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Neurobiology of the circadian clock: metabolism control & implications for Alzheimer's disease

Tese de Doutoramento em Biologia Experimental e Biomedicina, ramo de Neurociências e Doença, orientada pela Professora Doutora Ana Cristina Rego e pelo Professor Doutor Rodrigo Cunha e pelo Doutor John Jones e apresentada ao Instituto de Investigação Interdisciplinar da Universidade de Coimbra.

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Universidade de Coimbra
António Manuel Carvalho da Silva

Neurobiology of the circadian clock: metabolism control & implications for Alzheimer’s disease

Thesis submitted to the Institute for Interdisciplinary Research of the University of Coimbra to apply for the degree of Doctor in Philosophy in the area of Experimental Biology and Biomedicine, specialization in Neurosciences and Disease.
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“Hope lies in dreams, in imagination, and in the courage of those who dare to make dreams into reality”. - Jonas Salk
À criança que vive dentro mim, pois nunca deixou de perguntar os porquês...
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Mãe e Pai sem vocês nada disto faz sentido, quem e que vou citar quando precisar de pensar na vida, quem vou olhar e seguir como modelo de paz e calma.

“Não há machado afiado que corte a raiz ao pensamento.”—minha Mãe
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2H</td>
<td>Hydrogen-2 deuterium</td>
</tr>
<tr>
<td>2H2O</td>
<td>Deuterated water</td>
</tr>
<tr>
<td>3xTg-AD</td>
<td>Triple transgenic mouse model of AD</td>
</tr>
<tr>
<td>Acetyl COA</td>
<td>Acetyl Coenzym A</td>
</tr>
<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
</tr>
<tr>
<td>ADK</td>
<td>Adenosine kinase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AKT</td>
<td>protein-chinasi B o PKB</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPA</td>
<td>(\alpha)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>Amplex RED</td>
<td>10-acetyl-3,7-dihydroxyphenoxazine</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>Apoe4</td>
<td>Apolipoprotein E4</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>AR</td>
<td>Adenosinergic receptors</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid-β</td>
</tr>
<tr>
<td>AβPP</td>
<td>Amyloid-β Protein Precursor</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BMAL1</td>
<td>Brain and Muscle Arnt-like protein 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis1 (region in the hippocampus)</td>
</tr>
<tr>
<td>CA2</td>
<td>Cornu Ammonis2 (region in the hippocampus)</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>MOM</td>
<td>Mitochondrial outer membrane</td>
</tr>
<tr>
<td>MPOA</td>
<td>Medial Preoptic Area</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acids</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>NAMPT</td>
<td>Nicotinamide phosphoribosyltransferase</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
</tr>
<tr>
<td>NMDg</td>
<td>N-Methyl-D-glucamin</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDARs</td>
<td>N-Methyl-D-aspartate receptors</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-Methyl-D-glucamin</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic resonance</td>
</tr>
<tr>
<td>NOL</td>
<td>Novel object location</td>
</tr>
<tr>
<td>Non-Tg</td>
<td>Age-matched control mice</td>
</tr>
<tr>
<td>NOR</td>
<td>Novel object recognition</td>
</tr>
<tr>
<td>NR1D1</td>
<td>Nuclear Receptor subfamily 1 group D member 1</td>
</tr>
<tr>
<td>NREM</td>
<td>Non-rapid eye movement</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor (erythroid-derived 2)-like 2</td>
</tr>
<tr>
<td>NRFs</td>
<td>Nuclear respiratory factors</td>
</tr>
<tr>
<td>OD</td>
<td>Object displacement</td>
</tr>
<tr>
<td>OF</td>
<td>Open field</td>
</tr>
<tr>
<td>OFT</td>
<td>Open field test</td>
</tr>
<tr>
<td>OH·</td>
<td>Hydroxyl radicals</td>
</tr>
<tr>
<td>OLIGO/FCCP</td>
<td>Oligomycin</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PARP1</td>
<td>Poly [ADP-ribose] polymerase 1</td>
</tr>
</tbody>
</table>
PDK4  Pyruvate dehydrogenase lipoamide kinase isozyme 4
PEPCK  Phosphoenolpyruvate carboxykinase
Per1, Per2  Period Genes 1,2
PGC-1alpha  Co-activator-1 alpha
Peroxisome Proliferator-activated Receptor γ
PGC1α  Coactivator 1-α
PK  Pyruvate kinase
PKAII  Protein kinase AII
PPARgamma  Peroxisome proliferator-activated receptor gamma
PPARGC1A  PPARG Coactivator 1 Alpha
PPARγ  Peroxisome proliferator-activated receptor gamma
PVN  Paraventricular Nucleus of the hypothalamus
qPCR  Quantitative real-time polymerase chain reaction
REF  Inactive periods
REM  Rapid eye movement
NR1D1 (nuclear receptor subfamily 1, group D, member 1)
Rev-erb α  Nuclear receptor ROR-alpha
RHT  Retina hypothalamic tract
RI  Recognition index
RNA  Ribonucleic acid
ROR α.  Retinoic acid related Orphan receptor Response
RORE  Elements
RORs  Orphan nuclear receptors
ROS  Reactive oxygen species
SAH  S-adenosylhomocysteine
SAHH  Adenosylhomocysteinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SBREP-1</td>
<td>Sterol regulatory element-binding protein 1</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic Nucleus</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sirtuin 1</td>
</tr>
<tr>
<td>SIRT3-5</td>
<td>Sirtuin3-5</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricloroanisolo</td>
</tr>
<tr>
<td>TEST</td>
<td>Memory test</td>
</tr>
<tr>
<td>THFA</td>
<td>9-(tetrahydro-2-furyl)-adenine</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethylrhodamine methyl ester</td>
</tr>
<tr>
<td>ZT</td>
<td>Zeigeber</td>
</tr>
<tr>
<td>ΔΨm</td>
<td>Membrane potential</td>
</tr>
<tr>
<td>ω-3</td>
<td>OMEGA 3</td>
</tr>
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</table>
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ABSTRACT

To maintain their homeostasis, organisms developed a system of endogenous molecular clocks to anticipate the momentum of the day and couple several physiological processes. A dysfunctional clock may be involved in several diseases, including metabolic syndromes and neurological disorders. Alzheimer’s disease (AD) patients display abnormal circadian rhythms, well before the appearance of cognitive deficits. There is evidence that: (i) a dysfunctional clock is responsible for metabolic syndromes and alterations in glucose and insulin levels, (ii) insulin affects β-amyloid levels, one the AD-associated peptides and (iii) AD patients have an altered circadian clock.

Altered brain metabolism, namely impaired glucose utilization and energy metabolism, is observed early in AD progression. This occurs prior to a major hallmark of AD, the deterioration of cognitive functions. Therefore, energy failure appears to be one of the earliest symptoms of AD.

Aging underlies a decline in many capabilities and body functions. The molecular clock also slowly loses its pace. This loss is a progressive process and seems to be a derivative of aging. Importantly, many of those processes are orchestrated by the circadian clock, which ‘times’ the homeostasis, cell cycle, as well as memory and learning. The body may also lose the ability of accurately tracking time and coordinate body functions, such as metabolism in all its different forms, namely lipidogenesis, adipogenesis, glucose and insulin metabolism. Thus, the repercussions on the synchrony of metabolic processes are implicit and might be of pivotal importance in the onset of age-related neurodegenerative diseases such as AD.
The circadian system is a design model consisting of an oscillator responsible for the generation of the daily rhythm. It relates to input pathways, by which the environment and other components of the nervous system feedback information to the clock, and also to output routes, by which the oscillator provides temporal information to a wide range of physiological and behavioural processes. When cells are removed and maintained in a brain slice, they generate 24-hour rhythms of electrical activity, secretion, and gene expression. The amplitude of the electrical activity, gene expression and hormone-secreting activity decline in the aging brain. Impairment of circadian clock synchronization displays major alterations throughout the body. These include changes in memory formation, as well as hormonal and neurochemical changes, with consequences for neuroplasticity.

The triple transgenic (3xTg-AD) mouse model of AD displays abnormalities in circadian rhythmicity prior to AD pathology, making this mouse model instrumental to investigate the interplay between circadian clock, metabolism and the link to AD pathogenesis. Thus, in this study we aimed to unveil the impact of AD in biological rhythms relevant in pathology, as well as to gain a deeper understanding of the physiopathology.

Initially, this study evaluated the impact of the circadian profile on cognitive performance in 24 week-old 3xTg-AD mice versus age-matched control (Non-Tg) mice, explored how this correlated with hippocampal long-term potentiation (LTP) and how mitochondrial function, an indicator of brain metabolism, was impacted by circadian biology. Our analysis was performed at Zeitgeber (ZT) 04 (4 hours after lights on) and ZT 16 (4 hours after lights off).
The diurnal variation, in hippocampus-dependent learning tasks of 3xTg-AD and Non-Tg animals was assayed. The Morris water maze (MWM) results showed that, independently of the time of day, cognitive deficits were observed in 3xTg-AD; indeed, a correlation between ZT’s and genotypes was only possible when making use of the Spatial Reverse learning task. These results suggested that the pattern of circadian variation of memory performance depends on the type of task and the impact of circadian clock on AD pathology is better outlined in more complex tasks.

The amplitude of hippocampal LTP, an electrophysiological correlate of memory related processes, i.e. electrical signals recorded extracellularly at Schaffer fibers/CA1 pyramid synapses, exhibited a circadian profile in Non-Tg animals, which was not observed in 3xTg-AD mice.

Disturbances in mitochondrial function and oxidative stress have been implicated in the pathophysiology of AD. Therefore, mitochondrial membrane potential (ΔΨm) was measured in cortical synaptosomes; exposure to hydrogen peroxide (H2O2) revealed increased mitochondrial depolarization at Zt16, suggesting a ZT-dependent susceptibility to oxidative stimulus. Simultaneously, intracellular calcium (iCa) levels were measured, revealing an inability of 3xTg-AD synaptosomes to maintain iCa levels at Zt16; H2O2 stimulus did not show significant changes between ZT’s or genotypes. Data suggest that, when challenged/stressed, the mitochondria unravel to be dysfunctional in the AD mouse model. These findings are in agreement with the oxidative stress hypothesis for AD.

We concluded that, at this stage, there is impairment of the circadian rhythm on key traits of
AD pathogenesis; the study further outlines the prevalence of diurnal variation and pinpoints the need to better characterize the neurobiology of disease and integration of circadian rhythms as a crucial variable. Moreover, it supported the idea of mitochondrial dysfunction and increased susceptibility to oxidant stimulus.

At the age of 6 months (24 wks) the pathology in 3xTg-AD mice has been described to be established. Thus, in the second chapter we proved that at this age the cognitive decline, electrophysiological and mitochondrial dysfunction were present. Then, we posed a question regarding the circadian rhythm: Are biological rhythms impaired in AD? Data outlined the relevance of the diurnal variation studies in pathology. We showed that AD impairs biological rhythms, similarly to a circadian disruption.

To answer this questions we perform NMR analysis. In fact, NMR has been largely used as a method for longitudinal studies of metabolites and biochemical pathways. Therefore, metabolic analysis was also performed using this technique. This technique allows to measure, under baseline conditions, the direct pathway (hepatic glycogen synthesis from intact hexose) and indirect pathway (hepatic glycogen synthesis from gluconeogenic processing). The administration of deuterated water ($^{2}\text{H}_2\text{O}$) leads to positional 2H-enrichment of hepatic glycogen, rendering the effect of a disease (e.g. type 2 diabetes or other interventions on these fluxes (circadian disruptions) accessible to in vivo evaluation.
Animals were administered intraperitoneally with a bolus of 1% $^2$H$_2$O 24h prior to euthanazia and the liver used. mRNA and metabolites were extracted and analyzed, by qPCR and NMR, respectively. Biological liver samples of 3xTg-AD and Non-Tg mice were collect at ZT 04 & ZT 16. Our analysis showed alterations in already validated biomarkers at specific ZT’s. Metabolic alterations perceived in AD samples were accompanied by changes in gene expression. Moreover, rate-limiting enzymes presented a diurnal variation profile in Non-Tg animals, while 3xTg-AD animals displayed alterations in the circadian pattern of expression in some metabolic genes. Analysis of metabolic fluxes by NMR depicted changes in lipid metabolism, and differences in gluconeogenesis, which were coincident with metabolic alterations seen in type 2 diabetes mellitus, obesity or metabolic syndrome. Finally, analysis of canonical circadian genes revealed an alteration in the diurnal variation of core clock genes in the 3xTg-AD mice. In addition, AD animal model showed alterations in diurnal variation profile of metabolic networks. Those changes correlate with a chronodysfunction at the peripheral level.

In suma, in this thesis we show that AD-related pathology in the 3XTg-AD mouse model is linked to cognitive decline (Chapters 4.1 & 4.2) and metabolic alterations in brain and peripheral tissues (Chapter 4.2 & 4.3). In addition, this thesis ends by underpinning an AD-related chronodysfunction, thus raising the question of circadian dysfunction as a culprit for neuropathology.
RESUMO

De modo a manter a homeostasia, os organismos desenvolveram um sistema de relógios moleculares endógenos com vista a antecipar o momento do dia e assim sincronizar vários processos fisiológicos. Um relógio disfuncional pode estar envolvido em várias doenças, incluindo síndrome metabólica e doenças neurológicas. Pacientes com a doença de Alzheimer (AD) exibem ritmos circadianos anormais que precedem o aparecimento de défices cognitivos. Há evidências de que: (i) um relógio disfuncional é responsável por síndromes metabólicas e alterações nos níveis de glucose e de insulina, (ii) insulina afecta os níveis de β-amilólido, um dos péptidos associados com AD e (iii) pacientes com AD possuem uma alteração do relógio circadiano.

Alterações no metabolismo, nomeadamente alterações na utilização de energia e no metabolismo da glicose, precedem a deterioração das funções cognitivas. Estas alterações parece ser um dos primeiros sintomas de ADDA.

O envelhecimento é acompanhado de um declínio varias funções corporais. O relógio circadiano também perde lentamente o seu ritmo. Este é um processo progressivo e parece ser um derivado do processo de envelhecimento. É importante salientar que muitos desses processos são orquestrados pelo relógio circadiano, que regula temporalmente a homeostase, o ciclo celular, bem como os processos de memória e aprendizagem. O corpo também pode perder a capacidade de controle temporal e de coordenação e sincronização das funções corporais, tais como o metabolismo em todas as suas diferentes formas, nomeadamente lipidogenesis, adipogênese, metabolismo da glicose e insulina. As repercussões da falta de sincronia dos processos metabólicos são
implícitos e parece ser fundamental no aparecimento de doenças neurodegenerativas em que a idade é um factor de risco, tais como DA.

O sistema circadiano é um relógio molecular, que consiste de um oscilador responsável pela geração do ritmos biológicos diários. Através de vias de entrada, o ambiente e outros componentes do sistema nervoso enviam informação para o relógio, e também para as rotas de saída, através da qual o oscilador fornece informação temporal para uma grande variedade de processos fisiológicos e comportamentais. Quando as células são removidas e mantidas em uma fatia do cérebro, eles geram ritmos de 24 horas de actividade eléctrica, de secreção e da expressão do gene. A amplitude da actividade eléctrica, a expressão dos genes e a actividade hormona-secretora declina com o envelhecimento cerebral. A dessincronização do relógio circadiano apresenta grandes alterações em todos os sistemas biológicos. Estes incluem mudanças na formação da memória, bem como alterações hormonais e neuroquímicas, com consequências para a neuroplasticidade.

O modelo animal triplo transgénico (3xTg-AD) da AD exibe abnormalidades na ritmicidade circadiana que precedem alterações patológicas, tornando este modelo de doença instrumental para investigar a interação entre o relógio circadiano, o metabolismo e as possíveis conexões com a patologia. Sendo assim, este estudo teve como objetivo revelar o impacto de AD nos ritmos biológicos relevantes na patologia, bem como obter uma compreensão mais profunda da fisiopatologia.

Inicialmente, este estudo avaliou o impacto do perfil circadiano no desempenho cognitivo em murganhos 3xTg-AD de 24 semanas de idade versus
animais controle da mesma idade (Non-TG), explorou também como estas alterações são correlacionadas com a potenciação de longa duração (LTP) no hipocampo e como a função mitocondrial, um indicador do metabolismo cerebral, e alterada pela biologia circadiano. A nossa análise foi realizada no Zeitgeber (ZT) 04 (4 horas após a luz acesa) e ZT 16 (4 horas após a luzes apagadas).

A variação diurna, em tarefas de aprendizagem dependentes do hipocampo nos 3xTg-AD e nos Non-Tg foi avaliada. Os resultados do “Morris water maze” (MWM) mostraram que, independentemente da a hora do dia existiam défices cognitivos observáveis nos 3xTg-AD; uma correlação entre ZT e os genótipos só foi possível quando se realizou tarefa de aprendizagem espacial reversa no MWM. Estes resultados sugerem que o padrão de variação circadiana do desempenho da memória depende do tipo de tarefa e o impacto do relógio circadiano em patologia é mais visível em tarefas mais complexas.

A amplitude da LTP no hipocampo, uma correlação eletrofisiológica com processos de memória relacionada, ou seja os sinais elétricos, registrados extracelularmente em fibras de Schaffer/CA1 sinapses pirâmide, exibiu um perfil circadiano em animais Non-Tg, o que não foi observado em animais 3xTg-AD.

Perturbações na função mitocondrial e do stress oxidativo têm sido implicados na patofisiologia da AD. Por conseguinte, o potencial de membrana mitocondrial ($\Delta \Psi_m$) foi medida em sinaptossomas corticais; exposição ao peróxido de hidrogênio ($\text{H}_2\text{O}_2$) revelou aumento de depolarização mitocondrial em ZT 16, sugerindo uma susceptibilidade ZT-dependente ao estímulo oxidativo. Simultaneamente, os níveis de cálcio intracelular (ICA) foram
medidos, revelando uma incapacidade dos sinaptossomas de 3xTg-AD para manter os níveis iCa ao ZT 16; a exposição a estímulo não mostraram alterações significativas entre ZT's ou genótipos. Os dados sugerem que, quando desafiado / estressado, as mitocôndrias apresentam um comportamento disfuncional no modelo animal de DA. Estes resultados estão de acordo com a hipótese do stress oxidativo na DA.

Concluiu-se que, nesta fase, há comprometimento dos ritmos circadianos em características-chave na patogênese da DA; o estudo destaca ainda mais a prevalência de variação diurna e aponta a necessidade de caracterizar melhor a neurobiologia da doença e a integração de ritmos circadianos como uma variável crucial.

RMN tem sido largamente utilizado como um método para os estudos longitudinais de metabolitos e vias bioquímicas. Esta técnica permite medir, em condições basais, a via direta (síntese de glicogênio hepático de hexose intacta) e via indireta (síntese de glicogênio hepático do processamento gliconeogênica). A administração de água deuterada ($^2$H$_2$O) leva ao enriquecimento posicional 2H- de glicogênio hepático, tornando o efeito de uma doença (por exemplo, diabetes tipo 2 ou outras intervenções nestes fluxos (dessincronia de ritmos circadianos), acessíveis para avaliação.

Com a idade de 6 meses (24 semanas) a patologia em ratinhos 3xTg-AD foi descrito como estando estabelecida. Assim, no segundo capítulo, mostramos que a esta idade o declínio cognitivo, disfunção eletrofisiológico e mitocondrial estavam presentes. Em seguida, perguntamo-nos sobre o ritmo circadiano: estão os ritmos biológicos dessincronizados na DA? Os dados sublinham a relevância dos estudos de
variação diurna em patologia. Mostrámos que da prejudica os ritmos biológicos, de forma semelhante a uma dessincronização do ritmo circadiano.

Os animais receberam por via intraperitoneal um bolus de 1% 2H2O 24 horas antes de serem euthanaziados e o fígado removido. RNA e metabolitos foram extraídas e analisadas, por qPCR e RMN, respectivamente. As amostras biológicas de 3xTg-AD e ratos Non-Tg foram coletados ao ZT 04 & ZT 16. A análise revelou alterações em biomarcadores já validados em ZT’s específicos. AS alterações metabólicas percebidas em amostras de AD foram acompanhadas por mudanças na expressão gênica. Além disso, as enzimas limitantes apresentam um perfil de variação diurna em animais Non-Tg, enquanto nos animais 3xTg-AD apresentaram alterações no padrão circadiano de expressão em alguns genes metabólicos. Análise de fluxos metabólicos por RMN apresentam alterações no metabolismo lipídico e diferenças na gliconeogênese, que eram coincidentes com alterações metabólicas descritas em diabetes mellitus tipo 2, obesidade ou síndrome metabólica. Finalmente, a análise dos genes circadianos canônicos revelou uma alteração na variação diurna desses genes nos animais 3xTg-AD. Além disso, o modelo animal de DA mostrou alterações no perfil de variação diurna das redes metabólicas. Essas alterações parecem se correlacionar com um cronodisfunção ao nível periférico.

Em suma, nesta tese demonstramos que patologia relacionada com a AD no modelo 3XTg-AD está correlacionada com o declínio cognitivo (capítulos 4.1 e 4.2) e alterações metabólicas no cérebro e tecidos periféricos (Capítulo 4.2 e 4.3). Além disso, esta tese termina sustentando um cronodisfunção relacionadas com a AD, sugerindo a questão
da disfunção circadiana como um possível culpado para esta neuropatologia.
“What you need to know about the past is that no matter what has happened, it has all worked together to bring you to this very moment. And this is the moment you can choose to make everything new. Right now.” – Ajay SADH
Introduction
1.1 CIRCADIAN CLOCK: DEFINITION, FUNCTION, RELEVANCE

“Space and time are the framework within which the mind is constrained to construct its experience of reality”—Immanuel Kant

As you read these words, the Earth is rotating on its axis orbiting the Sun, creating a 24h cycle of light and dark periods which is present in almost our cells. This biological system is named the circadian clock; ‘circa’ means average and ‘diem’ means day, and like a clock it keeps track of time, allowing for the regulation of fundamental physiological functions and behavioural aspects, including body temperature, autonomic and endocrine function, and sleep-wake cycles. It has an approximate 24h periodicity, is self-sustainable and is capable of synchronizing to environmental cues [1].

This timekeeper is harboured in a region of the hypothalamus just above the optical chiasma and its form follows function. The Suprachiasmatic Nucleus (SCN), also known as Master clock, receives input via the retina hypothalamic tract (RHT) from the main entrainer of this system, i.e. light, conveying it in the form of neurotransmitters to a specific set of cells grouped together in a defined nucleus [2].

Thus, the SCN perceives light information from the environment and entrains these cells responsible for the generation of rhythms; by doing so synchronizes it to a specific light/dark cycle [3].

This clock uses light as timing cues or zeitgebers (German for time giver), which are used to entrain and synchronize internal rhythms to external environmental time. Light is not the only zeitgeber:
food, temperature and social interaction can also function as secondary entrainers [4].

By definition, zeitgeber time 0 (ZT 0) is the moment of exposure to first daily light and translates to real life as dawn. Analogously, ZT12 refers to the onset of night, if we consider a 12h/12h light/dark cycle.

The circadian clock or circadian oscillator is organized in a hierarchy of oscillators, with SCN being the master oscillator and responsible for coordinating independent peripheral oscillators, resulting in a coherent rhythm of physiological effects at the whole body level. All those clocks consist of an inner molecular machinery, which comprises a network of genes working together in transcriptional and translational feedback loops; these drive rhythmic, 24h mRNA profiles of core clock components, which protein products are necessary for the generation of rhythms in individual cells [5]. CLOCK (Circadian Locomotor Output Cycle Kaput) was the first mammalian locus found to regulate the circadian oscillator. The CLOCK protein together with BMAL1 (Brain and Muscle Arnt-like protein 1) form an heterodimer. This heterodimer comprises the positive limb of the feedback loop, which binds to E-box elements in the promoters of Period genes (Per1 and Per2) and Cryptochromes genes (CRY1 and Cry2) driving their transcription, and representing the negative limb of the feedback loop. Their transcription leads to an accumulation of their protein products in the cytoplasm, forming heterodimeric complexes that after post translational modifications translocate to the nucleus. Certain levels of CRY/PER inhibit CLOCK/BMAL1-mediated transcription, causing an indirect repression of CRY and PER transcription [6]. Those are considered the primary core loops but
there are also secondary negative loops mediated by other genes.

**FIGURE 1: CIRCADIAN CLOCK - A MOLECULAR TIMEKEEPER**

Illustrative diagram on the circadian clock, the entraining mechanism by which it synchronizes the inner milieu with external milieu, the molecular machinery behind this time keeping mechanism, the peripheral oscillators and their physiological output. Figure was adapted from doi:10.1038/nrneurol.2014.206

Secondary loops also play key roles in the circadian regulation. Rev-erb α or Nuclear Receptor subfamily 1 group D member 1 (NR1D1) is the mediator of this negative feedback loop connecting the positive limb
with the negative limb through activation of the CLOCK/BMAL1 heterodimer. Although Rev-erb α is not required for behavioural rhythms, its expression increases the precision and stability of the molecular clock by providing a second form of circadian regulation besides E-Box. This is because Rev-erb α is able to bind to another clock enhancer element, Retinoic acid related Orphan receptor Response Elements (RORE), thus inhibiting the activation of BMAL1 expression by transcription factors, ergo suppressing BMAL1 expression [7].

Rev-erb α has been considered a mediator of metabolism since 1985, when Hélène Duez and Bart Staels proposed that the nuclear receptor and core clock component Rev-erb α behaves as a gatekeeper to coordinate the circadian metabolic regulation and coordinate the timing of metabolic regulatory networks [8].

The TTFL model described above relies on transcription and translation and is considered to be the responsible mechanism for generating self-sustained rhythms in almost all cell types and almost all organisms. Nonetheless, in 2011 O’Neil and Reddy described a mechanism in human erythrocytes where peroxiredoxins show a 24h redox cycle [9]. Moreover, this study suggested a common ancestry for the circadian clock, linking the co-evolution of cellular time-keeping with redox homeostatic mechanisms.

Maintenance of transcriptional feedback loops of the SCN are sustained by 3′,5′-cyclic adenosine monophosphate (cAMP) signaling; this molecule is involved in maintaining amplitude, phase and period. Involvement of rhythmic cAMP-mediated cytosolic signaling enlarges the concept of the mammalian pacemaker beyond transcriptional feedback. Since
cAMP is a secondary messenger important in many biological processes, its importance becomes of relevance. cAMP derives from adenosine triphosphate (ATP) through the activity of adenilcyclase and used for intracellular signal transduction by neuronal receptors, being also involved in adenosinergic function[8].

The communication between cerebral temporal information and peripheral tissues is achieved not only by an output system composed of direct efferent innervation of specific brain nuclei and peripheral organs, but also via peptide, such as hipocretins, secretion and its actions [9]. These humoral factors are expressed in genes with regulatory elements targets of core clock proteins, which encoding genes are called clock controlled genes (CCG). Ten to twenty percent of all gene expression, in almost every given tissue has been estimated to be circadian regulated, either at the transcriptional or translational level. Furthermore, circadian regulation is also evident through post-translational protein modification [10]. Clock controlled genes create a network of pleiotrophic interactions between the molecular machinery of circadian clock and diverse processes and pathways relevant in metabolic homeostasis and health.

Peripheral oscillators work together to give a finely tuned homeostatic symphony, conveying output signals from the master clock (the SCN) and coordinating local circadian rhythms in intermediary metabolites (e.g. NAD+/NADH) or in key rate-limiting enzymes (e.g. phosphoenolpyruvate carboxykinase (PEPCK), pyruvate kinase (PK), glucose 6-phosphatase (G6Pase)) involved in lipid and carbohydrate metabolic pathways and in hormone secretion (e.g. insulin, leptin, cortisol, growth hormone).
Experimental studies have depicted different roles for different peripheral oscillators in complex physiological systems. These include adrenal gland regulation of gene expression profiles by the SCN causing alterations of plasma and brain corticosterone levels independent of activation of the hypothalamo-adenohypophysial axis, those are light-induced clock-dependent genes responsible for secretion of glucocorticoids. Ovulation and estrous cyclicity are also under circadian control. In the CLOCK mutant mouse not only is estrous cyclicity disrupted, but the reproductive efficiency is also compromised. Clock mutant females have irregular and lengthened estrous cycles where the luteinizing hormone (LH) does not coordinate with day of proestrus, as well as showing increased fetal reabsorption during pregnancy. These observations suggest that an appropriate circadian signal is mandatory for coordinating hypothalamic hormone secretion to support normal reproductive function. Adipose tissue and lipid metabolism are also tightly regulated by PER2, a core clock circadian gene. This includes direct control through the specific repression of PPARγ, a nuclear receptor critical in adipogenesis. Thus, PER2-knockout mice display altered lipid metabolism, reduced levels of total triacylglycerol and nonesterified fatty acids, resulting in pro-adipogenic activity in cultured fibroblasts. Pancreatic islets have self-sustained circadian gene and protein oscillations of the transcription factors CLOCK and BMAL1. Clock and BMAL1 mutant mice display impaired glucose tolerance and reduced insulin secretion, while pancreatic islets show alterations in size and proliferation. Moreover, a conditional knockout of Clock gene, tissue specific to the pancreas, causes diabetes mellitus, suggesting a role of circadian clocks in the regulation of insulin and glucagon secretion.
The effects of altered circadian regulation of pancreatic islet hormonal secretions can have significant effects on liver carbohydrate metabolism, and systemic glucose and lipid homeostasis. Those processes are abolished by deletion of *BMAL1* and are depressed in *CLOCK* mutants. Mouse studies showed that *BMAL1* and *CLOCK*, two core molecular clock genes, have a profound role in recovery from insulin-induced hypoglycaemia and suppress the diurnal variation in glucose and triglycerides [11-16].

In mammals the most accepted rationale for the origin of circadian clock, is based on the need to protect cellular processes such as DNA replication from sun ultraviolet radiation. This provides a clear evolutionary advantage, since it allows the anticipation and coordination of biological processes, as well as separation in time of reciprocal biological processes such as substrate oxidation and biosynthesis [17].
1.2 CIRCADIAN CLOCK IN NEURODEGENERATIVE DISEASES

The control and maintenance of synchrony between physiological processes is probably one of the most fundamental tasks of circadian clock. Thus, malfunction in the temporal organization of behavioural, physiological, cellular and neuronal processes has severe consequences at the organism level.

Aging affects the amplitude of the SCN rhythm, which can result in a dysfunction of clock control by a lack of synchrony between the neuron networks that constitute the master pacemaker. Therefore, it is not surprising that a dysfunctional clock is associated with diseases like metabolic syndrome, obesity, cancer and neurological disorders [18].

Circadian rhythms influence memory formation and are intertwined with alterations in metabolic functions. Circadian rhythm dysfunction, cognitive decline, namely memory loss, and alterations in metabolic processes are paired with natural aging. Despite our poor understanding on how those mechanisms occur, the hypothesis supported state that circadian rhythm disruption is part of the aging process and is driving the cognitive decline visible in elder people. These cognitive decline could be due to an array of different actions: epigenetic mechanisms, regulation of neuroendocrine pathways, alterations in metabolism. Those changes may have different causes subjacent to: chronic sleep deprivation, social jet-lag, metabolic entrainment, psychosocial stressor impacting adolescents, excessive artificial lightning, etc [19,20]. Nevertheless, due to the intricacy of these problems, our current knowledge has not allowed to discriminate the specific role
and the relative contribution of each of the factors involved.

Therefore, we must look at the convergence point, alterations of epigenetic mechanisms that occur in the SCN and in the hippocampus, bringing together circadian rhythm generation and memory formation, respectively [21].

Clock controlled genes are involved in the production and utilization of nutrient metabolites, which include the **nicotinamide phosphoribosyltransferase** (NAMPT), a rate-limiting enzyme in the NAD\(^+\) salvage pathway, a NAD\(^+\) biosynthetic enzyme. NAD\(^+\) has a central role in redox reactions and is a cofactor for SIRT1, SIRT3–5 and PARP1. The role of sirtuins in aging and disease, as well as their role as anti-aging genes has been established in animal models. In humans, sirtuin genes are a bridge between aging and metabolism; for instance SIRT1 regulates mitochondrial biogenesis and partly explain the effects of calorie restriction on lifespan, boost in sirtuin activity leading to less production of reactive oxygen species. SIRT1 was also discovered as the gene involved in the regulation of insulin sensitivity in muscle tissue that is directly regulated by CLOCK and BMAL1 actions.

CLOCK/BMAL1 heterodimer not only regulates NAMPT, but also leads to reduced NAD\(^+\) levels. NAD\(^+\) oscillations are dependent of core clock entrainment; interestingly, animals kept in constant darkness still present rhythmic expression of NAD\(^+\) [22].

Circadian amplitude dampening results in sleep fragmentation, which impact in memory consolidation [23]. Sleep patterns alterations are reinforced by changes in timing of light exposure, creating phase shifts in gene expression of core clock proteins.
Circadian rhythms have been linked to epigenetic function in the hippocampus, as some of these epigenetic mechanisms oscillate in the hippocampus and are considered to be circadian driven, namely AMP/MAPK/CREB signal transduction pathways [25]. Therefore, alterations of circadian gene expression will alter mnemonic mechanisms. Moreover, a reduction in homeostatic sleep drive, mediated by caffeine consumption and alteration of adenosine levels lead to the fragmentation of sleep and phase shifts via social and metabolic alterations [26].

The timing provided through the entrainment of peripheral oscillators ensures that nutrient transporters and metabolic enzymes are produced at the appropriate time, for example activating catabolic pathways during the active period to match increased energy demand. Moreover, it promotes a temporal separation of metabolic activities, such as glycolysis and gluconeogenesis. Thus, there is a fundamental interaction between circadian control and metabolism.

The role of metabolism in neurodegenerative disorders is accepted as an association between a) hyperinsulinemic states, b) insulin resistance and c) prevalence of diabetes among AD patients. This triad has been reported and recognized. Several biological factors support this hypothesis. Insulin-degrading enzyme is considered to modulate extracellular levels of the beta-amyloid protein; inactivation of glycogen synthase kinase 3 beta (GSK-3β) by insulin augments AD pathology as this protein phosphorylates tau protein. Epidemiological study (ERGO) reported abnormalities in insulin action or in insulin receptor of individuals in the early stages of AD; those patients had 45% lower levels of cerebral glucose utilization and 17–18% reductions in cerebral blood flow that correlated
with cerebral metabolic rate of oxygen consumption relative to controls.

Cognitive ability of AD animal models are improved by leptin administration; indeed, mechanistic studies suggest an upstream action linked to metabolic pathways modulated by leptin that positively impact on amyloid-β homeostasis and tau phosphorylation, contributing to amyloid plaque and neurofibrillary tangle (NFT) formation, respectively. Leptin can modulate 5' adenosine monophosphate-activated protein kinase (AMPK) activity following binding to the leptin receptor; activation of AKT by AMPK leads to the phosphorylation of GSK-3β [27].

Temporal organization of metabolic and replication cycles is advantageous and so circadian-clock dependent control of metabolic processes in peripheral tissues, such as the liver is crucial. SCN output rhythms are mainly propagated through neuro-endocrine and pre-autonomic motor neurons in hypothalamic regions. The SCN projects into some hypothalamic nuclei, being the Paraventricular Nucleus of the hypothalamus (PVN), the Dorsomedial Nucleus of the Hypothalamus (DMH) and the Medial Preoptic Area (MPOA), the most important and the ones with a clear defined function [28].

Neuro-endocrine circadian regulation by glucocorticoids hormones is highly relevant in energy metabolism; therefore, alterations in PVN innervation by SCN will impact the Hypothalamo-Pituitary-Adrenal (HPA) axis, as the corticotrophin releasing hormone (CRH) is synthesized in the PVN and responsible for the release of corticosterone [29].

Similarly, melatonin production is regulated by GABAergic and glutamatergic combined output from the
SCN to the PVN; this process allows, through GABAergic enervation to PVN, to produce low melatonin levels during the light period and an arrest of the light-induced activity of GABA inputs from the SCN combined with a continuous activation of glutamatergic inputs.

FIGURE 2: A BAD TIMING AND THE POSSIBLE CONSEQUENCES TO AGING AND NEURODEGENERATION

Circadian Clock function along with the physiological functions coordinated and regulated by the circadian molecular machinery; the deregulation of clock controlled genes and the impact on neurodegeneration, namely through metabolic homeostasis, mitochondrial regulation, neurohormonal regulation by the hypothalamic–pituitary–adrenal axis and the glucocorticoid hypothesis, reactive oxidative hypothesis, melatonin and other hormones circadian
secretion. All those pathways are circadian regulated and their impairment has deleterious consequences in aging and neurodegeneration. Figure was adapted from doi:10.1038/nrn3208 & doi:10.1038/nrm2995 & doi:10.1038/nrn1177 & doi:10.1152/ajpregu.00327.2006

The control of the autonomic nervous system by the SCN creates a daily rhythm in plasma glucose concentration [30]. This is especially relevant in the central nervous systems because a constant blood glucose level is mandatory to maintain neuronal function; indeed, neurons are unable to store the required amount of glucose as glycogen sources for normal cellular functioning. On the other hand the control of hepatic glucose production is performed by stimulation or blockade of glutamate or GABA receptors, respectively, in the PVN [31].

Phases of daily cycles of hepatic gene expression are imposed by SCN signaling, generating a local enzymatic activity synchronized with the metabolic demands of activity and sleep. This process is regulated as a whole, but also regulates the central pacemaker. These gene patterns are superimposed on changes of liver metabolic fluxes that occur as a result of the feeding to fasting transition, but they also suffer a feeding entrainment, thus working as a feedback mechanism.

These regulatory mechanism allows for the temporal organization of food-induced entrainment of the circadian clock at a specific time during the day. As an output, this create a food-seeking behaviour prior to normal feeding time, outlining the effects of circadian clock on food intake, and appetite hormones levels. This gains further relevance in the context of obesity and diabetes development, since altered nutrient intake in combination with modified hepatic metabolism is a co-factor in disease.
On the importance of brain glucose sensing mechanism and their interconnection with neurodegeneration and metabolism in general, one must consider the problem stepwise. Absorption of macronutrients from intestinal digestion is firstly sensed by the gastrointestinal tract (the gut brain theory), secondly the whole of chemoreceptors present in the hepatoporal system comprehend those metabolic signals, integrate them and communicate them to the brain. Nonetheless, the brain is also capable of sensing and acquire metabolic information on its own; an array of peptides such as insulin, leptin, adiponectin, or neuropeptide Y convey information from different organs such as gastrointestinal tract, pancreas, stomach or adipose tissue.

The liver releases a number of regulators of glucose and lipid metabolism, functioning at a paracrine and endocrine level. Brain and liver collect different types of information, they act as specialized separated structures. The brain converge different data to hypothalamic regions that function as integration centers, one of those is the PVN. Regarding the integration of humoral and neuronal signaling, the PVN is innervated by forebrain areas, the bed nucleus of the stria terminals and also by hypothalamic nuclei, thus it is strategically located in a position that allows the receipt and convey of information to autonomic and neuroendocrine outputs. Although the data is still scarce, a recent study provided a clear evidence for the involvement of neuronal pathways in communicating sensory information about energy metabolism, from the hypothalamus to the cerebral cortex [32].

Different neuronal functions require different energetic levels and certain brain functions occur at certain day phases. During active phase, gene
expression pattern must support brain functions such as acquisition, responsiveness and information processing; during inactive phase, gene expression pattern has to support memory consolidation and synaptic rewiring [33,34].

Hence, mice deficient in the circadian transcription factor BMAL1 exhibit reduced lifespan and show symptoms of premature aging: sarcopenia, cataracts, reduction in adipose tissue and muscle mass, organ shrinkage, and direct involvement in ROS homeostasis. All of those early aging phenotypes observed in BMAL1-/- animals correlate with increased levels of reactive oxygen species, which are important regulators of cellular metabolism. Thus, ROS homeostasis is controlled by circadian clock and provides an effective protection from damaging effects of oxidants, making these circadian mechanism relevant in the aging process as an imbalance in ROS levels can increase oxidative stress and augment the aging phenotype [35].
1.3 ALZHEIMER'S DISEASE - MAIN CHARACTERISTICS AND PATHOLOGICAL MECHANISMS

In 2014 there were worldwide nearly 44 million people with AD or a related dementia; projections forecast that by the year 2050 the prevalence of AD will be 106.8 million. Epidemiological data reveal that 2-in-3 people with AD are women; 30% of people with AD also have diabetes and hospitalization are three times more often than seniors without AD, show altered levels of cholesterol and display an array of metabolic changes [36].

AD is characterized by two pathologic hallmarks. Amyloidopathia, a condition associated to the formation of plaques by extracellular deposits of amyloid-β (Aβ) peptides derived by an abnormal cleavage of amyloid precursor protein (APP), and tauopathia a condition considered to be the result of tau hyperphosphorylation that aggregate intracellularly forming neurofibrillary tangles (NFTs). These abnormal protein aggregates result in cell damage; under these conditions neurons no longer function normally and may die, which in turn can lead to changes in memory formation and storage, altered behaviour and capacity to reason clearly. Synaptic deficits, alterations in neurovascularization, reduction in brain volume and death of neurons lead to an impairment to carry out basic bodily functions, resulting in a burden for the health care system and caregivers.

Although idiopathic AD is symptomatic. AD Symptoms are described in the Diagnostic and Statistical Manual of Mental Disorders (DSM5) by the American Psychiatric Association as: a) early clinical symptoms describe difficulty in remembering recent events, apathy and mild depression.; b) later symptoms include impaired communication,
disorientation, confusion, poor judgment, alteration in behaviour and changes in personality traits, c) terminal stage display difficulty in articulating words, swallowing and walking, becoming ultimately bed-bound [37].

Familiar cases of AD represent 1-10% of all AD patients; these are caused by three genes mutations: amyloid-β precursor protein (APP), presenilin 1 and presenilin 2, which are involved in the production of Aβ peptide. Age of onset in familiar AD cases is as early as 30 years of age, in comparison to 65 or later in other cases.

FIGURE 3: ALZHEIMER CLASSICAL VIEW VS AN ALTERNATIVE VIEW.
The pathophysiological traits of Aβ and Tau in Alzheimer’s disease. The physiological role of Aβ and Tau and how their levels might affect disease. Figure was adapted from DOI:10.5772/57398 & DOI:10.1242/jcs.01558

The focus in AD should not solely be in the pathophysiological alterations in targeting β-amyloid and tau proteins. The track record from clinical trials does not provide positive results to support such claims, showing an overall failure of more than 300 drugs and biological products that failed in clinical trials. This current trend in AD research points their beliefs and efforts on those two proteins as being the guilty partners in this devastating age-related neurological disease. Aβ and tau are without doubt part of the AD process, but perhaps mere consequences. Individuals who die of unrelated causes and show no neurological condition or cognitive impairment in post mortem evaluation display Aβ plaques and tau tangles. Nonetheless, this also suggests that Aβ and tau have different physiological roles in the brain at different stages of AD, counterwise to the predominant scientific consensus. Thus, raising the question of the physiological role of those proteins.

Sporadic AD is the gross amount of all AD cases, there are no mutations associated, leaving the rest as the possible culprit, if not nature than nurture. Note that ApoE4 (apolipoprotein E4) is a risk factor in sporadic AD, that is possible of being influenced by nurture. Behaviour and environmental factors lead to an increase in oxidation levels, commonly produced as a response to those insults. Thus, the concept of oxidative stress as a trigger of AD is recognized as a plausible hypothesis [38].
Oxidative stress is a direct downstream effect of the putative cause of AD, nurture. Neurons and other cells in the brain have mechanisms to protect that are regulated upon an increase in ROS levels and antioxidant mechanisms [39]. Aβ itself can play the role as an antioxidant agent, just like superoxide dismutase. Therefore, Aβ production can be interpreted as the brain counteracting to control the production of ROS and prevent oxidative damage.

Neurofibrillary tangles are composed by hyperphosphorylated Tau. This trait is not exclusive of AD, being observed in other pathologies such as chronic traumatic encephalopathy for instance [40]. Thus, tau appearance can be considered a protective one, upon insult tau protein act inside neurons as an antioxidant barrier trying to prevent damage; it seems that when large and prolonged events of oxidative damage occur, tau ability is overcome resulting in the classical NFTs seen in AD [41].

Interestingly, in early stages of AD there is more Aβ and tau in the brain, but the levels of oxidative damage in neurons are lower, unfortunately as the source and events keep on occurring, the insults keep on adding up and flood the defensive mechanism systems of the brain, leading to an increase in production of Aβ and tau. Thus, what was meant to be a defensive mechanism becomes one that attack cells.

Aβ vaccines have depicted the physiological importance of Aβ in maintaining the integrity of the blood brain barrier, as those treatments resulted in micro haemorrhages and subsequent encephalitis [42].

This cascade of events presupposes one main event, pivotal to this hypothesis. A deregulation in energy
homeostasis or energy usage. This could be a cause or a consequence, but what is generally observed is a metabolic syndrome status, mitochondrial dysfunction, high levels of ROS, neuronal damage and subsequent death. The causal nexus is then established, one just needs to find arguments/data supporting such an hypothesis.
1.4 IMPACT OF METABOLIC CHANGES IN AD NEURODEGENERATION - THE ROLE OF MITOCHONDRIA

Energy failure may be one of the earliest reversible hallmarks of AD. Evidence points to a central role for mitochondrion in neurodegeneration. The “mitochondrial cascade hypothesis” [43] proposes mitochondrial dysfunction as the trigger in AD pathology.[44]

CLOCK mutant mice display metabolic syndrome, and those alterations resemble the ones observed in AD. Therefore, malfunction of this system may underlie aspects of the pathology seen in psychiatric and neurological disorders. Longitudinal studies have shown that during aging brain metabolism declines prior to the deterioration in cognitive function, suggesting that brain hypometabolism observed in AD patients is but an intermediate step in the cellular and functional cascade of neurodegeneration.

Neurons have high metabolic energy demands and have a limited glycolytic capacity, making them very dependent on mitochondrial energy production. Mitochondria are distributed along the axons and dendritic trees. Due to the metabolic demand associated with synaptic transmission, the synapse is the place with more mitochondrial density in order to provide the ATP necessary for the biological processes, mandatory to neuronal firing.

Therefore, suboptimal glucose utilization may be a critical contributor to neuropathology. Patients with diabetes or presenting metabolic syndrome have excessive blood glucose levels. Considering the mitochondrial hypothesis, one can advocate that defects in glycolysis through glyceraldehyde 3-phosphate dehydrogenase (GAPDH) inactivation in response to oxidative stress susceptibility are operating to generate the observed outcome.
Moreover, the high glucose levels may per se promote oxidative stress and protein modification via glycation. This results in less ATP generation per unit of glucose. Mitochondrion is the main site for ATP production through oxidative phosphorylation (OXPHOS).

The decline in brain glucose metabolism, observed even before the clinical onset of AD, might be related with the aging process, as aged people may exhibit reduced brain glucose metabolism despite displaying or not a neurological disorder. Aging appears to be related to systemic glucose dysregulation and a gradual imbalance in the tight homeostatic equilibrium between brain glucose uptake and brain function. The homeostasis of brain energy levels gains further relevance than in peripheral organs. Neurons only use glucose and astrocytes are the only capable of glycogenolysis. In excitotoxicity processes the overactivation of glutamate receptors leads to impairment of calcium buffering, generation of free radicals and activation of the mitochondrial permeability transition pore, resulting in a vicious cycle of ROS production and a subsequent impairment in energy utilization.

Over time, some brain regions, like the hippocampus, become increasingly at risk of chronic fuel deprivation and gradually become fatigued, which in turn facilitates the neuropathological changes leading to AD. These changes create in the mitochondrial an altered a state, which at first the organelle can cope, but gradually lead to an increase in dysfunctional mitochondria, subsequently causing permanent alterations as those observed in AD. One of the processes essential for synaptic communication and that requires energy derived from mitochondria is the exocytotic release
of neurotransmitters accumulated in synaptic vesicles. Synaptic vesicles formation involve numerous steps which consume ATP, making this mechanism mitochondria dependent, moreover cytosolic calcium concentration also regulate vesicle formation and release, as depolarization of the plasma membrane triggers the opening of voltage-gated calcium channels, leading to calcium entry [45]. Simultaneously, mitochondria contribute to maintain calcium homeostasis by providing ATP to both plasma membrane or endoplasmic reticulum Ca$^{2+}$-ATPase, as well as to maintain synaptic ion homeostasis and phosphorylation reactions necessary for synaptic transmission [46]. Work by Li and co-authors provided the evidence that dendritic mitochondria contribute to long-term alterations in synaptic strength [47].

On the other hand, synaptic activity triggered by glutamate receptors have an effect ofon mitochondrial bioenergetics, seen in NAD(P)H levels at CA1 synapses of hippocampal slices [48]. Thus, alterations in mitochondrial function, namely mitochondrial membrane potential, intra-mitochondrial Ca$^{2+}$ levels and ROS production lead to alterations in DNA and RNA oxidation status, potentially impinging mutations in mitochondrial DNA. Thus, when mitochondria are damaged or the energetic demands are abusive, the antioxidant defenses present in cells surpass their capacity, leading to an increase in ROS levels, causing further damage to mitochondria and disabling the protective mechanism, generating more free radicals.

In this perspective, environmental factors play a major role than generally ascribed to the development of dementia, in general, and AD, in particular. Societies (e.g. japanese) or dietary
habits (mediterranean diet) in which the intake of processed foods is very low, show a low incidence of dementia, diabetes and other chronic disease conditions [49]. Indeed, obesity and diabetes are closely related with AD, as evidence points to perturbed cerebral glucose metabolism and subsequent brain insulin resistance scenario as a culprit in AD [50].

Metabolic dysfunction is one the first symptoms associated with AD. Importantly, the presence of Aβ in mitochondria was shown to strongly influence mitochondrial respiratory function, ROS production rates, and alter mitochondrial membrane potential in different brain regions of AD mouse models [51,52].

AβPP and AβPP/PS1 AD mouse models display an association between mitochondrial dysfunction and cognitive impairment; moreover, these mice also presented Aβ levels above control and the concomitant presence of mitochondrial dysfunction were found in brain regions linked with memory, namely the hippocampus and cortex [53]. These physiological alterations were only visible in regions exhibiting Aβ plaques, and the alterations consisted of decreased number in mitochondria, presenting some dystrophic and fragmented traits with reduced mitochondrial membrane potential. These experimental data pinpoint Aβ plaques as a cause of toxicity, causing structural and functional changes in mitochondria [54].

Moreover, in vitro studies performed in cell lines from chinese hamster ovarian cells stably expressing human wtAPP751(7WD4 cells) and APP751 mutation V717F (7PA2 cells) showed a functional impairment in mitochondrial complexes I and IV. In addition, ATP production by oxidative phosphorylation was
decreased up to ~25%, mitochondrial membrane potential was decreased and ROS levels increased [55].

Glucose intolerance and insulin resistance also characterize AD. Peripheral metabolic alterations have an impact on insulin signaling pathways at the central nervous system. Recent hypothesis consider AD as a “type 3 diabetes”. Insulin plays a critical role in glucose homeostasis, its action regulates glucose production by the liver and glucose uptake by muscle and adipose tissues, in a balance that must be maintain at all times. But insulin signaling also controls neuronal function in cortical and hippocampal areas, which are deeply involved in cognitive processes.

Considering all the hypotheses described so far for AD pathogenesis, in our view the whole is more than the sum of the parts, meaning that the “mitochondrial hypothesis”, the “Type 3 diabetes”, the “Glucocorticoids hypothesis” or even the “Circadian hypothesis”, are all plausible hypothesis playing a relative part in the onset and disease pathogenicity.

To understand further how these hypotheses may interconnect, one should not forget that mitochondria are also under circadian regulation. A recent study by Sassoni Corsi Lab revealed that mitochondrial proteins involved in metabolic pathways are under clock-driven acetylation, of pathways comprising the citric acid cycle, amino acid metabolism, and fatty acid metabolism. As mentioned earlier, the regulation of mitochondrial oxidative metabolism by the circadian clock occurs through the transcription control of nicotinamide adenine dinucleotide biosynthesis or ATP production. Moreover, mitochondrial respiration is
modulated by mitochondrial protein acetylation to synchronize oxidative metabolic pathways with energetic demands and feeding rhythms [56].
1.5. ADENOSINE: A MODULATOR OF NEUROPLASTICITY, NEURONAL SURVIVAL, AND A HOMEOSTATIC REGULATOR

ATP is used as an energy source, as a cell signaling molecule, extracellularly as well as intracellularly, and the ratio between ATP and AMP is used as an energy sensor allowing the control of metabolic pathways that produce and consume ATP. Furthermore, ATP also plays a relevant role in transcription, as synthesis of RNA relies on adenosine derived from ATP to be incorporated directly into RNA.

ATP is almost energetically perfect, the amount of energy released when the phosphate bond is broken closely matches the needs of most biological reactions, so little energy is wasted. ATP is formed as it is needed. Because oxidative processes in the mitochondria are the primary responsible for ATP production, in mitochondrion oxygen is consumed only when ADP and a phosphate molecule are available [56].

ATP is surely the main energy source, but metabolic imbalance is not signalled through ATP. Thus, adenosine acts as an intracellular signal of metabolism; the enzymatic reaction of adenylate kinase allows that minor changes in ATP concentration produce alterations in AMP concentrations of measurable fold changes [57]. AMP is then converted to adenosine by 5'-nucleotidase. Adenosine kinase phosphorylates adenosine to AMP. Hippocampal levels of extracellular adenosine increase by twofold upon adenosine kinase inhibition. AMP creates an amplified signal used for network communication. Newby in 1984 called adenosine a “retaliatory metabolite”, the end molecule of a metabolic pathway that senses minimal
intracellular ATP concentration alterations and turns into large changes in extracellular concentration of adenosine.

Before entering into more detail into this intriguing function of adenosine, emphasis should also be given to the role of adenosine derivatives in biological system that are primordial in the regulation and function of mammalian cells: a) the control of cell cycle by S-adenosylhomocysteine (SAH) and S-adenosylmethionine (SAM); b) redox states are a function of nicotinamide adenine nucleotides (NAD\(^+\) and NADPH); and c) energy status that, as mentioned above, are defined by the adenine nucleotides AMP, ADP, and ATP.

Breakdown of ATP is considered the major source of adenosine in the brain [58]. Extracellular adenosine derived from ATP exerts a peculiar role in stress conditions of different nature, acting as a metabolic switch by altering the metabolic rates of neurons; moreover, it also signals into protective mechanisms [59]. When considering AD-related pathogenesis, a direct exposure to Aβ was shown to impair the functional status of the mitochondrial electron transport chain (ETC), being ETC a part of the OXPHOS machinery, its correct functioning allows ATP production and complexes I and III are a major sources of ROS generation. Therefore, impairment in ETC may have a great repercussion on ATP production. So, one of the protective mechanisms is the neurohomeostatic role of the adenosinergic system.

Adenosine plays a role in several basal physiological functions of the nervous systems. Adenosine is a neuromodulator, but is also a nucleoside that bridges modulation through metabolism, by not only regulating cellular
metabolism [60], but also being linked to sleep homeostasis [61].

Diurnal variations in adenosine, as well as of its metabolizing enzymes were demonstrated to be increased at the beginning of the circadian rest period in rat cortical tissue [62]. Moreover, pharmacological blockade of adenosine kinase revealed a correlation of the inhibition of adenosine metabolism and prolonged sleep. Genetic studies in mice also suggested that polymorphisms in the genomic region responsible for encoding adenosine deaminase (ADA) and adenosylhomocysteinase (SAHH) genes, which contribute to the regulation of extracellular adenosine levels, are associated with modifications in the rate at which nonREM sleep need accumulates during the active period [63].

As a neuromodulator, adenosine controls the activity of neuronal circuits in the brain [64]. Increase in the firing frequency of neuronal circuits is associated with an increase in extracellular levels of adenosine. Adenosinergic receptors (AR) impact at a neuronal level. At the synapse, A1R control basal synaptic plasticity and A2AR control LTP. The distribution and relative density of these receptors is still debatable, either in pre-, post- and extrasynaptic sites, with different levels of expression and density for the different synaptic zones.

Synaptic levels of adenosine are under the control of the bi-directional activity of equilibrative nucleoside transporters, which have a preponderant role in extracellular adenosine levels. Experimental data indicate that A2AR activation facilitates the activity of the transporters, thus
modulating the levels of extracellular adenosine required for activating $A_1$R [64].

Interestingly, following increased neuronal activity, activation of excitatory synapses leads per se to a large consumption of ATP, necessary for synaptic transmission and to restore the ionic balance. In the context of pathology, such a scenario cause higher metabolic demands leading to increased production of adenosine. These increased levels of extracellular adenosine make of $A_1$R activation a homeostatic inhibitory signal, acting as a feedback inhibitory system to restraint excessive excitatory transmission [65]. This homeostatic signal is also neuroprotective, as adenosine via $A_1$R activation exerts not only a decrease in cell metabolism, but also refrains neurotransmitter release, preventing or modulating the propagation of neuronal damage by refraining the ability of neurons to amplify neurotoxic damage signaling cascades [66].

Epidemiological studies (CAIDE) show that caffeine consumption, which antagonizes adenosine receptors, results in a neuroprotective effect. A consumption of 3 to 5 cups a day at midlife showed an associated decreased risk of dementia/AD of 65% at late-life. This study proposed that caffeine might impact on mechanisms like antioxidant capacity and increased insulin sensitivity [67].

$A_2a$R are of special interest, considering their involvement in the mnemonic process of LTP and their biological characteristics. $A_2a$R are positively coupled to adenylyl cyclase, inducing the synthesis of cAMP. The role of cAMP is well established in cognition. Moreover, cAMP is necessary for the maintenance of circadian rhythms amplitude, as well as biological rhythm periodicity. Evidence shows
that proteins essential for cognitive mechanism display a circadian profile of expression. Thus, suggesting that long-term memory persistence might dependent on the cAMP/MAPK/CREB transcriptional pathway in the hippocampus, which might be affected by circadian disruption, as the above proteins are circadian modulated [68].

cAMP signaling is an integral component of the SCN pacemaker, thus altering the rate of cAMP synthesis affects circadian period. In an indirect manner, alterations in the levels of ATP or adenosine and/or adenosine derivatives, either through receptor interaction or through signaling mechanism, are able to influence circadian rhythms. Experimental data shows differential circadian effects of adenyl cyclase inhibitors: MDL-12330A, an irreversible inhibitor of adenylyl cyclase, reduces the concentration of cAMP to basal levels, producing a damping of circadian period; whereas 9-(tetrahydro-2-furyl)-adenine (THFA), a non-competitive AC inhibitor, slows the rate of Gsα-stimulated cAMP synthesis, produces a period lengthening. cAMP kinetics alteration results in the regulation of cAMP signaling transcriptional cycles. Thus, output from the current cycle constitutes an input into subsequent cycles[69].

Furthermore, the adenosinergic system is impaired along aging; under these conditions the release of ATP is decreased with a parallel decrease in ecto-ATPase activity. Adenosine released from ATP is also more efficient in aged rats, and the contribution of the ecto-nucleotidase pathway to enhance adenosine levels upon stimulation of hippocampal nerve terminals is increased in aged rats [70]. This increase in extracellular adenosine is associated to increased number, coupling and efficiency of A2AR;
contrasting with a decrease in the number of inhibitory A₁R.

This array of adenosine actions and the role as neuroprotective agent in cases of energetic dysfunction makes of adenosine receptors potential therapeutic targets to treat neurodegenerative human diseases, with a special relevance of those with a metabolic and cognitive culprit or origin as AD.
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2) Objectives

“Do not expect to persuade people of truth by the power of argument. The only arguments I accept come from practical experiments.” – Nullius in verba
Objectives
2.1 OBJECTIVES/ WORKING HYPOTHESIS

The underlying cause of AD remains unclear. Current hypothesis in biomedical research have almost all fall short on fully understanding the mechanistic behind, our even conceiving a therapy that proves to be effective. One aspect we can be certain, the number of patients suffering from this disabling neurodegenerative disease will continue to increase. Understanding each physiopathological traits separately is futile, no change/shift is a result of an isolated action. Primarily because every action causes a reaction, secondly cause in physiology we are speaking about an intricate system with pleiotropic networks, interconnected signalling/transduction networks. Therefore, new perspectives and more integrative views need to be persuade. Allow me just to start by stating that with this thesis, we attempt not to discover the cure for Alzheimer, not to claim that certain hypothesis is more accurate, but aim to unveil new ways of looking into a one-century-old known problem. Thus, we can envision an alternative scenario for AD, where all current hypothesis are integrated, play a substantial and defined part in the onset and disease development.

The physiological role of circadian clock in the generation and maintenance of biological rhythms has a clear relevance in the regulation of metabolism. Indeed, the synchronization of dissonant metabolic processes, the control of peroxiredoxin proteins as antioxidants counteracting an endogenous rhythm in the generation of cellular Reactive Oxygen Species (ROS) or the modulation of glucose homeostasis and tolerance, and many other cellular processes exhibit a diurnal rhythm.
Thus, the aim of this study was to investigate whether a dysfunctional clock is present in AD-related pathology. Specifically, we aimed to determine how alterations in the circadian control can influence homeostasis at different levels and lead to altered states of energy. Moreover, we aimed to correlate alterations in metabolic pathways involved in memory and learning, both at a molecular and functional level. By examining the role of the circadian clock on AD pathogenesis, which may affect the regulation of metabolic pathways and memory formation, we may improve the current understanding of this devastating neurodegenerative disorder and unveil novel potential therapeutic targets and strategies.

In order to understand whether biological rhythms are affected in AD, the 3xTg-AD mouse model of AD, developed by Frank la Ferla's lab, was used. This mouse model, presents the pathophysiological traits more commonly associated with AD: namely progressive deposition of extracellular Amyloid beta (Aβ) plaques; intracellular neurofibrillary tangles (NFT) formed by hyperphosphorylated tau protein, which result from the insertion of three genetic mutations (in APP Swedish and PSEN1 M146V) that are never present together in familial AD patients plus a tau mutation (MAPT P301L) that never occurs in AD.

As described previously in this thesis, AD is a progressive neurodegenerative disease closely associated with aging. It presents a heterogeneous clinical course with a number of common features that surplus the characteristic extracellular Aβ plaques and intracellular neurofibrillary tangles. Early symptoms are misinterpreted as part of aging, including the loss of short-term memory, metabolic alterations (e.g. diabetes), alterations in hormonal levels (e.g. melatonin), progressing to
irritability, confusion and loss of long-term memory. As disease progresses, neuropathological changes install, causing the loss of neurons and synapses in the hippocampus and cerebral cortex. Nevertheless, peripheral metabolic modifications have also been related with early AD stages. Therefore, we also aimed to establish a clear correlation between the circadian clock and peripheral modified circadian metabolic rhythms in AD. To determine whether a disrupted circadian clock system affects states of energy homeostasis, stable isotope tracer, Hydrogen-2 deuterium (²H), were used for metabolite quantification and assessment of the role of the TCA in hepatic glucose and lipid metabolism. This further aimed to establish a comprehensive understanding of the role of hepatic insulin resistance and its relationship with glucose and lipid metabolism.

By taking into account those multiple approaches, the following questions were posed:

☐ Does AD cause alterations in biological rhythms, associated and referred in AD-related physiopathology?

☐ Are those alterations associated with neuronal alterations in synaptic circuits, or lack of circadian rhythms in cerebral areas affected by the disease?

☐ Does AD condition alter the metabolic activity at the level of the synapse and related mitochondrial machinery?

☐ How can AD impact on peripheral metabolism diurnal oscillation?
By questioning the impact of AD in certain biological rhythms and ultimately understand if the alterations can be peripheral and metabolically driven, data generated in this work unravels the importance of studying both central and peripheral diurnal variation of pathophysiological mechanisms and their relevance in the correct comprehension of AD.
Material & Methods

3.1 NEUROBEHAVIOURAL EXAMINATION

“Erfahrung ist verstandene Wahrnehmung”-Immanuel Kant

The study of the nervous system is in fact the study of its functions, behaviour is per se a neural function, so behavioural analysis is a window to the work in action of the different brain areas coming together to generate a behavioural response.

Due to our current status in comprehending how the brain produces behaviour is mostly an associative perspective, many attempts have been made but it is difficult to create a direct relation of brain function to elicited behaviour, meaning one cannot state A+B=C. Therefore, testing method must be conceived as a descriptive attempt: general appearance, sensorimotor behaviour, immobility and reflex acts, locomotion, skilled movement, species-specific behaviours, and learning skills.

A comprehensive description of behaviour makes use of: a) endpoint measures, quantify the consequences of actions; b) but as “all roads lead to ROME” movements can also be differently executed, so Kinematics provide Cartesian representations of the same action, giving measures of distance, velocity and trajectories, all of which provide an extra layer to the quantification of movement; c) in order to reduce ambiguity description of movements are made using formal languages such as Eshkol Wachman Movement Notation.

Prior to any measurement, the researcher should be aware of the basal state of the animal in order to infer conclusions and perceive alterations.
TABLE 1: EXAMINATION PRIOR TO BEHAVIOURAL TESTING

In table 1 are described the main features of physical examination, this should be done first in the home cage and after handling in a individual basis. All the parameters have a specific value and provide valuable information which is crucial to understand and perform correctly, subtle alterations in those parameters can be indicative of other dysfunctions and be indicative of other test to be performed, moreover those should be considered and monitored along the course of an experimental study.

Performing a behavioural test can vary in degree of stress to the animal, in its homecage is a passive act with little or no stress, whereas others are performed in specific apparatus being the animal removed from its cage and therefore increasing stress levels. Therefore, prior handling of the animals as well as habituation to the researcher and the environment is crucial for getting a unbiased
answer. In order to not interfere with the sleep pattern of the animals and alter serum corticosterone levels, as well as the density of GluNR2A subunit of NMDARs at hippocampal synapses, all handling and animal maintenance were performed active period (ZT 12 - ZT 24) [1].

Before starting with behavioural testing, one must adequate the behavioural test to the animal model limitations, but also direct it to meet the desired goal. Meaning that certain tests evaluate certain areas, which are typically more affected in certain diseases and interconnected with the performance of certain tasks. In Fig. 1 we take an overlook on the most commonly used behavioural tasks in the study of Alzheimer disease, in different experimental mouse models of the disease [2].
**FIGURE 1: BEHAVIOURAL TEST AND COGNITIVE ASSESSMENT**

In this figure a brief summary of the behavioural test most widely used in cognitive deficit assessment; it is accompanied by a brief description of the technique, the cognitive domains required to execute the task and the respective bibliographic reference. Figure was adapted from Webster et al. 2014
3.1.1 ANIMALS

Non-Tg and 3xTg-AD mice were a kind offer from Dr. Frank Laferla, University of California, Irvine, USA. All animal procedures were in accordance with the approved animal welfare guidelines (FELASA) and European legislation (European directive 2010/63/EU) as well as Portuguese legislation (Decreto-Lei n°113/2013). Animals were housed under a 12 h light/12 h dark cycle, a constant temperature 23-27°C and 35% constant relative humidity. Animals were given ad libitum access to water and food.

3.1.2 BEHAVIOURAL TESTING

At the age of 8 weeks, all mice were housed in a 12h/12h light dark cycle and subject to habituation to handling and experimental user interaction. Animal maintenance was performed only in the dark cycle and all handling and experimental testing was performed under dim red light conditions, to avoid phase shifts in circadian regulation. Locomotor activity, emotional performance, cognitive tasks were evaluated using the open field (OF), novel location recognition test or object displacement (OD) and Morris water maze (MWM). All experiments were conducted, recorded and analyzed using the ANY-maze® software (Stoelting, USA).
3.1.3 OPEN FIELD TEST

This test represents a locomotor behaviour, it enters in the category of exploratory behaviour. Animal movements are observed by removing it from its cage and placing in open environment or field. Typically will pause, rear, turn, groom then will explore the rest of the open field. As it begins to examine the open field will explore the surrounding area usually by moving along the edge of the walls of the apparatus. These exploratory episodes will be interlaced with brief and slowly backing movements followed by longer outward excursions. Normally as a defense mechanism during the course of the test and while it explores, animals tend to choose a more protected location than the centre of the apparatus, place where it was first placed and considered to be the home base.

Home base can be identified by the pattern of movements the animal display: circles and grooming being performed after an exploratory trip. All those characteristic movements have been carefully described by Gollani [3,4].

Open field test was first designed for assessing emotional behaviour in rats, it was developed by Hall but later work by Maxwell has proven to be also effective in mice. A careful analysis of the results allows the researcher an insight into general locomotors activity, an initial screen for anxiety-related behaviour and ability of the animal to explore novel environment. Over time, animals will tend to show a reduction in open field activity, will shift behaviour status and will spend more time grooming or sitting immobile, this is called habituation[5,6].

Mice received a session of 10 min in the empty uniform square, gray polyvinylchloride open field
(38×38 cm x38 cm) arena. The arena was dimly illuminated by red light, luminosity over the open field measured 50 lux. The center area consisted of 1/9 of total area. Overall activity in the box was measured as well as the amount of time and distance traveled in the center area of the maze. The anxiety level was assessed as time spent in the center. This paradigm is based on the idea that mice will naturally prefer to be near a protective wall rather than exposed to danger out in the open. Motor activity was recorded as total path, number of lines crossed, and speed.
3.1.4 NOVEL OBJECT RECOGNITION OR OBJECT RECOGNITION MEMORY TASK

The study of the neural basis of learning suggests an independence in learning and memory systems, which does not imply that they do not interact at some point. Processing information (learning) the capacity to recall information posterior to an event (memory), are events that can typically be divided in short-term memory, object memory, emotional memory and spatial memory, all those categories have attributed brain areas which are dedicated and associated to the performance of this memory tasks.

Novel object recognition is a validated test for studying memory does not require positive or negative reinforcers, this method rely in the natural exploratory behaviour and a preference for novel objects. Such a paradigm evaluate recognition memory and object recognition memory, brain areas associated/involved are hippocampus and the perirhinal cortex, which play different roles in object recognition memory. In this thesis, the test was used to test the influence of genotype (ZT04 vs. ZT16), and the effect of A2aR antagonist as therapeutical agent (data not shown).

The general idea behind the test is direct: a mouse gets exposed to two or more objects, after a certain period of exploration one of the objects is replaced. A memory circuit, which is functioning normally, will elicit a behavioural response that traduces in more time exploring this novel object than it does exploring the familiar object. An equal exploration of both objects is interpreted as a memory deficit. Procedural experimentation consist of three phases: habituation, familiarization, and test; this was adapted from the work developed by Ennaceur and Delacour. [7,8]
By modulating the interval time between familiarization and test we can evaluate either short-term memory, intermediate-term memory, or long-term memory, this time represents the retention interval [9,10].

Measurements performed were presented as recognition index (RI) = (TN − TF) / (TN + TF) where TF is the time spent with the familiar object and TN is the time spent with the novel object (Antunes and Biala, 2012)
3.1.5 NOVEL OBJECT LOCATION OR OBJECT-LOCATION MEMORY TASK

Object-Location Memory task or also termed Novel Object Location is a behavioural designed to study cognition, by definition is intended to prove spatial memory and discrimination. This test like the aforementioned is based on the spontaneous exploratory behaviour of rodents, which reflects in a higher time exploring a novel object than a familiar object, but also the ability to recognize the spatial location of an object and the power to discriminate if it has been relocated.

Likewise the object recognition memory task, testing is performed in an open field arena, animals are first habituated to the arena, next day they are presented to similar objects in terms of material but presenting distinctable shapes when introduced to the arena, attention to detail is critical and spacing is as much as possible equidistant from objects and arena. Object recognition task memory and the study of spatial location exploration is associated with hippocampal and cortical regions.[11,12] Duration of the different phases of the test have been explored in order to conclude the time required for discrimination, the work of Dix was taken in consideration when elaborating our behavioural test. [13]

Measurements perfomed were presented as Displacement index(DI), was calculated using the following equation (Tnovel × 100) / (Tnovel + Tfamiliar); corresponding to the ratio of the time spent exploring the displaced object (Tnovel) and the time spent sniffing/exploring (i.e. being physically within 1 cm distance of all objects, including the objects in the familiar location - Tfamiliar) adapted from (Assini et al., 2009).[14]
3.1.6 Y MAZE SPONTANEOUS ALTERNATION TEST

Y Maze Spontaneous Alternation is a behavioural test based on the tendency to explore novel environments; investigation of a new arm is preferred over returning to an arm that was already visited. Several maze tests has been used to deal with spatial navigation, although they have different shapes and allow to survey more complex ideas the central idea in all those mazes is to use the willingness habit of rodents to explore. [15]

This test is defined as: a) a measure of spatial learning, uses spatial location information in order to discriminate navigation or other exploratory behaviour making use of spatial cues; b) a measure of spatial reference memory, the capacity to recall spatial location presented in training sessions is used to navigate; c) some researchers consider these test also a reward reinforced choice behaviour due to the fact of making a choice presents a higher probability of increased reward.

In this thesis we used a modified version of Y-Maze which does not use reward reinforced choice behaviour.

Y-shaped maze with three opaque plastic arms with a 120° angle, animal is introduced into the centre of the maze, the animal is allowed to freely explore two of the three arms, in trial phase. Several parameters are taken into consideration being the most relevant the number of arm entries and the time spent in each arm. In the test phase of the memory task, all arms are freely available for exploration, being expected an increase of exploratory behaviour towards the novel arm. This test was used to quantify cognitive deficits and evaluate drug effects on cognition [16].
Performance of this behavioural task requires an interplay of different parts of the brain: prefrontal cortex, hippocampus, septum [17].
3.1.7 MORRIS WATER MAZE

The behavioural task designed by Morris was primarily designed to measure spatial learning and memory recall. This test is one of the most widely used in behavioural neuroscience and allows researcher to study aging related memory problems, experimental drugs effects on cognition and in the study of neurodegenerative diseases such as Alzheimer’s Disease.

These behavioural processes require the acquisition of spatial cues which are then processed, packed, stored, and then recalled, those mnemonic actions must be all successfully performed in order to navigate and ultimately locate a hidden platform that provide an escape of the water milieu.

The study of cognitive decline is suitably performed by Morris water maze, the integrity of forebrain cholinergic systems are critical for efficient performance of the MWM; this fact appears to be consistently correlated with a disruption observed in AD patients [18].

Moreover Cortical and hippocampal projections from different brain regions are also clearly damaged in AD patients. The involvement of those brain areas in the neurobehavioural task and the observations of pathophysiological alterations makes it a most suitable test for cognitive deficit related to AD.

The hippocampus is an essential structure for place learning, with a subset of cells called the grid cells, adjacent structure entorhinal cortex is critically involved in cognitive mapping, context dependent behaviour and spatio-temporal.

The different phases required in the behavioural task: spatial orientation, navigation, learning, and recall; rely in visuospatial, visuoperceptual
and topographic orientation; all indicate that this is a complex task involving visual pathways and mnemonic processing [19,20].

For this reason in our experimental approach we perform a Day 0 consisting of a visible platform trial for assuring all animals possess a visual accuracy necessary to perform the task.

Morris water maze procedure consisted of circular pool filled with water rendered opaque by tempera paint, maintained at room temperature (i.e. 20°C-21°C); a fixed platform is hidden just below the surface water level. In order to navigate visual cues are placed in the testing room, hung on the wall as a means of topographical cues and help navigating in the maze. Animals are tested individually, placed into the various quadrants of the pool; time elapsed, distance travelled, mean speed, as well as other parameters are recorded.

In our methodology, each animal performed four trials per day for four consecutive days; in each trial, the experimental animal was allowed to swim for a period of 60 sec until it found the hidden platform; when it reached the platform, the animal was kept for 10 sec in the platform. On the 5th day two the platform was removed from the pool and animal is allowed to swim for a 60 sec period, several parameters are considered and plotted, namely: the time spent in the target quadrant, the number of crosses over the platform location, time elapsed till reach of the location of platform, mean speed, time spent in the opposite quadrant. In order to evaluate working memory procedures, the hidden platform was re-located to the opposite quadrant. This is called reversal spatial learning and allows to obtain information regarding the cognitive flexibility necessary to extinguish old memories and
form new memories also test the working memory of the animal.
3.2 MEMORY & SYNAPTIC PLASTICITY

"A MEMÓRIA É A CONSCIÊNCIA INSERIDA NO TEMPO"—Fernando Pessoa

The absence of memory or memory lapses in daily life, outlines the importance of these phenomena, posing to modern neuroscience as the next frontier. Memory is the brain ability to perform complex and integrative actions, store information acquired by experience and to retrieve it when required. In general cognitive functions dependent on learning and memory. Learning terms the concept of acquiring new information by the nervous system, store and when recalled elicit changes in behavioural output. Memory demands an encoding and transmission of information in interconnected networks of synapses in the brain.

Plasticity is the cellular mechanism that provide the current and most plausible cellular and molecular bases for information storage. Synaptic plasticity is a concept that defines the ability of the synapse, connection between two neurons, to modulate its strength. This process is a response to the activation or disuse of synaptic transmission [21].

Hebbian theory postulates the basic mechanisms for synaptic plasticity, stating that an increase in synaptic efficacy is the result from presynaptic part repeated and persistent stimulation of the postsynaptic part. Persistence and or repetition of a reverberatory activity leaves a "trace", which results into long lasting cellular changes, namely growth process or metabolic alterations, that alter the efficiency of firing of the parts involved [22]. "It takes two to Tango", and when presynaptic and postsynaptic parts listen to the music they dance.
On his own words "When one cell repeatedly assists in firing another, the axon of the first cell develops synaptic knobs (or enlarges them if they already exist) in contact with the soma of the second cell" [23].

This is the more classic approach, thought recent study demonstrate that relevant physiologically synapse modification mechanisms in mammals synaptic strengthening are induced by either Hebbian physiologically synaptic activity and non-Hebbian relevant mechanisms, such as local protein synthesis or synaptic scaling [24].
3.2.1 EXTRACELLULAR RECORDINGS HIPPOCAMPUS

The alteration of receptors located on a synapse cause plastic changes, this synaptic plasticity requires the cooperative action of several mechanisms, namely changes in neurotransmitters release into the synaptic cleft and changes in the efficiency of the cells response.

Synapse strength is regulated by a positive feedback loop dependent on stimulation or weakened by its lack, and by a negative feedback loop, called scaling and metaplasticity. For this thesis purpose Synaptic scaling is more relevant and will be briefly explained. This is a primary mechanism, which enables neurons to stabilize its firing rates, it helps to maintain the strengths of synapses relative to each other, a phenomenon that occurs gradually through changes in density of AMPA & NMDA receptors at the synapse [25].

The changes in neuronal networks affected by LTP/LTD and modified by scaling and metaplasticity are comprehended as memory, on the other hand changes at the level of the synapse are the ability to change a circuit and therefore part of the learning process.

The analysis and investigation of Central Nervous system neurobiology, focus mainly on the synapse. For that purpose one of the most studied preparations is the preparation of brain slices, namely hippocampal. The maintenance of neural circuitry, excitatory and inhibitory intrinsic circuits, as well as 3D structure containing all major cell types (neurons, astrocytes and microglia), is a technical and biological advantage. The experimental manipulation is also facilitated as hippocampal brain slices allow a reasonable number of slices and the modulation of
external/experimental variables (medium composition, Temperature, etc.).

**FIG. 2: HIPPOCAMPAL SYNAPTIC PLASTICITY**

![Fig. 2: a) Drawing of hippocampal slice and the neuronal networks; b) electrophysiological extracellular recording apparatus; c) field excitatory post synaptic potential](image)

**Fig. 2:** a) Drawing of hippocampal slice and the neuronal networks; b) electrophysiological extracellular recording apparatus; c) field excitatory post synaptic potential

The hippocampus is composed by three areas: CA1, CA2 and CA3, those were termed by Ramon & Cajal. In a hippocampal slice cut perpendicular to the longitudinal axis, we can preserve the main excitatory circuit, a tri-synaptic circuit that starts in the afferents from the enthorhinal cortex projecting through the perforant path to the dentate gyrus. In the dentate gyrus, the granule cells project through the mossy fibers to the pyramidal cells of the CA3 region, this cells then group in and send their axonal projections, named Schaffer fibers, to the CA1 pyramidal cells. This simple and visible architecture, simplify the technical placement of electrodes, making it relatively easy to study monosynaptic excitatory postsynaptic
potentials (EPSPs). Moreover its physiological stability makes it useful for uncovering changes in long term changes in synaptic strength [26,27].
3.2.2 LONG TERM POTENTIATION

Terje Lømo and Tim Bliss in 1973 described the most widely used and studied electrophysiological phenomenon termed long-term potentiation (LTP). They experimentally studied the synaptic connection between the perforant path and dentate gyrus in the hippocampi of rabbits, using a tetanic burst of 100 Hz as a stimulus, perforant path fibres caused a measurable and long-lasting augmentation in the post-synaptic response in the synapses of the dentate gyrus. Due to the owed role of the hippocampus in certain forms of memory, this was a very exciting tool and significant discovery [28, 29].

More recently our comprehension of the molecular mechanisms evolved, unveiled mainly by the by the Eric Kandel laboratories two molecular mechanisms for synaptic plasticity involving the NMDA and AMPA glutamate receptors.

NMDA channels opening a consequence of the level of cellular depolarization, leads to an augmentation in post-synaptic Ca\(^{2+}\) concentration. A strong depolarization of the post-synaptic cell completely removes the magnesium ions that block NMDA ion channels, therefore allowing a calcium influx to the cell. The activation of protein kinases phosphorylates excitatory AMPA receptors located post-synaptically, recruitment of additional receptors into the post-synaptic membrane. Those mechanisms increase post-synaptic excitation by a given pre-synaptic stimulus [30, 31].

Secondary messenger dependent mechanisms regulates gene transcription and changes in the levels of key proteins at synapses level. Activation of this second messenger pathways increase levels of CaMKII and PKAII within the dendritic spine, in the
dendritic spine these proteins have been associated with dendritic spine enlargement and LTP processes. Those processes require the addition of AMPA receptors to the plasma membrane and phosphorylation of ion channels, thus enhancing permeability. Furthermore, an influx of calcium derived from NMDA receptors is required for CaMKII activation, LTP modulation can be regulated by phosphodiesterase, for example, which breaks down the secondary messenger cAMP implicated in increased AMPA receptor synthesis in the post-synaptic neuron.

The density of NMDA and AMPA receptors on post-synaptic membranes changes in a dynamic process that is maintained in equilibrium, this balance can be altered by synaptic activity. High-frequency NMDA receptor activation, leads to an increase in the expression of PSD-95 protein, that increases synaptic capacity for AMPA receptors, thus leading to a long term increase and subsequently synaptic strength and plasticity.
3.2.3 SYNAPTIC PLASTICITY PROTOCOL

The extracellular electrophysiological recordings experiments were performed in acute transverse hippocampal slices from 3xTg-AD and Non-Tg animals.

Mice were deeply anesthetized with an intraperitoneal injection of Avertin (250 mg/kg) upon which transcardial perfusion was performed with 25–30 mL of room temperature carbogenated NMDG aCSF (NMDG aCSF: 92 mM NMDG, 2.5 mM KCl, 1.25 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 25 mM glucose, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM Na-pyruvate, 0.5 mM CaCl2·4H2O and 10 mM MgSO4·7H2O. Titrated pH to 7.3–7.4 with concentrated hydrochloric acid).

Following perfusion the mice were decapitated, and the brains gently extracted from the skull within 1 minute and placed into the NMDG-aCSF solution for an additional 1 minute. Hippocampi were dissected free in an ice-cold NMDG-aCSF solution and gassed with 95% O2 and 5% CO2. Slices with an averaging thickness of 300 to 400 nm were obtained with a McIlwain chopper. Recovery in a Harvard Apparatus resting chamber for 30 min at 35 ºC and for 30 min at room temperature, filled with aCSF solution (124 mM NaCl; 3 mM KCl; 1.25 mM NaH2PO4; 10 mM glucose; 26 mM NaHCO3; 1 mM MgSO4; 2 mM CaCl) gassed with 95% O2 and 5% CO2.

Individual slices were transferred to a 1 mL capacity-recording chamber kept at 30.5 ºC, continuously superfused at a rate of 3 mL/min with gassed aCSF. Recording were acquired with bipolar concentric electrode placed on the Schaffer collateral, appliance of rectangular pulses of 0.1 msec every 20 sec, and record through an extracellular microelectrode pipette filled with 4 M NaCl (2-4 MΩ resistance) of orthodromically-evoked
field excitatory postsynaptic potentials (fEPSP) placed in the stratum radiatum of the CA1 area. Readings were collected with a ISO-80 amplifier (World Precision Instruments, Hertfordshire, UK) and digitized using a ADC-42 board (Pico Technologies, Pelham, NY, USA). Individual data points represent an average of 3 consecutive responses. LTP induction represent changes in synaptic strength and are normalized to basal line readings previous to LTP. Long-term potentiation (LTP) protocol is evoked by a high-frequency stimulation protocol composed of 1 train of 100 Hz and a 1 sec duration, LTP duration was of 60 minutes. Quantification of long lasting effects was performed, by considering the average values of the last 10 minutes of recording.
3.3 MITOCHONDRIAL FUNCTIONAL STATUS

“Energy and persistence conquer all things”.- Benjamin Franklin

Mitochondria were first discovered in the 1800s, are found in nearly all eukaryotes, including plants, animals, fungi, and protists, are rod-shaped organelles and can be considered the power generators of the cell, converting oxygen and nutrients into adenosine trisphosphate (ATP). ATP is the chemical energy "currency" of the cell that powers the cell's metabolic activities.

Mitochondria in living cells resemble a continuous tubular network, constituted by two phospholipid bilayers with embedded proteins that define four compartments, where different metabolic processes occur: the mitochondrial outer membrane (MOM), the intermembrane space, the mitochondrial inner membrane (MIM) and the matrix.

The outer and inner membrane delimits the intermembrane space, protein content is mainly constituted by cytochrome c, involved in the physiological process of respiration, but that can be released under apoptotic conditions.

The MIM is compartmentalized into numerous cristae, which expand its surface area, enhancing its ability to produce ATP; the electron transport chain (ETC) complexes (complexes I-IV) and ATP synthetase (complex V) reside in the cristae. Mitochondria of cells that have a greater demand for ATP exhibit more cristae.

The matrix is the space enclosed by the inner membrane, containing a concentration of a mixture of hundred enzymes, accounting for 2/3 of the total content of proteins in a mitochondrion. The majority of the enzymes in the matrix are involved in
pyruvate and fatty acids oxidation, they are also responsible for the enzymatic reactions of the Krebs cycle [32].

The mitochondrial membrane potential (MMP), described by Klingenberg in 1975, is crucial for ATP synthesis. The electrochemical gradient that takes place in the inner membrane, it is the motor for oxidative phosphorylation, this is the process that allows the conversion of ADP into ATP; thus, maintenance of MMP is mandatory for ATP synthesis [33].

Mitochondrial function is critical for neuronal activity. Mitochondrial dysfunction include defects in oxidative phosphorylation, impaired calcium influx/accumulation and mitochondrial membrane potential dissipation; all of those are important cellular functions in which defective mitochondria may underlie both early and late-onset neurodegenerative disorders [34,35].
3.3.1 SYNAPTOSOMES

Synaptosomes were first isolated by Hebb and Whittaker in 1958, aiming to identify the intracellular distributions of acetylcholine and choline acetylase which remains when brain tissue is homogenized in iso-osmotic sucrose.

Gray and Whittaker later achieved this, when they showed that the acetylcholine-rich particles were synaptic vesicle-rich pinched-off nerve terminals. At this point Whittaker coined the term synaptosome to describe an isolated synaptic terminal from a neuron, obtained by mild homogenization of nervous tissue under isotonic conditions and subsequent fractionation using differential and density gradient centrifugation, detaching the nerve terminals from the axon and the plasma membrane reseals, they can often contain a piece of the attached postsynaptic membrane[36,37].

Synaptosomes are presynaptic nerve terminals osmotically sensitive; this biological structure can amount to a complete dendritic spine containing small and large dense core vesicles, as well as functional mitochondria. They are sealed structures able to perform biological functions, namely respiration, glucose consumption, translocate metabolites and ions, maintain a normal membrane potential, depolarize upon a stimulus, and storage/release/uptake of neurotransmitter(s) in a Ca\textsuperscript{2+}-dependent manner.

Thus, they are a biological structure most suitable for studying synaptic function, as they carry the morphological features and most of the molecular/chemical properties of the original nerve terminal [38,39].
In the work developed in this thesis, we performed a protocol of crude (P2) synaptosomes of cortical tissue collected from 3xTg-AD mice and Non-Tg at Zt04 and Zt16.

**FIGURE.3: SCHEMATIC DIAGRAM OF SYNAPTOSOMES PREPARATION**

Representative diagram of experimental procedure used in the preparation of the synaptosomes.
In brief, animal tissue upon isolation was placed into centrifuge tubes, previously rinsed and filled with 5 ml ice-cold 0.32 M sucrose solution.

Using a 15-ml Teflon-glass tissue grinder, cortical tissue was homogenized with approximately 12 even strokes of a motor driven pestle at 700-900 rpm.

Ice-cold Sucrose (1 ml) solution at 0.32 M containing 1 mM EDTA, 10 mM HEPES, 1 mg/mL bovine serum albumin (BSA), at pH 7.4. was added to the homogenates and centrifuged at 3,000 xg, for 10 min, at 4°C; after centrifugation the pellet was dispensed and the supernatant was centrifuged in a new tube at 14,000 xg, for 12 min at 4°C.

After this step, the pellet was homogenized in 1 ml 45% (v/v) Percoll in saline solution (156 mM NaCl, 3 mM KCl, 2 mM MgSO₄, 1.25 mM KH₂PO₄, 2 mM CaCl₂, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.35) and centrifuged at 14,000 xg for 2 min, at 4°C. The pellet was washed twice with saline solution and resuspended gently until homogenous solution was reached, followed by centrifugation at 7,500 xg for 1 min at 4°C.

The total amount of protein content in the synaptosomal samples was measured using the bicinchoninic acid (BCA) protein assay kit from Biorad.
### 3.3.2 FLUOROMETRIC PROBES

Different methodological approaches have been developed over the years to study mitochondrial function. Recent advances in the development of more sensitive methods to analyze mitochondria, fluorescent imaging technologies enhanced our ability to analyze mitochondrial morphology and dynamics and allowed to measure levels of certain metabolites and ions within its sub-compartmental regions (i.e., the mitochondrial membranes and matrix).

The use of fluorescent probes and potentiometric dyes had made possible the evaluation of overall mitochondrial number, membrane potential, oxidative stress, and Ca$^{2+}$ retention [40, 41].

The concentration and distribution of those markers across the MIM depends on the mitochondrial membrane potential, thus fluorescence changes depend on the cellular environment and are used to measure the mitochondrial membrane potential. Meaning that a more negative mitochondrial potential will translate into a greater accumulation of dye, for the researcher this is seen as a change in fluorescence.

![Figure 4: TMRM Fluorophore](image)

**FIGURE 4: TMRM FLUOROPHORE**
The panel on the left presents the molecular structure of TMRM fluorophore; the panel on the right presents the spectra of excitation and emission for TMRM fluorophore.

This change is a multi-factorial parameter that depends on the dye sensitivity to changes in membrane potential and consequent redistribution of dye and sensitivity to changes in the cellular environment [42].

In the experimental work developed in this thesis, we used tetramethylrhodamine methyl ester (TMRM), a cell-permeant, lipophilic and cationic fluorescent probe with an excitation spectra ranging from 515 to 555 nm and an emission spectra ranging from 575 to 590 nm, which enter active mitochondria. Mitochondrial respiration is not impaired when low concentrations are used [43].

Fluorescent dyes sensitive to Ca$^{2+}$ represent an opportunity to study cellular physiology, as this tool allows scientists to understand mechanisms involved in calcium homeostasis, but also to comprehend the role of cytoplasmic calcium in the regulation of various cellular functions.
Painel on the left presents the ratio excitation of calcium for the fluorophore FURA-2AM; painel on the right presents the spescotropic triation of the fluorophore FURA-2AM.

To measure the calcium levels we used the fluorometric probe Fura-2-acetoxymethyl ester, (Fura-2AM). Fura-2AM is cell membrane permeable due to 2-acetoxymethyl ester; after crossing the membrane the acetoxymethyl groups are cleaved by cellular esterases, generating Fura-2. Fura-2 is excited at 340 nm and 380 nm, the emission ratio at those wavelengths is directly proportional to the amount of intracellular calcium. Independently of the presence of calcium, Fura-2 emits at 510 nm. Measurement of fluorescence at both 340 nm and 380 nm allows for calculation of calcium concentrations based 340/380 ratios. This ratio allows to surpass certain limitations, that could otherwise lead to misreads due to artefacts when imaging calcium concentrations [44-46].
3.3.3 MITOCHONDRIAL MEMBRANE POTENTIAL

The mitochondrion is a double membrane-bound organelle. In the inner mitochondrial membrane different proteins with five types of functions coexist: proteins involved in the redox reactions of oxidative phosphorylation; ATP synthase involved in the generation of ATP in the matrix; Specific transport proteins, regulating metabolite exchange to the matrix, etc. Unlike the outer membrane, the inner membrane is impermeable to all molecules, thus nearly all ions and molecules require special membrane transporters. The electron transport chain (ETC) and the enzymes involved in this process form a membrane potential across the inner membrane.

Primordial function of the ETC is to produce electrochemical gradient. ETC consists of a spatially separated series of redox reactions in which electrons are transferred from a donor molecule to an acceptor molecule. In the mitochondrion ETC is the site of oxidative phosphorylation. These reactions are coupled to the creation of a proton gradient across the MIM, resulting in a transmembrane proton gradient used to make ATP via ATP synthase. Production of ATP from products of the citric acid cycle, fatty acid oxidation, and amino acid oxidation, require that electrons from NADH and/or succinate pass through ETC to a terminal electron acceptor, oxygen, which is then reduced to water. This is a continuous process that releases energy, which is then used to generate a proton gradient across the mitochondrial membrane by actively pumping protons into the intermembrane space. So, oxidative phosphorylation, represents the ADP phosphorylation to ATP using the energy of hydrogen oxidation generated by this continuous process briefly described above.
Note that there is a small percentage of electrons that do not complete the ETC; instead move directly to oxygen, resulting in the formation of the free-radical superoxide anion [47].

To measure mitochondrial membrane potential, P2 cortical synaptosomes were loaded with TMRM fluorophore, previously to loading protein was quantified by BSA method. In this measurements we used a total volume of 100 µl per well, with a protein content of 10 µg. Incubation of synaptosomes for 30 minutes, at 30°C, with 300 nM TMRM. Then we performed a spindown of 7,500 xg for 1 minute at room temperature, resuspend in basal saline medium and add 300nM TMRM. Plate in a 96 well plate and read in a fluorescence plate reader with 548 nm excitation and 573 nm emission. A 5-10 minute baseline was record previous to any treatment/condition.

Oxidative phosphorylation requires the coupling of nutrient oxidation for ATP production through a proton cascade across the MIM. Oligomycin, a stimulus used in our mitochondrial membrane potential studies in synaptosomes as well as used in intrasynaptical calcium levels, is an inhibitor of ATP synthase. In this study, it was used to inhibit ATP hydrolysis due to the potential reversal of ATP synthase when applying the protonophore mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP).

ROS are formed as by-products of the electron transport chain during the generation of ATP or due an imbalance in antioxidant defense mechanisms. ROS formed in mitochondrial complexes include: \( \text{O}_2^* \), hydroxyl radicals (OH·), and hydrogen peroxide (\( \text{H}_2\text{O}_2 \)). In this study the application of \( \text{H}_2\text{O}_2 \) was used as mimicking stimulus of ROS and the objective
was to indirectly evaluate the antioxidant defense mechanism and assess how the mitochondrial functional status can be differently by ROS experimental conditions.
3.3.4 INTRACELLULAR CALCIUM

Calcium ions (Ca\textsuperscript{2+}) are key players in physiology and biochemistry of the cell, as a second messenger in signal transduction pathways, or most relevant in the release of neurotransmitter from neurons.

Extracellular calcium is very important for maintaining the membrane potential difference in excitable cells of the nervous system and other tissues.

Calcium homeostasis is extremely important. At the cellular level, intracellular organelles, such as mitochondrion and the endoplasmic reticulum, accumulate Ca\textsuperscript{2+} and release it during certain cellular events, acting as intracellular Ca\textsuperscript{2+} storages.

Mitochondria can transiently store calcium, this ability to rapidly store in calcium for later release, works as a buffering system for cytosolic calcium, being primarily driven by the mitochondrial membrane potential. This Ca\textsuperscript{2+} influx to the mitochondrial matrix has recently been implicated into the bioenergetics of neurons, an increase in cytosolic and mitochondrial calcium act to synchronize neuronal activity with mitochondrial energy metabolism.

In case of excitotoxicity, a recurrent finding in neurodegenerative diseases, synaptic overactivity leads to the excessive release of glutamate triggering a number of postsynaptic cell membrane receptors; this activation causes a disturbance of the intracellular ionic environment, namely the influx of sodium and Ca\textsuperscript{2+}. Excessive Ca\textsuperscript{2+} ions influx activates intracellular Ca\textsuperscript{2+}-dependent signaling cascades, neurotoxic signaling pathways transduce Ca\textsuperscript{2+}-dependent excitotoxicity [48].
To measure intracellular calcium in the scope of this thesis we used of the fluorometric probe FURA-2AM and the P2 cortical synaptosomes, thus intracellular calcium will be termed as intrasynaptical calcium. Previously to loading protein levels, these were quantified by the BSA method. In this measurements we used a total volume of 100µl per well, with a correspondent protein content of 10µg. Incubation of synaptosomes for 30 minutes at 30°C with 10µM of FURA-2AM, followed by a spin down of 7,500g for 1 minute at room temperature, resuspended in basal saline medium. Plated in a 96 well plate and read in a fluorescence plate reader with 340 nm and 380 nm excitation and 510 nm emission. A 5-10 minute baseline was record previous to any treatment/condition.
3.3.5 MITOCHONDRIAL RESPIRATION

A respirometer or oxygraph is a device used to measure the rate of respiration of a living organism by measuring its rate of exchange of oxygen.

The Oxygraph Plus oxygen electrode system allows the measurement of oxygen uptake from mitochondria and this system consists of a highly sensitive SL Clark Type polarographic oxygen electrode disc. The electrode disc comprises a central platinum cathode and a concentric silver anode.

This electrode is mounted within a DW1/AD electrode chamber which connects to the electrode control unit, allowing to measure oxygen in liquid-phase samples of between 0.2 – 2.5 ml. This control applies a small polarising voltage between the platinum and silver electrodes. In the presence of oxygen, a small current is generated proportional to oxygen activity in the sample. Temperature maintenance of the sample and electrode disc is achieved by connecting the water jacket of the DW1/AD to a circulating heated water bath.

Biological sample were placed within a borosilicate glass reaction chamber, controlled access during experiments is obtained by the gas-tight adjustable plunger, with a central precision bore using a standard Hamilton type syringe.

This provides a sensitive and rapid response to small changes in oxygen tension within the sample.
3.3.6 AMPLEX RED

Reactive oxygen species (ROS) produced in isolated mitochondria, live cells or in biological structures such as synaptosomes, are of particular interest on the study of neurodegenerative diseases with associated mitochondrial dysfunction. The mitochondrial respiratory chain is a major source of ROS. ROS accumulation, in the form of superoxide anion or hydrogen peroxide contribute to cellular damage associated with AD. ROS production is part of normal cellular metabolism, recent studies suggest that hydrogen peroxide may function as a signaling molecule necessary for survival. Nonetheless, an imbalance is deleterious. Antioxidant enzymes, like glutathione peroxidase, catalase, and peroxiredoxins, eliminate hydrogen peroxide [49].

Measurements of mitochondrial hydrogen peroxide were made using the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine), a highly sensitive fluorogenic stable probe, has proven to be a reliable method. This probe in the presence of horseradish peroxidase reacts in a 1:1 stoichiometry with $\text{H}_2\text{O}_2$ and produces resorufin, that is detect as fluorometric emission.

ROS were measured as $\text{H}_2\text{O}_2$ using the amplex red assay. Oxidation of mithochondrial complexes substrates in the presence of antimycin A increases $\text{H}_2\text{O}_2$. Antimycin A binds to cytochrome c reductase that is essential in the ETC of oxidative phosphorylation. Thus, inhibition of this reaction disrupts the formation of the proton gradient, inhibiting the production of ATP through the ATP synthase complex. As a result, the levels of free radicals increase.

Fluorescence of resorufin was measured using a Molecular Devices SpectraMax M5/M5e microplate.
reader controlled by PC SoftMax Pro 5 software (Sunnyvale, CA), with an excitation at 530 nm and emission at 587 nm.

In this measurements we used a total volume of 100µl per well, with a correspondent protein content of 10µg. A working solution with 0.5 U/ml horseradish Peroxidase and 10 µM Amplex RED was prepared. Note: please weight peroxidase and prepare this solution freshly every time. Plate in a 96 well plate and read in a fluorescence plate reader. A 5-10 minute baseline was recorded previous to any treatment/condition. After baseline reading 2 µM antimycin A was added, and continued with readout of fluorescence for more 20 minutes.

**FIGURE 6: AMPLEX RED AND ROS DETECTION**

Amplex Red reagent and reactions principles behind. Oxidation of glucose by glucose oxidase results in generation of \( \text{H}_2\text{O}_2 \), which is coupled to conversion of the Amplex Red reagent to fluorescent resorufin by horseradish peroxidase.
3.4 PERIPHERAL METABOLISM

Peripheral rhythms are an output of tissue-based circadian regulation. The temporal coordination of metabolism have focused mainly on gene expression by analysing and describing extensively “circadian transcriptomes”. Circadian controlled genes represent 5%-10% of our all genes [50]. Thus, the contribution of circadian regulated transcripts to hepatic physiology is considerable, as a circadian disruption impact metabolic homeostasis [51].

Liver mRNA levels analysis by Quantitative real-time polymerase chain reaction (qPCR)

Isolated tissues were harvested and kept in RNAlater solution (Life Technologies) according to the manufacturer’s protocol. Total RNA of liver tissues was extracted using TRIzol reagent (Life Technologies). cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) with random hexamer primers. qPCR was performed using GoTaq qPCR Master Mix (Promega) on a CFX96 thermocycler (Bio-Rad, Munich, Germany). Relative gene expression was quantified using the ΔΔ threshold cycle (Ct) method with adjustments to the amplification efficiencies of individual primer pairs. Eeflα was used as the reference gene for all experiments. Primer sequences are shown in the table of oligos below.

<table>
<thead>
<tr>
<th>GENE</th>
<th>FWD-Sequence</th>
<th>REV-Sequence</th>
</tr>
</thead>
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<tr>
<td>ADK</td>
<td>GATGATTCAGGAGCCACAGAGCA</td>
<td>AGTGACATCATTTTCTCTGAGCCA</td>
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<tr>
<td>AGER</td>
<td>CAGGGTACAGAAACCAGG</td>
<td>ATTCAGCTCTGCAGTTTCT</td>
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<tr>
<td>SIRT3</td>
<td>GCTGCTCTGCGGCTCTTATAC</td>
<td>GAAGGACCTTCCAGAGACCGT</td>
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<tr>
<td>NFE2L2</td>
<td>TAGATGACCATGAGTCCCGTTC</td>
<td>GCCAAACTTGTCCATGTC</td>
</tr>
<tr>
<td>ADORA2A</td>
<td>TGGCTATTGCCATGAGTCCA</td>
<td>GGTCTTTTGTGGAGTCTCATCTT</td>
</tr>
<tr>
<td>PRKCA</td>
<td>AGAGGTGCCATGAGTCCGTA</td>
<td>GGCTTCCGTATGTGAGATTTT</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Oligo</td>
<td>Reverse Oligo</td>
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<tr>
<td>--------</td>
<td>---------------</td>
<td>---------------</td>
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<tr>
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<td>CGGTCTCGTTACCCTCCTACT</td>
</tr>
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<td>GCTCCCAGTAGCTTTCTCATCCCTTCTGT</td>
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<tr>
<td>REV-ERBα</td>
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<tr>
<td>EEF1A</td>
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<td>AATTCACCAACACAGCAGCA</td>
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<tr>
<td>BMAL1</td>
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<td>FOXO1</td>
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</tr>
</tbody>
</table>

**Table 2: qPCR Genes**

List of oligos sequences used in QPCR experiments, primers sequences for forward and reverse primers for each gene used.

**Analysis of metabolic fluxes by Nuclear Magnetic resonance (NMR)**

The use of stable isotopes in the study of metabolism, is a viable tool in biomedical research. The use of deuterium water for the Hepatic Glycogen Synthesis quantification by Direct and Indirect Pathways. Upon the administration of deuterated water the analysis of positional isotopomer allows the determination of specific lipid species. The analysis of those metabolic fluxes gains a biological importance in the study of disease with metabolic alterations associated.

Animals from each group received a loading dose of 99% 2H2O in saline solution at their respective Zeitgeber, 24h prior to euthanaziation. This bolus
administration by intraperitoneal injection represents 1% of the animal’s body weight. 70% of the body weight is constituted by water, thus such dose was designed to produce an enrichment of 3% body water. In order to maintain this level of enrichment, drinking water was substituted with 5% 2H2O enriched drinking water.

One hour before the correspondent Zeitgeber. Mice were deeply anesthetized with an intraperitoneal injection of Avertin (250 mg/kg) upon which transcardial perfusion was performed to obtain hippocampal slices by NMDG method. 0.5 ml of blood was collected from heath puncture and used to determine body water deuterium enrichment, blood was immediately centrifuged and plasma was stored at -20°C. The liver was excised and collected, being immediately freeze-clamped in liquid nitrogen and stored at -80°C.

Sample Preparation and Metabolite analysis

Each biological sample of liver was grinded to a fine powder in liquid nitrogen. Previous to glycogen extraction, samples were submitted to MTBE lipid extraction, followed by a lipidomic analysis as described previously in the work of Joao Duarte et al[52].

Detection of metabolites relative to hepatic glycogen synthesis pathways, were performed as follows. Each gram of liver was treated with 2 ml of 30% KOH, after 1h min at 70°C add 1 ml of 6% Na2SO4 invert the tube a couple of times and add 7 ml 99.9% ethanol were added and left overnight at 4°C in order to precipitate glycogen. Proceed with a centrifugation step (10.000 rpm for 5 minutes), discard the upper liquid phase and dry the pellet.
Resuspend in acetate buffer (2mL, 0.05 M pH4.5) with 20 µl of an aqueous solution containing 16 International Units (IU) of amyloglucosidase (reference: A7420, Sigma-Aldrich, Germany).

Incubate samples overnight at 55°C, proceed with a centrifugation step (10,000 rpm for 5 minutes) and collect the supernatant. Derivatize the glucose residue, after drying it, to monoacetone glucose (MAG).

To analyze $^2$H NMR spectrum of MAG, samples were dissolved in 0.5ml of 85%/15% v/v acetonitrile/dimethyl sulfoxide. Metabolic fluxes analysis were performed as described previously by Ana Soares et al [53]. To determine the contribution of newly synthesized glycogen to the hepatic glycogen pool, ratio of glycogen position 2 to body water $^2$H-enrichments was used. Secondly, the route of synthesis used to convert glucose to glycogen, was determined calculating the ratio of $^2$H-enrichment in position 5 relative to position 2 (D5/D2). This represents the indirect pathway to hepatic glycogen synthesis; direct pathway contribution was calculated as 1 minus the indirect pathway contribution.

$^2$H enrichment of body water

Body water enrichment was calculated using $^2$H NMR as previously described [54]. Briefly, 10 µl of plasma was added to 190 µl of acetone and a $^2$H NMR spectrum was acquired. Enrichments were calculated by comparing the ratio of the deuterium signal of acetone and water with the previously determined ratios of standards.
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4) Thesis Results

THESIS RESULTS

“It is not the answer that enlightens, but the question.” —Eugène Ionesco
4.1

EVALUATION OF PHENOTYPIC
TRAITS OF ALZHEIMER’S DISEASE
IN 12 WK OLD 3XTG–AD

“No princípio era o Verbo, e o Verbo estava com
Deus, e o Verbo era Deus” João 1:1–4
4.1

4.1.1 INTRODUCTION

AD is a progressive neurodegenerative disorder mainly affected by the aging process and exhibiting metabolic/mitochondrial dysfunction as an underlying cause. Many cell and humanized animal models of AD have been used to study AD pathogenesis.

3xTg-AD, in particular, is a mouse model for AD pathology developed by Frank La Ferla’s Lab. This model bears three mutations, two of them present in familial cases of AD, namely PSEN1 M146V, APP Swedish and MAPT P301L. In terms of pathological display this animal model presents both plaque and tangle progressive pathology, with an initial intracellular deposition of Aβ in cortical neurons, detected as early as at three to four months of age followed by extracellular Abeta deposition at later stages. Hyperphosphorylated tau occur later, at the age of twelve months in the hippocampus [1].

In order to understand the progressive character of the disease and establish a direct correlation with the aging process, we underwent a neurobiological characterization of the 3xTg-AD mouse model. In literature it was previously shown that at the age of three months (12 weeks of age) this mouse model did not present symptoms characteristic of the disease [2]. Moreover, some sexual differences were reported [3], so for the entire study we have only used males. Data suggest that biochemical and phenotypical characteristics of AD-like pathology seem to onset between the age of four and six months of age [4].
Considering that 3xTg-AD mice are presymptomatic at three months of age, such intermediate stage of the disease might underlie a dysfunction at some level and under certain conditions. Therefore, we designed a study aiming to study the phenotypical characteristics of the disease, in a top-down perspective at 3 months of age and ZT 04.
4.1.2 RESULTS

➤ Behaviour and cognitive deficits

AD probable diagnosis is primarily based on cognitive evaluation. Memory-based behavioural tests are a measure of cognitive processes, where some tasks are more prone to define the decline of certain brain structures affected in AD.

The Open field test (OFT), which measures locomotor activity and anxiety levels, was the first performed task. This behavioural test was performed by adapting the protocol developed by Calvin S. Hall.

![FIGURE 1: 12 WKS OLD 3XTG-AD OPEN FIELD TEST](image)

**a)** Motor activity displayed as total distance travelled during the entire duration of the test. **b)** Anxiety measure displayed as time spent in the center; *= P<0.05; n = 8 mice per group; data are presented as mean±SEM, Statistical
This initial assessment was performed at ZT 04, our observations show no change between the two experimental groups regarding the total motor activity (Fig. 1a). The statistical significant difference of time spent in the centre of the arena in relation to Non-Tg displayed by 3xTg-AD, represents a decrease in the levels of anxiety (Fig. 1b).

Memory is traditionally associated with hippocampal area. In order to evaluate the presence of cognitive decline in hippocampus-dependent learning tasks performance of 3xTg-AD and Non-Tg animals, Novel object recognition (NOR) and Novel object location protocol was performed.

**FIGURE.2:SPATIAL RECOGNITION SHORT-MEMORY TASK, NOL AND NOR BEHAVIOURAL TEST**

a,b) Displacement index represents the ratio of the time spent exploring the displaced object (T\textsubscript{novel}) and the time spent sniffing/exploring the familiar object (T\textsubscript{familiar})
plus T\textsubscript{novel}; b) Recognition index represents the ratio of the time spent exploring the novel object (T\textsubscript{novel}) and the time spent sniffing/exploring the familiar object (T\textsubscript{familiar}) plus T\textsubscript{novel}; Mice were allowed to explore freely the open field during the sessions, number of contacts and time spent interacting with the objects was counted. Data are presented as mean±SEM, \( n = 8 \) mice per group, **\( P<0.001 \). Statistical analysis performed by Student’s t-test (GraphPad Prisma 6.0 software).

The results depicted in Figure 2 require a careful interpretation, because some animals did not interact with the novel object during the test session, both for the NOL and NOR tests. Nonetheless, 3xTg-AD animals at this age apparently have cognitive deficits in spatial short term memory (Fig. 2a). For the NOR test there were no differences between the two experimental groups.

Cognitive deficits characteristic of the pathology are commonly can be evaluated by modified Y-maze behavioural test. This memory task allows monitoring the number of enters into previous closed arm, a measure of spatial working memory. Thus, the Y-maze can be used to measure short term memory and general locomotor activity. Animals with preserved cognitive function enter more often the previously blocked arm and will explore that one first on the test trial.
FIGURE 3: MODIFIED Y-MAZE IN 12 WKS OLD ANIMALS. EVALUATION OF SPATIAL WORKING MEMORY

a) total distance travelled in the test trial, b) number of entries in the novel arm expressed in terms of percentage, c) Time spent exploring the novel arm expressed in terms of percentage, d) Schematic drawings of the Y-maze and the experimental procedures. Y-maze room was formatted with different spatial cues. The sample trial and test trials were conducted for 8 min with a 90 min interval. Data are presented as mean±SEM, n = 8 mice per group, * P< 0.05. Statistical analysis performed by Student’s t-test (GraphPad Prisma 6.0 software)
Memory loss is the most common problem in AD patients. In our study, 3xTg-AD mice displayed behavioral deficits in hippocampus-dependent learning paradigms. Y-maze results show a significant deficit in spontaneous alternation in the Y-maze (Fig. 3). Spontaneous alternation is highly dependent on hippocampus function; this assay reveals the hippocampus-dependent deficits in learning and memory observed in Alzheimer's patients.

Another behavioral test commonly used to evaluate cognitive deficits in animal models of disease is the widely used Morris water maze (MWM) test. This test allows the researcher to enquire the spatial memory aspects, the ability to acquire a learned task and to retain it in the form of an acquired long term memory.

The MWM performance in the spatial reference memory test (TEST) was shown to be impaired in 3xTg-AD versus Non-Tg mice. The recording of the latency to reach the platform during the acquisition period (Fig. 4a), shows a higher performance of the learning ability in the Non-Tg animals. Analysis of the spatial learning test, revealed an impairment of the geo-spatial location memory ability in the 3xTg-AD animals. This result showed that Non-Tg animals travelled into the correct quadrant an higher number of times that was statistical significant when compared to 3xTg-AD(Fig. 4c); this occurrence was accompanied by a higher number of crossings (Fig. 4c), indicating a clear geospatial memory of the platform. A relevant observation is seen in the absence of difference for the time spent in the correct quadrant between the two experimental groups.
FIGURE 4: MORRIS WATER MAZE IN 12 WKS OLD ANIMALS, EVALUATION OF LONG-TERM SPATIAL REFERENCE MEMORY

a) Learning curves during Acquisition in the Morris water maze spatial learning task with hidden platform, latency for each mouse to reach the hidden platform over the 4 trials; Long-term memory performance during probe trial or test, b) latency to reach the annulus (would-be platform, c) Percentage time spent in the platform Quadrant, d) numbers of annulus crossings; All data are means ± SEM, n = 8 mice per group, (*P < 0.05, **P < 0.001, ***P < 0.0001). Statistical analysis performed by Student’s t-test (GraphPad Prisma 6.0 software)

In order to evaluate the ability to form new memories and extinguish old references, the platform was relocated to another quadrant (the opposite one) and after 24h another set of four trials was performed; under these conditions we may evaluate
different forms of memory impairment. Reversal learning in the MWM evaluates cognitive flexibility and also working memory.

**FIGURE. 5: REVERSAL SPATIAL LEARNING IN 12 WKS OLD ANIMALS, EVALUATION OF COGNITIVE FLEXIBILITY**

Behavioural test constituted of 4 trials where hidden platform is relocated into the opposite quadrant in relation to the acquisition trial; **a)** Mean swimming speed in reversal spatial learning test, **b)** Total distance swummed during the reversal spatial learning test, **c)** latency to reach the new location of the hidden platform. Data are presented as mean±SEM, n = 8 mice per group, *P<0.05. Statistical analysis performed by Student’s t-test (GraphPad Prisma 6.0 software)

When analyzing the data obtained in the Reversal Spatial Learning behavioural task, Non-Tg animals required less time to reach the new location of the hidden platform (Fig. 5c). Consequently, the total distance travelled during the duration of the period taken to reach the platform was higher for the 3xTg-AD than the Non-Tg mice (Fig. 5b). The mean swimming speed was also considerable higher for the 3xTg-AD mice (Fig. 5a) suggesting an alteration in anxiety levels.
Hippocampal synaptic plasticity

AD pathology has been characterized by a decrease/increase in induction of Long-term potentiation (LTP). At the age of six months the 3xTg-AD mouse model presents alterations in this electrophysiological parameters, however no data exist for the considered "presymptomatic stage," at 3 month of age.

Therefore, we performed a simple evaluation of ability to induce LTP. Behavioural testing showed a cognitive decline in some hippocampus-dependent tasks. By aiming to correlate electrophysiological changes with behavioural data, field potentials from hippocampal slices of 3xTg-AD and Non-Tg mice were performed as described in the section of Material & Methods. Field potentials from hippocampal slices were recorded and changes in basal synaptic transmission and plasticity were evaluated. Field excitatory postsynaptic potentials (fEPSP) records were obtained in CA1 stratum radiatum and stimulation in CA3 Schaffer collateral fibers.

Primarily, we tested for differences in basal synaptic excitability, namely an increase (or decrease) in basal synaptic strength by recording input-output (IO) series, prior to any plasticity protocol. Synaptic transmission was evoked by electrical stimulation of the Schaffer collaterals and recorded in the CA1 region using field potential recordings field fEPSP, the input-output (I-O) relationships for the respective fEPSP slope. After establishing a baseline at 40% of the maximum, a protocol of high frequency stimulation (HFS) was used to elicit LTP in dorso-medial hippocampal slices.
FIGURE 6: HIPPOCAMPAL SYNAPTIC PLASTICITY EVALUATION BY LONG TERM POTENTIATION

a) Input-output curves of hippocampal transmission; b) Graphical display of LTP time course; C) Quantification of changes after LTP induction; Data are presented as mean±SEM, n = 5-6 mice per group, *P<0.05. Statistical analysis performed by Student’s t-test (GraphPad Prisma 6.0 software)

The 3xTg-AD and the Non-Tg animals showed no differences in the analysis of the basal synaptic excitability, as seen by the analysis of the Input/Output curve (Fig. 5a). In terms of synaptic plasticity, the HFS protocol used to elicit LTP resulted in a statistical significant difference between the two experimental groups (Fig. 5b). In terms of induction, the 3xTg-AD mice reached an
average value of 176,522 %, whereas the Non-Tg group reached an average value of 145,129 % (Fig. 5c).

**Mitochondrial Function**

Mitochondrial function has been largely described to be impaired in AD. Mitochondrion is the main organelle responsible for energy production at the synaptic level. Failure of synaptic mitochondria to generate adequate ATP levels has been implicated as a leading event in AD, potentially preceding the loss of synaptic network. Therefore, nerve terminals were prepared from the brain cortex of 3xTg-AD and Non-Tg mice. Cortical tissue was collected and enriched nerve terminals were prepared. Cortical nerve terminals, at 12 weeks of age, was used to study mitochondrial functional status at the level of the synapse. Synaptic mitochondria failure to generate adequate ATP levels has been implicated as a leading event in neurodegenerative disease, in AD this dysfunction precedes the loss of synaptic networks.
**FIGURE 7: DOUBLE FLUOROMETRIC ASSESSMENT OF MITOCHONDRIAL FUNCTIONAL STATUS**

Representative Plot using cortical synaptosomes from Non-Tg and 3xTg-AD. Bar graphs quantify the maximum respiration in terms of MMP and calcium levels (average value of the last measurements after addition of OLIGO/FCCP). Data are presented as mean±SEM (n= 4 biological replicates each represent the average of 3 technical replicates); *P<0.05. Statistical analysis performed by Student’s t-test (GraphPad Prisma 6.0 software).

Analysis of TMRM+ fluorescence in cortical synaptosomes exposed to oligomycin plus FCCP (OLIGO/FCCP) revealed no significant changes in MMP between 3xTg-AD and Non-Tg nerve terminals (Fig. 8a). Regarding the levels of intrasynaptosomal calcium, there is a statistical significant higher level of calcium for the 3xTg-AD, when compared to Non-Tg animals, which reflected into higher mitochondrial Ca^{2+} retention.
In order to mimic oxidative stress conditions and evaluate the susceptibility of cortical synaptosomes to an oxidant insult, H$_2$O$_2$ was further tested on MMP and intracellular Ca$^{2+}$ measurements.

**FIGURE 8: DOUBLE FLUOROMETRIC ASSESSMENT OF OXIDATIVE DAMAGE IN MITOCHONDRIAL FUNCTION**

Representative Plot using cortical synaptosomes from Non-Tg and 3xTg-AD in an experimental condition of 1 M H2O2 exposure. Bar graphs quantify the change in MMP and calcium after Stimulus and maximum respiration alteration due to stimulus (average value of the last measurements after addition of H2O2 and OLIGO/FCCP, respectively). Data are presented as mean±SEM, n= 4 biological replicates each represent the average of 3 technical replicates; *P<0.05. Statistical analysis performed by Student’s t-test (GraphPad Prisma 6.0 software)

In this experimental setup we found that H$_2$O$_2$ induced a slight (but significant) lower release of TMRM+ in 3xTg-AD, when compared to Non-Tg mice, suggesting reduced depolarization caused by the oxidant
stimulus in AD animals (Fig. 8a), while overall MMP was not significantly affected, as observed after Oligo/FCCP stimuli. Intracellular calcium levels did not reflect the alterations seen in MMP, as H$_2$O$_2$ did not differentially affect the two experimental groups; nevertheless, and similarly to the conditions in the absence of H$_2$O$_2$ (Figure 7), intramitochondrial Ca$^{2+}$ retention was significantly higher in synaptosomes obtained from 3xTg-AD mouse cortex (Fig. 8f).

**FIGURE. 9: DETECTION OF HYDROGEN PEROXIDE**

a) Representative plots of the horseradish peroxidase activity in cortical synaptosomes activity for the control and Antimycin condition for Non-Tg vs 3xTg-AD groups; b) graph bars representing the fluorescence levels reached either at basal condition (basal) and the fluorescence level reached after antimycin appliance, calculated by subtracting the levels reached by Antimycin to Ctrl condition (ΔAA); Data are presented as mean±SEM, n= 4 biological replicates each represent the average of 3 technical replicates, **= p<0.01. Statistical analysis performed by Student’s t-test (GraphPad Prisma 6.0 software)
4.1.3 DISCUSSION

AD pathology is a progressive neurodegenerative disease with clear and defined pathophysiological traits. In this chapter, it was our scope to evaluate the "presymptomatic" characteristics of AD-related pathology using the 3xTg-AD mice.

Behavioral characterization of 3xTg-AD mice at presymptomatic stage

As a principal indicator of the cognitive deficit associated with disease, behavioural characterization is essential. This analysis revealed a deficit in spatial memory tasks especially in the long-term memory tasks. The short memory tasks, here illustrated by the NOR and the NOR behavioural tests, require a further and more refined interpretation that is not as straightforward as the results obtained in other tests.

In terms of anxiety and general locomotor activity behavioral tasks (Fig. 1), it is consensual that 3xTg-AD mice display an increase of time spent in the center of the arena, which can be interpreted in different forms [5]. Such observations replicate This trait is visible not only in the findings already described aforementioned behavioural task, but also in the modified Y-maze as the 3xTg-AD spend more time in the center and therefore exhibit less distance travelled. Such observations replicate the findings already described [6], although the age is a non-matching criteria between the two studies. In our interpretation of the data, probably this characteristic is not age-dependent, but rather reflects an intrinsic genetic characteristic. Emotionality is tightly co-related with the amygdala
activity and neuronal circuitry associated like the entorhinal cortex and the hippocampus. As the transgenes are expressed also in this region, the alterations seen could reflect a direct or indirect effect of the genotype, moreover it has been proposed by (Oddo et al 2003) that those regions are specially affected in early AD.

Continuing on the behavioural assessment performed and the evaluation of cognitive decline, the behavioural tasks designed to evaluate and characterize cognitive function have shown to be efficient and valuable in the intended function. Of relevance, there is no literature reporting a deficit or impairment in working or short-term memory at the age of three months. Recent published data support our findings [7]; if one considers that the modified Y-Maze test allows to evaluate working memory, with the correct and clear differences in mind between the two behavioural tests, we could claim that in the MYM 3xTg-AD mice made significantly worse performance in working and reference memory tasks (Fig.3). This result also supports the findings in short-term spatial memory (NOR and NOL), where we observed an impairment in reference memory for the 3xTg-AD mice at three months of age (Fig. 2). A possible explanation for this finding is the qualitative assessment of pathological progression by brain regions in male 3xTg-AD mice at 2 to 26 months of age [8]. In the work of this group aforementioned, there is a well described and thorough characterization it is well established the presence of elevated levels of hAPP, hAPP/Aβ, intracellular hAβ1–42, human Tau, microglial activation and astrocyte reactivity in brain areas pivotal to the memory performance, namely the primary motor cortex, hippocampus and the
entorhinal cortex. All of the above were present at 2 months of age.

The Morris water maze results (Fig. 4) depict a deficit in the learning ability in 3xTg-AD animals at three months of age, which was not described before. This deficit in learning was accompanied by a poor performance in the test/probe trial itself. This cognitive deficit in long-term spatial reference memory was accompanied by an impairment in the cognitive flexibility required to shift learning.

These findings support the aforementioned elevated levels of pathological and biochemical markers of disease [8]. The behavioral tasks require an integration and activate the brain areas mentioned.

➢ **Synaptic plasticity at 12 Wks.**

The electrophysiological activity of the hippocampus as a structure involved in memory processes has been considered a good indicator of cognition [9, 10]. Our observations support a different idea. A cognitive deficit or alteration in behavioral tasks presupposes a direct correlation of LTP impairment of 3xTg-AD when compared to Non-Tg mice. However, this was not the case, as recordings in brain samples from 3xTg-AD mice showed no alteration in basal synaptic excitability and a higher level of LTP induction in the protocol of synaptic plasticity (Fig. 6). This concept, accepted by most, proposes in accordance with a classic view, that a bigger is better. Recent findings in this scientific field, are nowadays reconsidering a more abrangent approach towards the interpretation of LTP mechanistics. Indeed, LTP
occurrence is still not an open box. The intricacy of recent findings have unravelled several layers to this process, including multiple LTP forms and pleiotropic molecular partners and cascades required for LTP occurrence. LTP is considered a direct correlation of learning and memory phenomena at the synapse level. [11]. A possible interpretation for this outcome assumes an initial excitatory role for the β-amyloid peptide in an initial phase of the disease. Such an idea is supported by the experimental findings of other researchers[12], in which they also demonstrate in a mouse model with a pre-plaque state of AD an increase in LTP potentiation. This poses the question of a progressive excitotoxicity role of Aβ and disruption of protective mechanisms that further lead to neuronal degeneration.

- Mitochondrial Functional Status at 12 Wks.

The role of mitochondria in AD is claimed to be central to disease onset and progression [13]. The oxidative stress hypothesis as a form of explanation for some of the molecular findings and phenotypical alterations observed in AD is supported by our findings. The cortical synaptosomes showed no difference between experimental groups, in terms of normal physiological functioning. The mitochondrial functional status, as assessed by the MMP, seems to be working equally between groups (Fig. 7). Nonetheless, upon an insult, here mimic by an \( \text{H}_2\text{O}_2 \) stimulus, the MMP results tell us a different story (Fig. 8). Alterations in MMP, are a prior event that relate with an inability to deal with an oxidative status or stress, leading to an cumulative status of damage that translates upon progression with aging and onset of the disease in a mitochondrial
dysfunction. These results and hypothesis gain further support with the measurement of ROS, suggesting early increased oxidative stress at this stage (Fig. 10).

In terms of intrasynaptosomal calcium, data were slightly more interesting, when compared to MMP. When comparing the two experimental groups the calcium levels were elevated in the 3xTg-AD synaptosomes under basal conditions (Fig. 8), meaning that the ability to handle calcium or the calcium entry through voltage gated channels was higher in the 3xTg-AD group. Those facts correlate with the observations of synaptic plasticity. Moreover, they summon up a scenario where the role of excitotoxicity mediated by calcium is an initial step on the onset of the disease.

In conclusion we must acknowledge that at the age of three months the 3xTg-AD animals displayed a spatial cognitive alteration in comparison to the Non-Tg group. The results are indicative of the presence of Aβ in brain structures related to the cognitive functions enquired.

Together these data shows that amyloidopathia is initially non-toxic to the mitochondria and causes an alteration in the oxidative defense system. A clear confirmation of the progressive trait of the disease with aging.
REFERENCES


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THE IMPACT OF CIRCADIAN CLOCK IN THE NEUROBIOLOGY OF ALZHEIMER'S DISEASE, ACTIVE VS INACTIVE PERIOD IN 3XTg-AD.

"Even a broken clock is right twice a day" - Marie von Ebner-Eschenbach
Chapter 2

4.2.1 INTRODUCTION

Alzheimer’s disease (AD) is a brain disease that mainly affects the elderly, having a major impact in modern society, with increase in lifespan expectancy. Despite recent advances in molecular biology and genetics, the etiology of AD is still undefined. Indeed, about ninety five percent of AD cases are sporadic, with no link to a gene or family history of AD [1,2]. AD has been considered a slowly progressive brain disease, which onset precedes the emergence of clinical symptoms. Aging per se has been related to a progressive decline in cognitive function, AD can also be considered as a disease of aging [3]. The aging process is accompanied by an incremental failure of normal cell and organ function, in the clearance of toxic compounds, or in regenerating new cells, in suma a gradual failure in maintaining homeostasis. Importantly, age-related decline is also described for the circadian clock. Aging impacts the amplitude of rhythmic behaviours and fragmentation of sleep patterns [4]. The circadian clock is a biological system responsible for maintaining the synchrony of processes and orchestrate homeostasis as an whole. [5]. Increasing evidence denotes that modifications of circadian rhythms are associated with neurological disorders, namely AD [6]. Moreover, circadian disturbances are reported early in the progression of AD [7]. The scope of our work was to evaluate the impact of AD in biological rhythms. For this purpose we used the genetic model of AD developed by La Ferla’s Lab, the triple transgenic AD mouse model (3xTg-AD) that harbours genetic mutations responsible for the
phenotypic hallmarks of the disease in humans [8]. 3xTg-AD mice display abnormalities in circadian rhythmicity prior to AD-related pathology [9]. These traits make this mouse model instrumental to investigate the interplay between circadian clock and a possible link to AD.

By definition Neurobiology is the assumption that behaviour meets a function, this function is associated with a brain region and the inner works of this process are governed by specific cells and regulated by certain proteins. Using this holistic approach, we looked at the 3xTg-Ad mouse model and created a working paradigm. Therefore, we analysed the most striking pathologic differences of active versus inactive phases, which can be translated to Human data as awake versus sleep. We interrogated the genotype about phenotypic characteristics affected in pathology and the circadian variation associated with.
4.2.2 RESULTS

To meet our aim we evaluated, at different times of the day, namely zeitgeber (ZT) 04 and 16 hours the behavioural cognitive performance, explored how this correlated with hippocampal long-term potentiation (a proposed neurophysiological correlate of learning and memory) and analyzed mitochondrial function, as an indicator of brain metabolism and an essential partner in synaptic strength. All of those studied aspects are known to be critically affected in AD [10, 11, 12].

Diurnal variation of behaviour and cognitive deficit in 3xTg-AD

Cognitive tasks are a measure of memory processes, some behavioural tests are more prone to elicit the role and decline of certain brain structures. The Object Displacement (OD) test and the Morris water maze (MWM) test are the most common and reliable tests used to evaluate AD-related cognitive deficits [13]. As many other processes, memory processing also follows a circadian profile [14], meaning that during active periods the brain, as a structure dedicated to reasoning, has higher levels of certain molecules related to memory processing [15].

In order to evaluate the changes in behaviour in 3xTg-AD versus non-transgenic mice at different times of the day, we have first performed the Open field test (OFT), which measures locomotor activity and anxiety levels. Importantly, AD patients exhibit alterations in sleep/awake cycles and alterations in activity patterns, which can be assessed by the OFT in rodents. Dyck RH. Lab has previously shown that 3xTg-AD mice displayed a desinhibitory behaviour, hence we have used this aspect as an internal control [16], bearing in mind this expected
result as an attempt to recapitulate relevant findings and a form of validation. This behavioural test was performed using/adapting the protocol developed by Calvin S. Hall [17].

**FIGURE 1 - DIURNAL VARIATION IN THE OPEN FIELD TEST**

a) Motor activity in 1 min displayed as total distance travelled (in m); b) Exploratory activity; c) Anxiety measure displayed as the time spent in the center d) Exploratorion index representative of locomotive activity paths; 3xTg-AD mice showed for ZT 04 a significantly greater locomotor activity, a reduced anxiety-like behaviour displayed as a significant increase in the time spent in the center. Statistical analysis was performed by 2-way ANOVA and revealed a significant interaction of genotype versus zeitgeber; **= P<0.001; n=8 mice per group. Data are presented as mean±SEM.

The observed decrease in time spent in the centre of the arena in relation to Non-Tg displayed by 3xTg-AD, was only considerable at ZT 04.
The diurnal variation in hippocampus-dependent learning tasks performance of 3xTg-AD and Non-Tg animals was assayed. Novel object recognition (NOR) protocol measures behavioural functions with a contribution that is not only hippocampal [18], was adapted from literature [19] and performed in Red dim light conditions. The performance of the task showed a clear circadian profile although no interaction between genotype and Zeitgeber was observed (Fig. 2).

FIGURE 2: EVALUATION OF SPATIAL RECOGNITION SHORT-TERM MEMORY BY NOVEL LOCATION RECOGNITION TEST
a) Methodological diagram for the one-trial object exploration task. Black squares represent the square open field. Mice individually explored the open field during the object exploration and displaced object exploration sessions with an intersession interval of 90 min. Two identical objects (A, B) were placed for the exploration session, and one object was displaced (B); b) Mice were allowed to explore freely the open field during the sessions; the number of contacts and time spent interacting with the objects was counted. Object displacement index represents the ratio of the time spent exploring the displaced object (Tnovel) and the time spent sniffing/exploring the familiar object.
(Tfamiliar) plus Tnovel, in 3xTg-AD and Non-Tg mice. Data are presented as mean±SEM. \( n=8 \) for each group, *\( P<0.05 \). Statistical analysis was performed by 2-way ANOVA.

Cognitive deficits, characteristic of the pathology, are present at this age (24 wks) as described by Billings et al. 2005, and were evaluated using the Morris Water Maze (MWM) behavioural test (Figure 3). The protocol developed in 1984 by Morris was adapted to a version with a 4 day training period named here as acquisition, with a day 0 of visible platform, a 5th day of spatial learning test, and 6th day which comprised a 4 trial assay of reversal spatial learning [20]. On day 0 (visible platform trial) there was no difference between experimental groups in latency to reach the platform and path length (data not shown) indicating that both groups have similar motor and visual capabilities. During the acquisition period (day 1 to 4 of hidden platform trials) data showed a significant difference in the escape latency (Fig. 3) between the groups--; Non-Tg mice performed significantly better than 3xTg-AD for ZT 04. Moreover, Non-Tg animals exhibited a circadian profile in spatial learning that reflected in a significant smaller escape latency time to reach the hidden platform for Non-Tg at ZT04. Analysis of 3xTg-AD mice showed no difference in learning curves between the two Zeitgebers.
FIGURE 3: CIRCADIAN VARIATION IN THE MORRIS WATER MAZE (MWM) LEARNING CURVES

MWM spatial learning task with hidden platform: the latency for each mouse to reach the hidden platform was recorded over the 4 trials. 

a) Escape latency to reach the hidden platform for the Non-Tg animals, at ZT 04 versus ZT 16; 

b) Escape latency to reach the hidden platform for the 3xTg-AD animals, at ZT 04 versus ZT 16; 

c) escape latency to reach the hidden platform for ZT 04 condition, Non-tg vs 3xTg-AD; 

d) escape latency to reach the hidden platform for ZT 16 condition, Non-tg vs 3xTg-AD; 

Data are presented as mean±SEM; n=8 mice per group, *P<0.05, **P<0.01. Statistical analysis was performed by 2-way ANOVA.

The MWM performance in probe trial (Spatial reference memory test) was impaired in Non-Tg versus 3xTg-AD, at both ZT04 and ZT16.
FIGURE 4 - EVALUATION OF LONG-TERM SPATIAL REFERENCE MEMORY IN MWM.

Long-term memory performance during probe trial a) Latency to reach the annulus (would-be platform); b) Number of annulus crossings; c) Percentage of time spent in the correct quadrant; Data are presented as mean ± SEM (*P < 0.05). Statistical analysis was performed by 2-way ANOVA. Note: dashed line in figure 4c represents the level of chance performance.

Analysis of the probe test or spatial learning test revealed an impairment of the geo-spatial location memory ability in the 3xTg-AD animals, especially at ZT16. Results of the probe trial showed that Non-Tg animals travelled to the correct quadrant (where the hidden platform had been previously placed) a statistical significant higher number of times when compared to 3xTg-AD mice (Fig. 4c). Moreover, 3xTg-AD ZT04 group reached the correct quadrant mainly by chance; values of % of time in the correct quadrant indicate an impairment in spatial reference
memory. The number of crossings indicated a clear geo-spatial memory of the platform, Non-Tg and 3xTg-AD mice showed a similar number of crossings at both ZTs, and a clear impairment, when comparing 3xTg-AD versus Non-Tg, independently of ZT. Then, by simply re-locating the platform to another quadrant (the opposite one), on day 6 we performed another set of four trials to evaluate different forms of memory impairment. This procedure is often called reversal learning. Reversal learning in the MWM can be informative about whether or not animals can extinguish their reference learning of the platform’s position and acquire a direct path to the new goal position, translating into a lower latency times to reach the new platform location [21]. In the Reversal Spatial Learning, statistical significant cognitive decline was only observed at ZT16. MWM data showed that independently of the time of day, cognitive deficits were observed in 3xTg-AD mice, though a correlation between ZT’s and genotypes was only possible when using the Reversal Spatial learning task. These results suggest that the pattern of circadian variation in memory performance depends on the type of task and that the impact of circadian clock on AD pathology is better outlined in more complex tasks. Supporting this fact, spatial reference memory is more than a learned task, and relates to a form of working memory and a behavioural flexibility ability [22]. More refined tasks require more integration of different brain areas; interestingly, the impact of active versus inactive is more pronounced in Non-Tg animals, causing a masking effect in the cognitive deficits exhibited by 3xTg-AD mice.
FIGURE 5—CIRCADIAN RELEVANCE IN REVERSAL SPATIAL LEARNING BEHAVIOURAL TASK IN 3XTG-AD MICE.

Reversal Spatial learning: behavioural test constituted of 4 trials, in which the hidden platform is relocated into the opposite quadrant in relation to the acquisition trial; a,b) Latency to reach the new location of the hidden platform; c,d) Latency to reach the new location of the hidden platform for each individual trial. Data are presented as mean±SEM; n=8 mice per group; statistical analysis: *P<0.05, **P<0.01. Statistical analysis was performed by 2-way ANOVA.

➢ Diurnal variation of hippocampal synaptic plasticity in 3xTg-AD

AD pathology is characterized by a decrease/increase in induction of Long Term Potentiation (LTP), which works as window into the synaptic ability to memory formation. During LTP, the pre- and postsynaptic densities modulate activity of neurotransmitters and modify the surface levels of glutamate
receptors, namely NMDA and AMPA, making the elicited synapse more prominent [22].

Behavioural testing showed a circadian profile disruption on a hippocampus-dependent task in 3xTg-AD mice, therefore we performed electrophysiological studies with the aim to correlate electrophysiological changes with behavioural data. We recorded field potentials from hippocampal slices and looked for changes in basal synaptic transmission and plasticity.

As a primary read out we tested whether ZT’s caused a lasting change in basal synaptic strength by recording input-output (I-O) series prior to any plasticity protocols.

The characteristics of basal synaptic transmission in the CA1 region of the hippocampus was studied as follows: synaptic transmission was evoked by electrical stimulation of the Schaffer collaterals and recorded in the CA1 region using field excitatory postsynaptic potential (fEPSP). The properties of synaptic transmission were then characterized by the input-output (I-O) relationships for the fEPSP slope. In these experiments, we varied the stimulus intensity and measured the resulting slope. Input/Output (I/O) relationship for evoked responses is considered indicative of a change in the sensitivity of the neuronal network to excitatory input. In the Non-Tg animals we observed a significant enhancement of the fEPSP in hippocampal slices from ZT 16 group when compared with slices from ZT 04 group. (Fig. 6b). Difference was characterized by a leftward shift in the I-O curve showing the relationship between fEPSP slope and stimulus intensity. Thus, outlining a circadian profile in neuronal excitability.
FIGURE 6- IMPAIRMENT OF DIURNAL VARIATION OF LTP INDUCTION IN THE 3XTG-AD MICE

a) Schematic diagram of synaptic plasticity experiments; b) graphic display of field-EPSP of hippocampal slices amplitudes as a function of stimulus intensity. Curves fit to the data were significantly different by global analysis F-test (P < 0.0001). Input-output curves showing reduced hippocampal transmission in Non-Tg mice at ZT 04; c) LTP time course for NON-Tg in the left panel, followed by graph bars quantifying the level of LTP induction for both groups, in the right panel LTP timecourse for 3xTg-AD. Data are presented as mean±SEM; n= 4-6 mice per group; Statistical analysis was performed by Student’s t-test, *P<0.05.

LTP as an electrophysiological process has been described as displaying a circadian oscillation [24], this variation suggest a vital role in memory consolidation and memory formation. Therefore, we applied a protocol of high frequency stimulation (HFS) to elicit LTP in dorso-medial hippocampal slices; field excitatory postsynaptic potentials fEPSP records were obtained in CA1 stratum radiatum and stimulation in CA3 Schaffer collateral fibbers.
This procedure was performed at the two different periods, in order to understand the impact of AD. After recording an input/output curve, we selected the intensity of the stimulus to evoke a fEPSP of about 40% of maximal amplitude, and built a stable basal line for 10 minutes and afterwards evoked an LTP. Furthermore, upon a HFS stimulation our recordings showed a circadian profile of LTP induction present in the Non-Tg and absent in the 3xTg-AD mice (Fig. 6c). Those changes in SC/CA1 synaptic connection strength are viewed as a primary model of activity-dependent changes in synaptic strength, which may ultimately be linked to learning and memory [25]. Assuming LTP as a neural correlate of learning and memory formation, circadian rhythms alterations known to modulate learning and memory, will hence influence LTP, depicting a positive correlation between the two phenotypical characteristics and an influence of circadian variation.

➢ **Diurnal variation of mitochondrial function in 3xTg-AD mice**

At a cellular level there is a growing body of evidence linking mitochondrial dysfunction, metabolic dysregulation and oxidative stress to AD [26]. The metabolic impairment observed in Circadian mutants is an indirect evidence of the impact of circadian clock on metabolism coordination [27]. Mitochondria are the main responsible for energy production at a local level, thus it has been reported that brain activity and consequently energy consumption is different during active in comparison with inactive periods, suggesting that mitochondria activity might also reflect those observations. Therefore, we used the cortical tissue of the same
animals to study pre-synaptic mitochondrial function. The use of cortex nerve terminals to evaluate mitochondrial function, at the age of 24 weeks old, and correlation with memory evaluation by hippocampal electrophysiology performance is plausible to be seen contradictory. As observed before in rat synaptosomes, this biological structure undergoes a diurnal variation in neurotransmitters content [28]. Recent data suggests that the hippocampus might not be the storage center that was thought to be and in that context, the cortex plays a greater role than previously assumed [29]. Indeed, we may consider that the behavioural output seen in the cognitive test is the result of the integration of multiple brain areas and that alterations in cerebral metabolism may be present in other interconnected areas such as the cortex.

Oxygen consumption was registered polarographically using a Clark oxygen electrode. Analysis of cortical P2 synaptosomes mitochondrial function by oximetry showed a circadian profile of oxygen consumption in Non-Tg mice, which was not observed in 3xTg-AD mice. In particular, Non-Tg synaptosomes showed low levels of oxygen consumption at ZT04 and higher levels at ZT16; moreover, FCCP clearly enhanced O2 consumption in Non-Tg synaptosomes at both ZTs.

![Graph A](image1.png)  
**Graph A**

![Graph B](image2.png)  
**Graph B**
Synaptosomal oxygen consumption rate in brain cortices of 3xTg-AD versus Non-Tg mice, at ZT04 and ZT 16. a) Respirometry graph of Non-Tg at ZT 04 vs ZT16; b) Respirometry graph of 3xTg-AD at ZT 04 vs ZT16; 1st set of bars represent basal oxygen consumption rate (OCR) at the 2 ZT’s, 2nd set represents the maximal stimulatory capacity of synaptosomes in response to the uncoupling agent FCCP. Data are presented as mean±SEM, n= 3-4 biological replicates each represent the average of 3 technical replicates, *= p<0.05. Statistical analysis performed by Student’s t-test (GraphPad Prisma 6.0 software).

Pre-synaptic nerve terminals demand high levels of ATP for proper synaptic function. Synaptic mitochondria failure to generate adequate ATP levels has been implicated as a leading event in neurodegenerative disease; in AD this dysfunction seems to precede the loss of synaptic networks. In order to assess mitochondrial status and verify the observed impairment, simultaneous fluorimetric analysis of intrasynaptosomal Ca2+ levels and mitochondrial membrane potential (MMP) were determined using Fura2-AM and TMRM, respectively. Our data revealed that after stimulation with OLIGO/FCCP, a higher polarization was shown in 3xTg-AD in comparison to Non-Tg, being only statistic significant at ZT16. (Fig. 8). Excitotoxicity is linked to high intraneuronal Ca2+ levels, which is taken up by mitochondria, as described before following exposure to the β-amyloid peptide (Ferreira and Ferreiro et al., 2015); therefore the analysis of Ca2+ levels in the presynaptic structure is of great relevance.

Intrasynaptical levels of calcium were maintained constant in the Non-Tg animals (Fig. 8c), in the 3xTg-AD animals the intrasynaptical levels of calcium were elevated at ZT16, showing an impairment.

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in maintaining calcium homeostasis. In all the experiments the calcium levels were in accordance with the observations measured in the mitochondrial membrane potential with the different stimulus.

**Figure 8- Simultaneous analysis of mitochondrial membrane potential and mitochondrial calcium content in 3xTg-AD versus Non-Tg cortical synaptosomes.** Representative traces of Fura-2 and TMRM+ fluorescence using cortical synaptosomes from Non-Tg and 3xTg-AD at ZT 04 and ZT16 is shown. Complete mitochondrial depolarization was achieved after addition of oligomycin (oligo) plus FCCP; the bar graphs depict the maximum levels of fluorescence of TMRM+ or Fura-2 observed after exposure of oligo+FCCP. Data are presented as mean±SEM (n= 5 biological replicates each represent the average of 3 technical replicates), *= p<0.05. Statistical analysis performed by Student’s t-test (GraphPad Prisma 6.0 software)

Oxidative stress has been described as a contributing factor to the onset of AD, since it has been implicated in Abeta/tau-induced neurotoxicity. The mechanistic behind this disruptive redox balance and the generation or clearance of free radicals remain unclear. Excessive reactive oxygen species (ROS) may proceed from mechanisms such as
mitochondrial dysfunction; concomitantly abnormal accumulation of Abeta and tau proteins appears to promote redox imbalance observed in AD. (REF) Enriched nerve terminals are able to generate hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) and possess the anti-oxidant machinery necessary to deal with it [30]. To mimic oxidative stress, \( \text{H}_2\text{O}_2 \) was added to cortical synaptosomes isolated from 3xTg-AD or Non-Tg mice at both ZT04 and ZT16 (Fig: 9). Exposure of \( \text{H}_2\text{O}_2 \) to synaptosomes prepared at ZT16 and subsequent stimulation with Oligo/FCCP showed no statistically significant differences; altered mitochondrial membrane potential only was observed in 3xTg-AD when compared to Non-Tg synaptosomes prepared at ZT04 and subjected to \( \text{H}_2\text{O}_2 \). The increase in TMRM+ induced by the oxidant stimulus (which is insufficient to cause complete mitochondrial depolarization as Oligo+F CCP) suggests \( \text{H}_2\text{O}_2 \)-induced depolarization.

**FIGURE 9— INFLUENCE OF HYDROGEN PEROXIDE EXPOSURE ON MITOCHONDRIAL MEMBRANE POTENTIAL AND CALCIUM LEVELS IN 3XTG-AD VERSUS NON-TG CORTICAL SYNAPTOSOMES** Representative traces using cortical synaptosomes from Non-Tg and 3xTg-AD, at ZT 04 and ZT16 in a experimental condition of 1M \( \text{H}_2\text{O}_2 \) exposure. Bar graphs quantify the change in MMP and calcium after Stimulus and maximum respiration alteration due to stimulus (average value of the last measurements after addition of \( \text{H}_2\text{O}_2 \) and OLIGO/FCCP). Data are presented as mean±SEM (n= 5 biological
replicates each represent the average of 3 technical replicates), *= p<0.05. Statistical analysis performed by Student’s t-test (GraphPad Prisma 6.0 software).

**FIGURE 10: DETECTION OF HYDROGEN PEROXIDE LEVELS IN 3XTG-AD AND NON-TG CORTICAL SYNAPTOSOMES at ZT 04 vs ZT 16**

Synaptosomal hydrogen peroxide levels under basal conditions and after exposure to antimycin A (AA) to selectively inhibit mitochondrial complex III; **a)** Graph bars for Non-Tg animals, representing the fluorescence levels reached both at basal condition and after AA exposure subtracted to internal controls without exposure to AA; **b)** Graph bars for 3xTg-AD animals, representing the fluorescence levels reached both at basal condition and after AA exposure subtracted to internal controls without exposure to AA. Data are presented as mean±SEM (n= 5 biological replicates each represent the average of 3 technical replicates), *= p<0.05. Statistical analysis performed by Student’s t-test (GraphPad Prisma 6.0 software).
4.2.3 DISCUSSION

Alzheimer pathology is defined as neuropsychiatric disease with clear and defined pathophysiological traits, is considered a progressive and neurodegenerative disorder. Evidence from recent epidemiological data on the role of circadian clock in neurodegenerative disorders points to circadian disruption as an outcome in AD patients [31]. Our data recapitulate previous published results and provided an answer to our question: “Does AD impair biological rhythms relevant in physiopathology?”

➢ Behavioural and cognitive output.

Apart from presenting the pathological hallmarks of AD, namely Aβ plaques and neurofibrillary tangles, the 3xTg-AD mouse model also show cognitive deficits, impairment in LTP/LTD and metabolic alterations. Classically, rodents display a circadian pattern regarding the locomotor activity. Moreover, the CLOCK gene has proven to be critical for behavioural output [32]. Interestingly, recent studies showed that 3xTg-AD mice display a phase shift phenotype prior to AD onset [33].

Although it has been shown that cognitive performance presents a circadian pattern, the impact of AD-related pathology on circadian behavioural output has not been evaluated so far [34]. AD pathology impacted our 24 week-old animals showing a cognitive deficit independently of the Zeitgeber. Indeed, a diurnal variation is present in the Non-Tg mice and is impaired in the 3xTg-AD mice. Indeed this offers the researcher a time window for achieving a statistical discriminating difference which otherwise would not be possible, a clear example of the aforementioned is present in Fig. 5.
This AD transgenic mouse model produces melatonin in a rhythmic manner [35]. Melatonin has been described as a neuro-hormone with several physiological functions; in particular, it regulates and is regulated by circadian clock, it impacts mood and behaviour and it has also a modulatory effect on LTP, thus also playing a role in cognition [36]. The anxiolytic behavioural displayed by 3xTg-AD (Fig. 1c) is compatible with a disturbance in melatonin night-time levels. Moreover, performing any action (behavioural testing) in the middle of the inactive phase is per se a stress factor that is different from the same action performed in the active phase of the animals. Data linking the cortisol levels and neurodegeneration correlate high levels of cortisol with dementia and mild cognitive impairment [37]. Cortisol levels in humans and corticosterone in rodents follow a rhythmic secretion. Upon awakening the levels of the hormone rise, a phenomenon termed cortisol awakening response (CAR), a product of the coordination of the hypothalamus-pituitary-adrenal (HPA) axis [38]; therefore, an interruption of the sleep-wake cycle of the animals will cause a rise in corticosterone levels at a specific time when its levels were not supposed to be secreting or animals awake. Data shows that during the period between the end of the inactive phase and the onset of the active phase, cortisol secretion is mostly under the control of circadian rhythmicity; interestingly, the amplitude of the diurnal variation is more influenced by sleep-wake timing [39]. These levels are relevant for the interpretation of learning and a circadian disruption is capable of altering the secretion of this hormone. The learning and memory process studied and inherent to the cognitive deficit assessed by the MWM behavioural test is a multi-stage task with different stages separated
temporally [40]. Procedural memory is best consolidated during REM sleep as well as emotional information, on the other hand NREM sleep is crucial for explicit and episodic memory consolidation, being important in spatial tasks [41]. Therefore, an impairment in circadian variation is capable of producing behavioural changes due to an impact of circadian clock coordination on different aspects of memory process, and on metabolic processes. Recently it was shown that the diurnal variation is a confounding factor in the analysis of cerebrospinal fluid biomarkers for AD [42]. These data gives further strength to the need for characterization of diurnal variation, of already considered solid phenotypic alterations in AD, in this case between the active and inactive phase. In order to precisely take conclusions, one needs to clearly understand the variables inherent to certain test and the associated biological factors, some of those under physiological control by the circadian clock. Confounding factors are more prone to have a masking effect at certain zeitgebers, thus a diurnal variation characterization reduces variability and allows drawing solid biological conclusions.

The learning and memory hippocampal dependent tasks, like the MWM, have been classically used to characterize the cognitive deficits [43]. Scientific community generally accepts that learning and memory impairment at the cellular/molecular level are also accompanied by behavioural changes in a sleep deprivation protocol [44]. Thus, supporting the importance of sleep patterns for the study of mnemonic processes, a 2-hour protocol as the one performed in MWM at ZT 04 over a week is detrimental to sleep/wake patterns and therefore to cognition, even in Non-Tg animals. Even with the use of dim red light, to prevent phase shifting of the internal clock of the animals,
interruptions in the sleep/wake pattern of the animals may impair learning and memory. Our findings support the fact that this action is more prominent in pathological conditions, opening the question of a less robust clock or an already disrupted circadian coordination for the 3xTg-AD animals and ergo in the pathology.

➢ **Circadian profile of synaptic plasticity.**

The observation of a circadian pattern in some behavioural tests raised the question of a similar pattern in hippocampal synaptic plasticity. Although the physiological impact of circadian rhythms in temporal regulation of the aforementioned molecular events is not so studied, several studies provide evidence that support the diurnal variation of hippocampal synaptic plasticity [45, 46]. Independent of the fact of being a central oscillator controlled process or not. Our measurements of basal synaptic excitability (I/O curves) confirmed in the Non-Tg animals the observation of others [47, 48], namely by outlining a basal response that is more pronounced for the Non-Tg and display a diurnal variation in excitability. Although MEL is very important for synaptic plasticity and learning behaviour it is not the sole key player in this rhythmic output; the experiments performed with the C3H and C57 mice by Dipesh Chaudhury and colleagues are prove of that. Several factors are known to modulate and have an impact on electrophysiological processes such as LTP [49]. The majority of those molecules are either circadian regulated or present a circadian oscillation, therefore the preponderance of circadian control on the induction of LTP is undeniable. Nonetheless, the reason why the diurnal variation of LTP observed in 3xTg-AD mice is
impaired is unclear and can be attributed to many different players. Interestingly we can still claim that the variation seen in behavioural test of cognitive functions are expressed also in the induction profile of LTP in hippocampal circuits. This lack of diurnal variation presents a scenario where the signal to noise ratio required to perform certain memory stages is impaired; based on this assumption the mechanisms of homeostatic scaling, that are pivotal to strengthen certain connections and reduce others in order to form clear and precise memory paths, are more prone to error due to this observed lack of differences in synaptic excitability during the 24 hour period.

➢ Mitochondrial function and calcium changes

Recently, it was suggested that BMAl1 is essential in mitochondrial fusion and fission; this dynamic mitochondrial activity influences metabolic changes and oxidative stress [50]. In our aim to understand: a) if there was a circadian variation in mitochondrial function; b) if mitochondrial function correlated/explained the observations seen in other parameters; c) if it replicates the metabolic alterations already described within changes in metabolic demands [51]. We found a clear diurnal variation in respiratory rate associated with a variation in mitochondrial membrane potential in the Non-Tg animals. Indirect evidence already pointed in this same direction, either through the oscillatory mechanism of peroxiredoxins or by metabolic coordination of other factors by the circadian clock [52, 53].

The brain is a highly energy demanding organ, requiring up to about 25% of our energy production [54]. Peripheral metabolic alterations seen in AD correlate with the mitochondrial dysfunction
already described for the mouse model [51], however there is only statistical significant in the inactive phase. This enhances the importance of energy demand and supply by the mitochondria, outlining also the relevance of circadian timing in research. The difference observed in basal synaptic excitability could also be translated into different basal mitochondrial function at the synaptic level, therefore an overdrive of this excitability will result in an extra effort to the mitochondria. This can be observed as an acute effect of circadian disruption, induced by AD genotype. The correlation between brain and metabolism and disruption of circadian timing leading to dysfunction of physiological and behavioural processes was already shown by others. Briefly, circadian disruption resulted in increased weight gain and increased leptin and insulin levels, an emotionality alteration and an inability to shift learned behaviours, as well as a reduction in neuron complexity [55]. Thus, the observed synaptic alterations in plasticity correlate with alterations in mitochondrial functioning, showing an inability to maintain calcium levels due to the high metabolic demand that burdens the mitochondria. Moreover, this fact supports the theory of calcium-mediated excitotoxicity. Nonetheless, we can only speculate on the reason for these increased levels of calcium. Oxidative imbalance has been consistently reported in AD [56].

The circadian clock evolved in order to maintain cell cycle protected from DNA damage, so it is plausible to assume that the ability to deal with oxidative damage might fall also into a circadian variation, this fact was registered for the Non-Tg. The fact that 3xTg-AD were unable to deal with oxidative insult, as also expected for the same
Zeitgeber (ZT 04) where synaptosomal mitochondria presented high mmp, can be interpreted as an imbalance towards the excessive production of ROS by high metabolic demands and consequently a burden into the mitochondria.

**In conclusion** we must acknowledge that the mystery of sleep is more than closing our eyes or go to bed, the disruption of sleep patterns impacts our daily life and we all have suffered the effects of a not so good night of sleep. Circadian clock proteins play a pivotal role in this homeostasis of arousal and sleep, 10% of all our genes expression is circadian coordinated. Thus, synchronizing physiological processes and generating circadian profiles are essential in many metabolic, endocrinal, neuronal, cognitive and behavioural outputs. Together these data show that circadian profiles are impaired in AD, moreover it pinpoints the relevance of thoroughly characterizing diurnal variation, as some findings are only measurable (statistically different) due to profiling at active versus inactive phase. The coordination or regulation of the aforementioned processes by the circadian clock is still an open question in the onset of the disease. Nonetheless, this work demonstrates that AD phenotype is related with circadian disruption, ablation of diurnal variation and metabolic abnormalities.
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[55] Ilia N. Karatsoreos, Sarah Bhagat, Erik B. Bloss, John H. Morrison, and Bruce S. McEwen Disruption of circadian clocks has ramifications for metabolism, brain, and behaviour.

Metabolic alterations and circadian regulation in 3xTg-AD

"It Don't Mean a Thing (If It Ain't Got That Swing)" - Duke Ellington
4.3

4.3.1 INTRODUCTION

A wealth of evidence indicates that impaired energy metabolism are implicated in the etiology and progression of neurodegenerative diseases. Neurodegenerative disorders such as Alzheimer’s are affected by metabolic disturbances, such as insulin resistance, diabetes, obesity, stress and neuroinflammation. All of those can be a result of impaired glucose metabolism, a result of an imbalance of peripheral metabolism aligned with brain function.

The integration of peripheral metabolic information by the brain and consequent regulation of peripheral metabolism through brain-regulated mechanisms is intricate and complex. Evidence states that reduced glucose utilization and alterations in energy metabolism is an early event in the onset of AD, along with abnormalities in insulin and insulin-like growth factor type I and II (IGF and IGF-II and I) signalling mechanisms in brains with AD suggests a role in AD. Due to the similarities this hypothesis was termed as "Type 3 Diabetes" or Brain diabetes [1].

The aforementioned receptors are distributed throughout the brain; Insulin actions on these receptors modulate peripheral metabolic actions, such as regulation of appetite, body temperature, hepatic glucose production, adipose tissue deposition as well as response to hypoglycaemia states. Let’s not forget that Insulin signalling
also modulates neurotransmitter channel activity impairing neuronal function and synaptogenesis, impacts on mitochondrial function and alters the synthesis of cholesterol [2].

The regulation of food intake is coordinated by numerous peripheral signals that act in accordance with energy expenditure to maintain energy homeostasis. Primarily the gastrointestinal tract is equipped with sensing receptors (mechanic and chemical) signalling to maintain satiety. Levels of blood glucose induce food intake, those actions are mediated by hypothalamic neurons responsive to glucose. Secondly, all those stimulus interact with neuro-endocrinal signals like insulin and hipocretins.

These peripheral signals, like Insulin and leptin are produced in different places and through response to different actions. Nonetheless, they are both transported to the brain to modulate feeding behaviour. Insulin and leptin concentrations decrease during fasting periods. Changes in diet composition can lead to obesity as dietary fat and fructose do not stimulate insulin secretion and leptin production leading to an increased in food intake [3].

The role of circadian clock in mammalian physiology is considered to be the synchrony of processes. The metabolic control and coordination performed by circadian clock is pivotal to align temporally the needs and demands in terms of energy, but also to produce the neuro - endocrine signals in the appropriate moment. Metabolic homeostasis is pivotal to a healthy status. When disrupted this balance between energy requirement and energy intake, the metabolic fuels are converted and storage as: liver glycogen, muscle protein or triglycerides. Rhythms in energy intake should match endogenous fluctuations in gene expression in order
to obtain metabolic homeostasis at the cellular level.

These rhythmic transcription profiles are tissue specific or at least oscillate in a tissue-specific manner. In this manner a certain tissue or organ satisfy their metabolic demands using regulatory molecules that are temporally and or spatially distinctive. Metabolic homeostasis is essential in normal physiology, the synchrony of processes is fundamental for a proper functioning. Energy intake must match the energy demands and correlate with endogenous fluctuations in gene expression. Circadian-regulated genes in the liver comprise among others, genes committed to energy and glucose metabolism. Moreover, most of these genes seem to be regulated directly by BMAL1.

Nature and nurture are capable of inducing circadian disruption, leading to metabolic disorders. Artificial lightning, night shift, rotating shift and social jet lag produce alterations in physiological and in our molecular rhythms. Epidemiological data shows that circadian disruptions contribute to metabolic disorders[4]. Metabolic oscillations in circulating blood glucose levels persist during a forced dyssynchrony protocol, highlighting that rhythms in carbohydrate metabolism are not simply secondary to alterations in physical activity and/or food intake during the day. Making of these 24 h oscillations in endocrine and metabolic parameters a compromise between environmental rhythms, as well as between endogenous mechanisms.

The use of non-invasive methodology to question intermediate metabolism, allow us to understand how energy is used and how the metabolic fuels are storage, and it is empowering research with new tools to understand the metabolic fluxes alterations. Comprehension of those metabolic networks alterations, present in circadian disruption, present in metabolic disorders and shared by neurodegenerative disorders, and allow the
design of a bigger framework, for tackling such a challenging disease as Alzheimer’s.

Nuclear magnetic resonance (NMR) is currently being used to study intermediary metabolism. In the words of Schoenheimer and Rittenberg, “If carbon-bound hydrogen of physiological substances is re-placed by deuterium, the metabolism of such compounds, after their administration to an animal, can be studied by tracing the route taken by the deuterium”. Hence, the use of stable isotopes such as deuterium is an appropriate indicator to study this physiological processes [5].

Neurodegenerative diseases share common biochemical pathways with metabolic disorders, such as altered cellular energy states, and impaired or decreased glucose metabolism. These may be factors underlying in the mechanism of neurodegeneration, aging and AD. By exploring intermediary metabolism and profiling the enzyme kinetics through the follow up of flux rates and the intermediates of metabolic pathways, NMR can unveil new biochemical and physiological information in pathological conditions.

Liver glucose metabolism in mammals, provide the circulating glucose levels required to maintain adequate brain function, interestingly AD show neuronal condition of glucose hypometabolism. The brain is extremely dependent on glucose, and the levels of blood glucose control feeding mechanism.[6] Glucose is not only the main source of energy but is also the main precursor for neurotransmitters. The brain is thus capable of up regulate anaerobic metabolic pathways and change into a more glycolytic profile in order to produce ATP. Glucose is stored in the form of glycogen; brain glycogen is in the order of a µmol/g, comprising the single largest energy reserve. Glycogen is an endogenous store for energy metabolism and is primarily localized in astrocytes. Astrocytic glycogen can be increasingly utilized when glucose supply is restricted, its use provide
the energy to perform the continued glutamate uptake by astrocytes and posterior conversion to glutamine in order to reduce glutamate excitotoxicity [7]. Hence, the maintenance of proper glucose metabolism and the rates of these metabolic fluxes are of crucial importance in neurodegeneration.

Thus, as a hypothesis: the pathophysiological mechanisms and intracellular signalling cascades are prone to be shared by the two conditions. Hence, peripheral metabolism and brain disease, are connected in ways that are seemingly overlooked. Interconnecting them, a circadian regulation of metabolism, which is gradually impaired with aging and possibly disrupted in AD.
4.3.2 RESULTS

From the characterization of the diurnal variation in 3xTg-AD of phenotypic characteristics affected by the pathology. Those findings raised a question on whether this hypothesis of Alzheimer perceived as brain diabetes or “Type 3 diabetes”, is due to an alteration in circadian regulation. Bearing in mind the premiss of circadian clock regulation of metabolism and that alterations in metabolism are a co-factor in disease.

Does alterations in circadian patterns at a central level (behavioural cognition, LTP induction, mitochondrial functional status) in 3xTg-AD, are accompanied by a de-regulation in peripheral metabolism diurnal variation?

For this reason, study of metabolic fluxes by NMR was used to evaluate these 3xTg-AD animals at the active and inactive phase and understand the metabolic states. Moreover, we were also driven to understand if circadian genes were altered and if metabolic gene network implied were altered.

Animals were given intraperitonealy a bolus of 1% of total body weight of deuterated water, 24h prior to euthanaziation and their liver was collected at ZT 04 and ZT 16. Biological samples(liver) were treated, mRNA and metabolites were extracted and analyzed, by qPCR and NMR respectively.

Apart from the original report by Alzheimer and a subsequent report by Perusini and Kraepelin, the pathological trait of glial cells increase with lipid granule accumulation, is not widely considered. These features recorded in the brain point to a deregulation in metabolism.

The hypothesis of Alzheimer as a form of Type3 diabetes puts glucose utilization in the center of
this theory. In fig: 1 we illustrate the study of glucose metabolism by NMR. Enrichment in deuterium levels of plasma body water, show similar levels between genotypes and zeitgebers, presenting 3.5% to 5% enrichment (fig. 1a). This percentage of body water enrichment as previously proven effective in labeling satisfactorily the metabolites, in order to perform an adequate $^2$H NMR analysis preventing toxic side effects and alterations in normal physiology. The spectrum obtained of Monoacetoneglucose (MAG), is a product of glucose conversion obtained from glycogen hydrolysis, is decently resolved with all seven aliphatic hydrogens being distinguishable. A diurnal variation is observed in newly synthesized glycogen (fig. 1b) being in average less synthesized at ZT 16 than at ZT 04, with no significant changes observed between genotypes. Basal metabolic rates are under circadian coordination, implying a greater depletion during active periods (ZT 16) and a greater synthesis in the inactive period (ZT 04). As depicted by the spectra readout of positional $^2$H-enrichment, position 2 is the one most enriched in all experimental groups. Direct and indirect pathway are neither different, presenting no changes in genotype or zeitgeber, meaning that glycogen synthesis was perform via direct and indirect pathway without any preference. These findings represent a normal situation in a healthy physiological state, where the contribution to hepatic glycogen is an equal contribution of either pathways.
**FIGURE 1: HEPATIC GLYCOGEN SYNTHESIS**

Glucose utilization and glycogen synthesis profile in liver of 3xTg-AD and Non-Tg animals at ZT 04 and ZT 16; data are presented as mean ± SEM (n=4-5 per group), *p < 0.05* Statistical analysis was performed by Student’s t-test.
Regarding the indirect pathway, the metabolic pathways used are the triose pathway or the Krebs cycle (fig. 1e, 1f). The glycogen that was produced through this pathway, comes from the conversion of F6P to G6P. The G6P can continue on the Krebs pathway or G6P can enter the pentose phosphate pathway. When analyzing the metabolic fluxes, t-student test show a significant change between the Non-Tg and 3xTg-AD only at ZT04, for the use of the glycolysis pathway. Thus, we can interpret the results, as a contribution of gluconeogenesis to the indirect pathway, pointing to a scenario where 3xTg-AD animals have higher energetic demands forcing them to shift to glycolysis for energy. Furthermore our analysis of mRNA levels of liver genes, showed no clear-cut differences in genes in glucose metabolism key step rate limiting enzymes. G6PC levels are elevated for the 3xTg-AD, presenting a tendency but are not statistically significant. Interestingly PEPCK1, a gene involved in glucose homeostasis but also relevant to lipid metabolism (fig. 5), is significant.

Thus, considering the above mentioned traits and the alterations in systemic metabolism, we evaluated the peripheral lipid metabolism. Metabolic alterations, such as insulin resistance are not solely due to glucose metabolism alterations but let’s not forget that metabolic alterations impact energy systems in an integrative way. Certain minor imbalance might repercute into considerable alterations in a related system. Therefore, we proceed with lipid synthesis and flux analysis, as a minor imbalance in glucose utilization might prompt alterations of use and synthesis in these correlated biological system. In Fig. 2 we can observe the lipidic profile of those animals. NMR evaluation allowed us to depict a defect in normal lipid metabolism. Lipid functions
include signaling and transcription regulation; lipids are part of the membrane structure, interact in synaptic function, allow protein processing and protein-protein interactions.

**FIGURE 2: LIPOGENESIS IN 3XTG-AD VS NON-TG ANIMALS**
Liver lipidomic profile of 3xTg-AD and Non-Tg animals at ZT04 and ZT16; Data are presented as mean ± SEM (n=4-5 per group), # = interaction in a 2 Way-Anova statistical test. (GraphPad Prisma 6.0 software).

Analysis of lipidic fluxes show in a 2Way-Anova test a significant interaction between the experimental groups. De novo lipogenesis (fig. 2a) does not present a diurnal variation but seems to be impaired in pathology, meaning that there is a significant decrease in the fatty acid synthesis in the 3xTg-AD animals. These facts are rather peculiar as 3xTg-AD animals do not show an increase in body weight, suppression of hepatic de novo lipogenesis affords
protection against obesity. Therefore, this reduced hepatic lipogenesis, is probably a compensatory mechanism to improve a metabolic dysfunction.

Triglyceride per se is not sufficient to cause systemic insulin resistance intermediate metabolites are more prone to cause damage. Newly desaturated fatty acids in liver do not show differences. In contrast, chain elongation presented a circadian pattern of lipid biosynthesis in either experimental groups (fig. 2d). Representing a clear diurnal rhythm of a biological process necessary for lipid homeostasis. In suma we found a reduction of de novo lipogenesis for the AD mouse model, meaning that their metabolic needs or metabolic abnormalities prompted them to be incapable of producing less new triglycerides in comparison to control.

Those changes in lipid biosynthesis pathways incited us to quantify the relative amount of lipid species. In fig. 3 we can appreciate the relative amount of lipid species present in our liver samples, between the two experimental groups and at the two zeitgebers. The health benefits of ω-3 are well known, essential in maintaining normal brain function, as well as the use in neurodegeneration as a neuroprotective strategy [8]. In our study we confirmed that the pathology alters the relative quantity of ω-3 and non ω-3 in the 3xTg-AD vs Non-Tg. Conventionally the levels of ω-3 should be higher for the Non-Tg, that was not the case, pathology is impacting physiology causing decrease in ω-3 species in Non-Tg.
FIGURE 3: RELATIVE AMOUNT OF LIPID SPECIES

Lipid species percentage in liver samples determined by $^2$H NMR of 3xTg-AD and Non-Tg animals at ZT04 and ZT16; Data are presented as mean ± SEM (n=4-5 per group), # = interaction in a 2 Way-Anova statistical test (GraphPad Prisma 6.0 software).

This is per se interesting but the alterations that led to this state are unknown and unclear. On top of fatty acid synthesis alterations (DNL decrease) coincident with Studies with animal exposed to a High Fat diet, contrastingly AD pathology alter elongation and relative amount of lipid species [9]. Mono unsaturated fatty acids lipid species, show significant interaction between all the experimental groups in a 2Way-Anova statistical test. Moreover, unsaturated and saturated fatty acids show an interaction between genotypes. Meaning that alterations in AD are not just a straightforward result of a metabolic alteration,
such as insulin resistance or obesity, but an intricate disease with severe alterations in different biological systems. Liver modifications to fatty acid structures such as elongation, desaturation or peroxissomal β-oxidation, all those processes perform an important role in lipid composition or storage, in generating signaling molecules or regulating transcription factors. So the observed low levels of Monounsaturated fatty acids (MUFA) are coherent with the effects of dietary MUFA on health: effects blood pressure and reduction of cardiovascular disease, improvement on insulin sensitivity and regulation of glucose levels [10]. Meaning that these is an important lipid specie with impact on disease and presenting a circadian interaction. It can afford its protection by modulating inflammation, or altering plasma membrane composition, having already been described as associated with a lower risk of mild cognitive impairment [11].

After these data analysis we went back to our initial question, and pose the question of whether these was a result (although to fully impinge this on circadian regulation same analysis had to be performed on Dark/Dark cycle) of a chronodysfunction. In (fig. 4) we see a profile of core clock genes and their oscillatory pattern de-regulated for the 3xTg-AD but also the profile of some secondary genes in circadian rhythms, with a clear role in metabolism coordination namely REV-ERB α and ROR α. The Non-Tg animals exhibited, for all genes, patterns of expression in accordance with the ones obtained when comparing against CIRCADIOMICS[12].
**FIGURE 4: PERIPHERAL OSCILLATOR SYNCHRONY**

Quantification by qPCR of mRNA liver levels of core clock and secondary oscillator genes in 3xTg-AD and Non-Tg animals at ZT04 and ZT16; Data are presented as mean ± SEM (n=4-5 per group)

Interestingly, a circadian gene mediates the control of lipid metabolism. In 3xTg-AD, the elevated levels of BMAL1 at ZT04, where consolidated by the elevated levels of DBP at ZT16, as this gene oscillate anty-phase to BMAL1. High levels of ROR α at ZT16, could potentially be an explanation for the alterations seen in lipid metabolism, as ROR α regulates gene expression of several genes, SBREP-1 for instance, that control lipid metabolism [13].

In order to validate our findings but also to understand if those, already accepted biomarkers, are also under circadian regulation we measured the mRNA levels of APOE, a widely accepted co-factor in AD, Nrf2 (a biomarker related to mitochondrial dysfunction), as well as some genes involved in the above metabolic pathways.
Quantification by qPCR of mRNA liver levels genes in 3xTg-AD and Non-Tg animals at ZT04 and ZT16; Data are presented as mean ± SEM (n=4-5 per group)

From the selected set of candidate genes previously chosen, only the above show a statistical significant change. These data (fig.5) underlines the relevance of the characterization of this circadian variation, as some genes are only statistical significant at a specific ZT for that gene, meaning that surveying the genome for biomarkers at only one time point can produce more variability and cause to lose discrimination power between Non-Tg and 3xTg-AD. Moreover and probably, more defining for the question raised and results found is the involvement of genes with a circadian regulation and a metabolic function, which is abnormal in this mouse model of disease.

Organisms have only periodic access to food to maintain a high ATP/ADP ratio. To maintain regular energy demand under fasting, cells convert sugars and fat into storage forms, respectively glycogen and triglycerides. Fat is essential for longterm storage. The fat stored in adipose tissue, in the morning as you awake up after an overnight fast, most of the needed ATP is generated by fatty acid oxidation.

Low glucose levels in the blood start the hydrolysis of the triacylglycerol molecules in fat droplets into free fatty acids and glycerol. After entering the bloodstream they are capture by the cell and transported into the mitochondria. The mitochondrion is the place where Acetyl COA is produced, its organelle where most of the oxidation reactions take place and where most of ATP is made. Indeed, the mitochondrion is the energetic center
towards which all processes converge, independently if they are sugars, fats or proteins.

The glycolysis and the TCA cycle produce important intermediates in biosynthetic reactions, namely oxaloacetate and ketoglutarate; these processes not only generate energy, but also provide the precursors required to synthesize many important biological molecules, e.g. Glutamate. The complexity and the intricacy of the cell as a chemical power plant is visible in the relations of glycolysis and TCA cycle to the other metabolic pathways. In theory, all cells throughout our body contain the required enzymes for glycolysis, lipid catabolic and anabolic, amino acid metabolism, and the TCA cycle; nonetheless, not all tissues require the same levels of those processes. Nerve cells keep almost no metabolic reserves and rely mainly on a supply of glucose; on the other hand, liver cells produce glucose and recycle the lactic acid back to glucose through the gluconeogenesis pathway. Therefore, a multi-trait network is responsible for coordinating the rates of all these reactions and articulate the cooperation of distinct cells to maintain tissue and organism homeostasis.

The hypothesis that insulin insensitivity might be linked to a dysfunctional clock added to the recent advances in the field which suggested that liver clock regulates the gluconeogenic pathway through Cry-mediated inhibition of CREB activity during fasting. Mitochondrial dysfunction has been also linked to transcription deregulation through disruption of the transcriptional co-activator peroxisome proliferator activated receptor gamma (PPARgamma) co-activator-1 alpha (PGC-1alpha).

The fact of presenting circadian variation in newly synthesized glycogen, reveals a normal
physiological function, nonetheless the analysis of glucose utilization allow us to hypothesize into a preferable usage of glycolysis at the inactive phase. Thus envisioning a scenario where the metabolic needs are provided by an increase in metabolic function, augmenting the mitochondrial dysfunction seen and increasing the ROS levels, all of those serious/recognized partners in the disease onset. Moreover, this scenario is partially supported by the mRNA levels, with the outcoming of several transcription factors and genes involved not only in mitochondrial function but in energy and metabolic pathways.

PEPCK1 gene works as a gateway in the regulation of gluconeogenesis, catalyzing the formation of phosphoenolpyruvate from oxaloacetate. Interestingly its expression can be regulated by insulin, glucocorticoids, glucagon, cAMP, and diet. The encoded enzyme is a rate-limiting step in the metabolic pathway that produces glucose from lactate and other precursors derived from the citric acid cycle.

PDK4 encodes a mitochondrial protein located in the matrix of the mitochondria and inhibits the pyruvate dehydrogenase complex contributing to the regulation of glucose metabolism, it also mediates cellular responses to a high-fat diet. Regulating both fatty acid oxidation and de novo lipogenesis.

FOXO 1 gene is a transcription factor that is the main target of insulin signaling and regulates metabolic homeostasis in response to oxidative stress. In hepatocytes, among its functions: promotion of gluconeogenesis by interacting with PPARGC1A, regulation of expression of adipogenic genes such as PPARG.
PGC1α is a transcriptional coactivator that regulates the genes involved in energy metabolism. Interaction with PPARg allows the interaction of this protein with multiple transcription factors, like nuclear respiratory factors (NRFs) or region orphan nuclear receptors (RORs). Thus, it is capable to regulate key mitochondrial genes, has an essential role in metabolic reprogramming as it coordinates the expression of a wide array of genes involved in glucose and fatty acid metabolism.

Adenosine kinase is an enzyme involved in the conversion of adenine derivatives, playing an important role in inflammatory processes. These alteration in liver expression levels, indicate that perhaps the neuroprotection that adenosine 2a receptors provide is also mediated by a peripheral effect. The blockade of A2a receptors in hippocampal slices of 3xTg-AD, modulate LTP. This pharmacological target has been considered as possible therapeutical approach [14]. By blocking A2aR, you prevent the activation of these receptors by ATP and you prevent the homeostatic function of this receptor at the synaptic level. Thus, improving the energy levels that are available for the synapse to perform properly. Elevated levels of ADK seen in the 3xTg-AD, represent an attempt to increase the availability of ATP due to higher energy requirements inherent to the pathology [15].
Energy seems to be at the center of these questions, we have come with a panel of genes involved in metabolic pathways, moreover we have provided direct evidence of an up-regulation of mitochondrial function (NRF2, FOXO1, SIRT3). We have constructed a clear image of the metabolic network affected in AD and were able to show that these genes interact with each other and are under circadian regulation (fig. S5).
4.3.3 DISCUSSION/CONCLUSION

As a final remark, this work presents itself as an initial step towards a metabolomic approach in Alzheimer’s. By performing an analysis at the active and inactive phase of the circadian cycle, we were able to create a better picture of the dynamic processes involved and their relative alterations/contribution in pathology. Moreover, these works supports the hypothesis of a selective form of insulin resistance causing a greater burden in lipid biosynthesis that in glucose utilization. Genetic analysis confirmed our suspicions of a circadian impairment and revealed a metabolic link from circadian regulation to AD. There are still many questions to be considered, for instance the therapeutical potential of a circadian intervention in AD. An extensive validation in AD patients of the results obtained, NMR and QPCR data, is required until we can come to the point of generating and establishing some of the genes analyzed as potential biomarkers of disease.
REFERENCES


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5) General Conclusions & Future Perspective

"I know you think you understand what you thought I said but I'm not sure you realize that what you heard is not what I meant" - Alan Greenspan
General Conclusions & Future Perspective

5.1 General Conclusions

The aim of studying the neurobiology of circadian clock, understanding the biological processes under a circadian regulation with a clear implication in Alzheimer’s pathology, is a daunting task. Alzheimer pathology has known several hypothesis; so far all theories have fall short form providing a reasonable inclusive explanation. Circadian biology links almost all of the theories, presenting itself as possible hypothesis. Therefore, we design a framework capable of providing a solid commitment to tackle this intricate question. By analyzing the behavioural output and the cognitive aspects; the brain and the memory circuit of the hippocampus; complementing with a molecular/biochemical/energetic analysis at the cellular level of the mitochondria.

This top down approach allowed us to make some conclusions. We have established that AD is indeed a progressive neurodegenerative disease, with a clear mitochondrial contribution to disease onset. The cognitive deficit is also a progressive trait, considerable more heterogeneous due to the considerable amount of brain structures required to elicit a behaviour. Hippocampal LTP is not impaired at three months of age, but is altered relative to the Non-Tg animals, argumenting in favour of an interpretation where Ltp induction levels should not be considered as a quantintative measurement, but included in processes of plasticity. Analysis of mitochondrial functional status, allowed to us
concluded that mitochondrial alterations are primary events in disease, likewise this solidify the idea of alterations in metabolism as a leading cause. Energy demands impinge on mitochondrial metabolism, causing an increase in regular functioning to meet the energetic needs, as well as an imbalance in ROS levels.

A single time point analysis does not provide a correct observation, in almost every scientific paper published the Zeitgeber at which the experiment was performed is neglected and comparisons are performed between data collected at different time points and considered to be representative phenomenon.

Thus, as we are speaking of physiological processes, dynamic by nature, with a circadian pattern established, we ought to assume variation and comprehend this diurnal variation.

Accepting these facts, we conclude that by dividing the cycle in active phase and inactive phase we could gain an overview of the physiopathology. Allowing us to confirm that Non-Tg animals display a diurnal/circadian variation in physiopathological traits, being this pattern disrupted in the 3xTg-AD.

The behavioural tasks require an integration and activation of brain areas affected in AD. More complex tasks provide clear results, as they require more integration and a proper functioning of hippocampus, cortex and other areas to perform the intended long term memory spatial test, being in consequence less affected by alterations in the emotional/anxiety profile. In terms of LTP induction, we found support for a progressive excitotoxicity mediated by Aβ. These progressive sate of disease starts with alterations in mitochondrial
functional status that are untraceable at normal physiological levels, interestingly they show an impairment in dealing with ROS levels.

After concluding on this progressive trait, leading to neurodegeneration by an alteration in mitochondria function and anti-oxidant defense system. We proceed to an established all symptomatic stage, already described and accepted, and evaluated the impairment of circadian traits by AD genotype, as well as confirm the existence of some diurnal variation profile of certain biological aspects, not yet described in literature.

Synchronization of physiological processes and the generation of circadian profiles are essential in many metabolic, endocrinal, neuronal, cognitive and behavioural outputs. In that context, our results show that circadian profiles are impaired in AD. We have proven that at 6 months of age, we have a mouse model of AD that display alteration in all the major pathological traits. In this experiment, we were able to differentiate changes between active and inactive phase in normal physiology and in pathology. Thus, revealing the importance of thoroughly characterizing diurnal variation, cause there are findings only measurable (statistically different) at certain ZT’s. The behavioural testing confirmed the cognitive deficit already seen at 3 months of age, revealed some clear circadian profiles in learning and memory tasks, most important it shows the need for complex testing to surpass alterations in emotionality.

The LTP induction profile in 3xTg-AD shows a loss of circadian profile, at ZT 04 they show an elevated potentiation, in accordance with the elevated levels seen at 3 months of age. Thus, plasticity is altered in AD at both age groups, and should be considered the utilization of protocols that allow the
evaluation of synaptic scaling mechanism or metaplasticity. Mitochondrial dysfunction and oxidative stress have been implicated in the pathophysiology of AD. The observations seen at 3 months coincide and reveal that an impairment of mitochondrial functioning is augmented with aging. In mitochondrial membrane potential the susceptibility to oxidative stress upon exposure to Hydrogen peroxide (H2O2) revealed a greater depolarization at Zt16, which correlates with a greater susceptibility to damage depending on the ZT. Inability to maintain iCa levels by 3xTg-AD at Zt16, relate to calcium exictotoxicity scenario.

The last chapter allowed us to conclude that AD animals have an impairment in circadian clock genes at a peripheral level, those genes are key players in the regulation of metabolic pathways. We could find also alterations in mitochondrial genes level, which support our previous findings in MMP.

In suma the hypothesis of a circadian dysfunction impacting Alzheimer’s at different levels is supported by our findings. All current hypothesis are in some way circadian impaired: circadian genes regulation of metabolic pathways, mitochondrial function, neuronal firing, excitotoxicity, ROS level or simply aging. With this work we had some other pieces to a rather intricate and complex puzzle, that has been intriguing science for more than 50 years. We should definitely consider the Circadian Clock as a research topic in AD and a co-factor in disease.

5.2 Future Perspectives
To clear enlighten the role of circadian clock in normal physiology the same set of experiments should be performed in dark/dark cycle regimen.

In order to establish circadian clock as culprit in AD. Although some pathology occurs relatively late in life, this mouse model is useful as it is well characterized regarding biochemical, pathological and behavioural characteristics. By crossing 3xTg-AD mice with Bmal1 or Cry1/Cry2 deficient mice, different states of ‘locked’ circadian clocks are combined with amyloidopathy. We will prove the paradigm by crossbreeding an AD transgenic mouse model with circadian mutant mice and assess the onset and progression of AD. Alternatively, we could use some circadian mutants and administrate Aβ oligomers directly into the brain. Finally, we could address the question of why do 3xTg-AD mice become arrhythmic before the onset of AD. Recently, LA Ferla’s Lab has shown a loss of rhythmicity previous to the onset of AD; in this context, it becomes relevant to discern if a loss in locomotor activity output is accompanied by electrical activity of SCN, and if so, how does the molecular clock respond in terms of gene expression.

The intricate network that controls biological rhythms has a determined circadian gene profile; indeed, the levels of certain genes might be relevant due to subsequent functions of those genes and their impact on metabolic traits. Another relevant question that needs to be assessed is if a loss of rhythmicity in the SCN is coupled to PVN deregulation. Quantitative PCR analysis of expression levels of different circadian genes (Rev-erb, CRY, Bmal1, CLOCK) and clock controlled genes will be performed
on SCN, liver and pancreas tissues from 3xTg-AD mice at different time points during the day, the more frames you have the more fluid the picture becomes.

Having in mind a translational perspective, circadian disruption are part of our daily habits, therefore we will determine how a shift or a total loss of circadian rhythms and related metabolic disruption may result in a hasten and/or earlier onset of amyloidopathological characteristics of AD. Sleep deprivation, restricted feeding, altering light/dark cycle or SCN ablation can be used to induce this disruption in 3xTg-AD.

Early detection are crucial in neurodegenerative disease outcome and can make a big difference in the treatment of neurodegenerative disease like Alzheimer’s, the candidate genes which have unveiled as potential biomarkers require validation. Thus, we aim to validate some of the most promising genes, in isolated RNA from patients blood.