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# Type 2 Diabetes Mellitus Acts as a Risk Factor for the Development of Early Stage Alzheimer's Disease

Men Su

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Signalisations et Réseaux Intégratifs en Biologie

Spécialité de doctorat : Aspects moléculaires et cellulaires de la biologie  
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Par

**Mme Su Men**

**Type 2 Diabetes Mellitus acts as a risk factor for the  
development of early stage Alzheimer's disease**

**Thèse présentée et soutenue à Orsay, le 6 Juin 2017**

**Composition du Jury :**

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<b>Pr. Taouis, Mohammed</b>	Professeur	University of Paris-Saclay	Président du jury



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*To my parents and brother,*



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## Preamble

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Alzheimer's disease (AD) is a chronic and progressive form of neurodegeneration, and is the leading cause of age-related dementia. It is characterized by early memory loss that rapidly progresses to deficits in speech and visuospatial perception, mental confusion and complete loss of intellectual function (Goedert and Spillantini, 2006). It increasingly impairs daily activities, induces mood disturbances and leaves the patient totally dependent on carers. Finally, there is loss of body and brain function that results in death.

The number of individuals diagnosed with AD today has already reached catastrophic levels. The World Alzheimer Report (2016) estimated that there are about 47 million people with dementia throughout the world and this is growing dramatically; it is predicted to increase to more than 131 million by 2050 (Prince et al., 2016) with much of the increase in developing countries. The total estimated worldwide cost of dementia was US \$604 billion in 2010 and a tentative estimate of an 85% increase in costs by 2030 has been made (Alzheimer's Disease International (ADI), 2010). In our current world of increased life expectancy and population growth this presents not only a huge global health concern, but has already become a massive socio-economic burden.

Despite knowing about the disease for over 100 years, currently there is no substantial treatment to prevent or stem its development. This is not from lack of experimental effort or financial support; over the past 10 years alone, PubMed registered 67, 465 publications on AD by December, 2016 and the amount of money poured into research has been US \$818 billion (World Alzheimer Report, 2016). Yet still the question as to how the disease is triggered remains unanswered. Partly this is due to the fact it is a notoriously difficult disease to diagnose at the early stages as it has a slow onset; and partly due to the prevailing Amyloid Cascade Hypothesis (Hardy and Higgins, 1992) that became the major driving force, channelling research down specific avenues to develop therapeutic strategies to treat the disease (Hansen et al., 2008; Wollen, 2010).

Alzheimer's disease was first described by Alois Alzheimer in 1901 when he encountered a 51 year old woman suffering 'strange' behavioural symptoms including short-term memory loss (Maurer et al., 1997). Post mortem examination of her brain in 1906 showed the presence of senile plaques or Amyloid Plaques (APs) associated with abnormal clusters of fibrils or Neurofibrillary Tangles (NFTs) in neurons. Later in 1912, Kraepelin defined 'Alzheimer's Disease' as a rare degenerative disease in 'young' subjects (Maurer et al., 1997; Goedert and Ghetti, 2007). Some 20 years later, based on assessment of several family pedigrees, AD was recognised as having, at least in some cases, a hereditary-based autosomal dominant form, Familial Alzheimer's Disease (FAD; Mullan et al., 1992) or what is known now as Early Onset Alzheimer's Disease (EOAD) (Boller et al., 2007). No substantial advances were made in understanding the pathology of the disease until the mid-1980's and early 1990's when the core component part of the senile plaque was identified as amyloid (Glennner and Wong, 1984) and localised to the amyloid precursor

protein (APP) gene on chromosome 21 (Levy et al., 1990). Later genes encoding Presenilin 1 on chromosome 14 and Presenilin 2 on chromosome 1 (Levy-lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995) were identified. Despite evidence from post mortem studies that suggested dementia follows the progression of NFTs and cell loss rather than APs; and that other pathologies, not specific to AD were detected post mortem, research would focus on amyloid pathology as the causal lesion of the disease and dementia. The first mouse model showing amyloid pathology and memory deficits was published in 1995 (Games et al., 1995); and was rapidly followed by the first indication that amyloid beta (A $\beta$ ) immunisation could attenuate plaque load in these mice in 1999 (Schenk et al., 1999). Quite remarkably the first clinical trial, based on preclinical results in mice was terminated in 2002 at phase II as several patients developed autoimmune meningoencephalitis (Orgogozo et al., 2003). Subsequent analyses of the brains of these patients showed that although plaque load was attenuated there was no improvement in cognition nor prolongation of life (Holmes et al., 2008); and between 1984 until 2014, more than 100 drug trials have resulted in failure (see review by Schneider et al., 2014). Furthermore, more than 50 compounds have been tested in phase II, but none has passed phase III of clinical trials (see Bachurin, et al., 2017).

Across the same period of time huge experimental input was made into (i) understanding the pathological processing of amyloid and the attendant proteins associated with the process; (ii) the discovery of different species of soluble A $\beta$ , (iii) development of biomarkers and live imaging techniques to identify plaques in demented and non-demented aged persons. This would result in accumulating evidence to suggest non-AD specific processes may have a more important role in the disease process than originally proposed; and epidemiological studies suggest the disease is subject to environmental risk factors (Chen et al., 2009; Nicolia et al., 2015). Despite the evidence that would potentially query the causal role of APs in the development of dementia; it was essentially ignored by a large and influential part of the scientific community, who doggedly adhered to the Amyloid Cascade Hypothesis supporting a causal role in development of the pathology and dementia. Only more recently is this being substantially challenged and alternative strategies to determine what triggers AD pathology being pursued (see Struble et al., 2010; Herrup, 2015). Most notably are the potential roles that lifestyle risk factors may have in creating a neuronal environment for the early development of AD, or at least the dementia associated with the disease; are coming to the forefront of research.

The introduction of my thesis is organised in 4 chapters; where the first chapter focuses on Alzheimer's disease. Here I describe the clinical features of this disease and development and anatomical distribution of the two major lesions that define the disease. Then, secondly; I take a relatively historical stance to describe how the driving force of research was based on the notion that the amyloid plaque was the critical causal trigger of the pathology and dementia. Thirdly, as the role of the amyloid plaque in the disease process and dementia collapsed; I describe our understanding of the processing of amyloid and how it may be implicated in the disease and more recent evidence from imaging studies in humans; that introduces the potential effect that risk factors may have in promoting the disease. The following 3 chapters

are dedicated to describing the major risk factors that have been associated with AD (chapter 2); with a focus on Type 2 Diabetes Mellitus (T2DM) as the most important factor of risk for AD after ageing (chapter 3) and lastly, I describe the common pathologies and deregulated functions between T2DM and AD and certain underlying mechanisms that may suggest T2DM has a critical role in promoting AD.



# Introduction

---

## 1 Alzheimer's Disease

### 1.1 Clinical Assessment of Alzheimer's Disease

Alzheimer's Disease (AD) has been, and to a certain extent still is, defined by the classic pathological lesions; the widespread presence of amyloid plaques (APs) and neurofibrillary tangles (NFTs); and brain atrophy due to neuronal loss (Gómez-Isla et al., 1996; Heininger, 1999; Dickson and Vickers, 2001). Until more recently, with the development of different imaging technologies to identify the presence of biomarkers of the development of these lesions (Johnson et al., 2012), clear-cut identification of the disease has only been post mortem. Therefore, assessment was solely based on clinical diagnosis. Currently assessment of AD follows a number of stages where memory impairment signals the first indication of the potential development of the disease with mild cognitive impairment (MCI; Petersen, 2004).

#### *Stages of Alzheimer's disease*

Memory deficits constitute the central element of the clinical assessment of AD; they are early and manifest themselves under a form of progressive forgetting of recent facts and episodic memories (Conway, 2009; Tromp et al., 2015).

These deficits implicate several memory systems (Heindel et al., 1989), which include working (Stopford et al., 2012; Kirova et al., 2015), and semantic (Hodges et al., 1992; Giffard et al., 2008) memory, whereas other systems such as procedural memory are conserved for a comparatively longer time (Cohen and Squire, 1980; Deweer et al., 1994). The major problem arises from attempting to understand the very early stages of the disease, where it is possible to dissociate what

#### **Box 1. Stages of Alzheimer's disease**

**Stage 1- Normal:** This is considered a non-AD stage, with no subjective or objective symptoms of memory deficits.

**Stage 2- Normal Aged Forgetfulness:** The earliest stage where memory deficits are as reported mild but are difficult to distinguish from healthy aged persons. Individuals report difficulties in recalling names or where they have placed objects. At this stage, there is no ability to predict whether an individual will develop AD or not.

**Stage 3- Mild Cognitive Impairment (MCI):** Memory deficits at this stage are subtle and can manifest themselves in the form of repeated queries, suggesting working memory deficits. Ability to perform executive skills required for job performance or mastering new skills, organizing social events and concentration may also be compromised. Evidence suggests that not all people with MCI with develop AD, it is considered a 'border stage', but those with memory deficits are predicted to go on and develop AD (Korolev et al., 2016). When symptoms become overtly noticeable, the subject is commonly midway or near the end of this stage.

**Stage 4- Mild AD:** Memory impairment at this stage is frank and overt. Deficits are prominent in episodic memory, where they have difficulty in recall of recent personal memories, such as a holiday or visit to a relative. Semantic memory, such as weather conditions or the name of famous persons at this stage is relatively intact. Because of these deficits, daily life, such as managing personal finances becomes compromised. There is a flattening of mood, denial of decline in intellectual capacity and withdrawal from social situations.

**Stage 5- Moderate AD:** Deficits are sufficient to make independent living difficult. Cognitively, there is further decline in episodic memory with a decline in semantic memory as well. Recall of remote memories becomes lost; spatial orientations and intellectual capacity starts to decline; for example, a tendency to get lost becomes apparent and ability to count back from 100 in 2's is compromised.

**Stage 6- Moderately Severe AD:** At the earliest, patients require assistance in dressing and bathing, and progresses into a decline in personal hygiene. Advancement in the stage results in urinary and then fecal incontinence. Cognitive deficits advance to severe levels with little or no knowledge of daily life events and complete loss of current events. Recall of personal information such as where they were born or former occupation is lost and identity of close family members is confused. At the later part of this stage patients suffer anxiety, and outbursts of aggressive and violent behaviour. Speech deficits are manifest where patients stutter and there is increasing paucity of speech.

**Stage 7- Severe AD:** This stage marks the decline into death; intellectual capacity is lost; speech is reduced to a minimal number of unintelligible words, followed by loss of ability to move. Patients now start to show physiological decline with development of joint deformities, and decline in neurological reflexes and the emergence of infantile or primitive reflexes, such as a grasping and sucking reflex. Most patients will then die of pneumonia but other causes are stroke, heart attack and cancer.



is described as normal ageing from pathological ageing. Reisberg and colleagues have described 7 stages of AD, existing in a continuum with normal ageing (Reisberg et al., 1999). No single age can pinpoint the start of the clinical syndrome, as at the earliest stage is difficult to distinguish 'normal' cognitive ageing from that which would develop into AD; and depending on the lifestyle history of the individual AD may start earlier or later (see also details in section 1.7). In general, however when individuals have been clinically diagnosed with AD they have of the order of 8-10 years to live (see Box 1; Reisberg et al., 1999; Feldman and Qadi, 2006).

## **1.2 Pathological assessment of AD**

That which defines AD over and above other forms of dementia and neurodegenerative diseases consists of the widespread presence of the amyloid plaques (APs), neurofibrillary tangles (NFTs) neuropil threads, and dystrophic neurites containing hyperphosphorylated tau (Terry, 1994; Mandelkow and Mandelkow, 1998; Trojanowski and Lee, 2000; Crews and Masliah, 2010). These are accompanied by astrogliosis and microglial activity (Itagaki et al., 1989; Beach et al., 1991) and the characteristic loss of neurons, neuropil and synaptic elements (Terry et al., 1991; Gomez-Isla et al., 1997; Scheff, et al., 2006).

Amyloid plaques (APs) are insoluble proteinaceous deposits of misfolded A $\beta$  peptides. These are released from the Amyloid Precursor protein (APP) in soluble forms, either A $\beta$ 40 or A $\beta$ 42 (LaFerla et al., 2007). A $\beta$ 42 is the species that has the greatest propensity to self-aggregate into fibrils that with time become compacted into dense cores surrounded by an outer sphere of A $\beta$  (Jarrett and Lansbury, 1993). They are intermingled with neuronal, astrocytic and microglial processes, known as dystrophic neurites and often contain packets of paired helical filaments and abnormal mitochondria (Hirai et al., 2001; Fiala et al., 2007). These are the classic APs that have been detected post mortem, but in addition there is the presence of diffuse plaques; amorphous deposits that lack a morphologically identifiable substructure and are not associated with a glial response or synaptic loss (Serrano-Pozo et al., 2011). Both morphologically defined  $\beta$ -amyloid plaque variants are now known to be present in both early and late stages of AD. Finally, more recently, other species of soluble and semi soluble A $\beta$  have been identified (see section 1.6.5).

The other major lesion is the neurofibrillary tangles (NFTs). Electron microscopy has identified NFTs as intracellular pairs of fibrils about 10 nm in diameter that are folded into helices. They are principally composed of abnormally phosphorylated Tau protein (Brion, 1985; Grundke-Iqbal et al., 1986; Kosik et al., 1986). This protein is normally expressed in axons, but its abnormal phosphorylation interferes with the stabilisation of the microtubule network and the proteins accumulate in the form of paired helical filaments (PHFs) in the cytoplasm (Xie et al., 1998; Sergeant et al., 2005). Dysfunctional Tau affects intracellular transport and the morphology and viability of neurons, ultimately driving them to death via apoptosis (Lassman et al., 1995; Smale et al., 1995; Troncoso et al., 1996). A number of kinases are known to phosphorylate Tau, such as GSK3 $\beta$  (Baum et al., 1998), CaMKII, cAMP-PKC (Litersky et al., 1996), PKA (Zheng-Fischer et al., 1998), Cdk5 (Maccioni et al., 2001) and DYRK2 and DYRK IA (Woods et al., 2001),

but their sequential action or the particular kinases responsible for excessive phosphorylation of Tau and dissociation of the microtubules are unknown.

### **1.2.1 Topographical Distribution**

Despite the widespread accumulation of APs and NFTs throughout the brain at end stages of AD, progression of the two lesions follow a different spatio-temporal pattern of distribution. APs start to accumulate in the isocortex; mainly in layers II-V (Braak and Braak, 1991; Thal et al., 2002). From then, according to Thal and colleagues there are another 4 stages; APs spread to allocortical regions such as the entorhinal cortex; hippocampal formation, amygdala, insular and cingulate cortex during stage 2 and are present in subcortical nuclei such as striatum, basal forebrain cholinergic nuclei, thalamus, hypothalamus and white matter by stage 3. By stage 4 they are present in brainstem structures including the red nucleus, substantia nigra, reticular formation of the medulla oblongata and superior and inferior colliculi. Finally stage 5 sees APs in the pons and molecular layer of the cerebellum (Thal et al., 2002).

The spatiotemporal pattern of progression of NFTs is much more predictable than that of the APs. Braak and colleagues (Braak and Braak, 1991; Braak et al., 2006) describe the progression in 6 stages, where they first appear in the transentorhinal cortex, then CA1 of the hippocampus by stage II. At stage III NFTs appear in the subiculum; and then spread to the amygdala, thalamus and claustrum in stage IV. Finally, by stage V and VI, they are present in all isocortical areas, with associative cortical areas being affected earlier and more severely in stage V than the later primary sensory, motor and visual areas in stage VI.

### **1.2.2 Clinicopathological correlations**

Numerous studies have shown that the level and distribution of NFTs correlate much better with the severity of AD than the APs (Arriagada et al., 1992; Gomez-Isla et al., 1997; Ingelsson et al., 2004). Moreover, the spatiotemporal appearance of NFTs matches the staging of cognitive deficits rather than their widespread distribution. For example, early impairment in episodic memory can be explained by the isolation of the hippocampus from the association isocortex. This is followed by NFT invasion of multimodal high order association isocortical regions that may account for the progressive loss of cognitive functions such as executive function, apraxia, visuospatial navigation, and semantic memory associated with full-blown dementia. By contrast, the late involvement of the primary motor, sensory and visual isocortical regions explains the sparing of these functions until the end stage of the disease (Hyman et al., 1984; Braak and Braak, 1991).

Closely associated with the classic pathology is the loss in brain weight and volume that affects many regions. Much of the loss of volume appears to be due to the shrinkage and loss of neuronal processes (Palop and Mucke, 2010). Regional and laminar neuronal loss matches NFTs rather than APs, but within the same region neuronal loss exceeds the number of NFTs. In addition to neuronal loss there is also loss of synapses, which is not only due to the loss of neurons as synapse loss can predate that of neurons, resulting in the loss of connections within neuronal circuits, suggesting synaptic density to be the best

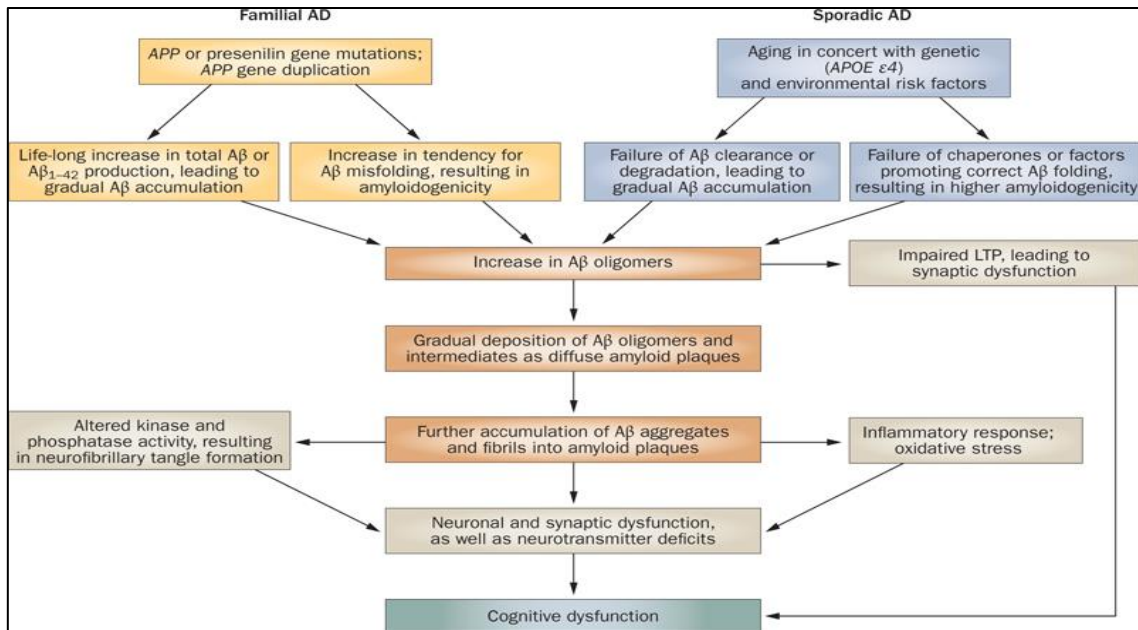
correlate of cognitive decline (Scheff et al., 1990; 1993; 2006; Masliah et al., 1994; Ingelsson et al., 2004). Longitudinal studies in communities of non-demented aged persons have shown at post mortem they have a high amyloid plaque load that matches the regional distribution of pathological changes seen in AD (Arriagada et al., 1992).

Together evidence at the time would show (i) a high amyloid load in non-demented aged individuals; (ii) progression of the two lesions do not follow each other, NFTs start in transentorhinal/hippocampal regions and progress to cortical regions; whereas APs follow the opposite progression, suggesting they may be independent pathological events; (iii) staging of clinical decline follows NFT lesions better than AP lesions; and (iv) memory deficits more closely correlate with cell and synapse loss than either of the two lesions. Together, the data could at the extreme level suggest that neither of the hallmark lesions may have a direct causal role in the cognitive deficits and dementia (see section 1.6.5). Nonetheless, despite even early evidence to suggest APs may not be directly linked with the dementia associated with AD, the Amyloid Cascade Hypothesis was unleashed on the scientific community in 1992 and would direct research over the next 20-25 years.

### **1.3 Identification of amyloid plaques and the Amyloid Cascade Hypothesis**

From the mid 1980's the core protein associated with amyloid plaques was identified (Glennner and Wong, 1984; Masters et al., 1985; St. George et al., 1987). Later, mutations in the APP and the Presenilin genes were identified in families with early onset FAD (Levy et al., 1990; Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995).

Based largely on these mutations identified in FAD; Hardy and Higgins postulated their Amyloid Cascade Hypothesis (1992). Quite simply the hypothesis stated that "the main component of the plaques is the causative agent of Alzheimer's pathology and that neurofibrillary tangles, cell loss, vascular damage and dementia follow as a direct result of this deposition" (Hardy and Higgins, 1992). Although this hypothesis would be applicable to the genetic form of the disease, which even in the 1990's was considered to be a rare form of AD, it was deemed to be of significant importance in understanding both the familial and sporadic forms of the disease (see Figure 1; Blennow et al., 2010). Subsequently this hypothesis would then become the driving force behind the generation of transgenic mice modeling different mutations in the familial form of the disease and lead rapidly to clinical trials designed to reduce plaque load in the brain. The key feature of this hypothesis was that it is longitudinal, where all pathological events observed in AD patients, including NFTs and cell loss; and the ensuing dementia; are all downstream of APs. Later versions of the theory would suggest soluble species of A $\beta$  is the key triggering mechanism. The main point being that the theory precludes other pathological events having a causal role in inducing the pathology and/or the dementia.



**Figure 1. The amyloid cascade hypothesis of AD.** According to the amyloid hypothesis, pathological processing of APP leads to increased brain concentration of Aβ oligomers, which in turn leads to amyloid plaque deposition. The plaques and/or more soluble Aβ can lead to synaptic dysfunction, neuronal death and dementia, tau pathologies and other pathologies like inflammation and oxidative stress (Blennow et al., 2010).

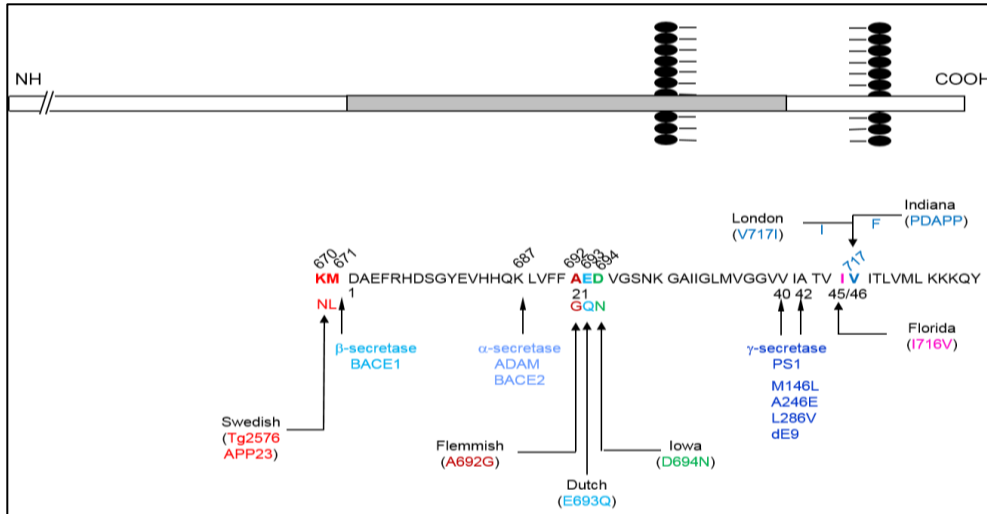
## 1.4 Rodent models of the disease

### *Development of the Pathology*

The first generation transgenic mice engineered to model amyloid pathology over-expressed wild-type APP, either as the full length protein (Quon et al., 1991; Wirak et al., 1991; Mucke et al., 1994) or as C-terminal, amyloidogenic fragments (Kawabata et al., 1991; Sandhu et al., 1991; Kammesheidt et al., 1992). Most of these transgenic mouse models had two features in common: they failed to show increased levels of APP RNA and the development of amyloid plaques (see Elder et al., 2010).

This 'benign' means of attempting to induce amyloid pathology was rapidly dropped in favour of engineering the second generation transgenic mice over-expressing mutated forms of APP or the Presenilins that mimicked different families with the genetic form of the disease (see Figure 2). To date a number of different murine models have been generated (see Table 1A), and in general can be classified as (a) point mutations on the APP and/or the Aβ peptide; (b) a combination of mutations in APP and Presenilin-1 (PS1), and (c) multiple mutations in APP, PS1 and Tau.

The 2 murine lines with point mutations in APP that have produced the most prolific data are the PDAPP over expressing the 'Indiana' mutation at position 717 (Games et al., 1995) and the Tg2576 model that over-expresses the 'Swedish' double mutation on APP695 on K670N and M671L (Hsiao et al., 1996). Both models show progressive deposits of APs, dystrophic neurites, apoptosis, synapse loss and gliosis.



**Figure 2. Different genetic APP and PS1 mutations found in FAD.** The amyloid peptide sequence is represented in gray and below shows the amino acid sequence. Numbering above the sequence represents the amino-acid numeration based on the full length of the APP and where point mutations have been identified in known cases of FAD. Numeration below the sequence represents that of the  $\beta$ -amyloid peptide. Small arrows indicate scission by the 3 secretases and the large arrows indicate the position and amino-acid substitutions of the different FAD families (modified from Davis and Laroche, 2003).

Frank fibrillar amyloid deposits appear around 6-10 months in hippocampus and cortical regions; although the development of pathology in PDAPP mice appears to be more aggressive than that in Tg2576 mice. At 6-9 months, when aggregated amyloid deposits are present they are already associated with astrogliosis and dystrophic neuritis and there is a decrease in synaptic activity indicated by reduced levels of synaptophysin (Games et al., 1995); however, this occurs later in Tg2576 mice, at 11 months (Irizarry et al., 1997). Despite the lack of Aps at an early age, both mouse models show certain pathophysiological events; such as a reduction in synaptic spine density in CA1 between 2-5 months in PDAPP mice (Lanz et al., 2003), and increased levels of A $\beta$ 40 (x5) and A $\beta$ 42 (x14) at 6 months of age in Tg2576 mice (Hsiao et al., 1996). The major features lacking in both models are the presence of NFTs, although both show hyperphosphorylated Tau (Masliah et al., 2001; Dobarro et al., 2013; Sherman et al., 2016), and cell loss (Irizarry et al. 1997; Masliah et al., 2001; Cuadrado-Tejedor et al. 2013; Sherman et al., 2016).

Based on Swedish families harbouring mutations in the Presenilin gene a number of double mutations in APP and PS1 were overexpressed (reviewed in Wisniewski and Sigurdsson, 2010; Marchetti and Marie 2011). In general, they show similar pathology to that observed in APP transgenic mice, but it is accelerated in these mice; an increase in A $\beta$ 42 starts as early as 6 weeks followed by age dependent increase in both A $\beta$ 42 and A $\beta$ 40 and widespread fibrillary deposits at 3-4 months and a further substantial increase by 6-7 months in the hippocampus and cortex (Borchelt et al. 1997; Holcomb et al. 1998). At 6-7 months, reactive astrogliosis appears around A $\beta$  deposits (Holcomb et al., 1998; Duffy and Hölscher, 2013) with accompanying dendritic spine loss, thinning of dendrites and breakages adjacent to APs (Tsai et al., 2004; Wisniewski and Sigurdsson, 2010). As with the APP transgenic mice there is no development of

NFTs but hyperphosphorylation of Tau (Ramos-Rodriguez et al. 2013; Ruan et al. 2016; Zhang et al. 2016) and some neuronal loss in hippocampus or cerebral cortex, in association with caspase dependent apoptosis (Long et al. 2013; Soto et al. 2016). Importantly, PS1 mutations alone selectively elevate A $\beta$ 42, but there is no overt plaque pathology (Duff et al., 1996).

The key feature of the 3xTg mice is in addition to mutations in APP and PS1, there is an additional mutation in Tau, despite the general consensus that Tau mutations are not associated with the pathology in AD. Nonetheless, as both mutations in APP and APPxPS1 do not result in NFTs or substantial cell loss that has always been associated with NFTs, these mouse models were developed in an attempt to model how the full-blown pathology may impact cognitive function and synaptic plasticity (Selkoe, 2001; Oddo et al. 2003). The first 3xTg AD mouse carrying the PS1M146V, APP<sup>swe</sup> and tauP301L human mutations (Oddo et al. 2003) produced the closest recapitulation of AD pathology, as there was progressive development of both classical lesions, with a similar spatio-temporal distribution as seen in humans. Expression levels of APP or Tau are doubled in the hippocampus and cortex by 4 months and extracellular A $\beta$  immunoreactivity is first detected in the neocortex at 3-4 months and subsequently in the hippocampus by 6 months. A $\beta$  deposits first become apparent at 6 months in the frontal cortex, and are readily evident in different cortical regions and the hippocampus by 12 months. By 18 months these deposits are thioflavin S positive and co-localise with reactive astrocytes (Oddo et al. 2003). Different stages of Tau pathology, such as hyperphosphorylation and subsequent NFTs are initiated in the hippocampus at 12 months and progress to the neocortex, showing amyloid pathology develops much earlier than Tau pathology (Oddo et al. 2003; Watamura et al. 2016).

#### *Development of cognitive deficits*

Several groups have tested memory capacity and synaptic transmission and plasticity in the models described above, however there are comparatively few studies and they lack consistency largely due to the different paradigms used, the age at which mice are tested and variations in mutations and promoters (see Table 1B). Three most commonly used memory tests in which memory deficits have been observed are working memory (Blodgett and Mccutchan, 1947; Vanderwolf, 1964), spatial learning and memory (Barnes, 1979; Morris et al. 1982) and recognition memory (Ennaceur and Delacour 1988; Dunnett, 1993; Davis et al., 2013; See review by Webster et al., 2014). The majority of electrophysiological studies has been conducted in the hippocampus, most frequently in CA1 *in vitro* (Larson et al., 1999; Jacobsen et al., 2006; D'Amelio, et al., 2011) and have shown deficits in both synaptic transmission (Larson et al., 1999; Mitchell et al. 2009; D'Amelio et al., 2011) and plasticity (Chapman et al., 1999; Giacchino et al., 2000; Trinchese et al., 2004; Hartman et al., 2005; See Marcheitti and Marie, 2011; Pozueta et al., 2013). Some studies have shown when memory deficits occur, deficits in synaptic transmission (Jacobsen et al., 2006; D'Amelio et al., 2011) and plasticity (Larson et al., 1999; Giacchino et al., 2000) also occur in the hippocampus. In general, there are few studies that show progressive impairment once the deficit occurs. Two studies in spatial memory have shown this; one has shown PDAPP transgenic mice have a deficit prior to the onset of amyloid deposits (4 months) that significantly deteriorates with age (17-19 months; Hartman et al., 2005).

Another has shown progressive exacerbation in spatial reference memory in Tg2576 mice after the age of 6 months until the age of 20-25 months (Westerman et al., 2002). Despite these 2 studies a major limitation of these models is that there is at least one, if not more studies that show no deficit in either memory (Arendash et al., 2001) or synaptic transmission (Chapman, 1999; Giacchino et al., 2000; Michell et al., 2009) or plasticity (Giacchino et al., 2000; Fitzjohn et al., 2001). Thus, although most studies show dissociation between ages at which mice show normal memory and then a deficit, there is little convincing evidence of a subsequent progressive decline with ages (see Table 1B).

APP/PS1 transgenic mice show similar deficits in memory and synaptic plasticity to other mouse models with the exception that; in general, they occur earlier (approximately 3 months). However, some inconsistencies occur with other studies showing deficit at later ages (Holcomb et al., 1999; Arendash et al., 2001). Discrepancies also occur in reports on whether LTP is impaired or not (Fitzjohn et al., 2010; Pozueta et al., 2013), and at what age it occurs (Gong et al., 2004; Gureviciene et al., 2004; Trinchese et al., 2004; Gengler et al., 2010). However, there is general consistency in as much as deficits in LTP are usually associated with memory impairment (Trinchese et al., 2004; 2008; Yoshiike et al., 2008) and appearance of amyloid pathology (Trinchese et al., 2004; Chang et al., 2006; Gengler et al., 2010).

3xTg mice show progressive memory deficits in passive avoidance, associative learning and spatial memory at 3 months. This is followed by deficits in alternation memory, fear conditioning (6-8 months), and recognition memory (9-11 months); with deficits in reference memory in Barnes maze at 12-14 months (Billings et al., 2005; Clinton et al., 2007; Halagappa et al., 2007). Although some reports have shown no memory deficits at early ages (3-5 or 9-11 months), there is consistency in the deficits that start from 12 months onwards (Gimenez-Llort et al., 2007; Pietropaolo et al., 2008). Few studies of synaptic transmission and plasticity have been conducted, but these show a decrease in basal glutamatergic synaptic transmission and LTP in CA1 between 5 and 6 months (Oddo et al., 2003; Giannopoulos et al., 2014).

In general, none of the mouse models that have faithfully mimicked human FAD have recapitulated the full-blown AD pathology, neither do they show progressive decline in memory deficits with age. Only the 3xTg mouse line, that is not a strict model of AD, more faithfully mimics the pathology and progressive memory decline. It is not surprising this model better mimics the pathology as it is designed to do that, but the better fit with memory decline, might better argue for a more important role for Tau in inducing memory deficits as suggested from human pathological studies. All models show inconsistent results in early stage deficits in memory and synaptic transmission and plasticity. Whether this would reflect early variability observed in humans developing the disease is difficult to assess as there are relatively few studies and much of the variability could be attributed to the different learning and memory paradigms used and the different ages at which they are tested. Key questions relating to the validity of these models, particularly in terms of Aps inducing dementia and exactly what stage of AD they were modeling were essentially ignored, in favour of developing treatments to clear plaque load.

**Table 1A. Pathology in the models of AD transgenic mice**

<b>Model</b>	<b>A<math>\beta</math> deposits</b>	<b>Tau pathology</b>	<b>Loss Neurons</b>	<b>Inflammation</b>
PDAPP Indiana	6-9 months Progressive	14 months phosphorylation	No	8 months Astroglyosis
Tg2576 Swedish	6 months Progressive	9-10 months phosphorylation	No	11 months Astro- microgliosis
APP/PS1 Swedish	3-4 months Progressive	7-8 months phosphorylation	14-23 months Hippocampus; Locus coeruleus	6-7 months Astroglyosis
APP/PS1KI	2.5 months Progressive			
3xTg AD Swedish; PS1; Tau	3-4 months Progressive	12 months phosphorylation + NFT	Ctx layer III: 4 months CA1: 8-11 months	3-18 months Astroglyosis
5 x FAD Swedish; Florida; London; 2xPS1	2 months Progressive	12 months phosphorylation + NFT	9 months Cortex, Subiculum	2-10 months Astro- microgliosis
APP23 Swedish	6 months Progressive	6-15 months phosphorylation	No	12 months Astro- microgliosis
Tg CRND8 Swedish, Indiana	5 months Progressive			
APP J20 Swedish, Indiana	6-7 months Progressive			
APP V717I London	10-12 months Progressive		No	
APP Femish	No		No	
APP Swedish; Dutch; Iowa	7-10 months Progressive			
PS1	No			
PS1 KI	None			
PS1 cKO	decrease A $\beta$ production			
PS cDKO	None	9 months phosphorylation	6 months Tau in cortex	6-10 months Astrogliosis, Apoptosis
APP/Tau P301L Swedish	10-12 months Progressive	14-18 months NFT: Hippocampus		
Tau (different types)	None	phosphorylation; NFTs	Yes, but not all	Axonal degeneration Astro- microgliosis

Table indicates the age (in months) at which pathology appears in the brains of models of AD (see Figure 2 for details on the transgenic mouse lines). Empty cells indicate where there is no available data. Table is based on reviews by (Wisniewski and Sigurdsson, 2010; Marchetti and Marie, 2011; Pozueta et al., 2013). And references included in reviews (Games et al., 1995; Holcomb et al., 1998; King and Arendash 2002; Flood et al., 2002; Oddo et al., 2003; Ohno et al., 2006; Sturchler-Pierrat et al., 1997; Chishti et al., 2001; Mucke et al., 2000; Moechars et al., 1999; Kumar-Singh et al., 2000; Davis et al., 2004; Duff et al., 1996; Guo et al., 1999; Yu et al., 2001; Saura et al., 2004; Terwel et al., 2008; Boekhoorn et al., 2006).



**Table 1B. Cognitive deficits and synaptic plasticity changes in AD mice models**

	A $\beta$ deposits (months)	Working Memory		Spatial Reference Memory		Recognition memory		Basal Synaptic Transmission		Paired Pulse Facilitation		LTP	
		No Deficit	Deficit	No Deficit	Deficit	No Deficit	Deficit	No Change	Decrease	No Change	Decrease	No Change	Decrease
PDAPP	6-9	3-9	13-21		3-19+	3-5; 6-19	6-11	3-5; 24+	4-6; 24+	24+	24+; 4-6	3-5; 24+	3-6; 17-20; 24+
Tg2576	6	1-2; 3-18	3-19	1-2	6-19	6-8; 15-18	12-18	2-9; 12-18	3-6; 12-13; 18-19	3-18	8-9	2-9; 12-19	4-18
APP/PS1	3-4	3-8; 15-18	3-5; 9-19	1-11	6-19	6-8	12-14	2-20	6-9	2-24+		2-20	3-13
APP/PS1KI	2.5	6-8	9-19+	6-11	15-19+			3-24+	9-24+	3-24+	3-7	9-24+	
3xTg AD	3-4	3-5	6-14	1-5; 9-11	3-18	6-8	9-18	1-2	6-7	1-2; 6-7		1-2; 9-13	5-13
5xFAD	2	1-5	5-14		3-11		3-14	3-5-4	6	6		3-5-4	6
APP23	6	15-18	19+	1-2	3-19		3-8	3-13; 24+	12-13; 18-19	3-5; 12-13		3-13; 18-19; 24+	3-5; 12-13
Tg CRND8	5	1-2	6-11		3-14		1-3	2-6		2-6	2-3	2-6	
APP J20	6-7	12-14	3-19+		3-14	3-5; 15-18	1-14	3-6	3-7	3-6	3-7	3-6	3-7
APP V717I	10-12				3								
APP-Flemish	None				3								
APP Swe/Dutch/Iowa	7-10				3								
PS1	None	12		4-9; 12		4-6					2-13; ??		??
APP/Tau P301L	10-12						4-6						4-6
PS1 KI	None			4-6	3						??		??
PS1 cKO	decrease A $\beta$ production			4-6	5-8			3-6		3-6		3-6	
PS cDKO	None				2								6

Deficits in different types of memory and aspects of synaptic transmission and plasticity in different transgenic models of AD. The deficit is observed and the range at which deficits in memory or decrease in physiological response appear. Values and “??” in red indicate age ranges where there are inconsistencies in the results. Those in blue indicate an increase in physiological response. It is important to note are some models show no evidence of frank amyloid pathology but still show deficits in memory or physiological response. Empty cells indicate where there is no available data. Table is based on reviews by (Wisniewski and Sigurdsson, 2010; Marchetti and Marie, 2011; Pozueta et al., 2013; Webster et al., 2014).

## 1.5 Immunisation Therapy and Clinical Trials

On the strength of the results from transgenic mouse models, it was deemed sufficiently important to focus on potential strategies for developing therapies to reduce or eliminate plaques. In the mid 1990's Solomon and colleagues demonstrated that monoclonal antibodies against A $\beta$  could dissolve aggregated plaques and prevented soluble A $\beta$  from aggregation *in vitro* (Solomon et al., 1996; Solomon et al., 1997). Thus, was born the target strategy for immunotherapy; using either active immunotherapy, where injections of fibrillar A $\beta$  would stimulate the immune system or passive immunotherapy using injections of monoclonal or polyclonal antibodies against A $\beta$ .

The first study was conducted in the PDAPP mouse using active immunization. The results showed a reduction in amyloid pathology in older mice, and prevent development of the pathology in younger mice (Schenk et al., 1999). A number of other papers followed showing similar reduction in amyloid pathology in other transgenic mice using both active (Morgan et al., 2000; Das et al., 2001) and passive immunization (Vevites et al., 2006; Zago et al., 2012). In most cases the reduction in amyloid pathology resulted in the rescue of cognitive deficits (Janus et al., 2000; Morgan et al., 2000). However, in some studies only certain memory deficits were rescued (Billings et al., 2005; Mably et al., 2015), and in others improvement in memory was observed using passive immunization but no reduction in pathology (Dodart et al., 2002). Thus, as with the characterization of behavioural deficits associated with Tg mutant mice, few studies have been conducted and there is variability in the results. This, then questions whether immunotherapy, either active or passive, would pose a valid therapy for AD.

The first human phase 1 safety trial (AN1792) was started in December of 1999 using active immunization against A $\beta$  plaques (Lemere and Masliah, 2010). The second phase was started in October 2001 with a total of 372 AD patients with mild to moderate AD, but was terminated in January 2002, as 6% of the patients developed aseptic meningoencephalitis and leukoencephalopathy and died (Orgogozo et al., 2003). The death however was due to the proinflammatory effect of the adjuvant (Lue and Walker, 2002; Nicoll et al., 2003; Monsonogo et al., 2006). Despite termination of the study, follow up testing was continued in the remaining subjects and a number of adverse effects were detected, including infection, confusion, accidental injury, irritability, etc. (see Bayer et al., 2005); whether these were due to the adjuvant or the treatment is not clear. However, possibly the most damning result from this trial showed that although amyloid plaques were cleared from the brain, there was no overall improvement in cognitive function or disease progression (Nicoll et al., 2003; Holmes et al., 2008).

Nonetheless, second generation active immunization and passive immunization treatments using mono- and polyclonal antibodies to target smaller fragments and small molecules were developed. Following the failure of the AN1792 trial some 53 clinical trials based on positive preclinical trials to reduce amyloid load were started. Thirteen have been positively discontinued, 10 are still in the pipe line, the majority have either not been registered for further Food and Drug Administration (FDA) approved clinical stages, while a small number have been approved for other treatment such as epilepsy (AGB101) and pain

(Ibuprofen). More importantly none has been approved for treatment of AD (see Mullane and Williams, 2013; St-Amour et al., 2016).

The failure of treatments was due mainly to lack of improvement in cognition, failure of efficient clearance of plaques or development of severe side effects. It was argued that drugs need to be refined and treatment should be started at an earlier age. However, the worst case in terms of the validity of the hypothesis that Aps have a causal role in the dementia; and the transgenic mice modelling human AD was that in which there was clearance of APs with no substantial improvement in cognition.

Based on these results, and the fact that the transgenic mice do not develop full AD pathology as predicted by the Amyloid Cascade Hypothesis, it would suggest that amyloid plaques have no causal role in triggering the downstream pathological events and the ensuing dementia in humans. However, ongoing research into understanding the processing of amyloid and live imaging in humans would suggest that if not the amyloid plaque then the amyloid peptide may have a functional role in the aetiology of the disease.

### 1.6 Biological processing of the Amyloid Precursor Protein (APP)

This is a large ubiquitously expressed, transmembranal glycoprotein of about 110-140 kDa (see Box 2 for details). It is synthesized in the endoplasmic reticulum and transported through the Golgi and Trans Golgi network (TGN) to undergo posttranslational modification during maturation and then is delivered to the plasma membrane in TGN-derived secretory vesicles (Koo et al., 1990). The Trans Golgi network (TGN) is the major site of resident APP in neurons (see Jiang et al., 2014).

The principle interest in the protein is that it harbours the A $\beta$  peptide that can be released from the APP by a series of cleavage events in an amyloidogenic manner, however it also undergoes non-amyloidogenic cleavage under so-called normal physiological processing.

#### Box 2. Amyloid Precursor Protein (APP)

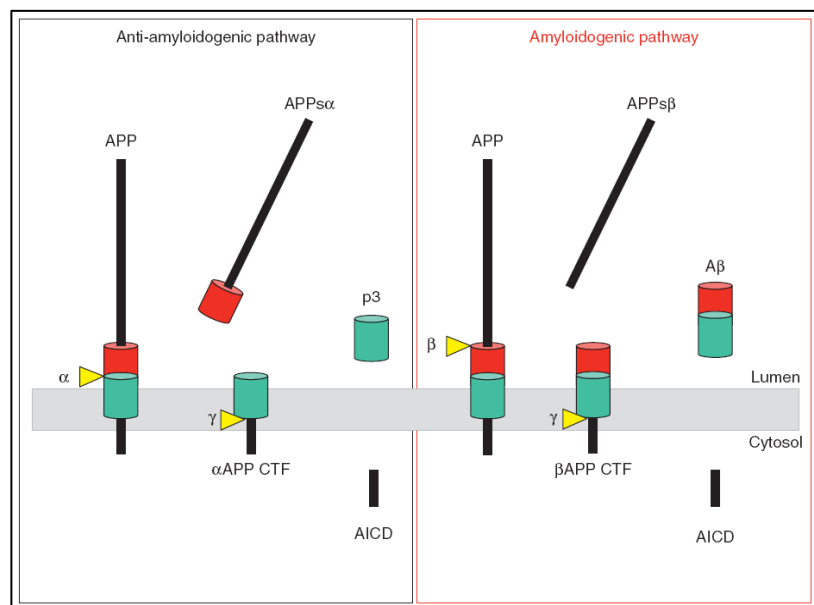
APP: a complex protein in terms of structure, function and how it is processed. It contains a number of motifs and cleavage sites for metabolic fragments that together endow the protein with a number of potential functions, be they beneficial or detrimental. In addition it can undergo alternative splicing at exons 7 and 8 to produce three major isoforms; APP695, APP751, and APP770 that are distinguished by size and the presence or absence of certain functional domains: APP751 lacks the OX.2 domain and APP695 lacks both the OX.2 domain and the KPI domain. In brain, APP695 is the predominant isoform expressed in neurons (Kang et al., 1987; Haass et al., 1991); whereas the longer forms are expressed in non-neuronal cells (Rohan de Silva et al., 1997).

Starting at the extracellular N-terminal domain there is a signaling peptide, followed by an E1 domain that has binding domains for heparin (Small et al., 1994), zinc (Bush et al., 1993) and copper (Hesse et al., 1994). Binding at these sites allows dimerisation that can stabilize the protein to allow cell adhesion and ligand binding (see Dawkins and Small, 2014); characteristics that suggest it could act as a receptor-like entity (Deyts et al., 2016). The longer isoforms; APP751 and APP770 contain the kunitz protease inhibitory (KPI) domain (Kang et al., 1987); potentially involved in wound healing (Van Nostrand et al., 1991) and/or neuronal cell growth (Wang and Reiser, 2003); and an OX.2 domain (Kitaguchi et al., 1988), that may be involved in cell surface recognition and binding (Dawkins and Small, 2014). This is followed by an E2 domain that also readily dimerises and may act in a similar manner to the E1 domain. Evidence suggests dimerisation of APP facilitates synapse formation (Wang et al., 2009; Baumkötter et al., 2014). The E2 domain is followed by the A $\beta$  peptide spanning part of the extracellular and the membranal domain, and finally a C-terminal cytoplasmic domain that contains a number of serine/threonine phosphorylation sites for at least PKC, CaMKII; JNK and GSK3 $\beta$ . Phosphorylation on these residues can modulate APP trafficking and signal transduction (Tamayev et al., 2009; Schettini et al., 2010). In general the full length protein under physiological conditions has been associated with cell adhesion, synaptic transmission and plasticity, neurogenesis, cell movement, neurite outgrowth and learning and memory and it has been putatively suggested it may act as a G-protein or receptor (Dodart et al., 2000; Soldano and Hassan, 2014; Deyts et al., 2016).

### 1.6.1 Cleavage of APP and its proteolytic fragments

APP undergoes a number of posttranslational modifications; the most classically defined is cleavage within 2 mutually exclusive pathways, the amyloidogenic pathway that leads to the production of A $\beta$ , and the non-amyloidogenic pathway which prevents generation of the full length A $\beta$  peptide and consequentially was described as a benign pathway (Haass et al., 2012). Cleavage in both pathways occur in 2 consecutive events by three major secretases: (i)  $\alpha$ -secretase, a potential member of the 'alpha-disintegrin and metalloprotease' (ADAM) family (Allinson et al., 2003); (ii)  $\beta$ -secretase or BACE-1 (Hussain et al., 1999; Sinha et al., 1999; Lin et al., 2000); and (iii)  $\gamma$ -secretase, a transmembranal complex of 4 protein subunits that contain Presenilin 1 (PS1) or 2 (PS2) and nicastrin (DeStrooper et al., 1998; Yu et al., 2000; Kimberly et al., 2003).

In the non-amyloidogenic pathway the first cleavage event is induced by  $\alpha$ -secretase, which sheds a soluble fragment, the APP $\alpha$  (APP $\alpha$ ) predominantly at the cell surface (Parvathy et al., 1999), leaving the remaining C-terminal fragment, CTF83 (LaFerla et al., 2007). It is subsequently cleaved by the  $\gamma$ -secretase in the membrane to release a P3 fragment containing the stub of the A $\beta$  peptide from the C-terminal APP intracellular domain (AICD; LaFerla et al., 2007; Haass et al., 2012; see Figure 3). If it is not cleaved, it is internalised into endosomal compartments where it is either recycled back to the cell surface or delivered to lysosomes for degradation (Norstedt et al., 1993; Caporaso et al., 1994). In the amyloidogenic pathway,  $\beta$ -secretase first sheds a soluble ectodomain, sAPP $\beta$  from the remaining C-terminal fragment, CTF99 (LaFerla et al., 2007) at the last amino acid of the A $\beta$ .  $\gamma$ -secretase then cleaves the full length A $\beta$  peptide at the C-terminal AICD domain (LaFerla et al., 2007; Haass et al., 2012). The  $\beta$ -secretase requires an acidic environment for optimal activity and the majority of  $\beta$ -cleavage occurs in the endosomes (Haas et



**Figure 3. Proteolytic processing of APP within the anti-amyloidogenic and amyloidogenic pathways.** APP can undergo proteolytic processing by one of two pathways. In non-amyloidogenic pathway enzymatic cleavage is mediated by  $\alpha$ -secretase. Cleavage by  $\beta$ -secretase occurs within the A $\beta$  domain, thereby preventing the generation and release of the A $\beta$  peptide. Two fragments are released, the larger ectodomain (sAPP $\alpha$ ) and the smaller carboxy-terminal fragment ( $\alpha$ APP CTF or C83), which further can undergo an additional cleavage mediated by  $\gamma$ -secretase to generate P3. In amyloidogenic pathway, it is cleaved by  $\beta$ -secretase, releasing an ectodomain (sAPP $\beta$ ), and retaining the last 99 amino acids of APP ( $\alpha$ APP CTF or C99) within the membrane, then subsequently cleaved 38–43 amino acids from the amino terminus to release A $\beta$ , by the  $\gamma$ -secretase complex. This cleavage predominantly produces A $\beta$ 40, and the more amyloidogenic A $\beta$ 42 at a ratio of 10:1. AICD, APP intracellular domain. (LaFerla et al., 2007; Haass et al., 2012).

al., 1992; Golde et al., 1992; Vassar et al., 1999), however, it can also be found at the cell surface (see review by Zhang et al., 2012). In addition to cleaving A $\beta$ ,  $\beta$ -secretase has other substrates, but as with APP all substrates are transmembranal proteins (Vassar et al., 2009). The  $\gamma$ -secretase is localised at the plasma membrane and endosomal/lysosomal compartments (see Haass et al., 2012), however it is not clear whether the different locations of cleavage are associated with the two different pathways.

### **1.6.2 Soluble ectodomains**

The shedding of soluble proteolytic fragments in either pathway can mediate beneficial or detrimental effect. The soluble APP $\alpha$  fragment can stimulate cell and neuronal growth (Araki et al., 1991; Milward et al., 1992); partly by regulation of stem cells (Caille et al., 2004; Kwak et al., 2006) and have protective effects against traumatic brain injury (Thornton et al., 2006) and ischaemic injury in CA1 (Smith-Swintosky et al., 1994). Furthermore, it is associated with memory formation and facilitation (Roche et al., 1994; Meziane et al., 1998; Anderson et al., 1999; Taylor et al., 2008) and synaptic plasticity (Taylor et al., 2008). More importantly, lower levels of sAPP $\alpha$  have been found in the cerebrospinal fluid (CSF) of AD patients compared with healthy subjects (Lannfelt et al., 1995) and their impaired spatial memory correlates with the level of sAPP $\alpha$  (Almkvist et al., 1997).

The soluble APP $\beta$  fragment cleaved in the amyloidogenic pathway is also endowed with some beneficial trophic properties; however, it has 100-fold lower potency than sAPP $\alpha$  in protecting hippocampal cells against excitotoxicity, A $\beta$  induced toxicity and glucose deprivation (Furukawa et al., 1996) and has no effect on synaptic plasticity (Taylor et al., 2008). As neurotrophic effects mediated by APP are at least in part mediated via the heparin binding sites, it is possible that the more potent effects of sAPP $\alpha$  over sAPP $\beta$  may be mediated in the first 16-17 amino acids of the A $\beta$  peptide.

### **1.6.3 P3 Fragment**

Following cleavage of APP in either the amyloidogenic or non amyloidogenic pathways, the second cleavage event is by the  $\gamma$ -secretase in both pathways. In the non-amyloidogenic pathway, the P3 fragment, which corresponds to a 3 kDa truncated A $\beta$  peptide is released from the AICD fragment and has been observed in diffuse plaques (Gowing et al., 1994; Iwatsubo et al., 1996), some dystrophic neurites around Aps and vesicle structures in the temporal lobes in AD brains (Higgins et al., 1996) and as fleecy deposits in the entorhinal cortex (Thal et al., 1999). Originally P3 was thought to be a benign form of amyloid as it was associated with diffuse plaques that lack amyloid with a fibrillary structure (Dickson 1997). However, it contains all structural requirements for fibril assembly (Higgins et al., 1996), adopts a  $\beta$ -sheet conformation and rapidly aggregates (Pike et al., 1995). P3 lacks the hydrophilic tail of the A $\beta$  peptide in the first 16-17 residues that permit them to stabilise in a soluble form. In fact, Dulin et al. (2008) have shown the highly hydrophobic P3 rapidly forms fibrils, passing through no intermediary soluble forms. P3 has been associated with apoptosis, although at a lower level of potency than A $\beta$  plaques (Wei et al., 2002) and promotes an inflammatory response (Szczepanik et al., 2001). Thus, as a fibrillary form of A $\beta$ , processed by the non-amyloidogenic pathway, it begs the questions of how benign this pathway is in AD pathology.

#### **1.6.4 AICD Fragment**

The intracellular AICD fragment is a product cleaved in both the amyloidogenic and non-amyloidogenic pathways by the  $\gamma$ -secretase. The dominant AICD fragment is 50 amino acids in length, however other species, AICD48/51/53 also exist that may be due to alternative cleavage at the  $\epsilon$ -site (Sato et al., 2003; Müller et al., 2008). This fragment of the APP can be involved in a number of functions that may potentially be related to different putative phosphorylation sites regulated by kinases such as CaMKII, PKC GSK3 $\beta$  and JNK. In addition, it has certain protein binding motifs that allow it to bind to G-protein subunits; a caspase cleavage motif; an endocytotic signalling motif; and a number of site for adaptor proteins to bind to enhance regulation at this fragment (see Deyts et al., 2016). Importantly when AICD is cleaved in the non-amyloidogenic pathway it is destined for degradation by proteolytic processes; however, in the amyloidogenic pathway phosphorylation at a Thr668 site induces conformational change in the endocytotic motif that makes it less vulnerable to degradation and cleavage by caspases. It promotes interaction with the adaptor protein Fe65 and subsequent translocation to the nucleus (Chang et al., 2006). And, although not fully confirmed; AICD could putatively translate a number of genes associated with AD pathology, including APP, BACE, GSK3 $\beta$  (von Rotz, 2004), EGFR (Zhang et al., 2007) neprilysin (Belyaev et al., 2009), low density lipoprotein-receptor related family proteins (LRP1; Liu et al., 2007) and p53 (Chechler et al., 2007).

#### **1.6.5 A $\beta$ Peptide**

The nexus of amyloid pathology is the A $\beta$  peptide. It is between 40-42/43 amino acids in length. The majority of the cleaved A $\beta$  peptide is 40 amino acids in length, only about 10% of the cleaved product is between 42-43 amino acids (Jarrett et al., 1993). Following cleavage, it is released from presynaptic sites and subsequently accumulates close to nerve terminals (Haass et al., 2012). It is released as a monomer and then proceeds to self-assemble into different soluble oligomers including dimers, trimers, putative dodecamer A $\beta$ -derived diffusible ligands (ADDLs) and annular protofibrils (APFs) prior to aggregating into the insoluble compacted plaques observed post mortem (see Glabe, 2008; Wang et al., 2016).

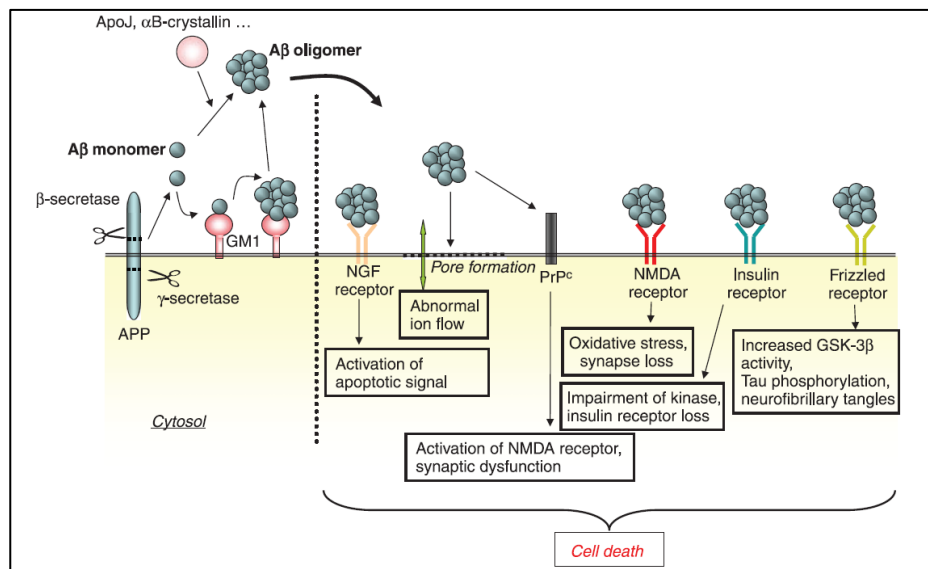
It was originally considered that the dense core amyloid plaque would grow in size and number with the progression of the disease and ultimately be the cause of the progressive decline in cognitive function. However, more contemporary research has shown that soluble, rather than dense-core plaques are more detrimental to the pathology. In fact, some have gone so far as to suggest that intracellular soluble oligomers are the most toxic forms of the peptide (Yankner et al., 1989; Lesne et al., 2006; Panza et al., 2011) and Aps might even be a protective pathway whereby A $\beta$  is exported from the cell and aggregates into fibrils to reduce the burden of intracellular oligomers (Lorenzo and Yankner, 1994; Tiwari and Kepp, 2015).

Monomeric species are considered to be relatively ineffective, however as with all species of A $\beta$  the neuronal environment can impact on its processing. For example, if monomeric A $\beta$  is exposed to high concentrations of A $\beta$  or form contact with metal ions it can form oligomers and fibrillary aggregations (Drago et al., 2008; Hung et al., 2010; Sharma et al., 2013). When fibrillary occurs, these soluble oligomeric species

have been shown to induce a slew of toxic and dysfunctional events that relate far better with memory deficits and synapse dysfunction than the aggregated fibrillary species. Evidence shows that soluble non-fibrillar A $\beta$  levels correlated strongly with the severity of the disease (McLean et al., 1999) and synaptic loss (Lue et al., 1999). Moreover, different species of soluble A $\beta$  appear at different times during life and are associated with different stages of AD. In humans, trimers are the earliest endogenous species of A $\beta$ , and can appear as early as 1 year of age in humans; as opposed to dimers that do not appear until about 50-60 years of age (Larson and Lesne, 2012). A $\beta$ \*56 a dodecamer, appears to peak during the preclinical stage of the disease and then decrease at the MCI stage and in AD patients, suggesting this may form in the ageing brain prior to and serve as a building block for amyloid plaques (Lesne, 2013). Some of these species have also been identified in transgenic mice, for example in Tg2576, A $\beta$ \*56 dodecamer appear around 6 months and remain relatively stable for up to 15 months of age, whereas other species; soluble monomers, insoluble monomers, dimers, trimers accumulate with monomers and dimers sharply increasing at 12 months (Larson and Lesne, 2012).

Experimental studies, infusing or injecting either extracts from human brains or synthetic fragments of the amyloid peptide can impair memory (Terranova et al., 1996; Cleary et al., 2005; Sipos et al., 2007; Eslamizade et al., 2016), synaptic plasticity and/or loss of synaptic function (Cullen et al., 1997; Walsh et al., 2002; Wang, et al., 2004; Townsend et al., 2006; Zhao et al., 2010).

Evidence suggests this can be mediated extracellularly, by binding to and rendering dysfunctional several receptor types, including AMPA, NMDA,



**Figure 4. Formation and toxicity mechanisms of extracellular A $\beta$  oligomers.** A $\beta$  is released extracellularly as a product of proteolytically cleaved, plasma membrane-localized amyloid precursor protein (APP). Extracellular A $\beta$  oligomers can be formed in the presence of GM1 ganglioside on the cell membrane. GM1 induces A $\beta$  oligomer-induced neuronal cell death mediated by nerve growth factor (NGF) receptors. Toxic nonfibrillar A $\beta$  is also produced in the presence of  $\alpha$ B-crystallin and ApoJ. A cellular prion protein (PrPC) acts as an A $\beta$  oligomer receptor with nanomolar affinity, and mediates synaptic dysfunction. Furthermore, the membrane pore is formed by A $\beta$  oligomers. The pores allow abnormal flow of ions, such as Ca $^{2+}$ , which causes cellular dysfunction. Binding of A $\beta$  oligomers to the NMDA-type glutamate receptor (NMDAR) also causes abnormal calcium homeostasis, leading to increased oxidative stress and synapse loss. Binding of A $\beta$  oligomers to the Frizzled (Fz) receptor can inhibit Wnt signaling, leading to cell dysfunctions such as tau phosphorylation and neurofibrillary tangles. Moreover, A $\beta$  oligomer can induce insulin receptor loss from the neuronal surface and impaired kinase activity related to long-term potentiation (Sakono and Zako, 2010).

metabotropic receptor (mGluR) associated with glutamatergic transmission (Li et al., 2009; Zhao et al., 2010; Brito-Moreira et al., 2011; Jurgensen et al., 2011). In addition they can also bind to or disrupt regulation of receptors associated with maintaining normal functioning of cells or homeostatic regulation that include receptor for Advanced Glycation End (RAGE), associated with oxidative stress (Wang et al., 2004; Eftekharzadeh et al., 2012); TNF receptors involved in inflammation (Eftekharzadeh et al., 2012; Morrioni et al., 2016) and insulin receptors, associated with homeostatic regulation of energy and normal function of the cell (Townsend et al., 2007; Zhao et al., 2008; De Felice et al., 2009; Sakono and Zako, 2010; Figure 4.). Intracellular A $\beta$  has been shown to disrupt vital functions associated with proteasomal activity (Young and Bennett, 2010), calcium deregulation (Resende et al., 2008; Jensen et al., 2013), autophagy (Caccamo et al., 2010; 2011) and can hyperphosphorylate Tau at pathological sites (Magdesian et al., 2008; Larson et al., 2012). Thus, soluble species of A $\beta$  are capable of mounting a pincer movement both from within and outwith the cell to disrupt physiological function (Wang et al., 2016).

However, as with APP, the A $\beta$  peptide has been shown to have a physiological function at picomol levels, albeit to date much of its function has been determined *in vitro*. Injection of picomol levels of both A $\beta$ 40 and A $\beta$ 42 increase the magnitude of LTP and mediates memory formation (Garcia-Osta and Alberini, 2009), whereas high nanomol levels impair LTP (Puzzo et al., 2008). Evidence suggests A $\beta$  may be strongly involved in vesicle release necessary for basal and activity regulated function at the synapse (Abramov et al., 2009). Physiological concentrations of A $\beta$  are also implicated in neurogenesis, defence against oxidative stress, maintaining the integrity of the Blood Brain Barrier (BBB), and influencing kinase signalling pathways, notably Insulin regulation of the PI3K-Akt pathway and calcium signalling (see reviews by Parihar and Brewer, 2010; del Cardenas-Aguayo et al., 2014).

Together these data suggest that cleavage to release the A $\beta$  peptide or parts of it are a lot more complex than originally thought and could have different consequential effects mediated by the different fragments of APP, that can result in or contribute to a physiological or pathological function. Therefore, whether it is the A $\beta$  peptide or the resulting fragments that have a causal role in the pathology becomes an important question. Nonetheless, the evidence that soluble species of A $\beta$  may be more detrimental than the AP has reached a level that can no longer be denied. It has a greater impact on synaptic function, now considered to be an important early event in the pathological processes of AD, and shows the best correlate with memory deficits. However, as strategies to combat AD are designed to develop treatments to eliminate production A $\beta$ , they risk creating other deficits by deregulating normal function of A $\beta$ . The optimal strategy would be to develop treatments that would maintain A $\beta$  within physiological levels.

### **1.7 Standardisation of early AD staging with objective biomarkers of the disease**

The NIA-AA (National Institute of Ageing and Alzheimer's Association Criteria) international workshop committee in 2011 would harness newly developed imagery and biomarker technology to



promote standardised objective measures to develop criteria for what they described as preclinical staging of AD, using a panel of live imaging and biomarkers (Sperling et al., 2011; Weiner et al., 2015; see Box 3).

### **Box 3. Imaging studies of AD biomarkers**

**CSF Biomarkers:** Levels of A $\beta$ 42 show in general, a 50% decrease in patients with moderate AD compared with age-matched controls (Counts et al., 2016). In contrast there is an increase of up to 300% levels of all Tau isoforms (Counts et al., 2016). As Tau is not specific to AD and elevated in patients suffering other forms of dementia (see Formichi et al., 2006), the ratio of increased total Tau to decreased A $\beta$ 42 in the CSF of patients with MCI, AD and controls present the most robust combination for predicting the transition of MCI to AD (Counts et al., 2016).

**Fluorodeoxyglucose Positron Emission Tomography (FGF-PET):** This form of imagery measures glucose metabolism to indicate synaptic activity and strongly correlates with synaptophysin in post mortem AD brains (Rocher et al., 2003). Hypermetabolic activity has been observed in a number of limbic and associated cortical structures, including at least the hippocampus, parietal and posterior cingulate gyri and portions of the temporal lobe and medial temporal cortices in AD patients, and worsens with age. At early stages hypometabolism occurs in regions that are highly vulnerable to A $\beta$  deposits (Klunk et al., 2004; Buckner et al., 2005) and are anatomically and functionally interconnected to form part of a distributed neuronal network known as the 'default mode network' (Raichle et al., 2001). Moreover FGF hypometabolism parallels cognitive function during the progression of the disease; from normal to preclinical to prodromal and established AD (Minoshima et al., 1997; Furst et al., 2010). Although plaque load has reached a plateau by the time AD is clinically established (Engler et al., 2006; Jack et al., 2009) FGF metabolism continues to decline in relation to cognitive function (Engler et al., 2006), most notably in the progression from MCI to AD (Chen et al., 2010). A major limitation to FGF retention is that it is not specific to AD and can be associated with inflammation and ischemia.

**Amyloid and Tau PET:** The development of PET radioligands have been used to detect fibrillary A $\beta$  deposits and intracellular Tau aggregates to show they precede clinical onset of AD by years or even decades (Counts et al., 2016). The Pittsburg compound B (PiB) binds with high affinity to  $\beta$ -sheet structured amyloid aggregates (Levine, 1995) rather than soluble species or diffuse amyloid plaques. It is retained longer in AD patients compared with non-demented age-matched controls and shows a strong concordance with levels of CSF A $\beta$ 42 in controls, MCI and AD patients; but not CSF levels of Tau (Fagan et al., 2006). A major limitation with this imaging is that currently it is known that non demented elderly people; and those with prodromal stages of the disease also show a relatively high amyloid plaque burden, therefore using this form of imaging to determine early stage AD or the potential transition to AD becomes difficult on its own. In addition a number of different studies show conflicting results; in part due to heterogeneity in conducting and interpreting the tests (see Villemagne et al., 2011; Zhang et al., 2014).

**Structural Magnetic Resonance Imagery (sMRI):** This is used for visualizing brain volume and thickness (Thompson et al., 2007) and to determine atrophy, thought largely to be due to dendritic and neuronal loss. AD is characterized by an inexorable progression of atrophy that first manifests in the hippocampus in AD patients (Scahill et al., 2002) and essentially follows the Braak and Braak staging of NFTs and cell loss (Johnson et al., 2012). When AD is clinically diagnosed, atrophy is already identified in temporal lobe regions. In presymptomatic and mildly effected individuals the hippocampal volume can be reduced by 15-20% and the entorhinal cortex by 20-30% (Johnson et al., 2012), whereas increasing rates of atrophy in the parietal cortex is observed at all stages of the disease (Scahill et al., 2002). Importantly, longitudinal studies show there is hippocampal loss at the MCI stage over 6 months that is accelerated over 1 year as the disease progresses (Ma et al., 2016). Although sMRI is a robust means of assessing brain atrophy it lacks the specificity of the pathological events inducing it.

**Functional MRI (fMRI):** This is a complementary form of imaging that identifies the functional integrity of brain networks by providing indirect measures of synaptic activity by measuring differences in blood oxygen level dependant (BOLD) MRI signals (Johnson et al., 2012). This can be done during resting states or during cognitive testing. Relatively few studies have been conducted using fMRI in MCI subjects or at risk individuals, but several studies have shown reduced activity in the medial temporal lobe in these subjects (Johnson et al., 2012). In contrast other studies have shown increased activities in individuals with very mild MCI or cognitively intact individual with genetic risk for developing AD (Dickerson et al., 2004; Celone et al., 2006; Lenzi et al., 2009; Fleisher et al., 2005; Fillipini et al., 2009). It has been suggested the hyperactivity may be present only in early stages of the disease and longitudinal studies show that early hyperactivity is a predictor of rapid cognitive decline (Brookheimer et al., 2000; Dickerson et al., 2004; Miller et al., 2008). Currently, there are few studies measuring activity in AD or preclinical subjects.

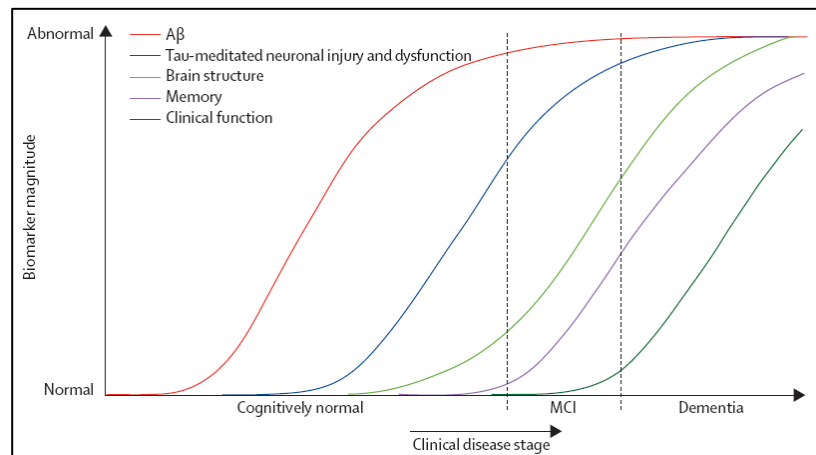
The term preclinical refers to a 'clinically silent stage of AD' and is part of a continuum between 'normal ageing' and different defined stages in presymptomatic AD. Stage 1 is characterized by evidence of A $\beta$  accumulation either by CSF assays or PET scans; Stage 2 is defined by evidence of amyloidosis and neurodegeneration either as elevated CSF Tau levels or abnormal structural or functional MRI. Stage 3 requires amyloidosis and neurodegeneration with evidence of subtle cognitive decline that does not yet meet the criteria of MCI (see Figure 5; Jack et al., 2010). In addition, there exists a category of clinically normal individuals that are positive for one or more biomarkers (see Berti et al., 2016).

However, no single marker gives an adequate assessment, for example CSF levels of A $\beta$  and Tau, gives no indication of the species of amyloid in the brain alone; sMRI and FGF-PET although robust measures of atrophy and metabolism reflecting neuronal activity, do not give specificity to the pathology; amyloid PET analyses APs and as such does not correlate with cognitive decline; and there are few studies analyzing activity using fMRI and there are reservations about interpretation of the results (Johnson et al., 2012; John et al., 2016; Koch et al., 2012; Tentolouris-Piperas et al., 2016).

Combined biomarkers and imaging studies have shown two very important aspects of the pathology and its progression. Firstly, CSF Tau changes have been shown to occur approximately 15 years before the onset of clinical AD (Bateman et

al., 2012; Fagan et al., 2014); and A $\beta$  accumulation can be observed around 20 years prior to onset of clinical symptoms (Jack et al., 2010). Moreover, using amyloid PET tracer accumulation; plaque load reaches a plateau in vulnerable brain regions, such as the hippocampus, in both sporadic and autosomal dominant AD and the estimated time between the ability to detect plaques with PET until saturation levels have been reached is about 15-20 years (see Jack et al., 2013).

Secondly, longitudinal studies in non-demented elderly people suggest that up to 45% would meet the criteria for AD based on imaging APs, had they been demented (Knopman et al., 2003; Bennet et al., 2006; Price et al., 2009; Schneider et al., 2009). Amyloid PET has shown positive retention of the radioligand in cognitively normal aged subjects (Mintun et al., 2006; Aizenstein et al., 2008) that show similar distribution to that seen in AD patients (Mintun et al., 2006; Aizenstein et al., 2008). This lends support to the notion that APs may not have a causal role in the dementia.



**Figure 5. Dynamic biomarkers of the Alzheimer's pathological cascade.** A $\beta$  is identified by CSF A $\beta$ 42 or PET amyloid imaging. Tau-mediated neuronal injury and dysfunction is identified by CSF Tau or fluorodeoxyglucose-PET. Brain structure is measured by use of structural MRI (Jack et al., 2010).

A major advancement in the NIA-AA directives was the setting down of guidelines for attempting to assess preclinical AD using specific biomarkers that should be consistent at the international level (Alexopoulos et al., 2016). A major challenge, however; despite the general consensus that soluble A $\beta$  would be an important target; is currently there is no biomarker or imaging assay that can detect this in the human brain. A second drawback that in fact might be more worrying is how the directives have classified the ever-growing evidence that cognitively normal aged individuals have a high amyloid load. On the one hand, as argued above, it might suggest that APs may not have a causal role in dementia; on the other hand, some within the NIA-AA working group (Vos et al., 2016) have suggested even cognitively normal nonagenarians with a heavy plaque load might be placed in a pre-stage 1 criteria. This carries the implicit understanding that everyone will enter the AD trajectory sooner or later; the question will not be whether an individual develops the disease, but when. Based on this, it further beggars the question of whether Alzheimer's disease is a disease or is it merely part of the ageing process. And if it is part of the ageing process, why do some aged persons with a high plaque load develop AD while others do not, and what determines the time point at which an individual will start the slippery slope into dementia.

Results from imaging studies in humans, although far from perfect, show two important findings; (i) that a growing number of aged persons that are cognitively normal have a high amyloid burden; and (ii) the increase in soluble A $\beta$  and plaques can start to increase 20 years or so before the first clinical signs of the disease are diagnosed. In fact, when people are first diagnosed with AD, plaque levels have reached a plateau. This together with drug trials that clear plaques but show no improvement in cognition must surely put the final nail in the coffin that suggesting the plaque is 'the' triggering mechanisms for the disease and ensuing dementia; which now has a growing consensus.

Not to dismiss the potential role of amyloid in the disease out of hand, soluble species have been shown to be toxic and can circulate throughout the brain. This allows us to rationalise how the differential distribution of amyloid and Tau; the two key-signature pathological markers of the disease may interact, and better fits with the slow insidious onset of the disease. However, as soluble species of amyloid interact with number of different receptor types it at least suggests dysfunction in AD can be induced in a parallel, not a linear manner as suggested in the Amyloid Cascade Hypothesis. Moreover, the process of shearing amyloid from APP creates a number of different fragments that potentially contribute to a slow ongoing dysfunctional process, well before plaques are detected; an important area of research that is not well documented to date.

An extremist point of view would be that amyloid is merely a by-product of other dysfunctional events (see Struble et al., 2010); others would suggest that although amyloid might be involved in the pathology and dementia, these processes are mediated by multifunctional effects (Frautschy and Cole, 2010; Herrup et al., 2010; 2013). Critical to both stand points is the fact that low levels of amyloid have a physiological effect; and this in turn asks the question as to what might trigger an increase in amyloid from a physiology level to a pathological level. In fact, it begs the question as to whether other pathological events, not specific

to AD may create a disequilibrium in functional homeostasis that together permit the development of the classic AD pathology and have a causal role in the ensuing dementia. Given the timeframe of the slow increase in soluble A $\beta$  and plaques, it brings into sharp focus the potential role that risk factors may have in the early development of the pathology.

## 2 Lifestyle risk factors for the development of Alzheimer's Disease

There are numerous risk factors for AD that have been identified; age being the most important. Other risk factors can generally be classified as either (i) genetic factors that mainly include mutations in A $\beta$ ; the APP or cleavage proteins observed in people with EOFAD; however excess copies of ApOE4 also pose a risk for the disease; (ii) metabolic diseases which include Type 2 Diabetes Mellitus (T2DM); thyroid dysfunction, hypertension, cardiovascular disease. (iii) Finally, a third category is lifestyle risk factors that include basically a wide range of behavioural habits that have a negative impact on health. The importance of this category of risk factors is that they are both modifiable and can be risk factors for other risk factors.

Evidence suggests that environmental and lifestyle factors can have both a positive and negative impact on general health and disease (see Table 2). For the most part evidence has largely been obtained from epidemiological studies and clinical interview. Understanding how these factors may impact brain and cognitive function is not easy research to conduct, as much of it is correlational, subjective, occurs slowly over time and has a lot of inbuilt variability. Despite this paucity in our knowledge, modeling these effects in rodents has expanded our understanding of certain mechanisms mediated by lifestyle that may be associated with early stages of the development of AD.

**Table 2. Risk factors of AD**

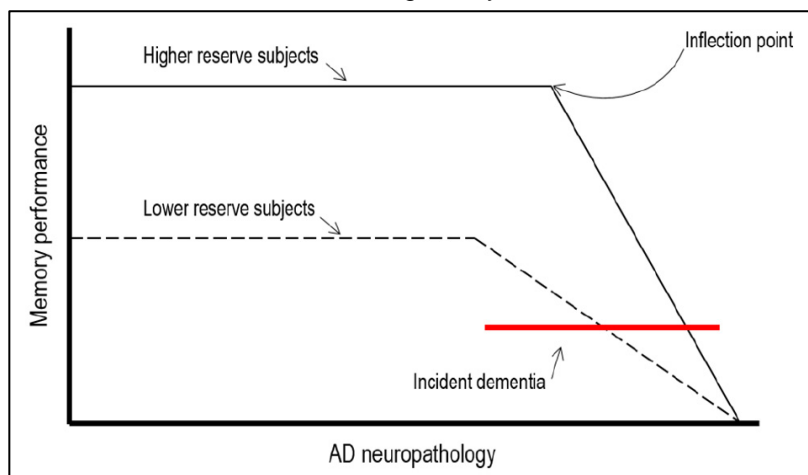
	<b>Risk factors</b>	<b>References</b>
Lifestyle factors	Smoking (-)	Launer et al., 1999; Almeida et al., 2002; Rusanen et al., 2011
	Diet and nutrition (+/-)	Kalmijn et al., 1997 ; Mattson et al., 2003 ; Nicolai et al., 2015
	Education, cognitive activity, social activity (+)	Kukull et al., 2002b ; Lindsay et al., 2002 ; Karp et al., 2004 ; Ngandu et al., 2007 ; Bickel and Kurz, 2009
	Exercise (+)	Rovio et al., 2005; Hamer and Chida, 2009; Laurin et al., 2001
	Caloric restriction (+)	Mattson et al., 2003; Van Cauwenberghe et al., 2016
Other disease	Obesity (-)	Whitmer et al., 2007; Luchsinger and Gustafson, 2009; Barnes and Yaffe, 2011
	T2DM (-)	Arvanitakis et al., 2004; Akomolafe et al., 2006; Kim et al., 2010; Profenno et al., 2010; Cheng et al., 2011
	Cardiovascular disease (-)	Stampfer, 2006; de Bruijn and Ikram, 2014; Ferreira et al., 2014
	Thyroidism (-)	Kapaki et al., 2006; Tan et al., 2008 ; Chaalal et al., 2014
Other factors	Hypertention (-)	Forette et al., 2002; Barnes and Yaffe, 2011; Ogunniyi et al., 2011
	Brain injury (-)	Lindsay et al., 2002; Plassman et al., 2000
	Gender: More risk in women	Andersen et al., 1999; Ruitenber, 2001 ; Liu et al., 2003

(+): Beneficial factor; (-): Detrimental factors

## 2.1 Ageing and Cognitive Reserve

The most obvious risk factor for developing sporadic AD is ageing and, unlike most of the other lifestyle risk, cannot be controlled. However, as already alluded to, there is a growing number of aged cognitively normal individuals that have a high plaque load (Hedden et al., 2009). Although the pro-amyloid hypothesis argues, regardless of their age they are relegated to the preclinical stage of AD; a viable alternative explanation, suggests these individuals build up what is described as ‘cognitive reserve’ (Stern, 2009). This remains to date a conceptual description, with few identified underlying mechanisms. In essence, the concept suggests that based on their lifestyle, these individuals are more resilient to brain damage or in the case of AD, the pathology before they begin to suffer the cognitive consequences (See Figure 6; Arenaza-Urquijo et al., 2015). Cognitive reserve is inextricably linked with intellectual capacity as epidemiological studies and clinical interview show the major influencing factors are education level, intellectual stimulation and socialization (Kukull et al., 2002; Karp et al., 2004; Ngandu et al., 2007; Bickel and Kurz, 2009), that can be beneficial to both young and aged adults (Piras et al., 2011; Ewers et al., 2013). Of particular importance are longitudinal studies that have been conducted in closed order religious communities; this allows elimination of other environmental factors such smoking and alcohol consumption; and their lifestyle over many years is well documented. Nuns were cognitively tested on an annual basis

and it was found in general those with higher educational standard were less likely to develop AD (Riley et al., 2005; Tyas et al., 2007; Bickel and Kurz, 2009). The concept suggests that greater intellectual stimulation promotes more plasticity in multiple neural circuits, such that in the event of a pathological insult in one neural circuit, cognition, memory and intellectual capacity can be maintained via other neuronal circuits.



**Figure 6. Theoretical illustration of how cognitive reserve may mediate the relationships between AD neuropathology and cognitive function.** Aged persons with a high cognitive reserve that develop AD, will not begin to show cognitive decline until the pathology has advanced much more than those with a lower cognitive reserve (Arenaza-Urquijo et al., 2015).

Comparative imaging studies between aged individuals that are cognitive normal, those with MCI and those with AD show that atrophy and glucose metabolism are key dysfunctional mechanisms associated with cognitive decline (Thompson et al., 2007; Lehmann et al., 2011; Schöll et al., 2011; Ma et al., 2016). Moreover, cognitively normal aged persons show less activity during cognitive processing, suggesting a more effective use of neural networks (Solé-Padullés et al., 2009). Finally, cognitively normal aged persons with a high amyloid plaque level, compared with AD patients, have significantly less loss of

neurons and lower levels of inflammation (Lue et al., 1996), and larger brain and hippocampal volumes, suggesting potential preservation of neurons and synapses (Erten-Lyons et al., 2009).

## **2.2 Environmental Enrichment in rodents**

The equivalent to intellectual stimulation in rodents is exposure to environmental enrichment (EE); and there has been a long history of studies showing EE improves cognitive performance in young and old rodents (see reviews by van Praag et al., 2000; Simpson and Kelly, 2011) and can at least partially rescue cognitive deficits in rodent models of neurodegeneration (Nithianantharjan and Hannan, 2006; Laviola et al., 2008; Hannan, 2014). However, the beneficial effects of EE on cognition can vary depending on a number of factors such as the duration of enrichment, the presence or absence of a running wheel, size of the environment, etc (see Simpson and Kelly, 2011). In general EE is known to induce neurogenesis, but this is specific to the dentate gyrus of the hippocampus (van Praag et al., 2000; Kempermann et al., 1998; Segovia et al., 2006; Mora et al., 2007; Rueda and Trejo, 2007). However, EE also (i) induces synaptogenesis (Levi et al., 2003; Kondo et al., 2012; Kelly et al., 2013) and angiogenesis (Ekstrand et al., 2008; Herring et al., 2008); (ii) increases brain weight, dendritic branching; (iii) prevents decreases in synaptic density and gliogenesis and (iv) positively changes the dynamics of several neurotransmitter system including glutamate and dopamine receptors (Foster et al., 2000; Mora et al., 2007; Laviola et al., 2008). Therefore, the mechanisms by which EE can have a beneficial effect are multifactorial and could mediate its effect in different mechanisms in different brain structures.

In view of the studies that suggest intellectual capacity can potentially affect the development or onset of AD in humans, a number of studies have been conducted in transgenic models of AD, but have yielded mixed results. For example, some studies report that EE improves cognition (Lazarov et al., 2005; Costa et al., 2007; Valero et al., 2011) and, in some cases, reduces A $\beta$  load (Lazarov et al., 2005; Costa et al., 2007) whereas others have reported an improvement in cognition in the absence of a change in A $\beta$  load (Arendash et al., 2004; Mirochnic et al., 2009) or even that EE induces an increase in A $\beta$  load (Janowski et al., 2005). Others have shown that EE needs to start prior to the onset of plaque load (Verrret et al., 2013) or that the enrichment period needs to be extensive (Cracchiolo et al., 2007). These differences may result from the variables described above, but how they may relate to the beneficial effects of intellectual stimulation and education level in human is not well understood.

Two of the major beneficial lifestyle factors that could have a protective effect against development of AD or its severity are basically factors that are associated with the maintenance of a basic healthy lifestyle, namely exercise (Laurin et al., 2001; Lindsay et al., 2002; Podewils et al., 2005; Catlow et al., 2008) and caloric restriction (Mattson et al., 2003; Cauwenbergh et al., 2016). Complementary evidence suggests that a sedentary lifestyle and excess weight gain and obesity put individuals at risk for developing dementia (Laurin et al., 2001; Podewils et al., 2005; Whitmer et al., 2007; Luchsinger and Gustafson, 2009; Barnes and Yaffe, 2011).

### 2.3 Exercise

Exercise has been shown to have general beneficial effect in humans across the life span (Prohaska and Peters, 2007; Rockwood and Middleton, 2007); studies reveal that being more physically active, especially as a teenager, is beneficial for cognitive performance in late adulthood (Middleton et al., 2010), and old age (Iwasa et al., 2012). Meta-analysis show that exercise intervention training in elderly people with cognitive impairment and dementia increases both physical and cognitive function (Heyn et al., 2004). Some have gone so far as describing exercise as having an inverse relationship to the risk of developing dementia in humans (Heyn et al., 2004; Rovio et al., 2005; Hamer and Chida, 2009). The effect on cognition is somewhat selective in as much as it improves executive-control processes (Colcombe and Kramer, 2003). Physical intervention has been shown to induce beneficial metabolic and neuropathological changes, such as increased brain tissue volume (Colcombe et al., 2006) particularly in the hippocampus (Killgore et al., 2013; Erickson et al., 2011; 2014) and maintenance of cortical plasticity (Erickson et al., 2007).

In contrast, sedentary lifestyle is detrimental to health and associated with obesity and cardiovascular disease, which may contribute to cognitive dysfunction and AD, but it is difficult to ascertain the precise direct effect of sedentary lifestyle on AD (Peterson et al., 2013; Ryan et al., 2015). Nonetheless, some studies have shown sedentary behaviour in elderly people may be responsible for decline in general cognitive function as exercise tends to improve these functions (Scherder et al., 2014; Falck et al., 2016).

In rodent studies, there is a vast bank of literature on the beneficial effects of exercise on memory processes and synaptic plasticity in both the young and aged (Farmer et al., 2004; van Praag, 1999b; 2005; Vaynman et al., 2004; O'Callaghan et al., 2007; Vivar et al., 2013) and in transgenic models of AD (Adlard et al., 2005; Hüttenrauch et al., 2016; Tapia-Rojas et al., 2016). In the dentate gyrus these beneficial effects are mediated largely by neurogenesis (Kitamura et al., 2003; Wu et al., 2008a; van Praag, 2009). This is mediated by inducing proliferation of neural progenitor cells, increasing the number of new neurons, and promoting survival of newborn cells in both young (van Praag et al., 1999a; Trejo et al., 2001) and aged animals (Fabel et al., 2003; Wu et al., 2008a). Some of the underlying mechanisms mediating exercised induced neurogenesis include growth and neurotrophic factors including BDNF (Cotman and Berchtold, 2002; Vaynman et al., 2004; Wu et al., 2008a), insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF) associated with the PI3K-Akt signalling pathway (Carro et al., 2000; Trejo et al., 2001; Bruel-Jungerman et al., 2009; Heras-Sandoval et al., 2014).

In addition to inducing neurogenesis in the dentate gyrus, exercise enhances spine density in hippocampus and entorhinal cortex and maturation of dendritic spine in newborn neurons (Redila and Christie, 2006; Zhao, 2006; Stranahan et al., 2007) promotes angiogenesis (Fabel et al., 2003; van Praag, 2005; Cotman et al., 2007) and synaptogenesis (van Praag, 2009; Lista and Sorrentino, 2010), and increases hippocampal volume (Fuss et al., 2014; Biedermann et al., 2016). As exercise induced neurogenesis is specific to the dentate gyrus its effect on other forms of genesis and underlying



mechanisms suggest that the beneficial effects of exercise are multifactorial and can be mediated via different neural circuits.

Exercise in AD transgenic animals reduces A $\beta$  load in hippocampus or cortex and improves the hippocampus dependent memory and novel object recognition memory (Adlard et al., 2005; Yuede et al., 2009; Liu et al., 2013; Moore et al., 2016). Voluntary exercise improves memory performance and attenuates AD pathology by decreasing A $\beta$  burden, tau phosphorylation and astrogliosis, and increasing neurogenesis, as shown in several AD mice (García-Mesa et al., 2011; Rodríguez et al., 2011; Marlatt et al., 2013; Tapia-Rojas et al., 2015). Exercise therefore protects the hippocampus against AD pathology and has been shown to be mediated via a PI3K-dependent mechanism (Isla et al., 2016).

## **2.4 Caloric restriction**

Caloric restriction (CR) is an evolutionary conserved mechanism from yeast to humans that promotes longevity (Fontana et al., 2010; Anderson and Weindruch, 2012; Mercken et al., 2012; Rizza et al., 2014). In humans, this basically equates to energy expenditure (the caloric intake versus the amount of physical output). Numerous studies have shown maintaining this balance reduces the risk of developing AD; whereas a high calorific diet is associated with a higher risk of developing AD, particularly with individuals carrying the apolipoprotein E4 allele (Spindler et al., 1996; Keene and Hope, 1997; Luchsinger et al., 2002). In our current, relatively sedentary environment, it has been shown that the 'Mediterranean Diet' (Morris et al., 2005; Scarmeas et al., 2006) or the 'Okinawan Diet' (Willcox et al., 2014) that is based on relatively high consumption of fruit, vegetables, cereals and fish; and low consumption of meat, cereals, unsaturated fatty acids, and processed food stuffs, have beneficial effects in both normal condition and reducing risk of developing disease.

CR has been shown to affect a number of different neuronal functions in the brain including resistance to oxidative stress, metabolic and excitotoxic injury and promotion of neurogenesis and synaptic plasticity (Hori et al., 1992; Contestabile et al., 2004; Kitamura et al., 2006; Gillettel-Guyonnet and Vellas, 2008). This can be mediated by stabilising glutamate receptors and synaptophysin required for excitatory transmission in hippocampus in rats (Adams et al., 2008) and also increasing levels of NMDAR, BDNF and genes associated with synaptic plasticity (Murphy and Dias, 2014).

In rodents, it has been shown to promote lifespan extension in different models at least in part by stimulating autophagy (Ferreira-Marques et al., 2016). In the brain, CR and autophagy is based on the availability of amino acids that determine how proteins are generated. When there are high levels of amino acids, the preferred mechanism for generating new proteins is protein synthesis and this is an energy expensive mechanism (Mortimore and Pösö, 1987; Mortimore et al., 1989). Under conditions of low levels of amino acid availability, the preferred mechanism is autophagic recycling of existing protein complexes as it is less energy-expensive (Mortimore and Pösö, 1987; Mortimore et al., 1989; Donati et al., 2001; Bergamini et al., 2007).

CR has shown to be neuroprotective and improve learning and memory performance in young and aged animals (Idrobo et al., 1987; Stewart et al., 1989; Sharma and Kaur, 2005; Qiu et al., 2012; Yang et al., 2014) and can restore memory deficits in Tg models of AD (Halagappa et al., 2007; Wu et al., 2008b). During normal ageing, autophagy declines and its inhibition can accelerate the ageing process; however, this can be prevented by CR (Donati et al., 2001; Bergamini et al., 2007). In transgenic mouse models of AD, CR has reduced levels of A $\beta$ 1–40, A $\beta$ 42 and phosphorylation of Tau in 3xTg mice (Halagappa et al., 2007) and prevented or reduced the accumulation of amyloid deposition in other models (Patel et al., 2005; Wang et al., 2005; Mouton et al., 2009).

To date few studies of CR on cognitive function in humans have been conducted, Witte and colleagues (2009) first showed the beneficial effect of CR on memory performance in aged healthy subjects, where a 30% reduction in caloric intake over 3 months resulted in improvement in memory performance, particularly in verbal memory tests, and this correlated with decreases in fasting plasma levels of insulin.

Although there are a number of known physiological dysfunctions that place an individual at risk for the development of AD, most notably metabolic disorders (see Table 2), these disorders are also subject to a maladapted lifestyle mainly in terms of excess weight and sedentary lifestyle. Following ageing, it is now considered that the greatest lifestyle risk factor for developing AD is T2DM (Arvanitakis et al., 2004; Janson et al., 2004; Akomolafe et al., 2006; Cheng et al., 2011), a metabolic disorder that manifests itself in middle age and is highly susceptible to obesity (see section 3). Clinical assessment suggests that patients with T2DM have approximately 65% higher risk of developing AD in later age (Janson et al., 2004). There is a huge database in human studies and circumstantial evidence to suggest that dysfunctional regulation of brain homeostasis precedes the development of AD, the most likely candidates to induce this could be risk factors associated with the disease (Mattson et al., 2003). Since diabetes usually precedes AD, it is believed that conditions in diabetics are conducive for the development of AD at the early stage but the mechanisms by which this occurs are poorly understood.

### 3 Type 2 Diabetes Mellitus (T2DM)

T2DM is one of a group of diabetes metabolic disorders (see Box 4) characterised by hyperglycaemia over a prolonged period, and dysfunctional insulin regulation (Alberti and Zimmet, 1998; Cersosimo et al., 2015). It is subject to a number of risk factors, of which the most prevalent is excess weight or obesity. In our current global climate in which there is increasing availability of highly processed foodstuffs and an overall increase in sedentary behaviours; the global incidence of the disease is increasing rapidly.

The International Diabetes Federation (IDF) reported that the number of people with T2DM in the world was estimated at 371 million in 2012 and will rise to 552 million by 2030. Four out of 5 people with T2DM are from low-income communities and their numbers in low and middle-income countries are on a rapid increase. As population studies show that all forms of diabetes at least double a person's risk of death, the global economic cost of T2DM in 2014 was estimated to be \$612 billion. The mechanism of pathogenesis, due to its complex and multifactorial nature (Aguiree et al., 2013; World Health Organization, 2014) are not fully understood and this is of major concern to socio-economic welfare on a global level.

#### 3.1 Diagnosis and Classification for T2DM

##### 3.1.1 *Diagnosis criteria*

The clinical diagnosis of all forms of diabetes is high levels of glycosuria usually present and often prompted by symptoms such as increased thirst and hunger, frequent urination, recurrent infections and drowsiness and coma in severe cases (World Health Organization, 1985). Most practical method for diagnosis of diabetes are based on traditional plasma (blood or serum) glucose criteria, either the fasting plasma glucose (FPG) or the 2-h plasma glucose (2-h PG) value after a 75-g oral glucose tolerance test (OGTT) (Nathan et al., 2009). Also factors such as elevated levels of triglycerides, blood pressure, body mass index (BMI) and family history of diabetes would be considered. The commonly used plasma glucose criteria, first suggested in 1995 and then renewed in 1998 by the WHO are described in Table 3.

**Table 3. Diagnosis criteria of diabetes**

	Fasting Plasma Glucose (mg/dL)	2-h Oral Glucose Tolerance test (mg/dL)
Normal	99 or below	139 or below
Prediabetes	100-125	140-199
Diabetes	126 or above	200 or above

Based on American Diabetes Association, 2015

##### 3.1.2 *Classification*

T2DM is the most common form of diabetes making up 85-90% of diabetic patients, also previously referred to as "non-insulin dependent diabetes mellitus" (NIDDM), which has a late-onset, mainly occurring in middle age. It is characterized by chronic insulin resistance and progressive loss of beta-cell mass, which lead to impaired insulin resistance and hyperglycemia. The predominant feature of T2DM is disorders of either insulin action or insulin secretion. Both are usually present at the time that this form of diabetes is

clinically manifest. The specific reasons for the development of these abnormalities are not yet known (American Diabetes Association, 2015). T2DM is also subject to risk factors; obesity is a major risk factor thought to confer increased risk for T2DM through the mechanism of associated insulin resistance (Butler et al., 2003).

#### **Box 4. Classification of diabetes mellitus**

There are 2 major forms of diabetes: Type 1 Diabetes Mellitus (T1DM) and Type 2 Diabetes Mellitus (T2DM). The classification of the two types of diabetes is based on a clinically descriptive criterion but more recently a third form, Gestational Diabetes Mellitus (GDM) that is a temporary form that is specific to pregnant females has more recently been included in the classification (World Health Organization, 1985; American Diabetes Association., 2015).

##### ***Type 1 diabetes mellitus (T1DM)***

Type 1 DM has an early onset, was previously referred to as "insulin-dependent diabetes mellitus" (IDDM) results from autoimmune mediated destruction of  $\beta$ -cells of the pancreas, where the pancreas failure to produce enough insulin. Its occurrence is most common in children and young adults (Humphrey et al., 1998). Individuals with T1DM eventually become dependent on insulin for survival and are at risk for ketoacidosis. There is a genetic predisposition to autoimmune destruction of beta cells, and it is also related to environmental factors that are still poorly defined (Willis et al., 1996). Although patients are rarely obese when they present with this type of diabetes, the presence of obesity is not incompatible with the diagnosis (Betterle et al., 1984).

##### ***Gestational diabetes mellitus (GDM)***

Gestational diabetes is carbohydrate intolerance resulting in hyperglycaemia of variable severity with onset or first recognition during pregnancy (Alberti and Zimmet, 1998). However, criteria for designating abnormally high glucose concentrations at this time have not yet been established. Individuals at high risk for gestational diabetes include older women, those with previous history of glucose intolerance; those with a history of large for gestational age babies; women from high-risk ethnic groups, and any pregnant woman who has elevated fasting, or blood glucose levels (World Health Organization, 1985; American Diabetes Association., 2015). Young offspring from GDM mothers show elevated risk of developing T2DM, however the role of GDM in T2DM is currently unknown (Vaag et al., 2014).

### **3.2 Insulin and glucose regulation**

#### **3.2.1 Glucose**

Glucose is one of the major sources of energy required for body, brain and nervous system activity. The body converts carbohydrates from food into glucose, as it requires levels of glucose to be maintained within a very narrow range of 70-110 mg/dL. Glucose is converted to glycogen and stored in tissues such as muscle, liver and fat cells for a backup energy source when blood glucose levels drop (Kreitzman et al., 1992; Ryder et al., 2001). Glucose entry into cells requires glucose transporter proteins to cross the plasma membrane. Five different glucose transporters (GluTs) have been identified, GluT 1-5, which have their own specific tissue distribution and mechanisms depending on the demands of each tissue (Ryder et al., 2001). It is reported that under insulin stimulation both GluT 1 and GluT 4 localize from an intracellular storage site to the cell surface, however GluT 4 plays a main role in insulin-stimulated glucose transport in skeletal muscle of rodents and humans (Lund et al., 1994; Ryder et al., 2001). The brain is the organ that consumes the highest levels glucose, most notably for synaptic transmission (Howarth et al., 2012) as it does not produce or store glucose itself (Cunnane et al., 2011). Glucose is taken up into the brain via the blood brain barrier (BBB) in a gradient dependent manner by the insulin-independent GluT 1. It requires no

energy for transfer from the blood but Glut1 transfers glucose from the compartment with the higher glucose concentration to the lower; therefore, the brain is highly susceptible to levels of glucose in the periphery.

### 3.2.2 Insulin

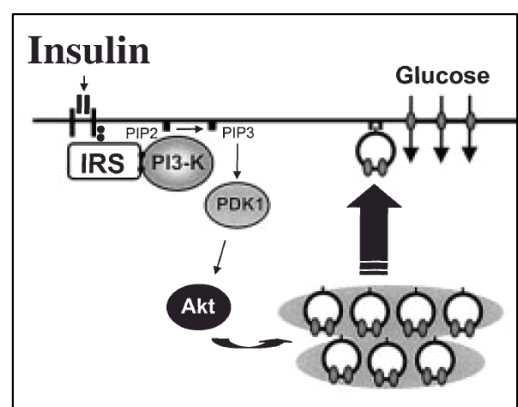
Insulin, discovered by Banting and Best in 1921, is a hormone produced by  $\beta$ -cells in the pancreas which reduces blood glucose levels by enhancing glucose uptake, mainly in liver, muscle and adipose tissue, thus promoting glucose oxidation and glycogen synthesis to regulate glucose homeostasis (Sonksen and Sonksen, 2000). It also inhibits lipolysis and hepatic glucose production and facilitates energy storage by promoting lipogenesis (So and Ng, 2000). There are four known specifically-named insulin receptor substrate (IRS) proteins. IRS 1 is the major IRS in skeletal muscle, IRS 2, is the main IRS in liver, IRS 3 is found only in adipose tissue,  $\beta$ -cells and liver and IRS 4 in thymus, brain and kidney (IRS 3 and 4 are less well characterised).

Up until 3 decades ago, insulin was considered only as a peripheral hormone and unable to cross the blood brain barrier (BBB). It is well established that insulin is taken up into the brain via the BBB through a saturable insulin transporter system; suggesting that high levels of blood insulin will not affect brain levels; however low levels will. More recently, some studies suggest that insulin can be synthesised in brain (see reviews by Duarte et al., 2012; Blazquez et al., 2014); however there is no consensus, as evidence supporting brain synthesis of insulin has been mainly conducted in lower order species or *in vitro* (Banks et al., 2012). Although it is well established that insulin plays an important role in brain function, notably during learning and memory (Park 2001; Duarte et al., 2012) and there are insulin receptors present in the brain (Werner and LeRoith, 2014); whether insulin mediated regulation of brain functions are due to insulin generated in the brain or whether it is taken up by the periphery is not clear.

### 3.2.3 Insulin-stimulated glucose regulation

In healthy conditions following the consumption of a meal blood glucose levels rise due to digestion of carbohydrates that triggers insulin secretion from  $\beta$ -cells in the pancreas. Insulin exerts its biological function by binding to its receptor on the cell surface of specific target tissues (So and Ng, 2000). The insulin receptor belongs to a class of tyrosine kinase receptors, containing two ligand binding  $\alpha$  subunits and two tyrosine kinase  $\beta$  subunits. Binding of insulin to one of the extracellular  $\alpha$  subunits results in autophosphorylation and activation of multiple tyrosine molecules in the intracellular  $\beta$  subunits (Ryder et al., 2001).

This intracellular domain plays a central role in the signalling cascade involved in glucose uptake and glycogen synthesis (So and Ng, 2000). The Insulin kinase receptor has specific cellular substrates, the family of insulin receptor substrate, IRS 1-4 (Chang and Chiang, 2005). The tyrosine kinase activity of the insulin receptor can induce phosphorylation of IRS-

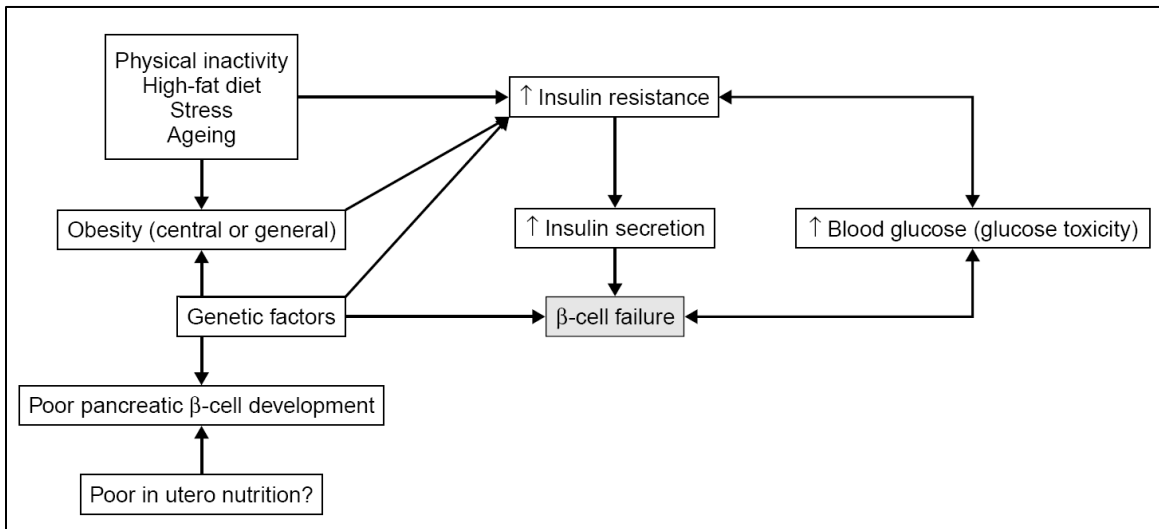


**Figure 7. Insulin-stimulated glucose transport is dependent on the activation of PI3K-Akt signaling pathway (Ryder et al., 2001).**

1 (Sun et al., 1991) which functions as an important scaffolding protein in various signalling pathways, such as insulin growth like factor (IGF-1), the PI3-kinase/AKT and MAPK pathways (del Rincón et al., 2004) in tissues such as, adipose tissue and muscle, to promote insulin stimulated glucose transport into cells (Figure 7; Ryder et al., 2001). Pharmacological inhibition of the PI3K-Akt signalling pathway prevents glucose transport, indicating a key role of this pathway in insulin-induced glucose transport.

### 3.3 Pathology of T2DM

The pathology of T2DM is principally due to a failure of insulin to clear and regulate levels of glucose in the blood. This can be due to a number of factors that include gene mutations associated with insulin function (Kahn et al., 1996; Brunetti et al., 2014); epigenetic deregulation that can be induced *in utero* (Swenne et al., 1992; Hales and Barker, 2013); but mainly by lifestyle and factors (McCarthy, 2015) that cause impaired  $\beta$ -cell insulin secretion, peripheral insulin resistance and hyperglycemia (Figure 8; So and Ng, 2000; Brunetti et al., 2014).



**Figure 8. Pathogenesis of type 2 diabetes mellitus** (So and Ng, 2000)

#### 3.3.1 Insulin resistance and hyperglycemia

Insulin resistance and hyperglycemia are the most characteristic features of T2DM. Insulin resistance is an impaired biological response to insulin by its target tissues, mainly in muscle, liver and fat (Brunetti et al., 2014). Unlike normal conditions, the cells of these target tissues become resistance to insulin and are not able to function as effectively, leading to high blood sugar. Resistance can be mediated via numerous insults including inflammation, lipid toxicity and dysfunction gut microbiota (Johnson and Olefsky, 2013), but can also be induced by insulin receptor desensitisation and receptor internalisation (Boothe et al., 2016). To balance the increase in glucose levels, pancreatic  $\beta$ -cells secrete more insulin, further contributing to hyperinsulinemia (So and Ng, 2000).

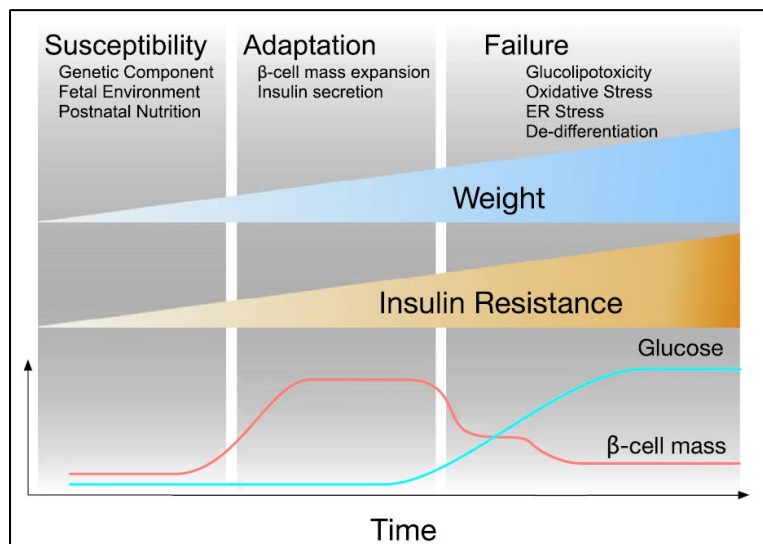
Although insulin resistance is a strong predictor of T2DM, it alone is not sufficient to cause diabetes. The majority of patients with T2DM have a moderate degree of insulin resistance (DeFronzo, 1988) and non-diabetic obese individuals who also have a moderate degree of insulin resistance do not necessarily become glucose intolerant, thus indicating that relative insulin deficiency is essential for hyperglycaemia which constitutes the primary hallmark of T2DM (Gola et al., 1988; Brunetti et al., 2014). Once hyperglycaemia has developed, glucose toxicity can induce further insulin resistance and impaired pancreatic beta cell function, thus putting in place a self-serving detrimental cycle. Therefore, insulin secretion and its action are closely interconnected, and a defect in one is likely to lead to defect in the other and thereby contribute to the development of late-onset diabetes (Taylor et al., 1994; So and Ng, 2000).

### 3.3.2 Pancreatic $\beta$ -cell failure

Beta cells ( $\beta$ -cells) are found in the pancreatic islets of the pancreas, producing insulin and glucagon. Four different types of cells have been identified in the pancreatic islets:  $\alpha$ -cells that secrete glucagon responsible for increasing blood glucose,  $\beta$ -cells that secrete insulin,  $\delta$  and  $\gamma$ -cells which are involved in regulation of  $\alpha$  and  $\beta$  cells and secretion of pancreatic polypeptides. All these act to control blood glucose through secreting glucagon to increase the levels of glucose, and insulin to decrease it in a homeostatic manner (Cabrera et al., 2006).

T2DM is characterized by relative insulin deficiency in response to an increase in insulin induced by insulin resistance. Experiments in humans and rodent models have shown failure of  $\beta$ -cells to increase mass and function is a key process in the development of T2DM. Hyperglycemia in insulin resistant individuals develops later when the  $\beta$ -cells fail to compensate for the decrease in insulin levels (Brunetti et al., 2014). All factors affecting normal  $\beta$ -cell function could contribute to the development of T2DM.

In a recent review, researchers divided the process of  $\beta$ -cell impairment in relation to obesity and diabetes into three phases: susceptibility, adaptation and failure (Figure 9; Alejandro et al., 2014). It is believed that genetic factors, fetal



**Figure 9. Natural history of the adaptation of  $\beta$ -cells to obesity and diabetes.** It is clear that the majority of the obese individuals develop insulin resistance. The  $\beta$ -cells adapt to insulin resistance by increasing mass and function. The  $\beta$ -cells compensates appropriately in the majority of obese individuals and hyperinsulinemia is a common finding in obesity. However, in a fraction of obese subjects  $\beta$ -cells fail to compensate appropriately with the development of hyperglycemia and diabetes. After the development of hyperglycemia, glucose acts synergistically with other factors to induce beta-cell failure. The evolution of  $\beta$ -cells in this process can be divided into three major stages: 1) Individuals with high risk of diabetes are born with increased susceptibility by genetic component, the fetal environment and the nutrient environment during the first years of life. 2) As individuals gain weight there is a phase of adaptation. 3) Individuals with increased susceptibility develop  $\beta$ -cell failure (Alejandro et al., 2014).

environment and exposure to certain postnatal nutritional regimes would have a causal role in the functional control of  $\beta$ -cell mass at early stages (susceptibility). In fact, most individuals will not develop T2DM unless they are exposed to an increase in insulin demand by conditions such as obesity-induced insulin resistance. In this case  $\beta$ -cells compensate for glucose homeostasis by enhancing insulin secretion and  $\beta$ -cell mass. However due to the lifestyle and diet or other factors,  $\beta$ -cells in obese people fail to properly compensate and they subsequently develop hyperglycemia (adaptation). The persistence of insulin resistance and hyperglycemia becomes harmful, and together with other metabolic abnormalities induced by obesity results in a progressive loss of  $\beta$ -cells and impairment in function (Alejandro et al., 2014).

### **3.4 Obesity as a risk factor for developing T2DM pathogenesis**

Although studies have shown there are certain genetic risk factors for developing T2DM, the known variants account for less than 10% of the overall estimated T2DM risk, suggesting in general environmental factors play a large part in inducing the disease (Alejandro et al., 2014); most notable is obesity (Srinivasan, 2005).

Most people with T2DM are overweight or obese at the point of diagnosis. Overweight and obesity represent a rapidly growing threat to the health of people in the world. Classification of overweight or obesity based on the body mass index (BMI) suggests that when BMI is between 25 to 30 people are considered overweight; and when it is higher than 30 it is considered to be an indication of obesity (World Health Organization, 2000). Statistics have shown in 2014 that 39% of adults were overweight with at least 2.8 million people dying each year as a result of it. Therefore, obesity poses a huge economic burden and health threat to people as it also increases the risk for developing many disease including cardiovascular disease, dementia, hypertension, stroke, heart disease, cancer and metabolic disorder especially T2DM. (World Health Organisation, 2014).

At the heart of obesity is dietary consumption; this is not solely based on the volume of food versus the expenditure of energy, but also the quality of foodstuffs consumed. Individuals with long-term excess diet, particularly that which is high in fat and sugar, have more chance of developing obesity, T2DM and other metabolic disorders such as cardiovascular disease (see Djoussé et al., 2010; Riccardi et al., 2004). Human diets are complex and include many specific dietary components, however long-term consumption of food that is high in sugar and fat, as is abundantly used in “fast food” are particularly detrimental as they have a high propensity for inducing obesity.

Carbohydrates are the main components of food that are responsible for postprandial glycemia and insulinemia and excess levels are related to the etiology of many chronic metabolic diseases, including T2DM and obesity (Bao et al., 2011). The type and amount of fat intake can differentially regulate insulin sensitivity; polyunsaturated fats appear to have beneficial effects on insulin action by enhancing peripheral glucose utilisation as measured in rodents (Lardinois and Starich, 1991; Okere et al., 2006), whereas monounsaturated fat impairs insulin sensitivity (Vessby et al., 2001). As stated above changes in fatty acids may influence insulin action in the body through many mechanisms that affect lipid composition, metabolism and signal-transduction pathways (De Ferranti and Mozaffarian, 2008).

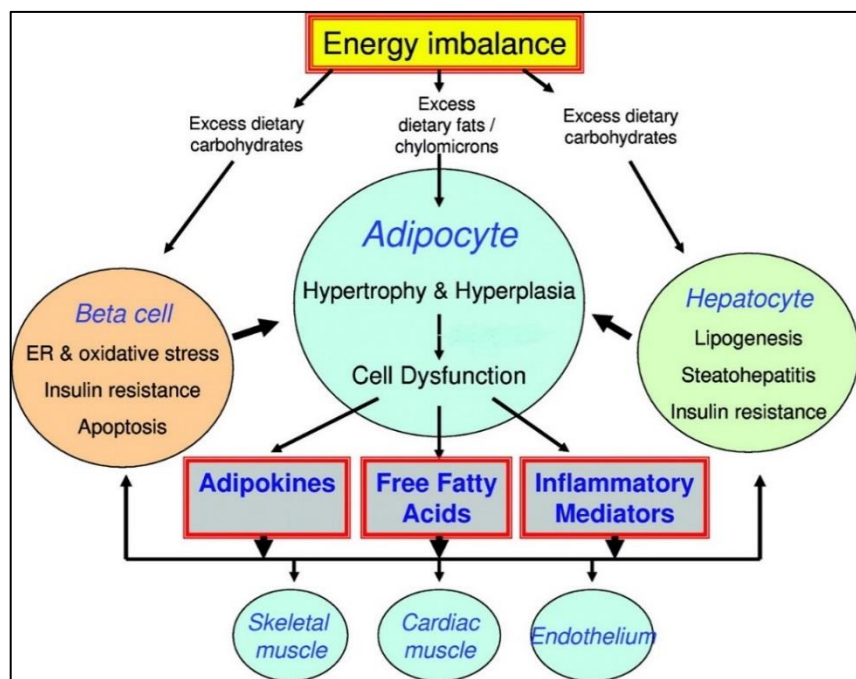


The etiology of obesity however is multifactorial, with the main cause considered to be energy imbalance, leading to storage of excess energy in adipocytes. This then can lead to both hypertrophy and hyperplasia leading to cell dysfunction associated with intracellular deregulation of (i) free fatty acid (FFA) and adipokines production; (ii) inflammation in the muscle and endothelium resulting insulin resistance, and (iii) endoplasmic reticulum and oxidative stress, leading to apoptosis in pancreatic  $\beta$ -cells. Exposure of hepatocytes and pancreatic  $\beta$ -cells to excess carbohydrates also leads to insulin resistance. Obesity and T2DM are associated with chronic inflammation in tissues involved in energy homeostasis in related tissue including fat, liver, muscle, and islets. Although inflammation can be induced by metabolic signals, how excess nutrition and obesity initiate and sustain inflammation in metabolically active tissues including the  $\beta$ -cells is not fully understood (Alejandro et al., 2014).

In contrast, certain types of dietary intervention can reduce body weight and reduce the risk of developing T2DM (Clark et al., 2016). Lifestyle intervention is an important way to prevent and slow the progression of chronic diseases. Diet is one of the main modifiable factors and reduction in caloric intake is effective for both prevention and reversal of obesity and T2DM and their associated health problems (McLellan, 2011). Caloric restriction has also been shown to be a very effective regime to prevent and delay metabolic and neurodegenerative disease and also improve learning and memory. Taken together excess caloric intake and less

physical activity, creates the perfect storm for the development of obesity (Figure 10; De Ferranti and Mozaffarian, 2008).

Modelling obesity in rodents has had a long history with the first diet-induced model of obesity being published in 1949 (Ingle, 1949). A number of other animal models have been developed and widely used.



Models focusing specifically on the effects of a high fat or a high sugar; have shown that high fat diets are more effective at inducing obesity than high sugar diets (Sleder et al., 1980). The normal laboratory rodent diet contains 5% fat; however, in numerous models of obesity the level of fat ranges between 30%-78% of total energy. In most studies this has been done by feeding rodents' calibrated high fat pellets; however some have used human high fat foodstuffs (see review by Hariri and Thibault, 2010).

Evidence shows several T2DM animal models are obese, reflecting the links between obesity related and the development of T2DM (Al-awar et al., 2016). The fact that obesity is a major risk factor for developing T2DM is supported by evidence from rodent models of obesity induced by diet (Von Diemen et al., 2006; Hariri and Thibault, 2010; Sim et al., 2014) and genetic models, such as ob/ob obese mice and Zucker rats (Al-awar et al., 2016).

### **3.5 Rodent Models of T2DM**

To date there are a number of different types of rodent models. The first of the main 3 categories are mice and rats with genetic modifications; in general, these models have been used to understand the underlying metabolic mechanisms associated with obesity and T2DM in peripheral organs. They are not the focus of my thesis however they are discussed in a number of important reviews (King and Bowe, 2016; King et al., 2016; Kitada et al., 2016). The other two categories are Diet-Induced Obesity (DIO) and the use of Streptozotocin (STZ), to kill  $\beta$ -pancreatic cells.

#### **3.5.1 Diet induced obesity (DIO)**

Diet induced obesity (DIO) is a straight forward model of obesity, there are a number of studies using high fat, high sugar diet induced animal models which show obesity and/or T2DM profiles (Setti et al., 2015). The majority of studies have used calibrated high calorific (HC)/high fat (HF) laboratory diet (Levin and Dunn-Meynell, 2002; Woods et al., 2003; Dourmashkin et al., 2006). Other studies have used high fat/sugar and human junk food (Cafeteria Diet: CD; Esteve et al., 1994; Sclafani and Gorman, 1977; Harrold et al., 2000; Levin and Dunn-Meynell, 2002). Models of diet induced obesity vary distinctly in different studies, mainly due to the duration of the feeding regime, the difference in fat/sugar content and different species (see Table 4).

The most successful diets that induce obesity are those in which the total energy intake contains 40% fat; whereas in control diet the fat content is 5% of total energy (Buettner et al., 2007; Hariri and Thibault, 2010). Rodents develop obesity and insulin resistance but rarely develop hyperglycemia that constitutes a partial T2DM phenotype (Storlien et al., 1991; Dourmashkin et al., 2006; Reuter, 2007). In contrast, high carbohydrate diet alone is less efficient (Sleder et al., 1980; Zavaroni et al., 1980; Hariri and Thibault, 2010), whilst with human junk food rodents gain weight more efficiently with short feeding duration (8-10 weeks; Harrold et al., 2000; Levin and Dunn-Meynell, 2002); show fat deposition and insulin resistance, but not hyperglycemia as seen with HF diet (Prats et al., 1989; Kretschmer et al., 2005).

One major limitation with these diet-induced models is usually the feeding regime has a long duration and there are no standard protocols. Calibrated HF/HC laboratory diet gives precise information concerning

the volume of fat, sugar, protein and calories, which is used to correlate with pathology or dysfunctional regulation of proteins. In contrast, CD lacks this precision but mimics human obesity more closely, as much as human foodstuffs contain preservative and chemical colorants etc., and is more palatable compared with calibrated laboratory diet (Rothwell and Stock, 1988; Shafat et al., 2009). In general, diet induced obesity can induce weight gain and insulin resistance, but not hyperglycemia.

### **3.5.2 STZ injected models**

Streptozotocin (STZ) is a broad-spectrum antibiotic that is used in cancer treatment in humans. However, it is widely used to induce diabetes mellitus in experimental animals (Rossini, 1977). STZ induces pancreatic  $\beta$ -cell death; by being taken into the cells via GluT 2, where it induces alkylation of DNA and DNA fragmentation leading to cellular toxicity and an immune response that cause hypoinsulinemia and hyperglycemia in animals (Szkudelski, 2001; Motyl and McCabe, 2009). STZ is most commonly used for inducing T1DM, but high doses are required (Motyl and McCabe, 2009; O'Brien et al., 2014; Al-awar et al., 2016; Kitada et al., 2016). In contrast, single or multiple low dose injections of STZ tend to induce T2DM, and importantly the dose of STZ will determine degree of glucose tolerance across time (Ito et al., 2001; Ventura-Sobrevilla et al., 2011). However, in general STZ induced diabetic rats develop hyperglycemia primarily by direct cytotoxic action on the  $\beta$ -cells and insulin deficiency rather than insulin resistance. Depending on dose and duration of the protocol, these effects can be reversed due to spontaneous regeneration of  $\beta$ -cells (Srinivasan and Ramarao, 2007; King et al., 2016). The toxic action is not specific to pancreatic beta cells and can cause damage to other tissues including the liver and kidney. In higher dose it may lead to mortality (Deeds et al., 2011).

### **3.5.3 STZ injection coupled with HF diet**

Diet influences the sensitivity of the effect of STZ for inducing diabetes in rodents, and several studies have developed models coupling low dose STZ that does not on its own induce T2DM with high fat diet. Reed et al. (2000) show rats with high fat diet coupled with a single low dose injection of STZ (50 mg/kg) show insulin resistance and hyperglycemia, but not insulin deficiencies and is confirmed by other studies (Srinivasan et al., 2005; Sugano et al., 2006). Other models using multiple injections coupled with high fat diet induces a mild impairment of insulin secretion and stable hyperglycemia, and inflammation-mediated destruction of the  $\beta$ -cells (Zhang et al., 2008) compared with rapid cell death induced by a single high dose of STZ (Goyal et al., 2016). Up to 2014, more than 30 studies using high-fat/carbohydrates diet coupled with low dose STZ were reported to successfully induce early or late stage T2DM in rats (Skovsø, 2014), where an increase in insulin response is initially triggered by high energy diet feeding and subsequently decreased by STZ injection and is accompanied by hyperglycemia. In addition to inducing long lasting hyperglycemia, polyuria, polydipsia, and polyphagia, dyslipidemia and diabetic complications such as hypertension are also observed (Srinivasan and Ramarao, 2007; Skovsø, 2014). This model more closely mimics natural history and metabolic characteristics of the human syndrome due to obesity and insulin resistance and is considered a better and stable model of T2DM compared to others (See Table 4). Although some genetic models recapture some of the phenotypes, they don't mimic the etiology of the

majority of patients who develop T2DM largely due to diet or lifestyle factors, and not always genetic modifications (Heydemann, 2016).

**Table 4. Comparison of DIO and STZ induced models**

<b>Comparison of DIO and STZ induced models</b>							
Model	HF	HF	CD	STZ	STZ	STZ+ HF	STZ+HF
Animal	Rat	Mice	Rat	Rat	Mice	Rat	Mice
Treatment (range)	36-85 % fat	40-72 % fat	13-52.5 % fat	25-45 mg/kg	35-80 mg/kg	30-58 mg/kg + 22-60 % fat	30-90 mg/kg+ 36-54 %fat
Ct (range)	6.5-10 % fat	6.1-13 % fat	9.2-9.6 % fat	Vehicle/ no	Vehicle/ no	Normal diet+ STZ/no	Normal diet+ STZ/no
Duration (w)	6-65	8-19	8-10	Single or multiple injection(s)	Single or multiple injection(s)	2-4 w HF + STZ injection(s)+ HF	3-6 w HF + STZ injection(s)+ HF
Weight gain	+	+	+	NC/-	NC/-	+	+
Insulin resistance	Yes	Yes	Yes	No	No	Yes	Yes
Hyperglycemia	Lack/NC/ NR	Lack/NC/ NR	Lack/NC/ NR	Yes	Yes	Yes	Yes
Advantage	Obesity induced, good standardization	Obesity induced, quicker than rats; good standardization	More efficiency to induce DIO in rat, mimic human food	Quick frank hyperglycemia; rats more sensitive	Quick frank hyperglycemia	Stimulate natural disease progression, better model of T2DM	Stimulate natural disease progression, better model of T2DM
Limitation	Long duration; chow not palatable ; variability	Relatively long duration; Chow not palatable	Less standardization, cost and time consuming for feeding	Less stable; reversible; T1 or T2?	Less stable; reversible; T1 or T2?	Dose dependent	Dose dependent; Less studies
References	Reuter, 2007; Harri and Thibault, 2010	Reuter, 2007; Harri and Thibault, 2010	Reuter, 2007; Harri and Thibault, 2010	Goyal et al., 2016; Radenković et al., 2016 ; King et al., 2016	Deeds et al., 2011; Goyal et al., 2016; Radenković et al., 2016	Skovso, 2014; King et al., 2016; Goyal et al., 2016	King et al., 2016; Zhang et al., 2016

Description: 1). Model: DIO, diet induced obesity; HF, High fat diet; CD, Cafeteria diet; STZ, Streptozotocin; STZ+HF, STZ injection(s) coupled with HF. T1or T2: Type1 or 2 diabetes. Treatment: range of fat contained in diet or STZ dose used in different studies. % fat: percentage fat of total energy in HF/CD food; mg/kg: dose of STZ injection based on body weight (i.p.); Ct: controls related to treatment group, for DIO: controls given normal laboratory diet chow, STZ injected experiments, controls received vehicle or no vehicle injection. Duration, range of treatment time by weeks (w). 3). T2DM phenotypes: changes compared to Ct: Body weight gain: + increase, - decrease, NC, No change. Hyperglycemia: lack, very few studies shown hyperglycemia; most of the cases NC, No change or NR, Not reported. Table based on summaries from several different reviews focused on different aspects and recent papers.

### **3.6 Cognitive deficits in T2DM**

Diabetes is associated with significantly faster decline in cognitive functions in older people, showing a high risk of mild and moderate cognitive impairment. This association may contribute to dementia as people with diabetes are at 1.5 times higher risk of developing dementia than those without it (Lu et al., 2009). However, the mechanism underlying this phenomenon remain unclear.

#### **3.6.1 Cognitive changes in T2DM patients**

There is not a lot of data concerning cognitive impairments induced by T2DM and the forms of cognitive dysfunction vary from subtle executive dysfunction to moderate memory deficits (Kirkman et al., 2012). A recent study also showed Mild cognitive impairment (MCI) and depression like behaviour in older people with T2DM, supporting T2DM as a risk factor for AD (Gorska-Ciebiada et al., 2014). Characteristic cognitive dysfunction in persons with T2DM mainly focuses psychomotor efficiency, attention, learning and memory, cognitive flexibility and speed, and executive function, while across studies the most common finding is impairment in processing speeding, attention and memory (Barbagallo and Dominguez, 2014).

A 3.25-year follow up study in the U.S. and Canada has shown poor performance in T2DM patients using Digit Symbol Substitution Test (DSST) which assesses a wide variety of executive functions including visual motor speed, learning capacity, sustained attention and working memory. This study also suggested an association between hyperglycemia and cognitive dysfunction (Punthakee et al., 2012). Similarly in a 5 and a half year longitudinal study, people with diabetes showed lower baseline scores on tests of episodic memory, semantic memory, working memory, visuospatial ability, but also a rapid rate of decline in perceptual speed (Arvanitakis et al., 2004)

Imaging studies have shown that brain anatomy is altered in people with T2DM and may be associated with cognitive dysfunction at different stages. For example, global moderate brain atrophy and volume loss that is similar to or up to three times the rate of atrophy rate in normal ageing (Ryan, 2014). Moreover, brain tissue loss is accelerated by expansion of the ventricles with regions round them, such as the subcortical gray or white matter and the hippocampus becoming comparatively smaller (Biessels and Reijmer, 2014). Although there is little evidence indicating the direct contribution of atrophy to memory deficits, some show reduced gray matter volume correlates with memory and executive function, while others show no direct relation between brain atrophy and cognition (Espeland et al., 2013). MRI studies show that white matter hyperintensity (WMH) and infarcts are associated with the slowing of processing speed and decrements in attention and executive functioning in T2DM patients, and progression of WHM has been linked to accelerated cognitive decline (Biessels and Reijmer, 2014). It has been suggested that white matter abnormalities occur in the early stage of the disease where disruption of white matter connections in the temporal lobe is associated with memory (Reijmer et al., 2013). Although the dysfunction mediating these deficits have been linked with inflammation and BBB disruption the underlying mechanisms have not been clarified (Biessels and Reijmer, 2014). Importantly, the severity of insulin resistance and longer duration of T2DM have been correlated with decrease in brain volume, confirming the long-term

harmful effects T2DM may have on the brain (Saczynski et al., 2009; Tan et al., 2011). Finally, fMRI studies show resting-state brain activity abnormalities in T2DM patients, showing decreased low frequency fluctuations in the post central gyrus and occipital cortex in the absence of structural brain changes, and this is related to poorer memory performance and executive functioning (Cui et al., 2013; Reijmer et al., 2013). These resting state networks strongly overlap with task related networks and are compromised in various cognitive disorders, such as mild cognitive impairment (MCI) and AD (Chhatwal and Sperling, 2013). Therefore, in summary, T2DM is associated with alterations in both in structural and functional brain connectivity that evolve with age; and are likely to start in prediabetic stages, contributing to the slow decline in cognitive function (Reijmer et al., 2013).

### ***3.6.2 Deficits in Memory and Synaptic Plasticity in models of obesity and T2DM***

There are limited studies on cognition in diet and/STZ induced rodent models of T2DM; the majority of studies have been conducted in rodents with genetic mutations. However, studies using models of obesity (Petrov et al., 2015; Cordner and Tamashiro, 2015) and T1-and T2DM (reviewed in Reagan, 2012) report memory deficits in hippocampus dependent spatial memory tested in water maze, radial arm maze and object recognition task, with more consistent deficits in diet-induced models (Mielke et al., 2006; McNay et al., 2010). In STZ induced diabetic rats (Sasaki-Hamada et al., 2012; Kalalian-Moghaddam et al., 2013; Thomas et al., 2013) and HF diet induced obesity (Woo et al., 2013; Liu et al., 2015), a deficit is observed in CA1 of the hippocampus and is accompanied by downregulation of insulin signalling (Grillo et al., 2011; Liu et al., 2015).

Taken together, studies in humans and animal models suggest T2DM can induce cognitive deficits. However, how T2DM pathologies in peripheral tissue cause an abnormal neuronal environment that leads to changes in synaptic plasticity and cognitive function is not well understood.

Significant advances have been made in recent years in our understanding of the pathogenesis of T2DM. This has significantly improved our knowledge of one of the most serious health threats in the world, allowing us to identify genes and pathways involved in the development and progression of the disease. Diabetes is associated with increased risk of multiple co-existing medical conditions in older adults, ranging from cardiovascular disease to dementia and cancer. T2DM is frequently associated with ageing and age related disease, such as Alzheimer's disease. As our understanding of the neural effects of T2DM advances; it provides inroads into understanding how it may promote AD.

## **4 Links between Type 2 Diabetes Mellitus and Alzheimer's Disease**

As described above AD is characterized by progressive impairment in memory and cognitive functions, while insulin resistance and hyperglycemia are the hallmarks of T2DM. Epidemiological and animal studies show a strong association between T2DM and AD. Persons with T2DM have a higher risk of cognitive decline and of developing all types of dementia including AD in later life compared with age-matched non-diabetic individuals (Barbagallo and Dominguez, 2014). Evidence suggests patients with AD share some classic pathologies associated with T2DM and, conversely those with T2DM share some pathologies associated with AD; many of which are linked with impaired insulin and PI3K signalling.

### **4.1 T2DM and AD in Humans**

Epidemiological studies suggest that the incidence of AD is 2-5 times higher in people with T2DM (Luchsinger et al., 2001; Desai et al., 2014). In a 5.5 year follow up study, people with diabetes were shown to have a 65% higher risk of developing AD compared with those without diabetes (Arvanitakis et al., 2004), and diabetic patients show similar cognitive deficits to those patients with early stage AD (Gorska-ciebiada et al., 2014). Imaging studies show patients with both T2DM and AD have increased cortical atrophy compared with the AD with non-T2DM group (Biessels et al., 2006). As with AD, T2DM patients show more hippocampus and amygdala atrophy compared to controls (Den Heijer et al., 2003).

In addition, tests conducted in the Mayo Clinic show 81% of patients of AD had either impaired fasting glucose (IFG, 110-125 mg/dL) or T2DM (Janson et al., 2004). Hyperglycemia is associated with reduced cognitive performance and reduction in the gray matter volume in brain in young and middle aged adults (Verdile, 2015). Post-mortem analysis of brains from people with T2DM show increased levels of neurofibrillary tangles and amyloid plaques (Peila et al., 2002; Jellinger, 2008; Mittal and Katare, 2016), and importantly once the cerebral amyloid is present, its accumulation correlates with the duration of T2DM (Janson et al., 2004). Moreover, T2DM patients have accumulation of amyloid plaques and hyperphosphorylated Tau in the pancreas (Miklossy et al., 2010) and increased levels of serum A $\beta$  (Kim et al., 2010). Conversely, AD patients have more frequent and extensive amyloid in the pancreas compared with non-AD individuals (Janson et al., 2004).

### **4.2 Animal studies**

Several animal studies also indicate the close pathophysiological link between T2DM and AD. For example diabetic phenotypes exacerbate memory impairment, cerebral amyloid accumulation, tau pathology and inflammation as seen in AD transgenic mice fed high fat diet (Ho et al., 2004; Julien et al., 2010; Knight et al., 2014; Walker et al., 2017). Conversely reducing fat intake in these models can reverse some of the AD type pathology (Walker et al., 2017). Further evidence shows in a model in which rats fed high fat diet were given STZ injections and subsequently injected with A $\beta$ 42 into hippocampus showed greater spatial memory deficits compared with rats injected with A $\beta$  alone (Ma et al., 2013).

More recently, studies where sub-diabetogenic doses of STZ were injected into the brain (icv) reveal induction of a form of insulin resistance and mice and rats shows many aspects of sporadic AD (Lannert and Hoyer, 1998; Chen et al., 2013; Salkovic-Petrisic et al., 2013; Grieb, 2016). STZ induces insulin resistance, decreased glucose and energy metabolism and cognitive deficits without changing peripheral insulin and glucose levels (Lannert and Hoyer, 1998; Kamat et al., 2016). In addition, STZ treated rats show vascular A $\beta$  deposits in the brain 3 months after injections and cerebral amyloid angiopathy between 6 and 9 months later; characteristic A $\beta$  that is frequently observed in sporadic AD patients (Salkovic-Petrisic et al., 2011); however, there is no evidence of APs or NFTs in the brain (Salkovic-Petrisic et al., 2011). Other effects induced by STZ are neuro-inflammation, altered synaptic proteins and insulin/IGF signalling and hyperphosphorylation of Tau (Lester-Coll et al., 2006; Chen et al., 2013) and changes in expression of a number of genes are consistent with those observed in AD brains. Importantly, most genes that are down regulated are those associated with insulin signalling and glucose metabolism (Chen et al., 2012), further suggesting impaired insulin signalling in T2DM might be a common trigger contribute to the development of AD.

This model mimics a number of pathological events observed in AD and induces insulin resistance in the brain. This has led some in the scientific community to describe AD as a form of diabetes; T3DM (De la Monte and Wands, 2008). However, referring to AD as T3DM might be considered somewhat of an over simplification; as insulin resistance largely refers to the relative inability of insulin receptors to respond to insulin in the brain. T2DM is characterized principally by hyperglycemia and hyperinsulinemia/insulin resistance; in AD patients, the brain is essentially hypoglycemic (Meneilly and Tessier, 2016) and there are no indications that there is an increase in levels of insulin even at early stages of the disease (Stanley et al., 2016). These differences do not negate the potential for T2DM to induce a dysfunctional neuronal environment that would promote or be implicated in the pathology associated with AD. What becomes important now is to understand how dysregulation of mechanisms associated with T2DM can promote AD.

#### **4.3 Mechanisms associated with common pathologies**

T2DM and AD increase with age and they share amyloid pathological features in the islets of the pancreas and the brain (Kahn et al., 1990), since amyloid in the pancreas is more frequent and extensive in patients with AD (Janson et al., 2004). They share other pathologies such as inflammation, apoptosis, autophagy, oxidative stress, advanced glycation end products (AGEs), impaired insulin signalling and glucose metabolism, many of which are mediated by insulin mediated PI3K-Akt signalling pathway; albeit in T2DM these are regulated in the periphery (see reviews by Cole et al., 2007; Kim et al., 2010; Verdile, 2015; Mittal and Katare, 2016).

##### **4.3.1 Insulin Regulation**

Insulin is hormone belonging to a super family of insulin related proteins that include the insulin-like growth factors (IGFs). Almost all types of cell respond to insulin, in particular, liver, muscle, adipose tissue



and brain are most sensitive (Duarte et al., 2012). Although still a moot point as to whether the brain can synthesis its own insulin; it is taken up through the BBB and, once in the brain, insulin binds to the insulin receptors that are highly enriched in olfactory bulb, cortex, hippocampus, hypothalamus and amygdala in rat brains (Havrankova et al., 1978). It has key roles in the CNS such as hypothalamus and other brain regions regulating glucose/energy metabolism, neuronal survival and longevity, synaptic transmission, learning and memory and is dysfunctional in neurodegenerative disease, particularly AD (see reviews by Park, 2001; van der Heide et al., 2006; Duarte et al., 2012). Similarly, IGF-1 and IGF-2 are distributed throughout the brain and play similar roles to insulin in the CNS functions, notably synaptic transmission and effects on learning and memory and cognition (Nelson and Alkon, 2005; Benarroch, 2012; O'Neill et al., 2012).

Insulin Degrading Enzyme (IDE) is the major mechanism by which misfolded or abnormal insulin is cleared. It is a highly conserved zinc metallopeptidase that is ubiquitously express in all tissues, and is particularly abundant in brain liver and muscle; tissues susceptible to insulin regulation. In general, it is localised in the cytosol, but is present to a lesser extent in mitochondria (Leissring et al., 2004). In addition to degrading misfolded insulin, IDE also degrades monomeric A $\beta$  to prevent formation of oligomers and aggregates, but also degrades the AICD fragment of APP preventing this fragment from translocating to the nucleus to transcribe genes associated with the disease (Llovera et al., 2008). Mice with mutated forms of IDE show increased A $\beta$  accumulation and AD phenotypes (Farris et al., 2003; Miller et al., 2003).

AD patients have decreased insulin clearance and elevated insulin stimulated A $\beta$  in plasma; and this abnormal regulation in the periphery may also contribute to a higher risk of developing AD (Kulstad et al., 2006). They also show a decrease in levels of leptin, an increase in adiponectin and insulin in the blood that positively correlate with the severity of dementia (Khemka et al., 2014). Peripheral hyperinsulinemia and insulin resistance in T2DM down regulate brain insulin uptake at the BBB, resulting in long-term reduction in brain insulin levels (Craft, 2005), and AD patients have reduced CSF to plasma insulin ratio compared with healthy controls (Craft et al., 1998). Intranasal administration of insulin has been shown to improve memory and cognition in people with MCI and early AD (Reger et al., 2006; 2008). In the AD brain the A $\beta$  degrading capacity of IDE is about 50% of the normal brain. Decreased IDE activity is found in the brains of both genetic and sporadic AD (Cook et al., 2003; Kim et al., 2007); however, in neurons adjacent to senile plaques, IDE is upregulated (Bernstein et al., 1999), suggesting they are actively trying to reduce A $\beta$ .

Experimental studies in animals show abnormal brain insulin/IGF-1 is associated with learning and memory impairment and age related neurodegenerative disease such as AD (O'Neill et al., 2012). Moreover, insulin promotes the release of intracellular A $\beta$  to the extracellular space (Gasparini et al., 2001) and regulates expression of IDE (Zhao et al., 2004). Conversely overexpression of IDE in mouse brains reduces A $\beta$  accumulation and slows the progression of AD (Leissring et al., 2003). IDE may also prevent amyloid fibril formation by serving as a 'dead-end chaperone', forming stable complexes with A $\beta$  to prevent

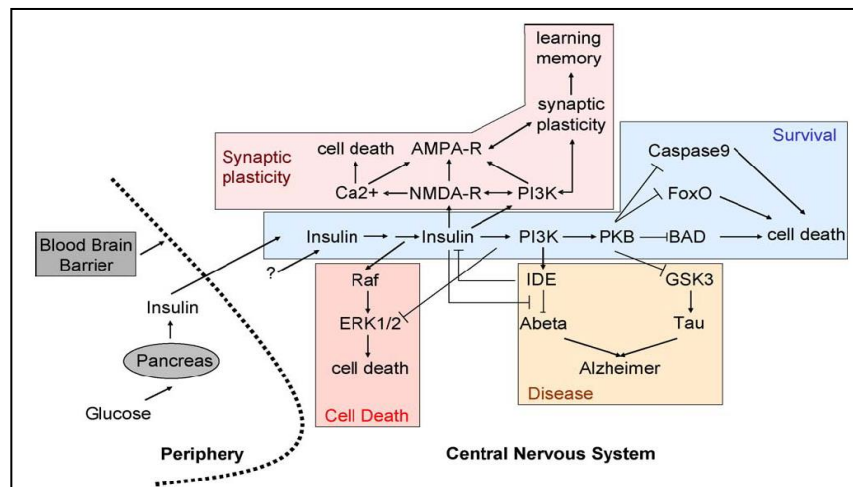
progression to fibrils (Llovera et al., 2008). Importantly, peripherally injected insulin directly influences the brain and causes rapid cerebral insulin receptor signal transduction and tau phosphorylation *in vivo*, suggesting the important link of T2DM and neurodegeneration through insulin signalling (Freude et al., 2005); however the exactly how T2DM leads to these dysfunctional mechanisms in the brain and whether they have a causal role, currently is not well understood.

Impaired insulin signalling in T2DM stimulates abnormal in several functions including glucose metabolism (Virally et al., 2007), autophagy (Glick et al., 2010), inflammation (Garcia et al., 2010), apoptosis (Liu et al., 2008a) and also deposition of amyloid and tau phosphorylation in peripheral tissues (Erol, 2008). Such common pathologies are also seen in the AD but the mechanisms of their interactions are not clearly understood.

#### 4.3.2 Insulin-PI3K/Akt signalling pathway

In the periphery, insulin-stimulated glucose transport is dependent on the activation of PI3K-Akt signalling pathway as described in Chapter 3. In a similar manner, insulin in the brain stimulates an increase in insulin receptor tyrosine phosphorylation and PI3K signalling. Under normal conditions Akt can be activated via a number of different sources in addition to insulin receptors such as growth factors (IGF, VEGF), neurotrophins (BDNF) (Singh et al., 2015). In turn it activates a number downstream targets associated with different functions, such as energy and glucose metabolism, apoptosis, autophagy and protein synthesis, inflammation and transcription (Yao et al., 2014; Singh et al., 2015). Thus, Akt acts as a major hub integrating signals from various sources mediated by different environmental inputs and redirecting appropriate responses via its downstream targets. Proteins regulated by Akt are also subject to regulation by other mechanisms, maintaining a finely tuned balance necessary for the well-being of cells and neurons. Therefore, the state of the cell, in terms of basal protein expression and phosphorylation could tip the balance in one direction or another when Akt is activated.

Abnormal insulin signalling pathway causes neuronal death and dysfunction in synaptic plasticity and cognition, which may possibly link T2DM with AD, as both of these diseases have shown abnormal regulation of PI3K-Akt signalling and their target proteins (Figure 11; van der Heide et al., 2006).



**Figure 11. Insulin signalling in the central nervous system (CNS).** Increases in peripheral glucose levels result in secretion of insulin by the pancreas. Insulin from the peripheral circulation crosses the blood-brain barrier to enter the CNS. In the CNS insulin activates the PI3K-PKB (Akt) pathway, which affects synaptic plasticity, survival, disease, and cell death (van der Heide et al., 2006).

Studies report a decrease in the levels and activities of several components of insulin-PI3K-Akt signalling in AD and T2DM patients (Schubert et al., 2004; Steen et al., 2005), and importantly this decrease is more severe in patients with both T2DM and AD cases (Liu et al., 2011). The levels and activation of the insulin-Akt signalling pathway negatively correlated with Tau phosphorylation, suggesting the important impact of the insulin-Akt signalling to neurodegeneration in AD and the possible molecular link between T2DM and AD (Liu et al., 2011).

#### **4.3.3 Autophagy**

Autophagy; a function necessary for eliminating misfolded proteins (Xie and Klionsky, 2007), and also recycling proteins under conditions of low nutrient availability (Backer, 2008), is dysfunctional in both T2DM and AD (Laplante and Sabatini, 2012; O'Neill et al., 2012). Under normal conditions, autophagic recycling of proteins requires a concerted balance between activity of mTOR and Beclin-1. Caloric restriction reduces mTOR activity and experimental reduction of mTOR by rapamycin (Yang et al., 2014), coupled with activation of Beclin-1 to promote longevity across a number of different species (Bergamini et al., 2007; Minina et al., 2013; Ntsapi and Loos, 2016).

Evidence suggests that a number of transcription factors that include FoxO3, NFκB, cJUN and E2F1 can drive Beclin-1 expression (Wirawan et al., 2012). In addition, anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-xL can interact with Beclin-1 via protein-protein interaction to inhibit autophagy, suggesting a regulated link between autophagy and apoptosis (Debnath et al., 2005; Maiuri et al., 2007).

Evidence suggests that the autophagic machinery is dysfunctional in obesity (Khamzina et al., 2005; Yang et al., 2010), T2DM (Ma et al., 2013) and AD (Ma et al., 2010; Laplante and Sabatini, 2012), either by causing an increase in mTOR activity and/or a decrease in Beclin-1 (Khamzina et al., 2005; Pickford et al., 2008) and may be instrumental in allowing Aβ peptides to aggregate (Pickford et al., 2008). Importantly, hippocampal mTOR levels and hyperphosphorylated Tau levels are increased in T2DM-AD rats compared with T2DM and AD alone, and it has been suggested that T2DM may activate mTOR excessively by impairing insulin signalling in the brain, thus increasing Tau phosphorylation and promoting AD development (Ma et al., 2013). In contrast, inhibition of mTOR signalling correlates with impairment in hippocampal synaptic plasticity in Tg2576 AD mice and hippocampal slices from wildtype exposed to exogenous Aβ42; while upregulation of mTOR signalling rescues LTP in AD mice and Aβ-induced LTP impairment (Ma et al., 2010). Given that mTOR is involved in mechanisms subserving both autophagy and protein synthesis; these opposing results may reflect the physiological status of the brain under slightly different conditions.

#### **4.3.4 Glucose metabolism**

In the cerebral cortex glucose consumption is used in neurons mainly to restore ion balance after some form of activity. For example, in theory, 55% is used following postsynaptic glutamate receptor activation; 21% on action potentials; 20% on resting potentials and 4% on transmitter recycling (Howarth et al., 2012). It consumes approximately 20% of the body's glucose that is largely taken up into the brain

from the periphery via the BBB, although some studies have suggested that gluconeogenesis could occur in the brain (Yip et al., 2016). Most of the glucose is processed by astrocytes either at the BBB or in the interstitial space to produce energy requirements for neurons (Pellerin, 2008) by producing lactate, which is then shuttled to neurons (Dringen et al., 1993).

Impaired cerebral glucose metabolism in the brain has been associated with a number of brain insults including stroke, head trauma and neurodegenerative diseases (Cunnane et al., 2011; Jalloh et al., 2015) and consequently it impacts on brain plasticity and cognitive processing. In AD glucose toxicity is associated with oxidative stress, mitochondrial dysfunction, autophagy, GSK3 activation and inflammation, which may partly be due to impaired insulin signaling in the brain (Chen and Zhong, 2013).

#### *Brain glucose and glucose transporter-1*

Glucose is transported into the brain by GluT 1, a member of a family of 14 different transporters. It is highly expressed in brain endothelial cells forming the blood brain barrier (BBB) as opposed to peripheral endothelia (Harik et al., 1990) and astrocytes (Virgintino et al., 1997). Imaging studies (FGF-PET) in AD patients have shown progressive reduction in glucose metabolism that correlates with the severity of the disease (Mosconi et al., 2006). Glucose hypometabolism in AD first arises in the hippocampus and entorhinal cortex; then progresses to parietal, temporal and posterior cingulate cortex. Impaired glucose metabolism results in early abnormalities in cognitive abilities (Mittal and Katare, 2016). The decrease in hippocampal glucose metabolism has also been shown to arise in MCI (Mosconi et al., 2006); GluT1 is decreased in the cortex of AD brains (Simpson et al., 1994; Mooradian et al., 1997; Liu et al., 2008).

#### *GSK3 $\beta$ and $\beta$ -Catenin*

Glycogen synthase kinase 3 $\beta$  (GSK3  $\beta$ ) is an unconventional kinase compared with others, as it is constitutively active and expression levels are relatively high; however, when cells are exposed to growth factors or insulin, GSK3 $\beta$  activity can be reduced to between 30 and 70% quite rapidly (Sutherland, 2011). This is achieved by phosphorylation of GSK3 $\beta$  on serine 9, principally by Akt (Cross et al., 1995), but possibly also by p90RSK (Eldar-Finkelman et al., 1995) and/or PKA (Fang et al., 2000). Constitutively active GSK3 $\beta$  mediates two downstream mechanisms: (i) it phosphorylates and inhibits glycogen synthase to prevent uptake and storage of glucose in cells (Cohen and Frame, 2001); (ii) it catalyses the phosphorylation and inhibition of eukaryotic protein synthesis initiating factor 2B (eIF2B) thereby inhibiting protein synthesis (Cohen and Frame, 2001). Therefore, inhibition of GSK3 $\beta$  by Akt would result in an increase in glucose uptake and storage and protein synthesis.

GSK3 also principally interacts with the Wnt signalling pathway in a rather unorthodox manner. A key downstream target of Wnt is  $\beta$ -Catenin, and in the absence of a Wnt signal, constitutive GSK3 forms part of a multiprotein 'destruction complex' with the scaffolding protein Axin that leads to hyperphosphorylation of  $\beta$ -Catenin and directs it towards degradation by proteosomal mechanisms (Cohen and Frame, 2001; Medina and Wandosell, 2011). By a series of phosphorylation steps triggered by Wnt,  $\beta$ -

Catenin is dissociated from the 'destruction complex', accumulates in the cytosol; and then can mediate two distinct functions: (i) it can complex with other proteins to form the adherent-junctions to create and/or maintain adhesion between cells, mainly epithelial layer cells (Brembeck et al., 2006) and/or (ii) translocate to the nucleus to activate a number of target genes, including cyclin D, involved in cell cycle progression; the multifunctional protein, c-myc involved in cell proliferation and differential and apoptosis; and Axin 2 that is part of the 'destruction complex' with GSK3. Therefore, inhibition of GSK3 by Akt would result in increased activity of the protein function signalled by  $\beta$ -Catenin (Zhao et al., 2005; Sutherland, 2011).

GSK3 however is a promiscuous protein and more recently has been shown to be involved in other putative functions such as microtubule stability, apoptosis, receptor trafficking and inflammation (Sutherland, 2011). This however largely depends on the proteins it targets or interacts with (Doble and Woodgett, 2003; Sutherland, 2011). Not surprisingly, given the number of functions it is associated with, dysfunctional regulation of GSK3 is observed in a number of psychiatric disorders, such as depression (Chen et al., 2015), metabolic diseases, such as diabetes (Eldar-Finkelman and Kaidanovich, 2002; Lappas, 2014) and neurological disease such as AD (Forlenza et al., 2011) to the point where therapeutic targets have been designed to reduce expression levels (De Sarno et al., 2002; Gould and Manji, 2002).

In AD, GSK3 $\beta$  has been shown to phosphorylate Tau at several pathological sites to promote the formation of paired helical filaments (Zheng-Fischhöfer et al., 1998; Phiel et al., 2003; Cole et al., 2007). However, it is not clear whether under normal conditions, GSK3 may interact with Tau to evoke a physiological response (Doble and Woodgett, 2003). In AD brains GSK3 $\beta$  expression is present in the cytoplasm of pre-tangle neurons and its over expression coincides with the development of neurofibrillary changes (Pei et al., 1999). In addition, it has been shown to facilitate A $\beta$  release by increasing the cellular maturation of APP, a process associated with early stage AD (Citron et al., 1994; Aplin et al., 1997). Loss of Wnt signalling is also associated with AD, via neurotoxicity of A $\beta$  deposition induced by increased levels of GSK3 $\beta$  (Chong et al., 2005). GSK3 suppresses glycogen synthase and insulin receptor substrate-1, two key targets of insulin action. GSK3 $\beta$  activity is increased and consequently reduced phosphorylation in peripheral tissues such as the liver in obese mice (Cai et al., 2005), T2DM models (Eldar-Finkelman and Kaidanovich, 2002) and in brains of T2DM patients (Liu et al., 2011). GSK3 $\beta$  activity is increased in blood of patients with MCI that have memory impairment and AD (Forlenza et al., 2011); and in white cells in patients with early AD (Hye et al., 2005) and the hippocampus of AD patients (Steen et al., 2005). In APP/PS1 transgenic mice GSK3 $\beta$  activity is decreased at 6 months of age but increased at 18 months (Jimenez et al., 2011), suggesting an age related biphasic regulation of GSK3 $\beta$ .

#### **4.3.5 Inflammation**

Neuroinflammation is one of the important pathologies associated with AD (Salminen et al., 2008; Hommet et al., 2014), and is detected by inflammatory markers in brain and CSF of AD patients post-mortem (Lue et al., 2001; Rösler et al., 2001) and brains of transgenic mouse models of AD (Guillot et al., 2016; Yin et al., 2016). Population based studies in the Netherlands show high levels of inflammatory

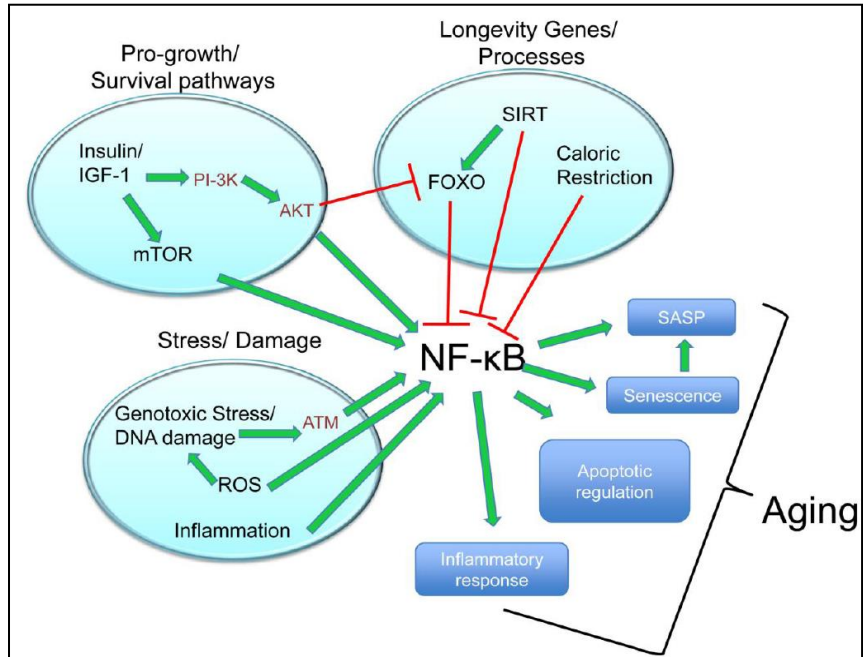
proteins in plasma are associated with increased risk of dementia and AD (Engelhart et al., 2004). Similar correlations between early inflammation and AD and or dementia was revealed by analysis of blood samples in a 25-year follow-up study of Japanese American men (Reinhold et al., 2002). Importantly this is independent of cardiovascular risk factor and disease, indicating the inflammation in the periphery contributes to the brain disease; possibly via disruption of the BBB, and these processes are detectable long before clinical symptoms occur (Reinhold et al., 2002). As with AD, T2DM, obesity and insulin resistance, are associated with inflammation, and with increased levels of inflammatory mediators such as interleukin-6 (IL-6), C-reactive protein and IKK/NF- $\kappa$ B (Hak et al., 2001; van de Ree et al., 2003; Zhang et al., 2008; Cai, 2013).

Nuclear factor- $\kappa$ B (NF $\kappa$ B) is a transcription factor regulating genes classically involved in the immune response, inflammation and oncogenesis in all tissues (Tornatore et al., 2012). It is poised in the cytoplasm ready to respond rapidly to signals from a number of sources, including metabolic stress, inflammatory cytokines and insulin signalling (Patel and Santani, 2009; Ye and Keller, 2010; Tornatore et al., 2012). There are 5 members of the NF $\kappa$ B family of transcription factors, RelA-p65, RelB and cRel and the precursor proteins p105 and p100 (Kistler et al., 1998), which can dimerise with each other. Under resting conditions NF $\kappa$ B is tethered in the cytoplasm by tight binding to inhibitory I $\kappa$ B kinases (Zandi et al., 1997; Hinz and Scheidereit, 2014). Stimuli leading to activation of NF $\kappa$ B are mediated via the Inhibitor of nuclear factor- $\kappa$ B (I $\kappa$ B) kinase (IKK) complex comprising the catalytically active kinases, IKK $\alpha$  and IKK $\beta$  and the regulatory I $\kappa$ B $\gamma$  (Zandi et al., 1997; Sakurai et al., 1997). They phosphorylate I $\kappa$ B's for ubiquitination and proteasomal degradation, thereby releasing NF $\kappa$ B dimers to translocate to the nucleus. This tripartite IKK structure is the most abundant form, however, *in vitro* studies have shown that I $\kappa$ B $\gamma$  (NEMO) can interact with IKK $\alpha$  and IKK $\beta$  homodimers (Hinz and Scheidereit, 2014) to induce a more diverse range of effects. The IKK complex is classically regulated by signals from cytokine receptors such as TNF and IL-1, antigen receptors and Toll-like receptors to activate NF $\kappa$ B. However, IKK $\alpha$  homodimers are crucial regulators of non-canonical NF $\kappa$ B signalling necessary for  $\beta$ -cell maturation and are involved in the maturation of dendritic cells and pancreatic homeostasis (Li et al., 2013; Mancino et al., 2013).

In addition to releasing NF $\kappa$ B to translocate to the nucleus, both IKK $\alpha$  and IKK $\beta$  have other substrates that are either common or specific to each kinase. For example, both kinases can interact with other proteins in, or associated with, Akt signalling such as  $\beta$ -Catenin, Akt-mTOR regulation of autophagy, and regulation of different proteins that ultimately increase or decrease transcriptional regulation of NF $\kappa$ B (Hinz and Scheidereit, 2014). In particular, IKK $\alpha$  participates in Akt-dependent regulation of mTORC1 (Dan et al., 2014). In addition, it enhances mTORC2 kinase feedback regulation of Akt and Akt-mediated regulation of FoxO3 and Gsk3 $\beta$  (Dan et al., 2014). Thus, the potentially rapid response that NF $\kappa$ B can make is tightly controlled by a number of positive and negative regulatory elements at multiple levels.

It is possible that different complex compositions may be associated with different stimulus inputs, different tissues and functions; however very little is known about this.

Although NFκB is best known for its function in immunity, inflammation, cell senescence, apoptosis and metabolism associated with ageing, more recently it has been shown to be involved in processes regulating life span, through its interaction with proteins associated with apoptosis, such as FoxO3 and autophagy, such as mTOR (Figure 12; Tilstra et al., 2011). Not surprisingly, aberrant regulation of IKK/NFκB has been associated with



**Figure 12. NFκB as a central factor in pro-ageing and longevity pathways.** Pro-growth survival pathways known to promote ageing phenotypes, specifically Insulin/IGF-1 and mTOR are known to stimulate NFκB as described. Insulin/IGF-1 acts via two mechanisms, AKT and mTOR signalling, to activate NFκB. However, through AKT, Insulin/IGF-1 signalling also interacts with known longevity processes by inhibiting FOXO. As with the other known longevity factors and signalling components, SIRT and caloric restriction, FOXO inhibits NFκB signalling as described. Additionally, stress/damage pathways known promote age-associated changes including genotoxic stress, ROS, and inflammation also activate NFκB. Secondary to activation of NFκB by pro-ageing pathways, NFκB then acts to promote ageing related changes by contributing to cellular senescence, senescence-associated secretory phenotype (SASP), apoptotic signals and inflammatory responses (Tilstra et al., 2011).

neurodegenerative diseases such as AD (Guillot et al., 2016), diabetes and obesity (De Souza et al., 2005; Zhang et al., 2008; Moraes et al., 2009); not least due to its role in inflammation (Patel and Santani, 2009; Tilstra et al., 2011; Tornatore et al., 2012).

#### 4.3.6 Apoptosis

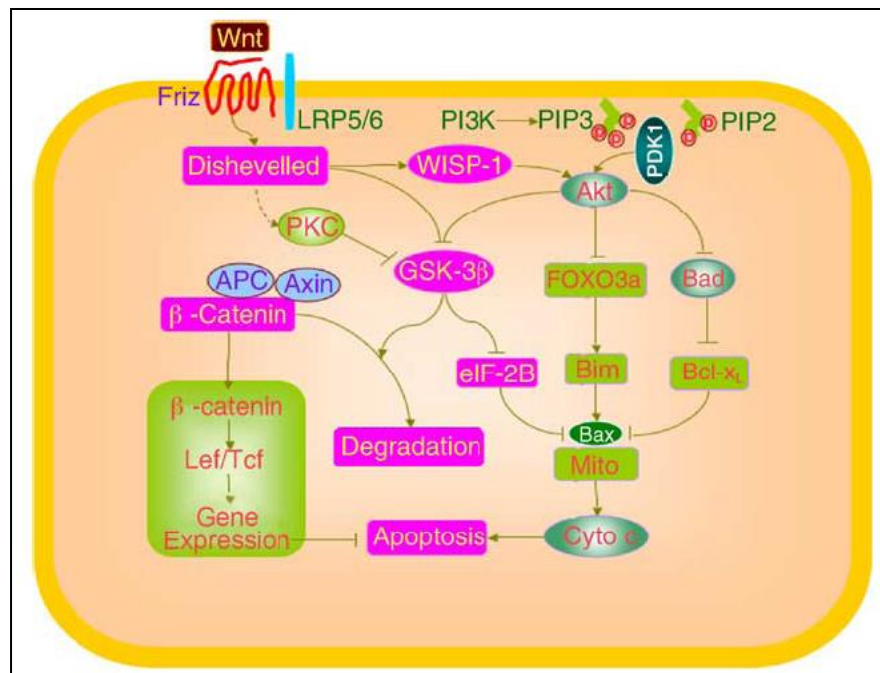
Apoptosis in normal physiological conditions plays a significant role as a counterpart to mitosis; particularly during development, as, critically, in the nervous-and immune systems there is overproduction of cells, and apoptosis rids the system of those cells that fail to establish functional synaptic connections or productive antigen specificities (Nijhawan et al., 2000; Opferman and Korsmeyer, 2003).

Bcl-2-associated death promotor (BAD) and FoxO3 promote apoptosis, albeit by different mechanisms. One mechanism common to both is that they can be phosphorylated by Akt, which tethers them in the cytoplasm with 14-3-3 proteins to prevent their downstream action in promoting apoptosis (Zhou et al., 2000; Li et al., 2001; Zhao et al., 2005). BAD belongs to the super apoptotic, Bcl-2 family and in response to stress signals, it translocates to the mitochondria where it forms heterodimers with the anti-

apoptotic proteins, Bcl-xL and Bcl-2 to prevent their anti-apoptotic action (Reed, 1996; Chao and Korsmeyer, 1998). Activation of Akt leads to inhibition of apoptotic pathway by phosphorylating and inactivating BAD at Ser136 (Datta et al., 1997). In contrast, activation of BAD results in apoptotic processes in the cells where cytochrome c is released to engage initiator caspases, such as caspase 9 (Figure 13; Zhao et al., 2005) and the activation of executioner caspases such as caspase 3 and caspase 7 to promote cell death (Galluzzi et al., 2016).

FoxO3 is a member of the O subclass of family of Forkhead transcription factors. As a transcription factor, upon activation by oxidative stress, FoxO3 will translocate to the nucleus to regulate pro-apoptotic genes such as Bim (Hagenbuchner et al., 2012; Kopycinska et al., 2013) and PUMA (Dey et al., 2014; Shukla et al., 2016) and

downregulate anti-apoptotic genes such as FLIP, but it can also disrupt mitochondrial membrane permeability to release cytochrome C (Yu et al., 2003) and caspase-induced cell death (Kraft, 2015). Similar to Akt inhibiting its translation to the nucleus, serum and glucocorticoid-inducible protein kinase (SGK) also induces nuclear exclusion, by phosphorylation at different sites to that of Akt (Leong et al., 2003). In addition to its role in apoptosis, FoxO3 has been associated with autophagy



**Figure 13. Modulation of apoptotic injury by Wnt and Akt pathways.** Akt inactivates BAD and FoxO3 to prevent apoptosis. Akt targets GSK3β through phosphorylation, resulting in the inactivation of GSK3β and blocking the degradation of β-catenin. Furthermore, phosphorylation of the translation initiation factor 2B (eIF2B) is prevented to prevent the release of cytochrome c (Cyto c) to inhibit apoptosis (Zhao et al., 2005).

and proteostasis but mainly in non-neuronal cells (Webb and Brunet, 2015). This is partly due to the autophagic genes it transcribes, including Beclin-1 in response to nutrient deprivation via positive activation of AMPK (Greer et al., 2007). Other evidence suggests that it can suppress the activity of raptor to lower the activity of the mTORC1 complex (Webb and Brunet, 2015). This combined ability of FoxO3, to regulate apoptosis and autophagy, has been suggested to at least in part, promote, longevity, as supported by population studies showing the presence of FoxO3 genetic polymorphisms in old people is linked with lower prevalence for cancer and cardiovascular disease and higher cognitive abilities (Martins et al., 2016). In addition to FoxO3 activation by Akt, it can be phosphorylated by SGK and IKKβ to induce nuclear exclusion































and also by AMPK in response to nutrient deprivation to positively regulate transcriptional activity (Dobson et al., 2011; Tzivion et al., 2011; Tzivion and Hay, 2011).

Furthermore, in ageing organisms, cells deteriorate at a faster rate and apoptosis will eliminate these to avoid pathology, neurodegeneration and autoimmune disease (Elmore, 2007). A $\beta$  is thought to induce apoptosis by causing oxidative stress and activation of extrinsic death pathways such as FasL pathway (Elmore, 2007). A number of apoptotic mechanisms have been shown to be dysfunctional in AD and obesity. For example, an activation of caspases has been observed post mortem in the brains of AD patients (Rohn et al., 2002; Pompl et al., 2003). Increased caspase 3 is found in either MCI or early stage AD (Gastard et al., 2003). Activated caspase 3 co-localises with PHFs in mild forms of AD (Rohn et al., 2002); and in exposure of cultured cells to A $\beta$  directly results in activation of caspase 3 and 9 (MCardoso et al., 2002; Tamagno et al., 2003). Finally, increased caspase activation is found in the hypothalamus of obese rats (Moraes et al., 2009).

In conclusion, evidence suggests that T2DM and AD do share a number of common pathologies and dysfunctional mechanisms, most notably impaired insulin and glucose regulation, disrupted autophagy, increased inflammation and apoptosis. Insulin stimulated PI3K-Akt signalling and their target proteins are strongly involved in these biochemical functions; however how these proteins interact with each other and contribute to the different functions are not clear. Chronic hyperglycemia and insulin resistance together may play key roles in the cognitive dysfunction and cerebral lesions in people with T2DM that may, at least in part, promote AD (Kodl and Seaquist, 2008).

**Table 5. Changes of key proteins in AD, T2D and obesity in literature**

**AD model:**

Protein	Change	Region	Detail	Ref
Akt		Frontal cortex	AD patients	Liu et al., 2011
		Hippocampus	3x Tg mice	Chen et al., 2013
pAkt Ser 473		Hippocampus	AD patients	Steen et al., 2005
		Hippocampus	3x Tg mice	Chen et al., 2013
GSK3 $\beta$		White cells	MCI and AD patients	Hye et al., 2005
		Hippocampus	3 x Tg mice	Chen et al., 2013
		Hippocampus	AD patients	Čaušević et al., 2010
pGSK3 $\beta$ Ser 9		Blood	MCI and AD patients	Forlenza et al., 2011
		Hippocampus	AD patients	Steen et al., 2005
	 6 M	Hippocampus	APP/PS1 mice	Jimenez et al., 2011
	 18 M	Hippocampus	APP/PS1 mice	Jimenez et al., 2011
mTOR		CA1	APP/PS1 mice	Guillot et al., 2016
pmTOR Ser2448		Temporal cortex	AD patients	Griffin et al., 2005
Bad	 18 M	Hippocampus	APP/PS1 mice	Jimenez et al., 2011
$\beta$ -Catenin	 6 M	Hippocampus	APP/PS1 mice	Jimenez et al., 2011
	 18 M	Hippocampus	APP/PS1 mice	Jimenez et al., 2011
p $\beta$ -Catenin	 18 M	Hippocampus	APP/PS1 mice	Jimenez et al., 2011
Beclin-1		Mid-frontal cortex	Early AD and MCI patients	Pickford et al., 2008
APP		Hippocampus Hypothalamus	AD patients	Steen et al., 2005
		Hippocampus	APP/PS1 mice	Guillot et al., 2016
		Ventral posterior cortex	APP/PS1 mice	Hiltunen et al., 2012
IDE		Hippocampus	Severe AD patients	Candeias et al., 2012
		Hippocampus	AD patients + APOE $\epsilon$ 4	Cook et al., 2003
		Hippocampus	APP/PS1 mice	Vepsäläinen et al., 2008
		Hippocampus	AD patients	Čaušević et al., 2010
GluT1		Cortex	AD patients	Simpson et al., 1994
		Frontal cortex	AD patients	Liu et al., 2008b
		Cortex	AD patients	Mooradian et al., 1997

Caspase 7	↑	Brain	AD/dementia patients	Pompl et al., 2003
Caspase 9	↑	Brain	AD/dementia patients	Pompl et al., 2003
pNFkB	↑	CA1 / DG	APP/PS1 mice	Guillot et al., 2016
FoxO3	↑	Hippocampus	P25 mice	Shi et al., 2016





















#### T2DM-AD models:

Protein	Change	Region	Detail	Ref
Akt	↓	frontal cortex	AD-T2DM patients	Liu et al., 2011
pAkt Ser 473	↓	frontal cortex	AD-T2DM patients	Liu et al., 2011
	↓	Hippocampus	Tg2576 + DIO mice (9 M)	Ho et al., 2004
	↓	Hippocampus	STZ-ICV rats	Xiang et al., 2015
GSK3β	↓	frontal cortex	AD-T2D patients	Liu et al., 2011
pGSK3β Ser 9	↓	frontal cortex	AD-T2D patients	Liu et al., 2011
	↓	Hippocampus	Tg2576 + DIO mice (9 M)	Ho et al., 2004
	↑	ventral posterior cortex	APP/PS1-IGF-2 Tg mice	Hiltunen et al., 2012
	↓	Hippocampus	STZ-ICV rats	De la Monte et al., 2008
mTOR	↑	Hippocampus	STZ-HF/HC+ Aβ injection	Ma et al., 2013
IDE level and activity	↓	Hippocampus	Tg2576 + DIO mice (9 M)	Ho et al., 2004
Glut1	↓	Forebrain	Slc2a1 <sup>+/-</sup> APP Tg mice	Winkler et al., 2015



**T2DM model:**

<b>Protein</b>	<b>Change</b>	<b>Region</b>	<b>Detail</b>	<b>Ref</b>
Akt	↓	frontal cortex	T2DM patients	Liu et al., 2011
pAkt Ser 473	↑	Kidney	Diabetes patients	Habib and Liang, 2014
	↓	Hippocampus	NIRKO mice	Schubert et al., 2004
	↓	Hippocampus	GK diabetic rat	Xiang et al., 2015
GSK3β	↓	frontal cortex	T2DM patients	Liu et al., 2011
pGSK3β Ser 9	↓	frontal cortex	T2DM patients	Liu et al., 2011
	↓	Hippocampus	NIRKO mice.	Schubert et al., 2004
	↑	Hippocampus	IGF- 2 Tg mice	Hiltunen et al., 2012
	↑	Ventral posterior cortex	IGF- 2 Tg mice	Hiltunen et al., 2012
mTOR	↑	Hippocampus	STZ (ip, 30 mg/kg) +HF/HC(8 W) rat	Ma et al., 2013
IDE	↓	Cortex	GK rat	Fakhrai-Rad et al., 2000
	↑	Ventral posterior cortex	IGF-2 Tg mice	Hiltunen et al., 2012
GluT-1	↓	Forebrain	SLC2A1 +/- GluT1 deficient mice	Winkler et al., 2015

**Obesity model:**

protein	Change	Region	Detail	Ref
pAkt Ser 473		Muscle	SD + HFD rats (15 W)	Yong et al., 2016
		Hippocampus	Hamsters + H-fructose (6 W)	Mielke et al., 2005
		Liver/ muscles	Wistar rats + HF/HS (4 W)	Khamzina et al., 2005
pGSK3 $\beta$		Liver	LIKKTg mice	Cai et al., 2005
mTOR		Muscle	SD rats + HF (15 W)	Yong et al., 2016
mTOR 1		Muscle	SD rats + HF (15 W)	Yong et al., 2016
mTOR 2		Muscle	SD rats + HF (15 W)	Yong et al., 2016
pmTOR Ser 2448		Liver	Wistar rats + HF/HS (4 W)	Khamzina et al., 2005
Beclin-1		Liver	ob/ob mice	Yang et al., 2010
		Liver	C57BL/6J mice + HF (5M)	Yang et al., 2010
NF $\kappa$ B		Hypothalamus	Wistar rats + HF (16W)	De Souza et al., 2005
		Hypothalamus	ob/ob mice	Zhang et al., 2008
		Hypothalamus	C57BL/6 mice + HFD	Zhang et al., 2008
		Liver	C57BL/6 mice + HF (8 W)	Cai et al., 2005
		Liver	LIKK Tg mice	Cai et al., 2005
IKK $\beta$		Hypothalamus	ob/ob mice	Zhang et al., 2008
		Hypothalamus	C57BL/6 mice + HF (8 W)	Zhang et al., 2008
IKK ( $\alpha$ and $\beta$ )		Liver; Isolated adipocytes	C57BL/6 mice + HFD (4 M)	Chiang et al., 2009
pIKK		Hypothalamus	Wistar rats + HF (8 W)	Moraes et al., 2009
Caspase 9		Hypothalamus	Wistar rats + HF (8 W)	Moraes et al., 2009

**Abbreviations:**

AD: Alzheimer's disease; T2DM: Type 2 diabetes mellitus; T2DM-AD: Both with T2DM and AD; MCI: Mild cognitive impairment; GK rat: Goto-Kakizaki diabetic rat; IGF-2: Insulin like growth factor-2 overexpressing transgenic mice; NIRKO mice: Neuron specific insulin receptor knock-out mice; Slc2a1: GLUT 1; LIKK: Liver IKK selectively active expressing mice; SD: Sprague Dawley rat; HFD: High fat diet; HC: High carbohydrate diet; HFHS: High fat high sucrose diet; STZ: Streptozotocin; W: weeks; M: months.  Increase;  Decrease.

## Objectives

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Sufficient evidence from a wide range of research now suggests that the main species of A $\beta$  that is more detrimental in the Alzheimer pathology are the oligomeric forms rather than the plaque. Moreover, this process starts much earlier than was originally thought; up to 20 years prior to the onset of the first clinical signs of cognitive deficits. This now suggests early interventive strategies would be more propitious (Jack et al., 2010; Liu et al., 2011; Hampel et al., 2011) even though this would be well before the onset of cognitive disturbances. The start of this slow increase in A $\beta$ , puts it in the right time frame for risk factors associated with AD to be implicated in potentially triggering the increase. Some evidence suggests that picomole levels of A $\beta$  have a physiological role and can in fact facilitate memory and synaptic plasticity. This then suggests metabolic destabilisation induced in risk factors, that themselves develop slowly across many years could provide a neuronal environment that tips the balance of physiological A $\beta$  production to a pathological level.

Following ageing, T2DM, is the main risk factor associated with AD; it is largely induced by obesity and manifests itself in middle age. Both T2DM and AD share a number of pathologies, including amyloid pathology; and disruption of cellular functions that are in part, regulated by PI3K-Akt signalling cascade, a pathway that responds directly to environmental input (Desai et al., 2014). The major difference between the two pathologies is that in T2DM it is mediated in the periphery, whereas in AD the pathology is in the brain. However, under physiological conditions the brain must access both glucose and insulin from the periphery; and as these are the two characteristic dysfunctions in T2DM, it can have an impact on the brain. *The specific aim* of the project therefore, is to understand how and by what mechanism T2DM may induce a favourable neuronal environment to promote dysfunction and cognitive deficits associated with AD.

To address this our strategy was to conduct studies that more closely resembled the human condition. Therefore, we conducted (a) longitudinal studies (over approximately 10 months) that would more closely mimic the slow onset of dysfunction as that seen with T2DM and AD; (b) a feeding regime using human junk food instead of calibrated high fat laboratory pellets; (c) a low dose infusion of soluble A $\beta$ 42 to ensure an increase in A $\beta$ ; (d) analyses of dysfunctional regulation of expression and phosphorylation of proteins in the PI3K signalling pathway in CA1. Therefore, our hypothesis was that any dysfunctional effect induced by A $\beta$  would be exacerbated by a T2DM phenotype. Our hypothesis is that T2DM pathology creates dysfunctional homeostatic regulation of glucose and energy metabolism and decreased insulin signalling in the brain; and will exacerbate any effect induced by A $\beta$  alone.

Currently, there are numerous models of T2DM, ranging from genetically modified mice or inbred rats; STZ injection, high fat feeding with calibrated pellets or the use of human junk food. Other models have used a combination of low dose injections of STZ coupled with high fat diet. We adapted this model but substituted high fat calibrated pellets for human junk food. The rationale for using low dose injections of STZ was based on evidence showing this protocol reduces lethality and added complications induced

by single high doses (see Goyal et al., 2016). In our protocol, we used 2 low dose injections that will kill a portion of the  $\beta$ -pancreatic cells and induce insulin resistance that is more associated with T2DM.

In addition, we used human junk food instead of calibrated high fat pellets. A limitation with this choice was that we were not able to have a precise analysis of the nutritional components of the food; however, this was not our major question. Our major interest was to feed rats a diet containing the same food stuffs that humans eat to mimic more closely how obesity and T2DM develop. To date we believe this particular model has not been reported in the literature.

Our rationale for infusing a low dose soluble form of A $\beta$ 42 is based partly on the fact that, as we are developing a relatively new T2DM model; we had no way of knowing whether our T2DM phenotype would alone would induce an increase in amyloid pathology. It is also based on our own preliminary results that show this dose of A $\beta$  induced a temporary deficit in spatial memory that recovered 6 weeks following infusion. In order to have a functional readout of the effects of the treatments, we conducted spatial memory testing repeatedly throughout the experimental period. We chose to use a spatial recognition memory task as it is susceptible to damage or dysfunction in the hippocampus (Vann and Albasser, 2011). Furthermore, as the task is based on natural exploration of objects and remembering their location within a spatial environment, it requires no appetitive reward or aversive conditions to promote learning; therefore, it is a task that is the least likely to affect subsequent learning and memory.

To monitor the development and evolution of T2DM we conducted repeated glucose tolerance tests; a standard test used in humans to assess diabetes. At the end of the experiment blood was harvested for analyses of insulin levels. This allowed us to measure basal insulin levels; and in relation to basal glucose levels to assess hyperinsulinemia; insulin resistance and  $\beta$ -pancreatic cell failure.

At the end of the experiment, CA1 was dissected for biochemical analyses of basal expression and phosphorylation of proteins in the PI3K signalling pathway, that are associated with a number of dysfunctions that are common to both AD and T2DM. In addition, other proteins associated with dysfunctional amyloid processing and associated with T2DM were examined. As our experimental protocol was relative novel, we chose to analyse a broad spectrum of proteins, rather than focusing on in-depth analyses of proteins mediating specific neuronal dysfunction induced by T2DM that could contribute to AD pathology.

Finally, to deconstruct the potential contributions of the T2DM phenotype or A $\beta$  alone to the memory deficits and regulation of proteins, a number of groups were tested: Control rats fed with laboratory diet (Ct), rats fed with cafeteria diet (CD), rats injected with Streptozotocin (STZ) and rats fed with cafeteria diet combined with STZ injections (STZ-CD). Twenty weeks after the start of feeding regimes, some rats in each group were infused with soluble A $\beta$ 42 to determine the effect of A $\beta$  alone and the impact of T2DM treatment on A $\beta$ . The deconstruction of these groups allowed us to identify the relative importance of each contributory effect to the endpoint effect on functional regulation of memory processing and proteins.

## Results (manuscript of article)

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## **Mechanisms by which Type 2 Diabetes Mellitus (T2DM) may act as a potential risk factor for the development of Alzheimer's disease (AD)**

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### **Abstract**

Type 2 Diabetes Mellitus (T2DM) is the most important risk factor after ageing for developing Alzheimer's disease (AD). It is a metabolic disorder characterised by hyperglycemia and insulin resistance that develops in middle age and is promoted largely by obesity. We conducted a longitudinal study (10 months) using a T2DM rat model to assess the potential impact T2DM may have on the development of pathologies associated with AD. Rats were fed cafeteria-style diet (CD) coupled with subdiabetogenic doses of Streptozotocin (STZ)(STZ-CD). STZ-CD treated rats showed classic signs of T2DM and a modest deficit in consolidation of spatial recognition memory.

To test whether this phenotype could exacerbate an effect of amyloid we infused intracerebroventricularly a soluble form of amyloid beta42 (A $\beta$ 42). The phenotype prolonged a deficit in spatial recognition memory induced by A $\beta$ 42 alone; and although environmental enrichment improved memory deficits, it was short lasting.

Biochemical analyses were conducted on proteins in-or associated with the PI3K-Akt signaling pathway and markers of A $\beta$  processing and glucose transport in CA1. The T2DM phenotype infused with A $\beta$  induced a number of long-lasting changes in proteins associated with aberrant functions identified in brains of AD patients and models of the disease. When we deconstructed the component parts of the T2DM phenotype and A $\beta$  alone, we found; although a number of changes were specific to the T2DM phenotype infused with A $\beta$ ; others were independently driven by either CD alone or A $\beta$  alone whereas STZ injections alone had a negligible effect. Most of the aberrantly regulated proteins are associated with functions such as autophagy, inflammation, apoptosis, glucose availability and amyloid processing. The data suggest T2DM may promote a dysfunctional neuronal environment that would allow A $\beta$  to induce pathological events associated with early stage of AD and cognitive deficits.

## Introduction

Alzheimer's Disease (AD) is an age-related form of neurodegeneration that has a slow and insidious onset. It is defined clinically by dementia and pathologically by the widespread presence of amyloid plaques and neurofibrillary tangles and cell loss (Heininger, 1999; Dickson and Vickers, 2001). Until recently, the focus of research into understanding the progression of the disease and the development of treatments to stem or arrest the pathology has been centred on the amyloid pathology. However; over the past 10 years or so, accumulation of evidence would now suggest that the hard-core, immobile amyloid plaque is not the key triggering mechanism of at least the dementia associated with AD. The main evidence supporting this is clinical trials using plaque removing drugs that do not improve cognition or prolong life in AD patients (Holmes et al., 2008); and that cognitively 'normal' aged persons have an equivalent plaque load as do those diagnosed with AD (Knopman et al., 2003; Oh et al., 2015; Mufson et al., 2016). Moreover, more recent research has shown that pre-aggregated species of amyloid may be more instrumental in inducing the dementia associated with the disease (Larson and Lesne, 2012; Lesne, 2014). These species are toxic, relatively mobile and, extracellularly, they can bind to and render dysfunctional a number of receptors (Sakono and Zako, 2010) and their subsequent regulation of signaling pathways. This can promote inflammation and apoptosis processes (Eftekharzadeh et al., 2012; Kumar et al., 2016) and also deregulate homeostatic processes such as autophagy and energy regulation (Townsend et al., 2007; Caccamo et al., 2011; See Wang et al., 2016). Experimental evidence in rodents suggests these amyloid species induce deficits in memory (Cleary et al., 2005; Sipos et al., 2007; Eslamizade et al., 2016) and synaptic plasticity (Walsh et al., 2002; Townsend et al., 2006; Zhao et al., 2008). Finally, evidence shows amyloid increases slowly over some 15 to 20 years prior to the onset of the first identifiable clinical signs and has reached a plateau at this time point (Jack et al., 2010; Jack et al., 2013; Swerdlow et al., 2014). Thus, there is a growing consensus that suggests pre-aggregated species may constitute a preclinical stage of the disease where development of interventional treatments may be the optimal strategy. However, in the absence of live markers of pre-aggregated amyloid in humans, it becomes difficult to dissociate mild memory deficits in normal aged persons from those that will develop AD.

One potential predictive marker for early development of AD is Type 2 Diabetes Mellitus (T2DM); a disease characterised by hyperglycemia and insulin resistance and/or hyperinsulinemia (Alberti and Zimmet, 1998; Brunetti et al., 2014). Following ageing, it is the most important risk factor for developing AD; the incidence of AD is 2-5 times higher in people with T2DM (Mayeux and Stern, 2006; Desai et al., 2014). As with AD, T2DM develops over time; displaying characteristic symptoms around the 5<sup>th</sup> decade of life (Aguiree et al., 2013; American Diabetes Association., 2015) and is also subject to risk factors, namely obesity (van Leiden et al., 2003; World Health Organization, 2000). Epidemiological and animal studies show a strong association between T2DM and AD and it is suggested T2DM can exacerbate the process of AD (Verdile et al., 2015a). Diabetic patients show lower levels of global cognition, episodic memory, semantic memory, working memory and perceptual speed (Arvanitakis et al., 2004). T2DM and AD also share some common pathologies, such as hippocampal atrophy; and structural and function neuronal

connectivity (Den Heijer et al., 2003; Reijmer et al., 2013, John et al., 2014); impaired fasting glucose (Janson et al., 2004) and the presence of neurofibrillary tangles and amyloid plaques (Peila et al., 2002; Mittal and Katare, 2016) albeit these key signature lesions are in the pancreas in T2DM patients. Moreover, studies have reported impaired insulin-PI3K-Akt signaling in AD and T2DM patients (Schubert et al., 2004; Steen et al., 2005) that could contribute to dysfunctions such as inflammation, apoptosis, autophagy, oxidative stress, impaired insulin signaling and glucose metabolism, many of which are mediated by insulin mediated PI3K/Akt signaling pathway as reviewed (Cole et al., 2007; Kim et al., 2010; Verdile, 2015b; Mittal and Katare, 2016). Although these common dysfunctions are principally observed in peripheral tissues in T2DM, it is known that the brain is wholly reliant on the peripheral system for glucose and the majority of its insulin; suggesting deregulation by T2DM will affect brain energy homeostasis.

Thus, given the common pathologies and dysfunctional mechanisms, the memory deficits observed in T2DM may constitute an early, fragile state, where a slight disruption of homeostatic regulation of neuronal mechanisms may tip the balance in favour of developing AD and the ensuing dementia. Furthermore, the age at which the disease manifests, places it in the right time frame for the start of the rise in amyloid in patients.

A number of studies have shown that sporadic AD is a metabolic brain disorder (Iqbal and Grundke-Iqbal, 2005; Heiss et al., 1991; Gong et al., 2006), where impaired glucose metabolism occurs prior to the appearance of AD symptoms and worsens with age (Heiss et al., 1991; Smith et al., 1992; Hoyer, 2004). In addition the AD brain shows insulin resistance (Riveral et al., 2005; Talbot et al., 2012; Liu et al., 2011) that is associated with decreased glucose metabolism (Liu et al., 2009; 2011). Some studies have coupled T2DM with AD, for the main part using AD transgenic mice (Ho et al., 2004; Hiltunen et al., 2012; McClean and Hölscher, 2014; Yeh et al., 2015) that have principally focused on amyloid and tau pathology (Ho et al., 2004; Takeda et al., 2010; Yeh et al., 2015) or T2DM pathology (Takeda et al., 2010; Hiltunen et al., 2012). Given the associations between the two diseases, the aim of our experiments was to uncover dysfunctional mechanisms in CA1 of the hippocampus mediated by T2DM that might exacerbate an early increase in amyloid in preclinical stages of AD. Although the amyloid cascade hypothesis (Hardy and Higgins, 1992; Selkoe and Hardy, 2016), a major driving force directing research in AD would suggest that all non-specific AD pathology is downstream of amyloid, a major underlying concept on our experiment is that chronic low level destabilization of brain function induced by T2DM promotes an environment to permit dysfunctional regulation of A $\beta$ . To this end, we developed a model of T2DM in rats that closely mimicked the human condition and conducted longitudinal studies. When characteristic symptoms of T2DM were established we infused a low concentration of soluble A $\beta$ 42 and monitored rats for a further 15 weeks. As a functional readout, we repeatedly tested spatial recognition memory across the experimental period. And, finally we examined regulation of proteins in the PI3K-Akt signaling pathway, as this is associated with the normal homeostatic regulation of functions that are shown to be aberrant in AD.

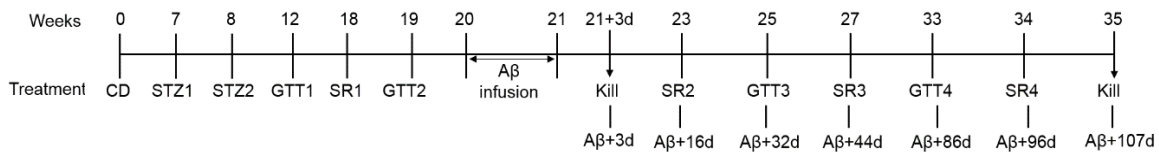
## Methods

### Animals

Male Sprague Dawley rats (200g-300g) were purchased from Charles River Laboratories, France. The animals were housed in standard cages (2 rats/cage) and maintained in a temperature and humidity controlled colony room with 12/12-hour light dark cycle with fresh water *ad libitum* and normal laboratory pellets (A04, SAFE, France) until the start the feeding protocol. All efforts were made to minimize the animal number and suffering during the whole experimental procedure. Experiments were conducted according to the European Communities Council Directive of 24 November 1986 (86/609/EEC), EU Directive 2010/63/EU, and the French National Committee (87/848), and approval of the local ethics committee (n°59).

### General protocol and Experimental groups

We conducted longitudinal studies over 8-10 months. Rats were first started on a feeding regime and injected twice with subdiabetogenic doses of STZ, one week apart. After 20 weeks, some of the rats were implanted with minipumps containing soluble A $\beta$ 42 and the experiment was continued for another 15 weeks. Throughout the experimental period, regular glucose tolerance tests (GTTs) and spatial recognition memory tests were conducted (see timeline below, Fig 1). There were 4 main experimental groups: Control rats fed with laboratory diet (Ct, n=21), rats fed with cafeteria diet (CD, n=8), rats injected with Streptozotocin (STZ, n=10) and rats fed with cafeteria diet combined with STZ injections (STZ-CD, n=9). A sub group of these groups were infused with soluble A $\beta$ 42 (See supplementary Figure 1).



*Figure 1. Time line of experimental protocol:* Protocol starts with feeding Cafeteria Diet (CD) at week 0. Thereafter different treatments across a period of 35 weeks are depicted. Abbreviations: STZ1 and 2 are the times at which low dose injections of Streptozotocin are given; SR1-4 depict the times at which spatial reference memory is tested; GTT1-4 depict when glucose tolerance testing is conducted; A $\beta$ + indicates the time following the end infusion of A $\beta$ .

### Feeding Protocol

After 2 weeks' adaptation to laboratory conditions, rats were randomly divided into two basic feeding regimes; calibrated laboratory diet (LD) at the recommended dose to maintain normal health and growth; or cafeteria diet (CD), comprising human 'junk' food (see supplementary Table 1) that was high in calories, sugar and fat. Moreover, these foods contained chemical components used as stabilisers, colorants, etc. A wide range of food products was offered to rats at the start of the experiment to determine their food preference and these foods stuffs were given in excess quantities on a daily basis. This feeding regime was continued throughout the experimental period. Body weight was recorded on a weekly basis.

Consumption of major nutritional components in CD was assessed each day over a 6-week period in the middle of the experiment.

#### *Streptozotocin (STZ) injection*

STZ (Sigma- Aldrich) was dissolved in citric acid (pH 6.0), prepared as required and protected from light. Rats were given 2 sub-diabetogenic doses (30 mg/kg i.p.) one week apart approximately 7 weeks after the start of the feeding regime.

#### *Surgical procedure and amyloid-beta (A $\beta$ ) infusion*

The A $\beta$ 42 peptide was specifically synthesised to be maintained in a soluble form (L. Fulöp, University Szeged, Hungary) and stored as lyophilised aliquots (0.1 mg) at -80° until required. Prior to use the peptide was resuspended in HyPure Molecular biology double distilled sterile water (Thermo; France), sonicated and diluted to a concentration of 50 mM in aCSF (Alzet protocol) and filtered.

Approximately 20 weeks after the start of the feeding regime half of the rats in each group underwent surgery to implant osmotic minipumps (Alzet, model 2004, USA) to infuse A $\beta$ 42 intraventricularly (icv) over 7 days (0.5  $\mu$ l/hour). Rats were deeply anaesthetised with Ketamine (1.5 ml/kg) and Domitor (0.5 ml/kg). They were placed in a stereotaxic frame, the skull exposed to allow a single hole to be drilled out to place a cannula in the lateral ventricle (Bregma 1.3 mm; midline 1.8 mm; depth 3.0 mm from the brain surface). The cannula was connected to osmotic minipump via flexible tubing and fixed in place with cement. The pump was then inserted subcutaneously between the scapula and the scalp incision was closed with surgical staples.

#### *Glucose tolerance test (GTT)*

At different time points during the experiment (see Fig 1), glucose tolerance tests were conducted. After overnight fasting, blood from the tail vein (approximately 4  $\mu$ l each time) was sampled prior to (0 min) and 15, 30, 60, and 120 minutes after a bolus injection of glucose (i.p, 2g/kg body weight, Sigma- Aldrich,). Blood glucose levels (mg/dL) were analysed using a glucometre (HemoCue<sup>R</sup>, Sweden). Statistical analyses using one-way ANOVA were conducted on basal (0 min) glucose levels and the area under curve (AUC) in response to the glucose challenge in each rat.

#### *Blood insulin assay*

At the end of the experiment after overnight fasting, whole blood was collected immediately following sacrifice. Plasma was separated by centrifugation (3500 rpm, 25min, at 4°C) and stored at -20°C until analysis. Plasma insulin was measured using a Rat/Mouse Insulin ELISA Kit (Cat # EZRMI-13K, Millipore, Germany) according to the manufacturer's protocol and analysed using spectrophotometry (Molecular Device, France) at an absorbance level of 450 nm. Sample insulin concentrations (ng/ml) were calculated based on standard insulin curves and one-way ANOVA was used to analyse group differences.

### *Spatial Recognition task*

The spatial recognition memory was conducted in a circular open field (diameter 90cm, height 50cm, painted black) in a room containing multiple 3-dimensional cues. Before training rats were habituated to the open field (5 min/day, for 3 days). Following habituation, rats were given a sample phase (3 sessions of 4 minutes with a 4-minute interval between sessions) where they explored three different objects constructed out of Lego™. Following a delay (24 or 72 hrs) one of the objects was changed to a new location and rats were allowed a single session of exploration (4 min). Time spent exploring the objects was recorded via a video tracking system (ANYMAZE, Stoelting Co., USA). ANOVA was conducted on the total time spent exploring objects during the sample phase to determine whether differences in exploration would indicate deficits in motor coordination or stress that may contribute to cognitive performance. Percent time spent exploring the novel location vs a mean of the two familiar locations was calculated to determine 50% as chance level and analysed with one-way ANOVA.

### *Environmental enrichment*

A mild form of environmental enrichment (EE); developed in the laboratory (Bruel-Jungerman et al., 2005) was given to a subset of rats in the STZ-CD and control groups infused with A $\beta$  or not. Groups of 4-5 rats were placed in a large wooden box (Length 100 cm; Width 80 cm; Height 60 cm) containing junk objects for 3 hours a day over 14 days.

### *Brain tissue preparation for biochemical analyses*

Rats were sacrificed by decapitation and CA1 of the hippocampus was dissected for analyses of expression and phosphorylation of proteins using immunowestern-blotting analyses. Dissected tissue was immediately frozen in liquid nitrogen and kept at -80°C for later use. Proteins were extracted from the frozen tissue in lysis buffer (described in Davis et al., 2000); and Complete Protease Inhibitor Cocktail and PhosSTOP (Roche, France). Homogenized samples were incubated on ice for 30 minutes and centrifuged at 15,000 rpm for 15 min at 4°C; then the supernatant was recovered and stored at -80°C. Protein concentrations were calculated using Bio-Rad protein assay (Bio-Rad, Germany) and samples were diluted with lysis buffer to give equal protein concentration of 1  $\mu$ g/ $\mu$ l.

### *Western-blotting*

The protocol was based on previous experiments (Kelly et al., 2003), briefly 20  $\mu$ l of sample was denatured in 5 x Laemmli sample buffer and boiled at 95°C for 5 minutes before loading onto gradient acrylamide gels (6-12%). Proteins were separated using constant voltage (150 V; between 1.5-3 hr) and then transferred onto a nitrocellulose membrane (Amersham, GE Healthcare, Germany) by electro-blotting at a constant voltage (100 V; 90 min). Membranes were blocked with 5% non-fat dry milk (Bio-Rad, France) in TBS-T (Euromedex, France) and incubated overnight in primary antibodies at 4°C with gentle shaking (see primary antibodies and dilution in supplementary Table 2). Membranes were washed 3 times (5min) in TBS-T and incubated in a horseradish peroxidase-conjugated anti-mouse/ rabbit immunoglobulin IgG

secondary antibody (Dilution 1:2000 – 1:10,000 in 5 % BSA, Amersham, GE Healthcare, France) for 1-1.5 hours at room temperature. Membranes were rinsed x 3 (5 min) in TBS-T and proteins were reacted with Chemiluminescence ECL solution (Amersham, GE healthcare, France), exposed to film (optimal exposure time for each antibody was maintained) and developed by hand. Membranes were subsequently washed and stripped (Re-blot plus; Millipore, Germany) and re-incubated with other antibodies using the same procedure as described above. Protein bands were quantified using Gene Tools software (SynGene, Cambridge, UK). Total proteins were normalised to  $\beta$ -Actin and phosphorylated proteins to corresponding total protein. The relative changes in expression and phosphorylation of proteins were represented as percent of control levels per gel and statistical analyses, using Students t-Test determined whether changes were significantly different from controls, and one-way ANOVA to determine group differences.

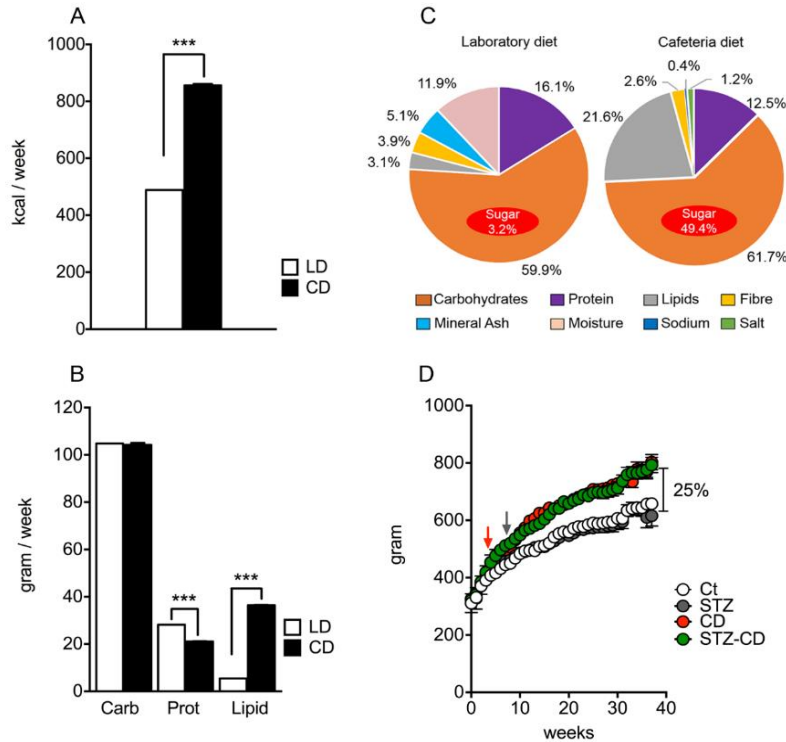
## Results

### *Food intake and weight gain*

We made cursory analyses of the food consumption over a 6-week period in the middle of the experiment in the groups fed laboratory diet (control and STZ alone) and cafeteria diet (CD alone and STZ-CD). We found rats fed CD consumed nearly twice the amount of weekly calories (853 kcal) compared with those fed laboratory diet (488 kcal;  $t=73.93$ ;  $p<0.0001$ ; Fig 2A). In terms of the major nutritional food groups rats fed CD consumed about the same amount of carbohydrates as those fed laboratory diet ( $t=0.7383$ ;  $p>0.05$ ); less overall protein content ( $t=72.29$ ;  $p<0.0001$ ; Fig 2B) and more lipids ( $t=204.2$ ;  $p<0.0001$ ). However, the amount of sugar contained in the CD was approximately 49.4% whereas in laboratory diet it was 3.2%. And the protein content in CD was mainly derived from animal proteins where as it was at least 66% vegetable proteins in laboratory diet. The other major differences in nutritional composition are shown in Fig 2C.

Weight was measured on a weekly basis and, of course at the start of experiment all rats had the same weight ( $F<1$ ). Not surprisingly, rats fed with cafeteria diet (CD alone and STZ-CD group) gained significantly more weight than those groups fed laboratory diet (Controls and STZ alone) across the experimental period. By the end of the experiment rats fed CD had gained approximately 25% more weight than those on the control diet ( $F(3,96)=12.78$ ;  $P<0.0001$ ; see Fig 2D). Although rats could not be considered obese compared with other models (Bray, 1977; Harishankar et al., 2011); some studies have suggested that weight gain between 10 and 25% is an indicator of obesity (Levin and Dunn-Meynell, 2002; Woods et al., 2003).

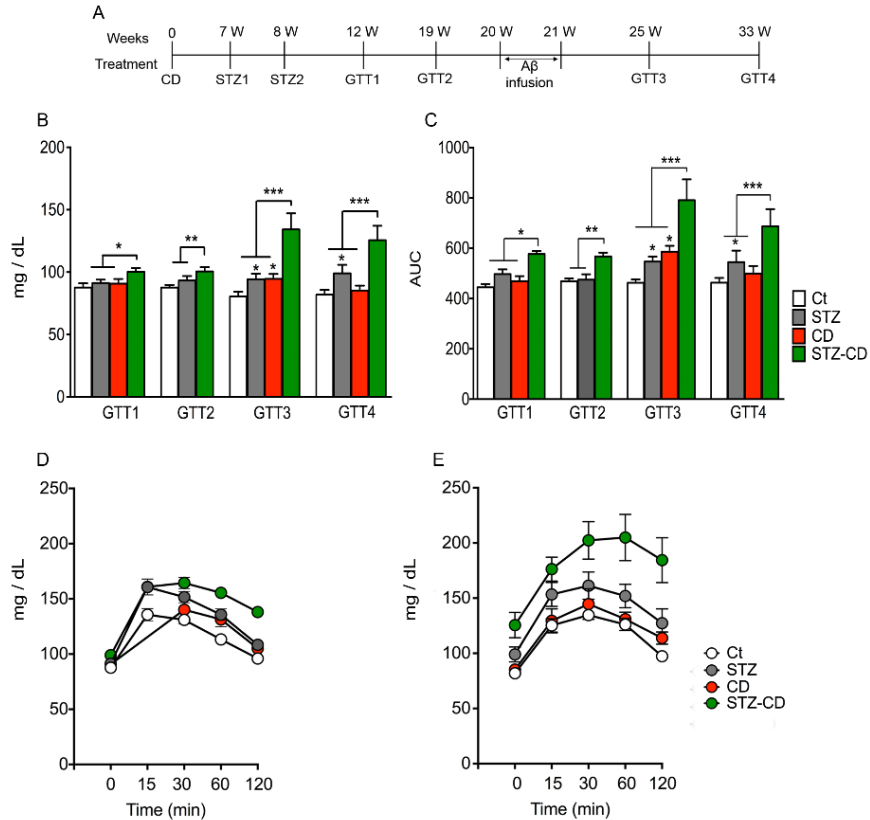




**Figure 2. Food intake and weight gain:** (A) shows the relative caloric intake in rats fed normal laboratory diet (LD) or cafeteria diet (CD). (B) Indicates the consumption of major food groups, Carb (Carbohydrates); Prot (Proteins); and Lipids. (C) Percentage of nutritional components in standard laboratory diet and Cafeteria Diet. (D) Weight gain in the 4 major groups: Ct (fed Laboratory Diet); STZ (fed Laboratory Diet), CD (fed Cafeteria Diet) and STZ-CD (fed Cafeteria Diet). Arrows indicate start of feeding regime (red) and the second STZ injection (grey). Abbreviations: STZ, Streptozotocin, CD, Cafeteria diet; LD, Laboratory Diet; CD. \*\*\*  $p < 0.001$ .

### Blood glucose levels

We conducted 4 glucose tolerance tests (GTT) at different time points throughout the experimental period (see timeline in Fig 3A). ANOVA and Tukey post hoc analyses conducted on basal glucose levels in the first test showed a significant difference between groups (GTT1:  $F(3,84)=3.041$ ;  $p < 0.05$ ) that was attributed solely to a modest increase in STZ-CD treated rats compared with controls (Tukey post hoc analysis;  $p < 0.05$ ). This effect was maintained in the second test ( $F(2,58)=5.23$ ;  $P < 0.1$ ) where the difference was attributed to STZ-CD treated rats (Tukey post hoc analysis;  $p < 0.05$ ). NB at this time point we did not test rats fed CD alone. However, by the third and fourth tests, basal glucose was greatly elevated in STZ-CD treated rats but there was also a modest increase in rats injected with STZ alone or fed CD alone GTT3:  $F(3,53)=10.56$ ;  $p < 0.0001$ ; GTT4:  $F(3,52)=6.729$ ;  $=0.001$ ; Fig 3B). Analysis of the AUC, in response to glucose challenge, showed a similar pattern to that observed with basal levels.



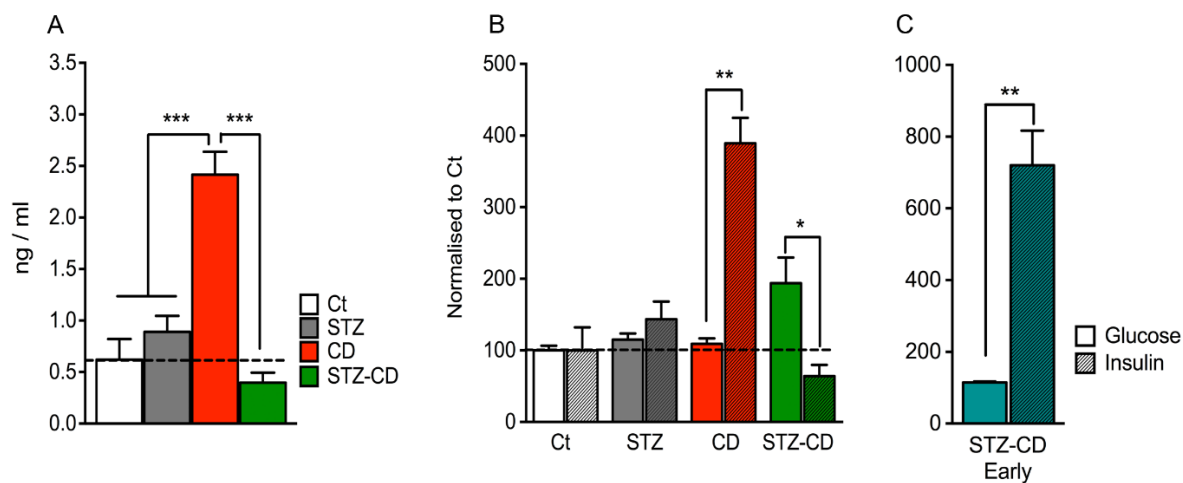
**Figure 3. Glucose regulation:** (A) depicts time line of the 4 glucose tolerance tests. (B) Shows evolution of fasted basal glucose levels (0-time point; mg/dL) across the 4 time points. (C) Shows Area Under Curve (AUC) of individual response curves to a glucose challenge. (D and E) show the mean group differences of the response curves in first test (D) and the last test (E) in mg/dL (Time after glucose injection). Data is represented as mean±SEM; \*p<0.05; \*\*p<0.01; \*\*\*p<0.001.

The first two tests showed only a significant increase in the STZ-CD treated rats (GTT1:  $F(1,3)=5.01$ ;  $p<0.01$ ; GTT2:  $F(1,2)=13.22$ ;  $p<0.001$ ). However by the 3<sup>rd</sup> and 4<sup>th</sup> test, STZ-CD treated rats showed an even greater increase; with STZ-alone and CD-alone showing an increase but to a much lesser extent (GTT3:  $F(1,3)=4.31$ ;  $p<0.05$ ; GTT4:  $F(1,3)=9.55$ ;  $p<0.001$ ; Fig 3C). Importantly both basal levels and AUC significantly increased across time in STZ-CD treated rats (Basal levels:  $F(3,71)=6.37$ ;  $p<0.001$ ; AUC:  $F(3,79)=7.49$ ;  $p<0.001$ ) and this is reflected in figures 3D and E showing glucose curves in mg/dL in the first and last test. In summary, only STZ-CD treated rats showed obvious hyperglycemia as they had a consistent increase in basal fasting plasma glucose and impaired glucose tolerance in response to glucose challenge that evolved with time.

#### Blood insulin levels

At the end of the experiment, plasma insulin was measured using ELISA. ANOVA showed very high levels in rats fed CD that is a common feature of obesity (see Templeman et al., 2017); with a slight

non-significant increase in rats injected with STZ alone, and a trend towards a decrease in STZ-CD treated rats ( $F(3,15)=18.14$ ;  $p<0.0001$ ; Fig 4A). To compare the relationship between glucose and insulin, we normalised blood insulin and glucose levels, in the last test to their corresponding controls (Fig. 4B). In rats injected with STZ alone although there was a slight increase in insulin to glucose levels it was not significant ( $t=0.88$ ;  $p>0.05$ ). In rats fed CD there was a huge and significant increase in insulin level compared with a relatively normal level of glucose ( $t=9.19$ ;  $p<0.01$ ) suggesting either insulin resistance and/or hyperinsulinemia. In contrast, STZ-CD treated rats had increased levels of glucose and a corresponding decrease in levels of insulin ( $t=6.24$ ;  $p<0.05$ ), suggesting  $\beta$ -pancreatic cell failure (Leahy, 2005; Alejandro et al., 2014).



**Figure 4. Insulin regulation:** (A) shows blood insulin level at the end of the experiment in the 4 major experimental groups. (B and C) show the relationship between glucose (solid bars) at the last test and insulin (hatched bars) when killed. Data are normalised to respective control levels. (B) shows relationship between glucose and insulin levels at the end of the experiment. (C) Depicts relationship between glucose and insulin in a subgroup of STZ-CD treated rats and control rats killed at the earlier time point of 21 weeks. Data is represented as mean $\pm$ SEM; \* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ .

Importantly we analysed a group of STZ-CD treated rats at an earlier time point (week 21) and found the relationship between glucose and insulin levels resembled that of the rats fed CD tested at the end of the experiment; where there was no significant difference in glucose levels between control and STZ-CD treated rats ( $t=0.91$ ;  $p>0.05$ ) but a large difference in insulin levels ( $t=8.43$ ;  $p<0.0001$ ) and a significant increase in insulin to glucose ( $t=6.03$ ;  $p<0.01$ ) suggesting hyperinsulinemia and/or insulin resistance (Fig 4C). Together the weight gain, hyperglycemia and impaired insulin that were specific to the rats treated with STZ-CD suggests this treatment at least promotes a number of characteristics associated with a T2DM phenotype. Moreover, the difference between the glucose and insulin levels at the earlier time point compared with the later time point suggests that the T2DM phenotype evolved with time and at the end of the experiment represents a more advanced stage of T2DM than that induced by CD alone.

## Memory deficits induced by T2DM

To test whether the T2DM phenotype, STZ injections and CD alone induced memory deficits we tested rats 4 times throughout the experimental period (see time line; Fig 5A). In all 4 tests, during the sample phase, rats in all groups showed equal exploration of the 3 objects (all  $F$  values  $<1$ ; data not shown), suggesting the different treatments had no discernible effect on locomotor activity or the normal tendency for exploration of the objects. When we tested consolidation of the memory 24 hour following the sample phase we found on the first test that the T2DM phenotype, STZ-alone and CD-alone showed significantly poorer memory compared with controls ( $F(3,36)=23.48$ ;  $p<0.0001$ , Fig. 5B). However, their performance was significantly greater than chance level (all  $p$  values  $<0.01$  compared with 50%). This same pattern of a modest deficit was also observed in the subsequent tests (Week 23,  $F(3,36)=23.35$ ;  $p<0.0001$ ); Week 27,  $F(3,27)=30.47$ ;  $p<0.0001$ ; Week 34,  $F(3,27)=25.45$ ;  $p<0.0001$ ). This suggests that each component of the T2DM phenotype is capable of affecting consolidation of recognition memory and maintaining a stable impairment across time (see Fig 5B).

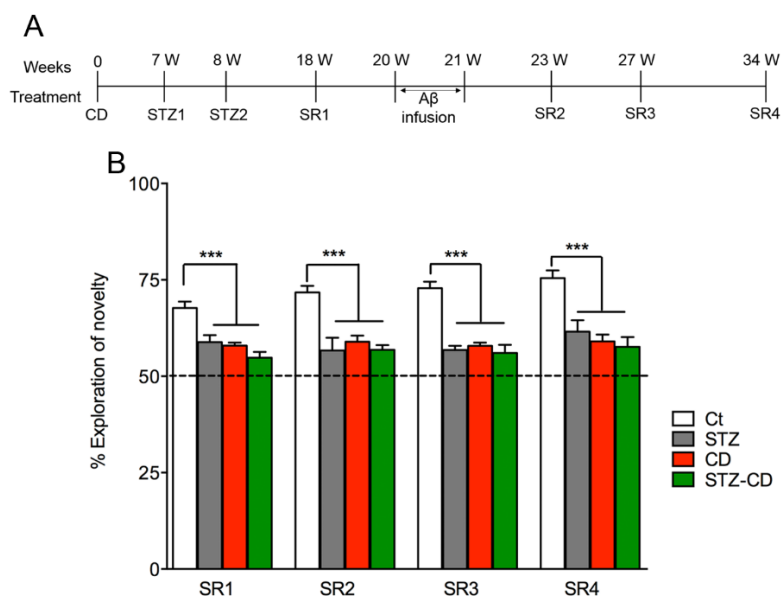
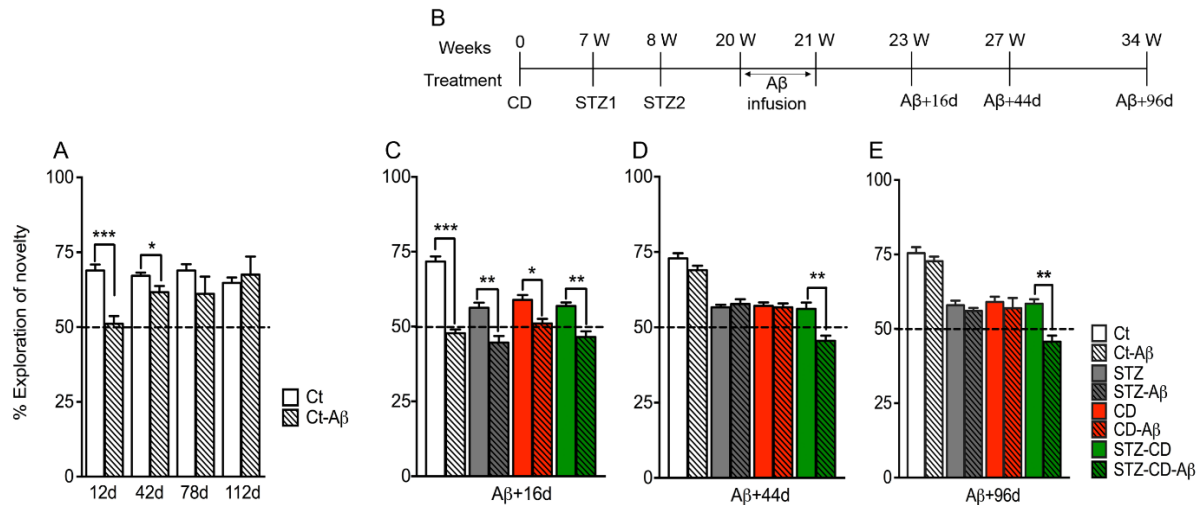


Figure 5. Effect of the T2DM phenotype (STZ-CD) and component parts, STZ injections alone (STZ) and rats fed cafeteria diet alone (CD) on spatial recognition memory. (A) depicts the time line of memory testing. (B) Shows the percent exploration of the novel location at the different time points tested (SR1-4). Data is represented as mean $\pm$ SEM; \*\*\* $p<0.001$ .

## T2DM prolongs the memory deficits induced by Aβ

At week 20 half the rats in each group were infused with soluble oligomeric Aβ42 peptide into lateral ventricle (icv) for 7 days. We conducted a first experiment to determine whether Aβ42 alone induced a deficit in spatial recognition memory. Here rats were either infused with aCSF or the Aβ peptide and tested 12, 42, 78 and 112 days following the end of infusion. During the sample phase all rats showed a comparable level of exploration in the 4 tests (all  $p$  values  $<0.05$ ; data not shown). Compared with control

rats, those infused with A $\beta$  showed a significant deficit in memory consolidation at day 12 ( $t=4.96$ ;  $p<0.0001$ ) with performance falling to chance level. Although slowly starting to recover by day 42, rats still showed significantly poorer performance when compared with non-infused rats ( $t=2.42$ ;  $p<0.05$ ); however, they were significantly above chance level ( $t=5.62$ ;  $p<0.01$ ). By day 78 the memory had recovered and showed no difference to non-infused rats ( $t=1.57$ ;  $p>0.05$ ) and this was maintained at day 112 ( $t=0.53$ ;  $p>0.05$ ). Furthermore ANOVA confirms the recovery of memory deficit across time ( $F(1,3)= 3.34$ ;  $p<0.05$ ; see Fig 6A).



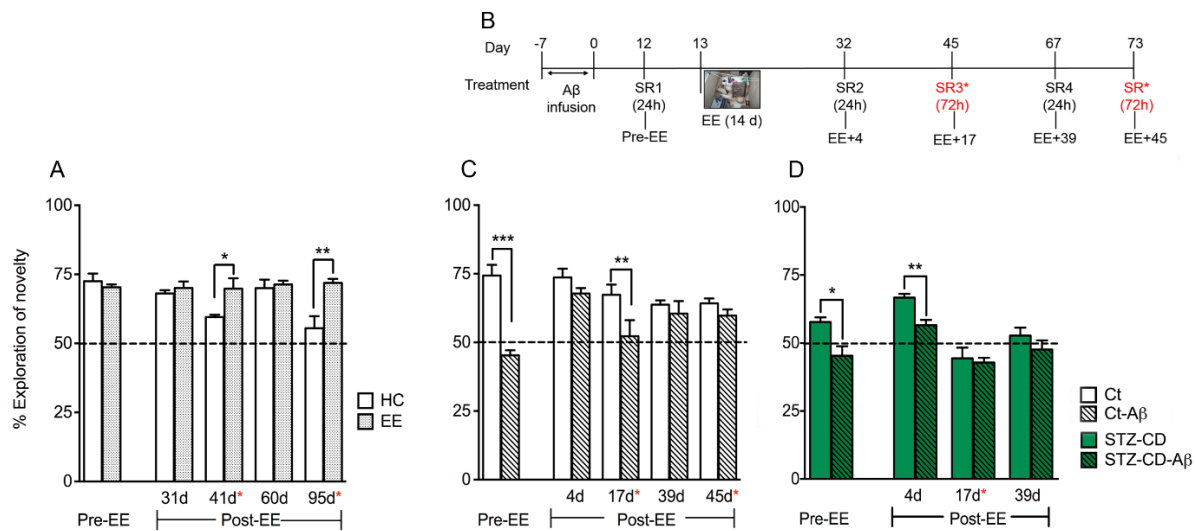
**Figure 6.** Effect of A $\beta$  alone and in conjunction with T2DM and component parts on spatial recognition memory: (A) Depicts a preliminary study testing the effect that A $\beta$  alone (hatched bars) compared with non A $\beta$  infused rats (white bars) tested repeated at different time points after infusion of A $\beta$ . (B) shows the time line of testing of the effect of infusion of A $\beta$  in STZ alone, CD alone and in combination (STZ-CD). (C-E) memory deficits induced in all groups at different times following infusion of A $\beta$  (hatched bars) compared with non-A $\beta$  infused (solid bars) groups. NB, non-infused groups are the same as those depicted in figure 5. Data is represented as mean $\pm$ SEM; \* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ .

We therefore tested rats in the different groups infused with A $\beta$ , 16, 44, and 96 days following the end of A $\beta$  infusion. This corresponds to the same time at which non-A $\beta$  infused groups were tested. In the first test (A $\beta$ +16 days) compared with non-infused controls, A $\beta$  induced a deficit ( $t=11.27$ ;  $p<0.0001$ ) and performance dropped to chance levels comparable to that we observed previously. Importantly, rats injected with STZ alone, fed CD alone or treated with STZ-CD also showed a deficit compared with their non-infused counterparts (STZ:  $t=4.18$ ;  $p<0.001$ ; CD:  $t=3.53$ ;  $p<0.05$ ; STZ-CD:  $t=4.65$ ;  $p<0.001$ ; Fig 6B). By day 44 following infusion, the control, STZ and CD groups recovered to the same level of performance to that of their non-infused counterparts (Ct:  $t=1.79$ ;  $p>0.05$ ; STZ:  $t=0.62$ ;  $p>0.05$ ; CD:  $t=0.26$ ;  $p>0.05$ ). In contrast, STZ-CD treated rats still showed a deficit ( $t=0.387$ ;  $p=0.0024$ ; Fig 6C) and this same performance in all groups was maintained by the last test; 96 days post A $\beta$ -infusion; (Ct:  $t=1.07$ ;  $p>0.05$ ; STZ:  $t=1.04$ ;  $p>0.05$ ; CD:  $t=0.54$ ;  $p>0.05$ ; STZ-CD:  $t=5.05$ ;  $p<0.001$ ; Fig. 6D). Importantly, the deficits observed in rats injected with STZ or fed CD alone, only recovered to their level of performance prior to infusion of A $\beta$ ,

suggesting there is no spontaneous recovery and suggesting STZ and CD alone independently induce a modest but stable deficit across time. However, the combined STZ-CD treatment exacerbates the deficit induced by A $\beta$  by prolonging it.

### *Beneficial effect of environmental enrichment on spatial memory*

As rats treated with STZ-CD showed a prolonged deficit in spatial recognition memory we tested whether environmental enrichment (EE) could have a beneficial effect on memory consolidation. We first established the effect of EE in control rats by comparing performance in rats given 14 days of EE with home-caged rats (HC). We conducted 5 spatial recognition tests, the first prior to EE, and then 4 tests following enrichment. Prior to enrichment both groups show equivalent memory consolidation ( $t=0.74$ ;  $p>0.05$ ; Fig 7A). Thirty-one days following enrichment we found both groups also showed good memory consolidation, significantly above chance level, and were not significantly different from each other ( $t=0.75$ ;  $p>0.05$ ). As spatial memory 24 hours after the sample phase in HC rats was relatively high, it suggests the potential beneficial effect of EE may be masked. Therefore we conducted another test 10 days later where we made the task more difficult, by increasing the delay between the sample and test phase to 72 hours. Here we found that the performance of home-caged rats; although greater than chance level ( $t=11.45$ ;  $p<0.001$ ; Fig 7A) was significantly poorer compared with those that were given EE ( $t=2.64$ ;  $p<0.05$ ). We conducted a second test with a 24 hr delay, 60 days post EE, and again both groups performed equally well ( $t=0.38$ ;  $p>0.05$ ).



**Figure 7. The effect of environmental enrichment (EE) on control and STZ-CD treated rats infused with A $\beta$ :** (A) An initial experiment determines whether EE impacts on home-caged (HC) rats (white bars) and rats exposed to EE (stippled bars) at different times following EE. Time-delay between the sample and test phase is either 24 hr or 72 hr (red asterisk). (B) Represents the time line of experimental testing for the effect of EE on STZ-CD treated and control rats. (C) Represents memory capacity in control rat infused with A $\beta$  or not; prior to and at different time points after EE. (D) Represent STZ-CD treated rats infused with A $\beta$  or not at the same time points as control rats. Hatched bars represent either control rats or STZ-CD treated rats that are infused with A $\beta$ . Data are represented as mean $\pm$ SEM; \* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ .

And finally, a second test with a 72 hr delay, 95 days post EE to test the duration of the effect of EE and again, the memory performance in home caged rats dramatically dropped compared with that of the enriched group ( $t= 3.59$ ;  $p<0.01$ ). Together these data show that that the beneficial effect of EE is long-lasting.

As the effect of A $\beta$  on memory consolidation was only prolonged in STZ-CD treated rats; we focused on this treatment (STZ-CD, STZ-CD+A $\beta$ ) and controls (Ct, Ct+A $\beta$ ). We conducted 5 tests; 12 days following infusion of A $\beta$  and prior to EE; and the others at different time points following EE (see time line in Fig 7B). In the first test prior to EE, both control ( $t=6.71$ ;  $p<0.0005$ ) and STZ-CD treated rats ( $t=3.2$ ;  $p<0.05$ ) that were infused with A $\beta$  showed poorer performance compared with their non-infused counterparts; as we have shown previously (see Fig 6C). Four days following EE at a time point when A $\beta$  still impairs memory, the control animals infused with A $\beta$  performed as well as their non-infused counterparts ( $t=1.56$ ;  $p>0.05$ ; Fig 7C). In STZ-CD treated rats we found those infused with A $\beta$ , although they had significantly poorer performance than their non-infused counter parts ( $t=4.21$ ;  $p<0.01$ ) were significantly better than in their previous test ( $t=2.83$ ;  $p<0.05$ ). The deficit compared with non infused STZ-CD treated rats was due to the fact that non-infused STZ-CD treated rats also benefited from EE as their memory capacity was significant better than their performance on the previous test ( $t=3.99$ ;  $p<0.01$ ; see Fig 7D).

As the time of testing was relatively close to the end of the time window in which A $\beta$  impaired memory performance in control rats, we conducted at test with a 72-hour delay, approximately 2 weeks later. Non-A $\beta$  infused control rats showed normal memory capacity as shown previously (see Fig 7A); however, with the longer delay A $\beta$  induced a deficit in memory performance; although not statistically significant when compared with non-infused controls ( $t=2.18$ ;  $p=0.07$ ) and their performance in the previous test ( $t=2.52$ ;  $p<0.05$ ); suggesting that even though memory consolidation over a 24 hour delay has recovered in control rats infused with A $\beta$ , either due to the time window or to EE; when the demand on memory is made more severe there is a residual effect of A $\beta$ . As expected memory performance in both STZ-CD treated rats whether infused with A $\beta$  or not; deteriorated to chance level (see Fig 7D).

A further test with a 24-hour delay was conducted; principally to determine whether the beneficial effect of EE on memory with a 24-hour consolidation period was maintained in the STZ-CD treated rats infused with A $\beta$ . This test was conducted 39 days following EE and memory performance in rats treated with STZ-CD, whether infused with A $\beta$  or not, had fallen back to about the same levels as that observed prior to enrichment with no significant difference between the two tests in STZ-CD treated rats ( $t=1.47$ ;  $p>0.05$ ) and those infused with A $\beta$  ( $t=0.47$ ;  $p>0.05$ ).

A final test with a 72-hour delay was conducted only in control rats at a time when we have previously shown that the effect of A $\beta$  on memory consolidation had washed out (67 days post infusion and 45 days post EE). Indeed we found the performance level in this group was now no longer different from non-infused control rats ( $t=1.56$ ;  $p>0.05$ ; Fig 7C) and was substantially better, although not significantly so from the previous test with a 72-hour delay ( $t=1.2$ ;  $p>0.05$ ), confirming the limited time window in which A $\beta$  can elicit

a deficit; however, it further suggests that task difficulty also plays an important role in the impact A $\beta$  can have on memory performance. In contrast in STZ-CD treated rats whether infused with A $\beta$  or not the beneficial effect of EE is more limited.

### ***Regulation of the Akt signaling pathway and associated proteins***

We conducted western-blotting analysis in CA1 on the expression and phosphorylation of the key proteins in the PI3K-Akt signaling pathway that are associated with a number of dysfunctions common to both T2DM and AD. To address this, we organized the analyses in a series of logical steps. All values of proteins examined are presented in Table 1 and sample blots for some proteins are shown in Supplementary Figure 2.

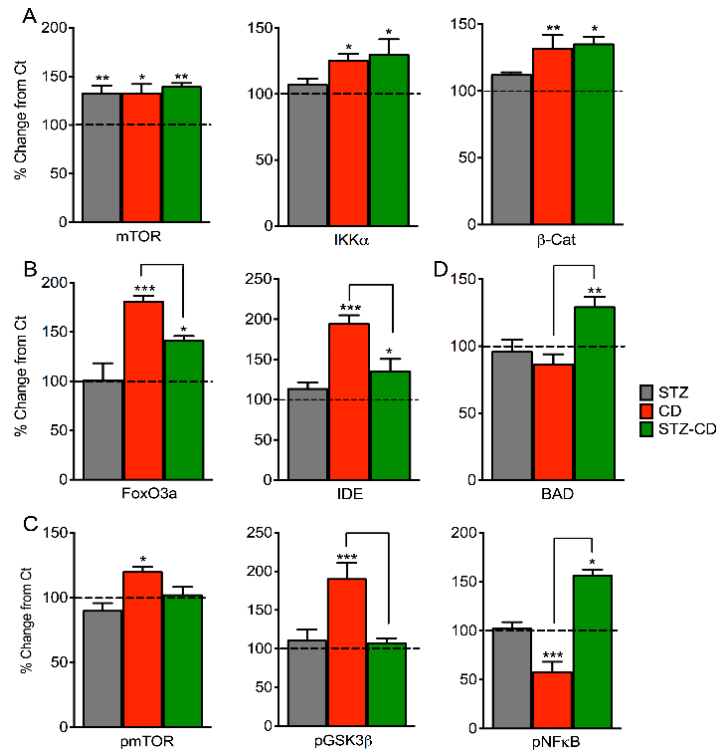
#### *T2DM phenotype induced dysregulation of proteins*

We first asked whether the T2DM phenotype induced lasting changes in expression or phosphorylation of proteins. Compared with controls, we found changes in certain key proteins directly associated with Akt signaling in STZ-CD treated rats. These include an increased expression of mTOR (t=3.0; p<0.01), BAD (t=3.28; p<0.01), FoxO3 (t=2.48; p<0.05), and IKK $\alpha$  (t=2.43; p<0.05); and proteins downstream of direct targets of Akt, such an increase in expression of  $\beta$ -Catenin (t=2.33; p<0.05) and phosphorylation of pNF $\kappa$ B (t=3.74; p<0.01). Finally, there was an increase in expression of IDE (t=3.01; p<0.01; see Table 1, panel A). We then asked whether the component parts of the T2DM phenotype; STZ and CD independently or in combination, regulated the same proteins. We found STZ injections alone had very little effect; only an increase in expression of mTOR (t=3.05; p<0.01; see Table 1, panel B). In contrast a number of proteins regulated by STZ-CD treatment were also regulated by CD alone; such as mTOR (t=2.75; p<0.05), IKK $\alpha$  (t=2.49; p<0.05), and  $\beta$ -Catenin (t=2.84; <p0.01; Fig 8A) to approximately the same level as in the T2DM phenotype.

The increase in expression of FoxO3 and IDE in the CD group however was significantly greater than that observed in the T2DM phenotype (FoxO3: t=4.79; p<0.01; IDE: t=3.22; p<0.05; Fig 8B). Importantly, however, some proteins were regulated by CD alone that were not regulated in STZ-CD treated rats; this was mainly hyperphosphorylation of pmTOR (t=2.52; p=0.05), pGSK3 $\beta$  (t=3.29; p<0.05) and as opposed to the increase in pNF $\kappa$ B seen in STZ-CD treated rats; there was a decrease with CD alone (t=7.15; p<0.0001; see Table 1, panel C and Fig 8C).

In general, STZ-CD treatment resulted in an increase in expression and/or phosphorylation of the proteins examined. Only the increased expression of BAD could be attributed solely to the T2DM phenotype (F(1,2)=5.43; p<0.05; Fig 8D); most of the changes could be potentially attributed to CD alone as STZ alone had little effect on proteins. This may be explained by the fact that injections were made some 27 weeks prior to analyses of proteins, and evidence suggests that STZ induced diabetes may not be stable over a long period of time (Goyal et al. 2016), possibly due to low level regeneration of  $\beta$ -cells (Afelik and Rovira, 2016).





**Figure 8. Proteins regulated in the STZ-CD group and effect of STZ and CD alone:** All groups are represented, but only when there is a significant difference from control levels of at least one group, indicated by \* p<0.05; \*\* p<0.01; \*\*\* p<0.0001 (see Table 1 for details). (A) Shows equal contribution of STZ and CD to the levels of mTOR in STZ-CD group. (B) Shows the significant elevation in FoxO3a and IDE with STZ-CD treatment is due to that regulated by CD alone. (C) Shows regulation of pmTOR and pGSK3 $\beta$  in CD in the absence of change with STZ-CD treatment and an opposing effect with pNF $\kappa$ B. (D) BAD is solely regulated by STZ-CD treatment. Data represents % change from controls indicated by the bar at 100%. ANOVA shows significant group differences and Tukey post hoc analyses indicate with bars that the differences are between CD alone and STZ-CD treatment.

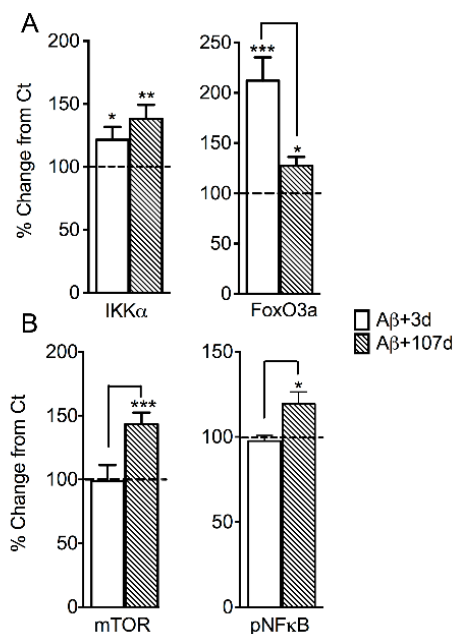
As CD was maintained until the end of the experiment, it is not surprising that the majority of changes could be attributed to CD in the T2DM phenotypic group. However, some changes specific to CD that were not observed with STZ-CD treatment are difficult to explain. One possibility is to suggest that the treatments alone regulate proteins differentially, and even in the absence of any change induced by STZ injections alone, in conjunction with CD may alter regulation mediated by CD alone. Another potential explanation is that, as we suggested, based on the difference in insulin levels in CD alone and STZ-CD, it may reflect a different stage in the evolution of T2DM.

#### *Effect of A $\beta$ alone on protein regulation*

As the effect A $\beta$  had on memory consolidation was temporally restricted we analysed proteins at 2 time points; one 3 days following the end of infusion; and the other at the end of the experiment to determine whether there was evolution of the effect A $\beta$  alone.

At the early time point (3 days-post infusion) A $\beta$  induced a number of changes in expression or phosphorylation of proteins (see Table 1, panel D), but for the majority of these changes they were only

temporary as they had returned to control levels by the end of the experiment (see Table 9 panel E; Fig 9A). Only the increase in expression of IKK $\alpha$  (t=2.26; p<0.05) and FoxO3a (t=7.15; p<0.0001) was maintained until the end of the experiment; albeit for FoxO3a the expression was significantly lower at the end of the experiment compared with that observed at the earlier time point (t=3.43; p<0.01). In contrast the increased expression of mTOR (t=4.27; p<0.001) and phosphorylation of NF $\kappa$ B (t=2.09; p<0.05) was specific to the late time point (see Fig 9). In summary, the majority of changes induced by A $\beta$  alone were not enduring and may have had some influence on the temporary deficits in memory consolidation. We cannot, however dismiss the possibility that early changes induced by A $\beta$  may contribute to longer term alterations in the T2DM phenotype or the independent component aspects of the phenotype. Importantly those changes in FoxO3a and IKK $\alpha$  and the late regulation of mTOR and pNF $\kappa$ B are similar to those observed in the T2DM phenotype.

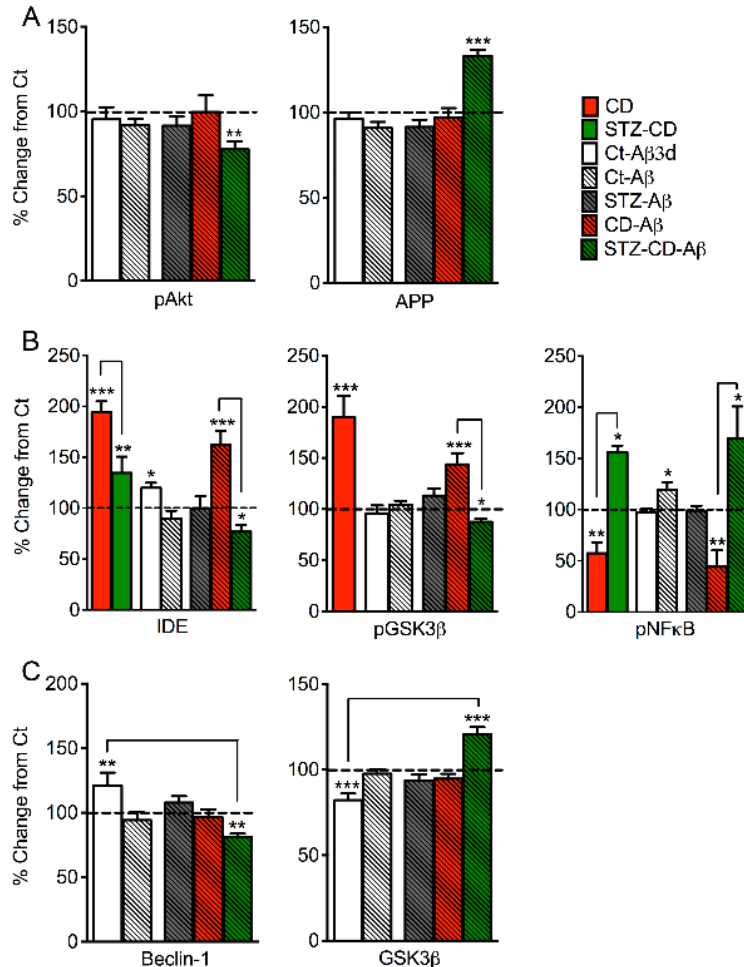


**Figure 9. The effect of infusion of A $\beta$  alone on protein regulation:** Protein regulation was analysed at 2 different time points; either 3 days (white bars) or 107 days (hatched bars) following the end of infusion of A $\beta$  (see Table 1 for details). Only proteins that were regulated at the end of the experiment are represented in the figure (for those represented at 3 days post infusion that contributed to protein regulation induced in the STZ-CD group infused with A $\beta$ , see figures 10B and C; and 11B and C). (A) shows proteins regulated at day 3 that were significantly elevated at the end of the experiment. (B) shows proteins regulated only at the later time point. Data represents % change from controls indicated by the bar at 100%. Significant changes from control levels are indicated by \* p<0.05; \*\* p<0.01; \*\*\* p<0.0001 and Students t-Test shows significant differences between the 2 time points indicated by bars.

#### *Effect of the T2DM phenotype on A $\beta$ regulation of proteins*

As our hypothesis was that the T2DM phenotype would exacerbate a temporary effect induced by A $\beta$ , we first examined protein changes in STZ-CD treated rats infused with A $\beta$ . This group induced more protein regulation compared with any of the other groups; and similar regulation of these proteins has also

been observed in the brains of AD patients and rodent models of the disease (see Table 1, panels F and I). When we compared regulation of expression and phosphorylation of proteins with the other groups there were only two changes that we could attribute solely to infused A $\beta$ ; namely a decrease in phosphorylation of Akt ( $t=3.61$ ;  $p<0.01$ ) and an increase in expression of APP ( $t=4.22$ ;  $p<0.001$ ; see Fig 10A) as none of the other treatments alone or in conjunction with A $\beta$  induced significant changes compared with control rats. Other changes in proteins in this group were mediated by a number of layers of complexity via the different treatments.



**Figure 10. Proteins regulated by STZ-CD treatment and infusion of A $\beta$ :** All proteins significantly regulated in this group compared with control rats are represented. Levels of protein induced by A $\beta$  (both time points) and in conjunction with STZ alone and CD alone are also represented. Non-A $\beta$ -infused STZ and CD alone groups are only represented when they show a significant change from control levels. (A) pAKT and APP are the only 2 proteins that are specifically regulated by STZ-CD+A $\beta$  treatment. (B and C) represent changes induced specifically by STZ-CD+A $\beta$ , but CD drives opposing regulation. Note the increase in phosphorylation of NF $\kappa$ B is driven by the non-A $\beta$  infused T2DM phenotype. (C) Opposing regulation of protein expression mediated via infusion of A $\beta$  measured 3 days post infusion. Data represents % change from controls indicated by the bar at 100%. Significant changes from control levels are indicated by \*  $p<0.05$ ; \*\*  $p<0.01$ ; \*\*\*  $p<0.0001$ . ANOVA shows significant group differences and Tukey post hoc analyses indicate differences between specific treatments by the bars.

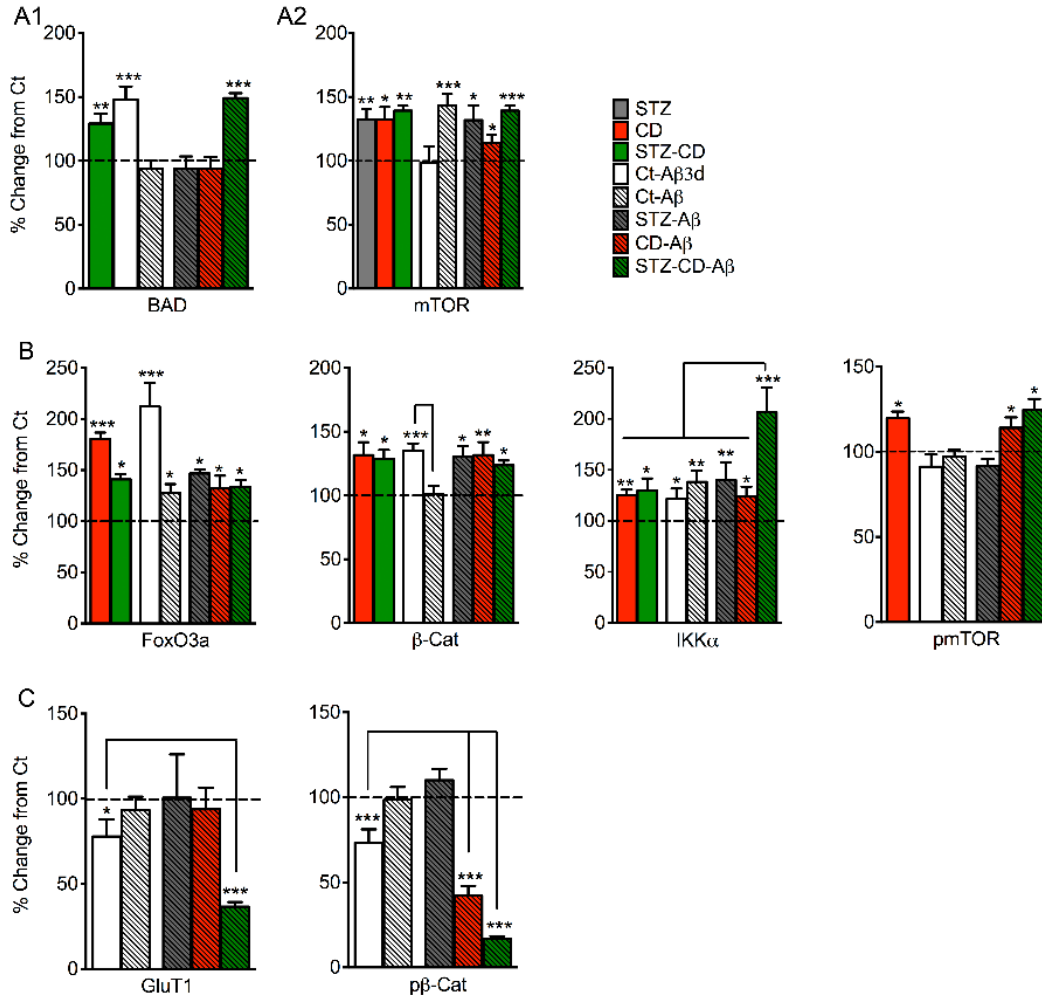
First we found the regulation of certain proteins was specific to the T2DM phenotype infused with A $\beta$ , but other treatments alone induced opposite regulation of the same proteins. For example, the decrease in expression of IDE (t=2.74; p<0.05), and phosphorylation of GSK3 $\beta$  (t=2.62; p<0.05) and the increase in pNF $\kappa$ B (t=3.0; p<0.001) were accompanied by an increase in IDE (t=9.76; p<0.0001) and pGSK3 $\beta$  (t=9.77; p<0.0001) and a decrease in pNF $\kappa$ B (t=3.14; p<0.01) induced by CD alone. This was specific to CD treatment as there was no significant difference in these protein levels in the corresponding group infused with A $\beta$  (IDE: t=1.8; p>0.05; pGSK3 $\beta$ : t=1.97; p>0.05; pNF $\kappa$ B: t=0.68, p>0.05; see Fig 10B).

We also found a similar opposing effect with A $\beta$  in expression of Beclin-1 and GSK3 $\beta$ . Whereas the phenotype infused with A $\beta$  induced a decrease in Beclin-1 (t=3.0; p<0.01) and an increase in GSK3 $\beta$  (t=6.54; p<0.001); infusion of A $\beta$  alone induced an increase in Beclin-1 (t=2.93; p<0.001) and a decrease in GSK3 $\beta$  (t=3.61; p<0.0001; see Fig 10C). However, regulation by A $\beta$  was observed at the early time point and these changes were short lasting, suggesting it has no impact on the effect observed in the phenotype infused with A $\beta$  at the later time point.

Although CD alone and A $\beta$  alone mediated opposing regulation of proteins specific to the phenotype infused with A $\beta$ , for the regulation of some proteins they appeared to be driving the changes observed in the T2DM phenotype infused with A $\beta$ . First we found the increase in expression of BAD may be attributed to the T2DM phenotype. Even though there was a substantial increase mediated by A $\beta$  observed at the early time point it was not lasting (see Fig 11A1). In contrast STZ, CD and infusion of A $\beta$  increase expression of mTOR in a parallel but independent manner (Fig 11A2).

CD accounts solely for the increase in phosphorylation of mTOR (F<1) and is a driving force for increasing the expression of FoxO3 (F(1,6)=6.29; p<0.0007);  $\beta$ -Catenin (F(1,6)=3.62; p<0.001) and IKK $\alpha$  (F(1,6)=4.21; p<0.01); however, infusion of A $\beta$  may have a contributory effect as it at least increases levels of the same proteins in the group injected with STZ. Of particular importance is the increase in expression of IKK $\alpha$  as it is increased by CD alone and A $\beta$  alone but is substantially elevated in the combined treatment compared with the independent treatments (see Fig 11B).

Finally, we found infusion of A $\beta$  alone strictly observed at the early time point may induce the decrease in expression of GluT 1 (t=3.2; p>0.5) and in conjunction with CD to elicit the decrease in phosphorylation of  $\beta$ -Catenin (F(1,4)= 15.19; p<0.0001) observed in the T2DM phenotype infused with A $\beta$  (Fig 11C).



**Figure 11. Positive contributory effects of separate treatments to protein regulation in STZ-CD+A $\beta$  treated rats:** All proteins significantly regulated in this group compared with control rats are represented. Levels of protein induced by A $\beta$  (both time points) and in conjunction with STZ alone and CD alone are also represented. Non-A $\beta$ -infused STZ and CD alone groups are only represented when they show a significant change from control levels. (A1) Increase in expression of BAD is driven by non-A $\beta$  infused phenotype as with pNF $\kappa$ B (Fig 10B), even though there is an increase in the A $\beta$  alone group at 3 days post infusion, this is not sustained and has no effect in STZ and CD alone groups. (A2) mTOR expression is driven by non-A $\beta$  infused STZ and CD alone in parallel. (B) Expression of FoxO3a,  $\beta$ -Catenin and pmTOR are driven by non-A $\beta$  infused CD and other treatments exacerbate the CD-induced increase in the expression of IKK $\alpha$  in STZ-CD+A $\beta$  treatment. (C) Decreased expression of GluT-1 and p $\beta$ -Catenin at the early time point after infusion of A $\beta$  is exacerbated by STZ-CD+A $\beta$  treatment. Data represents % change from controls indicated by the bar at 100%. Significant changes from control levels are indicated by \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.0001$ . ANOVA shows significant group differences and Tukey post hoc analyses indicate differences between specific treatments by the bars.

In summary, although there were numerous proteins regulated in the T2DM phenotypic group infused with A $\beta$ , few were specific to the group, the phenotype alone or infusion of A $\beta$  alone, which constitute the key effectors of the disease process. We did find certain changes in expression or phosphorylation of proteins specific to the T2DM phenotype infused with A $\beta$  in terms of the direction of change but these were accompanied largely by opposing effects mediated either by CD or by A $\beta$  at an early stage. When we

deconstructed the phenotype by analysing the effects mediated by STZ and CD independently, we found that many of the effects observed with the T2DM phenotype infused or not with A $\beta$  were mediated by CD alone. STZ injections alone had very little effect on the regulation of proteins, however when infused with A $\beta$  certain proteins were regulated in a similar manner to that seen in the T2DM phenotype with A $\beta$ .

**Table 1. Representation of all protein changes as a percent change from control rats**

	A	B	C	D	E	F	G	H	I	J
	STZ-CD	STZ	CD	A $\beta$ +3 days	A $\beta$	STZ-CD+A $\beta$ 42	STZ-A $\beta$	CD-A $\beta$	AD/models	Ref
Akt	101.7+/-3.1	104.3+/-6.2	105.9+/-3.1	97.7+/-2.8	105.9+/-2.0	108.9+/-4.6	105.9+/-7.0	110.+/-0.8	↓	[1][2]
pAkt	110.1+/-8.1	102.0+/-9.5	91.2+/-5.3	95.4+/-7.1	91.85+/-3.8	77.6+/-4.7* ↓	89.1+/-6.5	99.7+/-9.8	↓	[3]
mTOR	139.2+/-3.9** ↑	132.5+/-8.0** ↑	132.0+/-5.6* ↑	98.7+/-12.0	143.4+/-9.0*** ↑	159.2+/-9.3*** ↑	131.8+/-11.5* ↑	152.7+/-8.7* ↑	↑	[4]
pmTOR	101.8+/-6.3	89.7+/-6.0	119.6+/-3.9* ↑	91.2+/-7.1	97.0+/-4.1	124.7+/-6.3* ↑	91.6+/-4.1	114.0+/-6.2* ↑	↑	[5]
Beclin	93.8+/-0.5	114.8+/-8.4	91.1+/-6.1	121.1+/-9.9** ↑	94.3+/-6.2	81.8+/-2.8** ↓	115.4+/-7.6	96.5+/-6.1	↓	[6]
GSK3 $\beta$	104.4+/-11.8	100.2+/-4.9	106.4+/-4.5	82.2+/-3.9*** ↓	97.6+/-2.6	120.8+/-4.1* ↑	93.8+/-3.3	101.3+/-1.6	↑	[7]
pGSK3 $\beta$	107.2+/-6.3	110.4+/-12.2	190.1+/-9.0*** ↑	95.7+/-8.02	104.3+/-3.9	87.5+/-3.2* ↓	115.7+/-17.5	143.8+/-10.9*** ↑	↓	[3][8]
$\beta$ -Catenin	128.8+/-6.9* ↑	110.7+/-4.9	131.7+/-10.1** ↑	135.0+/-5.7*** ↑	101.3+/-6.2	123.7+/-3.7* ↑	130.4+/-8.3* ↑	134.2+/-1.9** ↑	↓	[8]
p $\beta$ -Catenin	108.6+/-8.2	83.9+/-9.5	88.1+/-24.6	73.3+/-7.8*** ↓	98.6+/-7.4	16.9+/-1.4*** ↓	110.1+/-6.6	42.2+/-5.6*** ↓	↑	[8]
BAD	123.7+/-10.4** ↑	99.8+/-12.5	86.2+/-7.9	148.0+/-10.2*** ↑	93.7+/-6.4	149.8+/-4.1*** ↑	93.7+/-9.8	94.8+/-9.2	↑	[8]
pBAD	90.8+/-1.71	104.2+/-6.8	105.4+/-7.0	93.4+/-9.1	106.7+/-4.7	102.2+/-9.3	108.9+/-8.0	115.0+/-2.7		
FOXO3a	141.0+/-4.9* ↑	103.9+/-18.8	180.6+/-6.1*** ↑	212.2+/-23.1*** ↑	127.5+/-5.2** ↑	133.8+/-6.5* ↑	146.8+/-3.7* ↑	132.1+/-12.5* ↑	↑	[9]
Caspase 7	89.7+/-2.6	90.9+/-6.2	97.2+/-2.6	112.9+/-8.7* ↑	106.9+/-3.3	103.0+/-5.0	95.16+/-7.6	100.7+/-11.1		
Caspase 9	87.5+/-0.5	106.6+/-9.4	108.5+/-4.2	87.9+/-4.2* ↓	93.6+/-3.0	98.26+/-3.2	90.6+/-5.3	101.1+/-5.3		
C-Caspase 9	97.7+/-7.4	96.3+/-6.0	108.7+/-10.1	108.8+/-10.5	102.9+/-5.7	92.7+/-7.9	105.6+/-0.5	100.1+/-6.7		
IDE	134.6+/-15.9** ↑	113.3+/-8.3	194.1+/-10.8*** ↑	120.0+/-4.9*** ↑	94.8+/-6.4	76.83+/-6.7* ↓	99.5+/-12.3	161.9+/-14.3*** ↑	↓	[10-13]
APP	90.0+/-4.5	93.4+/-5.5	93.3+/-6.7	96.4+/-3.6	90.9+/-3.6	133.2+/-3.7*** ↑	91.5+/-4.4	96.96+/-5.5	↑	[3][4][14]
NF $\kappa$ B	98.1+/-5.9	102.5+/-4.4	94.2+/-15.6	102.8+/-3.8	94.6+/-7.7	90.2+/-5.3	98.5+/-17.8	100.4+/-18.5		
pNF $\kappa$ B	156.1+/-6.3* ↑	102.1+/-6.7	57.4+/-10.8** ↓	97.58+/-3.3	119.3+/-7.1* ↑	169.8+/-20.9* ↑	98.7+/-4.88	44.1+/-16.2** ↓	↑	[4]
IKK $\alpha$	129.8+/-11.9* ↑	106.9+/-4.8	125.1+/-5.3** ↑	121.6+/-10.2* ↑	138.2+/-11.0** ↑	206.6+/-24.3*** ↑	140.1+/-17.4** ↑	124.3+/-9.1* ↑		
Glu-T1	94.4+/-21.0	92.6+/-6.1	105.2+/-9.3	77.8+/-10.1* ↓	93.2+/-7.9	36.5+/-2.8** ↓	100.5+/-25.5	93.8+/-12.7	↓	[15-17]

Analyses were conducted individually with Students t-Test in each condition (panels A-H). Arrows indicate the direction of change and asterisks indicate the level of significance; \*p<0.05; \*\*p<0.01; \*\*\*p<0.001. Panel (I) indicates changes and the direction of change in human studies and experimental models. (j) References: 1. Liu et al., 2011; 2. Chen et al., 2013; 3. Steen et al., 2005; 4. Guillot et al., 2016; 5. Griffin et al., 2005; 6. Pickford et al., 2008; 7. Hye et al., 2005; 8. Jimenez et al., 2011; 9. Shi et al., 2016; 10. Cook et al., 2003; 11. Vepsäläinen et al., 2008; 12. Čaušević et al., 2010; 13. Candeias et al., 2012; 14. Hiltunen et al., 2012; 15. Simpson et al., 1994; 16. Mooradian et al., 1997; 17. Liu et al., 2008. All references refer to studies in brains from AD patients with the exception of 2, 4, 8, 9, 11 and 14, that are conducted in AD transgenic mice.

## Discussion

The aim of our experiment was to identify whether proteins in-or associated with the PI3K signaling pathway may have a causal role in inducing a mild dysfunctional neuronal environment, which would exacerbate an effect induced by infusion of low dose A $\beta$ . Furthermore, as our main focus was on dysfunctional regulation of proteins, and we were unsure whether our T2DM model would induce elevated levels of A $\beta$  we chose to infuse a low dose of A $\beta$ 42 to address whether the T2DM phenotype would exacerbate an effect induced by A $\beta$  alone; thereby suggesting it as a risk factor for developing early AD-type dysfunction. To this end we attempted to mimic the human condition as closely as possible by

developing a model of T2DM coupling subdiabetogenic doses of STZ with human junk food; and conducting longitudinal studies. To our knowledge, a model such as this has not been reported in the literature previously.

Our model showed characteristic dysfunctions associated with T2DM; namely the development of hyperglycemia, hyperinsulinemia and subsequent  $\beta$ -pancreatic cell failure. Importantly, we found a hierarchical effect, where STZ alone induced the least dysfunction; followed by CD alone, which induced hyperinsulinemia in the absence of hyperglycemia; and STZ-CD treatment which induced an early state of hyperglycemia and hyperinsulinemia, followed by a later stage of hyperglycemia and  $\beta$ -pancreatic cell failure. This finds support in reports showing STZ injections alone do not induce insulin resistance or weight gain; are less stable and potentially reversible (Goyal et al., 2016; King et al., 2016). In contrast, high-energy diets induce weight gain, insulin resistance and lack of hyperglycemia (Reuter, 2007; Harri and Thibault, 2010). Studies coupling subdiabetogenic doses of STZ with high fat diet have reported mild weight gain, insulin resistance and hyperglycemia (Reuter, 2007; King et al., 2016; Skovso, 2014). Our model has two major advantages over the others inasmuch as we used human junk food over calibrated high energy pellets that mimics the human condition more faithfully; and, in the absence of frank obesity it suggests that the quality of processed human junk food might make a more detrimental contribution to T2DM than merely quantity of food intake. Secondly the duration of our experimental protocol exceeds those previously reported and with this we were able to see the evolution of T2DM characteristics; most importantly being that STZ-CD treated rats at an early stage showed a similar profile to that of CD at the late stage; but evolved to develop late stage T2DM characterized by  $\beta$ -pancreatic cell failure (Srinivasan and Ramarao, 2007; King et al., 2016; Skovso, 2014).

Despite the hierarchical effect of STZ-alone, CD-alone and the combined STZ-CD treatments we found STZ and CD independently induced a modest but remarkably stable deficit in spatial recognition memory across the entire testing period. Several studies in humans with T2DM have reported that cognitive dysfunction can vary from subtle executive dysfunction to moderate memory deficits (see Barbagallo and Dominguez, 2014; Kirkman et al., 2012). Rodent studies of obesity and T2DM have shown deficits in hippocampal dependent tasks such as spatial learning and memory and recognition memory (see McNay et al., 2010; Reagan et al., 2012; Petrov et al., 2015; Corder and Tamashiro, 2015); however, it is not clear what the degree of severity of the deficit was. What we found surprising, was that although STZ alone had a minimal impact on developing T2DM and protein regulation in CA1; it had an equivalent effect on memory to that observed in rats fed CD alone or the combined treatment. We can only argue that it may be mediated via mechanisms not examined in our experiment. Given that the increase in expression of mTOR was common to all treatments it suggests it may play a key role in mediating the deficits in memory.

The major effect of interest was whether dysfunctional neural mechanisms induced by T2DM could exacerbate a short-lasting effect induced by A $\beta$ 42. Functionally we found A $\beta$  alone induced a deficit in recognition memory within a specific time window of approximately 7 weeks following infusion. Numerous

studies have shown that soluble A $\beta$  induces deficits in learning and memory; including spatial learning and memory (Sipos et al., 2007; Eftekharzadel et al., 2012; Chang et al., 2015; Morroni et al., 2016) and recognition memory (Sipos et al., 2007; Souza et al., 2016; Morroni et al., 2016). However, there is a degree of variability in the results that is dependent on the source and type of amyloid species and the route of administration; type of learning and memory processes tested; and the time at which testing was conducted following administration (Selkoe, 2008; Ghasemi et al., 2014). The major point of importance in our study is that we repeatedly tested rats and established (a) the effect of A $\beta$  was not long lasting which suggests it is eliminated from the brain relatively rapidly, and (b) the time window in which a deficit in memory was observed. Most other studies have not tested effects later than approximately 3 weeks (Nabeshima and Nitta, 1994; Terranova et al., 1996; Malin et al., 2001; Sipos et al., 2007; Eftekharzadeh et al. 2012; Chang et al., 2015, Morroni et al., 2016; Nassireslami et al., 2016).

Infusion of A $\beta$  in rats injected with STZ alone, and CD alone or the combined STZ-CD treatment turned a modest deficit in spatial recognition memory into complete loss of memory. However infusion of A $\beta$  alone also induced complete memory loss; and when it recovered, so did the memory loss induced by STZ and CD alone when infused with A $\beta$ , suggesting the deficit could only be attributed to A $\beta$ . This is also reinforced by the fact that the deficits observed when A $\beta$  is coupled with STZ alone and CD alone recover to the same levels as prior to infusion of A $\beta$ . Although STZ alone and CD alone induced a mild deficit in memory independent of each other; independent treatments were not sufficient to prolong the effect of A $\beta$  as did in the manner that the combined STZ-CD treatment. Thus only in combination does the treatment serve as a risk factor for memory deficits. Moreover, we found environmental enrichment had a beneficial effect on memory in the T2DM phenotype group infused with A $\beta$ ; however it was short-lasting.

To date few studies have examined whether T2DM can exacerbate early memory deficits in AD. Studies have shown high fat diet fed to either SAMP8 mice (Mehla et al., 2014) or transgenic mice (Ho et al., 2004; Cao et al., 2007; Herculano et al., 2013; Maesako et al., 2013; 2012a; 2012b) accelerates deficits in different learning and memory tasks. However, other studies feeding AD transgenic mice high fat diet have shown that either the memory deficits induced by a high fat diet are not specifically associated with AD pathology (Knight et al., 2014); or subsequent adjustment of weight to that of control levels is not sufficient to reverse the memory deficits (Maesako et al., 2012b). This suggests that the dietary regime could induce irreversible changes in brain function that may contribute to the memory deficits. The authors show that exercise has a more beneficial effect than reverting the dietary regime and Neprilysin, an A $\beta$  degrading enzyme, mediates this. However, as the authors did not assess regulation of other proteins known to be associated with the beneficial effects of exercise (Carro et al., 2000; Trejo et al., 2001; Bruel-Jungerman et al., 2009) or caloric restriction (Contestabile, 2009; Pani G, 2015), it is difficult to know exactly what mechanisms are modified by high fat diet. In our experiments we used environmental enrichment that couples social and sensory stimulation with mild exercise that has been shown to have a beneficial effect on memory performance in healthy rodents (Bruel-Jungerman et al., 2005; van Praag et al., 2000) and can mitigate cognitive deficits in AD transgenic mice (Arendash et al., 2004; Costa et al., 2007; Hu et al., 2010;



Valero et al., 2011; Wolf et al., 2006; Verret et al., 2013). In these experiments EE had a beneficial effect on memory for at least 4 months after EE; as opposed to the short lasting effect we found in our experiment. However, the duration of exposure to environmental enrichment may account for this difference; in many of these reports mice have lived in the enriched environment from weaning until the onset of cognitive deficits; and even in reports suggesting transient exposure to EE was used, it was 2 months (Verret et al., 2013) as opposed to 3 hours a day for 2 weeks in our experiment.

An integral part of our experiment was to gain insight into the potential mechanisms induced by T2DM that could promote a neuronal environment to favour the development of pathologies associated with AD. We found a number of dysfunctionally regulated proteins in-and associated with the PI3K-Akt signaling pathway. Most protein regulation was observed in the T2DM phenotypic group infused with A $\beta$  that could be described in a number of different interconnected hubs associated with different functions.

Firstly we found the only 2 proteins regulated that were specific to the T2DM phenotype infused with A $\beta$  were a decrease in phosphorylation of Akt and an increase in expression of APP. Akt was associated with decreased phosphorylation of GSK3 $\beta$  that was accompanied by an increase in expression of the constitutively active protein, suggesting the protein is abnormally regulated as is observed in AD patients (Blalock et al., 2004). Over expression of GSK3 $\beta$  in mice prevents induction of hippocampal LTP and impairs spatial learning (Hernandez et al., 2002; Hooper et al., 2007); suggesting it is an important mechanism mediating memory deficits in AD. In addition overexpression of GSK3 $\beta$  can hyperphosphorylate Tau (Lucas et al., 2001) and mediate A $\beta$  production from APP, by binding to PS1 (Phiel et al., 2003). In our experiments although we did not measure A $\beta$  levels, we found increased expression of APP, which could indirectly support aberrant processing of APP mediated via GSK3 $\beta$ . In support of this we also found a decrease in IDE levels suggesting less clearance of aberrantly processed A $\beta$  as observed in AD patients (Cook et al., 2003; Causevic et al., 2010), AD transgenic mice (Vepsalainen et al., 2008) and those fed high fat diet (Ho et al., 2004).

Under normal conditions constitutively active GSK3 $\beta$  phosphorylates  $\beta$ -Catenin to signal its degradation by proteosomal activity. However we found a decrease in GSK3 $\beta$  induced phosphorylation of  $\beta$ -Catenin with a corresponding increase in the expression of the protein, suggesting it is in an active state. In general, most studies suggest that  $\beta$ -Catenin is decreased in AD (Zhang et al., 1998; He and Shen, 2009) and, in cell cultures, activation of Wnt signaling increases  $\beta$ -Catenin in a manner that is neuroprotective (Garrido et al., 2002; Inestrosa et al., 2002; De Ferrari et al., 2014). However; regulation of  $\beta$ -Catenin is complex and not well understood; a number of different signaling events compete with each other to regulate  $\beta$ -Catenin (Stamos and Weis, 2013) and certain evidence does suggest in AD transgenic mice there is biphasic regulation of the expression of  $\beta$ -Catenin, showing an early increase followed by a later decrease with age (Jimenez et al., 2011).

Secondly, we found an increase in both expression and phosphorylation of mTOR, coupled with a decrease in Beclin-1 that together would imply inhibition of autophagy over protein synthesis. Under healthy

conditions autophagy is a mechanism to recycle proteins that requires less energy than the synthesis of new proteins and is associated with longevity (Salminen and Kaarniranta, 2009; Glick et al., 2010). In addition, it is a necessary mechanism for clearing misfolded proteins that is a common dysfunction in age related diseases (Menziés et al., 2011). Inhibition of autophagy is reflective of excess proteins and is dysfunctional in models of T2DM and AD where there is an increase in mTOR activity and/or a decrease in Beclin-1 (Groffo, et al., 2005; Khamzina et al., 2005; Pickford et al., 2008; Ma et al., 2013). Conversely, in PDAPP transgenic mice, long-term inhibition of mTOR increases autophagy and is accompanied by a reduction in A $\beta$  levels and improvement in learning and memory (Spilman et al., 2010).

Thirdly, we found an increase in expression and phosphorylation of proteins associated with apoptosis (BAD and FoxO3) and inflammation (IKK $\alpha$  and phosphorylation of NF $\kappa$ B) that are also activated in AD (Bamberger and Landreth, 2002; Obulesu and Lakshmi, 2014; Heppner et al., 2015). As we found a decrease in phosphorylation of Akt; and as it normally prevents BAD entering the nucleus to transcribe proapoptotic genes (Zhao et al., 2005), this confirms that BAD may be in an active state. However; we found no regulation of several caspases that reflect key end-stage mechanisms of apoptosis. Apart from acting as a proapoptotic transcription factor, FoxO3 is implicated in a number of other functions, such as autophagy by its interaction with mTOR and Beclin-1 (Greer et al., 2007; Brunet, 2015) but it has also been shown to increase levels of A $\beta$  and induce astrocytosis in AD transgenic mice (Shi et al., 2016). In contrast inactivation of FoxO3 attenuates spatial memory deficits and amyloid pathology in Tg2576 mice (Qin et al., 2008), suggesting FoxO3 is closely linked with early pathological changes in AD. Dysregulation of IKK/NF $\kappa$ B is commonly associated with inflammation and has been identified in AD (Tilstra et al., 2011), diabetes and obesity (Patel and Santini., 2009). However, it is also implicated in apoptotic and autophagic processes (Tilstra et al., 2011) and *in vitro* studies have shown that IKK $\alpha$  can phosphorylate mTOR in the TORC1 complex contributes to Akt dependent regulation of mTORC1; FoxO3 and GSK3 $\beta$  (Dan et al., 2014).

Finally, we found a severe decrease in Glu-T1; the principle transporter of glucose across the blood brain barrier suggesting a hypoglycemic state in the brain (Brant et al., 1993; Ngarmukos et al., 2001). It has been shown there is a progressive reduction in glucose metabolism in the brain that correlates with the severity of the disease in AD patients (Mosconi et al., 2006). In total the differential regulation of proteins we find in our T2DM phenotypic group infused with A $\beta$  share common dysfunctions observed in AD.

When we deconstructed the independent treatments of the T2DM phenotype and A $\beta$  alone to determine their contribution to protein regulation observed in the T2DM phenotype infused with A $\beta$ , we found the interactive effects to be highly complex. Our original expectation was that STZ injections alone and CD alone would have an additive effect in the T2DM phenotype. This in turn we anticipated would exacerbate an effect induced by A $\beta$  alone; however this hypothesis did not hold true. On the contrary we found no proteins to be regulated in this manner. Instead, we found a number of proteins to be associated either with CD alone or with A $\beta$  alone, that appear to be acting in a parallel but independent manner. This would not necessarily be surprising if one considers AD pathology to be multifactorial and can be elicited

by numerous independent mechanisms occurring in parallel at different stages or in different neural circuits (see Herrup, 2015). In addition, we found CD alone induced excess or opposing expression and phosphorylation of proteins compared with the T2DM phenotype that may reflect an earlier stage of T2DM. Moreover we found A $\beta$  alone could induce early changes in protein expression and phosphorylation that were not always sustained, but may be instrumental in driving changes seen at the later time point in the T2DM phenotype.

A clear limitation of our results is that although we have identified a number of deregulated proteins that would implicate the PI3K-Akt signaling pathway, we can only speculate from the literature how they may interact with each other to induced neural dysfunction. Moreover, we did not measure key signaling lesions associated with AD pathology, such as amyloid levels and phosphorylation of Tau. However, this was not the aim of our experiments and our data now guide us for future experiments. Thus, in summary, our model of T2DM induced characteristic features of the disease and a modest but stable deficit in spatial recognition memory. Infusion of a soluble form of A $\beta$ 42 alone induced a temporary deficit in memory; but on the T2DM background this was protracted. Moreover, the T2DM phenotype infused with A $\beta$  induced dysregulation of proteins involved in functions such as autophagy, inflammation, apoptosis, and glucose and insulin metabolism; known to be aberrantly regulated in AD. More importantly these changes were present in CA1 at a substantial length of time after infusion of A $\beta$  (5 months), supporting the notion that T2DM or component parts can promote a dysfunctional neuronal environment to promote pathology associated with AD.

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## Supplementary data

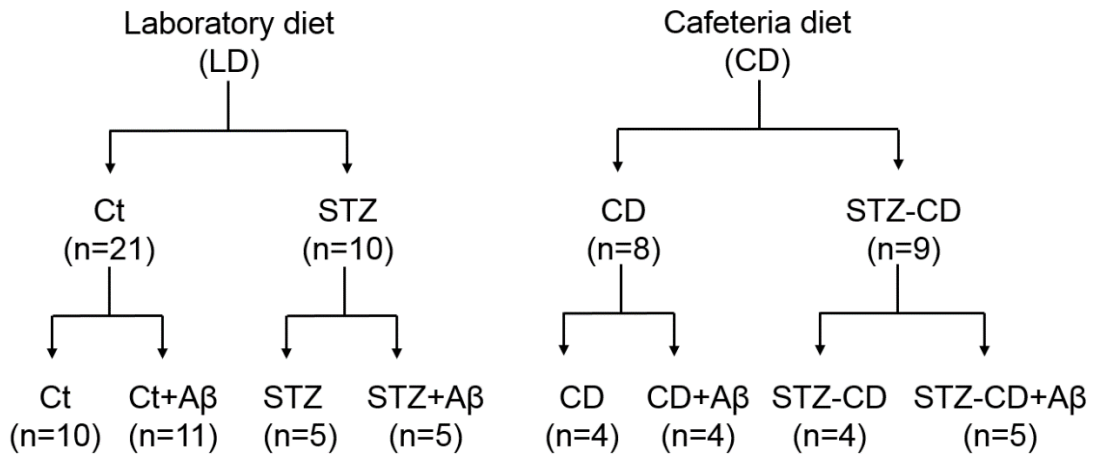
**Table 1: List of human 'junk' food given for cafeteria diet rats**

Food product		
Bacon	Fruit cake	Napolitain cake
Belin biscuits	Grated cheese	Pasta
Belin chips	Grissini sticks	Peanut butter
Belin Curves	Harrys bread	Pizza sandwich
Benenuts toasts	Jacquet bread	Popcorn
Breakfast biscuits	Ketchup	Processed bread
Buns	Kiri cheese	Ravioli
Butter	Lu biscuits	Rillette
Cereal Bars	Madeleine	Sausage
Chicken Nuggets	Marzipan	Smacks
Classic chips	Mayonnaise	sunflower seeds
Coca Cola	Mc chips	Sweet milk
Cream	Mini toasts	Trancetto cake
Croutons	Mini waffles	Tuc biscuits
Crusti Croc Flips	Muesli	

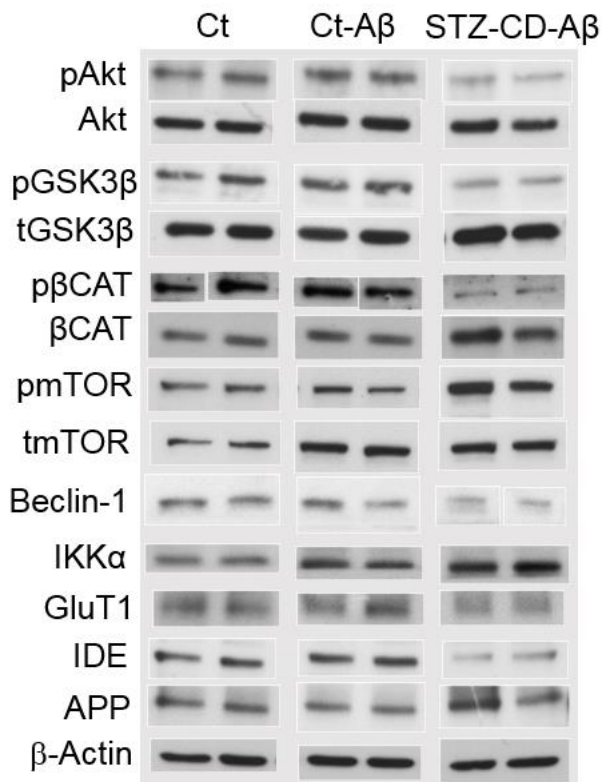
**Table 2. List of primary Antibodies for WB**

Antibody name	Supplier	Source	MW(kDa)	Dilution
$\beta$ -Actin	Sigma	mouse	42	1:10000
Akt	CST	rabbit	60	1:1000
pAkt (ser473)	CST	rabbit	60	1:1000
mTOR	CST	rabbit	289	1:1000
pmTOR (ser2448)	CST	rabbit	289	1:1000
Beclin-1	CST	rabbit	60	1:1000
GSK3 $\beta$	CST	rabbit	46	1:1000
pGSK3 $\beta$ (ser9)	CST	rabbit	46	1:1000
$\beta$ -Catenin	CST	rabbit	92	1:4000
p $\beta$ -Catenin (ser33/37)	CST	rabbit	92	1:1000
Bad	CST	rabbit	23	1:1000
pBad (ser136)	CST	rabbit	23	1:1000
FoxO3a	CST	rabbit	82	1:1000
Caspase7	CST	rabbit	35	1:1000
Caspase9	CST	rabbit	40/51	1:1000
IDE	Abcam	rabbit	118	1:1000
APP	CST	rabbit	100	1:1000
NF $\kappa$ B (p65)	CST	rabbit	65	1:1000
pNF $\kappa$ B (ser536)	CST	rabbit	65	1:750
IKK $\alpha$	CST	rabbit	85	1:1000
GluT1	Thermo	rabbit	55	1:1000

**Figure 1. Experimental groups and numbers of rats**



**Figure 2. Representative sample western-blots**



## General discussion

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### **Successful model of T2DM**

Several population studies and animal studies demonstrate a strong correlation and possible underlying links between type 2 diabetes mellitus (T2DM) and Alzheimer's disease (AD), many of which are linked via the PI3K-Akt signalling pathway (see reviews Li et al., 2015; Walker and Harrison, 2015; Mittal and Katare, 2016). However, the underlying mechanism of how T2DM leads to the development of AD is not clear. We developed a model to assess the impact T2DM may have on the development of AD. We modified an existing model, coupling low dose injections of Streptozotocin (STZ) with high fat diet, using STZ with cafeteria diet (CD). In order to see the individual effect of each treatment contributing to the model, we also had rats only fed with CD or injected with STZ. We found our STZ-CD treated rats showed hyperglycemia and impaired insulin levels in blood indicating a successful T2DM model.

Studies in rats fed a high fat/sugar diet to induce obesity coupled with low dose of STZ injection(s) could represent a good T2DM model; most of the studies used calibrated high fat diet (see Skovsø, 2014). However, in our study, rats were fed with processed food to induce obesity instead of calibrated high fat/sugar diet, which more closely mimics the development of the human disease. These foods are high in sugar and fat, and importantly the industrialized food enhances rewarding properties by manipulating salt, flavours and other food additives to make such foods more addictive (Cocores and Gold, 2009; Gearhardt et al., 2011; Carter et al., 2016).

Obesity is one of the major risk factors of T2DM. A diet rich in fat and sugar can easily induce obesity in humans and in animals as described in the introduction. After 35 weeks of cafeteria diet, our rats gained 25% more weight than the controls. In animal studies, there is no standard threshold for obesity such as body mass index (BMI) in humans. However, some studies suggest that around 10–25% more body weight over age-matched control rats fed laboratory diet is considered to be an indicator of “obesity” (Levin and Dunn-Meynell, 2002; Woods et al., 2003). We found that the food composition mainly contained carbohydrates, lipids and proteins, where carbohydrates comprised more than 50% of the total nutritional content. Interestingly, in human junk food, the percentage of sugar in carbohydrates is half, while the percentage of sugar was only 3.2% in normal animal diet. The main source of Carbohydrates is from food and they are responsible for postprandial glycemia and insulinemia and related to the etiology of many chronic metabolic diseases (Bao et al., 2011). We found that rats fed with cafeteria diet alone showed increased blood insulin levels, but no significant high glucose levels even at the end of the experiment, which may suggest insulin resistance with CD treatment alone. This result is consistent with data showing that high fat/caloric diet induces obesity and insulin resistance with hyperinsulinemia but rarely hyperglycemia (Dourmashkin et al., 2006; Templeman et al., 2017). However, rats for which CD and STZ injections were coupled showed both hyperglycemia and impaired insulin level.

STZ is widely used to induce diabetes in rodents (Radenković et al., 2016). The phenotype is dose dependent, where high dose injections can quickly induce T1DM, by impairing insulin secretion (for example 50 mg/kg body weight). In contrast, relatively low doses of STZ (25-50 mg/kg body weight) have been



shown to induce a mild impairment in insulin secretion; especially multiple low dose injections coupled with HF diet tends to induce T2DM (Skovsø, 2014). In our STZ injection model we used 30 mg/kg injections (twice, one week apart), a dose which has been reported to be able to induce stable hyperglycemia coupled with HF diet (Zhang et al., 2008). If compared with control rats, STZ injections alone induced mild increases in glucose level at the later time points, relatively higher insulin level but not significant at the end in our tests. This may be due to the fact that in general STZ induced diabetic rats develop hyperglycemia, primarily by direct cytotoxic action on the  $\beta$  pancreatic cells and insulin deficiency rather than as a consequence of insulin resistance (Srinivasan and Ramarao, 2007; King et al., 2016) and is less stable (Srinivasan and Ramarao, 2007). However, the most obvious stable hyperglycemia was shown in rats receiving the STZ-CD treatment, which was not observed in CD alone and STZ alone groups. A study using the same dose (30mg/kg) of STZ coupled with HF diet showed rats fed for 4 weeks with high fat diet had a 85% diabetic success rate and most importantly presented typical features of T2DM with insulin resistance, stable hyperglycemia and blood lipid disorders observed between 3 and 8 weeks following STZ injections (Zhang et al., 2008). Our STZ-CD rat model also showed hyperglycemia and impaired glucose tolerance 4 weeks after STZ injections, and showed stable hyperglycemia until the end of the experiment, 33 weeks after the start of cafeteria diet.

Importantly, STZ-CD rats showed hyperinsulinemia at an early time point (week 21), which is consistent with data showing that early stages of T2DM is associated with hyperinsulinemia; interestingly the increased insulin level was similar in the CD treatment alone group at late time point (week 35), which indicates that the combined treatment seems to accelerate or aggravate the development of the T2DM phenotype. However, STZ-CD rats showed hyperglycemia in parallel with relatively decreased insulin but not significantly different from controls at the end, suggesting the combined treatment may cause failure of  $\beta$ -cell to secrete sufficient insulin in response to the increased glucose level, leading to stable hyperglycemia and relative insulin deficiency as in the late stage of T2DM in rats (Skovsø, 2014). T2DM normally begins with insulin resistance, however as the disease progresses a lack of insulin may also develop, characterised by high blood glucose in the context of insulin resistance and relative insulin deficiency (Tripathy et al., 2012). Population based studies suggest in T2DM patients without obesity, mainly found in Asian countries such as Japan, China and Korea, the reduction in insulin secretion seems to be more important than the increase in insulin resistance. These defects may be caused by long-term hypersecretion of insulin in response to the intake of high caloric food owing to westernized food habits. The hypersecretion of insulin leads to depletion of the insulin stock and hence to the decline in insulin secretion from the  $\beta$  pancreatic cells (Nakamura et al., 2006).

In a study coupling HF diet with STZ injection (50 mg/kg, intravenously), prior to STZ injection, rats fed with HF diet for 2 weeks showed similar glucose level, but significantly higher insulin compared with rats fed with normal laboratory diet. Moreover, adipocyte glucose clearance was significantly reduced under maximal insulin stimulation, suggesting high fat diet induced insulin resistance (Reed et al., 2000), while STZ injection increased glucose and insulin levels and induced hyperglycemia with normal insulin function

compared with rats injected with STZ and fed normal diet 3 days after STZ injection (Reed et al., 2000). Studies show that the increase in plasma glucose can reach peak levels within 3 days after STZ administration and this is stable for at least 21 days and that insulin levels reach a peak after 24 hours and become stable 3 days after STZ, as reviewed recently (Goyal et al., 2016). However, in our experiment, the first glucose tolerance test was conducted 4 weeks after STZ injections. The STZ injections alone induced a mild increase in response to glucose compared with controls. This data is consistent with evidence showing severe hyperglycemia occurs 3 weeks after STZ injection, but only mild hyperglycemia has been observed 4 weeks following STZ injections in some studies (Ito et al., 2001; Ventura-Sobrevilla et al., 2011). But we also observed mild increased glucose level at later time points in STZ-injected rats, which suggests the STZ effect may still exist. However, in the STZ-CD treated rats we found the most obvious stable hyperglycemia across time, which was much higher than that induced by STZ alone, suggesting this combined CD and STZ treatment exacerbates glucose tolerance. Our insulin test was only conducted at the end of experiment; STZ alone induced a mild increase in blood insulin, which may be consistent with relative high glucose level in these rats. Importantly, some studies classified early and late stages of T2DM in STZ-HF induced models based on insulin level, of course they also show hyperglycemia. If the animals show higher glucose and insulin levels than controls, this is considered to mimic the early stage of T2DM; if their insulin levels are lower than, or the same as in controls, this mimics late stage of T2DM (Skovsø, 2014). Based on this classification, we conclude that rats treated with STZ-CD developed late stage T2DM by the end of the experiment.

Obesity is often associated with peripheral insulin resistance, while it is difficult to separate the phenomena of insulin resistance and hyperinsulinemia (Tabák et al., 2009). Insulin resistance is widely referred to as an impairment of glucose disposal in response to insulin stimulation (Biddinger and Kahn, 2006). Hyperinsulinemia refers to elevated basal and/or stimulated insulin secretion, which is nearly universal in the early stage of T2DM. Both insulin resistance and hyperinsulinemia are associated with impaired glucose tolerance. There is a hyperbolic relationship between insulin resistance and impaired insulin secretion, hyperinsulinemia has been considered to be a compensatory response to prevent elevated blood glucose when the body is failing to take in excess glucose due to conditions resulting from obesity (Shanik et al., 2008). While the mechanism of obesity induced insulin resistance is not clear, whether or not elevated insulin is a causal contributor to obesity and its associated complications remains unclear (Templeman et al., 2017).

### ***Does T2DM promote the development of memory deficits and AD?***

The next question we asked was whether T2DM could promote the development of AD; which is the link between these two diseases. To investigate this, half of the rats in each group were infused with soluble A $\beta$ 42 into the lateral ventricles to determine whether the T2DM phenotype would exacerbate the effect induced by A $\beta$  alone. This form of A $\beta$  alone does not form amyloid plaques and does not induce long-lasting

memory deficits. Throughout the experiments, spatial recognition memory, a hippocampal dependent memory task, was conducted regularly to test the onset and evolution of memory deficits.

We chose to use a spatial recognition memory task as it is susceptible to damage or dysfunction in the hippocampus (Vann and Albasser, 2011; Cordner and Tamashiro, 2015). Firstly, without A $\beta$  our STZ-CD treated rats showed consistent mild memory deficits throughout the whole period of the experiment, which could be mediated independently by injections of STZ alone or CD alone, rats in these groups also showed similar memory deficits. Our data are consistent with studies on learning and memory deficits in diet induced obesity models (Cordner and Tamashiro, 2015) and STZ induced diabetic model (Reagan, 2012) in several tasks, especially spatial memory. Few studies show learning and memory deficits in STZ-HF treated rats (Rahigude et al., 2012; Ma et al., 2013). The deficits were found either before the impairment of glucose and insulin levels (Kaczmarczyk et al., 2013) or after (Valladolid-Acebes et al., 2013). In our experiment, spatial memory deficits were seen after 4 month of CD feeding and 2 months after STZ injections in CD treated alone, STZ injected alone or STZ-CD treated rats. At this time point, we have observed increased glucose levels and impaired glucose tolerance in STZ-CD treated rats, but not in the CD treated alone rats or STZ alone injected rats, which indicates the memory deficits induced by different treatment may not be directly associated with changes of glucose level in the periphery.

Spatial memory deficits have been shown either in young and aged mice fed HF diet (Heyward et al., 2012; Carey et al., 2014) or AD transgenic mice fed with HF diet, where HF diet contributes to increased cerebral A $\beta$  levels and memory loss (Petrov et al., 2015). Several potential mechanisms have been proposed for memory deficits in diet induced obesity models. Cognitive deficits in animals fed HF diet have been linked with impairment in peripheral and central insulin signalling (Pintana et al., 2012) or inflammation (Jeon et al., 2012). Studies of chronic treatment with naringenin, an anti-oxidative, anti-inflammatory and anti-diabetic molecule, showed decreased blood glucose levels and improved memory, which might be through increasing insulin sensitivity and extra-pancreatic actions (Mulvihill et al., 2009; Rahigude et al., 2012). But the underlying mechanism of memory deficits in diet induced obesity models and STZ induced models is not clear. Acute administration of leptin was shown to enhance phosphorylation of Akt in controls but not in mice with HF diet, suggesting HF diet treatment triggers desensitization of this pathway (Valladolid-Acebes et al., 2013).

Our main focus was the effect of T2DM treatment on A $\beta$ -related dysfunction. A large amount of evidence shows a strong association between soluble A $\beta$  and memory impairments in animals (Cleary et al., 2005; Lesné et al., 2006; Townsend et al., 2006), which has been considered more toxic than the aggregated form. In our experiment, we used a soluble A $\beta$ 42 isopeptide, which alone induces memory deficits in a time dependent manner, but the deficits were not long lasting. Interesting, only the STZ-CD treatment exacerbated and prolonged the memory deficits induced by A $\beta$  infusion showing long lasting severe memory deficits in this group; STZ alone and CD alone rats infused with A $\beta$  showed temporary deficits and recovered as seen in A $\beta$  alone treatment. There is no similar study found in T2DM models.

Interestingly, only with STZ-CD treatment have we seen a typical T2DM phenotype, with stable hyperglycemia and possible reduced insulin production, which may be associated with the memory deficits. Most importantly, we observed the dysregulation of many key proteins in the PI3K-Akt signalling and APP processing pathways in this T2DM model coupled with A $\beta$ , as we examined in CA1. Similar changes of those proteins were found in studies of AD, especially at the same phosphorylation sites of key proteins. More interestingly, although there were fewer studies conducted, several changes in our STZ-CD treated rats infused with A $\beta$  were also observed in studies of AD patients with T2DM or in T2DM-AD animal models (Liu et al., 2011; Ma et al., 2013) (see summarized Table 5). These proteins are involved in autophagy, glucose metabolism and inflammation, many of which are mediated by insulin regulation of the PI3K-Akt signalling pathway. PI3K-Akt signalling has also been associated with memory consolidation (Horwood et al., 2006; Callaghan and Kelly, 2012) and several metabolic processes (De Felice, 2013; Mackenzie and Elliott, 2014), and was shown to be dysregulated in both brains of AD and peripheral organs or brains of T2DM patients (De Felice, 2013; Mittal and Katare, 2016).

Several studies showed a decrease in insulin-PI3K-Akt signalling in the CNS in T2DM and AD patients (Steen et al., 2005; Liu et al., 2011), such as reduction in pAkt and pGSK3 $\beta$ ; interestingly the deficits were more severe in patients with both T2DM and AD (Liu et al., 2011). Once insulin binds to subunits of neuronal insulin receptors (IR), insulin promotes phosphorylation of insulin receptor substrates (IRS) (Duarte et al., 2012), which in turn activates several signalling pathways, such as PI3K-Akt/GSK3 $\beta$ , Ras/Raf-1/extracellular signal-related kinase (ERK) (Duarte et al., 2012) and MAPK pathways (del Rincón et al., 2004). There is a decrease in levels of IRS, with an increase in inactivation of IRS-1 (Ser312 and Ser616) found in the cortex of AD patients. These increased levels of phosphoserine epitopes were colocalised with neurofibrillary tangles (Moloney et al., 2010). In our experiment, we didn't test brain insulin levels and there are limited studies of direct testing of brain insulin levels in the literature. However, some studies did show insulin changes in the brains of AD patients, with the majority showing a reduction in insulin/insulin receptor signalling (Stanley et al., 2016). A decrease in PI3K activity was also detected in the cortex in AD (Calon et al., 2004). It is known that active IRS proteins interact with PI3-kinase and activate Akt signalling (Chang and Chiang, 2005). We have shown a decrease in the activity of Akt that is consistent with results from the brains of AD patients (Steen et al., 2005), of patients with both T2DM and AD (Liu et al., 2011), and of Tg2576 mice fed with high fat diet (Ho et al., 2004). This suggest that the activity of Akt is dysregulated in the brains of AD and/or T2DM. Akt is a key protein in PI3K-Akt signalling pathway. Once Akt is activated, it phosphorylates several targets including mTOR (Taguchi and White, 2008; Jewell and Guan, 2013), GSK3 (Doble and Woodgett, 2003), BAD (Datta et al., 1997), transcription factors NF $\kappa$ B (through IKK $\alpha$ ) (Manning and Cantley, 2007), FOXOs (Brunet et al., 1999), regulating multiple processes. However, regulation of downstream proteins in the PI3K-Akt signalling pathway is complex; in addition to regulation by Akt, these proteins can be regulated by other signalling pathways and signalling events, and they also interact with each other.

Under normal conditions, insulin stimulation of Akt prevents downstream target proteins being functional such as GSK3 $\beta$ , BAD and FoxOs, whereas it activates others such as mTOR, IKK, where in concert with regulation by other proteins can modulate functions such as oxidative stress, glucose metabolism, apoptosis, inflammation, protein synthesis (Duarte et al., 2008). Dysregulation in glucose metabolism is an important feature of T2DM and AD. Imaging studies have shown progressive reduction in glucose metabolism that correlates with the severity of the disease in AD patients (Mosconi et al., 2006). Glucose transporter 1 (GluT 1) is insensitive to insulin and is the major glucose transporter in the blood brain barrier (Brant et al., 1993; Ngarmukos et al., 2001). *In vitro* studies have shown insulin treatment of neuronal cells could regulate GluT 4 levels and stimulate glucose uptake, which was dependent on PI3K activation (Benomar et al., 2006). Only in STZ-CD treated rats infused with A $\beta$  was there a significant decrease in GluT 1 expression in CA1, suggesting a decrease in glucose transport into the brain. Similar results have been found in the cortex of AD patients (Simpson et al., 1994; Mooradian et al., 1997) and the brains of GluT1 deficient mice over expressing APP (Winkler et al., 2015).

Glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) has been found to be dysregulated in the brains of T2DM and AD animal models (Ho et al., 2004; Jimenez et al., 2011) and AD patients (Steen et al., 2005; Liu et al., 2011). GSK3 $\beta$  is involved in Tau phosphorylation in AD, leading to the formation of paired helical filaments, the main component of neurofibrillary tangles (Cole et al., 2007). Overexpression of GSK3 $\beta$  coincides with development of neurofibrillary changes (Pei et al., 1999). In addition, GSK3 $\beta$  has been shown to facilitate A $\beta$  release by increasing the cellular maturation of APP (Citron et al., 1994; Aplin et al., 1997). In our experiments we found a decrease in Akt-mediated phosphorylation of GSK3 $\beta$  that is accompanied by an increase in the constitutively active form in STZ-CD treated rats infused with A $\beta$ , while there was decreased activity of GSK3 $\beta$  in CD rats, which suggests it is differently regulated in the brains of obese models. Dysregulation of GSK $\beta$ , either *via* its expression or phosphorylation, was found in the brains of both T2DM and AD patients (Steen et al., 2005; Liu et al., 2011).

GSK3 is involved in a number of functions such as microtubule stability, apoptosis, receptor trafficking, inflammation, that is largely depending on its target proteins or their interactions (Sutherland, 2011).  $\beta$ -Catenin is one of the key downstream proteins of GSK3 $\beta$ , which is involved in cell proliferation and apoptosis. Inhibition of GSK3 by Akt would result in increased activity of the protein function by blocking the degradation of  $\beta$ -Catenin (Zhao et al., 2005; Sutherland, 2011). In our experiment, the expression of  $\beta$ -Catenin was increased in the T2DM phenotype, which was mainly mediated by CD treatment; A $\beta$  infusion had no additional effect on CD treated and STZ-CD treated rats, only in STZ injected rats A $\beta$  exacerbated the increase. Interestingly, the phosphorylation level of  $\beta$ -Catenin was remarkably reduced in CD treated rats infused with A $\beta$ , which was exacerbated in T2DM phenotype infused with A $\beta$ , indicating a dysregulation of  $\beta$ -Catenin activity in these rats. Jimenez et al. found phosphorylation of  $\beta$ -Catenin was increased in the hippocampus of APP/PS1 mice at the age of 18 months but not at 6 months, while the total protein was increased at 6 months but decreased in 18 months (Jimenez et al., 2011) corresponding with the

phosphorylation of GSK3 $\beta$ , which was increased at 6 month of age, but markedly decreased later (18 months).

GSK3 $\beta$  is also involved in apoptosis. Constitutively active GSK3 $\beta$  results cell death, while inhibition of GSK3 $\beta$  prevents apoptosis (Pap and Cooper, 1998; Sanchez et al., 2003). Dysfunctional apoptotic mechanisms have been found in AD patients (Pompl et al., 2003) and obese rats (Moraes et al., 2009). In parallel with abnormal activation of Akt and GSK3 $\beta$ , we observed abnormal expression of proteins involved in apoptotic process such as increased BAD expression in our STZ-CD treated rats, but no changes in phosphorylation of BAD. Activation of Akt leads to inhibition of apoptotic pathway by phosphorylation of BAD (Datta et al., 1997). Activation of BAD results in apoptotic processes where cytochrome c is released to engage initiator caspases to promote cell death (Galluzzi et al., 2016). In our study, increased expression of BAD was only found in STZ-CD treated rats either with or without A $\beta$  infusion, while A $\beta$  infusion alone induced a temporary increase in expression of BAD. All this indicates the change in BAD expression is mainly mediated by T2DM treatment, while we haven't observed changes in phosphorylation of BAD to suggest inhibition of its activity by Akt. Although we did see changes in some of the caspases (an indicator of apoptotic activity) early after the end of infusion of A $\beta$  infusion these were not enduring. The apoptotic proteins are sensitive in response to stress signals and initiate the apoptotic process, while our western-blotting analysis was conducted a long time after the treatments; that may be one reason why we haven't observed many changes in these proteins.

The most striking effect was on the apoptotic related protein FoxO3a. We found an increased level of FoxO3a expression principally mediated by CD treatment; with increased expression in STZ-CD rats that was significantly lower than CD alone, while A $\beta$  infusion also increased the expression of FoxO3a independently. However, the increased level was most striking immediately after A $\beta$  infusion. Regulation of FoxO3a and the functions it has been shown to be involved in are complex (Zhao et al., 2005; Tilstra et al., 2011). Whilst some studies show that it plays a role in promoting longevity (Tilstra et al., 2011), it is not entirely clear how this is mediated. Moreover, it is also associated with autophagy (Greer et al., 2007; Brunet, 2015) and apoptosis (Shukla et al. 2016), suggesting it could be associated with a complex balance between survival and death of cells depending on the state of the cell and the type of regulation of FoxO3a. In terms of the increase in expression of FoxO3a that we observed in our experiment, other studies have shown that active FoxO3 increases A $\beta$ 1-42 levels and astrocytosis in p25 transgenic AD mice (Shi et al., 2016); while inactivation of FoxO3a activity was correlated with prevention of spatial memory deficits and attenuation of amyloid neuropathology in the Tg2576 mice, which was associated with insulin receptor activation by caloric restriction (Qin et al., 2008). Together it suggests FoxO3a is closely correlated with early pathological changes in AD.

In addition to inactivation of FoxO3 by Akt, it is also inactivated by Inhibitor of Nuclear factor- $\kappa$ B (I $\kappa$ B) kinase (IKK), IKK $\beta$  (Hu et al., 2004; Chapuis et al., 2010). IKK/NF $\kappa$ B complex is one of the important signalling pathway in inflammation, which can also be activated by Akt signalling (Sizemore et al., 1999).

Dysregulation of IKK/NF $\kappa$ B has been widely found in AD (Tilstra et al., 2011), diabetes and obesity (Patel and Santani, 2009). Our data showed remarkable increase in pNF $\kappa$ B (Ser536) in STZ-CD rats, A $\beta$  infusion had no additional effect; while A $\beta$  infusion alone induced a slight increase in controls, indicating increased inflammation. Increased pNF $\kappa$ B (Ser536) was seen in the hippocampus of APP/PS1 mice (Guillot et al., 2016). Activation of the transcription factor, NF $\kappa$ B is mediated by the IKK complex (Zandi et al., 1997; Sakurai et al., 1999). In addition to its role in inflammation, IKK/NF $\kappa$ B also interact with proteins associated with apoptosis and autophagy (Tilstra et al., 2011). In particular, IKK $\alpha$  contributes to the regulation of mTORC1, FOXO3 and GSK3 $\beta$  in an Akt-dependent way (Dan et al., 2014). An increase in IKK $\alpha$  was found in our CD and STZ-CD treated rats, while A $\beta$  infusion alone also increased the IKK $\alpha$  expression in controls and STZ injected rats. However, the most striking effect was on STZ-CD rats with A $\beta$  infusion. To date, most studies reporting on regulation of inflammation and IKK/NF $\kappa$ B mediated by obesity (De Souza et al., 2005; Chiang et al., 2009; Moraes et al., 2009) and T2DM (Cai et al., 2005; Zhang et al., 2008) have focused on peripheral tissues and/or the hypothalamus; they have shown increased in expression of IKK and NF $\kappa$ B (De Souza et al., 2005; Zhang et al., 2008; Chiang et al., 2009). Importantly, inflammation in the blood is associated with increased risk of AD (Engelhart et al., 2004), which suggests the inflammation in the periphery may contribute to the brain disease and the processes are detectable long before clinical symptoms occur (Reinhold et al., 2002). Increased inflammation induced by high fat diet in young rats is associated with impairments in hippocampus dependent memory (Boitard et al., 2014). Hyperinsulinemia promotes inflammation in adipose tissue, which contributes to metabolic disruption such as lipogenesis (Pedersen et al., 2015). While the mechanism of obesity-induced insulin resistance is not clear, energy imbalance induced changes in obesity are described, briefly, as excess exposure to high fat, high carbohydrates leads to cellular dysfunction, manifested as abnormalities in adipokines, increased circulating free fatty acids, inflammation and oxidative stress. These in turn affect lipid accumulation, peripheral insulin resistance and lipid deposition (De Ferranti and Mozaffarian, 2008).

mTOR is a kinase involved in autophagy and cell growth; mTOR1 has been considered to regulate metabolism in response to nutrient availability, and also regulates protein synthesis and autophagy (Wullschleger et al., 2006). mTORC1 activates its downstream target S6K, which is routinely involved in promoting protein synthesis via the elongation factor Eif4B (Backer, 2008; Díaz-Troya et al., 2008). However, it can also act as a negative feedback mechanism to turn off PI3K by phosphorylation of IRS-1 at sites that inactivate IRS-1 (Gual et al., 2005). The autophagic mechanisms are mediated by a complex balance between inhibition of mTOR1 activity and activation of Beclin-1 (Salminen and Kaarniranta, 2009), which constitutes a critical mechanism for recycling proteins under conditions of low nutrient availability (Minina et al., 2013) and also degrading misfolded proteins (Xie and Klionsky, 2007). STZ-CD treated rats showed an increase in mTOR level, which is consistent with studies in T2DM model (Ma et al., 2013); the change was also seen in STZ alone or CD treatment alone. Increased phosphorylation of mTOR was found in CD treated rats; increased mTOR activation was found in peripheral tissues of obese rats and *in vitro* studies show over activation of mTOR contributes to elevated IRS-1 phosphorylation leading to insulin

signalling dysregulation (Khamzina et al., 2005). However, the increased pmTOR and decreased Beclin-1 were dependent on A $\beta$  infusion in STZ-CD treated rats; in a similar manner to that seen in the brains of AD patients (Griffin et al., 2005; Pickford et al., 2008). In PDAPP mice, long-term inhibition of mTOR increased autophagy in neurons, which was accompanied by reduced A $\beta$ 42 levels and improved learning and memory indicating the autophagic response (Spilman et al., 2010). In addition, studies have also shown infusion of soluble A $\beta$  can hyperactivate mTOR in the hippocampus of wild type mice (Caccamo et al., 2011). Similarly, in our experiment we observed increased level of mTOR in controls rats infused with A $\beta$  (at 107 days). All these data suggest that the T2DM phenotype with A $\beta$  infusion caused dysregulation of proteins associated with autophagy and protein synthesis, which may lead to dysfunction in autophagy processes for clearance of misfolded proteins in this model, such as abnormal A $\beta$ .

Importantly, we found abnormal changes of proteins involved in markers of APP processing in T2DM phenotype rats infused with A $\beta$ ; such as increased APP levels and decreased IDE levels. APP is a key protein where A $\beta$  peptide is released by the amyloidogenic pathway. A consistent increase in APP has been found in the brains of AD patients (Steen et al., 2005) and AD models (Hiltunen et al., 2012; Guillot et al., 2016). Interestingly, decreased IDE level is also specific to STZ-CD treated rats infused with A $\beta$  in our experiments, which may suggest reduced clearance of A $\beta$ . A $\beta$  alone had no long-term effect on IDE. Similarly, decreased IDE level has been reported in the hippocampus of AD patients (Cook et al., 2003; Čaušević et al., 2010), APP/PS1 mice (Vepsäläinen et al., 2008) and Tg3576 mice fed with high fat diet (Ho et al., 2004). Mice with mutated forms of IDE show increased A $\beta$  accumulation and AD phenotypes (Farris et al., 2003; Miller et al., 2003); and conversely overexpression of IDE in mouse brains reduces A $\beta$  accumulation and slows the progression of AD (Leissring et al., 2003). Increased IDE was found CD rats and STZ-CD rats, which may correlate with abnormal insulin level induced by the diabetic treatment. Increased IDE was shown in cortex of IGF-2 transgenic mice (Hiltunen et al., 2012), but was decreased in Goto-Kakizaki (GK) diabetic rat (Fakhrai-Rad et al., 2000).

Many of the proteins examined here are closely related to the Akt signalling pathway, however is it due to the impaired insulin signalling and glucose metabolism in the brain induced by T2DM? As we did not measure insulin or glucose levels in CA1 directly; we can only speculate from the literature whether deregulation of the proteins we examined might be a result of dysfunctional insulin signalling and glucose availability in the brain. Evidence shows there is a decrease in mRNA levels of insulin in AD patients (Rivera et al., 2005; Steen et al., 2005) and a progressive reduction in levels of mRNA of insulin, IGFs and their receptors with advancing Braak staging in the frontal cortex of AD patients, that correlates with progressive increases in levels of APP (Rivera et al., 2005).

Hypoglycemia is often linked to cognitive deficits in human studies of T2DM patients and AD patients (Osborne et al., 2016; Sheen and Sheu, 2016). Interestingly, hyperinsulinemia could increase the risk of developing AD, and increased insulin levels are also found in AD patients (Luchsinger et al., 2004). Insulin resistance has been considered to increase A $\beta$  and Tau phosphorylation (Starks et al., 2015; Willette et al.,



2015). However, whether insulin is a cause or consequence of the disease and playing what kind of roles in AD brains are poorly understood (Stanley et al., 2016). It is still not clear what the mechanisms are that allow peripheral insulin to influence brain insulin signalling. It has been assumed that insulin resistance and chronic hyperinsulinemia in the periphery impairs insulin transport into the brain, resulting in long-term reduction of brain insulin levels (Craft, 2005; Duarte et al., 2012). More recently, studies also focused on the impairment and disruption of the blood brain barrier (BBB) in AD leading to impaired transporter function, which is associated with inflammatory cytokines, reactive oxidative stress (ROS), microglia activation and increased neuroinflammation as reviewed (Erdo et al., 2016). Some studies have pointed out the significant role of obesity and diabetes in inducing BBB disruption in central nervous system disorders (Prasad et al., 2014; Tucsek et al., 2014). Glucose changes in the blood have been associated with alteration in BBB transport functions and oxidative stress in the brain, which may be an important link between diabetes and AD due to impaired insulin and glucose transport (Prasad et al., 2014). The most convincing and consistent changes of insulin signalling in AD brains are lower levels of IRS-1 and higher levels of pIRS-1, which is thought to be a marker of insulin resistance in the brains of AD patients (Stanley et al., 2016). But there is no clear understanding about whether the insulin resistance in the brain is similar as that seen in the periphery.

Taken together, the possible reason for memory deficits induced by diabetes may be due to the disruption in glucose and energy metabolism (Cao et al., 2003), impaired insulin signalling (Steen et al., 2005) and increased advanced glycation end products (AGEs) and oxidative stress (Roriz-Filho et al., 2009; Mittal and Katare, 2016), and possible BBB dysfunction (Cordner and Tamashiro, 2015). All of these could cause changes in synaptic plasticity and transmission in the hippocampus (Biessels et al., 2002; Kamal et al., 2006) and lead to memory deficits in diabetic animals (Rahigude et al., 2012). These dysfunctional environments induced by diabetic phenotype may favour the abnormal A $\beta$  production, as the combination exacerbated the dysregulation in many functions associated with glucose metabolism, autophagy and inflammation, which are common to both AD and T2DM.

### ***Effect of enrichment***

In our study, we used environmental enrichment (EE) to try to rescue the memory deficits in STZ-CD rats infused with A $\beta$ . EE has shown beneficial effects in both diabetic (Beauquis et al., 2010; Pamidi and Nayak, 2014) and AD models (Lazarov et al., 2005; Cracchiolo et al., 2007) in memory performance (Cavallini et al. 2003) and synaptic plasticity (Lövdén et al., 2010). In our experiment, a 14-day EE period showed a beneficial effect on memory consolidation both in controls and STZ-CD treated rats. Enrichment rescued the memory deficits induced by STZ-CD treatment and/or A $\beta$  infusion. However, the beneficial effect on STZ-CD treated rats whether infused with A $\beta$  or not was not long lasting; while the beneficial effect of enrichment in controls was robust. Evidence shows that the length of time rats are housed in an enriched environment has an impact on the potential beneficial effects of EE. For example, 2 weeks of enrichment

did not improve recognition memory, whereas 3 and 6 weeks did. Moreover, the longer exposure also improved spatial learning and memory (Kelly et al., 2013). In a study comparing the effect of EE on spatial memory in young and aged mice using different enrichment protocols over a 6-week period (3hr/day or 24hr continuous complex enrichment); young mice outperformed aged mice (Harburger et al. 2007). In a study from our laboratory using same enrichment protocol (3hr/day for 14 days) in young rats, enrichment resulted in a marked facilitation of long-term memory either in 24-hr or 48-hr memory consolidation in novel object recognition task when tested 3 days after the end of enrichment (Bruel-Jungerman et al., 2005), that was mediated by EE induced increase in neurogenesis in the dentate gyrus (Bruel-Jungerman et al., 2005). In general EE is believed to be mediated to a large extent by neurogenesis in the dentate gyrus of the hippocampus (van Praag et al., 1999; Rueda and Trejo, 2007). Evidence also shows enrichment ameliorates A $\beta$ -deposition and memory deficits in APP transgenic mice fed with high fat diet (Maesako et al., 2012); mice exposed to enrichment showed 50% less brain A $\beta$  (Costa et al., 2007). Environmental enrichment manipulation before diabetic induction prevents the development of memory deficits induced by diabetes in STZ-treated rats, but does not correlate with cell proliferation in the dentate gyrus (Piazza et al., 2011). In most studies the beneficial effect of EE on neurogenesis, synaptogenesis and angiogenesis is assessed shortly after the enrichment period or after relatively long exposures to enrichment. For example, studies found increased nerve growth factors, cell survival and synaptogenesis in the dentate gyrus following 6 weeks of enrichment with a significant positive correlation between neurogenesis and recognition memory performance (Kelly et al., 2013).

## **Conclusion and Perspective**

In conclusion STZ-CD treated rats showed classic signs of T2DM, characterized by weight gain stable hyperglycemia with early hyperinsulinemia and later  $\beta$ -pancreatic cell failure. They also prolonged a deficit in spatial recognition memory induced by infusion of A $\beta$  that could be temporarily mitigated by exposure to environmental enrichment. In addition, a number of proteins in or associated with the PI3K-Akt signalling pathway were aberrantly regulated following infusion of A $\beta$ , supporting our hypothesis that T2DM can induce a dysfunctional neuronal environment that exacerbates an impairment in memory and potential underlying mechanisms observed in AD. Deconstruction of the component parts of the T2DM phenotype suggest that STZ injections alone contributed little to the long-term effects observed in the T2DM phenotype. In contrast, CD was an important contribution to the phenotype; most notably in regulation of the proteins that in fact could be attributed to the CD regime regardless of any potential effect of A $\beta$ . Moreover, regulation of certain proteins associated with CD was mediated in a converse manner compared with the combined STZ-CD treatment. As the characteristics of T2DM would suggest that STZ-CD treated rats may reflect a more advanced stage of the disease than rats fed CD alone; we can only suggest the differential regulation of proteins may similarly reflect different stages of the disease. One important effect was that where there was common regulation of proteins by different treatments, there was little evidence that they were mediated in a concerted manner. On the contrary, they seemed to be mediated in a parallel independent

manner.

The results we found, particularly in terms of potential mechanisms underlying the effects of the treatments are complex and to a certain extent it could be argued that they raise more questions than were answered. This is in part because of the longitudinal nature of the experiment and also as we wished to mimic more closely the disease in humans; we chose to substitute a calibrated high fat diet for the use of human junk food. Given we were not sure what biochemical changes may be induced and to date there is relatively few studies reported in the literature on the underlying mechanisms in the brain that would link T2DM to AD; we chose to assess a relatively wide panel of proteins associated with known dysfunctions in both AD and T2DM. The results therefore suggest a number of important future experiments that will allow us to 'drill' down into the dysfunctional mechanism.

Firstly, we did not measure A $\beta$  or insulin levels in the brain; two key markers of amyloid pathology and insulin resistance. The importance of measuring the level of A $\beta$  is particularly relevant as evidence has shown that picomole levels have a beneficial effect on synaptic plasticity and learning and memory (Garcia-Osta and Alberini, 2009). It becomes important to know now what are the levels in control animals and those displaying a T2DM phenotype as our hypothesis suggests that T2DM will promote an environment that would favour development of early A $\beta$  dysfunction.

A second important avenue to pursue is based on the dramatic reduction in levels of glucose transporter-1. As this transporter constitutes a major mechanism underlying the principle conduit for glucose to enter the brain, in depth biochemical and immunohistochemical analyses would clarify mechanisms by which a peripheral born dysfunction can impact the brain.

Finally, a third approach is based on the time limited beneficial effects of enrichment on memory performance in rats treated with STZ-CD and infused with A $\beta$ . A potentially more relevant treatment to attempt to reverse the memory deficits and potentially the dysregulation of proteins would be to change CD to normal laboratory diet, given that caloric restriction has beneficial effects and that proteins associated with nutrient sensing such as mTOR and Beclin-1 are dysfunctionally regulated. A further study attempting to rescue deficits pharmacologically and in particular *via* infusion of IGF-II, a growth factor that regulates insulin and insulin growth factor receptors, could be interesting. Currently we do have preliminary data showing in an experiment coupling infusion of A $\beta$  with pharmacological inhibition of Akt, that injection of a low concentration of IGF-II has a much more enduring beneficial effect than environmental enrichment.

In summary, our experiment has revealed a number of complex, yet important results that strongly support T2DM as a risk factor for developing early stage AD and the potential mechanisms that may play a critical role.

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## List of abbreviations

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AD : Alzheimer's disease	GlUTs : Glucose transporters
ADDLs : A $\beta$ -derived diffusible ligands	GSK3 $\beta$ : Glycogen synthase kinase 3 $\beta$
AGEs : Advanced glycation end products	GTT : Glucose tolerance test
AICD : APP intracellular domain	HC diet : High calorific diet
Akt : Protein kinase B (PKB)	HF diet : High-fat diet
AMPA : $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor	IDE : Insulin Degrading Enzyme
APFs : Annular protofibrils	IGF : Insulin growth like factor
APP : Amyloid precursor protein	IKK : Inhibitor of nuclear factor- $\kappa$ B (I $\kappa$ B) kinase
APs : Amyloid Plaques	IR : Insulin receptor
A $\beta$ : Amyloid beta	IRS : Insulin receptor substrate
BACE : Beta-site amyloid precursor protein cleaving enzyme	JNK : c-Jun N-terminal kinase
BAD : Bcl-2-associated death promotor	LTP : Long-term potentiation
BBB : Blood Brain Barrier	MAPK : Mitogen-activated protein kinase
BDNF : Brain-derived neurotrophic factor	MCI: Mild cognitive impairment
BMI : Body mass index	mGluR : metabotropic glutamate receptors
CaMKII: Ca <sup>2+</sup> /calmodulin-dependent protein kinase II	MRI : Magnetic Resonance Imagery
cAMP : Cyclic adenosine monophosphate	mTOR : Mammalian Target of Rapamycin
CD : Cafeteria Diet	NFTs : Neurofibrillary Tangles
Cdk5 : Cyclin dependent kinase 5	NF $\kappa$ B : Nuclear factor- $\kappa$ B
CNS : Central nervous system	NGF : Nerve growth factor
CR : Caloric restriction	NIDDM : Non-insulin dependent diabetes mellitus
CSF : Cerebrospinal fluid	NMDAR : N-methyl-D-aspartate receptor
CTF : C-terminal fragment	PET : Positron Emission Tomography
DIO : Diet induced obesity	PHFs : Paired helical filaments
DYRK : Dual Specificity Tyrosine Phosphorylation Regulated Kinase	PI3K : phosphatidylinositide 3-kinases
EE : Environmental enrichment	PKA : Protein kinase A
EOAD : Early Onset Alzheimer's Disease	PKC : Protein kinase C
ERK : extracellular signal-related kinase	PS : Presenilin
FAD : Familial Alzheimer's Disease	RAGE : Receptor for Advanced Glycation End product
FDA : Food and Drug Administration	STZ : Streptozotocin
FFA : free fatty acid	T1DM : Type 1 diabetes mellitus
FOXOs : Forkhead transcription factors	T2DM : Type 2 Diabetes Mellitus
FPG : Fasting plasma glucose	Tg mice : transgenic mice
GDM : Gestational diabetes mellitus	VEGF : Vascular endothelial growth factor
GK rat : Goto-Kakizaki diabetic rat	WMH : White matter hyperintensity



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ECOLE DOCTORALE N°568 : BIOSIGNE  
Signalisations et Réseaux Intégratifs en Biologie

Spécialité de doctorat : Aspects moléculaires et cellulaires de la biologie  
Neurosciences

Par

**Mme Su Men**

**Type 2 Diabetes Mellitus acts as a risk factor for the  
development of early stage Alzheimer's disease**

Thèse présentée et soutenue à Orsay, le 6 Juin 2017

**Composition du Jury :**

<b>Dr. Davis Sabrina</b>	Chargée de recherche	University of Paris-Saclay	Directrice de thèse
<b>Dr. Ferreira Guillaume</b>	Directeur de recherche	University of Bordeaux	Rapporteur
<b>Pr. Kelly Aine</b>	Professeur	University of Dublin	Rapporteur
<b>Dr. Daumas Stéphanie</b>	Maitre de conférences	University of Sorbonne	Examineur
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## Résumé

Les formes sporadiques de la maladie d'Alzheimer (MA) sont multifactorielles et représentent 99% des cas de MA. L'âge est le plus important facteur de risque, suivi par le Diabète Mellitus de Type 2 (DMT2), une maladie métabolique caractérisée par une hyperglycémie et une résistance à l'insuline qui se développe vers la cinquantaine et est fortement favorisée par l'obésité. Ces deux maladies partagent des événements pathologiques communs, en particulier liés à l'inflammation, l'autophagie, l'apoptose, la régulation du glucose et la signalisation par l'insuline ; mécanismes dont beaucoup impliquent la voie de signalisation cellulaire PI3K-Akt.

Nous avons exploré l'impact potentiel du DMT2 sur le développement de la MA dans une étude longitudinale (8-10 mois) chez le Rat. Pour cela, nous avons utilisé un protocole qui mime le développement d'un phénotype DMT2 chez l'Homme en couplant un régime alimentaire cafétéria (RC), qui induit une obésité, avec des injections de faibles doses de Streptozotocine (STZ) qui induisent une résistance pancréatique à l'insuline (STZ-CD). Cinq mois après le début du régime alimentaire, la moitié des rats reçoivent une infusion intracérébrale de peptides  $\beta$ -amyloïdes solubles ( $A\beta$ ) qui ne forment pas de plaques amyloïdes et ne conduisent pas à des déficits mnésiques durables. Ceci permet de rechercher si le DMT2 peut exacerber les effets du peptide  $A\beta$  seul. Durant toute l'expérience, on contrôle le gain de poids et l'hyperglycémie et on analyse en fin d'expérience le taux sanguin d'insuline. La mémoire de reconnaissance spatiale, une fonction dépendante de l'hippocampe, est évaluée régulièrement pour détecter le début et analyser l'évolution des déficits mnésiques induits par le DMT2 et/ou le peptide  $A\beta$ . Des analyses biochimiques dans CA1 ont été effectuées pour explorer des possibles altérations de la voie PI3K et de marqueurs de la cascade amyloïde.

Les rats STZ-CD montrent des signes classiques de DMT2 et des déficits légers de consolidation en mémoire de reconnaissance, déficits qui peuvent être induits indépendamment par les injections de STZ seules ou le régime cafétéria seul. L'infusion de peptides  $A\beta$  seule ou chez les rats CD ou STZ seuls induit des déficits mnésiques temporaires qui récupèrent en environ 6 semaines. Seul le phénotype DMT2 chez les rats STZ-CD exacerbe les déficits mnésiques observés avec le peptide  $A\beta$  en les prolongeant dans le temps. L'enrichissement environnemental pendant une période critique de 2 semaines après l'infusion d' $A\beta$  est capable de compenser les déficits mnésiques induits par la combinaison des traitements STZ-CD et  $A\beta$ , mais l'effet bénéfique de l'enrichissement est peu durable comparé à son effet bénéfique robuste chez les rats contrôles.

Des analyses biochimiques montrent que le peptide  $A\beta$  seul induit peu de changements durables de phosphorylation de protéines neuronales dans CA1, en concordance avec la récupération rapide des déficits mnésiques. Le phénotype DMT2 seul est associé à des changements pour quelques protéines-clé, largement en liaison avec le régime cafétéria. Par contre, la majorité des modifications dysfonctionnelles de protéines est observée chez les rats montrant un phénotype de type DMT2 et recevant le peptide  $A\beta$ . Ces altérations, similaires à celles rapportées chez des patients atteints de la MA et chez des modèles animaux de la MA, concernent notamment l'expression et/ou la phosphorylation de protéines de la voie PI3K-Akt impliquée dans des fonctions comme l'autophagie, l'inflammation et la cascade amyloïde. L'altération de ces protéines pourrait contribuer aux déficits mnésiques durables observés et mettre en lumière des mécanismes moléculaires induits par le DMT2 et favorisant un milieu neuronal favorisant le développement d'un stade précoce de la MA.

## Introduction

La maladie d'Alzheimer (MA) est une maladie neurodégénérative liée à l'âge qui a un début lent et insidieux. Elle est définie cliniquement par une démence et pathologiquement par la présence généralisée de plaques amyloïdes, de dégénérescences neurofibrillaires et de pertes neuronales (Heininger, 1999, Dickson et Vickers, 2001). Jusqu'à récemment, l'accent mis sur la recherche pour comprendre la progression de la maladie et pour le développement de traitements pour endiguer ou arrêter la pathologie était basé sur la pathologie amyloïde. Toutefois, au cours des 10 dernières années, l'accumulation de données suggère maintenant que les plaques amyloïdes ne sont probablement pas le principal mécanisme de déclenchement de la démence associée à la maladie d'Alzheimer. Les principales données à l'appui de cette vision sont, d'une part, les essais cliniques basés sur l'utilisation de médicaments éliminant les plaques qui n'améliorent pas la cognition ni prolongent la vie chez les patients atteints de la MA (Holmes et al., 2008) ; d'autre part le fait que les personnes âgées cognitivement « normales » ont une charge en plaques amyloïdes équivalente à celles diagnostiquées pour la maladie (Knopman et al., 2003; Oh et al., 2015, Mufson et al., 2016).

De plus, des recherches plus récentes ont montré que les peptides amyloïdes pré-agrégés peuvent être plus déterminants pour induire la démence associée à la maladie (Larson et Lesne, 2012; Lesne, 2014). Ces peptides sont toxiques, relativement mobiles et, de manière extracellulaire, peuvent se lier à un certain nombre de récepteurs neuronaux et rendre dysfonctionnels ces récepteurs et leurs voies de signalisation cellulaire (Sakono et Zako, 2010). Cela peut favoriser les processus d'inflammation et d'apoptose (Eftekharzadeh et al., 2012; Kumar et al., 2016) et aussi déréguler les processus homéostatiques tels que l'autophagie et la balance énergétique (Townsend et al., 2007; Caccamo et al., 2011; voir Wang et al., 2016). Les données expérimentales chez les rongeurs suggèrent que ces peptides amyloïdes solubles induisent des déficits de mémoire (Cleary et al., 2005; Sipos et al., 2007; Eslamizade et al., 2016) et de plasticité synaptique (Walsh et al., 2002; Townsend et al., 2006; Zhao et al., 2008). Enfin, des données montrent que la charge amyloïde augmente lentement pendant environ 15 à 20 ans avant que le début des premiers signes cliniques identifiables aient atteint un plateau (Jack et al., 2010; Jack et al., 2013; Swerdlow et al., 2014). Ainsi, il existe un consensus croissant qui suggère que les peptides pré-agrégés peuvent constituer un stade préclinique de la maladie où le développement de traitements interventionnels pourrait être la stratégie optimale. Cependant, en l'absence de marqueurs *in vivo* de l'amyloïde pré-agrégée chez l'homme, il devient difficile de dissocier les déficits de mémoire légers qui peuvent survenir chez les personnes âgées normales de ceux chez des patients qui développeront une MA.

Un marqueur prédictif potentiel du développement précoce de la MA est le diabète Mellitus de type 2 (DMT2), une maladie caractérisée par une hyperglycémie et une résistance à l'insuline et/ou une hyperinsulinémie (Alberti et Zimmet, 1998; Brunetti et al., 2014). Après le vieillissement, c'est le facteur de risque le plus important pour développer une MA. L'incidence de la maladie d'Alzheimer est de 2 à 5 fois plus élevée chez les personnes atteintes de DMT2 (Mayeux et Stern, 2006; Desai et al., 2014). Comme pour la MA, le DMT2 se développe avec le temps, montrant des symptômes caractéristiques vers la cinquantaine (Aguiree et al., 2013; American Diabetes Association, 2015), et est également soumis à des facteurs de risque, en particulier l'obésité (van Leiden et al., 2003; Organisation Mondiale de la Santé, 2000). Les études épidémiologiques et celles chez l'animal montrent une forte association entre le DMT2 et la MA et il a été suggéré que le DMT2 pourrait exacerber le processus de la MA (Verdile et al., 2015a).

Les patients diabétiques présentent des niveaux inférieurs de cognition globale, de mémoire

épisode, de mémoire sémantique, de mémoire de travail et de vitesse perceptuelle (Arvanitakis et al., 2004). Les patients atteints de DMT2 ou de MA partagent également certaines neuropathologies communes, telles qu'une atrophie de l'hippocampe et une altération de la connectivité neuronale structurale et fonctionnelle (Den Heijer et al., 2003; Reijmer et al., 2013), de la glycémie à jeun (Janson et al., 2004) et la présence de dégénérescences neurofibrillaires et de plaques amyloïdes (Peila et al., 2002, Mittal et Katare, 2016), bien que ces lésions clés soient dans le pancréas chez les patients atteints de DMT2. En outre, des études ont montré une altération de la signalisation par la voie insuline-PI3K-Akt chez les patients atteints de MA et de DMT2 (Schubert et al., 2004; Steen et al., 2005), qui pourrait contribuer à des dysfonctionnements neuronaux tels que l'inflammation, l'apoptose, l'autophagie, le stress oxydatif, la signalisation par l'insuline et le métabolisme du glucose, dont beaucoup sont médiés par la voie de signalisation insuline-PI3K-Akt (voir pour revue : Cole et al., 2007; Kim et al., 2010; Verdile, 2015b; Mittal et Katare, 2016). Bien que ces dysfonctionnements communs soient principalement observés dans les tissus périphériques dans le DMT2, on sait que le cerveau dépend largement du système périphérique pour le glucose et la majorité de son insuline, suggérant que la dérégulation par le DMT2 pourrait affecter l'homéostasie énergétique du cerveau.

Ainsi, compte tenu des pathologies communes et des mécanismes dysfonctionnels, les déficits de mémoire observés dans le DMT2 pourraient constituer un état précoce et fragile, où une légère perturbation de la régulation homéostatique de mécanismes neuronaux pourrait faire basculer l'équilibre en faveur du développement de la MA et de la démence. En outre, l'âge auquel ces maladies se manifestent les placent au moment où commence à apparaître une hausse de l'amyloïde chez les patients.

Un certain nombre d'études ont montré que les formes sporadiques de la MA sont des troubles du métabolisme cérébral (Iqbal et Grundke-Iqbal, 2005; Heiss et al., 1991; Gong et al., 2006), où l'altération du métabolisme du glucose survient avant l'apparition de symptômes de la MA et s'aggrave avec l'âge (Heiss et al., 1991; Smith et al., 1992; Hoyer, 2004). En outre, le cerveau de patients atteints de la MA présente une résistance à l'insuline (Riveral et al., 2005; Talbot et al., 2012; Liu et al., 2011) associée à une diminution du métabolisme du glucose (Liu et al., 2009; 2011). Certaines études ont couplé le DMT2 avec la MA, principalement sur la base de données chez des souris transgéniques modèles de la MA (Ho et al., 2004; Hiltunen et al., 2012; McClean et Hölscher, 2014; Yeh et al., 2015) qui ont surtout porté sur la pathologie amyloïde et tau (Ho et al., 2004; Takeda et al., 2010; Yeh et al., 2015) ou sur la pathologie du DMT2 (Takeda et al., 2010; Hiltunen et al., 2012).

Compte tenu des associations entre les deux maladies, le but de nos expériences était de rechercher des mécanismes dysfonctionnels médiés par le DMT2 dans l'aire CA1 de l'hippocampe qui pourraient exacerber une augmentation précoce de l'amyloïde telle que celle observée dans les stades précliniques de la MA. Bien que l'hypothèse de la cascade amyloïde (Hardy et Higgins, 1992; Selkoe et Hardy, 2016), qui joue un rôle moteur de la recherche en sur la MA, suggère que de nombreuses pathologies non spécifiques de la MA se trouvent en aval de l'amyloïde, un concept sous-jacent majeur dans notre recherche est que la déstabilisation de faible niveau, mais chronique, des fonctions cérébrales induite par le DMT2 pourrait favoriser un environnement neuronal permettant une régulation dysfonctionnelle de l'A $\beta$ . À cette fin, nous avons développé un modèle de DMT2 chez le rat qui imite de façon aussi proche que possible la condition humaine et mené des analyses comportementales et biochimiques longitudinales. Lorsque les symptômes caractéristiques du DMT2 ont été établis, nous avons infusé en intracérébral une faible concentration de peptide A $\beta$ 42 et avons suivi les animaux pendant 15 semaines. Pour les tests fonctionnels, nous avons examiné à plusieurs temps pendant toute la période expérimentale la mémoire de reconnaissance spatiale. Enfin, nous avons examiné la régulation de protéines de la voie de signalisation cellulaire PI3K-Akt associé à

la régulation homéostatique normale de fonctions qui sont altérées dans la MA.

## **Objectifs de la thèse**

Après le vieillissement, le DMT2 est le principal facteur de risque associé à la MA. Il est largement induit par l'obésité et se manifeste à l'âge moyen. Le DMT2 et la MA partagent un certain nombre de signes pathologiques, y compris la pathologie amyloïde, une altération des fonctions cellulaires en partie contrôlées par la cascade de signalisation PI3K-Akt, une voie qui répond directement à l'apport environnemental (Desai et al., 2014). La différence majeure entre les deux pathologies est que dans le DMT2 cette pathologie est observée à la périphérie, alors que dans la MA, la pathologie prend place dans le cerveau. Cependant, dans des conditions physiologiques, le cerveau doit accéder à la fois au glucose et à l'insuline de la périphérie et comme ce sont les deux dysfonctionnements caractéristiques du DMT2, ils pourraient avoir un impact sur le cerveau. Le but spécifique du projet était donc de comprendre comment et par quel mécanisme le DMT2 pourrait induire un environnement neuronal favorisant l'apparition de dysfonctionnements neuronaux et de déficits cognitifs associés à la MA. Pour répondre à cette question, notre stratégie a consisté à mener des études sur un modèle animal mimant au plus près la condition humaine en utilisant :

- Des études longitudinales (sur environ 10 mois) qui mimeront plus étroitement l'apparition lente des dysfonctionnements comme cela a été observé dans le DMT2 et la MA.
- Un régime alimentaire utilisant de la nourriture de type « junk food » humaine sous un régime de type cafétéria, au lieu de granulés calibrés de laboratoire à haute teneur en graisse.
- Une infusion intracérébrale de faibles doses de peptide A $\beta$ 42 soluble pour assurer une augmentation du taux de peptides A $\beta$ 42 dans le cerveau.
- Une analyse des dysfonctionnements de la régulation de l'expression et de la phosphorylation de protéines de la voie de signalisation PI3K-Akt dans l'aire CA1 de l'hippocampe.

Par conséquent, notre hypothèse était qu'un effet dysfonctionnel induit par l'A $\beta$  serait exacerbé par un phénotype de type DMT2. En particulier, que la pathologie de type DMT2 créerait un dysfonctionnement de la régulation homéostatique du glucose et du métabolisme énergétique et une diminution de la signalisation par l'insuline dans le cerveau, ceci exacerbant tout effet induit par le peptide A $\beta$  seul.

## **Méthodes**

### *Protocole général et groupes expérimentaux*

Nous avons effectué des études longitudinales sur 8 à 10 mois. Les rats sont d'abord nourris avec le régime alimentaire et sont ensuite injectés à deux reprises, séparées d'une semaine, avec des doses sous-diabétogènes de streptozotocin (STZ). Après 20 semaines, certains rats ont été implantés avec des mini-pompes osmotiques contenant de l'A $\beta$ 42 soluble et l'expérience a été poursuivie pendant encore 15 semaines. Tout au long de la période expérimentale, des tests réguliers de tolérance au glucose (GTT) et des tests de mémoire de reconnaissance spatiale ont été effectués (voir la chronologie ci-dessous, Figure 1). Quatre groupes expérimentaux principaux ont été utilisés : des rats témoins nourris avec un régime standard de laboratoire (Ct), des rats nourris avec un régime cafétéria (CD), des rats injectés avec la streptozotocin (STZ) et des rats nourris avec un régime cafétéria combiné à des injections de STZ (STZ-CD). Des sous-groupes

de ces différents groupes ont été infusés avec de l'Aβ42 soluble délivré dans les ventricules latéraux.

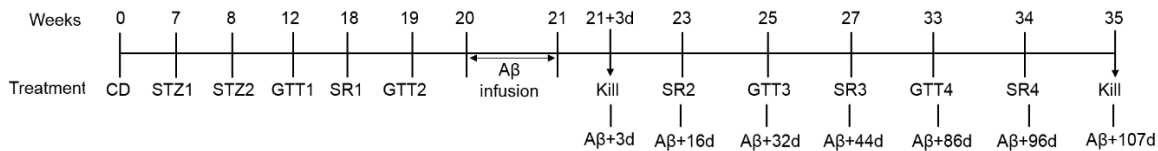


Figure 1. Déroulé du protocole expérimental : le protocole commence par l'alimentation avec le régime de cafétéria (CD) à la semaine 0. Ensuite, on distingue différents traitements pendant une période de 35 semaines. Abréviations : STZ1 et 2 sont les moments où des injections de faibles doses de streptozotocine sont administrées ; SR1-4 représentent les temps auxquels la mémoire de référence spatiale est testée ; GTT1-4 représentent les temps auxquels les tests de tolérance au glucose sont effectués ; Aβ+ indique le temps qui suit la fin de la perfusion d'Aβ.

- Protocole d'alimentation : Les rats sont nourris avec un régime de laboratoire calibré (LD) ou un régime cafétéria (CD) comprenant des aliments de type « junk food ».
- Injections de streptozotocine (STZ) : 30 mg/kg i.p., deux fois, 7 semaines après le début du régime d'alimentation.
- Procédure chirurgicale et infusion de peptides Aβ : infusion par mini-pompes osmotiques de peptides Aβ42 en intra-cérébroventriculaire (icv) pendant 7 jours.
- Test de tolérance au glucose (GTT) : Après une nuit de jeûne, le sang de la veine de la queue est prélevé avant (0 min) et 15, 30, 60 et 120 minutes après une injection en bolus de glucose.
- Test d'insuline dans le sang : l'insuline plasmatique a été mesurée à l'aide d'un kit ELISA d'insuline de rat / souris.
- Tâche de mémoire de reconnaissance spatiale : Utilisation d'une tâche de reconnaissance de la position spatial d'objets dans l'environnement.
- Enrichissement de l'environnement : les rats ont été placés en groupe, quelques heures par jours pendant 15 jours, dans une grande enceinte contenant un grand nombre d'objets qu'ils peuvent explorer à loisir.
- Préparation des tissus cérébraux pour les analyses biochimiques : les rats ont été sacrifiés et l'hippocampe a été disséqué et immédiatement congelé dans de l'azote liquide et maintenu à -80°C pour une utilisation ultérieure d'analyses biochimiques.
- Western-blot : Analyses de l'expression et de la phosphorylation des protéines.

## Résultats

### Caractéristiques du modèle de DMT2

#### Prise alimentaire et gain de poids

Nous avons effectué des analyses de la consommation alimentaire sur une période de 6 semaines au milieu de l'expérience pour les groupes alimentés en alimentation de laboratoire (contrôle et STZ seul) ou sous régime cafétéria (CD seul et STZ-CD). Nous avons constaté que des rats alimentés en régime CD consommaient près du double de la quantité de calories hebdomadaires (853 kcal) par rapport aux rats sous régime de laboratoire (488 kcal). En ce qui concerne les principaux groupes d'aliments nutritionnels, les rats nourris sous régime CD ont consommé environ la même quantité de glucides que ceux alimentés en alimentation de laboratoire ( $t = 0,7383$ ;  $p > 0,05$ ), moins de teneur globale en protéines ( $t = 72,29$ ;  $p < 0,0001$ ) et plus de lipides ( $t = 204,2$ ;  $p < 0,0001$ ). Cependant, la quantité de glucides contenue dans le régime CD était

d'environ 49,4% alors que celle du régime de laboratoire était de 3,2%.

Le poids des animaux a été mesuré sur une base hebdomadaire et, bien sûr, au début de l'expérience tous les rats avaient le même poids ( $F < 1$ ). Comme on l'attendait, les rats nourris avec un régime de type cafétéria (CD seul et STZ-CD) ont gagné beaucoup plus de poids que les groupes placés en alimentation de laboratoire (Contrôles et STZ seul) pendant toute la période expérimentale. À la fin de l'expérience, les rats alimentés sous régime CD avaient gagné environ 25% de poids en plus que ceux sous régime témoin.

### Taux sanguins de glucose et d'insuline

Les rats du groupe STZ-CD ont montré une hyperglycémie évidente qui se manifeste par une augmentation constante du glucose plasmatique basal à jeun et une tolérance au glucose altérée en réponse à un challenge de glucose qui a évolué avec le temps.

À la fin de l'expérience, l'insuline plasmatique a été mesurée à l'aide d'ELISA. L'ANOVA a montré des niveaux très élevés chez les rats nourris avec le régime CD, une caractéristique commune de l'obésité (voir Templeman et al., 2017), avec une légère augmentation non significative chez les rats injectés avec STZ seul et une tendance à une diminution chez les rats STZ-CD ( $F(3,15)=18,14$ ;  $p < 0,0001$ ; Figure 2A). Pour comparer la relation entre glucose et insuline, nous avons normalisé les taux d'insuline sanguine et de glucose du dernier test à leurs témoins correspondants (Figure 2B). Chez les rats injectés avec STZ seul, bien qu'il y ait eu une légère augmentation du taux insuline/glucose, ceci n'atteint pas le seuil de significativité. Chez les rats nourris avec le régime CD, il y a une forte augmentation significative du taux d'insuline par rapport à un taux relativement normal de glucose ( $t=9,19$ ;  $p < 0,01$ ), suggérant soit une résistance à l'insuline, soit une hyperinsulinémie. En revanche, les rats STZ-CD avaient des niveaux accrus de glucose et une diminution correspondante des taux d'insuline ( $t=6,24$ ;  $p < 0,05$ ), ce qui suggère une insuffisance des cellules  $\beta$  pancréatiques.

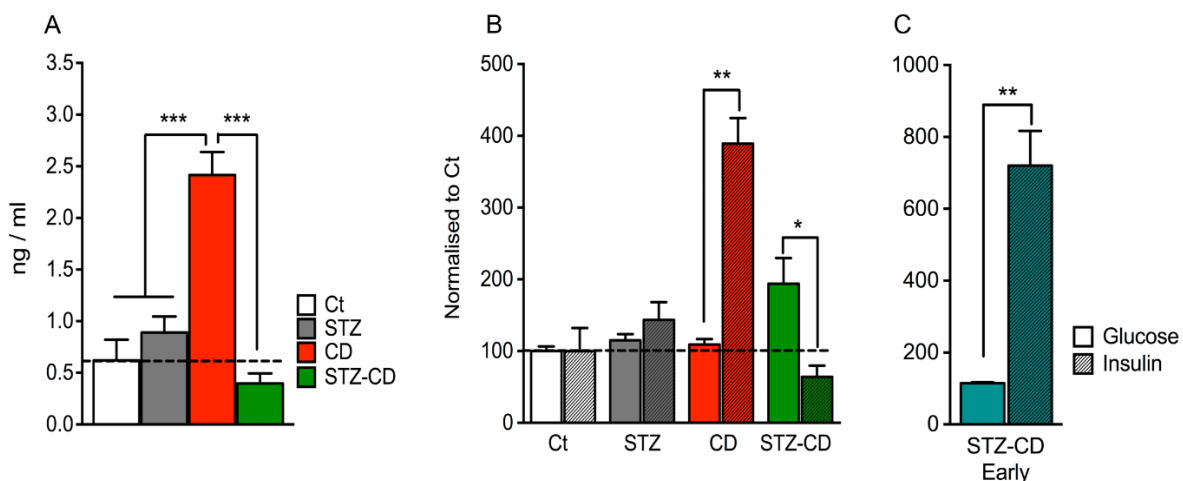


Figure 2. Régulation de l'insuline : (A) Montre le taux d'insuline sanguin à la fin de l'expérience dans les 4 groupes expérimentaux majeurs. (B et C) Montrent la relation entre le glucose (barres pleines) au dernier test et l'insuline (barres hachurées) en fin d'expérience. Les données sont normalisées par rapport aux niveaux de contrôle respectifs. (B) Montre la relation entre les niveaux de glucose et d'insuline à la fin de l'expérience. (C) Relie la relation entre le glucose et l'insuline dans un sous-groupe de rats traités par STZ-CD et chez des rats témoins examinés au temps inférieur de 21 semaines. Les données sont représentées par la moyenne  $\pm$  erreur standard, \* $p < 0,05$ ; \*\* $p < 0,01$ ; \*\*\* $p < 0,001$ .

Pris dans leur ensemble, le gain de poids, l'hyperglycémie et l'altération de l'insuline spécifiques aux rats traités par STZ-CD suggèrent que ce traitement favorise au moins un certain nombre de caractéristiques associées à un phénotype de DMT2. En outre, la différence entre les niveaux de glucose et d'insuline au temps antérieur suggère que le phénotype DMT2 a évolué avec le temps et à la fin de l'expérience représente un

stade plus avancé de DMT2 que celui induit par le régime CD seul.

### Déficits de mémoire induits par le DMT2 et par l'infusion d'Aβ

Les rats traités par STZ-CD ont montré des signes classiques de DMT2 et un déficit modeste dans la consolidation de la mémoire de reconnaissance spatiale, qui peut être observé indépendamment chez les groupes recevant des injections de STZ seul (STZ) ou soumis au régime CD seul (CD). La perfusion d'Aβ seule ou chez les rats alimentés par CD seul ou seulement injecté avec la STZ induit un déficit de mémoire temporaire qui a récupéré environ 6 semaines après la perfusion chez les rats témoins. Seul le phénotype DMT2 a exacerbé les déficits de mémoire induits par l'infusion d'Aβ en prolongeant le déficit pendant au moins 13 semaines (Figure 3).

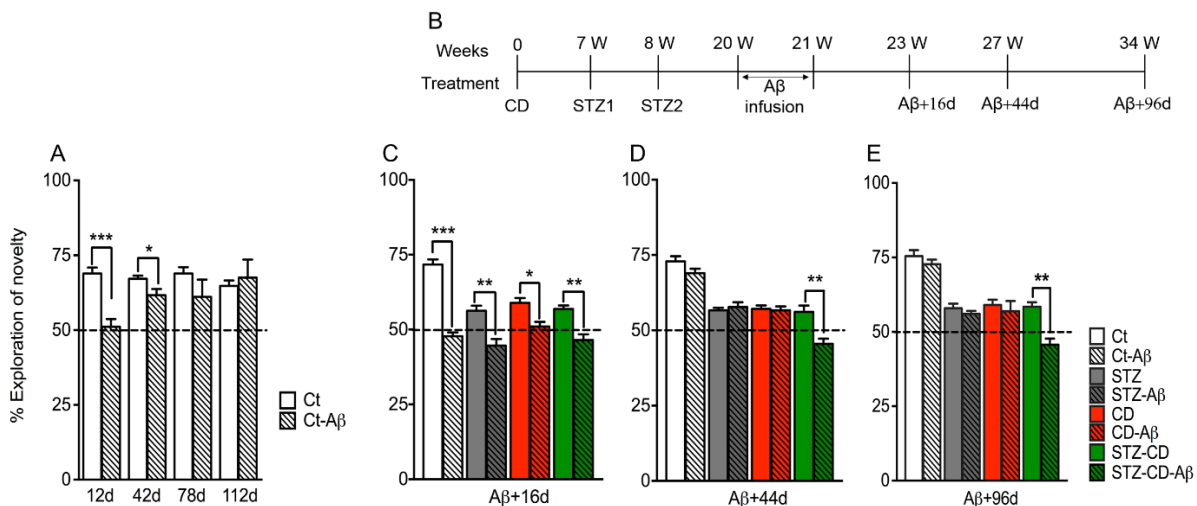


Figure 3. Effet de l'Aβ seul et en conjonction avec le DMT2 et les autres traitements seuls sur la mémoire de reconnaissance spatiale. (A) Dépeint une étude préliminaire testant l'effet que l'Aβ seul (barres hachurées) par rapport à des rats non infusés (barres blanches) testés à différents moments après l'infusion d'Aβ. (B) Montre le protocole de test de l'effet de la perfusion d'Aβ en STZ seul, CD seul et en combinaison (STZ-CD). (C-E) Déficits de mémoire induits dans tous les groupes à différents moments après l'infusion d'Aβ (barres hachurées) par rapport aux groupes non infusés (barres pleines). Les données sont représentées par la moyenne  $\pm$  erreur standard, \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

### Effet bénéfique de l'enrichissement environnemental sur la mémoire de reconnaissance spatiale

L'enrichissement environnemental (EE) a été mené quelques heures par jour pendant une période critique de deux semaines après l'infusion d'Aβ. Il a permis de récupérer des déficits de mémoire induits par un traitement Aβ et/ou STZ-CD, les rats présentant une capacité de mémoire améliorée lorsqu'ils sont testés immédiatement après l'exposition à l'enrichissement. Alors que l'effet bénéfique de l'EE sur la performance en mémoire de reconnaissance spatiale était robuste chez les rats témoins, étant toujours observé 45 jours après l'enrichissement, chez les rats traités par STZ-CD, cet effet bénéfique ne persiste pas à long terme.

### Régulation de la voie de signalisation PI3K-Akt et des protéines associées

Nous avons effectué une analyse par Western Blot dans l'aire CA1 de l'hippocampe sur l'expression et la phosphorylation de protéines clés de la voie de signalisation PI3K-Akt, protéines associées à un certain nombre de dysfonctionnements communs au DMT2 et la MA.

En résumé, bien qu'il y ait eu de nombreuses protéines régulées dans le groupe présentant un phénotypique de DMT2 infusé avec les peptides Aβ, peu étaient spécifiques du groupe, le phénotypique seul ou la perfusion d'Aβ seul, ce qui constitue les principaux facteurs du processus de la maladie. Nous avons trouvé



certaines changements dans l'expression ou la phosphorylation des protéines spécifiques au phénotype DMT2 ayant reçu des infusions de peptides A $\beta$  en termes de sens de changement, mais ceux-ci ont été accompagnés en grande partie par des effets opposés médiés par le régime CD ou par l'A $\beta$  à un stade précoce.

Les analyses biochimiques montrent que le peptide A $\beta$  seul induit peu de changements durables de phosphorylation de protéines neuronales dans CA1, en concordance avec la récupération rapide des déficits mnésiques. Le phénotype DMT2 seul est associé à des changements pour quelques protéines-clé, largement en liaison avec le régime CD. Lorsque nous avons déconstruit le phénotype en analysant les effets médiés indépendamment par STZ et par le régime CD, nous avons constaté que de nombreux effets observés avec le phénotype DMT2 infusé ou non avec les peptides A $\beta$  étaient médiés par le régime CD seul. Les injections de STZ seules ont eu très peu d'effet sur la régulation des protéines, mais lorsque les rats ont reçu de l'A $\beta$ , certaines protéines ont été régulées d'une manière similaire à celle observée dans le phénotype DMT2 avec infusion d'A $\beta$ . Par contre, la majorité des modifications dysfonctionnelles de protéines est observée chez les rats montrant un phénotype de type DMT2 et recevant le peptide A $\beta$ . Ces modifications concernant notamment l'expression et/ou la phosphorylation de protéines de la voie de signalisation cellulaire PI3K-Akt.

### **Conclusion**

En conclusion, les rats traités par STZ-CD présentaient des signes classiques du DMT2, caractérisés par un gain de poids, une hyperglycémie stable avec une hyperinsulinémie précoce et, ultérieurement, une insuffisance des cellules  $\beta$ -pancréatiques. Associé à l'infusion intracérébrale de peptides A $\beta$ , les rats STZ-CD montrent également un déficit durable de mémoire de reconnaissance spatiale, qui peut être atténué temporairement par l'exposition de quelques heures par jour à un enrichissement environnemental. De plus, un certain nombre de protéines de la voie de signalisation cellulaire PI3K-Akt, ou associées à cette voie, montrent une régulation anormale après la perfusion d'A $\beta$ , ce qui renforce notre hypothèse selon laquelle le DMT2 peut induire un environnement neuronal dysfonctionnel qui exacerbe les déficits de mémoire et les mécanismes moléculaires sous-jacents associés à la MA.

L'analyse séparée des composantes du phénotype DMT2 suggère que les injections de STZ seules ont peu contribué aux effets à long terme observés dans le phénotype DMT2. En revanche, le régime CD a eu une contribution importante au phénotype, plus particulièrement dans la régulation de différentes protéines neuronales qui, en fait, pourrait être attribuée au régime CD quel que soit l'effet potentiel de l'infusion de peptides A $\beta$ . De plus, la régulation de certaines protéines associées au régime CD va dans un sens inverse par rapport à celle observée après traitement STZ-CD combiné. Comme les caractéristiques du phénotype DMT2 suggèrent que les rats traités par STZ-CD correspondent à un stade plus avancé de la maladie que les rats alimentés par le régime CD seul, nous pouvons suggérer que la régulation différentielle de certaines de ces protéines peut également refléter différents stades de la maladie. Un effet important était que, lorsqu'il y avait une régulation similaire de certaines protéines par différents traitements, il y avait peu de données suggérant que ces régulations puissent être médiées de manière concertée. Au contraire, il semble qu'elles puissent être médiées d'une manière indépendante et parallèle.

Enfin, nos résultats montrent que les modifications de protéines observées chez les rats montrant un phénotype de type DMT2 et recevant le peptide A $\beta$  sont similaires à celles rapportées chez des patients atteints de la MA et chez des modèles animaux de la MA. Celles-ci concernent notamment l'expression et/ou la phosphorylation de protéines de la voie PI3K-Akt impliquée dans des fonctions comme l'autophagie, l'inflammation et la cascade amyloïde. L'altération de ces protéines pourrait contribuer aux déficits mnésiques durables observés et mettre en lumière des mécanismes moléculaires induits par le DMT2 qui pourraient

favoriser le développement d'un stade précoce de la MA.

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**Titre :** Le Diabète de Type 2 agit comme un facteur de risque dans le développement de stades précoces de la maladie d'Alzheimer

**Mots clés :**  $\beta$ -amyloïdes solubles, Voie PI3K-Akt, Dysfonctionnement, CA1, Mémoire spatiale, Obésité

**Résumé :**

Après le vieillissement, le diabète Mellitus de type 2 (DMT2) est le facteur de risque le plus important pour développer la maladie d'Alzheimer (MA). Le DMT2 est une maladie métabolique caractérisée par une hyperglycémie et une résistance à l'insuline qui se développe vers la cinquantaine et est fortement favorisée par l'obésité. Nous avons exploré l'impact potentiel du DMT2 sur le développement de la MA chez le Rat. Pour cela, nous avons utilisé un régime alimentaire cafétéria (RC) couplé à des injections de faibles doses de Streptozotocine (STZ) (STZ-CD). Les rats STZ-CD montrent des signes classiques de DMT2 et des déficits légers de consolidation en mémoire de reconnaissance spatiale. Afin d'imiter le développement des stades précoces de la MA, la moitié des rats reçoivent une infusion intracérébrale de peptides  $\beta$ -amyloïdes solubles ( $A\beta$ ) qui ne conduisent pas à des déficits mnésiques durables. Par contre, le phénotype DMT2 chez les rats STZ-CD exacerbe les déficits mnésiques observés avec le peptide  $A\beta$  en les prolongeant dans le temps. L'enrichissement environnemental pendant une période critique de 2 semaines après l'infusion d' $A\beta$  est capable

de compenser les déficits mnésiques induits par le peptide  $A\beta$  et/ou le traitement STZ-CD ; mais d'une manière limitée dans le temps. Des analyses biochimiques dans l'aire CA1 de l'hippocampe ont été effectuées pour explorer de possibles altérations de la voie PI3K, de marqueurs de la cascade amyloïde et du DMT2. Le peptide  $A\beta$  seul induit peu de changements durables ; le phénotype DMT2 seul est associé à des changements pour quelques protéines-clé, largement en liaison avec le régime cafétéria. Par contre, la majorité des modifications dysfonctionnelles de protéines est observée chez les rats montrant un phénotype de type DMT2 et recevant le peptide  $A\beta$ . Ces altérations, similaires à celles rapportées chez des patients atteints de la MA et chez des modèles animaux de la MA, concernent notamment des protéines de la voie PI3K-Akt impliquée dans des fonctions comme l'autophagie et l'inflammation et des marqueurs de la MA. L'altération de ces protéines pourrait contribuer aux déficits mnésiques durables observés et mettre en lumière des mécanismes moléculaires induits par le DMT2 et promouvant un milieu neuronal favorisant le développement d'un stade précoce de la maladie d'Alzheimer.

**Title:** Type 2 Diabetes Mellitus acts as a risk factor for the development of early stage Alzheimer's disease

**Keywords:** Soluble  $\beta$ -amyloid, PI3K-Akt signaling pathway, Insulin dysfunction, CA1, Spatial memory, Obesity

**Abstract:**

Following aging, type 2 Diabetes Mellitus (T2DM) is the most important risk factor of developing Alzheimer's disease (AD). It is a metabolic disorder characterised by hyperglycemia and insulin resistance that develops in middle age and is promoted largely by obesity. In this study, we used a T2DM rat model to assess the potential impact T2DM may have on the development of AD. Rats were fed cafeteria-style diet (CD) coupled with low dose injections of Streptozotocin (STZ)(STZ-CD). We found that STZ-CD treated rats showed classic signs of T2DM and a modest deficit in consolidation of spatial recognition memory. In order to mimic the development of early stage AD, half of the rats were infused with a soluble oligomeric amyloid beta ( $A\beta$ ), which alone was not sufficient to induce long-lasting memory deficits. Interestingly, the T2DM phenotype exacerbated the memory deficits induced by  $A\beta$  infusion by prolonging these deficits. Environmental enrichment during a critical two-week period following infusion of  $A\beta$  rescued memory deficits induced by  $A\beta$

and/or STZ-CD treatment; however, this was time-limited. Biochemical analyses were conducted mainly in proteins involved in the PI3K-Akt signalling pathway and markers of AD and T2DM in CA1 of the hippocampus.  $A\beta$  alone induced few long-lasting changes; T2DM phenotype alone induced some changes that were largely mediated by CD treatment alone; however, the majority of dysfunctional regulation of proteins was observed in rats showing a T2DM phenotype that were infused with  $A\beta$ . More importantly, many of these changes are similar to those reported in brains of AD patients or rodent models of the disease; notably key proteins in the PI3K-Akt signaling pathway that mediate functions such as autophagy, inflammation and markers of AD. Dysregulation of these proteins may contribute to the long-lasting memory deficits seen in this model, which may provide evidence of molecular mechanisms induced by T2DM that could promote a dysfunctional neuronal environment favouring the development of early stages of Alzheimer's disease.

