GGTTCATGGAGAAGGTGCCA-3'	NM_007698.3	131	101.6
Gapdh	5'AGGTCGGTGTGAACGGATTTG-3' 5'-GGGGTCGTTGATGGCAACA-3'	NM_001289726.1	95	120.5
Grm5	5'-CAGCTTAGATCGCAGCCACT-3' 5'- GGGTAAAATCACCAGGTGCG-3'	NM_001143834.1	124	110.8
Pgk1	5'-CTCCGCTTTCATGTAGAGGAAG-3' 5'-GACATCTCCTAGTTTGGACAGTG-3'	NM_008828.3	117	102.3

Abbreviations: *Actb*: beta-actin; *B2m*: beta-2-microglobulin; *Chrm1*: muscarinic acetylcholine receptor 1; *Gapdh*: glyceraldehyde-3-phosphate dehydrogenase; *Grm5*: metabotropic glutamate receptor 5; *Pgk1*: phosphoglycerate kinase 1.

2.5. qPCR

Quantitative PCR analyses on human brain samples were performed on a CFX96 real-time system (Bio-Rad, Hercules, California, USA). Cycling conditions were applied as recommended by Applied Biosystems. Assays were carried out in 20 µL reactions, containing 10 µL 2X TaqMan Fast Advanced Master Mix and 1 µL of the 20X TaqMan Assay mix, in addition to 2 µL cDNA (or 3 ng cDNA per well) and 7 µL ddH_2O. CFX Manager version 3.1 (Bio-Rad, Hercules, California, USA) and Microsoft Excel were used to calculate fold change relative to samples belonging to the CONTR group by applying the delta-delta Cq method. A replicate was excluded in case its Cq value was 0.5 units higher or lower compared to the other replicates, with minimum and maximum Cq values set at 15 and 35, respectively. A single reference gene, 18S RNA, was used as normalization factor.

Investigation of mRNA expression levels in mouse samples was carried out as described before, with identical cycling conditions, threshold settings, reagent volumes and quality control settings [582], with the exception of final primer concentrations, which were 333 nM for all assays. Samples to which no reverse transcriptase was added during cDNA synthesis (-RT control), were also analyzed in the same way and were all found to be negative (defined as an undetermined Cq

or if the difference between the -RT control and the corresponding cDNA sample was ≥10 Cq values). Since 60 samples needed to be analyzed in triplicate on 96-well plates, a calibrator sample was analyzed on each plate, to minimize inter-run variability. Assay efficiencies were determined using technical triplicates of 1:2 or 1:10 serial dilutions, consisting of up to six dilution points, of a representative cDNA sample and are displayed in Table 1. Calibrated normalized relative quantities (CNRQs) of *Chrm1* and *Grm5* were calculated using the qbase+ software (version 3.1, Biogazelle), implementing an efficiency-corrected delta-delta Cq method [583]. The geNorm application, embedded in the qbase+ software, was used to determine the best combination of reference genes as normalization factor.

2.6. Protein sample preparation

Hippocampi were thawed and covered immediately with RIPA buffer (45 µL/mg), consisting of 150 mM NaCl, 50 mM Tris base, 0.1% SDS, 10 mM iodoacetamide, 1% Triton X-100 and 0.5% sodium deoxycholate, and supplied with protease (1 mM phenylmethylsulfonyl fluoride, 1 mM Halt™ Protease Inhibitor Cocktail 100X and 1 mM EDTA (Thermo Fisher Scientific, Waltham, MA, USA)) and phosphatase inhibitors (Pierce Phosphatase Inhibitor Mini Tablets (Thermo Fisher Scientific, Waltham, MA, USA)). An electric roto-stator homogenizer (Bio-Gen PRO200, Pro Scientific, Oxford, Connecticut, USA) was used to lyse hippocampal samples on ice in three bursts of 10 s, with 10 s equilibration on ice in between bursts. Afterwards, samples underwent additional lysis on ice with the UP200St sonicator (Hielscher, Teltow, Germany), which was used at 25% amplitude during five cycles of 15 s, with 30 s in between. Samples were subsequently incubated and rotated during 60 min at 4°C, and centrifuged at 800 x g during 5 min, still at 4°C. The supernatant was transferred to a clean tube, and centrifuged at 10,000 x g during 10 min at 4°C. Afterwards, the supernatant was collected and divided into 60 µL aliquots which were stored at -80°C for further use. One aliquot was used in the bicinchoninic acid assay (BCA) (Pierce BCA Protein Assay kit, Thermo Fisher Scientific, Waltham, MA, USA) to determine protein concentration.

2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were thawed and incubated at 37°C during 30 min in the presence of 4X Bolt LDS sample buffer, 10X Bolt reducing agent (Thermo Fisher Scientific, Waltham, MA, USA) and ddH_20, so that 12-15 µg protein could be loaded in duplicate onto different Bolt 4-12% Bis-Tris Plus gels (Thermo Fisher Scientific, Waltham, MA, USA). These gels were submerged in 1X Bolt MOPS SDS Running buffer in the Mini Gel Tank (Thermo Fisher Scientific, Waltham, MA, USA).

Electrophoresis was performed at a constant voltage of 120 V during 105 min at 4°C.

2.8. Western blot

The SDS-PAGE gels were transferred onto polyvinylidene difluoride (PVDF) membranes with low autofluorescence (Immobilon-FL, Merck, Darmstadt, Germany), which were previously activated with methanol during 10 min. Western blot was performed at 4°C according to manufacturer's instructions, using 1X Transfer Buffer containing 10% methanol. Proteins were transferred at a constant current of 350 mA during 65 min. Membranes were washed briefly in ddH_20 and treated with a total protein stain (REVERT Total Protein Stain, Li-Cor, Lincoln, Nebraska, USA). Total protein content in each lane was subsequently imaged in the 700 nm channel of the Odyssey system (Li-Cor, Lincoln, Nebraska, USA). Afterwards, blocking was performed with the Odyssey Blocking buffer (TBS) during 60 min at room temperature, followed by overnight incubation at 4°C with rabbit anti-5-HT_6 antibody (ab103016, Abcam, Cambridge, United Kingdom), diluted 1:800 in Odyssey Blocking buffer (TBS) supplemented with 0.2% Triton X-100. Membranes were washed in TBS-T for four cycles of 5 min each, and incubated during 60 min with IRDye 800CW goat anti-rabbit IgG secondary antibody (LI 925-32211, Li-Cor, Lincoln, Nebraska, USA) diluted 1:15,000 in Odyssey Blocking buffer (TBS) supplemented with 0.2% Triton X-100 and 0.01% SDS. Four washing cycles of 5 min with TBS-T were conducted, and membranes were imaged in the 800 nm channel to obtain intensity measures of the protein band of interest. As such, normalized target signals were calibrated with respect to a standard control sample loaded onto every gel. Duplicate values were averaged to obtain the fold change compared to the control sample.

2.9. Double-labelling immunofluorescence

We tried to perform immunofluorescence analysis for 5-HT_6 in hippocampal brain slices of APP23 mice aged 6-8 weeks, 12, and 18 months to confirm its co-occurrence with neurons (neuronal nuclear protein; NeuN), and to investigate whether astrocytes (glial fibrillary acidic protein; Gfap) and microglia (ionized calcium-binding adapter molecule 1; Iba1) were present near this GPCR. Mice were anesthetized with an overdose of Nembutal (18 mg of a 60 mg/mL solution, Ceva, Brussels, Belgium) followed by transcardial perfusion with 50 mL PBS and 50 mL 10% formalin in PBS. Brains were collected and post-fixed overnight at 4°C in 10% formalin solution. These tissues were then washed three times with ddH_20, after which they were preserved in 70% ethanol until they were embedded in paraffin. Consecutive coronal microtome sections of 5 µm thickness were made from each tissue block and left to dry overnight at 37°C. Slides were deparaffinated and rinsed

in running tap water before heat-mediated antigen retrieval with citrate buffer (pH 6). As an additional antigen retrieval method, tissue sections were incubated in TBS-T during 5 min. Primary antibody incubation with rabbit anti-5-HT_6 antibody (ab103016, Abcam, Cambridge, United Kingdom) diluted 1:100 in TBS with 1% bovine serum albumin and either guinea pig anti-NeuN antibody (ABN90P; Merck, Darmstadt, Germany) diluted 1:1000, guinea pig anti-Gfap antibody (173 004, Synaptic Systems, Goettingen, Germany) diluted 1:500 or guinea pig anti-Iba1 (234 004, Synaptic Systems, Goettingen, Germany) diluted 1:500, was performed overnight at room temperature. After washing, slides were incubated during 60 min at room temperature with following secondary antibodies: highly cross-adsorbed goat anti-rabbit IgG conjugated with Alexa fluor 488 (A11034, Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:1000 and highly cross-adsorbed goat anti-guinea pig IgG conjugated with Alexa fluor 647 (A21450, Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:400. Protected from light, slides were washed in TBS, followed by incubation with 0.1% Sudan Black B (Abcam, Cambridge, United Kingdom) in 70% ethanol during 20 min. After a final washing step, tissue sections were mounted using Fluoroshield with 4′,6-diamidino-2-phenylindole (DAPI) (Merck, Darmstadt, Germany) and allowed to dry in the dark. Confocal imaging was performed with an Olympus BX61WI microscope (Olympus, Tokyo, Japan). Excitation of Alexa fluorophores was accomplished using an ANDOR AMH-200-F6J light source and resulting images were processed with ANDOR IQ3 software (Andor, Belfast, United Kingdom).

2.10. Statistical analysis

Since the (log-transformed) fold changes of *HTR1A*, *HTR7* and *ADRB3*, as well as storage time were characterized by non-normal distributions, nonparametric Mann-Whitney U tests were applied. In contrast, age at death was analyzed using the independent-samples T-test and chi-square statistics were adopted to assess the association of diagnostic group (AD or CONTR), gender and psychotropic medication use. Spearman's rank order correlation was applied to verify the association between fold changes of *HTR1A*, *HTR7* and *ADRB3* and storage time, behavioral data, and monoaminergic data in corresponding brain regions.
Log-transformed CNRQs obtained after RT-qPCR analyses on mouse brain samples were normally distributed and allowed the use of two-way ANOVA with age and genotype as fixed factors. Alternatively, if the assumption of homogeneity of variances was violated, separate Welch's ANOVA and independent-samples T-tests were conducted. Corresponding post-hoc analyses for age were performed using independent samples T-tests with Benjamini-Hochberg correction for multiple comparisons, and Games-Howell tests, respectively. Finally, separate Kruskal-

Wallis and Mann-Whitney U tests were performed on the protein expression data for 5-HT_6. All statistical analyses were performed using SPSS 25.0 for Windows. Initially, we planned to perform a colocalization algorithm on the confocal images to establish the co-occurrence of 5-HT_6 and neuronal, astrocytic and microglial cell markers, but due to time constraints and methodological issues, we were unable to do so. Therefore, confocal images will be presented here as a qualitative representation of this experiment.

3. RESULTS

3.1. Part I: human study population

3.1.1. Demographics

Demographic characteristics of the human study population are presented in Table 2.

Table 2. Demographics and clinical data.

Parameter	AD	CONTR	Test statistic
Age at onset (years)	75.4 ± 11.6 (n=42)	N/A	N/A
Age at death (years)	80.2 ± 10.0 (n=42)	74.7 ± 9.5 (n=18)	t(58)=1.995 *P*>0.05
Male/female (n)	25/17	10/8	X^2=0.082 *P*>0.05
Storage time (years)	2.9 (7.7) (n=42)	9.3 (3.0) (n=18)	U=166.500 *P*≤0.001
Postmortem delay (hours)	3.4 (1.3) (n=42)	5.4 (3.3) (n=18)	U=227.500 *P*<0.05
Taking/not taking psychotropic medication (n)	23/18	7/11	X^2=1.482 *P*>0.05
Anti-Alzheimer's	5/36	0/18	Fisher's Exact=2.398 *P*>0.05
Anti-psychotics	14/27	0/18	Fisher's Exact=8.059 *P*<0.05
Anti-Parkinson's	2/39	0/18	Fisher's Exact=0.909 *P*>0.05
Antidepressants	13/28	4/14	X^2=0.549 *P*>0.05
Hypnotic, sedative and anxiolytic medication	5/36	4/14	Fisher's Exact=0.973 *P*>0.05
Anti-epileptics	0/41	1/17	Fisher's Exact=2.317 *P*>0.05
Dementia stage: GDetS (/7)	6 (1) (n=41)	N/A	N/A
MMSE (/30)	11.0 ± 6.5 (n=25)	N/A	N/A
BEHAVE-AD (/75)	7.0 (11) (n=41)	N/A	N/A

Parameters characterized by a normal distribution are depicted by mean ± standard deviation, while non-normally distributed data are depicted by median with interquartile range between brackets. Postmortem delay indicates the number of hours between time of death and storage of the brain at -80°C. Abbreviations: AD: Alzheimer's disease; BEHAVE-AD: Behavioral Pathology in Alzheimer's disease rating scale; CONTR: control subjects; GDetS: Global Deterioration Scale; MMSE: Mini-Mental State Examination; N/A: not applicable.

3.1.2. Expression of HTR1A, HTR7 and ADRB3 in human brain

A significant (2.49 ± 3.77)-fold and (2.20 ± 2.33)-fold higher expression of *HTR1A* was observed in hippocampus (U=142.000; $P<0.05$) and amygdala (U=94.000; $P<0.05$) of AD subjects compared to CONTR. In addition, *HTR7* mRNA was found to have an approximately fivefold (0.22 ± 0.21) lower expression in AD versus CONTR (U=108.000; $P\leq0.001$), while relative expression of *ADRB3* was (3.68 ± 3.69)-fold higher in the diseased group (U=50.000; $P\leq0.001$) (Figure 1).

3.1.3. Correlation of expression of HTR1A, HTR7 and ADRB3 with cognitive and behavioral data

Of note, correlation analysis with behavioral data could only be performed in the AD group, since no such data were available for CONTR subjects. No significant Spearman's rank order correlations could be detected for global dementia staging using the Global Deterioration Scale or cognitive performance on the Mini-Mental State Examination (MMSE). Significant associations were observed between hippocampal *HTR1A* expression and scores on the BEHAVE-AD cluster G, which corresponds to anxieties and phobias ($rs(32)=-0.485$, $P<0.005$), between *HTR1A* levels in amygdala and total Middelheim Frontality Score for frontal behavior ($rs(20)=-0.476$, $P<0.05$), and finally, between *HTR7* levels in BA10 and BEHAVE-AD cluster B (hallucinations) ($rs(28)=0.444$, $P<0.05$). However, only the first association remained significant after Benjamini-Hochberg correction for multiple comparisons.

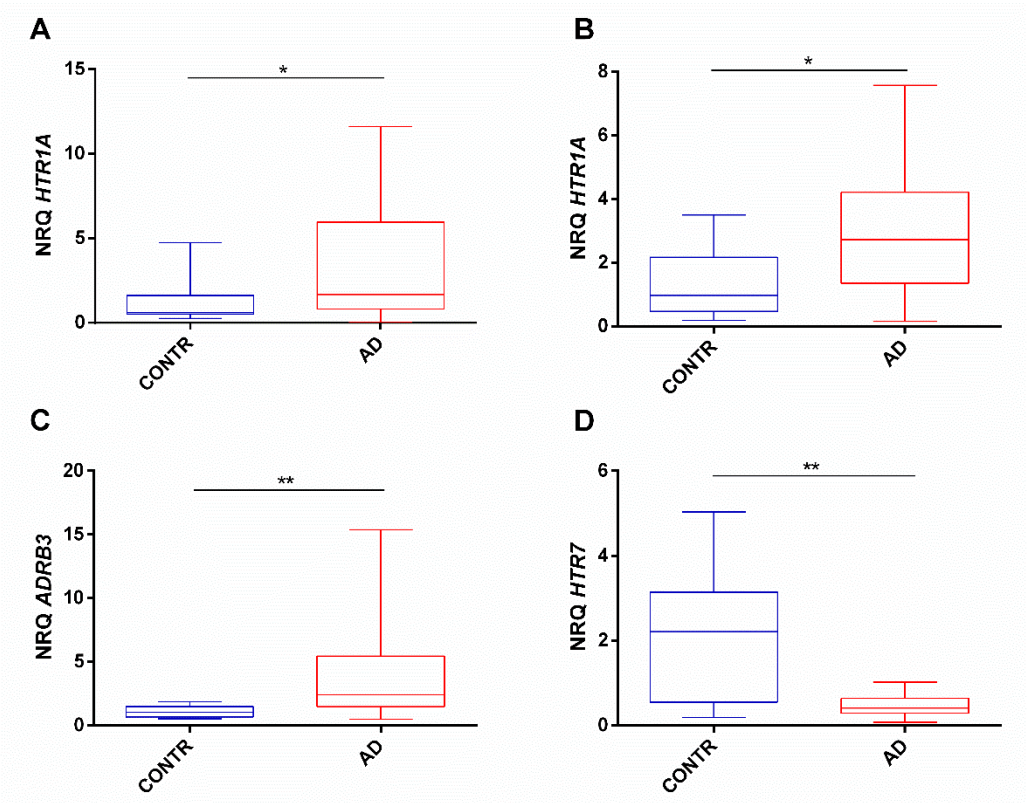

Figure 1. NRQs of significantly different amplicons between AD and CONTR. Data are depicted as median with minimum-maximum ranges. Statistically significant differences after Mann-Whitney U tests are indicated by one or two asterisks if $P<0.05$ or $P\leq0.001$. A, B: Differences in *HTR1A* expression in hippocampus (CONTR: n=14; AD: n=35) and amygdala (CONTR: n=16; AD: n=23), respectively. C: NRQs for *ADRB3* in hippocampus (CONTR: n=11; AD: n=30). D: Differential expression of *HTR7* in BA10 (CONTR: n=17; AD: n=31). Abbreviations: AD: Alzheimer's disease; *ADRB3*: β3 adrenergic receptor; BA: Brodmann area; CONTR: control subjects; *HTR1A*: serotonin receptor 1A; *HTR7*: serotonin receptor 7; NRQ: normalized relative quantities.

3.1.4. Correlation of expression of HTR1A, HTR7 and ADRB3 with monoaminergic concentrations

Spearman's rank order correlation analyses were performed on receptors and monoamines/metabolites (Table 3) showing significant differences between AD and CONTR subjects. Thus, we found a negative correlation between hippocampal 5-HT levels and NRQs of *HTR1A* ($rs(44)=-0.323$; $P<0.05$). Conversely, a positive association was observed between *ADRB3* in hippocampus and DOPAC levels in the amygdala ($rs(42)=0.336$; $P<0.05$). Negative relationships were observed when *HTR7* levels in BA10 were correlated with both MHPG concentrations in this brain region ($rs(45)=-0.311$; $P<0.05$), and DOPAC levels in the amygdala ($rs(42)=-0.311$;

P<0.05). However, none of these correlations remained significant after post-hoc correction for multiple comparisons. Finally, no associations were found between *HTR1A* levels in the amygdala and any of the monoamines/metabolites included in the analysis.

Table 3. Monoamine neurotransmitters and metabolites in AD and CONTR

Parameter	AD	CONTR	Test statistic
Hippocampus			
5-HT (ng/g)	55.93 (48.64) n=40	83.10 (41.87) n=17	U=191.000 *P*<0.05
Amygdala			
DOPAC (ng/g)	24.28 (24.25) n=37	18.74 (14.38) n=17	U=203.000 *P*<0.05
HVA (ng/g)	639.67 (298.69) n=37	931.93 (682.41) n=17	U=188.000 *P*<0.05
5-HT (ng/g)	133.51 (125.53) n=37	230.81 (116.47) n=17	U=162.000 *P*<0.05
DOPAC/DA	0.49 (0.44) n=37	0.27 (0.21) n=17	U=173.500 *P*<0.05
BA10			
MHPG (ng/g)	672.60 (608.49) n=41	245.68 (312.63) n=18	U=149.000 *P*<0.001
MHPG/NA	51.28 (97.01) n=37	14.93 (23.57) n=18	U=200.000 *P*<0.05

Only statistically significant differences after Mann-Whitney U analyses are presented. Data are depicted as median with interquartile range between brackets. Abbreviations: 5-HT: 5-hydroxytryptamine or serotonin; AD: Alzheimer's disease; BA: Brodmann area; CONTR: control subjects; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; MHPG: 3-methoxy-4-hydroxyphenylglycol; NA: noradrenaline.

3.1.5. Influence of medication, sample handling and age

Identical analyses were performed on AD and CONTR subjects who did not take any type of psychotropic medication. Mann-Whitney U analyses on NRQs of all investigated GPCRs in the brain, confirmed our previous findings of higher hippocampal *HTR1A* and *ADRB3* levels in AD (U=21.000, *P*<0.05 and U=10.000; *P*<0.005, respectively), although the difference for *HTR1A* in the amygdala did not remain significant. Finally, a trend for lower expression of *HTR7* in BA10 of AD subjects (U= 20.000; *P*=0.052) was observed in the medication-free subgroup.

In the medication-free subgroup, higher levels of MHPG in BA10 (U=31.000, *P*<0.05), as well as DOPAC levels (U=21.000; *P*<0.05) and DOPAC/DA ratios (U=27.000; *P*<0.05) in the amygdala could confirm our findings in the total study population. Nevertheless, no serotonergic differences were found in subjects who did not take medication. Instead, higher MHPG levels in amygdala (U=25.000; *P*<0.05) and hippocampus (U=29.000; *P*<0.05) were noted in AD compared to

CONTR. Finally, when we correlated monoamines/metabolites/ratios and receptors with significant differences between AD and CONTR in the medication-free subgroup, we only found positive associations between DOPAC levels in the amygdala and hippocampal *HTR1A* expression (rs(20)=0.430; $P<0.05$), as well as hippocampal *ADRB3* mRNA (rs(18)=0.501; $P<0.05$), of which none remained significant after post-hoc corrections for multiplicity.
No associations were observed between storage time, postmortem delay and age of onset, and any of the GPCRs with differential expression between AD and CONTR, apart from a trend for the negative correlation between hippocampal *ADRB3* expression and postmortem delay (rs(39)=-0.306; $P=0.052$). However, a positive correlation was found for *HTR1A* levels in the amygdala and age of death (rs(37)=0.462; $P<0.005$).

3.2. Part II: murine study population

3.2.1. Expression of Chrm1 and Grm5 in mouse brain

Of the four analyzed reference genes, *Pgk1* and *B2m* were the most stable according to the geNorm algorithm, despite the fact that none of these genes reached the stability limit of 0.5. This prompted us to include *Pgk1*, *B2m* and *Gapdh* in the normalization factor for the subsequent calculation of relative mRNA quantities. Expression levels of *Chrm1* and *Grm5* showed a steady increase until the age of 12 months, followed by a decrease in the 18- and 24-month-old groups, while in general, HEM animals showed almost equal expression levels compared to WT mice. The assumption of homogeneity of variances was violated for *Chrm1*, which necessitated the use of Welch's ANOVA to test the effect of age on expression levels and independent samples T-tests to assess the effect of genotype. A significant age effect was observed (Welch(5, 24.155)=4.966; $P<0.05$), while no such influence of genotype could be detected (t(57)=-0.800; $P>0.05$) (Figure 2. E). Hemizygous animals showed (1.25 ± 1.00)-fold higher expression of *Chrm1* compared to WT mice. Post-hoc Games-Howell tests for age indicated that animals aged 3 months had lower *Chrm1* levels than animals in the 12-month-old group ($P<0.05$), while CNRQs of this amplicon were lower in the 24-month-old animals when compared to the 12- and 18-month-old groups ($P<0.05$ for both tests) (Figure 2. C). When separate T-tests were performed in each age group, *Chrm1* levels in 6-month-old HEM animals were (3.33 ± 1.63)-fold higher than those of WT animals in this age group (t(8)=-4.945; $P\leq0.001$) (Figure 2. A).
To assess expression levels of *Grm5*, a two-way ANOVA test was conducted, which pointed out a significant age effect (F(5,46)=2.782; $P<0.05$), while no significant genotype effect (Figure 2. F) nor an age x genotype interaction were found (F(1,46)=0.024; $P>0.05$ and F(5,46)=0.075; $P>0.05$). Our results showed that HEM mice pooled over all age groups had an almost equal (1.04 ± 0.70) expression of

Grm5 compared to WT mice. To further investigate the age effect, post-hoc independent-samples T-tests were carried out. Due to the correction for multiple testing, no significant differences between any of the age groups could be retained (Figure 2. D). Finally, T-tests were performed to assess the genotype effect in separate age groups, which pointed out (2.16 ± 1.22)-fold higher *Grm5* levels in HEM animals of aged 6 months ($t(8)=-2.921$; $P<0.05$). However, this result did not remain significant after Benjamini-Hochberg correction for multiple comparisons.

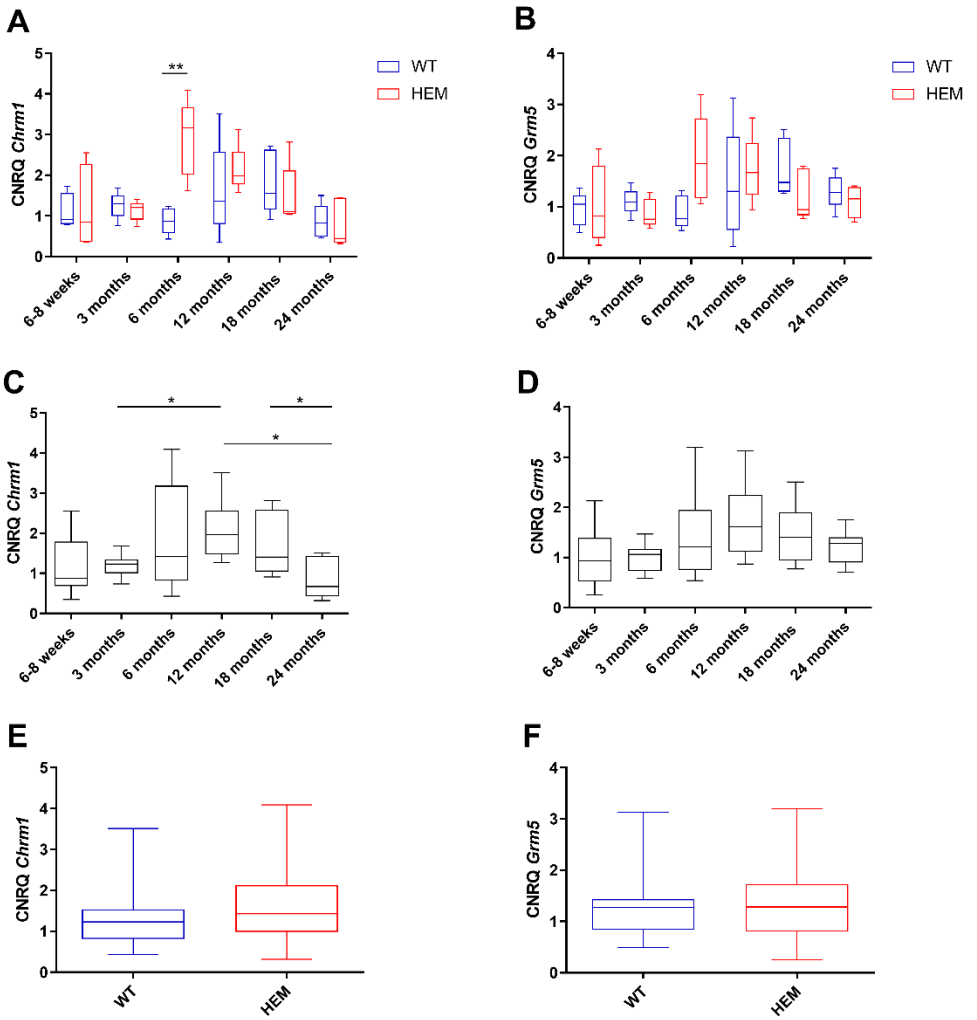

Figure 2. CNRQs across age- and genotype groups. Data are depicted as median with minimum-maximum ranges. Statistically significant differences are indicated by one or two asterisks if $P<0.05$ or $P\leq0.001$, respectively. A, B: *Chrm1* and *Grm5* levels per age and genotype, respectively. C, D: *Chrm1* (n=10, 10, 10, 9, 10, and 10 for 6-8-week old, 3, 6, 12, 18, and 24-month old animals, respectively) and *Grm5* (n=10, 10, 10, 9, 10, and 9 for 6-8-week old, 3, 6, 12, 18, and 24-month old animals, respectively)

levels of pooled WT and HEM animals across age groups. E, F: *Chrm1* (n=29 and n=30 for WT and HEM animals, respectively) and *Grm5* (n=29 for both WT and HEM animals) levels of pooled age groups across genotypes. Abbreviations: *Chrm1*: muscarinic acetylcholine receptor 1; CNRQ: calibrated and normalized relative quantities; *Grm5*: metabotropic glutamate receptor 5; HEM: hemizygous; WT: wild-type.

3.2.2. 5-HT$_6$ expression in mouse hippocampus

Non-parametric tests revealed that there was no significant effect of age (H=9.990; *P*>0.05), nor genotype (U=325.000; *P*>0.05) on the protein expression of 5-HT$_6$ in hippocampal tissue of the APP23 mouse model (Figure 3. B, C). Pooled over all age groups, HEM mice showed an approximately equal (1.05 ± 0.29) expression of hippocampal 5-HT$_6$ compared to WT mice. Again, separate Mann-Whitney U statistics were applied within each age group, although no differences in fold changes were statistically significant (Figure 3. A).

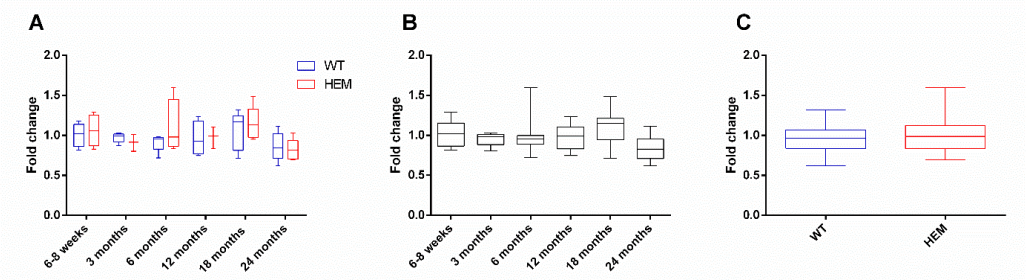

Figure 3. Fold changes of 5-HT$_6$ protein expression across age- and genotype groups. Data are depicted as median with minimum-maximum range. A: 5-HT$_6$ levels per age- and genotype group. B: pooled data from WT and HEM animals across age groups (n=9, n=8, n=9, n=7, n=10, n=10 for 6-8-week-old, 3-, 6-, 12-, 18- and 24-month-old groups, respectively). C: pooled data from all age groups across genotype (n=29 and n=24 for WT and HEM mice, respectively). Abbreviations: 5-HT$_6$: serotonin receptor 6; HEM: hemizygous; WT: wild-type.

3.2.3. Co-occurrence of 5-HT$_6$ with neuronal, astrocytic and microglial markers

Since we could not perform quantitative analyses on confocal images, we present them here as a qualitative representation of the co-occurrence between 5-HT$_6$ and neuronal, astrocytic and microglial markers. In all photographs, 5-HT$_6$ appears to be present in the apical dendrites of pyramidal cells in the stratum radiatum and stratum lacunosum-moleculare (Figure 4). Since we aimed to image an overview of the hippocampal region containing the dentate gyrus and cornu ammonis regions, it was not possible to focus on the presence of neuronal cilia. Therefore, it is difficult to conclude whether 5-HT$_6$ is also present on these protrusions on cell bodies.
Further, it appears that 5-HT$_6$ does not co-localize with glial cells, especially microglial cells (Figure 4. B), although a merged image of 5-HT$_6$ and Gfap, a marker

for astrocytes, seems to indicate that there could be a co-occurrence of these proteins (Figure 4. C).

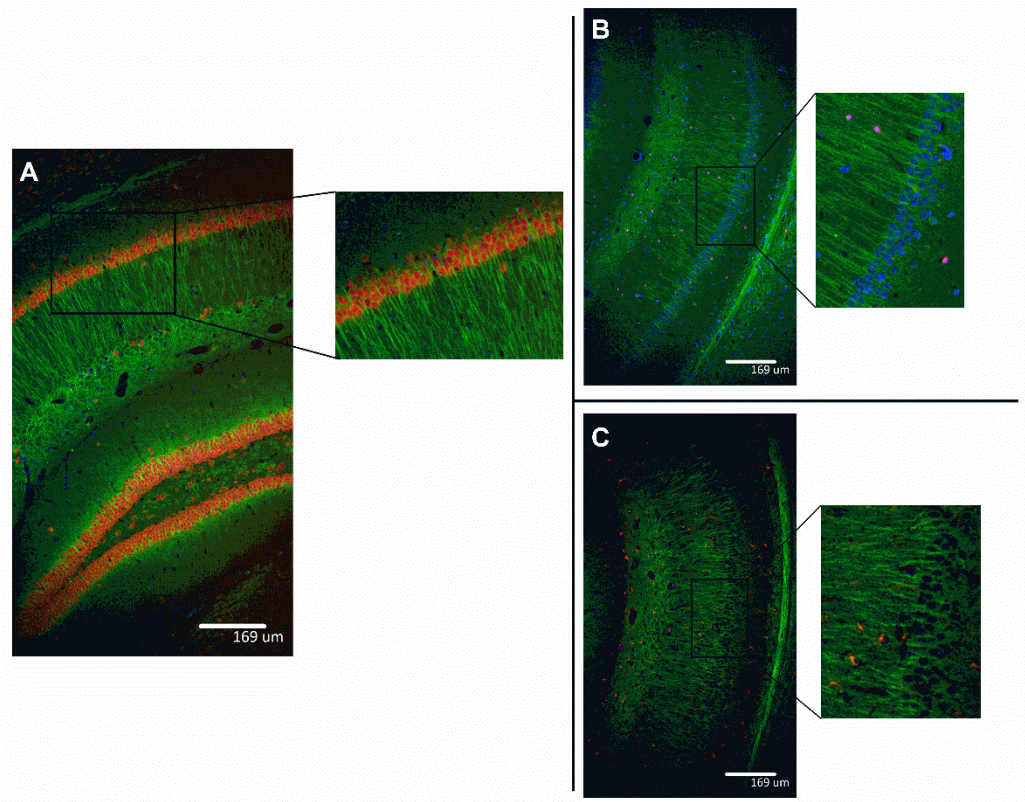

Figure 4. Confocal images of 5-HT_6 and neuronal, microglial and astrocytic markers in the hippocampus of APP23 mice. A: representative confocal image of dual labelling of 5-HT_6 (green) and NeuN (red) in a 12-month-old APP23 HEM mouse. B: representative confocal image of dual labelling of 5-HT_6 (green) and Iba1 (red) in a 12-month-old WT mouse. C: representative confocal image of dual labelling of 5-HT_6 (green) and Gfap (red) in a 6-8-week-old APP23 HEM mouse. DAPI signal is shown in blue in every image. Abbreviations: 5-HT_6: serotonin receptor 6; DAPI: 4',6-diamidino-2-phenylindole; Gfap: glial fibrillary acidic protein; HEM: hemizygous; Iba1: ionized calcium-binding adapter molecule 1; NeuN: neuronal nuclear protein; WT: wild-type.

4. DISCUSSION

4.1. Strengths and limitations

The human study population was well characterized, both clinically and neuropathologically. In addition, it was beneficial to generate paired neurotransmitter- and receptor measurements in brain regions of the same subjects. Sample handling, apart from postmortem delay, did not appear to have confounded our results, nor did age of AD onset. Nevertheless, medication effects might have influenced our (serotonergic) results, so caution should be exerted while interpreting these findings. Another limitation of the human study population was the imbalance in AD and CONTR group sizes and the fact that nonparametric statistics needed to be used, which decreased the power of the analyses. Only one reference gene was analyzed to normalize *HTR1A*, *HTR7* and *ADRB3* expression levels to, which might have biased these results.
It was also interesting to perform RT-qPCR and western blot analyses in the hippocampus of six distinct age groups of APP23 mice to be able to map the progression of receptor disturbances. Furthermore, hippocampi of the same animals were used for RT-qPCR and western blot experiments, which reduced variability and the (unnecessary) use of additional laboratory animals. Although geNorm analysis was performed to obtain the best normalization factor, none of the analyzed reference genes displayed a highly stable expression. To account for this, we combined the three reference genes with the lowest stability values and thus, most stable expression, into the normalization factor. A better approach would have been to analyze more reference genes, however, this was precluded due to limited sample availability. Furthermore, western blot conditions were not fully optimized at the time of performing these experiments, which might also have confounded our results. We were not able to apply a colocalization algorithm on the confocal images. Therefore, we were only able to present qualitative results, with a loss of (possible) valuable information. Finally, it would have been more interesting to investigate the same GPCRs analyzed in the human study population as well. However, this chapter comprises the combined results of two separate projects in time.

4.2. Neurochemistry in the human study population

The increased expression levels of *HTR1A* in hippocampus and amygdala of AD subjects conflicted with the existing literature (reviewed in [33]), while we were able to provide new information regarding *HTR7* and *ADRB3* expression levels in AD. However, latter result should be interpreted with caution since the CONTR group had a significantly longer postmortem delay which may have resulted in a type I error regarding increased *ADRB3* levels in hippocampus of AD subjects. Since

we identified an influence of psychotropic medication on expression of *HTR1A*, this may partly account for our findings. It is also necessary to mention that patients in our AD study group were in an advanced dementia stage, which may have contributed to the atypical finding of higher hippocampal *HTR1A* expression. Another result was the negative correlation between the presence of anxieties and phobias and hippocampal *HTR1A* expression, suggesting that the 5-HT_{1A} receptor has a possible protective function against this type of behavioral disturbances in AD. Indeed, knock-out mice for this receptor showed increased anxiety-like behavior [584, 585], supporting our findings. In addition, abovementioned hypothesis regarding *HTR1A* levels in the hippocampus of AD subjects could not be confirmed in a medication-free subpopulation, although this may also be caused by a considerable reduction in sample size. Nevertheless, it should also be mentioned that this theory is only based on mRNA and 5-HT levels, and no receptor binding, nor western blot analyses have been performed. Preferably, these techniques should also be performed to confirm abovementioned hypothesis.

4.3. Neurochemistry in the murine study population

Regarding RT-qPCR on mouse hippocampi, it is striking that expression levels of *Chrm1* are upregulated in HEM animals aged 6 months, which is the age when the first neuropathological lesions arise and behavioral disturbances are observed in the APP23 model [251-253]. For *Chrm1*, most studies have reported unchanged receptor levels in RT-qPCR experiments [504, 506], and our results also seem to confirm the absence of an overall genotype effect. An age effect, on the other hand, was present as an increase in *Chrm1* between 3- and 12-month-old animals, while at later ages, a decrease was observed. This could point to a general decrease in responsivity of cholinergic neurotransmitter system starting at the age of 12 months, as has been postulated before [586, 587]. Furthermore, in the APP23 model, cognitive performance in the Morris water maze also decreased with age [588]. The sudden rise in *Chrm1* levels in HEM animals aged 6 months might then reflect an attempt to restore cholinergic neurotransmission in response to the first Aβ plaques, as agonism of this GPCR has been reported to improve memory [498], and to promote the production of sAPPα [502, 503] in conjunction with a decrease in tau phosphorylation [499]. For *Grm5*, a similar age-related expression pattern was observed, although no statistically significant post-hoc age effects were apparent. No genotype effect could be confirmed either. Pathological overexpression of mGluR5 in AD has been previously established at the protein level [523, 524], but it is unclear why we only observed statistically non-significant overexpression of *Grm5* mRNA in the 6-month-old group of the APP23 mouse model, since we would also expect this overexpression in the older age groups. In general, it remains difficult to speculate about the pathophysiological meaning of

these RT-qPCR results, since various posttranslational modifications or binding site alterations may take place, which we could not investigate in our study. Ideally, (at least) radioligand binding experiments using specific ligands for these GPCRs should be performed to obtain a better understanding of the role of these receptors in the APP23 model.

Our western blot results for hippocampal 5-HT_6 expression in APP23 revealed no effects of age or genotype, while decreased levels in frontal and temporal regions of AD subjects were previously observed [553, 554]. Since various methodological difficulties were encountered while performing the western blot experiments, e.g. large coefficients of variation for duplicate samples, these issues may partly explain our findings. Either a larger number of animals should be included, or further optimization of the technique, perhaps by replacing the total protein stain by a conventional housekeeping protein as normalization method, should be implemented in order to obtain more certainty regarding these results. Of note, the disadvantage of latter proposition would possibly lead to unreliable protein expression results if the housekeeping protein is not stably expressed across age- and genotype groups, which was the reason we initially opted for a total protein stain. Finally, optimization is also necessary for the double-labelling immunofluorescence experiments we performed to investigate the colocalization between 5-HT_6 and neuronal, astrocytic and microglial markers. It seems that we can confirm its presence on neuronal cells, and additionally, a co-occurrence with astrocytes may also be present in the hippocampus of the APP23 model. However, caution should be exerted when interpreting these qualitative results, as color-merging is no reliable measure for colocalization [589]. We included negative controls in which slides were incubated with secondary antibody only, but analyses on knock-out tissue for 5-HT_6 would have provided more reliable information regarding its presumed expression on apical dendrites of hippocampal pyramidal cells, and on astrocytes. Overall, results of western blot and immunofluorescence experiments remain doubtful until further optimization.

4.4. Future perspectives

Since we only determined expression levels of receptors and concentrations of neurotransmitters possibly playing a role in AD, we still have limited information regarding the functionality of the interaction between receptors and their ligands. Radioligand binding experiments on postmortem brain material could therefore be initiated. Alternatively, in the APP23 model, the functional impact of GPCR (ant)agonists could be evaluated in cognitive and behavioral tasks, followed by regional brain collection and subsequent molecular analyses. Next to the previously proposed optimization of the techniques implemented in this work, it

would be beneficial to include a larger sample of medication-free subjects in future neurochemical analyses on human postmortem brain material.

5. CONCLUSION

In general, we found an upregulation of *HTR1A* mRNA in hippocampus and amygdala, and of hippocampal *ADRB3* mRNA levels in AD compared to CONTR. Conversely, levels of *HTR7* were lower in the frontal cortex of AD subjects. An inverse correlation was established between hippocampal *HTR1A* expression and the presence of anxieties and phobias, which might point to a possible protective role of this GPCR in anxiety-like behavior. Further, an age effect of *Chrm1* and *Grm5* was present in hippocampus of the APP23 model, with reduced expression of these receptors starting at the age of 12 months. Western blot and double-labelling immunofluorescence experiments confirmed the presence of 5-HT_6 in hippocampal neurons of the APP23 model, however, further optimization of these techniques is necessary to obtain conclusive results. In terms of future research, we suggest focusing on the receptor-ligand functionality rather than on individual receptor/ligand levels.

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General Discussion

This general discussion will first be centered around the aims presented in **Chapter II.** I will discuss the accomplishment of these aims, and their potential impact on the research field. Secondly, shortcomings associated with the studies presented in this work will be addressed, along with proposed solutions and alternatives. This discussion will eventually end with contemplating the direction of future research regarding the neurochemistry of neurodegenerative disorders (NDDs).

1. DISCUSSION OF AIMS AND RESULTS

The **first objective** of this thesis was to address pre-analytical procedures related to the presence of a circadian rhythm and rostrocaudal concentration gradient (RCG) for reversed-phase ultra-high performance liquid chromatography with electrochemical detection (RP-UHPLC-ECD) analysis of monoamines and their metabolites. These phenomena were previously hypothesized to confound comparisons between distinct NDDs, and between NDDs and control subjects (CONTR). We were able to identify a circadian rhythm for plasma homovanillic acid (HVA) with similar characteristics compared to that of melatonin. Evidence for 24-hour fluctuations of cerebrospinal fluid (CSF) HVA and 5-hydroxyindoleacetic acid (5-HIAA) was less convincing, while no rhythm could be detected for other monoamines and metabolites. With these results, we could indeed partly corroborate previous findings related to monoaminergic circadian rhythmicity. Moreover, we were able to propose a standardized sampling time (between 12:00 and 15:00 hours) for CSF and plasma based on time-related variations in monoaminergic compounds.
Conversely, HVA and 5-HIAA did show a rostrocaudal concentration gradient in aspirated CSF. These findings, along with the notion that 3-methoxy-4-hydroxyphenylglycol (MHPG) does not show such a gradient, were also in accordance with previous research. Thus, regarding the RCG, we could propose analyzing the first CSF fraction to account for the influence of the RCG when measuring multiple monoaminergic compounds. In addition, this study might also provide valuable information for projects with prospective CSF/serum sampling for monoamine analysis, hopefully facilitating inter-laboratory comparisons.

The **second aim** related to methodology, was to verify the applicability of mouse short interspersed nuclear elements (SINEs) as a normalization approach for reverse-transcription quantitative PCR (RT-qPCR) in brain tissue of the APP23 model for Alzheimer's disease (AD). Commonly used housekeeping or reference genes were previously shown to display unstable expression levels across experimental conditions [202, 211, 212]. Therefore, implementing these genes in a normalization approach for RT-qPCR, may severely impact the obtained results. As SINEs, among which B elements, are located in introns and untranslated regions of

various genes, their content in the transcriptome was thought to be largely unaffected by experimental conditions [224, 225]. While we identified B1 and B2 as stable transcripts in the hippocampus, these repeat elements had a less stable expression in the cortex. Thus, our results supported the use of B elements as reference genes in hippocampus of the APP23 model, although other genes would be more suitable for normalization of gene expression data in the cortex of these mice. At the time of writing, no other studies had reported the use of these repeat elements in mice. One paper investigated their use as normalization strategy in a rat model of temporal lobe epilepsy [225], but still we were able to present relatively novel information. With the corresponding paper, we also underlined the need for extensive validation of suitable reference genes for each new RT-qPCR setup.

Characterizing distinct NDDs with reference to their neurochemical profile represented the **third aim** of this work. Even though providing a comprehensive overview of neurotransmission-related changes in NDDs would certainly be of great value to future research, this would take up too much work for a single PhD project. We therefore predominantly focused on the monoaminergic systems, and in the case of the amyotrophic lateral sclerosis (ALS) - frontotemporal dementia (FTD) continuum, we shed light on alterations in compounds of the kynurenine pathway (KP) as well. In the **General Introduction**, we hypothesized that NDDs might all be characterized by a different monoaminergic (and/or kynurenergic) profile in the brain, possibly reflected in body fluids. These different neurochemical patterns could aid in the identification of biomarkers, or help to provide evidence for a therapeutic approach targeting these pathways.

Regarding monoaminergic differences between NDDs in brain material, we can only discuss AD and behavioral variant FTD (bvFTD). From the two datasets regarding monoamine levels in AD included in this work, and previous research by other groups [526, 571], it appears that limbic serotonin (5-hydroxytryptamine; 5-HT) concentrations are persistently lower in AD when compared to bvFTD and CONTR. bvFTD can be distinguished from CONTR by decreased 5-HIAA/5-HT and MHPG/noradrenaline (NA) ratios in frontal and temporal lobes, while increased 5-HT and NA levels in the limbic system and temporal cortex seem to be determining for the bvFTD-AD comparison. We hypothesized that the relative higher degree of frontal atrophy in bvFTD could result in increased 5-HT levels, in turn causing disturbed glutamate neurotransmission, while a dysfunctional inhibitory control of 5-HT on the locus coeruleus (LC), might account for the increased NA levels.

Remarkably, we found that respective increased and decreased MHPG levels in CSF and serum were characteristic of dementia with Lewy bodies (DLB) relative to AD, bvFTD and CONTR. We linked this to a higher degree of neuropathological damage to the LC of DLB/Parkinson's disease dementia (PDD) patients when compared to

other NDDs included in the study, with a potential subsequent compensatory mechanism increasing noradrenergic turnover. Notably, in a previous study of our group [144], lower MHPG concentrations were found in the amygdala and thalamus of DLB patients with concomitant depression versus AD subjects with the same comorbidity, thus controlling for the confounding influence of depression. These findings are difficult to reconcile with increased MHPG levels in CSF and decreased levels in serum of DLB/PDD subjects. As this metabolite readily passes the blood-brain and blood-CSF barriers (BBB) [191, 192], the storage of MHPG into a CSF sink is highly unlikely. Furthermore, in the temporal cortex of DLB/PDD subjects, increased MHPG concentrations were found, while the LC did not show any difference in MHPG levels between DLB/PDD and AD or CONTR [144]. Thus, while our group and others [145, 146] have identified MHPG as a possible biomarker for DLB, the direction of change of this compound in body fluids remains controversial. Finally, it should be noted that sample handling considerably affects concentrations of this metabolite [196], which would compromise its utility as an inter-laboratory CSF biomarker for DLB/PDD.

In another study, we investigated the monoaminergic and kynurenergic pathways in the ALS-FTD continuum. Here, we found that a dopaminergic disturbance, particularly with regard to decreased CSF and serum 3,4-dihydroxyphenylacetic acid (DOPAC) levels, was typical of frontotemporal lobar degeneration (FTLD) and ALS compared to CONTR. We tried to explain these findings by the hypothesis that a dysfunctional presynaptic dopamine (DA) reuptake mechanism could result in higher extracellular DA levels and decreased DOPAC concentrations, as monoamine oxidase (MAO), mediating DA catabolism, is a mitochondrial (and thus intracellular) enzyme [590]. These dopaminergic disturbances could also be observed in our previous study on CSF and serum biomarkers with predominant focus on DLB/PDD, although non-significant decreases of CSF and serum DOPAC levels in FTLD versus CONTR were noted. Furthermore, in our study comparing brain monoaminergic content between AD and bvFTD, higher frontal DA levels compared to AD and CONTR were noted, which was in line with subsequent findings related to this neurotransmitter in CSF of FTLD subjects. In addition, patients suffering from ALS appeared to have similar dopaminergic characteristics as FTLD in serum, although less so in CSF. We found this surprising since neither DOPAC, nor DA cross the BBB [417, 418]. Our dopaminergic findings across studies including FTLD and bvFTD seem to suggest that these (sub)groups are characterized by the same neurochemical alterations versus CONTR, albeit only with respect to the DA system. Although this may be explained by the simple fact that the FTLD group investigated in the study on the ALS-FTD continuum, also comprised bvFTD subjects, it would also be interesting to have a look at possible dopaminergic disturbances in semantic, nonfluent and logopenic variant primary

progressive aphasias. Altogether, we propose that a shared dopaminergic disturbance is characteristic of FTLD and ALS, although more evidence should first be provided by other studies before these compounds can be considered potential biomarkers. Direct and indirect serotonergic control mechanisms on DA-release have previously been proposed (reviewed in [591]), so that it is difficult to speculate about the possible processes resulting in increased and unchanged DA and 5-HT levels, respectively. Nevertheless, ongoing work on brain tissue of neuropathologically confirmed ALS subjects will hopefully shed light on the apparent absence of 5-HT disturbance in biofluids. In anticipation of these results, we formulated a hypothesis linking glutamatergic and dopaminergic alterations observed in ALS and PD, respectively, to disturbed serotonergic control.

Regarding the kynurenine pathway (KP), almost no differences between FTLD, ALS and CONTR groups could be distinguished. Given the striking involvement of the KP in (neuro)inflammation [393, 421, 592], we expected to observe altered KP compounds at least in FTLD and ALS versus CONTR. Maybe these negative results point to a relatively early disease stage with discrete alterations in KP metabolism, or CONTR subjects may have had subclinical immune activation, potentially masking any differences. Furthermore, as the study groups were all age-matched and the KP is altered with aging [421, 422], it could be possible that this pathway predominantly reflected aging in this study. In an ongoing follow-up study on brain material, possible alterations might be more apparent.
In general, when we combine most significant monoaminergic alterations observed in this thesis into **Table 1**, we can conclude that:

- Only DLB/PDD and FTLD (including bvFTD) showed consistently altered monoamines/metabolites in both brain tissue and body fluids.
- Except for dopaminergic alterations in FTLD and ALS, the direction of change is variable.

Table 1. Overview of key monoaminergic changes in NDDs.

Sample type	AD	FTLD (including bvFTD)	DLB/PDD	ALS
Brain	5-HT ↓	5-HIAA/5-HT ↓ MHPG/NA ↓ DA ↑	MHPG ↑, ↓, =	Spinal cord: (5-HT ↑), NA ↑
CSF	NA ↓, DA ↑, MHPG/NA ↓ DOPAC/DA ↓ HVA/DA ↓	DA ↑, DOPAC ↓, DOPAC/DA ↓	MHPG ↑	DOPAC ↓
Serum	/	DA ↑, DOPAC ↓, DOPAC/DA ↓	MHPG ↓	DA ↑, DOPAC ↓, DOPAC/DA ↓

Alterations in monoaminergic compounds are all as observed versus CONTR groups. Cells indicated in gray contain data not included in previous chapters of this thesis [144, 449]. Abbreviations: 5-HIAA:

5-hydroxyindoleacetic acid; 5-HT: 5-hydroxytryptamine or serotonin; AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; bvFTD: behavioral variant frontotemporal dementia; DA: dopamine; DLB: dementia with Lewy bodies; DOPAC: 3,4-dihydroxyphenylacetic acid; FTLD: frontotemporal lobar degeneration; HVA: homovanillic acid; MHPG: 3-methoxy-4-hydroxyphenylglycol; NA: noradrenaline; PDD: Parkinson's disease dementia.

Like the work performed on circadian rhythmicity and the RCG, this chapter does not comprise novel research. Yet, our aim was to characterize distinct NDDs based on monoaminergic (and kynurenergic for FTLD and ALS) profiles, keeping in mind the possible identification of biomarkers for these diseases, and establishing potential pathophysiological roles. Moreover, our study populations were, in most cases, well characterized. In this respect, the fundamental work performed in this chapter did bring about new insights which may be of interest to future research.

Finally, **the fourth aim** was to pinpoint pathophysiological expression of G protein-coupled receptors (GPCRs) in AD using RT-qPCR, western blot and double labelling immunofluorescence. Except for the 5-HT_3 receptor, all monoaminergic receptors belong to the GPCRs and thus represent interesting research targets. Our main findings in AD subjects versus CONTR were increased transcript levels of the 5-HT_{1A} receptor (*HTR1A*) in hippocampus and amygdala, and of the β_3 adrenoceptor (*ADRB3*) in hippocampus. Decreased expression of *HTR7* was observed in Brodmann area (BA)10. We also tried to couple these findings to neuropsychiatric symptoms (NPS) and alterations in monoamine levels in these brain areas, which led us to conclude that *HTR1A* levels in hippocampus were inversely correlated with the presence of anxieties and phobias. A possible protective effect of the corresponding receptor for this type of NPS could thus be hypothesized, corroborating existing literature [584, 585]. Nevertheless, we did find contradicting expression levels of *HTR1A* in AD with respect to the existing literature. Although various methodological parameters could explain this paradox, we could rule out the confounding effect of medication.
Regarding the expression levels of cholinergic (*Chrm1*), glutamatergic (*Grm5*) and serotonergic (5-HT_6) GPCRs in hippocampal tissue of the APP23 mouse model, our results failed to provide convincing evidence for a pathological expression or role in AD. In the case of western blot and double-labelling immunofluorescence for the 5-HT_6 receptor, certainly methodological issues requiring further optimization have hampered a sounder understanding of this receptor's expression and localization in the brain of an AD mouse model.
The analysis of GPCR transcript levels and monoaminergic compounds in the brain of the same clinically well-characterized subjects was quite unique, certainly when coupled to behavioral alterations. However, as the part performed on murine brain remains largely inconclusive, these results could mainly be used as an indication

for future research strategies and could be helpful for the optimization of the used techniques.

2. LESSONS AND CONSIDERATIONS

It is clear that fundamental, descriptive work was performed in this thesis. Consequently, integrating our results into the pathophysiology of NDDs is often complicated by the lack of supporting, mechanistic evidence irrefutably linking the analyzed neurochemicals to the pathophysiological molecular mechanisms. Nevertheless, we may not lose sight of the initial goal of this thesis, which was to characterize NDDs based on their neurochemical profile in brain and body fluids. Here, I discuss some lessons learned, culminating into considerations for follow-up studies.

2.1. What constitutes a good biomarker?

Next to our main goal of determining monoaminergic characteristics of several NDDs, we also wanted to verify whether these neurochemical patterns could serve as biomarkers. Could we argue that, since the observed monoaminergic alterations in CSF and serum do not fully comply with the alterations in brain, these compounds do not carry any potential as biomarkers for NDDs? Most definitions imply that a biomarker should be objectively measured, serve as an indicator of a biological/disease process or a pharmacological response, and predict the outcome of these variables [593]. While the analysis of monoaminergic compounds in NDDs meets the first criterium, it is less clear whether the second and third assumptions are fulfilled as well. In some cases, it is plausible that monoaminergic compounds, such as MHPG in the case of DLB/PD(D), mimic the pathological process, although an unequivocal causal disease process does not appear to explain other findings observed in this work (**Table 1**). Indeed, the precise role of other biomarkers which can reliably be measured, e.g. microRNAs for cardiovascular disease [594-596], is not well understood. Consequently, correlating levels of such biomarkers with clinical variables may be highly significant, although evidence for a causal role of these analytes cannot be guaranteed [593]. Thus, the general lack of mechanistic evidence explaining our neurochemical observations, complicate their evaluation as biomarkers for NDDs. In conclusion, we were able to identify altered components of monoaminergic neurotransmitter systems in several NDDs, while establishing those analytes as suitable biomarkers will require more extensive research towards a clear-cut pathophysiological role. Conversely, if no such role can be determined, the validity of monoamines as biomarkers for NDDs will probably remain unsatisfactory. In this case, these neurotransmitters and/or their metabolites might still prove useful in the differential diagnosis of NDDs in combination with other biomarkers.

2.2. Are monoamines/kynurenines markers for NDDs or comorbidities/confounding factors?

Related to the previous paragraph, is the consideration that monoamines/kynurenines may predominantly reflect comorbidities such as depression or other NPS, rather than the actual NDD. In the **General Introduction**, it was stated that drugs targeting monoaminergic systems are mainly used to treat symptoms instead of the disease itself. As an example, the 'serotonin hypothesis' is a widely known theory implicating reduced activity of serotonergic pathways in the pathophysiology of depression [597, 598], while the 'dopamine hypothesis' attributes psychosis in schizophrenia to an imbalance in DA receptors 1 and 2 (D1 and D2) functioning [599]. Selective serotonin reuptake inhibitors for the treatment of depression, and antipsychotics (non-exclusively) targeting D2 receptors are thus pharmacotherapies interrelated with these two hypotheses. As depression and psychosis may manifest as additional symptoms in patients suffering from NDDs, how can we distinguish neurochemical alterations attributed to the disease itself from changes due to comorbidities? A good start would be to prospectively include study subjects with and without certain NPS, sample biological fluids during several follow-up visits, and finally investigate postmortem brain tissue. As such, a previous study by our group has examined monoaminergic alterations in brains of DLB/PDD subjects with and without depression and psychosis [144], pointing to serotonergic and dopaminergic disturbances in DLB not ascribed to NPS.

Medication and diet represent two possible sources of bias when measuring monoaminergic concentrations. Throughout this dissertation, we have addressed the influence of medication effects by repeating the analysis in a medication-free subgroup, or by verifying the increasing/decreasing effect of certain drugs on monoaminergic concentrations. As such, we identified a confounding effect of medication on MHPG/NA and 5-HIAA/5-HT ratios in FTLD brain (**Table 1**). A better approach would be to adopt regression models incorporating medication use when looking at the relationship between levels of a certain neurochemical and a NDD. However, strict assumptions should be met for linear, and to a lesser degree, logistic regression, hampering its all-round implementation.
Lastly, standardization of the timing of and nutrients included in every meal should also be aspired when analyzing monoamines, as high-carbohydrate or high-protein meals might alter brain monoaminergic content [190]. Of course, such levels of standardization and control can only be attained in the case of prospective studies with a high degree of compliance to the study setup.

2.3. Selection of study populations

All studies included in this work were retrospective. As discussed before, important disadvantages entail a lack of control on diet, medication and lifestyle. Moreover, although we identified optimal sampling times/methods for monoamine analysis in biological fluids, most samples had already been included in the NeuroBioBank before this study and consequently, we could not always implement our own suggestions. We could, however, still control for sampling time, diet, age at sampling or death, and gender. Perhaps the most important advantages of (some) retrospective studies included in this work, are the possibility to exclusively assess samples of subjects who received neuropathological confirmation of their clinical diagnosis, and to include larger sample sizes than would be possible in the case of a prospective study. This certainly contributed to the reliability of our findings, in particular with respect to the confounding influence of misdiagnosis. Thus, although prospective studies offer the possibility of high levels of standardization, they would take up too much time for a single PhD project, and importantly, they would increase the level of uncertainty brought about by analyzing probable instead of definite NDD cases.

Another consideration is the inclusion of an appropriate CONTR group. Each project presented in this work comprised its own CONTR subjects, with different characteristics across studies. One could question whether comparisons of each investigated NDD versus its own CONTR can be generalized to a virtually universal CONTR group, at least age- and gender-matched for all NDDs studied in this work. In most studies, exclusion criteria for CONTR subjects were similar, although for the work performed on neurochemical disturbances in the ALS-FTD continuum, peripheral neuropathy was an additional reason for exclusion. This consideration might account for the inconsistency regarding neurochemical alterations compared to CONTR across studies. Therefore, an elaborate clinical (and if possible, neuropathological) characterization of CONTR groups is indispensable when comparing such alterations between studies.

2.4. Selection of sample type: brain, cerebrospinal fluid or serum/plasma?

Throughout this thesis, brain, CSF and serum/plasma samples were analyzed for neurochemical content. However, alterations in brain and biological fluids did not seem to be consistent for (i) the type of neurotransmitter system displaying (pathophysiological) changes and (ii) the direction of change. Thus, several factors appear to complicate a straightforward comparison between different compartments.

With respect to brain and CSF, it is generally accepted that neurochemical processes in the former compartment are reflected in the latter given their close

anatomical contact [600-602]. Nevertheless, some authors have argued against this assumption with the notion that discrete alterations in confined brain areas may not be measurable in a larger compartment such as CSF [319]. Another reason which may explain the often observed discrepancy between alterations in brain and CSF, is that we homogenized brain tissue as part of the sample preparation protocol. In this manner, both extra- and intracellular neurotransmitters and metabolites are obtained and measured simultaneously. However, an alternative would be to either use microdialysis, having limited feasibility in a clinical setting, or to use radioligand binding assays to analyze receptor binding potential instead of receptor densities. Earlier on in **Chapter V**, we discussed that we could not distinguish between pre- and postsynaptic GPCR densities. This problem could possibly be solved by implementing imaging techniques such as single photon emission computed tomography (SPECT) or positron emission tomography (PET) using a specific ligand to pre- or postsynaptic receptors. Most of these applications to date have been performed to measure D2 density or blockade and DA transporter (DAT) binding in the differential diagnosis of parkinsonian syndromes [412, 413, 603]. Of course, the interpretation of these results highly depends on the specificity of the ligand, for both *ex vivo* radioligand binding assays, as well as *in vivo* SPECT/PET.

In contrast to the brain-CSF comparison, we would expect dissimilarities of monoaminergic content between brain and serum/plasma, since none of the catecholamines, nor 5-HT, cross the BBB [414, 604]. In this respect, blood biomarker potential of monoamines could be impacted, as changes in the brain could not be reliably measured in the blood compartment. Furthermore, each monoamine also has its own peripheral synthesis, metabolism and functions, possibly interfering with the measurement of centrally derived levels. Taking into account all of the above, should we use brain, CSF or serum/plasma to perform neurochemical analysis? Each sample type has its advantages and limitations, and it would therefore be ideal to include all of them, in conjunction with a measure of BBB integrity such as CSF albumin/serum albumin ratios [605]. As stated previously, one should also take into account the feasibility of such setups. In conclusion, it would probably be a more fundamentally sound approach to capture neurochemical changes in CSF, in spite of the invasiveness of a lumbar puncture. Here, confounding effects of peripheral monoamines are limited in case of an intact BBB, and extracellular concentrations are measured.

2.5. Mimicking Alzheimer's disease: the APP23 model

In **Chapters III and V** of this thesis, the APP23 amyloidosis mouse model was implemented to mimic AD. Whereas a considerable amount of practical work was attempted on *ex vivo* tissue, we could only draw conclusions pertaining to the use

of B elements as reference genes and the brain-region specific *APP* overexpression in HEM animals of different ages. One could argue that overexpression of *APP*, in the presence of merely hyperphosphorylated tau without the formation of NFTs [251], has limited (patho)physiological meaning with respect to pathological processes in AD. However, patients with Down syndrome carry an extra *APP* allele and are more likely to develop early-onset AD than the general population [606]. On the other hand, NFTs are present in Down syndrome with concomitant AD, with a very similar distribution pattern to AD [606, 607]. So why does the APP23 model, as well as most other APP-based mouse models, aged canines and senescence-accelerated mice [608-613], fail to display NFTs? Although the answer is still elusive, it has been established that a three-dimensional model containing human neurons bearing mutations in *APP* and *PSEN1*, shows amyloid pathology as well as NFTs [614]. Could the development of tau pathology second to *APP* overexpression be species-specific? If so, it would be tempting to shift AD-research from animal models to *in vitro* models containing human-derived cells, such as cerebral organoids [615, 616], particularly when studying molecular mechanisms. However, a key disadvantage would be the missed opportunity to monitor cognitive and behavioral responses upon administration of a therapeutic agent, favoring the use of animal models.
The fact that GPCR density analyses using western blot and immunofluorescence did not yield conclusive results, was primarily due to insufficient optimization. However, although APP23 mice constitute a partial AD model displaying some aspects of construct, face and predictive validity [253, 612], it still needs to be elucidated whether or not the APP23 model is suitable for the analysis of neurotransmitter/receptor disturbances, certainly with respect to monoaminergic systems [617].

3. FUTURE DIRECTIONS

Where do we go from here? While this doctoral thesis provided a couple of significant contributions with respect to methodological aspects of measuring neurochemical changes in NDDs, as always, some other aspects of this work could be improved and some techniques should be further optimized. These suggestions are listed in previous paragraphs.

Among all tested neurochemical compounds, altered CSF and serum MHPG levels as a characteristic for DLB/PDD probably show the most (biomarker) potential. Consequently, the implementation of this metabolite in a prospective longitudinal study on DLB/PDD would shed more light on its external validity, i.e. in other study populations, and on the possible progressive changes of this metabolite, which

would provide more certainty of a close interrelationship with the pathophysiological process.

Furthermore, seemingly negative and/or conflicting results regarding alterations in monoamines or kynurenines might also be due to the use of suboptimal analysis methods. As an example, radioligand binding, surface plasmon resonance or imaging techniques could provide more valuable information on disturbed neurotransmission than analyzing raw levels of either ligand or receptor, without an indication about the functional interaction. If this challenge could be overcome, we would immediately face another one: how could we link the alterations in one or a few brain regions to the pathological process? Are neurochemical alterations rather a result of the pathology, or are they able to induce pathology? Probably, the answer to this question is a combination of both. Investigating intracellular signaling upon exposure to Aβ peptides in GPCR cell lines, or using methods such as chemo- or optogenetics to control the activation of certain GPCRs ('Opto-GPCRs' [618]), followed by studying downstream effects on NDD pathology, might represent mechanistic strategies to solve this problem. Combining said methods into a multidisciplinary approach, could help to gain a sounder understanding of the pathophysiological implication of GPCRs in NDDs. Furthermore, these interventions may also lead to the identification of (ant)agonism effects on the pathological process, which could subsequently be translated into a therapeutic option. In turn, these new therapeutic strategies, which may consist of multiple ligands exerting their effects on distinct receptors, may then be tested in animal models for their influence on the symptomatology of NDDs. All of the above represents a tremendous amount of work which can only be achieved by combined international efforts from multiple laboratories.

Regarding AD, alterations in various neurotransmitter systems were found, hampering the definition of a clear-cut therapeutic implication. Nevertheless, the use of selective serotonin reuptake inhibitors (SSRIs) or serotonin noradrenaline reuptake inhibitors might counter the reduced 5-HT and NA concentrations we observed in this dissertation. Clinical evidence supports this suggestion [619], although an earlier meta-analysis regarding the use of SSRIs in AD could not corroborate a positive effect of this treatment on depressive and cognitive symptoms [620]. In FTLD, it may be worthwhile to implement a 5-HT_{1A} antagonist, such as WAY100635, as this may restore frontal glutamatergic neurotransmission. In addition, the dopaminergic disturbance in terms of increased DA levels, and decreased DOPAC concentrations and DOPAC/DA ratios in this disorder may also be accounted for. As such, the administration of WAY100635 was previously shown to decrease DA release in the prefrontal cortex, possibly mediated via glutamatergic pyramidal neurons [621]. Conducting a first study in an FTLD animal

model, such as the Tau58/4 or Tau58/2 models available in our lab, could verify the potential beneficial effects of this serotonergic receptor antagonist on executive function, e.g. reversal learning [622], and other behavioral disturbances such as aggression and disinhibition. Regarding DLB/PD(D), engrafting embryonic stem cells or induced pluripotent stem cells to reverse damage to dopaminergic and noradrenergic nuclei, has previously shown preclinical benefit [623-625]. However, clinical results are contradictory and concerns regarding the tumorigenic potential of stem cell therapy, as well as ethical concerns, may impede its widespread clinical application [625]. Our findings in ALS and FTLD indicated a decreased dopaminergic metabolism, which we ascribed to a potential dysregulation of DAT in presynaptic terminals. Thus, exploring the effect of increasing the functionality and/or expression of this transporter may be of added value. Activating extracellular signal regulated kinase via (partial) agonism of D2, which also interacts with DAT to promote its cell surface expression [626, 627], might reduce extracellular DA levels. Alternatively, since we hypothesized that FTLD and ALS may be characterized by a shared dopaminergic disturbance, WAY100635 might also be implemented in ALS. Finally, it should be noted that all of these suggestions should first be thoroughly investigated in a preclinical setting.

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Curriculum vitae

PERSONAL INFORMATION

Name	Jana Janssens
Birth date	02/04/1992
Nationality	Belgian
Living area	Lier
E-mail	jana.janssens2@gmail.com

EDUCATION

November, 2015 - present

PhD student at the Laboratory of Neurochemistry & Behavior

University of Antwerp – Institute Born-Bunge

Thesis "Identifying dysfunctional monoaminergic neurotransmission in neurodegenerative disorders: methodological considerations and fundamental insights"

Promotors: Prof. dr. P.P. De Deyn and dr. D. Van Dam

October, 2013 – June, 2015

Master in Biomedical Sciences – Neurosciences

University of Antwerp – Institute Born-Bunge

Laboratory of Neurochemistry and Behavior

Thesis "Defining the prefrontal monoaminergic neurotransmission in frontotemporal dementia compared to Alzheimer's disease: association with neuropsychiatric symptoms"

Promotors: Prof. dr. P.P. De Deyn and dr. D. Van Dam

- Great distinction

October, 2010 – June, 2013

Bachelor in Biomedical Sciences

University of Antwerp

Thesis "NEBs and electron microscopy: past, present and future" (Dutch)

Promotor: dr. I. Pintelon

- Greatest distinction

CERTIFICATES

October – November, 2014	**FELASA Category C Certificate** University of Antwerp Basic course in Laboratory Animal Science
February – June, 2017	**French level 5 Certificate (CEFR B1-B2)** Linguapolis – University of Antwerp - 90%; ECTS A
April, 2018	**Multivariate Statistics** STATUA – University of Antwerp
June, 2019	**Categorical data analysis using logistic regression** STATUA – University of Antwerp

INTERNATIONAL CONFERENCE PARTICIPATIONS

16th – 19th July, 2017	**Poster presentation at AAIC 2017, London.** Biological fluid monoamine levels to explore the neurochemical continuum between frontotemporal dementia and amyotrophic lateral sclerosis.
25th – 30th March, 2019	**Oral presentation at AD/PD 2019, Lisbon.** The kynurenine pathway in amyotrophic lateral sclerosis and frontotemporal dementia: disparities of xanthurenic acid levels in cerebrospinal fluid.

PUBLICATIONS

2016	Vermeiren Y.*, Janssens J.*, Aerts T., Martin J-J., Sieben A., Van Dam D., De Deyn P. P. Brain Serotonergic and Noradrenergic Deficiencies in Behavioral Variant Frontotemporal Dementia Compared to Early-Onset Alzheimer's Disease. Journal of Alzheimer's disease 53(3): 1079-96.
2018	Janssens J.*, Vermeiren Y.*, Fransen E., Aerts T., Van Dam D., Engelborghs S., De Deyn P. P.

Cerebrospinal fluid and serum MHPG improve Alzheimer's disease versus dementia with Lewy bodies differential diagnosis

Alzheimer's and Dementia: Diagnosis, Assessment and Disease Monitoring 10: 172-181.

2018 Vermeiren Y., Janssens J., Van Dam D., De Deyn P. P.

Serotonergic dysfunction in amyotrophic lateral sclerosis and Parkinson's disease: similar mechanisms, dissimilar outcomes

Frontiers in neuroscience - ISSN 1662-453X-12.

2019 Janssens J.*, Atmosoerodjo S. D.*, Vermeiren Y., Absalom A. R., den Daas I., De Deyn P. P.

Sampling issues of cerebrospinal fluid and plasma monoamines: investigation of the circadian rhythm and rostrocaudal concentration gradient

Neurochemistry International 128: 154-162.

2019 Crans R. A. J.*, Janssens J.*, Daelemans S., Wouters E., Raedt R., Van Dam D., De Deyn P.P., Van Craenenbroeck K., Stove C. P.

The validation of Short Interspersed Nuclear Elements (SINEs) as a RT-qPCR normalization strategy in a rodent model for temporal lobe epilepsy

PLoS ONE - ISSN 1932-6203-14:1.

2019 Janssens J.*, Crans R. A .J.*, Van Craenenbroeck K., Vandesompele J., Stove C.P., Van Dam D., De Deyn P. P.

Evaluating the applicability of mouse SINEs as an alternative normalization approach for RT-qPCR in brain tissue of the APP23 model for Alzheimer's disease.

Journal of Neuroscience Methods 320:128-137.

*Shared first authors.

SUBMITTED MANUSCRIPTS

2019 Van Erum J., Faulí Perpiñá S., Janssens J., Van Dam D., De Deyn P.P.

Sleep architecture changes in the Tau58/4 mouse model of tauopathy.

Neurobiology of Disease

2019	Janssens J., Vermeiren Y., van Faassen M., van der Ley C., Kema I. P., De Deyn P. P. Monoaminergic and kynurenergic characterization of frontotemporal dementia and amyotrophic lateral sclerosis in cerebrospinal fluid and serum. Neurochemical Research

EDUCATIONAL ACTIVITIES

Academic years 2016-2017, 2017-2018 & 2018-2019	Research methodology for students in the 2nd Bachelor year Rehabilitation sciences and Physiotherapy. "Pitfalls when interpreting data" (Dutch) "Critical evaluation of published articles" (Dutch)
Academic years 2016-2017, 2017-2018 & 2018-2019	Behavioral neuroscience for students in the 1st Master year Biomedical Sciences – Neurosciences "The cerebellum: a focus on non-motor functionality"
Academic years 2016-2017, 2017-2018 & 2018-2019	Practical assistant laboratory animal sciences for students in the 2nd Master year Biomedical sciences and Biochemistry & Biotechnology

SUPERVISION OF BACHELOR/MASTER PROJECTS

Academic year 2016-2017	**Bachelor's thesis Susan Gomes, Biomedical Sciences (University of Antwerp)** GPCR heterodimerization in Alzheimer's disease: pathophysiology and therapeutic possibilities (Dutch) **Bachelor's thesis Eline Goffin, Chemistry-Life Sciences (University of Hasselt)** The effect of hemolysis on biogenic amine concentrations in mouse serum samples
Academic year 2017-2018	**Master's thesis Anna Helsen, Biomedical Sciences-Neurosciences (University of Antwerp)** Monoaminergic characterization of delirium among elderly patients with(out) cognitive impairment following hip fracture

Master's thesis Filipa Alves Pita Pilkington Ferro, Biomedical Sciences-Neurosciences

Circadian rhythm effects on CSF and plasma monoamines in healthy volunteers: relationship with melatonin and investigation of the rostrocaudal concentration gradient

Academic year 2018-2019

Master's thesis Charlotte Jacob, Biomedical Sciences-Neurosciences (University of Antwerp)

Unraveling the norepinephrinergic pathophysiology in Parkinson-plus syndromes

Bachelor's thesis Lara De Deyn, Biomedical Laboratory Technology (Artesis-Plantijn College, Antwerp)

Techniques used to unravel the density of neurotransmission-related G protein-coupled receptors in APP23-mouse model

Bachelor's thesis Clara Bartra Cabré, Biology (University of Barcelona)

Serotonergic receptor 6 quantification and localization in the APP23 amyloidosis mouse model for Alzheimer's disease

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"Oh, well, I don't mind if you don't mind

'Cause I don't shine if you don't shine

Before you go, can you read my mind?"

- The Killers

Based on previous experience, I know this part of my thesis will probably be the most popular one. Honestly, I never thought I would reach this stage. Many lows and a few highs have brought me here, and I would like to sincerely thank the people who made me put the lows in perspective and helped me appreciate the highs.

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PhD-related stress home. I'm sorry you couldn't do so many things you wanted to because of me. Evidently, I can't thank you enough for always being there for me and for providing a comforting atmosphere at home. Thank you for everything you do for me. I hope our future together will be Wonderful Wonderful.