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Serotonin System in Alzheimer’s Disease: from a Molecular and Biomarkers Perspective

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The cover picture of this thesis is designed by Mustafa Kamil Mustafa form an HPLC-chromogram of 5-HT metabolites provided by Prof. Takashi Yoshitake.
Serotonin System in Alzheimer’s Disease:
from a Molecular and Biomarkers Perspective

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my beloved family,
ABSTRACT

Serotonin, 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter with a wide range of biological functions. It contributes to the integrity, connectivity and function of the central nervous system (CNS). Here in this thesis, we aim to understand molecular and clinical changes of the serotonergic systems in Alzheimer’s disease (AD) and related neurodegenerative diseases.

We studied several aspects of the serotonin system, focusing on the 5-HT1B receptor and related molecules. Different models and samples were used to explore this, such as cell lines, transgenic animals, human platelets and brain tissues.

In paper I, we showed that the 5-HT1B receptor, s100A10 (p11), 5-HT, 5-HIAA and MAO-A enzyme activity were altered in the neuroblastoma cell line, SH-SY5Y stably transfected with the human APP (amyloid precursor protein) gene with the double APP Swedish mutation (APPswe). There was a reduction of extracellular 5-HT levels measured in cell medium. Moreover, higher 5-HT degradation indices were observed in APPswe cells. A similar pattern of proteomic changes was observed in the mice model of AD (Tg2576). Expression of 5-HT1B and the serotonin transporter (SERT) are reduced in 2-year-old female mice. These findings suggest that the decrease in 5-HT1B in these models might be a compensatory biological mechanism to the decreased extracellular 5-HT levels. Together these findings indicate an effect of APPswe mutation on the serotonergic system, modulating 5-HT1B and 5-HT metabolites.

In paper II, we showed that the normal compensatory upregulation of SERT gene expression, after the pharmacological blockade of 5-HT1B receptor, is lost in APPswe cells. The effect of 5-HT1B modulations on p11 gene expression is complex. Treatment with 5-HT and 5-HT1B antagonist decreases p11 gene expression in both APPswe cells and control cells. 5-HT also increases MAO-A gene expression and the production of 5-HIAA in APPswe cells. In addition, our results suggest that the MAPK signaling pathways is affected differently in APPswe by 5-HT and 5-HT1B.

In contrast to control cells, sertraline, a selective serotonin reuptake Inhibitor (SSRI), reduces intracellular 5-HT levels and increases indices of 5-HT breakdown (5-HIAA/5-HT ratio) in APPswe cells. However, an inhibitory effect of sertraline on MAOA-A activity was also observed in APPswe cells. Importantly, no change on extracellular 5-HT or 5-HIAA was seen. We suggest that these changes are SERT and MAO-A-independent and could be attributed to biochemical interactions between Aβ peptides and 5-HT pathway molecules.

The results, in this study, illustrate the differences in 5-HT1B-related molecules in AD and physiological models when extracellular 5-HT levels and 5-HT1B receptor are modulated.
In papers III and IV we explored the association of the serotonergic markers to cognitive decline, synaptic biomarkers and pathological changes in people with dementia. In paper III, we demonstrated that AD patients have lower 5-HT levels in their medium-density platelet fractions compared to the group with subjective cognitive impairment (SCI). In the SCI group, those with low platelets 5-HT have higher cerebrospinal fluid (CSF) total tau and tau/\(\alpha\)B42 ratio than those with high 5-HT.

These findings suggest that platelet 5-HT has a potential for being an early proxy for CSF biomarkers in AD.

In paper IV, our results showed alteration in 5-HT1B levels in the postmortem prefrontal cortex (PFC) of people with AD and other dementias, including dementia with Lewy bodies (DLB) and Parkinson’s disease dementia (PDD). Higher levels of 5-HT1B were observed in AD and DLB groups. Furthermore, 5-HT was reduced in DLB, and 5-HIAA was low in all dementia groups. There were significant associations between 5-HT and pathological markers and synaptic proteins in DLB. Higher 5-HT1B levels were associated with more rapid cognitive decline in AD, PDD and combined dementia group.

The study indicates an association between prefrontal 5-HT1B levels and cognitive decline in dementia. Furthermore, it suggests a potential benefit of 5-HT1B antagonists in enhancing memory function in dementia.

PMID: 25841787


**Pharmacological modulations of the serotonergic system in a cell-model of familial Alzheimer's disease.**

PMID: 27163811


PMID: 27163811

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LIST OF ABBREVIATIONS

5-HT 5-Hydroxytryptamine
5-HIAA 5-Hydroxyindoleacetic acid
5-HP 5-Hydroxytryptophan
5-HT1A 5-Hydroxytryptamine (serotonin) receptor 1A
5-HT1B 5-Hydroxytryptamine (serotonin) receptor 1B
5-HT1BKO 5-HT1B receptor Knockout models
5-HT1D 5-Hydroxytryptamine (serotonin) receptor 1D
5-HT2A 5-Hydroxytryptamine (serotonin) receptor 2A
5-HT3 5-Hydroxytryptamine (serotonin) receptor 3
5-HT4 5-Hydroxytryptamine (serotonin) receptor 4
5-HT5 5-Hydroxytryptamine (serotonin) receptor 5
5-HT6 5-Hydroxytryptamine (serotonin) receptor 6
5-HT7 5-Hydroxytryptamine (serotonin) receptor 7
AChE Acetylcholinesterase
AD Alzheimer's Disease
ADHD Attention Deficit Hyperactivity Disorder
APCs Antigen Presenting Cells
APP Amyloid Precursor Protein
ATD Acute Tryptophan Depletion
Aβ Amyloid-β
BBB Blood Brain Barrier
cAMP cyclic Adenosine mono-Phosphate
CA1 Region I of hippocampus proper
CNS Central Nervous System
DBS Dorsal Brain Stem
DSM Diagnostic and Statistical Manual of Mental Disorders
DLB Dementia with Lewy Bodies
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>DRN</td>
<td>Dorsal Raphe Nucleus</td>
</tr>
<tr>
<td>ECD</td>
<td>Electrochemical Detection</td>
</tr>
<tr>
<td>FGFR1</td>
<td>Fibroblast growth factor receptor 1</td>
</tr>
<tr>
<td>FTD</td>
<td>Frontotemporal Dementia</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal Tract</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>GP</td>
<td>Globus Pallidus</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G protein–coupled receptors</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>ICD 10</td>
<td>International Classification of Disease, Tenth Edition</td>
</tr>
<tr>
<td>ISHH</td>
<td>In Situ Hybridization histochemistry</td>
</tr>
<tr>
<td>LTM</td>
<td>Long-term memory</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinases</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine Oxidases</td>
</tr>
<tr>
<td>MAOIs</td>
<td>Monoamine Oxidase Inhibitors</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild Cognitive Impairment</td>
</tr>
<tr>
<td>MDD</td>
<td>Major Depressive Disorders</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MMSE</td>
<td>Mini–Mental State Examination</td>
</tr>
<tr>
<td>mRNA</td>
<td>messanger Ribonucleic Acid</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NKCs</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary Tangles</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>OCD</td>
<td>Obsessive–Compulsive Disorder</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's Disease</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal Cortex</td>
</tr>
</tbody>
</table>
p-tau  Phosphorylated tau
PTSD  Posttraumatic Stress Disorder
RAB3A  Ras-related protein
RBCs  Red Blood Cells
RES  Reticuloendothelial system
RN  Raphe Nuclei
SCI  Subjective Cognitive Impairment
SERT  Serotonin Transporter
SN  Substantia Nigra
SNAP25  Synaptosomal-associated protein 25
SSRIs  Selective Serotonin Reuptake Inhibitors
STM  Short-term memory
S100A10  S100 calcium-binding protein A10
TCA  Tricyclic Antidepressant
Tph  Tryptophan hydroxylase
VMAT  Vesicular monoamine transporter
VaD  Vascular Dementia
vWF  von Wilbrand Factor
WHO  World Health Organization
1 INTRODUCTION

The human central nervous system (CNS) consists of heterogeneous populations of neuronal and non-neuronal cells with complex interactions and evolutions[1]. Synapses connect cells and therefore are important for anatomical, structural and functional integrity of CNS. Synaptic loss and synaptic protein alterations are implicated in the pathophysiology of neurodegenerative diseases[2]. Studying the neuropathology associated synaptic dysfunctions and neurotransmitters alterations of dementia are important in understanding the clinical presentation or designing a new disease-modifying drug.

Complex neurochemical signaling, pre and postsynaptic membrane related events constantly follow the release of a neurotransmitter. These eventually lead to generation and propagation of an action potential, which is the electrical message between neuronal cells at synaptic spaces. There are different types of neurotransmitters that are well characterized in terms of structure, physiological function and neuroanatomical localization in CNS. Epinephrine, acetylcholine, dopamine and serotonin (5-HT) are some examples of these neurotransmitters.

1.1 Serotonin

1.1.1 Biological roles of serotonin

Serotonin or 5-hydroxytryptamine (5-HT) has the old name enterochromaffin substance. The word (entero) refers to the gastrointestinal tract (GIT), which is the organ where 5-HT expression is most abundant[3]. 5-HT was first proposed as a neurotransmitter after studies confirmed the availability of 5-HT is the human brain[4]. 5-HT as a neurotransmitter mediates and modulates several physiological functions.

From a physiological point of view, 5-HT is associated with cognition[5], locomotor functions[6], mood stability[7] and the regulation of the sleep/wake cycles[8]. In addition, it is important in the regulation of many visceral and vital functions such as gut, bladder, ejaculatory and cardiovascular control[9] and the regulation of platelet aggregation in blood hemostasis[10]. It is also associated with a wide range of behavioral modalities[11] like impulsive aggression[12], suicidal thoughts and events[13] and alcohol or substance addiction[14, 15].

Similar to the dopamine system, recent pharmacological and electrophysiological studies have demonstrated a role of 5-HT system in the emotional and cognitive mediation of reward representation[16].
1.1.2 Neurochemistry of the 5-HT pathway

5-HT has the “molecular formula C_{10}H_{12}N_{2}O and a molecular weight about 176 gram per mole” PubChem Compound Database. The chemical structure of serotonin is shown in Figure 1.1

![Chemical structure of serotonin](image)

Figure 1.1 The chemical structure of serotonin. Taken from the open chemistry database Pubchem, National Center for Biotechnology Information. PubChem Compound Database; CID=5202. https://pubchem.ncbi.nlm.nih.gov/compound/5202 (accessed June 23, 2016).

1.1.2.1 Serotonin biosynthesis

Serotonin (5-hydroxytryptamine, 5-HT) is derived from the essential amino acid L-Tryptophan after two main enzyme-dependent chemical conversions[17].

1. The first main step is achieved by the enzyme tryptophan-5-hydroxylase (Tph), where tryptophan is converted to 5-hydroxytryptophan (5-HP). Conversion of L-Tryptophan to 5-HP is the first and rate-limiting step in 5-HT biosynthesis. However, it includes a complex process composed of more than four sub-biochemical reactions.

2. The second step in 5-HT biosynthesis is a decarboxylation reaction for 5-HP into 5-HT.

The hydroxylation of L-Tryptophan is regulated by different chemical, physical and biological conditions. First, the intracellular levels of L-Tryptophan can modulate Tph activity[18]. Increased supply of the substrate L-tryptophan in animal infusion studies lead to increase 5-HT synthesis in different body organs including the brain and GIT[18]. Intuitively, factors such as concentrations of cofactors and enzyme inhibitors also influence L-Tryptophan hydroxylation.

The extracellular level of 5-HT is an important regulator of 5-HT synthesis process. This represents a negative auto-regulation at the presynaptic membrane level, which has a dual effect on intracellular 5-HT biosynthesis and extracellular 5-HT release[11]. 5-HT1A and 5-HT1B are
particular 5-HT receptor subtypes that are involved in this process. Therefore, molecular and physiological factors affecting their expression would indirectly influence 5-HT hemostasis[19]. After synthesis, 5-HT is taken into the intracellular vesicles via vesicle monoamine transporters (VMATs), which facilitate 5-HT uptake and storage into specialized presynaptic vesicles.

1.1.2.2 5-HT turnover

5-HT is degraded to 5-hydroxyindolacetoacetic acid (5-HIAA) by the neuronal monoamine oxidase (MAO) enzyme. First discovered by Hare MLC in 1928 and given a name of tyramine oxidase, MAOs catalyze the metabolism of a wide range of primary, secondary and tertiary amines[20]. In contrast to acetylcholine metabolism, 5-HIAA cannot be recycled into the 5-HT pool.

MAO-based 5-HT metabolism is an oxidation reaction that requires both oxygen and water. The localization of MAO in the outer mitochondrial membrane is functionally relevant, since it makes the enzyme prone to competition for oxygen by other oxygen consumer enzymes such as cytochrome C[21]. Moreover, MAO is not specific to 5-HT and has other monoamine substrates such dopamine, noradrenaline (NA) and adrenaline[22]. There are two different MAO isozymes, which are encoded by two distinct genes[23], and the enzyme is classified according to the substrate specificity and inhibitors sensitivity into isozyme A and B[21]. Beside the similarity in functions and the types of substrates, MAO-A and MAO-B are structurally identical in about 70% of the amino acid sequence[24].

Although MAO-A is relatively more specific to 5-HT and the main enzyme in 5-HT metabolism, 5-HT is also metabolized by MAO-B in brain and the GIT. Different biochemical factors determine the isozyme-switch between MAO-A/MAO-B in 5-HT metabolism. In addition to the type of biological tissue, the preference of the isozyme subtype in 5-HT metabolism depends on the availability of 5-HT. In brain, 5-HT is exclusively metabolized by MAO-A in conditions of low concentrations. Even in high 5-HT concentrations, MAO-B contributes by only 10% to 5-HT metabolism[21]. The expression of MAO-A and MAO-B has significant variations across different types of tissues. This variability in expression reflects the functional variability in serotonin metabolism and transmission depending on body systems[25]. Platelets are example of tissue that expresses only MAO-B.

MAO is subjected to a group of endogenous inhibitors that modulate the enzyme kinetics[26, 27]. For example, the small molecule neurocatin was shown to maintain 5-HT levels and inhibit the catabolism of 5-HT by inhibiting MAO[28, 29]. Moreover, two distinct isoquinolinium molecules show a significant inhibitory effect on MAO-A[30]. Similarly, smoking was also reported as inhibitor for both MAO-B[31] and MAO-A[32]. Inhibition of MAO is an
old and well-known strategy in the treatment of depression. The monoamine oxidase inhibitors (MAOIs) are classical class of antidepressants. Moreover, the efficacy of MAOIs in posttraumatic stress disorder (PTSD) indicates that stressful condition might contribute to the modulation of MAO[24, 33].

However the clinical application of MAOIs is limited by, among others, the interactions with milk products, smoking and alcohol [34]. A pattern of bidirectional interactions between MAO subtypes and alcohol was observed from previous reports. Alcohol is associated with modulation of MAO activity. On other hand, certain MAO-A gene mutations are associated with alcohol addictions[35]. Moreover, lower MAO-B activity is demonstrated in Alcoholism[36].

5-HIAA is an indicator of 5-HT turnover or serotonergic neuronal activity, and is considered as a valuable biomarker in 5-HT over-production disorders. For example, in case of 5-HT-secreting tumors such as carcinoid tumors typical clinical symptoms, radiological evidence of gastrointestinal mass and increased levels of 5-HIAA in the urine are the diagnostic triad of the tumor diagnosis[37]. Since the balance between 5-HT biosynthesis and degradation is an important measure in 5-HT hemostasis, 5-HIAA/5-HT ratio could also be a better reflection of the 5-HT turnover than levels of 5-HIAA alone[21]. The superiority of 5-HIAA/5-HT ratio comes from the fact that it takes into consideration the amount of 5-HT available in a particular system.

1.1.3 Neuroanatomy of serotonin

Anatomical considerations are essential in understanding the function of 5-HT system in both physiological and pathological states. Of note, the serotonergic system is not an isolated entity and has dense interactions with other non-serotonergic systems.

1.1.3.1 Serotonergic neuron in CNS

The presence of the serotonergic cell bodies in the brain stem was proposed by Dahlström and Fuxe in 1965[38]. With serotonergic neurons originating in the raphe nucleus (RN) in the brainstem, the serotonergic system represents the most abundant monoamine system in the CNS[39]. In a non-overlapping manner, serotonergic fibers form wide projections to different brain regions and the spinal cord[40, 41]. There are ascending serotonergic projections to septa, thalamus, hypothalamus, putamen, hippocampus and different cortical areas[41, 42]. These ascending projections are separated according to the origin from the RN. The median raphe nucleus projects to the forebrain and the medial part of septa, while the dorsal raphe nucleus innervates the frontal cortex, hippocampus and the striatum[42].
1.1.3.2 5-HT1B receptor mapping in CNS

Currently, 14 subtypes of 5-HT receptors are characterized in the human brain[43]. Each 5-HT receptor has a specific expression pattern in CNS. This multiplicity adds to the functional, physiological and pharmacological complexity of 5-HT system.

5-HT1A and 5-HT1B receptors are expressed in both pre and postsynaptic neurons, with a predilection to the presynaptic expression[44-47]. The 5-HT1B receptor is localized to both cell bodies and axon terminals. The receptor protein is trafficked from cell bodies through the axons to neuronal terminals[48]. In 5-HT1B knockout (5-HT1BKO) mice, overexpression of 5-HT1B in the striatum leads to specific transport to the globus pallidus (GP) and the substantia nigra (SN)[44, 49]. In agreement with these findings, binding and immunohistochemistry studies have showed that 5-HT1B has a dense distribution in the basal ganglia nuclei such as GP, SN and the hippocampal area subiculum[44, 50-53]. Lower 5-HT1B receptor densities were observed in the hypothalamus, striatum, hippocampus, spinal cord and amygdala[44, 53-55]. However, there is a discrepancy between the receptor protein and messenger RNA (mRNA) distributions evaluated with In Situ Hybridization histochemistry (ISHH)[44].

In contrary to the 5-HT1B receptor protein expression, mRNA binding studies showed no binding in SN and GP[45], and low levels in RN and striatum[56]. 5-HT1B has 5 major locations in terms of availability according to the majority of gene expression reports[44]:

- Striatonigral pathway.
- Raphe nucleus.
- Other basal ganglia nuclei (putamen, caudate and globus pallidus).
- Cerebellum.
- Hippocampus: pyramidal neurons of region I of hippocampus proper (CA1)[57].

1.1.3.3 Serotonin transporter (SERT, SLC6A4)

The function of 5-HT transporter (SERT) is to reuptake serotonin from the synapses or extracellular space to the intracellular space. It represents the high affinity return system of 5-HT, which is a sodium (Na) and chloride (Cl) dependent mechanism. Using 5-HT1BKO animals, it was shown that the mechanism by which SERT regulates 5-HT transmission is complex and includes collateral signaling with other molecules such as 5-HT1B (Figure 1.2). In addition, 5-HT1B antagonism, using the 5-HT1B selective antagonist SB224289, decreases SERT function[58].
SERT is expressed in brain, pulmonary vasculature, placenta and platelets. However, studies have shown that the brain SERT is the main site of action of antidepressants[59, 60].

Functionally, SERT has also a neurodevelopmental role in adjustment of the cortical sensory mapping during the critical period of brain development[61]. The distribution of SERT is also relevant to the neuropathology of affective disorders. Lower densities of SERT were reported in patients diagnosed with depression. Similarly, SERT densities are lower in winter compared to summer in healthy subjects, which suggests a role for SERT distribution in affective seasonal mood disorders[62].

Following the 5-HT neuronal projection, SERT protein is widely expressed in the human brain. It represents a marker for 5-HT projection mapping. All brain areas express more or less SERT, with the cerebellum being the lowest SERT expression brain area[63]. Binding studies have shown that the distribution of SERT in the raphe nucleus is similar to that of serotonergic neurons[64]. Relevant to the memory and memory processing, the density of SERT is high in the temporal entorhinal cortex[65]. In addition, SERT is abundant in limbic system and the levels here correlate to the 5-HT neuronal innervation[65, 66]. Intuitively, levels of SERT expression contribute to the binding of selective serotonin reuptake inhibitors (SSRIs) in these memory-related brain areas. Furthermore, brain areas that have high density of SERT protein like hypothalamus and forebrain would have higher receptor occupancy[66].

1.1.4 Pathophysiological implications of 5-HT system

The serotonergic system is linked to many physiological functions. It significantly influences the function of different body systems, like the musculoskeletal, CNS, and cardiovascular system.
It has also complex interactions with other neurotransmitters and intracellular molecules. 5-HT receptors belong to seven different families (5-HT1 to 5-HT7) and subdivided into further 14 different subtypes[67]. 5-HT3 is a ligand gated ion channel receptor, while other 5-HT receptors exert their downstream signaling through a G-protein coupled pathway[68].

### 1.1.4.1 Serotonin and cognition

5-HT plays an important role in the cognitive function. Serotonergic system modulators are essential in the treatment of many neuropsychiatric disorders[69, 70]. High-order brain functions, mapped to the prefrontal cortex (PFC) such as learning, working memory and cognitive flexibility are classified as executive brain functions[71]. The dense 5-HT innervation and expression of 5-HT receptor subtypes in PFC, together with the anatomical and functional connections between PFC and raphe nucleus indicate the impact of 5-HT in memory[71].

Beside cognitive flexibility, 5-HT contributes to the function of short-term memory (STM), and long-term memory (LTM)[41]. Cognitive decline is an age-related phenomenon. Impairments in memory and learning are associated with aging[72], and with both cholinergic and serotonergic disturbances[73].

Genomic analysis of hippocampal 5-HT receptor subtypes, using DNA microarray technique, has shown differential gene expression changes. This was dependent on the type of behavioral paradigm and time points during task performance in two different behavioral tasks, the water maze learning and the passive avoidance conditioning. This experiment illustrates modulations of 5-HT in memory and learning[74].

Different subtypes of receptors, according to specific localization in the brain, have different contribution to age-related memory decline. 5-HT1A is associated with memory for object location in human[75]. Learning difficulties, such as working memory impairment and task difficulty-dependent delay, were reported in 5-HT1BKO mice[76].

Disturbances in serotonin system can lead to disturbances in cognitive flexibility and difficulty to adapt to changes in task environment[77]. For example, animal models with low brain 5-HT levels, produced by acute tryptophan depletion (ATD) or destruction of the ascending serotonergic projections, have low cognitive flexibility indices and impulse control[78].

ATD, as a temporary method to reduce brain 5-HT concentration, affects not only cognitive function but also emotion processing. It was shown that ATD affect BOLD response, which is a measure of emotion processing, in brain areas relevant to depression like the amygdala[79]. In addition, 5-HT can influence cognitive functions in both direct and indirect ways[41]. 5-HT can affect the memory indirectly by modulating other neurotransmitters like dopamine, acetylcholine and GABA.
5-HT depletion in the prefrontal cortex (PFC) leads to cognitive inflexibility[80]. This effect is seen in dementia, schizophrenia[81] and obsessive-compulsive disorders (OCD)[82]. However, involvement of 5-HT in OCD pathophysiology is also related to the implication of 5-HT in controlling impulsive behavior[83]. These findings are consistent with the data that shows positive effects of serotonin-enhancing drugs, such as tricyclic antidepressant (TCA) and SSRIs, in treating OCD symptoms[80]. Furthermore, brain prefrontal 5-HT dysfunction is specifically associated to preservative symptoms[80]. However, these clinical symptoms are not necessarily attributed to PFC-5-HT dysregulation alone.

Other pathophysiological factors, such structural and biochemical changes, might play part as well. Structural brain changes, inflammatory process and other neurotransmitters disturbances are involved in pathophysiology and symptomatology of these disorders that affect the PFC[84].

5-HT was shown to modulate neuronal activity in PFC in both in vitro and in vivo studies. In contrast to 5-HT2A, which has a cortical excitatory effect[85], activation of 5-HT1A leads to inhibitory effect on pyramidal neurons due to hyperpolarization of the neurons[86]. In vivo studies also have demonstrated the effect of 5-HT on modulating the PFC neuronal activity. Yet, it is difficult to isolate individual effect of 5-HT on 5-HT subtypes of receptor since the in vivo effect refers to activation of a group of receptors. Like the in vitro models, 5-HT1A is inhibitory to PFC pyramidal neurons, while 5-HT2A is an excitatory receptor in vivo[87]. The detailed mechanism is still unknown despite the large number of electrophysiological reports studying the 5-HT action on the PFC pyramidal network in different behavioral states[71, 88-90].

The complex relationship between 5-HT system and cognitive functions is attributed to different aspects. Firstly, the effect of 5-HT is brought by activation of both pre- and postsynaptic neurons, which leads to a complex and different signaling depending on the type of receptor being activated[73]. Secondly, there is a well-documented interaction between 5-HT receptors and other neurotransmitters. Finally, results extracted from receptor ligands are inconsistent and dependent on behavioral state.

1.1.4.2 Serotonin and the mood

5-HT system is central in the pathogenesis and the therapeutics of depression[91]. The monoamine hypothesis of depression presumes an imbalance of 5-HT transmission in the synaptic space leading to the causation of clinical syndrome of depression[92]. The concept of treating depression or enhancing the availability of 5-HT in brain has moved strategically between different developmental milestones. It started in the 1960 by blocking MAO enzyme to decrease 5-HT turnover, and moved to systematically manipulate the intracellular monoamine
signaling[93]. The desired outcomes in the strategy of augmenting extracellular 5-HT concentrations are enhancement of neuronal plasticity and synaptic transmission.

There are additional factors that influence the clinical presentation and severity of depression such as dysfunctional hippocampal neurogenesis[94], inflammation[95] and neuroendocrine stress[96]. Modulations of 5-HT1B and 5-HT1A activities are addressed in the therapeutic strategies for major depressive disorders. Moreover, the activation of 5-HT1B and 5-HT1A during the treatment with SSRIs represents a major challenge for conventional antidepressant pharmaceuticals[97].

1.1.5 Physiological Aspects of selected 5-HT Receptor subtypes

1.1.5.1 5-HT1A

The very first successful attempt to clone a 5-HT receptor was reported in 1988 by Fargin et al, using a β-adrenergic receptor probe after being targeted by preceding molecular attempts to characterize 5-HT1A[98]. It has a dense expression in the RN, which is predominately auto-regulatory presynaptic type that negatively regulate the 5-HT release and extracellular 5-HT levels[99]. In contrary, the postsynaptic receptor is expressed in the limbic system, neocortex, hypothalamus[100] and cortical brain areas[101].

5-HT1A has an additional regulatory role on dopaminergic and cholinergic transmission[73]. The co-localization and co-expression of 5-HT1A and cholinergic markers in cholinergic neurons have been reported[102]. The functional consequences for this co-expression are the integrated role of cholinergic and serotonergic system in learning and memory. The associated deficit of 5-HT and acetylcholine in age-related memory deficit and brain neurodegenerative disorders are reported[102]. A dense expression of 5-HT1A is also seen in the hippocampus. Removal of 5-HT1A in 5-HT1A knockout mice impairs the hippocampal-dependent learning. This indicates the important role of the 5-HT1A in hippocampal function[103].

The therapeutic potential of targeting 5-HT1A has been widely tried in different neurocognitive, mood and behavioral disorders such as anxiety, depression, schizophrenia and Parkinson’s disease[104]. Although studies from 5-HTA full or partial agonists have inconsistent results regarding the outcomes in memory[105], some 5-HT1A antagonists have shown potential benefits in treating cognitive dysfunction[106]. The old generation of selective 5-HT1A agonist, S20244, produces anxiolytic effect on mice[107]. Similar effect is also noted in the partial 5-HT1A-agonist buspirone. However, buspirone is not selective to 5-HT1A since it alters GABAergic and monoaminergic pathways[108]. Moreover, the non-specific 5-HT1A agonists LY228729 and 8-OH DPAT have more predilection to 5-HT1A than 5-HT1B and 5-HT7.
respectively[109]. In 2013, vortioxetine, a non-selective 5-HT1A agonist and SSRI, was approved for the treatment of major depressive disorders (MDD)[110]. In addition, 5-HT1A agonists are also shown to be beneficial in the treatment of neuroleptics-induced extrapyramidal side effects[111].

One of the challenges with SSRIs treatment is the activation of 5-HT1A that would consequently lead to a negative feedback inhibition on terminal 5-HT release and delayed SSRIs effects. SSRI-induced 5-HT1A activation was shown to be corrected by pindolol, a mixed 5-HT1A selective antagonist and β-adrenoceptor antagonist[112]. The delayed effect of SSRIs is partly attributed to the time needed for desensitization of 5-HT1A receptor. That is why the 5-HT1A receptor antagonists were proposed as adjuvant therapy to the SSRIs to potentiate the increase in extracellular 5-HT and accelerate the onset of SSRIs effect[113]. Among the candidate drugs were the selective 5-HT1A antagonists WAY100635 and NAD-92. These drugs have the advantages of their high potency, ability in achieving high brain concentrations and improving cholinergic-related cognitive dysfunctions in AD[114]. Consistent with this notion, compensation of the cholinergic activity involved in working memory by the blockade of 5-HT1A using the antagonist NAN-190 was shown in rat[115].

5-HT1A is proposed as a molecular marker for cognitive function and a target for treatment in cognitive decline[116]. Moreover, interference with the 5-HT1A-gene expression through siRNA-mediated suppression of 5-HT1A produced beneficial outcomes in depression[97]. This leads to increased extracellular 5-HT levels, augmented SSRIs effect and showed promising results in behavioral studies in mice[97]. These results are in agreement with other reports that indicate the benefit of 5-HT1A antagonists in depression. 5-HT1A antagonists modulate complexes formation between 5-HT1A and other intracellular molecules. For instance, 5-HT1A forms complexes with brain derived fibroblast growth factor receptor-1 (FGFR-1), which enhance hippocampal neuroplasticity[117]. Interestingly, the antidepressant effect of SSRIs is partially mediated through this complex due to alteration of hippocampal plasticity and 5-HT system in the limbic system[118]. Such findings suggest an indirect role of 5-HT1A in regulation of SERT function and SSRIs effects.

The effect of 5-HT1A antagonist is derived from its implication in the pathophysiology of depression[119]. Higher density and activity of presynaptic 5-HT1A predispose to affective disorders, severe depressive episodes and poor response to antidepressant[120]. Consistent with this, it was shown that the activation of the presynaptic receptor decreases 5-HT release[121]. In addition, 5-HT1A agonist might facilitate dopamine release ventral tegmental area[122]. This
reflects the significance of 5-HT1A to neuroleptics used in the treatment of psychotic disorders[123].

1.1.5.2 5-HT1B

This receptor was classified as one entity together with 5-HT1D because of their high homology, pharmacological similarities and the fact that both subtypes of receptors inhibit adenylyl cyclase[124]. However, this relationship was latterly elaborated in terms of phylogenic basis. It was shown that rat 5-HT1B receptor is the homolog and species variant to the human 5-HT1D receptor[125].

There are few differences between human 5-HT1D and rat 5-HT1B in terms of pharmacological characterization[44]. For example, the rat receptor has more binding affinity to certain ligands such as the β-adrenergic antagonist propranolol. This is attributed to a difference in a single amine acid (threonine in position 335 in human 5-HT1D and asparagine in the same position in rodent 5-HT1B) in the receptor putative transmembrane domain[44, 126].

5-HT1B, similar to many of 5-HT subtypes including 5-HT1A, belongs to Gαi-coupled receptors. These receptors exert their downstream signal transduction through inhibition of adenylyl cyclase and subsequently lead to reduction of the second messenger cyclic adenosine monophosphate (cAMP)[127, 128]. This canonical signaling pathway is named the signaling pathway. Here, signals for complex and diverge biochemical and physiological effects are transduced[128]. However, a separate cAMP-independent intracellular signaling was also characterized for the down stream effect of 5-HT1B. Reports have demonstrated the coupling of 5-HT1B to the mitogen-activated protein kinase (MAPK) Erk-2 and the related kinase p70 S6 in non-sequential signal transduction pathways[129].

5-HT1B has both presynaptic and postsynaptic distributions. The cellular localization of 5-HT1B is variable depending on the tissue type[44]. In neuronal cells, axonal expression is the predominant and 5-HT1B transported to axon terminal from cell bodies[45, 49, 130]. The variability of 5-HT1B expression across different brain areas was reported by binding, immunohistochemistry and in situ hybridization studies of 5-HT1B mRNA[44, 56].

Higher levels of 5-HT1B were found in the basal ganglia nuclei globus pallidus (GB) and substantia nigra (SN), with the preferential transport of the receptor’s protein along the serotonergic projections to these nuclei[44]. Lower receptor protein levels were detected in the cerebral cortex and hippocampus[44, 50, 54]. This was confirmed by in situ hybridization studies, using cDNA probe encoding 5-HT1B. This has shown that 5-HT1B mRNA is expressed in the median and lateral raphe nucleus and CA1 area of the hippocampus[57].
Similar to the 5-HT1A, the presynaptic 5-HT1B is an auto-regulatory receptor that control 5-HT release through a negative feedback mechanism[16, 131]. The increased activity of these receptors lead to inhibition of 5-HT release, and thus takes part in the regulation of the extracellular 5-HT levels together with SERT[58]. However, the caudate nucleus, where 5-HT1B increase 5-HT release, is the only exception for this inhibitory effect of 5-HT1B on 5-HT[132]. This indicates that the mechanism of 5-HT1B in modulating extracellular 5-HT is more complex in basal ganglia’s circuits[132].

The interaction between the extracellular 5-HT and the activity or expression of both 5-HT1A and 5-HT1B are relevant to depression[133]. Although the desensitization of these receptors is required for higher synaptic 5-HT levels and thus antidepressant effect, 5-HT1B and 5-HT1A are mandatory for a long-term antidepressant effect[133]. 5-HT1BKO mice have decreased sensitivity of 5-HT1A to the SSRIs and the animal fail to modify stress adaption[133]. In addition, 5-HT1B also modulates SERT function in terms of 5-HT reuptake from the extracellular compartment under physiological conditions, which is necessary for the SERT-mediated SSRI function[58]. These findings suggest a functional connectivity between 5-HT1B, 5-HT1A and SERT. However, the exact mechanisms by which 5-HT1B modulate 5-HT1A and SERT functions, or vice versa, are not yet well understood. Beside the negative autoregulatory mechanism of 5-HT transmission and synthesis, 5-HT1B influences the transmission of other transmitters and thus acts as both autoreceptor and heteroreceptor[44]. For example, 5-HT1B is inhibitory to acetylcholine release in hippocampus and interference with 5-HT1B modulates acetylcholine levels in the hippocampus[134].

5-HT1B has significant implication on behavior as shown in animal studies[16]. Inhibition of 5-HT1A and 5-HT1B in the dorsal raphe nucleus facilitate rewarding and increasing the receptors activities, by selective 5-HT1A and 5-HT1B agonists, facilitates avoidance or averseness[16].

Studying the genetic variation of the single exon 5-HT1B gene has shown significant implication on cognitive domains such as motivation and memory. Additionally, 5-HT1B genetic polymorphisms associates with neuropsychiatric disorders and symptoms[135]. Some mutations of 5-HT1B gene, affecting the binding domain or the regulatory region of 5-HT1B, could influence the efficacy of certain antidepressant and antipsychotic medications[135, 136].

1.1.5.2.1 5-HT1B and memory

5-HT1B is relevant to memory. Activation of 5-HT1B in hippocampus impairs both working and reference memories and affects the spatial memory tasks in rat[135, 137]. Similarly, the 5-HT1BKO mice have selective facilitation of task difficulties related to spatial memory[76]. 5-HT1BKO mice showed impairment in task related to intermediate memory and the delay-
dependent working memory[138]. However, 5-HT1BKO animals perform better in tasks that require aspect of memory flexibility[135]. Additionally, due to the resultant negative tone on acetylcholine, 5-HT1BKO animals lack the visual inhibition that could explain the inattentive behavior observed in them[135]. This indicates the relevance of this receptor in attention deficit hyperactivity disorder (ADHD). These reports, together, reflect the complexity of the 5-HT1B contributions to memory function particularly when situation factor or cognitive demand is required[76]. It might also indicate the variation in 5-HT1B function depending on the 5-HT1B localization in different brain networks and areas[135].

1.1.5.2.2 5-HT1B and anxiety

Reduction of fear or stress is achieved by increasing 5-HT1B functions through either overexpression of the 5-HT1B or the use of a selective 5-HT1B agonist[16, 139]. However, in a different report 5-HT1B stimulation exacerbates anxiety in rats. This effect is reversed by the concomitant administration of a selective 5-HT1B antagonist, while no isolated anxiolytic effect of the antagonist alone was noted[140]. Except for the 5-HT1B antagonist propranolol, the results from other 5-HT1B antagonists on anxiety state showed contradicted results. Previous reports did not demonstrate correlation between the anxiolytic effect and changes in the levels of 5-HT after 5-HT1B modulations[41, 141].

The association of 5-HT1B and anxiety could be an indirect effect through influence of 5-HT1B on neurotransmitters other than 5-HT such as acetylcholine and GABA[44, 142]. Because of the inhibitory tone to acetylcholine release in cholinergic neurons in the dorsal hippocampus and acetylcholine activity has an anxiolytic effect[143], it is suggested that heterogeneous 5-HT1B receptors mediate the anxiety behavior through the inhibition of acetylcholine[44]. Similar to that with memory, the association with anxiety and fear is dependent on the anatomical localization of the 5-HT1B in the brain. Diffuse overexpression of 5-HT1B in brain reduced anxiety in mice, while selective overexpression of the receptor in the dorsal raphe nucleus (DRN)-amygdala circuit had increased anxiety in wild type but not 5-HT1BKO mice[144]. This suggests that the control of anxiety response through 5-HT1B is circuit-specific and partially controlled by the DRN and the upstream projections to the hippocampus[44]. However, no change in 5-HT1B gene expression was observed after subjecting animal to sudden or prolonged stress stimuli[145].

1.1.5.2.3 5-HT1B and depression

5-HT1B dysfunction is linked to the pathophysiology of depression[44]. In addition, 5-HT1B genetic polymorphisms, particularly G861C and C129T, are related to the development of mood disorder[146, 147] and response to antidepressant[148]. Electrophysiological studies in rats
revealed that 5-HT1B stimulates activating signals that contribute to the pathophysiology of depression[149]. Although these presynaptic excitatory signals are not specific to 5-HT1B, 5-HT1B has significant interaction with other molecules that influence these signals such as glutamate[149-151]. Moreover, the expressions of 5-HT1B and its adaptor protein p11 are reduced in human brain and animal models of depression[152, 153].

The use of antidepressant decreases the gene expression of the presynaptic 5-HT1B in DRN in a reversible pattern[154]. This would reflect a particular phenomenon that an antidepressant has to overcome the negative tone of 5-HT1B on 5-HT transmission in order to produce a clinical effect. In other words, desensitization and inhibition of 5-HT1B function is mandatory for an antidepressant action[155]. In agreement with this, PET studies have showed that the 5-HT1B binding in brain is reduced in the dorsal brain stem (DBS) after effective treatment of MDD[156]. However, this requires a relatively long time to occur and could probably explain the delayed-onset of improvement in depressive symptoms following administration of antidepressant[157]. However, an antidepressant-like effect was observed for the 5-HT1B agonist (CP94253) in mice in the forced swimming test and the mechanism is postulated to be through activation of postsynaptic 5-HT1B together with modulation of dopamine and adrenaline[157, 158]. Of note, the data from studies investigating the role of pharmacological modulations of 5-HT1B and its association with antidepressants effect are not showing conclusive results[157]. Such data would depend on the duration of 5-HT1B modulations and presence of antidepressant-like effect[159].

1.1.5.2.4 5-HT1B and aggression

5-HT deficiency is postulated as a contributing mechanism for aggression and suicidal events[44, 160, 161]. Therefore, increasing the level of 5-HT in brain through pharmacological modulation of 5-HT1A and 5-HT1B was a tempting strategy to control aggressive life-threatening events and symptoms[162]. However, this is challenged by the fact that 5-HT1B has complex association with different behavioral neuropsychiatric disorders[135]. Aggressive behavior was observed in 5-HT1BKO mice[163] and after the administration of the 5-HT1B antagonist, while it could be reduced by using the selective 5-HT1B agonist[162]. Similar to memory and anxiety (see section 1.1.5.2.1 and 1.1.5.2.2), the anatomical localization of 5-HT1B may influence aggression. 5-HT1B receptor expressed in the non-serotonergic neuron in the frontal cortex during development were shown to be associated with the impulsivity[164].
1.1.5.2.5 5-HT1B and substance abuse

Serotonin system in general and particularly 5-HT1B are implicated in alcohol dependence and related alcohol uptake disturbances[165-167]. The effect of 5-HT1B autoreceptor on alcohol intake and 5-HT1B heteroreceptors through their role in pathophysiology of impulsivity and aggression were reported[164]. Particular 5-HT1B ligands, depending on 5-HT1B subtype and being auto or hetero-receptor, have a potential in both modulating 5-HT1B function and altering alcohol intake behavior[167]. Reports discussing the association between 5-HT1B and alcohol intake are not conclusive. However, early reports suggest increased alcohol intake in 5-HT1B KO mice together with lower densities of 5-HT1B in rat with higher alcohol intake indices[167-169]. The discrepancy from the data derived from animal models of 5-HT1B is attributed to age and sex of animals[170]. Interestingly, stimulation of 5-HT1B by the selective agonist CP94253 reduced the aggressive behavior associated with alcohol abuse[171].

The expression and activity of 5-HT1B in different brain circuits are relevant to cocaine abuse. 5-HT1B and p11 reduction can be a risk factor or a consequence of cocaine intake. Their downregulation in certain brain area might predispose to higher susceptibilities to cocaine intake[172, 173]. Conversely, some reports suggested that the upregulation of 5-HT1B gene expression, in nucleus accumbens and dorsal striatum, could be a molecular marker for failure to keep cocaine abstinence after long-term withdrawal[174].

1.1.5.2.6 5-HT1B and migraine

Serotonergic disturbances are also implicated in the pathogenesis of migraine[44]. The extracellular levels of 5-HT metabolites are generally reduced in patients with migraine[175]. Increased activity of 5-HT1B in vascular system of territories supplied by the trigeminal artery is linked to vasodilation, increased blood supply and aggravation of symptoms seen in typical attacks of migraine[176].

Similar to 5-HT1D, 5-HT1F and 5-HT7, 5-HT1B receptor agonists are clinically preferable to the conventional analgesics and ergotamine. This is attributed to the neurovascular distribution in the head, higher safety index, well-documented pharmacodynamics mechanisms and their efficacy in alleviating the symptoms of migraine[177].

1.1.5.2.7 5-HT1B-adaptor protein p11

S100A (p11) acts as an adaptor protein for 5-HT1B receptor[153]. It belongs to a family of calcium-binding proteins that adjusts the target protein response to levels of intracellular calcium and work to place protein to their corresponding functional membranous localization[178]. Also, it increases 5-HT1B membranous expression, which has positive influence on the mood and
facilitates the antidepressant action[153]. In addition, neuronal plasticity resultant from the positive effect brain-derived neurotrophic factor (BDNF) on spine and dendritic growth are mediated by p11 in hippocampal cells[179]. They have shown, in different models, how p11 through 5-HT levels and BDNF facilitates an antidepressant action. In addition, removal of the p11 from the system produces a depression-like behavior in animal[153]. p11 is not an exclusive functional and structural protein for 5-HT1B. There is a similar association between p11 and 5-HT4[180]. As seen in case of 5-HT1B, p11 enhances membranous expression of 5-HT4 and promotes an antidepressant effect through this 5-HT receptor[180]. Moreover, the biological effect of p11 through 5-HT1B is not restricted to depression. It contributes to different physiological and pathological aspects of 5-HT1B function such as memory, anxiety, locomotor function, aggression and substance abuse[181].
1.2 Alzheimer’s disease

This disease represents the most prevalent type of dementia and is considered as a major global health problem[182]. According to the Delphi consensus on dementia, there are currently more than 20 million people are affected by AD worldwide and estimated to increase to more than 80 million after 30 years[182, 183]. The disease has a significant impact on the quality of life of the affected patients, contributes to the increase of mortality rates in elderly age groups and represents a high burden on health care systems and caregivers’ quality of life[184].

1.2.1 Epidemiology

AD is an age-associated disease and the incidence therefore depends on how old or young the studied population is. Other factors such as the adopted diagnostic protocol, the incidence of vascular risk factors and lifestyle play a role in the variation of epidemiological measures across ethnic and geographical group[185].

There is uncertainty regarding the worldwide incidence of AD. Epidemiological studies with acceptable methodological quality in Alzheimer’s disease research are conducted in restricted areas such as Japan, USA, Europe, Australia and Canada[183]. However, the global distribution of the diseases and the rise of the disease in developing countries necessitate an effective and well-integrated international program for epidemiological data from all over the world[183]. In the developed countries like USA, AD represent the 5th common cause of death in the age group over 65 years following heart attacks, cerebrovascular accidents, cancer and chronic obstructive pulmonary diseases[186]. The financial burden includes cost of treatment in health care institutions and the working hours of the caregivers, which is an estimated loss of more than 250 billion dollars[184]. More than 24 million were affected by AD and the number is expected to reach more than 80 millions after 25 years[182].

1.2.2 Risk factors

Different genetic, biological, environmental and behavioral factors have been proposed as risk factors or preventive factors. Beside age and genetic factors, AD is associated with wide range of vascular risk factors such as hyperlipidemia, diabetes mellitus, hypertension, unhealthy life style and obesity[187]. However, combined epidemiological and pathological studies have not shown an association between these vascular risk factors and the severity of AD[188]. Studies have shown that modification of risk factor could affect the onset and progression of dementia. This is dependent not only on the amount or duration of the exposure to a particular risk factor[189], but also to the presence or absence of certain genetic risk factors such as ApoE ε4 allele[190].
Table 1.1 shows risk and protective factors in AD[191].

**Table 1.1 Risk and protective factors in AD**

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Protective factors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>Genetic</td>
</tr>
<tr>
<td><strong>Genetic</strong></td>
<td>Different genes (e.g. APP, ApoE ε2) have been proposed (<a href="http://www.alzgene.org">www.alzgene.org</a>)</td>
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<tr>
<td>Familial aggregation</td>
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<tr>
<td><em>ApoE</em> ε4</td>
<td></td>
</tr>
<tr>
<td>Different genes (e.g. <em>CRI, PICALM, CLU, TREM2, TOMM40</em>) have been proposed (<a href="http://www.alzgene.org">www.alzgene.org</a>)</td>
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<tr>
<td><strong>Psychosocial factors</strong></td>
<td></td>
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<tr>
<td><strong>Vascular and metabolic</strong></td>
<td></td>
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<tr>
<td>Cerebrovascular lesions</td>
<td>High level of complexity of work</td>
</tr>
<tr>
<td>Cardiovascular diseases</td>
<td>Rich social network and social engagement</td>
</tr>
<tr>
<td>Diabetes mellitus and pre-diabetes</td>
<td>Mentally stimulating activity</td>
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<tr>
<td><strong>Midlife positive association but late-life negative association</strong></td>
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<tr>
<td><strong>Hypertension</strong></td>
<td>Moderate alcohol intake</td>
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<tr>
<td>High BMI (overweight and obesity)</td>
<td>Mediterranean diet</td>
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<tr>
<td>High serum cholesterol</td>
<td>PUFAs and fish-related fats</td>
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<tr>
<td><strong>Lifestyle</strong></td>
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<tr>
<td>Smoking</td>
<td>Antioxidant vitamins (A, C and E)</td>
</tr>
<tr>
<td>High alcohol intake</td>
<td>Vitamin D</td>
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<tr>
<td><strong>Diet</strong></td>
<td></td>
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<tr>
<td>Saturated fats, Homocysteine</td>
<td>Antihypertensive drugs, Statins, HRT, NSAIDs</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td>Depression, Traumatic brain injury, Occupational exposure (heavy metals, ELF-EMFs), Infective agents (herpes simplex virus type I, <em>Chlamydiophila pneumoniae</em>, spirochetes)</td>
<td></td>
</tr>
<tr>
<td><strong>Combined effect</strong></td>
<td></td>
</tr>
<tr>
<td>Increased risk</td>
<td>Decreased risk</td>
</tr>
<tr>
<td><strong>Genetic and environmental factors in midlife</strong></td>
<td><strong>Genetic and environmental factors in midlife</strong></td>
</tr>
<tr>
<td><em>ApoE</em> ε4 magnifies the effect of high alcohol intake, smoking, physical inactivity and high intake of saturate fat</td>
<td>High education level reduces the negative effect of <em>ApoE</em> ε4</td>
</tr>
<tr>
<td><strong>Vascular and metabolic factors in midlife</strong></td>
<td>Physical activity counteracts the risk due to <em>ApoE</em> ε4</td>
</tr>
<tr>
<td>Co-occurrence of hypertension, obesity, hypercholesterolaemia and/or physical inactivity</td>
<td>Environmental factors in midlife</td>
</tr>
</tbody>
</table>
| High level of complexity of work modulates the
has an additive effect; increased dementia risk due to low level of education

**Vascular and metabolic factors/diseases in late-life**

- Higher risk in individuals with brain hypoperfusion profile: chronic heart failure, low pulse pressure, low diastolic pressure
- Higher risk in individuals with atherosclerosis profile: high systolic pressure, diabetes mellitus or prediabetes, stroke

**Genetic and environmental factors in late-life**

- Active leisure activities or absence of vascular risk factors reduces the risk due to ApoE ε4

“Risk and protective factors for dementia and Alzheimer’s disease have been investigated, and there are greater and lesser degrees of evidence to support these various factors. APP, amyloid precursor protein; ApoE, apolipoprotein E; BMI, body mass index;CLU, clusterin; CR1, complement component receptor 1; ELF-EMF, extremely low-frequency electromagnetic field; HRT, hormone-replacement therapy; NSAID, non-steroidal anti-inflammatory drug; PICALM, phosphatidylinositol binding clathrin assembly protein; PUFA, polyunsaturated fatty acid; SES socioeconomic status; TOMM40, translocase of outer mitochondrial membrane 40 homolog; TREM2, triggering receptor expressed on myeloid cells” (courtesy Dr Francesca Mangialasche). The table is reprinted from the review article Dementia prevention: current epidemiological evidence and future perspective, Mangialasche et al, 2012 in Alzheimer Research & Therapy Journal[191].

### 1.2.3 Pathophysiology of Alzheimer's disease

The gross pathological feature in dementia in general and AD in particular is a widespread brain atrophy due to massive neuronal loss. Extracellular amyloid plaques formation and intracellular neurofibrillary tangles (NFT) represent the main pathological hallmark of AD[182]. They are not completely two separate pathological entities, since studies have shown that amyloid pathway could enhance hyperphosphorylation of the tau protein, which is a main and early step in the formation of NFT[192].

The amyloid cascade hypothesis, postulated by John A. Hardy and Gerald A. Higgins in 1992, suggested that one of these multiple amyloid species, resulted from the amyloidogenic APP-processing pathway, initiates a neurotoxic sequence of events that eventually leads to neuronal loss and accumulation of the amyloid species in plaques[193]. This sequence of events in amyloid hypothesis is illustrated in Figure 1.3.

This hypothesis has several supporting data during the timeline from the introduction of the hypothesis till the most recent clinical trials for the emerging disease-modifying pharmaceutical agents[192]. For example, it was shown that mutations affecting the rate of cleavage of the APP could lead to the familial early onset type of AD[194]. Another support to this hypothesis, is the high incidence of AD-like neuropathology in the trisomy 21 (Down syndrome), where 3 copies of the APP gene are carried in chromosome 21[195]. Finally, the increased clearance of amyloid species reduces amyloid aggregations and improvement in cognitive decline in patient treated with the immunotherapy Aβ monoclonal antibodies[192]. The genetic polymorphism (A673T) in
the gene encoding for APP, results in lower loads of Aβ peptides and better cognitive outcomes in both healthy and AD patients[196].

The microtubule-associated protein, tau, is the second main protein implicated in the pathogenesis of AD[197]. In AD, the hyperphosphorylated tau aggregates in the cytoplasm. This consequently affects the assembly of the microtubules that accumulate in the intracellular space in form of NFT and eventually leads to synaptic dysfunction and neurotransmission disturbances[197]. The release of tau to the extracellular space cleft is proposed as a mechanism that links pathology and serotonergic disturbances in AD[198]. Different staging systems are used to classify the hierarchical involvement of AD neuropathology in the brain. Currently, the Braak system for the NFT progression[199], the National Institute on Aging (NIA), Thal amyloid staging[200], and the Consortium to Establish a Registry for Alzheimer Disease criteria (CERAD) for neurotic plaques are used for staging of AD neuropathology[201].

Disturbances in the neuro-inflammatory pathways, lipid metabolism, neurotransmitter and oxidative stress play an important role in the pathogenesis of AD as shown in Figure 1.4
1.2.4 Clinical features and clinical diagnosis

The pathology of AD starts long before the appearance of the clinical symptoms, and the preclinical period of AD makes the onset of the disease like an iceberg phenomena\[202\]. In dementia, this period is followed by a phase of symptomatic decline in cognitive functions called mild cognitive impairment (MCI). Subjective cognitive impairment (SCI) and MCI are two stages preceding the full-blown picture of dementia. SCI is defined as a period where the objective assessment is not showing evidence of cognitive impairment despite the subjective complaints from the patient or his family.

Recently, “the consecutive 3-stage model” proposed SCI as an early stage of dementia that lead to MCI and eventually dementia of AD\[203\]. The proposed time-line for different AD stages compared with the course of cognitive decline in normal aging is shown in Figure 1.5.
AD is characterized by the disturbances in cognition, behavioral and impairment in functions needed for daily activities and complex tasks. The clinical diagnosis enables the physicians to reach a probable diagnosis and the definite diagnosis is only possible after postmortem histopathological studies[182, 204].

Multiple mental functions are affected in AD dementia. Importantly, the disease affects higher brain functions such as planning, problem-solving ability and judgment. Other cognitive domains that might be affected in AD are memory, language, the ability to identify objects even in the lack of sensation disturbances (agnosia) and the ability to perform familiar task (Apraxia)[204]. Objective decline in the neurocognitive domain measured by standardized neuropsychological tests are required for AD diagnosis[204].

In the updated protocol for clinical diagnosis of AD and according to the new guidelines from the Diagnostic and Statistical Manual of Mental Disorder from the American Psychiatric Association 5th edition (DSM-V), the memory deficit is not mandatory for inclusion in dementia, which is now named neurocognitive disorders (NCDs). In this new protocol, the demarcation between the severity classes is the preservation of an acceptable functional level[205]. The emergence of the term NCDs and the removal of the necessity for memory deficit in diagnosing NCDs are the main differences between DSM-V and DSM-IV[205]. The criteria for AD-dementia in ICD 10, the international classification of diseases adopted by the World Health Organization WHO, require cognitive decline in the absence of consciousness disturbances, gradual onset of symptoms and also the absence of structural brain lesions together with the exclusion of other organic cause of other dementia[206].

Many neuropsychological tests are commonly performed to evaluate and diagnose dementia. The clinical evaluation starts with the history taking and recording of the subjective cognitive-
related complaints from the patients. However, objective cognitive assessments and supplementary biomarkers including brain imaging are important to differentiate AD from other types of dementia. The revised international working group (IWG-2) criteria for typical AD include objective impairment in the episodic memory, PET evidence of amyloid brain pathology, typical AD CSF profile and autosomal dominant mutation of familial AD[207].

The biomarker profiles are indicative of underlying pathology (see section 1.2.5). However, there are some limitations to the diagnostic utility of biomarkers and neuropsychological test. Cognitive status is usually screened by the Mini-Mental State examination (MMSE), which provides a simple, rapid, practical and cost-effective clinical evaluation for the cognitive function. The test has a total of 30 points score and 24 represent the cut-off point for dementia. At the cut-off 24: the test has about 80% sensitivity and 90% specificity with modified version of the test available in clinical practice to enhance the detection rates such as addition of the clock test and the 20-point test[208-210]. MMSE is influenced of depression, the education levels and the Intelligence quotient of the participant[211]. The clinical dementia rating (CDR) is a scale used to objectively stage the severity of dementia. It is a semi-structured interview contains questions that evaluate the subjective memory complains from the informant, orientation, judgment, problem solving, abilities to independently perform household tasks and the interactions between the informant and his/her society[212]. There are wide ranges of brief cognitive tests; and their use is dependent on the reference standards, e.g. ICD10 or DSM-V, the type of dementia diagnosis and the country own national guidelines[210].

There is wide range of clinical tests depending on the diagnostic protocol in different health institutions. Tests that basically designed to be used at primary care services include Memory Impairment Screen (MIT)[213], specialized dementia clinics such as Brief Alzheimer screen (BAS)[214], Montreal cognitive Assessment (MoCA)[215] and abbreviated mental test (AMT), while validated for dual use in both primary and specialized settings like Addenbrook Cognitive examination (ACE) and the Clock drawing test[210].

Behavioral and psychiatric symptoms of dementia (BPSD) are frequently observed in AD and may require pharmacological intervention[216]. Their types and severities depend on the stage of AD. For example depression, anxiety, apathy and irritability are the most common BPSD symptoms in early AD, while delusion, agitation, sleep disturbances, hallucination and aggressive behavior are mainly observed in the intermediate to advanced stages of AD after more than 3-5 years following the onset of dementia[216].
1.2.5 Alzheimer's disease biomarkers

A biomarker is defined as measurable indicator for biological or pathological process used in clinical and research setting to monitor the progress of a disease or a response to an intervention[217]. In AD, biomarkers that reflect the disease pathology could be broadly divided into biochemical biomarkers and imaging biomarkers. Combination of these is used nowadays in memory clinics to confirm the clinical suspicion when AD diagnosis is probable[218].

The core biochemical markers for AD are Aβ42, total tau (t-tau) and phosphorylated tau, (p-tau) while promising biochemical markers for AD derived from synaptic structures, neuronal axons or derived from the cell bodies of the neuron (soma) are currently investigated[218].

CSF represents the biomarker compartment for neurodegeneration in general and AD in particular. Accumulating reports indicate the validity of the CSF tau and Aβ42 in the detection of AD[219]. AD is characterized by low CSF Aβ42 levels, which might reflect the deposition of this peptide in the brain and not necessarily the neurodegeneration process[218-220]. However, this pattern of low CSF Aβ42 is not specific to AD and is found in other neurocognitive diseases such as DLB, vascular dementia (VaD) and FTD[219]. In addition, other β-amyloid species are also used as CSF biomarkers for AD and the reduction of the ratio Aβ42/Aβ40 is used as early marker of AD[219].

The CSF total tau (t-tau) level, a marker of neurodegeneration, is increased in AD[221, 222]. This pattern, similar to Aβ42 pattern, is not pathognomonic for AD. Higher CSF t-tau levels are reported in VaD and FTD[220, 222]. Although NFL is not a core CSF biomarker is, among others, an emerging AD CSF biomarker[223].

As, phosphorylation of tau protein is believed to be the earliest step in tau aggregation and the subsequent formation of NFT[197], CSF-phosphorylated tau is high in AD[220]. Common sites of tau phosphorylation’s are at the threonine amino acid at residues 181 and 231, serine at positions: 199, 235, 396 and 404[219, 224]. CSF p-tau is not elevated in FTD and so p-tau has the advantage from other AD CSF-biomarker in its ability to discriminate AD from other type of dementia[220, 221]. The levels of CSF biomarkers are affected by different factors such as the neurodegenerative process, neuropsychiatric symptoms such as depression and the ApoE genotype[225]. People homozygous for ApoE ε4 allele have lower CSF Aβ42 compared to heterozygous and normal control[226]. Moreover, the interpretation of CSF findings in cases with mixed pathology, such as combinations of DLB and AD, is an additional challenge to their clinical applications.

Imaging techniques are used to evaluate structural alterations and functional characteristics in AD research and diagnosis[227]. Atrophy of the medial temporal lobe, particularly the
hippocampal, is strong anatomical clue for AD. Structural brain studies using magnetic resonance imaging (MRI) have revealed a correlation between CSF biomarkers and hippocampal atrophy in AD[228]. However, the volume of the hippocampus is affected by depression, which is a common risk factor, symptom or consequence of depression[229]. Functional imaging also has a promising role with the discovery of new tracers specific to tau[230] or Aβ[231], as well as the improvement in the resolutions of the new generations of PET scanners. The relationship between AD-biomarkers and the disease progression is shown in Figure 1.6. The figure illustrates a hypothetical model that describes changes of AD imaging, laboratory biomarkers across the progression phases of AD dementia[232].

![Figure 1.6 The interactions between biomarker and cognitive decline in AD[232]. Reprinted with permission from Elsevier, copyright (2016), from Jack CR Jr. et al. 2010, Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade, The lancet Neurology. 2010 (9): 119-128. PubMed PMID: 20083042](image)

**1.2.6 Treatment of Alzheimer’s disease**

The management of AD could conventionally be divided into pharmacological and non-pharmacological treatments. The non-pharmacological treatments include cognitive training and cognitive stimulation strategies. Despite the significant safety and efficacy profiles of pharmacological treatment, challenges such as the caregivers lower motivation and lack of resources might interfere with the outcome[233].

Commonly used agent in the medical practice are symptomatic treatments[233]. Drugs that increase brain acetylcholine levels, through inhibition of the degrading enzyme the acetyl choline
esterase, such as donepezil or galantamine, are used in AD with a good clinical effect in terms of cognition and function[234].

Symptoms such as agitation and aggression during the course of disease require optimal control with antipsychotic medication for patients and caregiver safety[182]. This usage is limited by both acute and chronic side effect of both classical and atypical neuroleptic medications[182]. Antidepressant are also frequently used in AD[182], and the prolonged use of SSRIs promotes neurogenesis in mice models of AD[235]. The use of SSRIs is associated with lower cognitive decline and higher levels of brain 5-HT metabolites in AD. However, a multi-center, double blinded and randomized clinical trials indicate not only the lack of effect when using sertraline but also increase incidences of side effects[236]. 5-HT6 blockade is an emerging cognitive-enhancing strategy and the objective is to indirectly increase the acetylcholine release by blocking the inhibitory receptor 5-HT6, resulting in dual antidepressant and beneficial cognitive effect[237].

Immunotherapy for AD implies the use of passive or active immunizations to take advantage of antibodies against tau or β-amyloid proteins in order to facilitate the clearance, reduce the aggregations of amyloid protein and reduce the brain amyloid and tau deposition[234].

Until very recently, no promising results were revealed from strategies of disease modifying drugs targeting Aβ synthesis by inhibiting the gamma-secretases and β-secretase or by inhibiting tau phosphorylation[238]. This might be attributed to the diffuse functions of these enzymes and to multiple systemic side effects when they are pharmacologically modulated. However, a recent Phase 2b study showed that amyloid immunotherapy might not only reduce the level of amyloid plaques but also showed promising clinical improvements[239]. Immunotherapy against tau is relatively new compared to the preceding research and trials for β-amyloid proteins[238].

In addition to the high cost of immunotherapy drug development, factors such as difficulties in designing a pharmaceutical that can cross the blood brain barrier and the high failure rates due to issues related to patient safety and drug tolerability, contribute to the innovation gap in this field[238, 240]. Many amyloid-based immunotherapy clinical trials were stopped due to the development of aseptic meningitis and meningoencephalitis. Although the incidence of this side effect is not high, it is a serious life-threatening condition that may require termination of the trial due to patient safety concerns[238]. Pharmaceutical agents licensed for the clinical use or on clinical trial in AD are summarized in Table 1.2[182].
Table 1.2 Treatment of AD

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Status</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Symptomatic treatments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholinesterase inhibitors</td>
<td>Donepezil, rivastigmine, galantamine</td>
<td>Licensed for mild-to-moderate Alzheimer's disease</td>
</tr>
<tr>
<td>NMDA receptor antagonist</td>
<td>Memantine</td>
<td>Licensed for moderate-to-severe Alzheimer's disease</td>
</tr>
<tr>
<td><strong>Treatments for neuropsychiatric symptoms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atypical antipsychotics</td>
<td>Risperidone, quetiapine, olanzapine, aripiprazole</td>
<td>Risperidone licensed for short-term treatment of severe aggression in Alzheimer's disease; other treatments are used off licence</td>
</tr>
<tr>
<td>Antidepressants</td>
<td>Citalopram, sertraline</td>
<td>All antidepressants used off licence in Alzheimer's disease</td>
</tr>
<tr>
<td>Anticonvulsants</td>
<td>Carbamazepine</td>
<td>Used off licence</td>
</tr>
<tr>
<td><strong>Proposed disease-modifying treatments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunotherapy</td>
<td>Bapineuzumab</td>
<td>In phase 3 clinical trials</td>
</tr>
<tr>
<td>Sectretase inhibitors</td>
<td>Tarenflurbil, semagacestat</td>
<td>In phase 3 trials</td>
</tr>
<tr>
<td>Amyloid aggregators</td>
<td>Tramiprosate</td>
<td>Discontinued</td>
</tr>
<tr>
<td>Copper or zinc modulators</td>
<td>PBT2</td>
<td>Phase 2 clinical trials</td>
</tr>
<tr>
<td>Tau aggregation inhibitors</td>
<td>Methylthioninium chloride</td>
<td>Phase 2 clinical trial</td>
</tr>
<tr>
<td>GSK3 inhibitors</td>
<td>Lithium</td>
<td>Early-phase clinical trials</td>
</tr>
<tr>
<td>Natural products and vitamins</td>
<td>Vitamin E, ginkgo biloba, omega 3 fatty acids, and docosahexaenoic acid</td>
<td>Phase 2 and phase 3 clinical trials</td>
</tr>
</tbody>
</table>
trial did not show any advantage of ginkgo biloba compared with placebo.
A large randomised controlled trial of omega 3 fatty acids did not report any benefit on function or cognition, but did suggest some possible benefit on neuropsychiatric symptoms in a post-hoc subgroup analysis. A National Institute on Aging phase 3 trial of docosahexaenoic acid is in progress.


1.2.7 Peripheral biomarkers in Alzheimer's disease

There are many advantages with peripheral biomarkers extracted from blood. This could provide an easy, non-invasive and cost affordable measure to diagnose or monitor AD pathology. However, the biological partition made by blood–brain barrier will hinder the assumption of correlation between CNS and blood levels of such biomarkers. Moreover, CNS-derived proteins will be subjected to more degradation when they are released into blood than when they are confined to CNS.

Due to alterations in the biological characteristics of different blood cells and blood-related molecules in AD, many blood constituents are studied for a peripheral biomarker candidate. These alterations might be in the amyloid content, production of particular blood cell type or in other intracellular biological pathway such as the inflammatory pathways, immune response and neurotransmitter systems. Again, these changes are not specific for AD.

The non-specificity of these biomarkers is due to the fact that wide range of physiological and pathological conditions are associated with alterations in the inflammatory biomarkers at the blood level. Furthermore, ageing is associated with disturbances in the balance between the inflammatory and the resolution mechanism of inflammation, which could be detected in the blood[241]. This imbalance might be exaggerated in AD, where more neuroinflammation and less effective brain-specific resolution of the inflammatory response are observed.

1.2.7.1 Plasma biomarkers and Alzheimer’s disease

The plasma, through blood brain barrier (BBB) and pial arteries around the meninges, represents the compartment for amyloid clearance from brain and CSF[242, 243]. Several plasma proteins are investigated for a possible peripheral biomarker potential[244].

Plasma levels of Aβ40 and Aβ42 increase with age and correlate with their counterpart CSF levels, which indicates sequential handling of Aβ peptides from the brain to the plasma via the CSF[245]. In addition, plasma levels of Aβ40 and Aβ42 are high in early AD and also high in
asymptomatic subjects with high risk for dementia[245]. Moreover, elevated levels of plasma Aβ42 are associated with increased mortality in AD[246].

Interestingly, reports have shown also an association between the ApoE genotype and the plasma Aβ. This relationship depends on the type of Aβ and absence or presence of AD pathology[247]. In agreement with this, the meta-analysis performed by Hye A et al, 2014 has revealed that plasma ApoE levels correlate with both hippocampal and whole brain atrophy[244].

ApoE ε4 allele-positive AD patients have higher plasma Aβ40, but not Aβ42, compared to the elderly non-demented controls[247]. However, plasma Aβ40 is not associated with MMSE and other AD biomarkers in AD in this study. Plasma biomarkers are not yet clinically useful in AD detection despite the compelling evidence of the association between plasma and CSF Aβ. Moreover, CSF Aβ biomarkers are superior to that of plasma in the prediction of conversion from MCI to dementia[248].

Plasma tau and p-tau were also investigated for a potential peripheral biomarker role in AD. In contrast to lack of significant difference in tau levels between healthy controls and MCI group, the AD-CSF pattern shows significantly higher t-tau levels compared to both MCI and healthy controls[249]. These findings suggest that total tau levels in plasma are not an early disease biomarker of AD and the benefit of it could be restricted for diagnostic purposes[249]. Limitations of the plasma AD biomarkers include the technical difficulties in their measurements due to their low concentrations in plasma, high cost and the variability of results between different laboratories[247].

1.2.7.2 Plasma and serotonin metabolites in Alzheimer’s disease

High performance liquid chromatography apparatus followed by electrochemical detection (HPLC-ECD) provides a robust and reproducible mean for measurement of body fluid 5-HT and 5-HIAA[250]. The seasonal variation in plasma 5-HT metabolites levels should be taken into consideration to avoid possible misinterpretation[251]. Other factors such as ethnic variations, type of diet, nutritional status, underlying chronic disease and use of particular medication could influence the plasma 5-HT levels. There are different neuropsychiatric conditions with reported changes in plasma 5-HT metabolites concentration such as depression, bipolar disorder and autism, which can coincide with dementia or present as differential diagnosis in the clinical workup of dementia[252, 253].

Although there is no compelling evidence indicating that plasma 5-HT correlates with CSF 5-HT level in human, a correlation between 5-HIAA and the 5-HT turnover, i.e. the 5-HIAA/5-HT ratio, between plasma and CSF has been reported[254].
The plasma as extracellular compartment contains different biological systems with a wide range of molecules, including signaling molecules, and host complex interactions between these different systems. The 5-HT metabolites are involved in biological functions produced mainly by other plasma systems such as immune function. Plasma levels of tryptophan decrease with aging and inversely correlate to levels of particular cytokines immune mediators such as interleukin 6 and 8 (IL6 and IL8)[255].

These findings illustrate the importance of the interaction between 5-HT and immune system in an inflammatory and age-related disorder such as AD[255]. Surprisingly, Higher plasma 5-HT levels were shown in AD, while no change in 5-HIAA is detected in one report[250].

1.2.7.3 Leucocytes and Alzheimer’s disease

Lymphocytes are central in the pathogenesis of chronic inflammatory conditions and the lymphocyte subtypes CD40 and CD40L are recruited centrally for the amyloid-induced microglial activation process[256, 257]. Levels of the soluble CD40 in the blood are higher in AD compared to non-AD dementia[258]. Lymphocytes, particularly CD95, are also associated with alteration in apoptotic activity and oxidative stress in AD and their peripheral levels are elevated in AD[257, 259]. In addition, there are non-conclusive reports discussing alteration in the central and peripheral levels of CD4 and CD8 in AD[257, 260]. Taken together, these reports together reflect the antigenic over-stimulation of the adaptive immune system in AD[261]. This is important to the interleukin and antibody profiles that modulate T-helper, T-suppressor and T-cytotoxic cells and regulate both the humoral and cellular immunity[257]. Although both T-lymphocytes and B-lymphocytes are reduced by AD pathology[262], no correlation was found between the total lymphocyte count and the cognitive decline in AD[263].

B-lymphocytes mature into plasma cells and produce clone-specific antibodies in response to stimulation with non-self antigens presented to them by the antigen presenting cells (APCs) in association with the major histocompatibility complex type 2 (MHCII). Levels of circulating autoantibodies from Immunoglobulin G (IgG) directed against AD-related pathological antigen are high in AD, which suggest an autoimmunity-like mechanism in AD due to lack of tolerance for particular self-antigens[264]. Profiles of phenotypic changes in blood cells could be dependent on the progression of cognitive symptoms and severity of dementia. Higher levels of lymphocytes and lower polymorph nuclear cells (neutrophils) are seen in MCI compared to age and sex matched healthy controls[262].

Alteration in other types of leucocytes, in terms of functional and expression profiles, are also reported in AD. Other peripheral cells affected by AD-pathology are natural killer cells (NKC), red blood cells and platelets[257].
1.2.7.4 Red blood cells and Alzheimer’s disease

Red blood cells (RBCs) or erythrocytes are non-nucleated cells characterized by wide-spanning cell membrane surrounds the cell in a biconcave-shape manner[265]. RBCs, due to the hemoglobin pigment, represent the body intravascular delivery system for the oxygen and nutrient to the tissue[265]. Patients with abnormalities in the synthesis of RBCs or hemoglobin have inadequate oxygen supply to tissues and present clinically with hypoxia. In addition, chronic hypoxia implies an oxidative stress for neuronal cells.

The oxygen-carrying capacity and gaseous exchange efficiency of RBCs are impaired by the peripheral β-amyloid (Aβ) peptides in the blood[242]. This is due to the fact that soluble forms of Aβ peptides lead to hemolysis of RBCs in contrast to non-soluble forms[266]. However, enhancing polymerization of Aβ peptides into fibrillar structure prevents this amyloid-induced hemolysis of RBCs[242, 266].

Reports have shown that human erythrocytes contain higher Aβ40 and Aβ42 peptides levels than those in plasma, which suggest that RBCs represent the subsequent clearance pool for plasma Aβ[242]. The interaction between plasma amyloid species and erythrocytes is relevant to AD pathology. Levels of Aβ peptides in RBCs are associated with oxidative stress[242]. Furthermore, significant correlations were shown between the plasma levels and erythrocytes levels of Aβ42 and Aβ40. Relevantly, binding of these Aβ peptides to RBCs increases with aging[242]. In AD, this interaction is more active in low-density RBCs[267]. Intuitively, the interaction between plasma and RBCs amyloid pools depends also of the physical and biochemical characteristics of Aβ peptides such as lipid solubility, presence of hydrophobic amino acids and the plasma concentrations[242, 268].

1.2.7.5 Physiological aspects about platelets

Platelets are derived from the bone marrow precursor, the megakaryocyte cell. Similar to RBCs they are enucleated peripheral blood cells. They are involved in different physiological and pathological process such as coagulation hemostasis, inflammation and hypersensitivity reactions and atherosclerosis[10].

Functional and structural molecules are localized in the platelet’s cell membrane, plasma, dense granules, α-granules and the platelets-surface associated glycoproteins[10]. The platelet 5-HT is transported from the cytoplasm to be stored in the dense granules through specialized transporters[269]. In particular situations, platelets are activated to release the stored vasoactive amines after exposure to von Willebrand factor (vWF) in the reticuloendothelial system.
P-selectin is contained in the α-granules and considered as a marker for platelets activity that is conventionally measured by flow cytometry or ELISA[271].

1.2.7.6 Platelets and Alzheimer’s disease biomarkers

Platelets have less proteomic variability, more stable and reproducible AD biomarker profiles compared to plasma[272]. Platelets are considered as peripheral neuropharmacological model with many biological similarities to neurons[273]. The advantages of this model is that they can be obtained by less invasive means and can be characterized with simple laboratory methods.

The similarities between platelets and neurons are relevant to AD. Platelets can be used in studying expression of relevant amyloid species, inflammation, oxidative stress markers, 5-HT trafficking, transmission and pharmacological modulations[274]. The serotonergic similarities to neurons include 5-HT uptake from the plasma into platelets intracellular compartment, 5-HT re-uptake modulation by antidepressant and antipsychotics, modulation of the intracellular granules by the pharmacological modulations of 5-HT receptors[273, 274].

Still, there are a number of limitations for using platelets as a neuropharmacological model in AD. First, the amyloid content of the platelets depends on the interaction between platelets and collagen and hence amyloid levels are not homogenous in all platelets sub-populations[275]. In addition, platelets activity influence platelet-derived AD biomarkers, 5-HT biomarkers and the pharmacological modulation of platelet 5-HT[273]. Important differences between platelets and neurons are the inability of platelets to synthesize or degrade 5-HT due to the lack of MAO-A[276].

The biological roles of Aβ in platelets are diffuse and not fully understood yet. However, it is known that platelets release Aβ peptides, which stimulate platelet activity[276, 277]. Although different candidate biomarkers were investigated for potential platelets-derived biomarker, only amyloid precursor protein APP and MAO-B are classified among the most reliable AD-related platelets biomarkers[276].

Platelets express all secretase enzymes that are needed to produce the similar neuronal amyloid processing. Moreover, the pattern of inhibition of the non-amyloidogenic pathway that takes place in platelets in early AD is similar to that occurring in brain[276]. Because of the downregulation of the activity of the responsible enzyme α-secretase, less production and release of soluble APP species is seen in platelets in early stages of AD[278]. This is mirrored by increased activity of the amyloidogenic pathway including increased β-secretase activity in AD[278]. The proteomic findings from APP isoforms are consistent with the gene expression
profiles of APP mRNA[274]. The potential platelet-derived AD biomarkers are shown in Figure 1.7[276].

![Figure 1.7 Platelets-derived Alzheimer’s disease biomarkers. “Adopted from Veitinger et al, 2014, Acta Neuropathologica Communicatian. Open access through The Creative Commons Public Domain Dedication waiver, © Veitinger et al.; licensee BioMed Central Ltd. 2014”][276].

1.2.7.7 Platelets serotonin in Alzheimer’s disease

Accumulating data suggest the reliability of platelets 5-HT as a peripheral biomarker in AD[276]. This is attributed to the fact that 5-HT transmission, packing in granules or vesicles, expression of 5-HT receptors and response to antidepressant and antipsychotic are comparable between platelets and neuronal cells[274, 276].

Although the main impact of AD on serotonergic system is seen in RN, serotonergic disturbances are not limited to neuronal cells[41]. Alterations of serotonergic pathway in platelets are repeatedly reported in AD, which include alteration in 5-HT2A binding, SERT uptake of 5-HT and the 5-HT metabolites profiles[276]. However, results from 5-HT levels in platelets in AD showed inconsistent data. The findings extracted from the literature fluctuate between higher, lower or no differences between platelets 5-HT in AD and their corresponding normal controls[250, 279, 280]. Similar inconsistent data is also reported regarding alteration of 5-HT uptake by platelets in AD[276]. Different factors affect 5-HT uptake or concentrations in AD such as depression, psychotic symptoms, platelets density and platelets activity[280-282]. Reports suggest an association between cognitive decline in late AD stages and lower platelets 5-HT levels[277, 279]. Moreover, platelets 5-HIAA levels are not reduced in AD[250]. While some reports indicate that the activity MAO-B is increased in AD in both platelets and brain neurons[250, 276, 281, 283], other reports indicate no alteration in MAO-B activity in AD[284, 285].
1.3 Brain serotonergic system in Alzheimer's disease

There are multiple factors that contribute to the brain serotonergic alterations in AD. First, there is loss of 5-HT cell bodies and damage to 5-HT neurons from RN and disturbances in 5-HT transmission[41]. Moreover, alterations of 5-HT receptors expression and/or function are observed in AD[41, 286]. Reduction in SERT expression, change in frequency of SERT genetic polymorphism and change of serotonin reuptake pattern could also contribute to disturbances of 5-HT hemostasis in AD[287]. The molecular aspects of the positive influence of 5-HT on neuronal protection against AD pathology are illustrated in Figure 1.8.

Figure 1.8 The neuroprotective mechanism of 5-HT in AD. Adopted from The serotonergic system in ageing and Alzheimer’s disease, Volume 99, page number 26 Rodriguez et al, 2012, Progress in Neurobiology. Copyright (2016), with permission from Elsevier
1.3.1 Serotonergic neuronal loss in Alzheimer's disease

Postmortem brain tissue and genetic animal models were used to understand how AD pathology affects 5-HT neurons. The methods used to illustrate brain expression of 5-HT markers include classical staining, IHC and PET. In contrast to normal aging where the 5-HT neurons are relatively stable until late in life[288], significant loss of 5-HT neurons was observed in AD[289]. However, age is an important factor in determining the impact of AD on serotonergic neurons. The more the advanced age at the onset of AD, the more the severe the impact of AD on RN[41].

RN which represents the anatomical location for the cell bodies of serotonergic neurons[42], is significantly affected by AD pathology and its involvement is associated with AD symptoms progression[290]. Furthermore, 5-HT neurons projecting to the hippocampus from the DRN are among the highest affected serotonergic projections by NFT-AD pathology[41, 291]. However, there is increased 5-HT neuron sprouting, detected by SERT staining, in hippocampus suggestive of compensation for 5-HT neuron loss[292, 293].

On the other hand, alteration in the serotonin content and 5-HT transmission might influence the amyloid-related pathology[294]. Triple transgenic AD mice subjected to high tryptophan diet showed lower amyloid burden in the hippocampus CA1 area compared to animal subjected to normal or low tryptophan diet[294]. 5-HT modulates the excitatory neural activities in the entorhinal cortex[295]. Therefore the availability of 5-HT affects no only AD pathology but also physiological functions of memory related brain areas.

1.3.2 Alterations of 5-HT and 5-HIAA in Alzheimer's disease

Reduction of 5-HT and 5-HIAA are consistently reported from different brain area and shown to correlate with dementia progression and particular BPSD[41, 296]. Brain areas with lower 5-HT and 5-HIAA levels include hippocampus, basal ganglia, frontal cortex, temporal cortex and amygdala[41]. However, other reports suggest equal to higher levels of 5-HIAA in AD in the frontal, parietal and temporal cortex in human[297]. The data from 5-HT metabolite in AD shows significant variation across different models and intervention techniques. Higher 5-HIAA levels are reported in rat model injected with Aβ40 in the dorsal hippocampus, while low levels are reported in AD mice model[298, 299].

Table 1.3 summarizes the human and animal studies highlighted 5-HT and 5-HIAA in AD[41].
Table 1.3: AD-associated changes in the levels of 5-HT/5-HIAA: post-mortem and animal model studies.

<table>
<thead>
<tr>
<th>Marker/Technique</th>
<th>Age (years)</th>
<th>N (AD)</th>
<th>Brain region/levels of 5-HT/5-HIAA in % of the control</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT (HPLC)</td>
<td>82</td>
<td>46</td>
<td>FC – 65 for 5-HT, TC – 49 for 5-HT</td>
<td>Palmer et al. (1987)</td>
</tr>
<tr>
<td>5-HT (HPLC)</td>
<td>76</td>
<td>17</td>
<td>A – 59 for 5-HT, 50 for 5-HIAA</td>
<td>Burke et al. (1990)</td>
</tr>
<tr>
<td>5-HIAA (HPLC)</td>
<td>81</td>
<td>13</td>
<td>DR – 215 for 5-HT, 118 for 5-HIAA</td>
<td>Nazarali and Reynolds (1992)</td>
</tr>
<tr>
<td>5-HT (HPLC)</td>
<td>81</td>
<td>20</td>
<td>TC – 37 for 5-HT, 63 for 5-HIAA</td>
<td>Chen et al. (1996)</td>
</tr>
<tr>
<td>5-HIAA (HPLC)</td>
<td>82</td>
<td>17</td>
<td>CN – 45 for 5-HT, 51 for 5-HIAA</td>
<td>Lai et al. (2002)</td>
</tr>
<tr>
<td>5-HT (HPLC)</td>
<td>81</td>
<td>20</td>
<td>A – 51 for 5-HT, 57 for 5-HIAA</td>
<td>Garcia-Alloza et al. (2005)</td>
</tr>
<tr>
<td>5-HIAA (HPLC)</td>
<td>74</td>
<td>9</td>
<td>P – 64 for 5-HT, 81 for 5-HIAA</td>
<td>Bowen et al. (2008)</td>
</tr>
<tr>
<td>5-HT (HPLC)</td>
<td>81</td>
<td>20</td>
<td>FC – 94 for 5-HT, 144 for 5-HIAA</td>
<td></td>
</tr>
<tr>
<td>5-HIAA (HPLC)</td>
<td>82</td>
<td></td>
<td>FC – the paper indicates that the 5-HT levels were decreased but does not provide quantification</td>
<td></td>
</tr>
<tr>
<td>5-HT (HPLC)</td>
<td>81</td>
<td>20</td>
<td>FC – 45 for 5-HT, 47 for 5-HIAA</td>
<td></td>
</tr>
<tr>
<td>5-HIAA (HPLC)</td>
<td>81</td>
<td>20</td>
<td>FC – 48 for 5-HT, 40 for 5-HIAA</td>
<td></td>
</tr>
<tr>
<td>5-HT (HPLC)</td>
<td>74</td>
<td>9</td>
<td>FC – 75 for 5-HT, 99 for 5-HIAA</td>
<td></td>
</tr>
<tr>
<td>5-HIAA (HPLC)</td>
<td>81</td>
<td>20</td>
<td>TC – 118 for 5-HT, 140 for 5-HIAA</td>
<td></td>
</tr>
<tr>
<td>5-HIAA (HPLC)</td>
<td>82</td>
<td>17</td>
<td>PC – 55 for 5-HT, 87 for 5-HIAA</td>
<td></td>
</tr>
</tbody>
</table>

Animal studies

<table>
<thead>
<tr>
<th>Marker/Technique</th>
<th>Brain region/levels of 5-HT/5-HIAA in % of the control</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HIAA (HPLC)</td>
<td>Transgenic mouse model of AD with Aβ pathology</td>
<td>Liu et al. (2008)</td>
</tr>
<tr>
<td>5-HIAA (HPLC)</td>
<td>Intra-hippocampal injected Aβ rat model</td>
<td>Verdun et al. (2011)</td>
</tr>
</tbody>
</table>


1.3.3 5-HT1A and Alzheimer’s disease

5-HT1A plays a role in the memory consolidation; it mediates particularly the consolidation of learning in both physiological state and cognitive impairment[300]. 5-HT1A is evaluated with binding studies, positron emission tomography (PET), or IHC, see down Table 1.4[41].

Whereas 5-HT1A antagonists were proposed to improve memory, 5-HT1A agonist (tandospirone) showed impairment of explicit memory. Negative correlation was observed between explicit memory and 5-HT1A hippocampal expression[301]. In addition, injection of Aβ40 in the dorsal hippocampus in a rat model produces a transient increase of 5-HT1A expression in astrocytes[298]. Consistent with this data, a negative correlation between the hippocampal 5-HT1A binding potential and explicit memory was shown[301]. Another study showed that reduction in 5-HT and increased densities of 5-HT1A are correlated with cognitive decline in AD[302]. 5-HT1A bindings in the hippocampus are associated with neuropsychiatric symptoms, where low 5-HT1A binding particularly associated with depression in AD[303]. Taken together, these reports reflect the negative effect of postsynaptic 5-HT1A on memory function...
through inhibition of hippocampal 5-HT[301]. An increase of 5-HT1A binding in the frontal cortex is also reported in AD; however, no correlation with the disease duration, pathological markers or neuroleptic medication was found[304]. In contrast, reduction in 5-HT1A densities in hippocampal and parahippocampal regions in AD was reported using PET[305]. The same study showed increased whole brain 5-HT1A densities in MCI and reduction in AD, which suggest 5-HT1A as a demarcating molecular marker to differentiate MCI from AD[305].

Table 1.4: AD-associated changes in 5-HT1A receptors: binding, PET and immunohistochemical studies.

<table>
<thead>
<tr>
<th>Radioligand/Marker</th>
<th>Age (years)</th>
<th>N (AD)</th>
<th>Brain region/5-HT1A receptor % of the control</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding studies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]-5-HT</td>
<td>nr</td>
<td>nr</td>
<td>FC – 52</td>
<td>Bowen et al. (1983)</td>
</tr>
<tr>
<td>[3H]-5-HT</td>
<td>79</td>
<td>nr</td>
<td>PC – 74</td>
<td>Perry et al. (1984)</td>
</tr>
<tr>
<td>[3H]8-OH-DPAT</td>
<td>72</td>
<td>nr</td>
<td>FC – 53</td>
<td>Middlemiss et al. (1986)</td>
</tr>
<tr>
<td>[3H]-5-HT</td>
<td>78</td>
<td>13</td>
<td>TC – no changes</td>
<td>Cross et al. (1986)</td>
</tr>
<tr>
<td>[3H]-5-HT</td>
<td>72</td>
<td>5</td>
<td>TC – no changes</td>
<td>Cross et al. (1988)</td>
</tr>
<tr>
<td>[3H]-5-HT</td>
<td>77</td>
<td>8</td>
<td>H – no changes</td>
<td>Jansen et al. (1990)</td>
</tr>
<tr>
<td>[3H]8-OH-DPAT</td>
<td>81</td>
<td>33</td>
<td>FC, TC – no changes</td>
<td>Lai et al. (2003a)</td>
</tr>
<tr>
<td>[3H]8-OH-DPAT</td>
<td>82</td>
<td>35</td>
<td>TC – no changes</td>
<td>Tsang et al. (2010)</td>
</tr>
<tr>
<td>Positron emission tomography studies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[18F]MPPF</td>
<td>77</td>
<td>14</td>
<td>H – 87 (aMCI)</td>
<td>Kepe et al. (2006)</td>
</tr>
<tr>
<td>[18F]MPPF</td>
<td>70</td>
<td>10</td>
<td>H, InfOG – decreased binding potential</td>
<td>Truchot et al. (2008)</td>
</tr>
<tr>
<td>Immunohistochemical studies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1A</td>
<td>83</td>
<td>8</td>
<td>PN – reduced density</td>
<td>Yeung et al. (2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VN – reduced density</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R – reduced density</td>
<td></td>
</tr>
<tr>
<td>5-HT1A</td>
<td>77</td>
<td>11</td>
<td>H – reduced immunoreactivity</td>
<td>Mizukami et al. (2011)</td>
</tr>
</tbody>
</table>

1.3.4 5-HT1B, 5-HT1D, 5-HT2A, 5-HT4 and 5-HT6 in Alzheimer’s disease

Despite the importance of 5-HT1B in regulation of multiple neurotransmitters, only one report investigating the binding of 5-HT1B in frontal and temporal cortex and implication of 5-HT1B in cognitive decline is available[286]. The study showed lower density of 5-HT1B in frontal and temporal cortex in AD. In addition, they reported a correlation between increase in 5-HT1B binding and MMSE decline[286]. However, in this study they used [3H]-GR-125743, which is a non-specific radiolabelled antagonist that binds both 5-HT1B and 5-HT1D[306]. The use of PET would provide a powerful tool to evaluate the antemortem levels 5-HT1B in dementia. Reduction of cortical 5-HT1B is attributed to normal aging in one PET study[307]. 5-HT6 pharmacological modulations showed potential therapeutic effects in depression, anxiety and metabolic disorder despite the lack of information about the detailed mechanism of 5-HT6 in neuropsychiatric disorders[308]. 5-HT6 antagonist is a newly emerging strategy of cognitive enhancement in AD[308]. This is attributed to the modulation of cholinergic, GABA, glutamate systems and a complex downstream signaling pathway. The proposed molecular mechanism of 5-HT6 blockade in cognitive enhancement is shown in Figure 1.9[309].


Brain 5-HT2A expression in AD was assessed by autoradiography, PET and single-photon emission computed tomography (SPECT). Consistent reductions of 5-HT2A levels were reported in temporal cortex with significant correlation with MMSE decline[41, 310].
Increased activity of 5-HT4 stimulates the non-amyloidogenic pathway through the activation of the α-secretase enzyme that favors the production of soluble Aβ species[311]. These findings are consistent with promising data from 5-HT4 agonist alone or in combination with acetylcholinesterase (AChE) inhibitor as a candidate drug for AD[312, 313].

1.3.5 SERT in Alzheimer’s disease

The genetic polymorphism of SERT influences the pattern of 5-HT transport and thus it might aggravate 5-HT deficit in AD[314]. Despite the repeated reports suggesting changes of SERT genetic variability to be a predisposing factor to late onset AD[315], no compelling evidence that SERT polymorphism plays a significant role in determining the clinical syndrome in terms of BPSD in AD exist[316]. Reports about the expression of SERT in human postmortem studies indicate a reduction of SERT in frontal, temporal and parietal cortex[41]. However, in a triple transgenic model of AD upregulations of SERT and SERT-expressing neurons were reported in CA1 area of the hippocampus[292, 293].
1.4 Serotonergic system in Lewy body dementia (LBD)

The term Lewy body dementia (LBD) include both Parkinson disease dementia PDD and DLB[317, 318]. As discussed above, serotonergic disturbances exist in different type of neuropsychiatric disorders, regardless the underlying pathology. They are seen in VaD, FTD and motor neuron disease such as amyotrophic lateral sclerosis (ALS)[41]. Although depression represents a logical link between dementia symptoms and serotonergic disturbances, independent serotonergic disturbances occur also in dementia patients without depressive symptoms[319].

1.4.1 Serotonergic system in Parkinson disease (PD)

Parkinson’s disease (PD) is the second most common neurodegenerative disorder in elderly. It is characterized clinically by mixture of motor and non-motor symptoms[320]. The motor symptoms comprise the classical triad of rigidity, tremor and bradykinesia. Dementia represents the most important and common non-motor consequence of PD[321]. PDD is a well-known sequel in PD during the disease clinical course. The prevalence of PDD in PD is about 31.1%, it accounts for 3% of all dementia cases and 0.5% of population above 65 year old[317, 321].

The pathological hallmark of PD is the degeneration of the dopamine-releasing neurons in the basal ganglia particularly the degeneration of the striatonigral pathway (SN). Moreover, multiple neurotransmitters such as 5-HT and acetylcholine are implicated in the pathogenesis of PD[322]. Of note, the basal ganglia receive dense serotonergic projections from RN, which inhibit the dopamine release[42, 323]. These projections are affected by the PD-pathology and the impact is more severe in PD patients with depression[324]. Basal ganglia’s serotonergic alteration in PD is shown in Figure 1.10[324].

Figure 1.10 Comparison of 5HT levels in PD and normal subjects. GPe: globus pallidum pars externa, GPI: globus pallidum pars interna, SN: substantia nigra and STN is subthalamic nucleus. Reprinted with permission from Elsevier, copyright (2016), from Hout et al. The serotonergic system in Parkinson's disease. Progress in Neurobiology. 2011 (95):163-212. PubMed PMID: 21878363
Lower CSF levels of 5-HT and 5-HIAA and the inverse correlations with the progression and severity of symptoms in PD indicate the serotonergic deficit in PD[325]. Beside the serotonergic deficit in the basal ganglia, cortical areas in the prefrontal cortex such as Brodman area9 (BA9), anterior cingulate cortex, entorhinal cortex and hippocampus have lower 5-HT levels in PD[324]. This serotonergic deficit is associated with both motor and non-motor symptoms in PD as well as the dyskinesia following dopamine enhancement therapy[317, 322]. This is consistent with the data from the *in vivo* 11C-DASB PET study that showed reduction in the cortical, brain stem and RN binding of the serotonergic marker in PD[326]. In addition, treatment with levodopa favors the conversion of Tryptophan into dopamine instead of 5-HT, which further aggravate the preexisting serotonergic dysfunction[322].

Although there are contradictory results about SERT expression in PD, SERT reduction independent of PD duration, treatment and severity of symptoms is likely[322]. However, alterations of SERT in PD depend on the neuroanatomical expression of SERT. The brain mapping of SERT pattern in PD is summarized in Figure 1.11[324].

![Figure 1.11 Alteration of SERT in PD. SERT is reduced in the temporal cortex (dark yellow), frontal (light yellow), orbitofrontal (green), visual cortex (light blue), cingulate (dark blue), insular cortex, hippocampus and basal ganglia. In depressed PD patients, SERT is increased in Prefrontal cortex (orange in A). Reprinted with permission from Elsevier, copyright (2016), from Hout et al. The serotonergic system in Parkinson’s disease. Progress in Neurobiology. 2011 (95):163-212. PubMed PMID: 21878363](image)

5-HT1B is expressed in pathways that are relevant to PD such as striatonigral and raphe-nigral pathways[167]. No compelling evidence indicates modulation of 5-HT1B levels in brain by PD pathology alone. The 5-HT1B reductions in orbitofrontal area in PD brains, assessed by PET[327], are attributed to physiological reductions due to aging in another report with similar methodology[307].
Significant correlation was found between 5-HT1B measured by PET and creativity in normal individuals but not PD[328]. However, reports from human studies and animal models of PD indicate increased 5-HT1B and p11 in the basal ganglia nuclei[324]. The PET findings of 5-HT1B, like other receptors, depend on the biochemical characteristics of the ligands, sensitivity of the methods, degree of brain atrophy and the type of system model used to evaluate 5-HT1B brain expression. For example, no alteration in 5-HT1B striatal expression was found in unilateral 6-OHDA-lesioned rat, a model of PD[329].

Other serotonin receptors such as 5-HT1A and 5-HT2A are also altered by PD pathology[324]. This was studied in primates model of PD and human, using PET or binding studies. In PD, 5-HT1A is increased in the prefrontal area, the temporal BA36, and in RN[324]. Additionally, 5-HT1A agonist is extensively studied in the dopamine-related extrapyramidal side effects of neuroleptics medication and dyskinesia[330].

1.4.2 Serotonergic system in dementia with lewy bodies (DLB)

There is an overlap between DLB and PD in different pathological and therapeutic aspects[318, 321]. Both neurodegenerative disorders are related to the accumulation of the misfolded α-synuclein, inclusion bodies and neuronal degeneration[318]. In addition, both PDD and DLB have amyloid and tau pathology. DLB is frequently described as a mixed pathological entity between AD and PD[318, 331]. The proportion of DLB among dementias is variable between 0-23% depending on the type of the study whether it is a community-based or a clinical hospital–based study[318, 321].

Definite DLB is a histological diagnosis and the disease is frequently misdiagnosed as AD. Presence of particular clinical symptoms such as visual hallucination, extrapyramidal features, cognitive fluctuation and visuospatial impairment might help in early differentiation of DLB[332]. The frequent symptomatic use of antipsychotics in dementia to alleviate BPSD and the sensitivity of DLB patients to antipsychotics in terms of development of side effects require correct diagnosis of dementia before commencing neuroleptic medications[333].

The 5-HT system has rarely been studied in detail in DLB. Clinical studies showed that DLB patients with hallucinations have higher 5-HT turnover in the frontal and temporal cortex compared to AD[334]. Moreover, reduction of global cognitive function in DLB has been attributed to 5-HT deficit[334, 335].
2 AIMS

2.1 General aims

- To investigate the serotonergic and related molecular alterations in cell models of AD.
- To understand the relationship between serotonergic, pathological and synaptic changes in neurodegenerative dementias.
- To examine platelet serotonin metabolites in AD.

2.2 Specific aims

- **Paper I:**
  
  To assess alteration in 5-HT1B and related molecules in a familial AD model (APPswe mutation).

- **Paper II:**
  
  To characterize the molecular outcomes after pharmacological modulations of the 5-HT system in an APPswe-cell model.

- **Paper III:**
  
  To compare the platelets 5-HT metabolites profiles between AD and SCI and correlate these to CSF biomarkers and cognition.

- **Paper IV:**
  
  To evaluate expressions of 5-HT1B, 5-HT and 5-HIAA in post-mortem PFC in AD, DLB, and PD.  
  To study the association of these serotonergic markers with cognition, neuropathology and synaptic protein biomarkers of dementia.
3 METHODOLOGY

3.1 Ethical considerations

All studies in thesis are conducted according to the Helsinki declaration, the local ethical review board and Karolinska Institutet guidelines in research with animal and human materials. Study I was approved by Stockholm Södra Djurförsöksköetiska Nämnd, study III and VI from The Regional Ethical Committee in Stockholm (Regionala Etikprövningsnämnden i Stockholm). Study II was conducted entirely in an in vitro model, so no ethical permit was required.

3.2 Materials

In papers I and II, Taqman gene expression was performed to study gene expression profiles of selected 5-HT pathway molecules. We used predesigned assays for 5-HT1B receptor gene, SERT (SLC6A4), p11 (S100A10), MAO-A and the housekeeping genes PRLP0 and GAPDH were purchased from (Life Technologies, Sweden). For paper I[336], antibodies for 5-HT1B and S100A10 from (Abcam, Cambridge, UK), and SLC6A4 from (Lifespan Bioscience, USA) were used for Western blotting. The enzyme activity kit for MAO-A, MAO- Glo™, was purchased from (Promega, USA).

For paper II[337], we used 5-HT, serotonin creatinine monophosphate from (Sigma, USA), the 5-HT1B antagonist (SB224289) and sertraline Hydrochloride (sertraline HCl) from (Tocris, UK). We also used the human untagged 5-HT1B cDNA cloned into pCMV6-XL4 vector for the in vitro receptor overexpression from (Origene, USA). In addition, we purchased antibodies for MAPK pathways with their corresponding positive controls and β-Actin from (Cell signaling, USA). For visualization of relative phosphorylations of MAPK pathway we used Proteome Profiler™ Array, Human phospho-MAPK array kit was purchased from (R&D systems, UK).

3.3 Aspects about experimental models we used

3.3.1 Subjects and clinical assessments

The study cohort is derived from subjects referred to a university-based memory clinic in Karolinska Huddinge hospital in Stockholm. We have selected 2 diagnostic groups of patients: An AD group fulfilling the ICD 10 criteria for AD[338] (n=15), and a comparison group with normal performance on cognitive tests and normal biomarkers but with memory complaints, i.e. subjective cognitive impairment (SCI) (n=20). The diagnostic and evaluation work-up includes structured comprehensive clinical, biochemical and imaging workup for the patients as previously described[338].
Screening for cognitive function using the Mini-mental state examination (MMSE) and neuropsychological assessment were performed[208]. The Cornell Scale for Depression in Dementia (CSDD), with the recommended cut-off of 8/9, was used to classify the depression severity on the cohort[339, 340] and/or a modified version of the Geriatric Depression Scale (GDS) with 20 items, cut-off 5/6[341]. Moreover, patients were interviewed according to previous history of depression or use of anti-depressants. Depression was defined as having a depression score above the cut-off, a history of depression or the use of antidepressants. In case of discrepancy between the scales, CSDD was given priority.

The clinical evaluation was performed blind to the platelet measurements by a clinician with many years of experience in clinical dementia research. Patients with a history of physical diseases, which according to the clinician influenced the cognitive functioning, were excluded. Participants signed an informed consent. Table 3.1 summarizes the basic characteristic of subjects in paper III.

In paper IV, cognitive data includes the Mini-Mental State Examination (MMSE) data (available for 41 of the 57 patients). The majority of patients were longitudinally followed (for average 9 years period) and annual rate of decline on MMSE was calculated. The last MMSE score was taken maximum two years prior to death. The final diagnoses for patients were made according to clinicopathological consensus diagnoses incorporating the 1-year rule to differentiate DLB and PDD[342]. The baseline, pathological and clinical characteristics of the study population are shown in Table 3.2.
### Table 3.1 Baseline, laboratory, clinical and medication characteristics of the two study groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SCI (n=20)</th>
<th>AD (n=15)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong> n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>11 (55.0%)</td>
<td>7 (46.7%)</td>
<td>0.625#</td>
</tr>
<tr>
<td><strong>Age</strong> (yr) - median (IQR)</td>
<td>61 (14)</td>
<td>74 (17)</td>
<td>0.006¶</td>
</tr>
<tr>
<td><strong>MMSE score</strong> - median (IQR)</td>
<td>29 (2)</td>
<td>24 (9)</td>
<td>&lt;0.001¶</td>
</tr>
<tr>
<td><strong>Medications</strong> n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSRIs</td>
<td>3 (15.0%)</td>
<td>4 (26.7%)</td>
<td>0.430§</td>
</tr>
<tr>
<td>ASA</td>
<td>3 (15.0%)</td>
<td>2 (13.3%)</td>
<td>1§</td>
</tr>
<tr>
<td>Anticoagulants</td>
<td>2 (10.0%)</td>
<td>0</td>
<td>0.496§</td>
</tr>
<tr>
<td>Statins</td>
<td>5 (25.0%)</td>
<td>2 (13.3%)</td>
<td>0.672§</td>
</tr>
<tr>
<td>Antihypertensive</td>
<td>5 (25.0%)</td>
<td>6 (40.0%)</td>
<td>0.344#</td>
</tr>
<tr>
<td>Anti-diabetes</td>
<td>0</td>
<td>1 (6.7%)</td>
<td>0.429§</td>
</tr>
<tr>
<td>Neuroleptics</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><strong>ApoE ε4</strong> n (%)</td>
<td>5 (29.4%)</td>
<td>7 (50.0%)</td>
<td>0.242#</td>
</tr>
<tr>
<td><strong>Depression</strong> Frequency n (%)</td>
<td>12 (60.0%)</td>
<td>3 (20.0%)</td>
<td>0.037§</td>
</tr>
<tr>
<td><strong>CSF Biomarkers</strong> mean (SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ42</td>
<td>1274.0 (280.2)</td>
<td>548.5 (171.0)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Total tau (T-tau)</td>
<td>250.0 (110.2)</td>
<td>600.1 (309.4)</td>
<td>0.001*</td>
</tr>
<tr>
<td>Phosphorylated tau (P-tau)</td>
<td>47.2 (16.7)</td>
<td>79.2 (36.0)</td>
<td>0.009*</td>
</tr>
<tr>
<td>T-tau/Aβ42 ratio</td>
<td>0.19 (0.07)</td>
<td>1.24 (0.80)</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>


Shapiro-Wilk test was used to check for the normality of the distribution of numeric variables in each study group. Statistical significant differences (two-tailed p-value < 0.05) are bolded.

# Pearson Chi Square statistics
¶ Mann-Whitney U-test
§ Fisher’s Exact test
* Independent Samples T-test
Table 3.2 Baseline, pathological and clinical characteristics of the study groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>AD</th>
<th>DLB</th>
<th>PDD</th>
<th>All Dementia</th>
<th>Control</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=17)</td>
<td>(n=19)</td>
<td>(n=21)</td>
<td>(n=57)</td>
<td>(n=21)</td>
<td></td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>6 (35.3)</td>
<td>13 (68.4)</td>
<td>9 (42.9)</td>
<td>28 (49.1%)</td>
<td>12 (57.1)</td>
<td>0.182</td>
</tr>
<tr>
<td>Age at death, mean (SD)</td>
<td>87.5 (7.2)</td>
<td>81.5 (7.0)</td>
<td>81.8 (5.0)</td>
<td>83.4 (6.8)</td>
<td>79.7 (7.8)</td>
<td>0.006 (AD vs. Control) &lt;0.001 (AD vs. DLB, AD vs. PDD)</td>
</tr>
<tr>
<td>Duration of dementia, mean (SD)</td>
<td>9.7 (2.9)</td>
<td>5.2 (2.7)</td>
<td>2.9 (2.1)</td>
<td>6.5 (4.1)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Last MMSE score prior to death, mean (SD)</td>
<td>9.2 (7.5)</td>
<td>16.3 (9.3)</td>
<td>15.1 (7.6)</td>
<td>13.6 (8.4)</td>
<td>-</td>
<td>0.058</td>
</tr>
<tr>
<td>Rate of annual MMSE decline, mean (SD)</td>
<td>3.9 (3.9)</td>
<td>3.8 (3.8)</td>
<td>1.7 (1.2)</td>
<td>2.8 (3.0)</td>
<td>-</td>
<td>0.065</td>
</tr>
<tr>
<td>Braak stage, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Control vs. DLB, AD) &lt;0.001 (Control vs. PDD, DLB, AD) AD vs. DLB)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (4.8)</td>
<td>1 (1.8%)</td>
<td>6 (37.5)</td>
</tr>
<tr>
<td>1/2</td>
<td>0</td>
<td>2 (10.5)</td>
<td>16 (76.2)</td>
<td>18 (31.6%)</td>
<td>8 (50.0)</td>
<td></td>
</tr>
<tr>
<td>3/4</td>
<td>3 (17.6)</td>
<td>10 (52.6)</td>
<td>3 (14.3)</td>
<td>16 (28.1%)</td>
<td>2 (12.5)</td>
<td></td>
</tr>
<tr>
<td>5/6</td>
<td>14 (82.4)</td>
<td>7 (36.8)</td>
<td>1 (4.8)</td>
<td>22 (30.1%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CERAD code, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Control vs. PDD, DLB, AD) PDD vs. AD AD vs. DLB)</td>
<td></td>
</tr>
<tr>
<td>0 (none)</td>
<td>0</td>
<td>5 (26.3)</td>
<td>7 (33.3)</td>
<td>12 (26.7%)</td>
<td>13 (86.7)</td>
<td></td>
</tr>
<tr>
<td>1 (sparse)</td>
<td>0</td>
<td>7 (36.8)</td>
<td>8 (38.1)</td>
<td>15 (33.3%)</td>
<td>2 (13.3)</td>
<td></td>
</tr>
<tr>
<td>2 (moderate)</td>
<td>2 (40.0)</td>
<td>1 (5.3)</td>
<td>2 (9.5)</td>
<td>5 (11.1%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3 (frequent)</td>
<td>3 (60.0)</td>
<td>6 (31.6)</td>
<td>4 (19.0)</td>
<td>13 (28.9%)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lewy body score (frontal lobe), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (none)</td>
</tr>
<tr>
<td>1 (sparse)</td>
</tr>
<tr>
<td>2 (moderate)</td>
</tr>
<tr>
<td>3 (frequent)</td>
</tr>
</tbody>
</table>

AD: Alzheimer’s disease; DLB: dementia with Lewy bodies; PDD: Parkinson’s disease dementia; SD: standard deviation; n: number; MMSE: mini-mental state examination; CERAD: Consortium to Establish a Registry for Alzheimer’s Disease; NIA-AA: National Institute on Aging-Alzheimer Association; ChEIs: cholinesterase inhibitors. Univariate comparisons have been performed using one-way ANOVA or Chi square tests wherever appropriate. Bonferroni post hoc and pair-wise tests were used in case of significant p-value of the one-way ANOVA or Chi square tests. All statistical significant differences are bolded (p-value<0.05). * None of the pairwise comparisons are statistically significant.
3.3.2 Human brain
As previously described[343], brain tissues from patients and normal healthy controls were obtained from different centers in UK and Norway; the MRC London Neurodegenerative Diseases Brain Bank, the Thomas Willis Brain Collection and the Newcastle Brain Tissue Resource, University Hospital Stavanger and stored in the UK brain banks as part of the Brains for Dementia Research Network. An informed consent was taken from all participants for their tissue to be used in research. This study was ethically approved from The Regional Ethical Committee in Stockholm (Regionala Etikprövningsnämnden i Stockholm). Patients were diagnosed as AD (n=17), DLB (n=21), or PDD (n=19) during life according to consensus criteria[343], and most subjects were followed prospectively as part of longitudinal cohort.

3.3.3 Platelet fractionation
Platelets as peripheral model for AD biomarker were used in paper III. Immediately after the venipuncture, fractionation of whole blood according to cell density was performed as previously described[280]. A total of (7.5 mL) of venous blood was anticoagulated with 2.5 mL 0.129 M disodium citrate. Fractionation of platelets according to density was performed using linear Percoll™ (GE Healthcare Bio-Sciences AB, Sweden) gradient[344-346]. We have chosen platelets having particular density in this study as standard, to reduce variability and include a homogenous platelets population. Linear Percoll™ gradients covering the density span 1.040 to 1.090 kg/L were used, particularly plasma–free platelets having a specific density of 1.064 kg/L are used from all cases. In addition, the procedure includes a blocking solution to avoid in vitro platelet activation and subsequent granule release. Platelet counts were determined electronically. Then, the detergent (Triton X-100 final concentration 0.1%) from (Sigma-Aldrich, Missouri, U.S.) was used for platelets lysis. Subsequently, cell debris was removed by a short centrifugation and samples were stored in -70 until further processing. For internal validation and platelet homogeneity determination, the intracellular soluble P-selectin was used as a measure reflecting platelet α-granules content, here a commercial immunoassay kit from (R&D Systems, UK) was used[345].

3.3.4 CSF biomarkers
We used CSF biomarkers in paper III as a reference in order to examine the ability of peripheral serotonin biomarkers in differentiating SCI from AD. CSF measurements for Aβ42, t-tau and p-tau were used from memory clinic records at Karolinska Huddinge Hospital, Stockholm. The collection and storage of CSF were performed according to local guidelines and recommended procedures. The CSF biomarkers tau, p-tau and Aβ42 concentrations were
determined with xMAP technology and the INNO-BIA AkzBio3 kit (Innogenetics)[347, 348]. The cutoff values for the laboratory to classify normal or abnormal values were: Aβ42>550 ng/l, p-tau<80 ng/l and t-tau<400 ng/l. We used the t-tau/Aβ42 ratio as a measure of AD pathology[349].

3.3.5 Transgenic mice

For paper I, we used hippocampus from the female Tg2576 mice. These transgenic mice are widely used as a genetic animal model of the familial AD mutation (APPswe mutation) in AD research since they overexpress the human APP gene with the APPswe double mutation (K670N/M671L). At 3 months, they display soluble Aβ aggregates and cognitive dysfunction and develop amyloid neuritic plaques after 12 months[350]. Hippocampus samples from different age groups were used in this study.

3.3.6 Cell Culture

The human neuroblastoma cell line, SH-SY5Y cells (ATCC, USA), were stably transfected with the human APP that has the Swedish KM670/671NL double mutation (APPswe) using cytomegalovirus promoter-constructed vector as described previously[351]. SH-SY5Y cells with the stable transfection of the empty vector p-cDNA 3.1 were used as control cells (Empty vector cells).

Cells were cultured according to the provider’s instruction in MEM (Minimal Eagle Medium) (Gibco, life technologies, USA), supplemented with 10% fetal bovine serum. Geneticin, G418 sulphate, 10µl per ml (life technologies, USA) was used for selection. Cells were grown under standard condition of 37°C temperature and 5% CO2 supply. Prior to treatments with chemicals of interest, cell medium was replaced with serum-free media and incubated overnight. At 80-90% cell confluence, cells were then treated with 5-HT or vehicle alone[352]. The concentrations and durations of treatments are shown in Table 3.3

<table>
<thead>
<tr>
<th>Substance</th>
<th>Company</th>
<th>ID or catalogue #</th>
<th>Working concentration</th>
<th>Duration of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>Sigma Aldrich</td>
<td>H7752</td>
<td>10µM</td>
<td>30 minutes</td>
</tr>
<tr>
<td>SB224289</td>
<td>Tocris Bioscience</td>
<td>1221</td>
<td>1µM</td>
<td>2 hours</td>
</tr>
<tr>
<td>Sertraline HCl</td>
<td>Tocris Bioscience</td>
<td>1032</td>
<td>10µM</td>
<td>20 minutes</td>
</tr>
</tbody>
</table>
3.4 Experimental techniques

3.4.1 High performance liquid chromatography followed by electrochemical detection

Concentrations of 5-HT and 5-HIAA in cell culture medium, cell lysate, platelets fractions and human brain homogenates were measured using HPLC with electrochemical detection previously described[353, 354]. 5-HT and 5-HIAA measurements were conducted separately and then 5-HIAA/5-HT ratio was computed as an index for 5-HT turnover and serotonergic activity. In summary, for 5-HT measurement we used a HPLC system: HTEC500 (Eicom, Kyoto, Japan), and a CMA/200 Refrigerated Microsampler (CMA Microdialysis, Stockholm, Sweden) connected with 20 µl loop and operating at +4°C. The potential of the glassy carbon-working electrode was +450mV vs. the Ag/AgCl reference electrode. The separation was achieved on a 200 x 2.0mm Eicompak CAX column (Eicom, Kyoto, Japan). The mobile phase is a mixture of methanol and 0.1M phosphate buffer (pH6.0) (30:70, v/v) containing 40mM potassium chloride and 0.13mM EDTA-2Na[354].

A separate HPLC system with electrochemical detection (HTEC500) is used for measurement of 5-HIAA concentrations. The potential of the glassy carbon-working electrode was +750mV vs. the Ag/AgCl reference electrode. The separation was performed on a 150 x 3.0mm Eicompak SC-5ODS column (Eicom, Kyoto, Japan). The mobile phase was a mixture of methanol and 0.1M citrate and 0.1M sodium acetate buffer solution (pH3.5) (16:84, v/v) containing 210mg/L Octanesulfonic acid sodium salt and 5mg/L EDTA-2Na. The chromatograms were recorded and integrated by use of the computerized data acquisition system Clarity (DataApex, The Czech Republic)[354].

3.4.2 Western blotting

In paper II and I, the Western blot technique is used as a proteomic method for semi-quantitatively compare certain makers across different experimental groups. The method was performed as described before[336, 355].

Briefly, proteins from cells or mice hippocampus tissues were extracted using a standardized lysis buffer supplemented with anti-protease and anti-phosphatase cocktail (Sigma Aldrich, MO). Samples were then centrifuged to 13000 rpm for 15 minutes. The supernatant (the extracted protein) was transferred to clean 1.5 ml polypropylene tube and stored at -20° C until use. The determination of the total protein concentration was done with BCA kit (Pierce, Rockford, IL, USA). The supernatant was mixed with NuPAGE® LDS sample buffer (4X) (Life technologies) and heated to 100° C for 5 minutes and loaded on 10% acrylamide SDS-PAGE gels for protein separation. An overnight transfer to a nitrocellulose membrane (0.2 µm pore size membrane
Protran® (BA83, Whatman, GE Healthcare) was done at 4°C. Membranes were then blocked with 5% milk (BCR685 FLUKA, Sigma Aldrich) for 1 hour. The different primary antibodies (diluted in TBS-Tween buffer) used for paper I are shown in Table 3.2. For paper II, primary antibodies for p70S6, phospho-p70S6 (Thr389), phospho-p70S6- (Thr421, Ser424), p70S6 and pMAPK (42-44), total MAPK[352] from Cell signaling, USA were used.

Each experiment was done 3 times in separate biological material. In cell culture experiments we used the same cell culture passage to control for variability between passages and for each experiment n=6. Table 3.4 shows details about some antibodies used in paper I and II.

**Table 3.4 Primary antibodies used for Western blotting**

<table>
<thead>
<tr>
<th>The Antigen</th>
<th>Company</th>
<th>Cat #</th>
<th>Dilution</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT1B</td>
<td>Abcam</td>
<td>ab13896</td>
<td>1:500</td>
<td>Rabbit</td>
</tr>
<tr>
<td>S100A10 (p11)</td>
<td>Abcam</td>
<td>ab76472</td>
<td>1:5000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>SLC6A4 (SERT)</td>
<td>Life Span Bioscience, Inc,</td>
<td>LS-C156102</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Enzo</td>
<td>ADI-CSA-335-E</td>
<td>1:5000</td>
<td>Mouse</td>
</tr>
</tbody>
</table>

### 3.4.3 Gene expression analysis

Total RNA from SHSY5 cell cultures and Tg2576 mice hippocampus tissues were isolated using the RNeasy mini kit (Qiagen, USA) after DNase treatment (RNase-Free DNase Set, Qiagen, USA). Subsequently, extracted RNAs were separated on agarose gels to exclude genomic DNA contamination and ensure the integrity of extract RNA.

This is further followed by reverse transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). For the amplification reaction analysis, we used Relative Standard Method supplied by Applied Biosystem[356]. A set of relative standard curves from human and mice samples were created and the relative quantities were measured and compared across the plates by plotting the threshold cycle (Ct) of each sample with that of the standard curve run in the same PCR plate. Different series of real-time PCR reactions were determined to quantify the both target genes and endogenous control sequences (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or ribosomal protein lateral stalk subunit P0 (RPLP0) or both).

Thermo-cycling and fluorescence detection was performed according to TaqMan®Gene Expression Assays Protocol using ABI PRISM® 7000 Sequence Detection System. PCR Master Mix (Applied Biosystems), and TaqMan® Gene Expression Assays (Applied Biosystems) were used in the amplification reaction. The stability of endogenous control, GAPDH and RPLP0, was measure by determining RNA copy numbers and estimating normalization factor (NF). This was performed using the software GeNorm 3.3 visual basic application for Microsoft Excel.
Finally, mRNA copy numbers of all target transcripts were adjusted by a normalization factor (NF).

### 3.4.4 MAO-A enzyme activity

MAO-A activity was determined using MAO-Glo™ kit (Promega, USA)[357]. Adding equal volumes of 4X prepare the reactions MAO substrate preparations to of 4Xtest preparation to initiate a luciferase reaction. Then, 25 μl of MAO-A was added to each reaction. Two different types of negative controls were used; either 25 microliters of MAO-A enzyme or 12.5 microliter of test preparation were added. After incubation for 1 hour at room temperature, stabilized luminescence signals were obtained when of luciferase detection solution was added and incubated again at room temperature for 20 minutes.

Luminescence was measured at an integration time of one second per well in a plate reader (Tecan Safire II). Relative light units (RLUs), expression for MAO-A activity, were calculated by subtracting values from negative controls without MAO-A enzyme from test preparations[352].

### 3.4.5 MAPK protein arrays

The Proteome Profiler™ Array, Human phospho-MAPK array kit (R&D systems, UK), to determine the relative phosphorylation of more than 20 members of the human MAPK[352, 358]. First, membranes were blocked with array buffer; this is followed by addition of 150 micrograms of protein. The membranes together with the antibody detection cocktail are incubated overnight at 4°C. The membranes were then washed incubated in streptavidin HRP, diluted in buffer 5, for 30 min. Membranes were washed again and final brief wash is carried out three times. Blot images were visualized with CCD camera.

### 3.4.6 ELISA

To quantify levels of 5-HT1B in brain homogenate in Paper IV, we used the human 5-HT1B receptor ELISA kit from EIAab®, China and according to the manufacturer’s instructions. We extracted data of the synaptic proteins Rab3A, SNAP25 and neurogranin from previously published report[359]. Beside ELISA, Aβ42, Aβ40, tau, p-tau and α-synuclein were measured by IHC as described before[360].

### 3.5 Statistical analysis

All statistical analyses were performed using SPSS software, version 22 (Armonk, NY: IBM Corp.). For paper I and paper II, the data was assessed for normal distribution by Kolmogorov-Smirnov test. For comparison of two groups T-test or Mann-Whitney test was used. Similarly,
one-way ANOVA or Kruskall Wallis test followed by post hoc Tukey’s or Dunn’s tests were used for multiple group analysis. Level of significance is taken at p-value of $\leq 0.05$ was. Data are either expressed as mean $\pm$ SEM or as median $\pm$ interquartile range (IQR) in addition to plots showing the distribution according to normality of data[361].

For paper III, the concentrations of 5-HT and 5-HIAA were adjusted to the fraction’s protein concentration and fraction’s platelet count. For description of normality of distribution or skewness of the data, mean and standard deviation (SD) or median and interquartile range (IQR) were used respectively. Like statistical analysis in paper I and II, Shapiro-Walk test was used to examine the normality of distribution for numeric variables. The unadjusted univariate comparisons between SCI and AD groups were evaluated using independent samples T-test, Mann-Whitney U-test, Pearson Chi square and Fisher’s exact tests were used for numeric, ordinal, and nominal variables wherever appropriate. As the main analysis, we applied multivariate ordinal logistic regression to adjust for differences in tertiles of platelets serotonin indicators between SCI and AD groups for participants’ age, depression status (yes/no), and P-selectin level. Adjusted odds ratio (OR) and their 95% confidence interval (CI) were reported for each regression model. Correlations were performed using Spearman test, and adjusted for age using partial correlation. Based on the inspection of the data, the SCI group was divided into two subgroups based on the median value of the protein-corrected 5-HT level. Mann-Whitney U test and linear regression model were used to compare the CSF biomarkers between the SCI with high 5-HT and low 5-HT. A $p$-value of $<0.05$ was considered statistically significant.

For paper IV, Continuous numeric and categorical variables are described using mean (standard deviation (SD)), median (interquartile range (IQR)) and frequency (percentage), respectively. For univariate between-group comparisons, we used Kruskal-Wallis, one-way ANOVA or Chi square wherever appropriate. Bivariate correlations between different continuous measurements have been assessed by means of Spearman non-parametric test due to the skewed distribution of numeric variables.

The multivariate analyses was performed using either linear (for numeric values of biomarkers and rate of decline in MMSE as the outcome) or ordinal logistic (for tertiles of biomarkers’ concentration as the outcome) regression models adjusted for age at death and sex where control group has been defined as the reference condition. A two-tailed $p$-value of $<0.05$ indicated statistically significant differences or associations. A number of comparisons were made without attempts to adjust, and thus findings need to be interpreted with caution and need replication in larger studies.
4 RESULTS AND DISCUSSION

This chapter summarizes the main results of the thesis and their implications in the context of relevant literature; the rest of the results and a more comprehensive discussion can be viewed in the individual papers.

4.1 Aspects of 5-HT1B in Alzheimer's disease

4.1.1 5-HT1B expression in Alzheimer's disease models

Few reports about 5-HT1B in AD were available prior to my work, and to our knowledge, we have the first report discussing 5-HT1B expression in familial AD models (APPSwe).

Reduction in the binding of 5-HT1B in the frontal and temporal cortex of sporadic AD was reported in postmortem study in human[286]. The levels of 5-HT1B in other AD brain areas, transgenic animal and cell models of AD still remain unknown. Based on that and the unknown functional implications of 5-HT1B in dementia, 5-HT1B has a lot of novelty aspects in this paradigm.

In paper I, we have studied the expression of 5-HT1B in both in vitro and ex vivo model of AD. We used SH-SY5Y neuroblastoma cell line that stably transfected with the familial mutation APP Swedish (APPSwe) together with hippocampus homogenate from the transgenic mice Tg2576 that overexpress APPswe[361]. In addition, we studied the postmortem levels of 5-HT1B levels in the PFC (BA9) in a mixed dementia cohort with AD, PDD, DLB and healthy subjects in Paper IV.

SH-SH5Y cell line is a neuronal cell line that has a moderate expression of 5-HT1B, while the hippocampus represents one of the most abundant brain areas in 5-HT1B. In paper I, our results suggested reduction of 5-HT1B protein and mRNA in APPswe cells (Figure 4.1) and reduction of the hippocampal 5-HT1B in Tg2576 mice (Figure 4.2)[361]. Tg2576 mice are acceptable model of AD. Although they develop the behavioral symptoms of cognitive impairment after 6 months, obvious biochemical characteristics of AD are seen at about one year of age. These biochemical changes include amyloid plaques, excessive inflammation, significant oxidative stress, tauopathy and synaptic degeneration[362].
Figure 4.1 Comparison between APPswe cells and control cells in the levels of selected serotonin markers. A. Western blot bands for 5-HT1B in APPswe and control cells (Empty vector). B, C and D are relative quantification of 5-HT1B, p11 and SERT respectively. E. Shows gene expression of APP in control cells and APPswe cells. Data is expressed as SEM, n=3-4. (*p<0.05, **p<0.01 and ***p<0.001) Reprinted with permission from Elsevier, copyright (2016).
Figure 4.2. Expressions of 5-HT1B (A, B and C) and SERT (A, B and D) in hippocampus of female Tg2576 mice in different age groups. Data is expressed as SEM, n=3-4. (*p<0.05, **p<0.01 and ***p<0.001). Tg: Tg2576. WT: wild type mice. Reprinted with permission from Elsevier, copyright (2016).

The physiological factors related to 5-HT regulation and subsequent effects on cognition might determine the expression of 5-HT1B in dementia. 5-HT1B is not only a negative regulator of 5-HT and acetylcholine release under physiological conditions but also is relevant to 5-HT and cholinergic deficit in AD[296]. Furthermore, the use of selective 5-HT1B agonists leads to negative outcomes in memory compared to the selective antagonists. Together, these indicate
that increased activity of 5-HT1B is related to memory impairment[137]. Reduction of 5-HT1B levels in AD might be attributed to the neuronal loss during the neurodegenerative process. Alternatively, it could be due to compensatory mechanisms to reinforce acetylcholine and serotonin levels by releasing the inhibitory tone of 5-HT1B by decreasing its expression[286].

There are some limitations in our cell and mice models used in paper I. For the in vitro model, we used control cells in form of SH-SY5Y cells transfected with the empty vector, which are acceptable control. While the most suitable control to show the exact effect of a mutated APPswe gene is cells transfected with the APP gene without APPswe mutation (APP wild type). In addition, in the mice experiment in paper I, we did not provide identical age groups in Tg2576 and the control group. We included age groups 6 months and 2 years in the wild type mice, while additional 9-month-old and 12-month-old age groups were included in Tg2576 mice.

Another limitation is that we used female only Tg2576 mice and this is due to a technical difficulty in including the male Tg2576 mice. The reason for using female, as in most other studies as well, is that one cannot have male mice together since they will fight and may end up killing each other. The alternative would be to keep them in separate cages and that causes stress, since they do not live alone in their natural habitat. Still, using only female mice might raise issues regarding the sex-hormones effects on 5-HT system, particularly the effect of estrus cycle on brain metabolism. For example comparing 6-month-old to 12-month-old group mice is a comparison of two different phases in the reproductive life, with two different sex-hormones profiles.

It is important to note that loss of 5-HT1A binding has already been reported in two transgenic mice models of AD[41]. Therefore, the changes in 5-HT transmission in AD cannot be explained entirely by modulation in expression and activity of 5-HT1B alone.

Treating APPswe cells with exogenous Aβ42 peptide produced no effect on 5-HT1B protein levels in control cells (result not shown here). Of note, the mechanism of 5-HT1B alterations is unknown and not investigated thoroughly in this thesis.

Aging affects the expression of 5-HT1B in brain. After twenty years of age, a gradual decrease of 5-HT1B binding potential by about 10% for each decade of life, in all human brain regions, was shown[307]. These findings are in line with results from animal models that showed reduction of 5-HT1B mRNA in forebrain areas, striatum, piriform cortex and septum but not in RN in 2-year-old rats compared to 4-month-old[363]. Importantly, reduction of 5-HT1B in brain is associated with the early onset of age-related dysfunctions. Premature age-related motor dysfunction, decreased survival and increased gene expression of age-related markers are demonstrated in 5-HT1BK0 mice[364]. The densities of 5-HT1B, measured by the binding of
the 5-HT1B/1D antagonist ([³H]-GR-125743), are reduced in temporal and frontal cortical areas in sporadic AD[286]. This study reported more reduction of 5-HT1B density in the temporal cortex (BA20) (37% reduction) compared to 25% reduction in the frontal brain area (BA10)[286].

To assess the pattern of 5-HT1B in different dementia sub-groups and relate this to the pathological markers of dementia, we used data derived from a cohort consist of three neurodegenerative diseases (AD, DLB, PDD) and a control group. The demographic factors and dementia neuropathological staging for this cohort are shown in Table 3.2.

Data for the pathological profiles for amyloid protein, tau and α-synuclein as well as selected synaptic proteins are used. In paper IV, pathological and synaptic biomarkers data was extracted from data published before for the same cohort[359, 365]. Our objective in this study is to investigate 5-HT1B levels in PFC (BA9 brain area) in dementia and to study their relation to pathological and synaptic markers. As shown in Table 4.1, we found that all the dementia groups have higher levels of 5-HT1B in BA9 brain area than the age and sex-matched control group. Significant difference was observed in the AD group (p=0.012).

In contrast to previous studies in AD, no available data indicates the downregulation of 5-HT1B in the PFC in PDD or DLB. PET studies suggested age-related changes of 5-HT1B in the brain stem and limbic system in PD, still no changes is detected in any of the cortical areas[327]. Levels of 5-HT1B could be affected by dopaminergic treatment in PD, particularly in the striatum-related neuronal projections[329]. In our cohort 90% of the PD subjects were covered by anti-Parkinson’s medications. This might influence 5-HT1B levels despite the difference in the brain areas studied between our study and the previous report. Both depression and its treatment are associated with 5-HT1B and the adaptor protein p11[153]. Furthermore, another limitation to this study is that 5-HT1B levels were not adjusted for the depression scores. Therefore, changes in 5-HT1B levels could be attributed to depression in this study cannot be excluded. The study also lacks correction of the data to the antidepressant usage, educational assessment for participants, their ethnic backgrounds and past medical health conditions data. The adaptor protein (p11) impacts not only the depression phenotype, but also the response to antidepressant and the antidepressant-related 5-HT1B alterations[153]. When we divided the dementia sub-groups into tertiles according to their 5-HT1B concentrations, 52.9% of AD and 47.4% of DLB subjects were in the 3rd tertile with the highest 5-HT1B levels, compared to only 14.3% in the control group. Results for 5-HT1B in BA9 are shown in Table 4.1.
Table 4.1 Serotonergic biomarkers in prefrontal brain BA9 area in different groups of study

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>AD (n=17)</th>
<th>DLB (n=19)</th>
<th>PDD (n=21)</th>
<th>Control (n=21)</th>
<th>Univariate p-value</th>
<th>Multivariate p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT1B concentration, median (IQR)</td>
<td>1068.9 (1313.3)</td>
<td>835.8 (583.8)</td>
<td>767.6 (623.4)</td>
<td>606.0 (391.9)</td>
<td>0.087</td>
<td><strong>AD: 0.012; DLB: 0.107; PDD: 0.328</strong></td>
</tr>
<tr>
<td>HT1B tertiles, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>5 (29.4)</td>
<td>4 (21.1)</td>
<td>7 (33.3)</td>
<td>10 (47.6)</td>
<td></td>
<td><strong>AD: 0.053; DLB: 0.020;</strong></td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>3 (17.6)</td>
<td>6 (31.6)</td>
<td>9 (42.9)</td>
<td>8 (38.1)</td>
<td>0.069</td>
<td><strong>PDD: 0.366</strong></td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>9 (52.9)</td>
<td>9 (47.4)</td>
<td>5 (23.8)</td>
<td>3 (14.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT concentration, median (IQR)</td>
<td>7.2 (2.3)</td>
<td>6.0 (2.5)</td>
<td>5.7 (4.5)</td>
<td>8.0 (3.8)</td>
<td>0.193</td>
<td><strong>AD: 0.309; DLB: 0.046; PDD: 0.245</strong></td>
</tr>
<tr>
<td>5-HIAA concentration, median (IQR)</td>
<td>39.8 (47.4)</td>
<td>42.5 (21.6)</td>
<td>31.2 (33.5)</td>
<td>67.0 (81.9)</td>
<td>&lt;0.001</td>
<td><strong>AD: 0.004; DLB: &lt;0.001; PDD: &lt;0.001</strong></td>
</tr>
</tbody>
</table>

Regarding the number of valid data for each biomarker, there are some missing cases for each of the comparisons ranging from n=0 to n=7. AD: Alzheimer’s disease; DLB: dementia with Lewy bodies; PDD: Parkinson’s disease dementia; SD: standard deviation; n: number. All univariate comparisons have been performed using Kruskal–Wallis non-parametric test. All statistical significant differences are bolded (p-value<0.05). Multivariate comparisons have been done using either linear or ordinal logistic regression models adjusted for age at death and sex where control group has been defined as the reference condition. All statistical significant differences are bolded (p-value<0.05).
The associations between 5-HT1B levels and the annual rate of MMSE decline or last MMSE prior to death are shown in Table 4.2 and Table 4.3.

Our multivariate regression model in Table 4.2 indicates that subjects in the highest tertile of 5-HT1B in BA9 from AD, PDD and combined dementias groups have greater decline of MMSE compared to the subjects belong to the first tertile of 5-HT1B (lowest 5-HT1B or reference group). Despite alterations of 5-HT1B levels in BA9 in DLB, association between 5-HT1B and rate of MMSE decline is insignificant in this group. In addition, no associations between 5-HT or 5-HIAA and the rate of MMSE in BA9 were observed (Table 4.2).

As shown in Table 4.3, when we replaced the rate of MMSE decline by the MMSE score prior to death in the model, no similar results were found. Of note, being in the 2nd tertile of the 5-HT1B in the AD group is associated with an average of -8.3 lower last MMSE score compared to those AD patients in the 1st tertile of the 5-HT1B. In contrast to 5-HIAA in AD, higher 5-HT levels in PDD are associated with lower MMSE scores prior to death.

Unexpectedly, no associations between 5-HT1B and 5-HT, 5-HIAA or 5-HIAA/5-HT ratio were observed in any of the diagnostic groups (results not shown).

<p>| Table 4.2 Multivariate regression model to investigate the association between rate of MMSE decline and concentration of serotonergic biomarkers in the prefrontal brain BA9 area in patients with AD, DLB and PDD |</p>
<table>
<thead>
<tr>
<th>Measurement</th>
<th>Group</th>
<th>1st tertile</th>
<th>2nd tertile</th>
<th>3rd tertile</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT1B</td>
<td>AD</td>
<td>Reference</td>
<td>3.58 (-1.28 – 8.43), p=0.149</td>
<td>4.66 (1.22 – 8.10), p=0.008</td>
</tr>
<tr>
<td></td>
<td>DLB</td>
<td>Reference</td>
<td>-0.69 (-6.38 – 5.01), p=0.813</td>
<td>-1.45 (-6.56 – 3.67), p=0.580</td>
</tr>
<tr>
<td></td>
<td>PDD</td>
<td>Reference</td>
<td>0.53 (-0.31 – 1.57), p=0.214</td>
<td>1.23 (0.18 – 2.28), p=0.022</td>
</tr>
<tr>
<td></td>
<td>All (n=41)</td>
<td>Reference</td>
<td>0.79 (-1.33 – 2.92), p=0.464</td>
<td>2.49 (0.44 – 4.54), p=0.017</td>
</tr>
<tr>
<td>5-HT</td>
<td>AD</td>
<td>Reference</td>
<td>-0.15 (-6.12 – 5.82), p=0.962</td>
<td>-2.15 (-8.92 – 4.62), p=0.534</td>
</tr>
<tr>
<td></td>
<td>DLB</td>
<td>Reference</td>
<td>14.99 (-1.70 – 31.67), p=0.078</td>
<td>-9.15 (-18.46 – 0.16), p=0.054</td>
</tr>
<tr>
<td></td>
<td>PDD</td>
<td>Reference</td>
<td>0.17 (-0.95 – 1.28), p=0.768</td>
<td>0.55 (-0.43 – 1.53), p=0.271</td>
</tr>
<tr>
<td></td>
<td>All (n=35)</td>
<td>Reference</td>
<td>2.01 (-0.43 – 4.45), p=0.106</td>
<td>-0.76 (-3.35 – 1.82), p=0.563</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>AD</td>
<td>Reference</td>
<td>-3.84 (-8.33 – 0.65), p=0.093</td>
<td>-6.09 (-12.33 – 0.15), p=0.056</td>
</tr>
<tr>
<td></td>
<td>DLB</td>
<td>Reference</td>
<td>0.36 (-4.25 – 4.97), p=0.879</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>PDD</td>
<td>Reference</td>
<td>0.44 (-0.56 – 1.44), p=0.391</td>
<td>0.30 (-0.81 – 1.41), p=0.595</td>
</tr>
<tr>
<td></td>
<td>All (n=35)</td>
<td>Reference</td>
<td>0.17 (-2.16 – 2.51), p=0.886</td>
<td>-1.19 (-4.23 – 1.85), p=0.441</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>AD</td>
<td>Reference</td>
<td>0.13 (-7.08 – 7.34), p=0.972</td>
<td>-2.00 (-7.39 – 3.39), p=0.467</td>
</tr>
<tr>
<td></td>
<td>DLB</td>
<td>Reference</td>
<td>-0.36 (-4.65 – 3.92), p=0.868</td>
<td>-4.28 (-9.51 – 0.95), p=0.109</td>
</tr>
<tr>
<td></td>
<td>PDD</td>
<td>Reference</td>
<td>-0.12 (-1.27 – 1.03), p=0.839</td>
<td>-0.08 (-1.08 – 0.92), p=0.873</td>
</tr>
<tr>
<td></td>
<td>All (n=35)</td>
<td>Reference</td>
<td>-0.05 (-2.62 – 2.52), p=0.972</td>
<td>-1.31 (-3.84 – 1.23), p=0.314</td>
</tr>
</tbody>
</table>

Regarding availability of data for MMSE and the biomarkers, there are some missing cases for each regression model. Number of valid cases included in each model is mentioned in parentheses.

All regression models have been adjusted for age at death and sex. All statistical significant coefficients are bolded (p-value<0.05). CI: confidence interval. * There are only two patients with the 3rd tertile of 5-HIAA concentrations in the DLB group and the model could not calculate regression coefficient.
in the DLB group and the model could not calculate regression coefficient. All regression models have been adjusted for age at death and sex. Regarding availability of data for MMSE and the biomarkers, there are some missing cases for each regression model. Number of valid cases included in each model is mentioned in parentheses.

All regression models have been adjusted for age at death and sex. All statistical significant coefficients are bolded (p-value<0.05). CI: confidence interval. * There are only two patients with the 3rd tertile of 5-HIAA concentrations in the DLB group and the model could not calculate regression coefficient.

### Table 4.3 Multivariate regression model to investigate the association between the last MMSE score before death and concentration of serotonergic biomarkers in the prefrontal brain BA9 area in patients with AD, DLB and PDD

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Group</th>
<th>1st tertile</th>
<th>2nd tertile</th>
<th>3rd tertile</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT1B</td>
<td>AD</td>
<td>Reference</td>
<td>-8.3 (-15.2 – 1.5), p=0.018</td>
<td>-2.0 (-6.9 – 2.8), p=0.418</td>
</tr>
<tr>
<td></td>
<td>DLB</td>
<td>Reference</td>
<td>5.9 (-7.0 – 18.9), p=0.368</td>
<td>3.3 (-7.2 – 13.8), p=0.537</td>
</tr>
<tr>
<td></td>
<td>PDD</td>
<td>Reference</td>
<td>0.1 (-6.1 – 6.3), p=0.980</td>
<td>-6.3 (-14.1 – 1.5), p=0.111</td>
</tr>
<tr>
<td></td>
<td>All (n=45)</td>
<td>Reference</td>
<td>-0.1 (-5.6 – 5.5), p=0.983</td>
<td>-3.5 (-8.6 – 1.6), p=0.183</td>
</tr>
<tr>
<td>5-HT</td>
<td>AD</td>
<td>Reference</td>
<td>8.1 (1.7 – 14.5), p=0.013</td>
<td>5.4 (-1.4 – 12.2), p=0.123</td>
</tr>
<tr>
<td></td>
<td>DLB</td>
<td>Reference</td>
<td>-8.7 (-18.3 – 0.0), p=0.076</td>
<td>6.5 (-3.7 – 16.8), p=0.211</td>
</tr>
<tr>
<td></td>
<td>PDD</td>
<td>Reference</td>
<td>-4.5 (-12.1 – 3.0), p=0.240</td>
<td>-7.0 (-13.6 – -0.3), p=0.040</td>
</tr>
<tr>
<td></td>
<td>All (n=39)</td>
<td>Reference</td>
<td>-4.5 (-10.5 – 1.5), p=0.138</td>
<td>-6.6 (-7.0 – 5.7), p=0.845</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>AD</td>
<td>Reference</td>
<td>3.4 (-2.1 – 8.9), p=0.227</td>
<td>9.4 (1.7 – 17.1), p=0.017</td>
</tr>
<tr>
<td></td>
<td>DLB</td>
<td>Reference</td>
<td>-9.8 (-18.1 – -1.5), p=0.021</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>PDD</td>
<td>Reference</td>
<td>-2.4 (-9.5 – 4.8), p=0.518</td>
<td>-5.5 (-13.4 – 2.5), p=0.178</td>
</tr>
<tr>
<td></td>
<td>All (n=39)</td>
<td>Reference</td>
<td>-3.3 (-8.9 – 2.3), p=0.244</td>
<td>-0.3 (-8.0 – 7.4), p=0.938</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>AD</td>
<td>Reference</td>
<td>1.6 (-7.3 – 10.4), p=0.727</td>
<td>2.2 (-5.0 – 9.4), p=0.550</td>
</tr>
<tr>
<td></td>
<td>DLB</td>
<td>Reference</td>
<td>-0.9 (-10.9 – 9.0), p=0.854</td>
<td>0.9 (-14.8 – 16.6), p=0.914</td>
</tr>
<tr>
<td></td>
<td>PDD</td>
<td>Reference</td>
<td>2.1 (-6.3 – 10.6), p=0.620</td>
<td>-0.3 (-7.6 – 7.0), p=0.939</td>
</tr>
<tr>
<td></td>
<td>All (n=39)</td>
<td>Reference</td>
<td>1.9 (-4.0 – 7.7), p=0.534</td>
<td>1.4 (-5.1 – 7.9), p=0.676</td>
</tr>
</tbody>
</table>

4.1.2 Associations between 5-HT1B, synaptic and pathological markers

We used a multivariate analysis adjusted for age at death and sex to investigate the association between 5-HT1B and synaptic markers in BA9. As shown in Table 4.4, four diagnostic groups (AD, DLB, PDD and control) and different pathological and synaptic biomarkers are included in this model.

No correlation was found in any of the dementia groups, between Aβ42, Aβ40 or the Aβ42/Aβ40 ratio and 5-HT1B. Conversely, in the control group we found significant positive correlation between 5-HT1B and the amyloid based-biomarker Aβ42, Aβ40 and the Aβ42/Aβ40 ratio (p=0.001, 0.017 and 0.017 respectively, (data not shown). However, no association between 5-HT1B and tau, α-synuclein or any of the synaptic markers was seen in the control group. Significant positive correlation between 5-HT1B and α-synuclein was observed in DLB (adjusted correlation coefficient = 0.73, p=0.01), see Table 4.4.
Table 4.4 Multivariate correlation between serotonergic, synaptic, and proteinopathy

<table>
<thead>
<tr>
<th>Variables</th>
<th>AD (n=17)</th>
<th>DLB (n=19)</th>
<th>PDD (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α-synuclein</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1B</td>
<td>0.32 (0.346)</td>
<td><strong>0.73 (0.010)</strong></td>
<td>0.02 (0.952)</td>
</tr>
<tr>
<td>5-HT</td>
<td>-0.15 (0.651)</td>
<td>0.36 (0.274)</td>
<td>-0.40 (0.141)</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>0.10 (0.763)</td>
<td>0.03 (0.930)</td>
<td>-0.25 (0.365)</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>0.26 (0.438)</td>
<td>-0.19 (0.580)</td>
<td>0.17 (0.552)</td>
</tr>
<tr>
<td><strong>Aβ42</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1B</td>
<td>0.32 (0.308)</td>
<td>-0.08 (0.774)</td>
<td>0.14 (0.594)</td>
</tr>
<tr>
<td>5-HT</td>
<td>-0.34 (0.283)</td>
<td>-0.06 (0.829)</td>
<td>-0.02 (0.929)</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>-0.41 (0.191)</td>
<td>-0.05 (0.854)</td>
<td>-0.06 (0.802)</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>-0.06 (0.862)</td>
<td>0.03 (0.928)</td>
<td>-0.08 (0.763)</td>
</tr>
<tr>
<td><strong>Aβ40</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1B</td>
<td>-0.05 (0.875)</td>
<td>0.19 (0.472)</td>
<td>0.04 (0.873)</td>
</tr>
<tr>
<td>5-HT</td>
<td>-0.52 (0.085)</td>
<td>-0.27 (0.310)</td>
<td>0.05 (0.855)</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>-0.17 (0.589)</td>
<td>0.18 (0.498)</td>
<td>-0.08 (0.755)</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>0.54 (0.071)</td>
<td>0.39 (0.131)</td>
<td>-0.11 (0.664)</td>
</tr>
<tr>
<td><strong>T-tau</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1B</td>
<td>0.04 (0.891)</td>
<td>-0.22 (0.415)</td>
<td>-0.05 (0.856)</td>
</tr>
<tr>
<td>5-HT</td>
<td>-0.12 (0.710)</td>
<td>0.34 (0.195)</td>
<td>0.24 (0.329)</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>-0.18 (0.564)</td>
<td>0.07 (0.795)</td>
<td>0.19 (0.458)</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>-0.14 (0.668)</td>
<td>-0.17 (0.523)</td>
<td>0.04 (0.880)</td>
</tr>
<tr>
<td><strong>P-tau</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1B</td>
<td>-0.29 (0.360)</td>
<td>0.12 (0.648)</td>
<td>0.13 (0.600)</td>
</tr>
<tr>
<td>5-HT</td>
<td>-0.40 (0.204)</td>
<td>-0.12 (0.658)</td>
<td>-0.33 (0.179)</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>-0.17 (0.603)</td>
<td>0.08 (0.760)</td>
<td>-0.39 (0.106)</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>0.20 (0.536)</td>
<td>0.13 (0.627)</td>
<td>-0.40 (0.096)</td>
</tr>
<tr>
<td><strong>Rab3A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1B</td>
<td>-0.28 (0.358)</td>
<td>-0.21 (0.427)</td>
<td>-0.04 (0.863)</td>
</tr>
<tr>
<td>5-HT</td>
<td>0.12 (0.700)</td>
<td>-0.48 (0.063)</td>
<td>0 (1)</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>-0.29 (0.339)</td>
<td>0.15 (0.588)</td>
<td>0.02 (0.937)</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td><strong>-0.59 (0.034)</strong></td>
<td>0.38 (0.147)</td>
<td>0.09 (0.728)</td>
</tr>
<tr>
<td><strong>SNAP25</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1B</td>
<td>-0.39 (0.182)</td>
<td><strong>-0.61 (0.012)</strong></td>
<td>-0.11 (0.670)</td>
</tr>
<tr>
<td>5-HT</td>
<td>-0.40 (0.174)</td>
<td>0.13 (0.645)</td>
<td>0.37 (0.135)</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>-0.08 (0.805)</td>
<td><strong>-049 (0.052)</strong></td>
<td>0.42 (0.085)</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>0.13 (0.684)</td>
<td><strong>-0.58 (0.018)</strong></td>
<td>-0.02 (0.932)</td>
</tr>
<tr>
<td><strong>Neurogranin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1B</td>
<td>-0.16 (0.611)</td>
<td>-0.19 (0.471)</td>
<td>0.23 (0.357)</td>
</tr>
<tr>
<td>5-HT</td>
<td>0.30 (0.312)</td>
<td><strong>-0.59 (0.016)</strong></td>
<td>-0.21 (0.397)</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>0.35 (0.247)</td>
<td>0.42 (0.106)</td>
<td>-0.43 (0.073)</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>-0.01 (0.970)</td>
<td><strong>0.70 (0.002)</strong></td>
<td><strong>-0.57 (0.014)</strong></td>
</tr>
</tbody>
</table>

Regarding the number of valid data for each biomarker, there are some missing cases for each of the correlations ranging from n=1 to n=4. AD: Alzheimer’s disease; DLB: dementia with Lewy bodies; PDD: Parkinson’s disease dementia; Aβ: amyloid-beta; P-tau: phosphorylated tau; T-tau: total tau. All multivariate correlations have been performed using partial correlation adjusting for age at death. Data are presented as adjusted.
Particular synaptic proteins such as the postsynaptic protein neurogranin, the synaptosomal-associated protein 25 (SNAP25) and the presynaptic protein Rab3A are associated with cognitive decline and dementia neuropathology[359].

In this study, we used previously published quantitative proteomic data for selected synaptic proteins[359] to test the hypothesis that these proteins correlate with serotonergic markers in dementia. The data suggests not only expression modulation but also the potential of neurogranin, SNAP25 and Rab3A in detecting cognitive decline in AD, PDD and DLB[359].

The multivariate analysis, partially adjusted to age of death, indicates an inverse correlation between 5-HT1B and SNAP25 in DLB (adjusted correlation coefficient=-0.61, p=0.012). In the DLB group again, negative correlations were observed between 5-HIAA and SNAP25 (adjusted correlation coefficient=-0.49, p=0.052), and the 5-HIAA/5-HT ratio and SNAP25 (adjusted correlation coefficient=-0.58, p=0.018). In addition, another inverse correlation was observed between 5-HT and neurogranin (adjusted correlation coefficient=-0.59, p=0.016) in DLB.

A positive correlation was also noticed between the 5-HIAA/5-HT ratio and neurogranin (adjusted correlation coefficient=-0.70, p=0.002) in DLB.

In PDD, a negative correlation was observed between the 5-HIAA/5-HT ratio and neurogranin (adjusted correlation coefficient=-0.57, p=0.014). However, no association was found between serotonergic markers and any of the selected synaptic proteins in AD group (Table 4.4).

In contrast to AD and PDD, our results suggest that the pathological marker α-synuclein is associated with the prefrontal expression of 5-HT1B in DLB group. Provided that DLB is a disorder of mixed α-synuclein and amyloid pathology, this suggests their integrated effect on 5-HT1B brain expression.

The predominant negative correlations between SNAP25 and serotonergic markers might indicate a compensatory synaptic activity to 5-HT deficit in DLB. Moreover, the relationship between serotonergic markers and synaptic proteins cannot be described by a unified trend. For instance, SNAP25 is associated with decreased serotonergic activity (5-HIAA/5-HT ratio) in contrary to neurogranin.
4.2 Modulations of the serotonergic system in Alzheimer's disease

4.2.1 Modulations of SERT

Depression is common and relevant to dementia and can present as prodromal symptom or symptom during the course of full-blown dementia[319]. Despite the previous knowledge about the positive effect of SSRIs on cognitive function and brain 5-HT levels, recent reports indicate that antidepressant, such the SSRI sertraline has no clinical effect on depression in AD[236]. Moreover, multiple reports demonstrated the downregulation of SERT expression in AD shown in different brain areas[41, 361].

We treated APPswe cells with sertraline to compare the serotonergic outcomes between in vitro AD model and control cells (Figure 4.3). First, we checked SERT gene expression in both types of cells after sertraline HCl treatment to exclude that changes in outcomes after sertraline treatment are due to modulation of SERT gene expression (Figure 4.3 B). No significant difference in SERT gene expression was observed in any of the experimental groups after sertraline treatment.

There is an increase in 5-HT1B gene expression in APPswe following sertraline HCl treatment compared to control cells, which is independent of the basal 5-HT1B and SERT gene expression differences between APPswe and control cells (Figure 4.3 A).

We further measured 5-HT, 5-HIAA and calculated the serotonin turnover index 5-HIAA/5-HT both in the cell lysate and cells’ medium to determine the intra and extracellular effects of sertraline on 5-HT metabolites (Figure 4.4). Importantly, sertraline decreased intracellular 5-HT levels in APPswe (Figure 4.4 B). Although higher levels of intracellular 5-HIAA were observed in both APPswe and control cells after sertraline treatment (Figure 4.4 D), a significant difference in the intracellular 5-HIAA/5-HT ratio was observed in APPswe cells but not control cells (Figure 4.4 F). No changes in 5-HT and 5-HIAA levels, measured in the cell medium or 5-HIAA/5-HT ratio, were noted after sertraline treatment in APPwe or control cells (Figure 4.4 G-L).

Because of the lack of effect on extracellular 5-HT metabolites, this data indicates acute inhibition of intracellular 5-HT and stimulation of 5-HIAA productions by sertraline in APPswe in a SERT-independent mechanism. Animal infusions and human functional imaging studies are needed to confirm and elaborate on these initial findings. The role of sertraline-dependent modulation in 5-HT1B expression and function in AD can be speculated as a contributing factor to these changes (Figure 4.3 A). Our findings show that lower MAO-A activities in APPswe cells treated with sertraline, which also indicate that these changes are MAO-A-independent modulations (Figure 4.5).
Figure 4.3 Gene expression profiles in APPswe after pharmacological modulations. A and D; 5-HT1B gene expression. B and C; SERT gene expression. E; p11 gene expression. Experimental conditions are shown in Table 3.3.
Figure 4.4 Intracellular (A-F) and extracellular (G-L) 5-HT and 5-HIAA measured after sertraline treatment and the 5-HT1B antagonist (SB224289) in APPswe and Empty vector (control cells). Experimental conditions are described in Table 3.3.
The interaction between 5-HT1B and SERT in the regulation of 5-HT transmission was discussed before[58]. SB224289 inhibits SERT function in mice and this could explain the compensatory increase in SERT gene expression after SB224289 treatment[58]. In addition, normal 5-HT1B expression and function are necessary for the SERT-mediated 5-HT uptake (Figure 1.2). The high upregulation of SERT gene expression, after blockade of 5-HT1B by SB224289 in AD models, could be partially due to abnormal function and expression of this receptor in AD.

Of note, the interactions between SERT and 5-HT1B, at the genetic and functional levels, could modify the inhibitory tone of 5-HT1A and thus add further influence on 5-HT release[58, 366]. 5-HT transmission can be affected by particular SERT genetic polymorphisms. However, no convincing data suggests higher prevalence of particular SERT polymorphisms in AD[314]. Moreover, SERT and 5-HT1B interactions in AD are not studied yet.

Increased adverse side effects and lack of benefits in treating depression in AD were shown in antidepressants particularly sertraline and mirtazapine in a randomized, multicenter, double blind, placebo-controlled trial[236]. We suggest that the refractoriness of SSRIIs in AD could be due to SSRI-induced molecular events in AD that might, early, activate or increase expression of 5-HT inhibitory receptors such as 5-HT1B and 5-HT1A instead of the desired desensitization of these receptors[155]. Consequently, activation of these receptors would results in lower intracellular 5-HT levels and subsequent decrease in the release of 5-HT in longer time scales.

On other hand, 5-HT1B reduces 5-HT clearance in hippocampus and the blockade of 5-HT1B by the selective antagonist SB224288 produces more inhibition of 5-HT clearance than the SSRI fluvoxamine[367]. However, this reuptake inhibition of SB224289 is dependent on the
integrity of 5-HT1B, which is still not studied in AD. The blockade of 5-HT1B is a potential adjunct to SSRI for 5-HT reuptake modifications[368]. Although SB224289 showed increased 5-HT levels of 5-HT both in vivo and in vitro by the selective blockade of 5-HT1B[369], our results showed no effect of this compound in APPswe or control cells in modulating 5-HT metabolites levels. This could be due to the type of cell model or the experimental conditions we used.

One limitation of this study is that it didn’t show the detailed mechanism of elevation of 5-HT1B mRNA following sertraline HCl treatments in APPswe cells. Although the validation experiments and previous data from the cell line we used have demonstrated higher levels of amyloid species in APPswe, a modification of 5-HT1B gene promoter by one of these amyloid peptides can be speculated. Another speculation is that the upregulation of 5-HT1B in APPswe cell could be a compensatory mechanism to the global, intra and extracellular, 5-HT deficit and increased 5-HT turnover in APPswe model prior to sertraline treatment[361] (Figure 4.1).

4.2.2 Modulations of Extracellular 5-HT concentrations

Measurement of Basal MAO-A activity and 5-HT metabolites in APPswe cells revealed lower extracellular 5-HT, higher 5-HIAA/5-HT index (Figure 4.6 C, D and E) and higher MAO-A activity (Figure 4.5). Of note, no difference in the basal levels of MAO-A gene expression between APPswe and control cells was found (Figure 4.5). The same pattern of 5-HT metabolites was observed after the measurement of intracellular levels from cells lysate (Figure 4.6 F, G and H).

To investigate the effect of modulating extracellular 5-HT levels on MAO-A and 5-HT turnover, we exposed both types of cells to a medium of 10 µM concentration of 5-HT for 30 minutes. Significant increase of MAO-A gene expression, 5-HIAA production and 5-HIAA/5-HT ratio, both extracellular and intracellular, were found in APPswe cells (Figure 4.7). No change of MAO-A activity following the 5-HT challenge in APPswe cells was observed.

Our results are in agreement with previous reports that suggest low 5-HT and increased MAO activity in AD[41]. Despite the fact that these results are relevant to 5-HT deficit phenomena in AD, they are descriptive and they do not provide a mechanistic evidence of how APPswe overexpression alters MAO-A gene expression and 5-HIAA production. Similar to what have been mentioned in paper I, another limitation to paper II is that the results are based on a single stable transfected cell line. Thus, it is not clear whether the data partially or totally depends on differences between cell lines or an APPswe overexpression. Moreover, there is lack of control that overexpresses the APP wild type (APPwt), which is considered the most appropriate control for APPswe cells and a better control than cells transfected with the empty vector alone. Additionally, the effect of sertraline on MAO-A gene expression is not included in the
experiments. MAO-B is also relevant to this area, and measurement of MAO-B was not performed in any of the sub-studies of this thesis.

Figure 4.6 Basal levels of 5-HT1B gene expression and related serotonergic markers
Figure 4.7 Modulation of A. MAO activity B. gene expression and 5-HIAA productions after 5-HT treatment both in cells medium C and intracellular D.

We suggested that increased 5-HIAA productions and 5-HT turnover after elevating the extracellular 5-HT is due to the combined effect of higher basal line activity of MAO-A and the 5-HT-induced stimulation of MAO-A synthesis at mRNA levels. Of note, SSRIs’ effects on 5-HT pathway are not identical to the conditions where extracellular 5-HT is elevated rapidly. Intuitively, longer periods are needed for SSRIs to increase extracellular levels of 5-HT.
Therefore, addition of animal models to the study could have given more integrated and mechanistic information. Similarly, a proteomic confirmatory data, preferably at in vivo model level, is needed. Such data could help in understanding the reasons behind the lack of efficacy of SSRIs on treating depressive symptoms on AD[236].

4.2.3 Modulations of 5-HT1B

In control cells, the 5-HT1B antagonist SB224289 increased the gene expression of SERT. Higher SERT gene expression profiles are observed in the group subjected to the concomitant antagonist SB224289 and 5-HT treatment compared to any of them alone (Figure 4.3 C). Although it is not statistically significant, a similar response is also noted in APPswe cells. SB224289 has no effect in 5-HT or 5-HIAA in both cell types. These maybe due to the fact that the modification of SERT gene expression is more sensitive than modification in SERT function. SERT function modifications and subsequent behavioral response need longer time scales. Therefore, an animal model is needed to study this. We investigated 5-HT1B blockade effects on MAPK in APPswe. 5-HT1B was shown to activate MAPK signaling pathway[370]. As previously shown, the concentration we used of 5-HT (10 M) is the most optimal concentration to inhibit cAMP[370]. SB224289 inhibits MAPK in both APPswe and control cells (Figure 4.8). The combination of SB224289 and 5-HT activates p-70S6 kinase in APPswe. A trend of increase is also seen in Western blotting in two different p-S70S6 phosphorylations; phospho-p70S6 kinase (Thr389) and phospho-p70S6 kinase (Thr421-Ser424) (Figure 4.9). These conclusions should be taken with caution due to the difficulties in the quantifications of blots either in the Western blotting or MAPK protein arrays.

S100A10, p11 is a relevant protein to 5-HT1B and depression[153]. Our results from Western blotting in APPswe indicate a reduction of p11 protein (Figure 4.1), while gene expression analysis indicates a marked increase in p11 mRNA compared to control cells (Figure 4.3 E). This discrepancy between protein and gene expression in our results could be attributed to either methodological limitation of Western blotting or compensation in form of upregulation of the p11 gene expression due to reduced 5-HT1B protein levels. More proteomic methods such as ELISA or IHC are needed to confirm the p11 protein data. Another limitation is that these experiments were conducted with one experimental condition for SB224289, 5-HT or their combination. Moreover, interesting combinations such as SSRI and 5-HT1B antagonist were not tried.
Figure 4.8 MAPK phosphorylation after 5-HT and 5-HT1B blockade. 150 microgram of protein are incubated with each paper[352].
Figure 4.9 MAPK, ERK and p70S6 assessed by Western blot after SB224289, 5-HT or both. V=vector or control cells and S=APPswe cells. C=Control cells treated with vehicle alone. S=5-HT, Am=SB224289 and San=SB224289+ 5-HT
4.3 Platelets and serotonin metabolites in Alzheimer's disease

The platelets represent an interesting and accessible opportunity to study the serotonin system with relevance to brain. Of note, adjustment for density is important when measuring 5-HT in AD, since both 5-HT levels and the species of platelets-derived amyloid are dependent on platelets density[275, 280]. Platelets with dual coating, coated with collagen and thrombin, have higher capacity in preserving the full length of APP compared to those with single coating[275]. We compared SCI group to AD group in a cohort obtained from memory clinic, Karolinska Hospital, Huddinge- Stockholm. As shown in Table 3.1, AD group is older and has lower MMSE scores. We investigated the association between platelets 5-HT and AD-CSF biomarker in both groups. Lower levels of 5-HT (p=0.016) and higher levels of 5-HIAA (p=0.044) in AD group are found when unadjusted non-parametric analysis was performed (Table 4.5 and Figure 4.10).

Table 4.5 Univariate and age-adjusted comparisons of serum serotonin level between the two study groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>SCI (n=20)</th>
<th>AD (n=15)</th>
<th>Univariate p-value</th>
<th>Adjusted Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Serotonin (5-HT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude level (nM/mL)-median (IQR)</td>
<td>65.600 (117.575)</td>
<td>1.000 (12.200)</td>
<td>0.016</td>
<td>B=9.4 § (95% CI: -126.2-107.3) (p-value=0.867)</td>
</tr>
<tr>
<td>5-HT nM/ng of protein-median (IQR)</td>
<td>17.615 (37.165)</td>
<td>0.238 (1.630)</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Tertiles Category-n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st tertile</td>
<td>2 (10.0%)</td>
<td>10 (66.7%)</td>
<td>OR=0.11*</td>
<td></td>
</tr>
<tr>
<td>2nd tertile</td>
<td>8 (40.0%)</td>
<td>3 (20.0%)</td>
<td>(p-value=0.032)</td>
<td></td>
</tr>
<tr>
<td>3rd tertile</td>
<td>10 (50.0%)</td>
<td>2 (13.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT nM/platelet count-median (IQR)</td>
<td>0.342 (0.908)</td>
<td>0.008 (0.121)</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>Tertiles Category-n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st tertile</td>
<td>3 (15.0%)</td>
<td>8 (53.3%)</td>
<td>OR=0.18*</td>
<td></td>
</tr>
<tr>
<td>2nd tertile</td>
<td>6 (30.0%)</td>
<td>5 (33.3%)</td>
<td>(95% CI: 0.03-1.21)</td>
<td></td>
</tr>
<tr>
<td>3rd tertile</td>
<td>11 (55.0%)</td>
<td>2 (13.3%)</td>
<td>(p-value=0.077)</td>
<td></td>
</tr>
<tr>
<td>Platelet 5-HIAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude level (nM/mL)-median (IQR)</td>
<td>0.000 (0.710)</td>
<td>0.560 (0.680)</td>
<td>0.044</td>
<td>B=0.44 § (95% CI: -0.25-1.13) (p-value=0.195)</td>
</tr>
<tr>
<td>5-HIAA nM/ng of protein-median (IQR)</td>
<td>0.000 (0.255)</td>
<td>0.193 (0.169)</td>
<td>0.178</td>
<td></td>
</tr>
<tr>
<td>Tertiles Category-n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st tertile</td>
<td>11 (61.1%)</td>
<td>3 (20.0%)</td>
<td>OR=10.55*</td>
<td></td>
</tr>
<tr>
<td>2nd tertile</td>
<td>2 (11.1%)</td>
<td>6 (40.0%)</td>
<td>(95% CI: 1.23-90.63)</td>
<td></td>
</tr>
<tr>
<td>3rd tertile</td>
<td>5 (27.8%)</td>
<td>6 (40.0%)</td>
<td>(p-value=0.032)</td>
<td></td>
</tr>
<tr>
<td>5-HIAA nM/platelet count-median (IQR)</td>
<td>0.000 (0.005)</td>
<td>0.005 (0.007)</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>Tertiles Category-n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st tertile</td>
<td>11 (61.1%)</td>
<td>3 (20.0%)</td>
<td>OR=24.64*</td>
<td></td>
</tr>
<tr>
<td>2nd tertile</td>
<td>3 (16.7%)</td>
<td>5 (33.3%)</td>
<td>(95% CI: 2.55-238.48)</td>
<td></td>
</tr>
<tr>
<td>3rd tertile</td>
<td>4 (22.2%)</td>
<td>7 (46.7%)</td>
<td>(p-value=0.006)</td>
<td></td>
</tr>
</tbody>
</table>


Shapiro-Wilk test was used to check the normality of distribution of numeric variables in each study group. Multivariate linear and ordinal regression models were applied to adjust between-group comparisons for participants’ age, depression and p-selectin level. Statistical significant differences (two-tailed p-value<0.05) are bolded.

# Mann-Whitney U-test § Multivariate linear regression * Multivariate ordinal logistic regression
Due to the small sample size of the study, we divided each group (AD or SCI) into three equal tertiles according to 5-HT or 5-HIAA levels. Of note, 5-HT and 5-HIAA levels were adjusted to platelets count, protein concentrations, P-selectin levels and presence or absence of depression. The use of tertiles also was intended to lower the potential floor effect and compare the rank of each individual rather than the mean of the continuous values. Multivariate analyses indicate 66.7% of AD patient belong to the lowest first 5-HT tertile after protein adjustment (Table 4.5). In agreement with this result, 53.3% of AD patients are in the first tertile of 5-HT adjusted to platelets count. Interestingly, 5-HIAA showed reciprocal pattern of higher 5-HIAA in AD in both protein and platelets count adjusted analysis.

We then studied the relationship between platelets-derived 5-HT or 5-HIAA and CSF-derived AD biomarkers. The unadjusted Spearman correlation analysis showed negative correlation between 5-HT and T-tau/Aβ42 ratio in SCI, which indicate less AD-like CSF profile with the increase in platelets 5-HT content. However, these correlations were insignificant when the model was partial-age adjusted. As shown in Figure 4.10, 5-HT levels separate SCI group into two groups, one with high 5-HT levels (SCI-HS) and the other with relatively lower 5-HT (SCI-LS). SCI-LS group has higher CSF t-tau and t-tau/Aβ42 ratio compared to SCI-HS. This result indicates that SCI-LS has more AD-like CSF biomarker than SCI-HS (Figure 4.11).

Figure 4.10 Crude, protein and platelets-adjusted 5-HT and 5-HIAA in SCI and AD.
In this study we used a single platelet fraction from SCI and AD group. Inclusion of one fraction could represent a limitation in this study, since alteration of platelets 5-HT in more than one platelets density fraction was reported[280]. The study did not include normal control or subject with mild cognitive impairment (MCI), which is an interesting intermediate group and could add more information about 5-HT levels in relation to AD severity stages. Measurement of platelets amyloid levels and 5-HT metabolites in CSF are also relevant to this area and can be done in future studies. The small sample size (15 AD subjects and 20 SCI) is a major limitation of this study, especially when they are later distributed into two groups based on 5-HT levels. Nonetheless, even with a total sample size of 35, our study showed significant differences between the two main groups and the subgroups. Another limitation in this study is the lack of platelets-derived MAO-B expression and activity data.

Platelets lack MAOA-A, but express MAO-B and a possibility is that in the absence of MAO-A maybe MAO-B takes over the conversion of 5-HT to 5-HIAA. In addition, no inverse correlation between 5-HT and 5-HIAA, which implies that 5-HIAA inside the platelets is not a directly derived from platelets 5-HT.
5 CONCLUSIONS, LIMITATIONS AND FUTURE PERSPECTIVES

5.1 Conclusions

In this thesis, we investigated the relationship between 5-HT pathway and clinical and pathological changes associated in AD, using cell models, animal models, post-mortem brain tissue and platelets from living patients. Alterations of 5-HT system in AD and modulations of AD pathology by some 5-HT receptors such as 5-HT4 receptor were previously reported[311]. However, there is a gap of information regarding the role of 5-HT1B in both AD and other dementia subtypes.

We show that 5-HT1B protein and gene expressions are reduced in APPswe mutation transfected SH-SY5Y neuroblastoma cell line. Consistent with these findings, lower hippocampal 5-HT1B levels in the two-year-old female transgenic Tg2576 mice compared to controls were observed. However, in the human postmortem brain tissues, our results suggest an increase in 5-HT1B levels in PFC area BA9. This variation in results between the two models maybe attributed to glial expression of 5-HT1B in response to neuronal loss in dementia. Another explanation is that increased expression 5-HT1B might be one of the molecular mechanisms of cognitive decline. Increased activity of 5-HT1B is associated with impaired working, reference and spatial memory tasks[76]. Consistent with this, our result indicates that in AD, PDD and combined dementia groups patients with high PFC-5-HT1B levels have more rapid decline of cognition.

The outcomes of pharmacological modulations of the serotonergic system differ between APPwe and wild-type cells. 5-HT1B receptor antagonist increased SERT gene-expression in control cells but not in the APPswe cells. Increased extracellular 5-HT levels lead to more MAOA-A gene expression and 5-HIAA productions in APPswe cells. Furthermore, sertraline increases the gene expression of 5-HT1B in APPswe cells. Our results suggest alterations of MAPK phosphorylations, particularly p70S6 and Erk in APPswe. However, no effects of sertraline or the 5-HT1B antagonist SB224289 were observed in intracellular or extracellular 5-HT levels. These results deserve a detailed biochemical and behavioral follow up studies to understand the reported lack of SSRIs and serotonin and norepinephrine reuptake inhibitors (SNRIs) response in AD[236].
We have studied differences in platelets serotonin between SCI and AD. Density of platelets, platelets count, and differences in protein concentrations were biochemical factors taken into consideration in our study. In medium-density platelets fractions, AD patients show significantly lower levels 5-HT and higher levels of 5-HIAA. In addition, SCI patients with lower platelets 5-HT levels have higher CSF t-tau and t-tau/Ab42 ratio. This reflects the association between AD-like CSF biomarker pattern and platelets serotonin in early stages of AD.
5.2 Limitations

In *paper I*:

- Changes in 5-HT and 5-HIAA levels cannot be explained by alteration of 5-HT1B expression alone. For example, 5-HT1A is another autoregulatory receptor for 5-HT. The study lacks the correlation between 5-HT1B expression and 5-HT metabolites level, which would provide an evidence of the relationship.

- The study consists of only one genetic model for familial AD, APPswe mutation. Inclusion of other genetic models such as presnilin-1(PSEN1) and presiniln-2 (PSEN2) mutations would give more information in this area.

- We did not show the effect of APPwt on 5-HT metabolites. However, our preliminary results have shown no difference in 5-HT1B levels between control cells and cells that overexpress the non-mutated wild type of APP (APPwt cells).

- The discrepancy between proteomic and gene expression data of p11 could be due to inherited limitations in the Western blot. The study does not provide confirmatory proteomic methods such as ELISA or immunofluorescence to support Western blotting results.

- Results from mice hippocampal 5-HT markers lack information from 9 months and 12 months age-groups in the wild type mice. Moreover, mice included in this study were only female and that represents a limitation in generalizing the serotonergic results. This is because of logical metabolic and neuroendocrine differences between sex groups such as influence of sex hormone in 5-HT pathway. Although data from 5-HT1B hippocampal expression is relevant to AD, inclusion of other areas such as neocortex would have shown the pattern of 5-HT1B expression in mice brain. Finally, the levels of 5-HT metabolites in mice hippocamus are not measured.

- Only one cell line was used in this study.

In *paper II*:

- The results are descriptive, i.e., there are no mechanistic insight on how overexpression of APPswe alters 5-HT pathway.

- Similar to *paper I*, APPwt cells are not included in this study.

- Studies conducted with transgenic mice should be performed in order to demonstrate that all AD models have similar results.
• We used one time and concentration point for each experimental condition. Inculson of different points would explain the time and dose-related effect of experimental conditions in 5-HT pathway modulations. Furthermore, it would be interesting if the combined effect of sertraline and 5-HT1B antagonist was tested.

In Paper III:

• The study represents a comparison between only two groups AD and SCI. Addition of healthy controls and patients with MCI would have added better insight about platelets 5-HT metabolites in different dementia stages.
• The study has a relatively small sample size which makes adjustment to different clinical variation difficult.
• No correlation is provided between CSF and platelet 5-HT metabolites was shown.
• The analytic assays seem to display a “floor effect” with a significant number of samples scoring very close to zero. However, we checked the crude values and found that in 6 cases, 17.1%, the level of 5-HT is quite low with a value of 0 for at least three decimals. Of these, 5 were in the AD group, which thus lends support to the finding of low levels in AD. The major contribution to this variation and the low 5-HT levels are related to the additional purification step, i.e., sorting cells in a gradient and selecting the right fraction. This makes the whole assay more vulnerable than a typical measurement of 5-HT levels in plasma-free platelet samples. In addition, we decided to also transform continuous values into tertiles to lower the potential floor effect and compare the rank of each individual rather than the mean of crude continuous values. However, consistent results after different type of adjustment lend support to our conclusions.
• Both study groups differ in age and depression frequency. After adjustment to these variables, no association between 5-HT or 5-HIAA levels and CSF biomarkers. We believe this is mostly due to the rather small sample size. However, in the analyses using tertiles the association remained statistically significant even after adjustment.

In paper IV

• Results in this study are not adjusted to relevant factors to 5-HT system such as depression scales and serotonin-modulating drug intake.
• There is no inclusion of other brain regions relevent to memory functions such as hippocampus.
• There is a need for a confirmatory proteomic method such as Western blotting or IHC. This was done for both pathological and synaptic markers but not for 5-HT1B.

• Similar to paper III, the sample size of individual dementia sub-group is small, added to the missing information about particular variables in subjects (range from 0 to 7), these factors affect the power of the study.

• There is a lack in information about baseline MMSE scores in the participants. Baseline MMSE might influence both the last MMSE prior to death and the annual rate of MMSE decline, which further affects the results we obtained in Table 4.3 and Table 4.4.

5.3 Future perspectives

More work is needed in order to understand how the 5-HT system is altered in neurodegenerative diseases, their clinical and therapeutic relevance. So, we suggest the following:

• Mechanistic studies elaborating in detailed interactions between pathological changes including amyloid and tau pathologies and alteration in the 5-HT pathway are needed. For example, computational chemistry analysis for structural interactions between amyloid peptides and MAO-A would improve our understanding for mechanism of increased 5-HT degradation in in vitro AD models.

• Using the new neuroimaging tools such as PET in human and novel ligands binding to serotonergic receptors in animal models to investigate expression of 5-HT1B and other receptors in AD and correlate the findings to clinical and biomarkers information.

• Analysis of S100A10 (p11) in AD brain samples would also expand our understanding about the clinical relevance of 5-HT1B and 5-HT4, since the protein is modulating these receptors and has significant implication in depression and antidepressant effect.

• The bidirectional relationship between 5-HT pathways and AD neuropathology should be explored in details, since 5-HT receptors influence the processing of amyloid precursor protein and amyloid peptides, and vice versa, amyloid neurotoxicity affects the brain expression of 5-HT pathway molecules.

• Finally, and most importantly, improved understanding of 5-HT1B and other 5-HT molecules can lead to better treatment of both cognitive and affective changes associated with AD and other neurodegenerative diseases.
6 ACKNOWLDGMENTS

This doctoral thesis has been conducted at the Department of Neurobiology, Care Sciences and Society (NVS), Karolinska Institutet-Centre for Alzheimer Research, Division of Neurogeriatrics. It would have been not possible without the help, support and encouragement I received from many people. Each and every day of this doctoral education period was a separate learning experience and each and every person I met has positively added to me. Here, I would like to acknowledge as many as I can remember but the list is definitely not exhaustive:

First, my supervisors: I am really lucky and grateful that I have this perfect combination of four competent, dedicated and knowledgeable supervisors that are experts in their fields. Working with you helped me not only to improve my skills in designing studies or critically appraise them but also it has re-shaped my view about the philosophy of science.

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