Role of pro-inflammatory S100A9 protein in amyloid-neuroinflammatory cascade in Alzheimer’s disease and traumatic brain injury

Chao Wang
致我的家人
To my family
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ABSTRACT

Background Traumatic brain injury (TBI) is a complex disease with a spectrum of symptoms and disabilities. Over the past decade TBI has become the focus of research due to growing epidemiological and clinical evidences that TBI incidences are strong risk factors for Alzheimer’s disease (AD). Major pathological hallmarks of AD are massive accumulations of amyloid-β peptide (Aβ) toxic oligomers and plaques. Neuroinflammation is also considered as a common denominator in AD and aging. The epidemiological and experimental studies have supported that non-steroidal anti-inflammatory drugs markedly reduce the age-related prevalence of AD and can slow amyloid deposition by mechanisms that still remain elusive. S100A9 is a multifunctional cytokine with diverse roles in the cell signaling pathways associated with inflammation and cancers. A widespread expression of S100A9 was also reported in many other ailments involving inflammatory processes, such as AD, malaria, cerebral ischemia and TBI, implying that S100A9 may be a universal biomarker of inflammation. The distinctive feature of S100A9 compared to other pro-inflammatory cytokines is its ability to self-assemble into amyloids, which may lead to the loss of its signaling functions and acquired amyloid cytotoxicity, exceeding that of Aβ.

Methods S100A9 properties was studied under various ex vivo and in vitro conditions. First, human and mouse tissues with TBI and AD were subjected to microscopic, immunohistochemical and immunofluorescent techniques. Then, aged mouse treated with native, oligomeric and fibrillary S100A9 was also studied by using behavioral and neurochemical analysis. Moreover, S100A9 was established as a biomarker of dementia progression and compared with others such as Aβ42 and tau proteins, by studying cerebrospinal fluid (CSF) samples from different stages of dementia. Finally, in vitro experiments on S100A9 amyloidogenesis, co-aggregation with Aβ40 and Aβ42, digestion and cytotoxicity were also performed by using spectroscopic, atomic force microscopy and cell biology methods.
**Results** S100A9-driven amyloid-neuroinflammatory cascade serves as a link between TBI and AD. We have found that S100A9 contributes to the plaque formation and intraneuronal responses in AD, being a part of the amyloid-neuroinflammatory cascade. In TBI we have found that extensive S100A9 neuronal production and amyloid self-assembly is triggered immediately after injury, leading to apoptotic pathways and neuronal loss. S100A9 is an integral component of both TBI precursor-plaques, formed prior to Aβ deposition, and AD plaques, characterized by different degree of amyloid maturation, indicating that all plaques are associated with inflammation. Both intra- and extracellular amyloid-neuroinflammatory cascades are intertwined and showed similar tendencies in human and mouse tissues in TBI and AD. *Ex vivo* findings are further supported by *in vitro* experiments on S100A9 amyloidogenesis, digestion and cytotoxicity. Importantly, being highly amyloidogenic itself, S100A9 can trigger and aggravate Aβ amyloid self-assembly and significantly contribute to amyloid cytotoxicity. Moreover, the CSF dynamics of S100A9 levels matches very closely the content of Aβ$_{42}$ in AD, vascular dementia and mild cognitive impairment due to AD, emphasizing the involvement of S100A9 together with Aβ in the amyloid-neuroinflammatory cascade in these ailments.

**Conclusions** The conclusions of this thesis is that the inflammatory pathways and S100A9 specifically represent a potential target for the therapeutic interventions during various post-TBI stages and far prior AD development to halt and reverse these damaging processes.
LIST OF PAPERS

This thesis is based on the following articles


Contributions to the following articles, not included in the thesis, were also made


# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>Aβ</td>
<td>amyloid-β</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>h-FTAA</td>
<td>heptameric formic thiophene acetic acid</td>
</tr>
<tr>
<td>H-tau</td>
<td>total human tau</td>
</tr>
<tr>
<td>MCI-AD</td>
<td>mild cognitive impairment due to Alzheimer's disease</td>
</tr>
<tr>
<td>NFTs</td>
<td>neuronal neurofibrillary tangles</td>
</tr>
<tr>
<td>P-tau</td>
<td>tau phosphorylated at Thr181</td>
</tr>
<tr>
<td>SMCI</td>
<td>stable mild cognitive impairment</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
</tr>
<tr>
<td>VaD</td>
<td>vascular dementia</td>
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</table>
INTRODUCTION

1. Protein folding and misfolding

1.1. Protein and structure

Proteins are molecules in the living organisms and perform multiple functions, including facilitating biochemical reactions, transmitting signals, transporting molecules, keeping the cell structures and storing amino acids. In order to fulfil their functions, suitable folding status is very important, which is dependent both on the surrounding environments where the protein is produced and also on the sequences of amino acids in the polypeptide chain (Anfinsen, 1973).

Essentially, 20 different amino acids make up all proteins on earth. By changing the permutations and combinations of these amino acids in the polypeptide chain, all the existing proteins can be produced. The spontaneous transition from a disordered polypeptide chain to a functional protein with a unique three-dimensional structure is defined as protein folding.

Proteins have four distinct levels of structure. The primary structure refers to the linear sequence of amino acids in the polypeptide chain, being held by the covalent bonds and determined by the specific gene sequences of the proteins; the secondary structure refers to two main stable secondary structure elements: \( \alpha \) helix and \( \beta \) sheet. They are distinguished by patterns of hydrogen bonds between the main-chain peptide groups. Meanwhile, some units do not have a stable secondary structure. It was defined as random coil; those secondary structures are further folded into a protein tertiary structure (protein domain) by special tertiary interactions, such as hydrogen bonds, hydrophobic interactions, Van der Waals interactions, salt bridges and disulfide bonds; and finally, when several protein domains make up into a complex, the quaternary structure is formed.
1.2. Protein folding, misfolding and amyloid formation

There are quite many molecular chaperones, folding catalysts involving to control the correct protein folding. Alternatively complex degradation pathways are involved to clean the misfolded and unfolded proteins. The ability of a protein to maintain its corrected and functional folding status is essential for its biological activity, while the unfolded and misfolded proteins will lose their biological functions. In some cases, these misfolded proteins may escape from the degradation system and further form into aggregates. Amyloid is one of the specific types of aggregates.

Amyloid or amyloid fibrils are distinguished by cross-β-sheet structures which are stabilized by hydrogen-bond interactions between groups in the polypeptide backbone as well as the characteristic tinctorial properties. Diazodye – Congo red will give a characteristic apple green birefringence under the polarized light when it binds with amyloid fibrils (Klunk et al., 1989; Steensma, 2001); the fluorescence intensity of the benzothiazole dye – Thioflavin T (ThT) will increase significantly when it interacts with amyloid fibrils (LeVine, 1993, 1999; Vassar and Culling, 1959).

The amyloid cross-β-sheet structures have a distinctive X-ray diffraction pattern which contains two predominate reflections. There are at 4.7 Å and 10 Å reflections, corresponding to the distance between the constituent β-strands and the space between two layers of β-sheets, respectively (Aoki et al., 1978; Eanes and Glenner, 1968; Glenner, 1980; Herczenik and Gebbink, 2008; Stromer and Serpell, 2005) (Figure 1).
Figure 1: (A) X-ray fibril diffraction pattern from partially aligned amyloid fibrils formed in vitro from amyloid-β(42) (Aβ_{42}) showing the characteristic cross-β diffraction signals on the meridian and equator at 4.7 Å and 10 Å, (B) schematic presentation of the hydrogen-bonded β-sheet structure (Figures were adopted from (Stromer and Serpell, 2005) and (Herczenik and Gebbink, 2008)).

1.3. Kinetics of amyloid formation

The amyloid formation is a nucleation-dependent polymerization process, and its kinetics can be easily described by a sigmoid curve (Figure 2). It consists of three stages – lag or nucleation phase, elongation or growth phase and saturation or plateau phase (Iannuzzi et al., 2013). In the first lag phase, soluble proteins, often monomers, which are present in the destabilized and partially unfolded states with higher aggregation propensity than the native state (Gillam and MacPhee, 2013), are associated with each other to form nucleus. Due to the thermodynamically unfavorable characters of the transition from native state to nucleus, this is normally considered as the rate-limiting step. The duration of the process depends on many factors, including the intrinsic properties of the specific protein as well as the surrounding incubation environment. When it comes to the growth phase, once the nucleus are formed, the growing species become thermodynamically favorable and the process becomes accelerated. Protofilaments are quickly formed through
monomers or oligomers joining to the nuclei. Finally, during the last saturation phase, amount of mature fibrils reach the highest level (Morozova-Roche et al., 2000). The whole amyloid conversion from soluble peptide or protein into mature fibrillar can be visualized by using different amyloid specific florescence dyes, such as Congo red and ThT.

Figure 2: The kinetics of amyloid formation (Figure was adopted from (Iannuzzi et al., 2013)).

2. Amyloid related diseases

2.1. General

To date, large number of proteins and peptides are known to have ability to form amyloids, causing multiple types of diseases in humans (See Table 1). The localization of the amyloid deposits and protein/peptide involved are largely determining the specific disease. They can be broadly distributed into three clusters. First, neurodegenerative diseases are the most well-known amyloid diseases, happening in the center nerve system. Alzheimer’s disease (AD) is one of the common neurodegenerative diseases, driven by the
production and deposition of Aβ peptide. Second, systemic amyloid diseases are a group of amyloid diseases affecting many different organs and tissues. Lysozyme amyloidosis is due to lysozyme gene mutations and affecting many different locations, such as symptomatic gastrointestinal tract, liver and kidney. Last, there are also localized amyloid diseases where the amyloid deposits are found closed to protein production. S100A8/A9 complexes are expressed due to inflammation existing in prostate and then formed into amyloids in the corpora amylacea of the aging prostate.

<table>
<thead>
<tr>
<th>Type</th>
<th>Main clinical settings</th>
<th>Aggregating protein or peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurodegenerative diseases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD</td>
<td></td>
<td>Aβ</td>
</tr>
<tr>
<td>Parkinson's disease</td>
<td></td>
<td>α-Synuclein</td>
</tr>
<tr>
<td>Dementia with Lewy bodies</td>
<td></td>
<td>α-Synuclein</td>
</tr>
<tr>
<td>Frontotemporal dementia with Parkinsonism</td>
<td></td>
<td>Tau</td>
</tr>
<tr>
<td>Huntington's disease</td>
<td></td>
<td>Huntingtin with polyQ expansion</td>
</tr>
<tr>
<td>Spongiiform encephalopathies</td>
<td></td>
<td>Prion protein or fragments thereof</td>
</tr>
<tr>
<td>Nonneuropathic systemic amyloidosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Familial Mediterranean fever</td>
<td></td>
<td>Fragments of serum amyloid A protein</td>
</tr>
<tr>
<td>Finnish hereditary amyloidosis</td>
<td></td>
<td>Fragments of gelsolin mutants</td>
</tr>
<tr>
<td>Lysozyme amyloidosis</td>
<td></td>
<td>Mutants of lysozyme</td>
</tr>
<tr>
<td>Fibrinogen amyloidosis</td>
<td></td>
<td>Variants of fibrinogen α-chain</td>
</tr>
<tr>
<td>Icelandic hereditary cerebral amyloid angiopathy</td>
<td></td>
<td>Mutant of cystatin C</td>
</tr>
<tr>
<td>Senile systemic amyloidosis</td>
<td></td>
<td>Wild-type transthyretin</td>
</tr>
<tr>
<td>Nonneuropathic localized disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type II diabetes</td>
<td></td>
<td>Amylin</td>
</tr>
<tr>
<td>Atrial amyloidosis</td>
<td></td>
<td>Atrial natriuretic factor</td>
</tr>
<tr>
<td>Cutaneous lichen amyloidosis</td>
<td></td>
<td>Keratins</td>
</tr>
<tr>
<td>Pulmonary alveolar proteinosis</td>
<td></td>
<td>Lung surfactant protein C</td>
</tr>
<tr>
<td>Medullary carcinoma of the thyroid</td>
<td></td>
<td>Calcitonin</td>
</tr>
<tr>
<td>Prostate deposits</td>
<td></td>
<td>S100A8/A9</td>
</tr>
</tbody>
</table>

(Table was referenced from (Chiti and Dobson, 2006)).
2.2. AD

AD is a progressive neurodegenerative disorder and the most common form of dementia. The disease destroys the memory, language ability, causes the difficulties with the activities and has a fetal consequences (Burns and Iliffe, 2009). According to the 2015 dementia report from the World Health Organization, 47.5 million people have dementia and Alzheimer’s disease may take up 60-70% of the cases.

AD was first described by the German psychiatrist and pathologist Alois Alzheimer in 1907 and brain atrophy is the main feature of AD (Figure 3, right part). The disease mainly affect the hippocampus and the temporal lobe (Hatchett et al., 2007). In the most severely disease affected areas such as the hippocampus and neocortex, abundant neuronal cell loss can be found which will lead to cognitive and psychiatric symptoms.

**Alzheimer’s disease**

![Comparison between healthy (left part) and AD human brain (right part) cross-sections (Figure was adopted from Alzheimer’s association).](image)

**Figure 3:** Comparison between healthy (left part) and AD human brain (right part) cross-sections (Figure was adopted from Alzheimer’s association).
There are two well-known pathologic features of AD which are amyloid plaques in the extracellular space as well as the intraneuronal neurofibrillary tangles (NFTs). The major component of the plaques – Aβ peptide was identified almost 30 years ago in 1984 (Glenner and Wong, 2012); meanwhile, 2 years later, the hyperphosphorylated tau was determined as the major constituent of the NFTs (Grundke-Iqbal et al., 1986). It has been more than one century passed since Dr. Alois Alzheimer described AD. However, there are still many unknowns in front of us. No efficient therapy method was developed to date and also no early diagnostics was established since the pathological changes such as neuronal degeneration and profound neuroinflammation have occurred 10 to 20 or even more years before the AD symptoms are shown up (Holtzman et al., 2012).

2.3. Traumatic brain injury (TBI)

The term of TBI refers to “an insult to the brain caused by an external force that may produce diminished or altered states of consciousness, which results in impaired cognitive abilities or physical functioning” as given by National Head Injury Foundation in 1988. From being a neglected disorder, over the past decade TBI has become the focus of increasing attention due to frequent injury incidents in modern society and sports. There are approximately 300,000 U.S. soldiers who have suffered TBI in Iraq and Afghanistan (Miller, 2012). The incidence rates of TBI are about 100/100,000 and 235/100,000 in the USA and in Europe, respectively (Maas et al., 2008; Roozenbeek et al., 2013). The real incidence rate should be even higher due to quite many unrecorded mild TBI cases.

Importantly, TBI is a long-lasting pathological process, not only an simple short event (Masel and DeWitt, 2010). As many as 15% of people with mild TBI will live with the post-TBI symptoms lasting for one year or even more. It initiates a chronic disease process by an initial injury event causing the biochemical and cellular changes which in turn lead to chronic inflammatory situation in the central nerve system or neuroinflammation (Morales et al.,...
Despite extensive efforts to develop short and long-term neuroprotective strategies, these are not yet satisfactory and a better understanding of underlying pathologies is required to focus on the specific therapeutic targets.

2.4. TBI is a risk factor for AD

The amyloid cascade hypothesis considers the deposition of the Aβ peptides as a central event of AD pathology. However, the reason of Aβ aberrant accumulation in the central nerve system in the old age still remain mysterious. Neuroinflammation, which is always connected with TBI, can potentially serve as the cues and thus attracted increasing attentions nowadays. More and more evidences show that in AD neuroinflammation is not passive event activated by amyloid plaques or NFTs, but instead in some extent, it promotes the pathological processes (Heneka et al., 2015). Epidemiological studies found that long term use of non-steroidal anti-inflammatory drugs in patients with rheumatoid arthritis produced a protective effect against the development of the disease, delaying the onset of the symptoms and reducing the risk of the disease occurrence (McGeer et al., 1990). This is also supported by observations that important microglial-expressed receptor genes, such as TREM2 and CD33 (Heneka et al., 2015; Ulrich and Holtzman, 2016), act as strong factors promoting AD.

Epidemiologically TBI is a strong risk factor for AD (Figure 4) (Fleminger et al., 2003; Magnoni and Brody, 2010). By examining the brain tissues from the patients with the post-TBI survival times varying from 1 to 47 years, nearly 30% of them were found to possess Aβ plaques. Moreover, Aβ deposits can be found in the TBI patient brain as early as few hours after the trauma (Ikonomovic et al., 2004). After inducing trauma in the AD transgenic mice that develop amyloid Aβ plaques, the accumulation of the plaques was tremendously enhanced (Abrahamson et al., 2009; Breunig et al., 2013; Hartman et al., 2002; Smith et al., 1998; Uryu et al., 2002).
Figure 4. Different consequences of TBI leading to higher risk for AD and dementia (Figure was adapted from (Sivanandam and Thakur, 2012)).

2.5. S100A9 protein

S100A9, a protein with 14 kDa molecular mass, belongs to S100 family which contains two EF-hand domains that can bind Ca$^{2+}$ (Markowitz and Carson, 2013). S100A9 is localized in both cytoplasm and nucleus of various types of cells. As a pro-inflammatory protein, it is constantly expressed by neutrophils and myeloid dendritic cells as well as inducible in various type of cells, particularly macrophages and microglial cells (Table 2) (Goyette and Geczy, 2011). Besides the cells shown in the table (Champaiboon et al., 2009; Healy et al., 2006; Hessian et al., 1993; Kumar et al., 2003; McCormick et al., 2005; Shepherd et al., 2006; Zreiqat et al., 2007; Zwadlo et al., 1988), increasing evidences have shown that neuronal cells can be also a potential source for S100A9. In the mouse model with endogenous oncogenic Kras expression in the post-mitotic neurons, S100A9 was found to be overexpressed in neurons (Ryu et al., 2012).
Table 2. Expression of S100A9 in various cell types

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Human S100A9</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>Constitutive</td>
<td>Hessien et al., 1993;</td>
</tr>
<tr>
<td>Monocytes/macrophages</td>
<td>Inducible</td>
<td>Hessien et al., 1993; Zwadlo et al., 1988</td>
</tr>
<tr>
<td>Myeloid dendritic cells</td>
<td>Constitutive</td>
<td>Kumar et al., 2003</td>
</tr>
<tr>
<td>Osteoclasts</td>
<td>Constitutive</td>
<td>Zrelqat et al., 2007</td>
</tr>
<tr>
<td>Microglia</td>
<td>Inducible</td>
<td>Shepherd et al., 2006</td>
</tr>
<tr>
<td>Platelets</td>
<td>Constitutive</td>
<td>Healy et al., 2006</td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td>Constitutive</td>
<td>Healy et al., 2006</td>
</tr>
<tr>
<td>Microvascular endothelial cells</td>
<td>Inducible</td>
<td>McCormick et al., 2005</td>
</tr>
<tr>
<td>Keratinocytes</td>
<td>Inducible</td>
<td>Champaiboon et al., 2009</td>
</tr>
<tr>
<td>Hypertrophic chondrocytes</td>
<td>Constitutive</td>
<td>Zrelqat et al., 2007</td>
</tr>
</tbody>
</table>

(Table was referenced from (Goyette and Geczy, 2011))

S100A9 is a multifunctional cytokine with diverse roles in the cell signaling pathways associated with inflammation and cancers. A widespread expression of S100A9 was also reported in many other ailments involving inflammatory processes, such as AD (Shepherd et al., 2006; Wang et al., 2014), malaria (Schluesener et al., 1998), cerebral ischemia (Postler et al., 1997), TBI (Engel et al., 2000), obesity (Nagareddy et al., 2013) and cardiovascular disease (Ma et al., 2012), implying that S100A9 may be a universal biomarker of inflammation. The abundance of S100A9 mRNA was also identified as a strong feature of aging in various mammalian tissues, including the central nervous system, and a novel mechanism of age-associated inflammation sustained by S100A9 was suggested (Swindell et al., 2013). The distinctive feature of S100A9 compared to other pro-inflammatory cytokines is its ability to self-assemble into amyloids, which may lead to the loss of its signaling functions and acquired amyloid cytotoxicity, exceeding that of Aβ (Wang et al., 2014). Therefore, the rising S100A9 level during inflammation may lead to its amyloid formation and deposition as we have shown in AD (Wang et al., 2014), aging prostate (Yanamandra et al., 2009) and also in cell model for protein amyloid aggregation (Eremenko et al., 2013). Moreover, the CSF
dynamics of S100A9 levels matches very closely the content of Aβ in AD, vascular dementia and mild cognitive impairment (Horvath et al., 2016), emphasizing the involvement of S100A9 together with Aβ in the amyloid-neuroinflammatory cascade in these ailments. We suggested that S100A9 in combination with Aβ and tau proteins can be used as a robust biomarker differentiating early stages of cognitive impairment in AD (Horvath et al., 2016). Interestingly, S100A9 knockdown attenuated memory impairment and reduced amyloid plaque burden in an AD mouse model (Ha et al., 2010), suggesting that S100A9 can be a prospective target for therapeutic interventions.

3. Methods used to study amyloids

The investigation of amyloid species with extraordinary speed, high accuracy and sensitivity is of great importance. Multiple techniques are developed and widely used in our lab.

3.1. Atomic force microscopy (AFM)

Nowadays, AFM scanning is a well-developed and widely used technique to visualize and even characterize the amyloid structures. Comparing with scanning electron microscopy, both of them can provide a nanometer resolution in the structure analysis. However, AFM does not required extra pre-chemical treatment for the soft biological samples, it has ability to characterize the physical properties of the structures, such as stiffness, elasticity and adhesion. It can even work efficiently in the liquid conditions. Thus, AFM scanning become more and more popular. In our lab, besides the observation of amyloid structures on the surface of the mica (Figure 5A and B), we have also developed the method to scan the human and mouse brain tissues on the common glass slides which can provide higher resolution of pathological structures such as precursor-plaques in the human TBI brain tissues (Figure 5C and D).
Figure 5. Examples of AFM application to study amyloids. (A and B) AFM height images of S100A9 amyloid clumps and protofilaments, respectively, formed in 10 mM PBS, pH 7.4, and 37 °C during 12 h, under shaking with glass beads. (C and D) AFM topographic images of S100A9 precursor-plaques in human traumatic brain injury tissues. Scale bars are 250 nm in (A and B) and 10 µm in (C and D). In (C and D), AFM z-heights correspond to a color gradient from 0 µm (dark brown) to 1.7 µm (yellow light) (Figure adopted from Wang C et al, 2016, manuscript).

3.2. Amyloid specific molecular probes

Different molecular probes were designed to bind specifically to cross-β-sheet structure of the fibrillar backbones. Invention of amyloid specific florescence dyes as well as antibodies were two major developments.

3.2.1. Heptameric formic thiophene acetic acid (h-FTAA)

Different florescence dyes were designed to detect the amyloid species. Among them, Congo red and ThT are two most popular florescence dyes. However, both of them have limitations. They cannot efficiently detect small pre-fibrillar species or heterogenic populations of protein aggregates (Klingstedt et al., 2011) and the specificity of Congo red has also been doubted (Bousset et al., 2004; Khurana et al., 2001).

h-FTAA is a luminescent conjugated oligothiophene dye (Figure 6A). Its fluorescence intensity is about one magnitude higher than Congo red. In addition, it is able to detect amyloid species at four magnitudes lower concentration, which is already outside the detection range of Congo red and
ThT (Sjolander et al., 2016). Histological staining of pathological tissues samples with amyloid deposits showed that besides the Congo red positive amyloid deposits, h-FTAA also stained small amyloid-nature protein aggregates, which were Congo red negative (Sjolander et al., 2016). Thus, h-FTAA is considered as a fluorescent hypersensitive and useful tool for recognizing amyloid structures. In my studies, h-FTAA was used in the detection of in vitro amyloids as well as the ex vivo TBI and AD associated protein aggregates in the brain tissues. Excitation at 480 nm (Figure 6B) and emission at 535 nm were used (Figure 6C) (Klingstedt et al., 2011).

![Figure 6. Chemical structure (A), excitation spectrum (B) and emission spectrum (C) of h-FTAA fluorescence dye. The spectrum graphs show the excitation and emission spectra of h-FTAA in PBS buffer – in blue dot line, in PBS with soluble Aβ42 – in magenta dash line and in PBS with fibrillar Aβ42 – in black solid line (Figure was adapted from (Klingstedt et al., 2011)).](image)

3.2.2. Amyloid detection by antibodies

Using antibodies specific to different types of amyloid structures is a biomolecular detection method, broadly used in classification of amyloids. The sensitivity of antibody detection is considered to be higher than that of florescence dye and the most importantly, they can distinguish the amyloid oligomers and fibrils by recognizing different generic conformation epitopes (Kayed et al., 2007; Kayed et al., 2003; O'Nuallain and Wetzel, 2002) under different amyloid assembly stages. Anti-fibrillar specific OC and anti-oligomeric specific A11 antibodies were widely used in my studies. The specific amyloid conformational states which A11 and OC recognize are briefly described in figure 7.

13
Figure 7. A11 and OC antibodies reactive with entire different amyloid conformational states formed by yeast Sup35’s prion-determining region (NM) (Figure was adapted from (Krishnan et al., 2012)).

3.2.2.1. Sequential immunohistochemistry

Visual localization of different antigens in the same paraffin-embedded tissue can provide the most direct evidence regarding the protein localization and co-localization. Multi-color immunofluorescence and peroxidase or alkaline phosphatase-linked chromogenic immunohistochemistry are two major choices. However, both of them have several limitations. Concerning multi-color immunofluorescence, auto-fluorescence signal from paraffin-embedded tissue has always been a big problem; in addition the limited number of available fluorescent tag combinations and impossibility to use primary antibodies from the same species were limiting factors in the visualization of more than two or three antigens; moreover, co-localization of the signals is normally examined in localized areas due to lack of high resolution fluorescence whole tissue scanner (Kim et al., 2012; Robertson et al., 2008). Regarding the chromogenic immunohistochemistry methods, double staining is still the most common application and in some conditions it is very difficult to demonstrate the co-localization due to the darker chromogens masking the contributions from the lighter colors. (Glass et al., 2009).
In our TBI and AD tissue staining, the major goal was to visualize the localization of S100A9 and Aβ in both intracellular and extracellular depositions. The amyloid assembly states as well as the connections with the surrounding cells were also analyzed. Thus, sequential immunohistochemical staining (Figure 8) was used in our studies as a primary method. By using this method, the tissue can be stained by four or five antibodies in a specific order according to the binding affinities of antibodies; 25 mM glycine-HCl, 10% SDS, pH 2 (Pirici et al., 2009) was used for denaturing and eluting the bound antibodies and every cycle of the staining pattern was captured by panoramic slide scanner 250 (3D Histech).

Figure 8. Sequential immunohistochemical visualization of five antigens in mouse cerebellum. Calbindin staining is shown in red, S100-beta – in green, GFAP – in yellow, MAP2 – in Magenta and NF-M – in blue (Figure was adopted from (Glass et al., 2009)).
RESULTS AND DISCUSSION

Paper I. The role of pro-inflammatory S100A9 in Alzheimer's disease amyloid-neuroinflammatory cascade.

Here, by using sequential immunohistochemistry method, we have shown that S100A9 is highly abundant in AD patients’ hippocampi and forms co-aggregates with Aβ peptide in the amyloid plaques. These aggregates are reactive with anti-fibrillar OC antibodies. By contrast, another well-known brain inflammatory protein S100B was not detected in plaques, but in surrounding amyloid plaque astrocytes. It is interesting to note that S100A8 protein, which was always forming hetero-complexes with S100A9 in many diseases and tissues, was not found in the AD tissues. This indicates that only S100A9 is contributed to AD plaque formation.

By using immunohistochemistry staining, we checked the brain tissues from two TBI patients with survival time less than 72 h. Abundant S100A9 immunopositive plaques throughout the whole hippocampi and the surrounding tissues were detected. These plaques were not reactive to Aβ, OC and S100B antibodies, but instead, were reacted with A11 anti-amyloid oligomeric antibodies. This indicates that S100A9 is not only rapidly secreted, but also aggregates into plaques with amyloid nature within this very short period.

The immunohistochemistry results also showed that S100A9 was intraneuronally present in AD, TBI and aged brain tissues. It is important to note that some immunopositive S100A9 staining was co-localized with Aβ immunopositive staining pattern, which demonstrates that S100A9 and Aβ can also co-aggregate within the neuronal cells.

By using in vitro amyloid formation and AFM scanning, we have demonstrated that highly amyloidogenic S100A9 is able to form amyloid structures as quickly as Aβ. In the physiological conditions in vitro (pH 7.4,
37 °C), S100A9 formed a plethora of amyloid complexes, including linear and annular amyloid protofilaments as well as flexible fibrils. S100A9 and its amyloids are also more hydrophobic than Aβ as shown by 1-anilinonaphthalene-8-sulfonic acid binding assay which indicates that S100A9 can be a good candidate for the role of plaque-forming protein, able to sequestrate amyloid species on its sticky hydrophobic surfaces. Moreover, S100A9 also readily co-aggregates with both Aβ40 and Aβ42 and promotes their amyloid deposition, displaying smooth surfaces and significantly thicker and loner fibrils. Therefore, the plaques of S100A9 rapidly developed in TBI brain, potentially, can serve as the precursor template of AD amyloid plaques, linking TBI and AD via the amyloid-neuroinflammatory cascade mechanism.

Cytotoxicity of the S100A9 amyloid species, formed individually as well as the co-aggregates formed together with Aβ40 and Aβ42, were studied by using WST-1 assay. The results demonstrated that S100A9 amyloid protofilaments are cytotoxic and even more cytotoxic than the corresponding amyloid species from Aβ40 and Aβ42. Co-aggregation of S100A9 with increasing concentrations of Aβ40 and Aβ42 could reduce and even eliminate the S100A9 amyloid cytotoxicity.

**PAPER II. The misfolded pro-inflammatory protein S100A9 disrupts memory via neurochemical remodeling instigating an Alzheimer's disease-like cognitive deficit.**

Memory deficits are a common feature of aged people and AD patients. Neuroinflammation and amyloidogenesis are considered as two contributory factors involving in memory deficits.

In this study, dual properties of S100A9 protein as a pro-inflammatory and amyloidogenic agent was explored in the passive avoidance memory task along with neurochemical assays in the prefrontal cortex and hippocampus of aged mice. S100A9 oligomers and fibrils were produced in vitro. Native S100A9, S100A9 oligomers and fibrils and their combination were
intranasally administered over 14 days to the aged mice, followed by behavioral and neurochemical analysis.

The results show both oligomers and fibrils evoked amnestic activity which correlated with disrupted prefrontal cortical and hippocampal dopaminergic neurochemistry. Meanwhile, the oligomer-fibril combination produced similar but weaker neurochemistry to the fibrils administered alone but without passive avoidance amnesia. Native S100A9 did not modify memory task performance even though it generated a general and consistent decrease in monoamine levels (DA, 5-HT and NA) and increased metabolic marker ratios of DA and 5-HT turnover (DOPAC/DA, HVA/DA and 5-HIAA) in the prefrontal cortex.

PAPER III. Pro-inflammatory S100A9 protein as a robust biomarker differentiating early stages of cognitive impairment in Alzheimer's disease.

In this study we focused on the detection in the CSF of S100A9 and compared its contents with established AD biomarkers including Aβ_{42}, total human tau (H-tau) as well as tau phosphorylated at Thr181 (P-tau). Studied patients were divided into five subgroups, including non-demented controls, stable mild cognitive impairment (SMCI), mild cognitive impairment due to Alzheimer's disease (MCI-AD), AD, and vascular dementia (VaD). Our findings have interestingly revealed that S100A9 is involved in the disease pathology and even already detectable as early as the SMCI stage.

By using immunohistochemistry staining of the brain tissues from a patient diagnosed with SMCI, both Aβ_{42} and S100A9 immunopositive plaques were detected. However, they were not co-localized. Both S100A9 and Aβ immunopositive neurons were abundantly observed. The co-localization pattern was also detected among the part of the immunopositive neurons. By contrast, S100A8 was also not found in either extracellular plaques or intracellular deposits at this stage of dementia. This again indicates that only
S100A9, but not S100A8, plays an important role in the amyloid-neuroinflammatory cascade in AD.

By using dot-blot and enzyme-linked immunosorbent assay methods, the precise level of S100A9 in the different subgroups was also studied. The results revealed that the content of S100A9 was already significantly decreased in the SMCI stage compared with the controls. In the MCI-AD group, its level was further reduced, effectively reaching those characteristic for AD individuals. AD and VaD groups gave similar S100A9 levels. It is important to note that the level changes of Aβ42 followed exactly the same trend as S100A9 throughout the whole dementia stages.

To the contrary, the CSF levels of H-tau and P-tau did not change in SMCI and showed an opposite tendency compared with S100A9 and Aβ42 at later stages of the dementia progression, but not in the subgroup with VaD.

**PAPER IV. S100A9-driven amyloid-neuroinflammatory cascade in traumatic brain injury as a risk factor for Alzheimer’s disease.**

The connection between TBI and AD is the subject of current scrutiny, but still remains unclear. For the first time we have presented here that the S100A9-driven amyloid-neuroinflammatory cascade can link the amyloid and inflammatory events triggered in TBI into a continuous process leading to AD.

By using immunohistochemistry, we have demonstrated that pro-inflammatory cytokine and highly aggregation-prone protein S100A9 is highly abundant both intra and extracellularly compared to Aβ in TBI, indicating that S100A9, but not Aβ, may play a leading role in amyloid aggregation.

If previously the AD pathology was related to the appearance of Aβ plaques in some TBI cases, we showed that in all studied young and old patients the numbers of S100A9 precursor-plaques were overwhelmingly higher. Following the dynamics of S100A9 and Aβ involvement in TBI, we showed that both S100A9 and Aβ precursor-plaques were fresh lesions, not possessing
yet amyloid structures and their number reduced dramatically with increasing post-TBI time. The remaining depositions, however, may represent the risk for AD and pursuing this we examined the AD brain tissues in humans and mouse model. In the human AD tissues we revealed two types of plaques: S100A9 meso-plaques and S100A9-Aβ senile plaques. The former is the first evidence of the amyloid plaques containing only S100A9. In the AD mouse model the amyloid plaques were constituted of both S100A9 and Aβ, implying that both polypeptides are essential for their formation.

By AFM, fluorescence and immunohistochemistry, we have demonstrated that all plaques in TBI and AD are characterized by diffused depositions of proteinaceous material spreading either from the center or condensing along circumference, which reflect their common mechanisms of formation around multiple initiation centers. Thus, a continuum of proteinaceous depositions undergoing transformation and maturation during post-TBI and AD were observed, which can be ranked from (a) non-amyloid S100A9 and Aβ precursor-plaques in TBI to (b) S100A9 amyloid oligomeric meso-plaques and (c) typical Aβ-S100A9 senile plaques in AD. Among them, the S100A9 meso-plaques can be the most hazardous providing an abundant source of neurotoxic amyloid oligomeric species.

In TBI hippocampi we have found that S100A9 is prevalent both in neurons and microglial cells, but their responses occur on different time scales. If neurons produce S100A9 immediately after injury, microglial cells – only in 4 day after post-TBI. With increasing post-TBI time the number of S100A9-immunopositive neurons and microglial cells become similar, and they both can sustain high S100A9 levels in the brain tissues. Remarkably, the same pattern of neuronal and microglial production of S100A9 was observed in the TBI mouse model, demonstrating that similar underlying cellular mechanisms are involved in both humans and mice.

Graph-analysis revealed that there is a correlation between S100A9 production, oligomerization and activation of apoptotic markers such as Bax
and caspase-3 in neuronal cells. The probability or chance of intraneuronal S100A9 oligomerization is 73%, and 60% of cells containing amyloid oligomers have a chance to enter the apoptotic cascade manifested in caspase-3 activation. This revealed the link between the S100A9 amyloid-neuroinflammatory cascade and apoptotic pathways in TBI, which may lead to tissue neurodegeneration.

Brain tissue acidification and fever were implicated in post-TBI, especially in the cases with unfavorable neurological outcomes. We found that S100A9 amyloid self-assembly was significantly enhanced by acidification and rising temperature, while proteinase K digestion of S100A9 amyloids was slowed down, leading to the prolonged life-span of toxic species. The initial stage of digestion produced an even larger population of short and cytotoxic S100A9 amyloids, which may lead to the secondary damage of the brain tissues. The removal of calcium or addition of reducing agent increased the rate of S100A9 amyloid formation. All together the in vitro experiments demonstrated that physiologically relevant environmental factors can regulate S100A9 amyloidogenicity, proteinase clearance and amyloid cytotoxicity.
CONCLUSIONS AND PERSPECTIVES

Here we presented compelling evidence for the critical role played by pro-inflammatory cytokine S100A9 in the amyloid-neuroinflammatory cascade in both TBI and AD. The S100A9 post-TBI responses were found to be similar in humans and mice as well as further amyloid developments in the AD human and mice tissues, implying that similar mechanisms underlie these pathologies. *Ex vivo* findings in the human and mouse TBI and AD brain tissues were supported by *in vitro* studies on the S100A9 amyloid formation, proteinase digestion and cytotoxicity under various environmental and stress conditions, demonstrating highly hazardous nature of the S100A9 amyloid self-assembly process. Moreover, being highly amyloidogenic itself S100A9 can trigger and aggravate Aβ amyloid self-assembly and significantly contribute to amyloid cytotoxicity. Therefore the S100A9-driven amyloid-neuroinflammatory cascade may serve as a mechanistic link between TBI and AD and TBI can be viewed as a precursor state for AD.

These findings present also an opportunity to target the inflammatory pathways and S100A9 specifically in therapeutic interventions during various post-TBI stages and far prior AD development to halt and reverse these damaging processes. Moreover, S100A9, Aβ_{42} and tau proteins taken together can be used as highly potent biomarkers able to differentiate accurately various stages of dementia starting as early as SMCI, providing a high accuracy, and also potentially differentiate AD from VaD. Our studies also suggest that amyloid species of S100A9 create deleterious effects principally on the dopaminergic system and this novel finding might be potentially exploited during dementia management through a neuroprotective strategy.
ACKNOWLEDGEMENTS

Exactly four years study at the Department of Medical Biochemistry and Biophysics, it was a very happy, busy fulfilling and contented life. I think all of you in the department have crossed my path at one time or another, here or there. I would like to warmly thank all of you, past and present.

Here comes the person I need to special mentioning:

First of all, I want to express my greatest gratefulness to my supervisor Prof. Ludmilla Morozova-Roche for giving me the opportunity of conducting my PhD education in your group and the constant encouragement, guidance and invaluable support during the passing four years’ time. I am really lucky and truly grateful to spend these years working in your lab under your guidance.

I also would like to thank my co-supervisor Jonathan Gilthorpe for the support, discussion and suggestion for the projects.

Many thanks to the current and past members of Prof. Ludmilla’s group:

Istvan H, being my colleague for more than two years, taught me quite a lot such as protein purification, dot-blotting and DLS. You are really an erudite biochemist. Really enjoy the discussion and collaboration with you. Wish you all the best with your new life in Göteborg! Xueen J and your family, introducing me into the AFM work, so many talking and discussion in and after work. I will always remember Xian’s food. I have always considered she is the best Chinese cooker in Umeå. Igor I, rescuing me from the AFM calibration, function extension as well as the mathematic calculation, statistic assay. You are really a very good biophysicist, computer-man, typesetting experts and illustrator. Roman M and your wife, I will never forget that tradition special chip fat. That’s my first time in my life eating fat meat. Also special thanks to you and your wife for participating my wedding registration in Stockholm City Hall. Kiran Y, never meet each other, but you really help
me quite a lot with my post-doc application with so many good suggestions. Great appreciate and wish you and your family all the best. The former master guiding by me, Hoa D, you were my first guiding master student and hope you have a good life in Italy. John K, we spent only half a year in Ludmilla’s group, but our communication is keeping. Sincerely hope you can find a PhD position soon. Marie-sofia, unfortunately have not included any results from your staining, but your name will be acknowledged in my second first-author paper. Also, thanks to Lovisa, Jason, Jesper, Nasibh, Raza, for all the help and good time in the lab.

Yue Shen and your family, both me and Binglei miss you and your family. You introduced me to the department life, helped me everywhere from experimental conducting, funding application and post-doc searching. Really hope we have chance to meet in Vancouver.

Shaodong J and your family, always remember the first day you arrived the department and the story with the unopened elevator. Truly happy for you find the life suitable for you and settle down in a nice city.

ZiQing K and your family, really enjoy the time with you both in and after work. So many talking, discussions, sports and hiking with you. Good luck with your future life and work.

Kun S, I am starting to count the exact number of fish we received from you. So many times conversations, hiking, playing poker as well as the splendor.

Clas, you are such helpful and happy friend! Congratulations for becoming a father! Jenny, Anna, Ingrid, you are also great.

Anders Olofsson’s group, Irian and Kristoffer, when I started my PhD study, you gave a lot kind help.

Rafil, you are really an expert in the protein purification and thanks a lot for all your kind help.
Phong, Tohid and Thomas W, it is always interesting talking to you. By the way, all of you should enjoy the weekend life. Remember how many times met you in the labs during the weekends.

Olena R and Sushma, also thank you for giving me many suggestions and help with my experiments.

Teaching labs, Farahnaz, Josefin, Yevgen, Mahsa, Khalil, Josefin, Parham, Lars, Ulf, Jonna and Jani, special thanks to all of you.

Innebandy participates, it is really a lot fun with all of you!

Jikui Guan, you are a good researcher and teacher. Under your guiding, I learned not only technics, but also the sprints in the research: scrupulous, systematical and realistic.

Also give thanks to my Chinese friends, in Umeå, Sun Kun, Yongguang Tong, Yaozong Li and Chaojun Tang, Huang Yang, Wanzhong Wang, Xiaolian Gu, Yangbin M and Guangxiang Zang, Minde Wang, Lingyu Meng, Shanshan Qian, Lingyan Shi and Zhihan Lv, Jianfeng Wang and Linghua Zhou, Mingquan Liu, Zhao Wang, Wei Huang, Lingling Gao, Chun Du, Zhiqiang Chen, Dengguo Wei and Yan Fang, Bin Yang and many many others.

最后，我想用中文感谢我生命中最重要的一些人：我的父母，我的岳父母和我的哥哥一家对我一直以来的支持和鼓励。现在我是我们家族里学位最高的人啦（好吧，截至 2016 年 9 月），哈哈！最后的最后，感谢我的妻子，韩冰磊，是你默默的陪我走过了在于默奥的时光，是你给了无数次的鼓励，是你熬了许多的夜做我们出行的攻略，是你为了我学会了做菜......永远爱你！
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