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The puzzle: absence seizures, astrocyte complexity, and memory impairments

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“All truths are easy to understand once they are discovered; the point is to discover them”.

Galileo Galilei

“The important thing is to never stop questioning”.

Albert Einstein

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meu colega de casa, de curiosidade sem fim.**

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Resumo

A epilepsia de ausência infantil é uma síndrome não convulsiva, caracterizada por crises de ausência relatadas como breves interrupções de consciência e movimento. É descrita como uma gênese multifatorial, afetando 2 a 8 crianças em cada 100 000 com idade inferior a 15 anos. As crises de ausência iniciam-se entre os 3 e 8 anos, podendo ocorrer múltiplas vezes por dia, podendo a sua frequência ser superior a 200 por dia. De entre todos os casos de epilepsia identificados em crianças em idade escolar, 10 a 17 % devem-se à epilepsia de ausência infantil. É diagnosticada por padrões de disparo neuronal anómalos e síncronos detetáveis por eletroencefalograma na rede talamocortical. A nível molecular, vários genes estão envolvidos na etiologia desta patologia, tais como os genes que codificam para os canais de cálcio tipo T, genes que codificam para as subunidades dos recetores GABA_A; bem como o gene *SLC6A1*, que codifica para o transportador de GABA do subtipo 1 (GAT1). Em crianças com mutação no gene *SLC6A1* o fenótipo de crises de ausência é o mais comum.

Para além das crises de ausência, descrevem-se dificuldades cognitivas em 60 % das crianças com epilepsia de ausência infantil, tais como deficiências de atenção, hiperatividade, dificuldade de leitura, ou na realização de tarefas visuoespaciais. No entanto, dados relativos a perturbação de memória são inconsistentes, uma vez que nem todos os estudos englobam comparações com crianças desguarnecidas de tratamento.

Relativamente à terapia farmacológica da epilepsia de ausência, esta baseia-se na administração individual de etosuximida, ácido valpróico ou lamotrigina, ou na combinações de dois destes fármacos. No geral, a ausência de resposta é de aproximadamente 30%, sendo que o ácido valpróico, quando administrado, está associado a efeitos neuropsiquiátricos adversos. Assim mesmo, quando o tratamento farmacológico permite o controlo das crises de ausência, défices cognitivos são ainda relatados em 70% dos casos, não sendo claro se os fármacos anti-crisis (*antiseizure*) têm efetivamente efeitos naqueles. Já os mecanismos patofisiológicos não são completamente identificados sabendo-se, no entanto, que as crises de ausência têm o seu início na rede talamocortical.

Uma transmissão GABAérgica anormal, aumentada nos neurónios talamocorticais, está envolvida no processo das crises de ausência, sendo a transmissão GABAérgica regulada por transportadores de GABA (GATs), como por exemplo o GAT1, que são responsáveis pela recaptação de GABA da fenda sináptica. A disfunção do GAT1 nos astrócitos do tálamo observa-se em animais com crises de ausência, levando ao aumento da inibição tónica, sendo este aumento necessário e suficiente para a geração daquelas. Ainda, no tálamo, o GAT1 é expresso exclusivamente nos astrócitos sendo a recaptação de GABA quase exclusiva destas células da glia.

Uma vez que esta síndrome está associada a défices cognitivos e a ausência de resposta a fármacos é comum, torna-se essencial a identificação de novos fármacos para esta doença. O Fator Neurotrófico Derivado do Cérebro (*Brain-derived neurotrophic factor* - BDNF) pode ser um novo alvo terapêutico para esta patologia, uma vez que é essencial para plasticidade sináptica, um processo necessário na aprendizagem e memória. O BDNF tem também a capacidade de aumentar o transporte de GABA mediado por GAT1 para os astrócitos, podendo contribuir para uma diminuição na inibição tónica.

Como tal, neste trabalho utilizaram-se dois modelos animais de epilepsia de ausência: os ratos GAERS e respectivos ratos controlo não epilético (NEC), e ratos Wistar (como segundo controlo); murganhos Stargazer (STG) e respectivos controlo WT. Os ratos GAERS é um modelo animal de epilepsia de ausência, apresentando *spike and wave discharges* bilaterais de 7-9 Hz, com interrupções de movimento a partir do dia pós natal 30 e respondem de forma semelhante a fármacos anti-crisis comparativamente

com humanos. O modelo animal de murganho, os STG, apresentam *spike and wave discharges* de 6-7 Hz, com interrupções de movimento, caracterizadas também por disfunção do canal de cálcio *CACNG2*, bem como comportamentos maladaptativos e ataxia.

Os objetivos principais deste trabalho foram: 1) identificar as comorbilidades comportamentais dos GAERS e dos STG, avaliando ansiedade, bem como aprendizagem e memória; 2) estudar a plasticidade sináptica em fatias do hipocampo, bem como avaliar a função do BDNF na potenciação a longo-prazo (LTP) nos GAERS e STG; 3) identificar diferenças a nível molecular e celular entre GAERS, Wistar e NEC, determinando a expressão de proteínas por western blot, nomeadamente transportadores de GABA, GAT1, GAT3 e GFAP (*Glial fibrillary acidic protein*). Recorrendo a imunohistoquímica, a quantificação de alterações de complexidade morfológica dos astrócitos foi também analisada em regiões cerebrais envolvidas na rede talamocortical e /ou memória.

Os resultados obtidos contribuem para uma definição fenotípica precisa do modelo mais representativo de epilepsia de ausência infantil. Confirmaram-se deficiências de memória em animais GAERS jovem adultos e adultos a nível de memória de trabalho (Y Maze, acquisition Barnes), memória de reconhecimento de novos objetos (NOR), memória cross-modal contemplando memória textural e visual (CMOR) e ainda memória de referência espacial (Barnes Maze). Foram também observados défices na memória de referência espacial (Barnes Maze) para o modelo animal de murganho. De igual forma, a ausência de níveis de ansiedade (Open Field, EPM, Emergence Test) demonstra que os resultados observados não foram enviesados por deficiências de locomoção e/ou ansiedade.

De modo a complementar os resultados observados *in vivo*, foi induzida potenciação a longo prazo em fatias de hipocampus de animais GAERS, Wistar e NEC, e registaram-se potenciais excitatórios pós-sinápticos de campo (fEPSP) nas fibras colaterais de Schaffer na região CA1 do hipocampo. A plasticidade sináptica foi avaliada, e identificadas diferenças significativas entre os animais controlo e os epiléticos a nível de LTP, sendo significativamente reduzida nos GAERS, corroborando os resultados comportamentais. Deficiências de LTP no hipocampo foram também observadas nos murganhos STG. Nos GAERS, o BDNF recuperou a perda de magnitude da LTP para valores semelhantes ao controlo.

Visto que o GAT1 está associado à perda de função e contribui para um aumento da inibição tónica nos animais epiléticos, e que no tálamo é meramente expresso em astrócitos, foi importante confirmar esta expressão única nos astrócitos talâmicos, visualizar a expressão destes transportadores nos astrócitos, bem como perceber se a morfologia dos astrócitos estaria alterada nas diversas áreas envolvidas nas crises de ausência, nomeadamente o córtex motor, somatosensorial, visual, talamo e ainda hipocampo, fundamental nos circuitos subjacentes à memória. Observou-se um aumento da complexidade morfológica dos astrócitos a nível do córtex motor, visual, hipocampo e tálamo, relativamente ao número de interseções à volta do soma, número de processos e comprimento total das ramificações nos GAERS.

Foi ainda fundamental compreender se existem diferenças de expressão dos transportadores de GABA dos astrócitos. GAT3 também foi analisado, servindo de controlo, bem como análise de GFAP, visto que se observaram diferenças significativas a nível morfológico. A expressão de GAT1 nos GAERS não está alterada em nenhuma área cerebral, exceto no córtex motor, que é acompanhada por um aumento significativo de expressão de GFAP comparado com ambos os controlos. A expressão de GFAP está ainda significativamente aumentada no hipocampo, no talamo comparado com ambos os controlos, e no córtex visual, comparado com os Wistar. Um aumento do rácio do peso cérebro- corpo foi ainda observado para os animais epiléticos.

Em suma, os resultados deste trabalho permitem inferir déficits de memória de nível comportamental em modelos animais de epilepsia de ausência, em circuitos subjacentes à rede talamocortical, nomeadamente córtex e tálamo, bem como as conexões dependentes do hipocampo. A nível de circuitos intrínsecos do hipocampo, uma reduzida indução de LTP foi observada nos animais epiléticos e esta função sináptica deficiente recuperou-se em perfusão com BDNF em fatias do hipocampo. Observou-se a nível molecular que no tálamo a expressão de GAT 1 é meramente observada em astrócitos e ausente em neurónios, e que a expressão de GAT1 e GAT3 não está alterada entre os animais epiléticos e os controlos, excepto no córtex motor. Finalmente, a nível celular foram ainda identificados aumentos de complexidade morfológica astrocítica, bem como de expressão de GFAP em diversas áreas cerebrais de GAERS, envolvidas nas crises de epilepsia de ausência e nos circuitos de memória alterados.

Palavras Chave: Epilepsia de Ausência Infantil; Astrócitos; GAT1; Fator Neurotrófico derivado do Cérebro (BDNF); Memória

Abstract

Typical absence seizures (ASs), characterized by 3-4 Hz spike-wave discharges in thalamocortical network, are the hallmark of Childhood Absence Epilepsy (CAE). In ASs animal models, dysfunction of thalamic GABA transporter-1 (GAT1), exclusively expressed in thalamic astrocytes and responsible for GABA reuptake, enhances tonic inhibition. This enhancement is essential and sufficient for the generation of ASs. A neuropsychological impairment is observed in 60% of children with CAE. Using an ASs animal model, the Genetic Absence Epilepsy Rat from Strasbourg (GAERS), the respective Non-Epileptic control (NEC), and Wistar rats, as well as STARGAZER mice, behaviour tests were carried out to assess anxiety-like behaviour, learning, and memory. Hippocampal synaptic plasticity was evaluated by performing long-term potentiation (LTP) experiments where field excitatory postsynaptic potentials were recorded in control hippocampal slices and perfusion with a possible target to CAE, BDNF (Brain-derived neurotrophic factor). Immunohistochemistry and Western blot assays were used to assess molecular and cellular morphological differences between epileptic and non-epileptic animals. In Novel object recognition, Cross-modal object recognition, Y-Maze, and Barnes Maze tests, we observed significant differences between GAERS and controls, suggesting an impairment in the prefrontal cortex, thalamus, parahippocampal regions, and hippocampal-dependent memory. LTP magnitude in GAERS was significantly lower than from Wistar and NEC, being rescued in perfusion with BDNF. GAERS display an increase in astrocytic morphological complexity, mainly in the hippocampus, thalamus, motor cortex, and visual cortex, and increase GFAP expression levels, compared to Wistar and NEC. No differences were found for GABA transporters expression. This work results suggest memory deficits in GAERS, especially in spatial working memory, object recognition, long-term memory, and reference memory, in line with the reduced LTP magnitude. Since GAT1 is expressed in astrocytes and there is an increase in morphological complexity and GFAP expression in GAERS, the involvement of astrocytes in the pathology of ASs is reinforced.

Keywords: Childhood Absence Epilepsy; Astrocytes; GAT1; Brain Derived Neurotrophic Factor; Memory.

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Abbreviations

ADHD	Attention Deficit Hyperactivity Disorder
ASs	Absence seizures
ASDs	Anti-Seizure Drugs
ATP	Adenosine Triphosphate
BDNF	Brain-Derived Neurotrophic Factor
BM	Barnes Maze
BSA	Bovin serum albumin
CACNA	Calcium Voltage-Gated Channel Subunit
CACNG	Calcium Voltage-Gated Channel Auxiliary Subunit
CAE	Childhood Absence Epilepsy
CIN	Cortical Initiation Network
CM	Centromedial
CMOR	Cross-Modal Object Recognition test
CNS	Central Nervous System
CREB	cAMP-Response Element Binding Protein
DG	Dentate Gyrus
DNMTP	Delayed Nonmatching Place To Task
EDTA	Ethylenediamine Tetraacetic acid
EEG	Electroencephalogram
EPM	Elevated Plus Maze
fEPSP	Field Excitatory Postsynaptic Potentials
ERK/MAPK	Extracellular Signal-Regulated Kinase 1/2/ Mitogen-Activated Protein Kinase
ET	Emergence Test
ETX	Ethosuximide
FBS	Fetal bovine serum
TrkB.FL	Full-Length Receptor Tyrosine Kinase
GABA	γ -aminobutyric Acid
GABAR	GABA Receptor
GABRB3	GABA Type A Receptor Beta3 Subunit
GABRG2	GABA type A Receptor Gamma2 Subunit
GAD	Glutamic Acid Decarboxylase
GAERS	Genetic Absence Epilepsy Rat from Strasbourg
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAT1	GABA Transporter 1
GAT3	GABA Transporter 3
GFAP	Glial Fibrillary Acidic Protein
GLAST	Glutamate Transporter
GLUT1	Glucose Transporter
GTCS	Generalized Tonic Clonic Seizures
HPC	Hippocampus
IGE	Idiopathic Generalized Epilepsy
IL1 β	Interleukin 1 Beta
ILAE	International League Against Epilepsy
IPSP	Inhibitory Postsynaptic Potential
ITI	Intertrial Interval
JAE	Juvenile Absence Epilepsy
JME	Juvenile myoclonic epilepsy
KO	Knock-out
dLGN	Dorsal Lateral Geniculate Nucleus
LTD	Long-Term Depression
LTG	Lamotrigine
LTM	Long-Term Memory
LTP	Long-Term Potentiation
MAP2	Microtubule Associated Protein 2
MBT	Marble Burying Test
MD	Mediodorsal thalamus
CaMKIV	Calcium/Calmodulin-Dependent Protein Kinase IV

MWM	Morris Water Maze
NEC	Non-Epileptic Control
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NMTP	Nonmatching To Place Task
NOP	Novel Object Placement
NOR	Novel Object Recognition
NRT	Thalamic Reticular Nucleus
OFT	Open Field Test
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PKC	Protein Kinase C
PMSF	Phenylmethylsulfonyl Fluoride
PRC	Perirhinal Cortex
PSD	Postsynaptic Density Protein
PTP	Post-Tetanic Potentiation
PVDF	Polyvinylidene Difluoride
RE	Nucleus Reuniens of the Thalamus
RIPA	Ristocetin Induced Platelet Agglutination
RM	Reference Memory
RT	Room Temperature
S100B	S100 Calcium-Binding protein B
SEM	Standard Error of the Mean
SIC	Slow inward currents
SKF	N-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic acid
SLC6	Solute Carrier 6 Family
SNr	Substantia Nigra
SOC	Slow outward currents
STG	Stargazer
STM	Short-Term Memory
SWD	Spike and Wave Discharge
SWM	Spatial Working memory
TARP	Transmembrane AMPAR Regulatory Protein
TBS	Tris-Buffered Saline
TC	Thalamocortical
TRN	Thalamic reticular nucleus
VB	Ventrobasal complex
VPA	Valproic acid
WB	Western Blot
WM	Working memory

1| Introduction

1.1| Types of epilepsy

1.1.1| Focus on CAE (ILAE) and Epidemiology

Epilepsy is a brain disorder characterized by an enduring predisposition to produce unpredictable epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this pathology (Fisher *et al.*, 2005). Worldwide, epilepsy affects about 50 million people, being one of the most common neurological diseases, and if properly diagnosed and treated, it is estimated that up to 70% of people could live seizure-free (WHO, 2019). Idiopathic generalized epilepsy (IGE) is a subtype of generalized epilepsy consisting of overlap of childhood absence epilepsy (CAE), juvenile myoclonic epilepsy (JME), juvenile absence epilepsy (JAE), and generalized tonic-clonic seizures (GTCSs, Fig. 1.1A). A seizure is defined as “a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain”(Fisher *et al.*, 2017.). Two unprovoked seizures occurring more than 24 hours apart, one unprovoked seizure with a high recurrence risk (60%), and a diagnosis of an epilepsy syndrome constitute a verdict of epilepsy. Identifying the type of seizure (focal, generalized, unknown) is the first step. The next step is to identify the epilepsy type (focal, generalized, combined generalized and focal, and unknown), and the final step is to determine if epilepsy syndrome can be found (conditions with identifiable features such as seizure type, imaging, and electroencephalography (EEG)) (Fig. 1.1B).

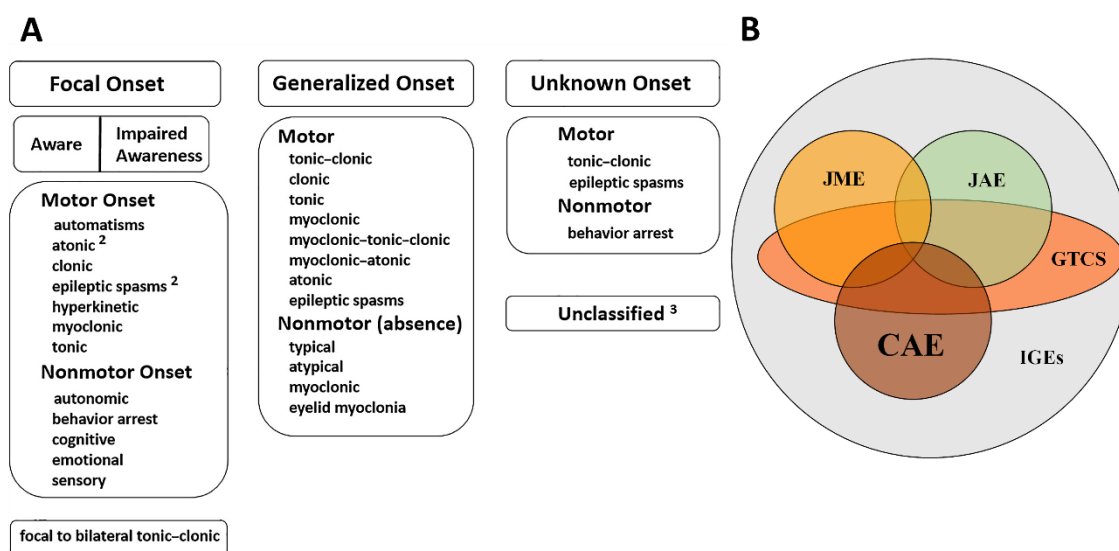


Figure 1.1 A) The expanded ILAE 2017 operational classification of seizure types and B) Overlap of the complex phenotypes of idiopathic generalized epilepsies.

A focal aware seizure corresponds to the prior term simple partial seizure. Focal-onset seizures are defined as “originating within networks limited to one hemisphere”. Generalized from onset seizures are defined as “originating at some point within, and rapidly engaging, bilaterally distributed networks. Seizures should be classified by the earliest prominent feature, except that a focal behavior arrest seizure is one for which cessation of activity is the dominant feature throughout the seizure. A seizure may be unclassified due to inadequate information or inability to place the type in other categories (Fisher *et al.*, 2017), A). There is a clear overlap between CAE, JAE, JME and GTCSs (Crunelli & Leresche, 2002).

Generalized seizures are divided into motor and non-motor (absence) seizures, having the highest prevalence with seizures of unknown etiology (Fiest *et al.*, 2017). Currently, typical absence seizures are an integral part of several IGEs, defined in the current classification of the International League Against Epilepsy (ILAE).

CAE is a type of idiopathic, non-convulsive generalized epilepsy that has a multifactorial genetic cause. It is the most common childhood epilepsy syndrome, representing 10-17% of epilepsy in school-aged children (Berg *et al.*, 2000), affecting 2 to 8 in 100,000 children under the age of 15.

Absence seizures (ASs) start between 3 and 8 years of age (peak 6–7 years) and usually occur several times a day (up to 200 per day) (Crunelli & Leresche, 2002). An AS is a brief ictal episode (4 to 30 seconds), during actual seizure activity, in which there is no awareness or feedback, with impairment of consciousness which is accompanied by abrupt onset and cessation of behaviour as well as no post-ictal symptoms and can be induced by hyperventilation.

There is a peak incidence of JAE in children between 10 and 12 years of age, with absence seizures being the most common phenotype, composed of a reduced impairment of consciousness. Generalized tonic-clonic seizures (GTCSs) are more common in patients with JAE, occurring in almost 80% of cases, and both perioral and hand automatisms persist, being hyperventilation, and tiredness triggering factors. There is a peak incidence of JME between the ages of 12 and 16 years, characterized by myoclonic seizures, GTCSs, and absence seizures, possibly lasting to 26 years of age. Photosensitivity, fatigue, and sleep deprivation are pouring factors. GTCs onset is variable and precipitating factors are analogous to JME (McWilliam, M., & Al Khalili, Y., 2021).

1.1.2 | Childhood Absence Epilepsy

In 1705, the first description of absence seizures was detailed by a mentioned Monsieur Poupart (possibly François Poupart 1661–1709):

“In that occasion, Mr. Poupart added that he was aware of a case of a young female child with epilepsy, who at the onset of the seizure sits down in a chair, and there remains immobile, without speaking and senseless. Her eyes are open, and after the episode she does not absolutely remember having fallen into this state. If she had previously begun a talk that the seizure interrupted, she takes it up again exactly at the same point at which she stopped talking, and she thinks she has talked without interruption.” (Poupart, 1705)

In 1824, Calmeil introduced the term “absences”, and in 1838, Esquirol, for the first time, used the term “petit mal” to describe an absence seizure (Brigo *et al.*, 2018). The term evolved in the following decades, and in 1924 Adie coined the term “pyknoleptic” to describe the disease's major manifestations (*“heads up, closely packed, aggregated attacks”*) (Adie, 1924). It was thought to be a kind of childhood epilepsy with a favourable prognosis at the time, in the early twentieth century. Gibbs, Davis, and Lennox first reported a link between impaired consciousness and 3-Hz spike-and-slow-wave complexes on electroencephalograms (EEGs) in 1935 (Gibbs *et al.*, 1935). However, it was only in 1945 that, based on EEG recordings, pyknolepsy was recognized as an epileptic entity, with seizures corresponding to the same generalized Spike and Wave Discharges (SWDs) pattern encountered in other typical absences.

In 1981, the Commission on Classification and Terminology of ILAE classified absence seizures among generalized seizures, and a specific syndrome classification was assessed. More recently, according to ILAE’s 2017 operational classification of epilepsy, the absence typical seizures were described as a *“sudden onset, interruption of ongoing activities, a blank stare, possibly a brief upward deviation of the eyes. Usually, the patient will be unresponsive when spoken to. Duration is a few seconds to half a minute with very rapid recovery. Although not always available, an EEG would show generalized epileptiform discharges during the event. An absence seizure is by definition a seizure of generalized onset. The word is not synonymous with a blank stare, which also can be encountered with focal onset seizures”* (Blume *et al.*, 2001; Fisher *et al.*, 2017).

1.2| Historical mechanistic theories of SWDs

The mechanisms of generalized SWDs, the thalamocortical electrical circuitry involvement at the molecular and cellular level in the initiation of the generalized absence event, as well as its ictogenesis are fundamental to address disease pathophysiology. The original theory described the midline thalamus as a pacemaker and the nucleus reticularis of the thalamus (NRT) as the main generator and initiator of the cortical rhythms, which were first described as epileptic discharges from the brainstem and diencephalon (Meeren *et al.*, 2005), giving rise to the Centrencephalic Theory (Fig. 1.2). Jasper and Williams controversy began in 1947, when CAE was considered a psychoneurolepsy, around the question of whether the structure responsible for the ASs was in the cortex, the thalamus, or both. In 1952, a new theory emerged the Cortical Theory, which proposed that abnormalities in the cortex were the leading cause of ASs (B.K.B., 1954). These studies supported the idea that generalized ASs were caused by abnormalities in the cortex, while the thalamus played a secondary role.

As early as 1968, the Corticoreticular theory was proposed, stating that both the cortex and the reticular system, comprising the thalamus and brainstem, were involved in the emergence of ASs. However, the specific mechanisms involving the cortex and thalamus were still in dispute (GLOOR, 1968).

In 1991, Buzsáki proposed the thalamic clock theory, hypothesizing that NRT contained pacemaker cells for the thalamus, and phasic bursts in NRT excited glutamatergic thalamocortical (TC) relay neurons, which then activated the entire thalamic network, thus forcing the rhythm onto the cortex (Buzsáki, 1991).

Models of ASs animals (Genetic Absence Epilepsy Rat from Strasbourg (GAERS) and WAG/Rij rats) support this theory (Seidenbecher *et al.*, 1998). In these models, NRT served as a pacemaker while TC relay neurons drove cortical cells, with the thalamus being regarded as the generator of cortical discharges. After non-linear association analysis of multi-site field potentials measurements in the cortico-thalamic regions of WAG/Rij rats, a cortical focus theory was formulated in the 21st century. According to Meeren *et al.*, 2002, ASs began in the perioral region of the primary somatosensory cortex, with the cortical focus always preceding the thalamic counterpart in the first seconds (~500 ms) of seizures. The cortex and thalamus *lead and lag* the discharges afterwards.

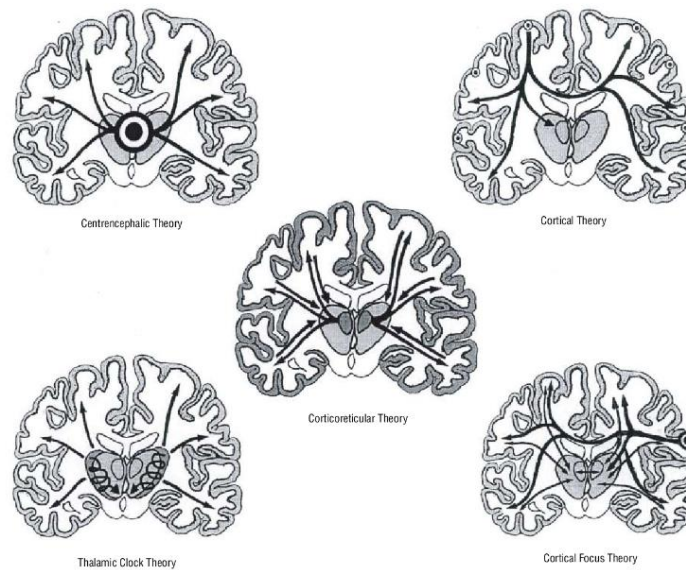


Figure 1.2 Schematic representation of the theories on the ictogenesis of generalized absence seizures.

On the left, thalamic theories, namely the centrencephalic theory of Penfield and Jasper and the thalamic clock theory of Buzsáki. On the right, the cortical theories: the cortical theory of Bancaud, Lüders, and Niedermeyer and the cortical focus theory of Meeren. In the middle is the corticocortical theory of Gloor (From Meeren et al., 2005).

1.3| ASs initiation /Ictogenesis

Additional evidence for the Cortical Focus Theory has been found in GAERS rats SWDs originating from the Somatosensory Cortex in the barrel field region, in pyramidal neurons of layers 5/6 (Polack *et al.*, 2007; Studer *et al.*, 2019a) as well as in a pharmacological ASs model originating from the prefrontal cortex (Venzi *et al.*, 2015). As a consequence, these cortical regions can be referred to as the cortical initiation network (CIN), ending the controversy about whether absence seizures are triggered by cortical or thalamic activity. In the thalamocortical loop, ASs are caused by promptly generalized, highly synchronous interactions between neurons. Key cellular elements of the thalamocortical networks include pyramidal cells and interneurons of different cortical layers, that excite the thalamocortical TC neurons of sensory thalamic nuclei and their main inhibitory input (ie. the GABAergic neurons of the NRT), driving NRT cells to impose bursts of inhibition on TC cells, which in turn sends synchrony excitatory feedback to cortex and NRT.

1.3.1| Initiation in humans

In humans, absence seizures start with paroxysmal activation of discrete frontal and parietal regions, which spreads to cortical regions and the thalamus (Bai *et al.*, 2010) with a 40ms time lag before other cortical regions are involved (Pavone & Niedermeyer, 2000). According to Bai and Moeller *et al.*, 2010, the preictal increase in BOLD amplitude in cortical areas precedes ASs by at least a minute, accompanied by a decrease in synchrony in the occipital cortex. Around 10 seconds prior to SWDs, several cortical regions show a significant increase in synchrony, even 20 sec after the end of the electrographic activity (Tangwiriyasakul *et al.*, 2018). This is not observed in rodent ASs animal model, due to differences in cortical functions or anaesthetics procedures used during animal imaging (Crunelli *et al.*, 2020). However, in general, data from animal and human studies indicate altered cortical networks as the generator of ASs. However, in animal models, thalamic regions are also responsible for generating ASs.

1.4| Pathophysiology- Genetic, Mutations, Heritability

CAE familial clustering studies indicate a complex non-mendelian mode of inheritance, not resulting from a single gene defect. Though, autosomal dominant inheritance has been shown for the EEG traits of bilaterally 3Hz SWDs (Panayiotopoulos, 2008). In this way, the aetiology of Absence Epilepsy (AE) is considered to be genetic with complex and multiple gene defects, although the mode of inheritance is still unknown. In 1951, Lennox reported that 66% of monozygotic twins showed an EEG pattern of 3Hz SWDs, being the possibility of carrying this epilepsy from 70-85% among monozygotic twins (Scheffer & Berkovic, 2003). In some cases, familial genetic defects are linked with abnormal changes in membrane receptors and channels (Crunelli & Leresche, 2002), being also considered a channelopathy. Genes associated with ASs are mainly involved in GABAergic neurotransmission or t-type calcium channels. Mutations in the $\alpha 1$ subunit of GABA A receptor were reported in patients with ASs (Maljevic *et al.*, 2006). In a cohort affected only by CAE, mutations exist in the *GABRB3* gene, encoding the $\beta 3$ subunit (Tanaka *et al.*, 2008) and in the *GABRG2* gene encoding the $\gamma 2$ subunit (Wallace *et al.*, 2001), representing the first polymorphism associated with CAE. Other studies also reveal that calcium channels, *CACNA1G*, and *CACNA1H* genes contribute to thalamocortical dysrhythmia, being activated not by depolarizations, but by membrane hyperpolarisations resulting from inhibitory inputs, being these inputs to thalamocortical neurons from GABAergic neurons in the NRT (Hughes, 2009). Mutations in *CACNA1H* are susceptible in human cohorts and also in GAERS and *CACNG3* mutations are also linked to absence seizures. Interestingly, mutations in *CACNA1* genes which encode for the $\alpha 1A$ subunit of p/Q-type high voltage-gated Ca^{2+} channels were reported in CAE patients as well as in mouse models of ASs and ataxia (Wakamori *et al.*, 1998). Finally, *SLC6A1* genes which encode the electrogenic sodium and chloride coupled γ -aminobutyric acid (GABA) transporter, GAT1, when mutated in children, are related with absence seizures as the most common epileptic phenotype (Mattison *et al.*, 2018b). The merging link between GABA and Ca^{2+} channels mutations on the thalamocortical network, although no single gene is ubiquitously associated and the underlying causes of excitability in CAE are indefinable, a dysfunction of GABA inhibition in the thalamocortical circuit plays a key role.

1.5| EEG features and Clinical representation

Diagnosis relies on ictal electroencephalographic findings of bilateral, synchronous symmetrical SWD of usually 3-4 Hz (Matricardi *et al.*, 2014), on a normal background interictal activity with a gradual decline in frequency during the seizure (Blume *et al.*, 2001), consisting of a high amplitude spike, positive transient and a slow wave (Fig. 1.3A). These SWDs ranges between 4-20 seconds (Crunelli & Leresche, 2002). The majority of the discharges consisted of typical spike and wave morphology, with one or two spikes per wave but children with photoparoxysmal response may have three to four spikes per wave. Hyperventilation is often performed during the EEG, given that children without having been submitted to treatment, will often have seizures with this stimulation (Berg *et al.*, 2000). The impairment of consciousness is the essential feature of absence seizures in CAE, with loss of awareness, unresponsiveness, and behavioural arrest (Blumenfeld, 2005). Immediately before and after the seizures, mild impairment has been reported, however transient peri-ictal deficits are still debatable (Blumenfeld, 2005). Regarding other ictal clinical features of CAE, staring and 3Hz eye movement and opening in an inconsistent manner during seizures in which eyes are initially closed, as well as automatisms in longer seizures and during hyperventilation. Mild clonic or tonic movements often occur during the first seconds of the absence seizure, whereas atonic falls never occur (Matricardi *et al.*, 2014). The seizure duration is influenced by hyperventilation (83%) or photic stimulation (21% of the patients), sleep deprivation or medication, emotional intellectual or nycthemeral and metabolic stimuli, being an average of 9.4 seconds (Matricardi *et al.*, 2014; Sadleir *et al.*, 2006).

1.6| Thalamocortical loop

1.6.1| T type calcium channels

T-channel bursts of cortical and NRT neurons are involved in the ictal synaptic interactions with TC neurons, whereas T-channels bursts of TC neurons do not play a role in the ictal interactions of these neurons with cortical and NRT neurons, given that blocking T-channels in the NRT and the CIN of GAERS drastically decreased ASs, whereas blocking in VB thalamus did not affect absence seizures (McCafferty, Connelly, *et al.*, 2018). So, T-channels of VB thalamocortical neurons (express Cav3.1 channels) are not essential for ASs in GAERS, whereas the cortical regular-spiking excitatory pyramidal neurons (RS, express Cav3.1, Cav3.2, Cav3.3 channels) in CIN and NRT neurons (express Cav3.2, Cav3.3 channels) neurons are crucial for ASs generation. These results are compatible with the gain of function mutations of the Cav3.2 subtype of T channels observed in some children with ASs, as well as in GAERS rats (Y. Chen *et al.*, 2003; Powell *et al.*, 2009; Talley *et al.*, 2000), which are not expressed in TC neurons (Fig.1.3B).

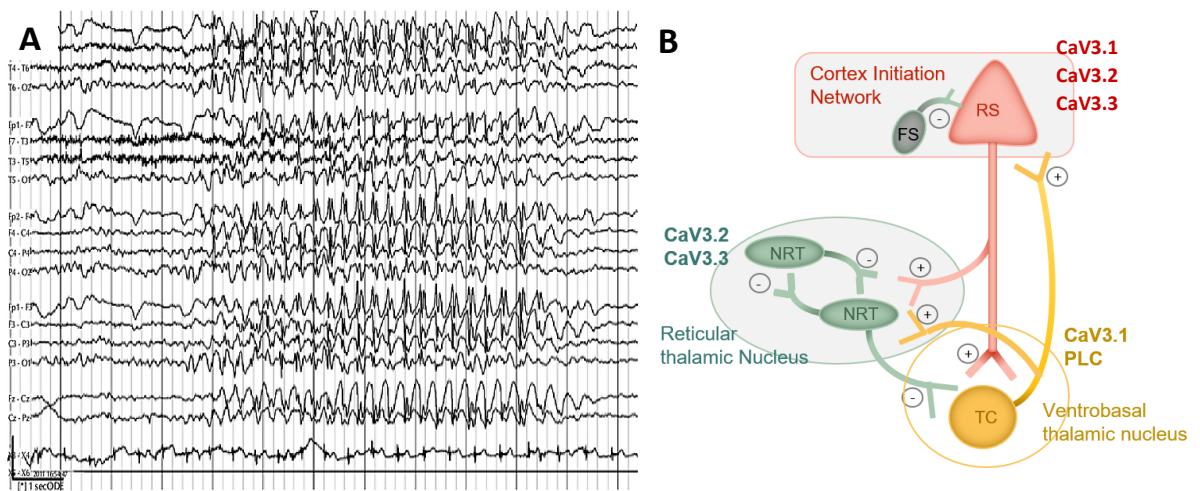


Figure 1.3 A) Child EEG during an absence seizure showing 3-Hz generalised regular spike-wave activity. B) Schematic representation of the thalamocortical circuit and T-type Ca^{2+} channel expression.

Cav3.2 and Cav3.3 channels are mainly expressed in NRT neurons; Cav3.1 channels are highly expressed in TC neurons. All three T-type calcium channels are found in overlapping layers of the neocortex. Alterations in level of expression or biophysical properties of the T-type calcium channel genes in the thalamocortical circuit may potentially induce absence seizures (Adapted from Crunelli & Leresche 2002 and Crunelli *et al.*, 2020)

Interestingly, two sources of increased ictal GABAergic inhibition embrace TC neurons of thalamic nuclei during ASs: one source from substantia nigra (SNr) and the other from the NRT. Phasic GABA_A inhibition comprises a tight temporal control of the firing of those neurons via activation of GABA_A receptors (McCafferty, David, *et al.*, 2018) and a persistent decrease in excitability, via tonic inhibition, by persistent membrane hyperpolarization with increased conductance due to GABA activation of perisynaptic and extrasynaptic GABA_A receptors (Cope *et al.*, 2005; Crunelli *et al.*, 2020). This increase in the inhibition of TC neurons contributes to a decrease in total firing, and of single action potential firing, and increases T channel-mediated burst firing. So, the balance between SNr derived inhibition and excitation may play a role in different ictal firing in TC neurons of thalamic nuclei. GABA_B receptors may also be involved in the generation of GABA_B receptor-mediated synaptic potentials (Crunelli & Leresche, 1991), their direct modulation of extrasynaptic GABA_A receptors (Connelly *et al.*, 2013), or direct activation of GABA_B receptors on thalamic astrocytes (Crunelli *et al.*, 2020).

1.6.2| Astrocytes and Absence Seizures

Cope and colleagues in 2009 observed that extrasynaptic GABA_A receptor-mediated tonic inhibition, might contribute to the generation of ASs in GAERS and stargazer mice, joining NRT and SNr derived ictal increase in GABAergic inhibition of TC neurons, possible enhanced by GAT1 loss of function providing additional restraints to the thalamocortical neuron firing. It is expected that GAT1 dysfunction reduces GABA clearance, leading to increased GABA levels, both synaptically and extrasynaptically. This increased GABA levels will lead to the overstimulation of extrasynaptic GABA_A, which are responsible for tonic inhibitory responses and reduction in GABA_A synaptic signalling (Fig. 1.4) (Cope *et al.*, 2009). Increased GABA_A-mediated tonic inhibition can lead to neuronal hyperpolarization and burst pattern firing in TC neurons, which can promote the generation of SWDs (Mattison *et al.*, 2018a). In GAERS, ASs are due to enhanced tonic inhibition within the VB thalamus, as a result of a failure in GAT1 reuptake of extracellular GABA, being clear after postnatal day 17 until day 30 when SWDs appear (Cope *et al.*, 2009) (Fig. 1.4). The same was observed in the ASs mouse model stargazer after postnatal day 19-21. Indeed, in GAERS and stargazer mice, GAT1 loss of function leads to increase GABA levels in the thalamus (Richards *et al.*, 1995).

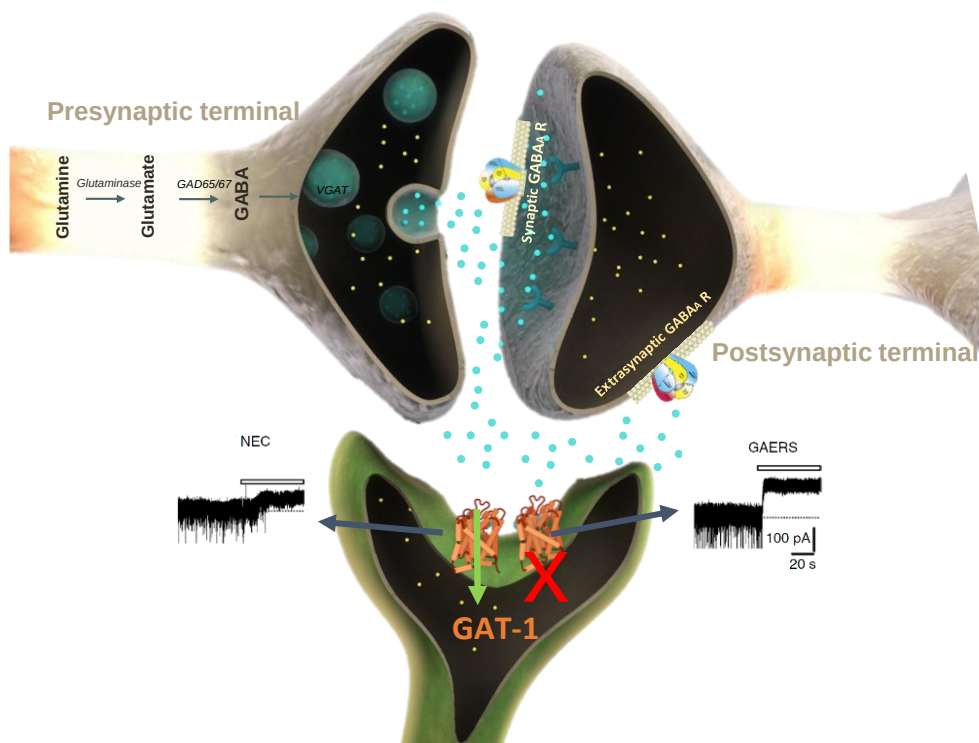


Figure 1.4 GABAergic Transmission.

GABAergic synapse in adult rat brain where GABA is released exocytotically and acts upon specific post-synaptic receptors. The signal is terminated by removal of GABA from the synaptic cleft by transport of GABA back into the nerve terminal or astrocytes by the plasma membrane GAT1. Representative current traces of nonepileptic control (NEC) and GAERS, showing that aberrant GABA uptake by GAT1 loss of function underlies enhanced tonic inhibition in GAERS .

The increased tonic GABA_A receptor-mediated inhibition, in thalamocortical neurons of absence epilepsy animal models, is necessary and sufficient for the generation of ASs.

GABA transporter current has been measured in thalamic astrocytes from GAERS as well as GABAergic astrocyte-to-neuron signaling by recording slow outward currents (SOCs) in the thalamocortical neurons, which was shown to display slower kinetics and smaller amplitude in GAERS. Being SOCs function slowing and continuously acting, contributing to thalamic tonic GABA_A-mediated inhibition (Pirttimäki *et al.*, 2013). These studies in ASs models are validated by high GABA levels in

ipsilateral thalamus, but not found in the cortex of one child with CAE, ASDs that increase GABA levels, both in humans and animals such as tiagabine and vigabatrin, worsen or induce absence seizures. In addition, as already mentioned, the majority of known human mutations in *SLC6A1* lead to a GAT1 loss of- function and are correlated with childhood epileptic phenotype (Crunelli *et al.*, 2020; Mattison *et al.*, 2018a). Given that in the thalamus GAT1 is only expressed in astrocytes, this disease is expected to be astrocytic specific.

1.6.3| GABA receptors and GABA transporters

GABA is the main inhibitory neurotransmitter in the brain, being its actions mediated by ligand-gated ion channels and metabotropic receptors (GABA_A and GABA_B respectively (Farrant & Nusser, 2005). GABA_A receptors are pentameric assemblies of subunits that form a central ion channel, comprised of different subunits ($\alpha 1$ – $\alpha 6$, $\beta 1$ – $\beta 3$, $\gamma 1$ – $\gamma 3$, δ , θ , ϵ , and π), that opens upon GABA binding, increasing membrane permeability to both bicarbonate and chloride ions (Farrant & Nusser, 2005). This occurs during a transient rise in GABA concentration within the synaptic cleft and the activation of postsynaptic receptors, caused by the release of GABA from presynaptic terminals. This change in membrane conductance is the underlying factor in phasic GABAergic inhibition and the generation of a classical inhibitory postsynaptic potential (IPSP). In this case, both the increase in conductance and the hyperpolarisation contribute to the inhibitory effect of GABA, thereby reducing the probability that an action potential initiates.

In some receptors, the $\gamma 2$ subunit can be switched to a δ subunit, resulting in receptor expression in the extrasynaptic or perisynaptic membrane (Cope *et al.*, 2005). The δ and $\alpha 4$ subunits of extrasynaptic receptors are expressed in thalamic nuclei, such as the TC neurons of the dorsal lateral geniculate nucleus (dLGN) and ventrobasal (VB) complex (Cope *et al.*, 2005). In addition to phasic inhibition, it has been shown that GABA_A receptor activation in the hippocampus, the cerebellum, and the thalamus can occur in a much more spatially and temporally diffuse manner, where low concentrations of GABA in the extracellular space, can activate persistently extrasynaptically GABA_A receptors, resulting in a tonic increase in membrane conductance. The peri and extrasynaptic receptors are sensors for extracellular GABA, with a higher affinity for GABA (Stell & Mody, 2002) compared to synaptic receptors.

GABA levels are controlled by the enzymes responsible for their production (GAD65 and GAD67), catalyzing the synthesis of GABA from glutamate, the release mechanisms, and the transporters that mediate their uptake (GAT1 and GAT3), mainly in neurons. GAT1 and GAT3 are responsible for the removal of GABA from the synapse after its release from presynaptic terminals. GAT1 is the most highly expressed GABA transporter in the mammalian cerebral cortex (Conti *et al.*, 2004), also localized to astrocytic membranes at GABAergic synapses (Minelli *et al.*, 1995), in some astrocytic processes and responsible for regulating GABA levels during sustained neuronal activity. GAT3 is distributed primarily in astrocytes and regulates GABA levels in extrasynaptic areas (Melone *et al.*, 2014). GAT1 transporter is exclusively expressed in astrocytes in the thalamus of both humans and rats. Furthermore, GAT1^{-/-} mice show spontaneous ASs, higher thalamic tonic GABA_A current, impaired hippocampal Long-Term Potentiation (LTP), and deficient novel object recognition (NOR) (Cope *et al.*, 2009; Gong *et al.*, 2009).

1.7| Treatment for ASs and pharmaco-resistance

1.7.1 | Pharmacoresistance, outcome, and prognosis

The pharmacological therapy of ASs, particularly CAE, generally relies on ethosuximide, valproic acid, lamotrigine, or the combinations of two of the previous drugs (Penovich & James Willmore, 2009). The absence of response is about 30% in children treated with first and second monotherapy (Crunelli *et al.*, 2020) (Fig.1.5). Earlier studies showed that notably, children initially

treated with ethosuximide (45%) exhibit better long-term outcomes compared with lamotrigine (21%) for a 12 months treatment (Berg *et al.*, 2014). Similarly, valproic acid treatment was reported to be highly efficient (45%), however, is associated with strong neuropsychiatric attention deficits (Glauser *et al.*, 2013). In the 70% of cases where seizure control is efficient with the available antiepileptic drugs, cognitive deficits are still present. Not being clear whether current antiepileptic drugs do affect cognitive deficits.

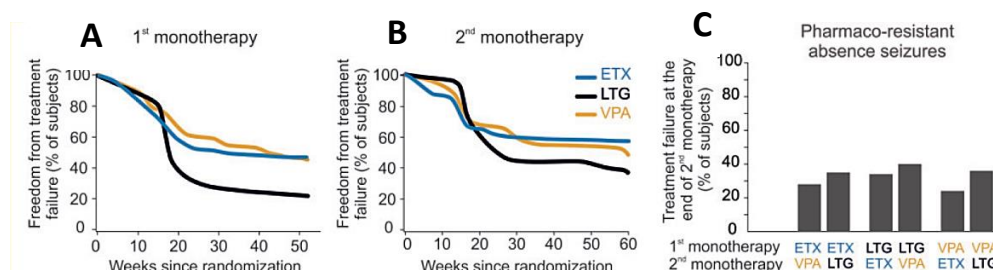


Figure 1.5 Pharmacoresistance of absence seizures.

The first 12-month-long monotherapy with either ethosuximide (ETX- modify the thalamic low-threshold calcium channels, reducing the number or conductance of the channels) or valproic acid (VPA- raise levels of GABA and affects sodium and calcium channels) is more effective than that with lamotrigine (LTG- blocker of voltage-sensitive sodium channels) in controlling absence seizures (A). Similar results are obtained when children unresponsive to the initial monotherapy undergo a second 12-month-long monotherapy with a different drug (B). The combined results of the first and second monotherapy (C) show that about 30% of children with childhood absence epilepsy have pharmaco-resistant absence seizures (Crunelli *et al.*, 2020).

1.8| Comorbidities and neuropsychology aspects

The comorbid conditions that accompany absence seizures are available since the early 1960s from studies with mixed-age, mixed-epilepsy populations with generalized SWDs, mainly under pharmacological treatment. Recent studies have shown that ~60% of children with CAE have neuropsychiatric comorbidities (Crunelli *et al.*, 2020). In drug-naïve cohort, attentions deficits were linked to “*loss of focus on a task more than impulsivity of a response*“(Masur *et al.*, 2013). Neuropsychological studies demonstrate that patients with CAE have cognitive and linguistic impairments as well as behavioural disorders (Caplan *et al.*, 2008). The cognitive difficulties involve mainly the attentional domain (35-40%) and executive functions and visual attention, as well as verbal learning and visuospatial memory (Cheng *et al.*, 2017; D’Agati *et al.*, 2012; Henkin *et al.*, 2005), even when seizures are medically controlled. Language and reading disabilities are also present (Caplan *et al.*, 2008; Vanasse *et al.*, 2005), attention deficit hyperactivity disorder, and affective disorders such as depression and anxiety disorders (Caplan *et al.*, 2008). Psychiatric comorbid conditions, in particular attention deficits, may precede the first absence seizure and the epilepsy diagnosis (J. E. Jones *et al.*, 2007). Caplan and collaborators describe specifically that 25% of the children with ictal absences had subtle cognitive deficits, 30% a behavioural disorder, 43% linguistic difficulties, and 61% a psychiatric diagnosis, particularly attention deficit hyperactivity disorder (ADHD) (Barnes & Paolicchi, 2008). Jacquin *et al.* concur that if left undetected and untreated, the ictal bilateral SW discharges can potentially “*cause significant brain and behavioural dysfunctions, interfere with information processing and contribute to mental alteration status*” (Hughes, 2009; Jacquin *et al.*, 2007)

There is still a debate whether behavioural problems are a part of the epileptic syndrome or develop as a consequence of disease-related factors. The presence of comorbidities may impact social difficulties and contribute to poor scholastic achievement in patients with CAE. Early evaluation and detection of these comorbidities can lead to specific interventions such as cognitive behavioural therapy, educational and didactic support.

1.9| Animals models of Absence Seizures

As neurological disorders can be an ethically difficult study in humans, animal models become useful to study and better understand such disorders. In this project, two ASs animal models were used, GAERS rat and STG mouse.

1.9.1| GAERS

GAERS and WAG/Rij are the two most frequently used rat polygenic models of absence epilepsy (Akman *et al.*, 2010). GAERS are the most validated and predictive models for this pathology, developed by selective breeding of the 30% of Wistar rats displaying spontaneous SWDs, until the strain resulted in 100% rats having seizures (3/4 generations) (Rudolf *et al.*, 2004). 100% of animals show the EEG and behavioral characteristics of ASs at 3-month-old, being the first detectable SWDs observed around 30 days of age. These rat models feature 7–11 Hz SWDs (Fig. 1.6) with EEG similar to the ictal waveforms seen in patients with ASs (Sitnikova & van Luijtelaar, 2009) and behavioural arrest. The majority of SWDs occur in both strains during quiet wakefulness as well as in transition between wakefulness and slow-wave sleep (Leresche *et al.*, 2012).

Genetic studies on GAERS rats (Rudolf *et al.*, 2004) showed polygenic control of SWDs and also the differential effect of gene variants on the average duration and number of SWDs. A CACNA1H mutation plays a significant role in the ASs phenotype in GAERS in terms of number and cumulative duration of seizures.

It has been observed an increase in the number of astrocytes and GFAP protein (astrocyte marker glial fibrillary protein) expression levels in the somatosensory cortex (SSCx), centromedial (CM), ventrobasal (VB), reticular (TRN), and dorsal lateral geniculate nuclei of the thalamus of GAERS compared to Wistar control animals, confirming the involvement of astrocytes in the mechanisms underlying absence seizures (Çavdar *et al.*, 2019).

Control animals of GAERS are the NEC (Non-epileptic control) or/and Wistar rats.

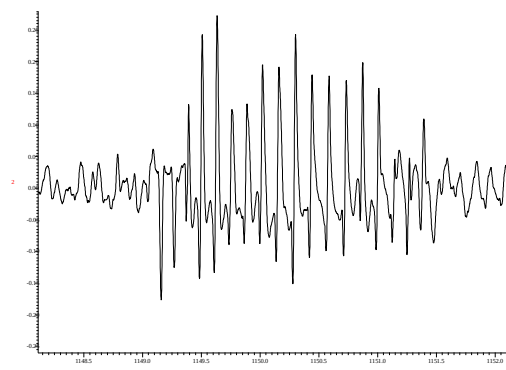


Figure 1.6 The SWDs recorded in a male GAERS of Lisbon colony.

1.9.1.1| Behaviour deficits in GAERS

In addition to the presence of SWDs, behavioural arrest, and response to ASDs, GAERS display behaviour abnormalities compared to NEC that resemble clinical aspects of CAE. GAERS demonstrated increased anxiety-like behaviour (Bouilleret *et al.*, 2009; N. C. Jones *et al.*, 2008; Marks, Cavanagh, *et al.*, 2016; José Eduardo Marques-Carneiro *et al.*, 2014; Powell *et al.*, 2014) and altered social interactions (Henbid *et al.*, 2017). However, reduced anxiety-like behaviour has also been observed (Studer *et al.*, 2019a). Regarding memory performance in GAERS, in MWM was observed delayed

learning, weaker working memory capacity although being capable of establishing an operational reference memory, and preferential use of egocentric strategies in a radial maze (J. E. Marques-Carneiro *et al.*, 2016). Although cognitive alteration may be subtle, not easily detected, and task-specific, more severe cognitive impairment has been observed in males, making more errors during visual discrimination and reversal learning task (Roebuck *et al.*, 2020). GAERS displayed exaggerated cued and contextual Pavlovian fear conditioning and impaired fear extinction. Male GAERS also showed impaired latent inhibition in a paradigm using Pavlovian fear conditioning, consisting of lack of selective attention found in children with CAE (Marks *et al.*, 2016). In addition, GAERS demonstrated recognition memory deficits in visual attention and crossmodal memory (Marques Carneiro *et al.*, 2016, Marks *et al.*, 2016).

In these rodents, SWDs are shown to be initiated in the barrel field primary somatosensory cortex responsible for coding whisker-related information. This is essential for the interaction between rodents and their environment. Impaired sensory-information processing was found in GAERS, with a delayed sensory-evoked potential and duplicated neurological responses to whisker stimulation after *in vivo* extracellular recordings in the epileptic barrel field primary somatosensory cortex of GAERS. Despite this, GAERS present no defaults in a texture discrimination task, suggesting a compensatory mechanism in the epileptic network (Studer *et al.*, 2019a).

1.9.2| Stargazer Mouse

The stargazer mouse model displays 6-7 Hz SWDs with behavioural arrest (Noebels *et al.*, 1990). This strain has a deficiency in the calcium channel $\gamma 2$ -subunit gene *CACNG2*, reducing the expression of stargazin protein (Letts *et al.*, 1998; Noebels *et al.*, 1990). Stargazin is a transmembrane AMPAR regulatory protein (TARP) critical for synaptic targeting and membrane trafficking of AMPARs at excitatory glutamatergic synapses (L. Chen *et al.*, 2000). Defects of the stargazin protein cause the loss of AMPARs which impairs excitation at glutamatergic synapses, possibly leading to the loss of function of inhibitory neuron synapses and altering thalamocortical circuit oscillations.

Impaired AMPA receptor regulation in the Somatosensory Cortex and thalamus may also contribute to absence seizures. In addition, GABA level and GABAR expression (Seo & Leitch, 2015) were found to be upregulated in the thalamocortical circuits of stargazers, which might contribute to ASs. Along with behavioural arrest, stargazer mice also display maladaptive behaviours, ataxia, and head tossing (Letts, 2005).

1.10| Astrocytes

1.10.1| Tripartite synapse and GABAergic signalling

Astrocytes are the most numerous glial cell type in the CNS, termed by Michael von Lenhossek in 1885, not presenting electrical excitability nor generating action potential, mediating their activity by ionic changes in the environment, intracellular Ca^{2+} transients, and oscillations (Perea *et al.*, 2009). The astrocytic filopodia are very thin and closely wrap synapses, forming a structure called the tripartite synapse (Araque *et al.*, 1999; Perea *et al.*, 2009). The tripartite synapse composes a morphological and functional unit where astrocyte-neuron interactions combine to shape synaptic transmission of information in the CNS (Araque *et al.*, 1999), allowing astrocytes response to surrounding synaptic activity with Ca^{2+} signalling, causing feedback regulation of neuronal activity and synaptic strength through gliotransmitter release, being involved in cognition, emotion, motor, and sensory processing (Oliveira *et al.*, 2015).

In an active astrocyte, glutamate, D-serine, and/or adenosine triphosphate (ATP) are released. Astrocyte glutamate release can induce slow inward currents (SIC) by activating N-methyl-D-aspartate (NMDA) receptors on postsynaptic neurons and stimulating pyramidal cells, controlling neuronal synchronization (Araque *et al.*, 1998; Shigetomi *et al.*, 2008). The effect of gliotransmitters is dependent on the activated receptors and sites of action, enabling a high degree of complexity and efficiency of tripartite synaptic communication. Astrocytes are also able to communicate directly through gap junctions created by connexins (i.e. cx 30 and 43), forming a syncytium (Pannasch & Rouach, 2013).

By taking up GABA through astrocyte GABA transporters, astrocytes can shape neural circuit activity by shaping signal transmission at inhibitory synapses. Astrocytic GABA is released by a specific anion channel called Best-1 which contributes to tonic inhibition (B. E. Yoon *et al.*, 2014) which was shown to affect LTP.

1.10.2| Astrocyte's morphology, types, different staining for different processes

The primary neural stem cells to produce neurons and glia originate in the neuroepithelium and are called radial glia and their posterior heterogeneity is due to their derivation from regionally patterned RG cells and specialization according to the brain location. Based on morphology, the most common division is between protoplasmic astrocytes found in grey matter, spongiform profile, central soma and dense ramification of fine processes, and fibrous astrocytes in white matter with long fiber-like processes (Kessaris *et al.*, 2008).

Different markers are used to study the molecular identity and heterogeneity of astrocytes. Glial fibrillary acidic protein (GFAP) is the most commonly used astrocytic marker, with major intermediate filament composing cytoskeleton, immunolabelling 15 % of the total astrocyte volume (Bushong *et al.*, 2002). S100 β , a Ca²⁺ binding peptide abundant in the cytoplasm, and nucleus of astrocytes, is involved in cell cycle regulation and cytoskeleton modification, however, S100 β is also expressed in a few neurons (Donato *et al.*, 2009). NDRG2 is specifically expressed in astrocytes and is widely expressed in the cerebral cortex, olfactory bulb, midcerebral, hippocampus, and thalamus (Flügge *et al.*, 2014). Other markers such as ALDH1L1, GLAST, GLT-1, CD44, and vimentin, are used to label astrocytes (Zhang *et al.*, 2019).

Importantly, in the healthy mouse brain, hippocampal astrocytes have a higher GFAP expression than cortical, thalamic, or striatal astrocytes, however, it does not mean more reactivity. An increase in GFAP expression may reflect physiological adaptive plasticity rather than a reactive response to a pathological stimulus (Escartin *et al.*, 2021). For clear terminology including morphological analysis (hypertrophic, atrophic), molecular markers and functional readouts, brain region or other relevant sources of heterogeneity should be merged (Escartin *et al.*, 2021).

1.11| Taxonomy of memory

The only proof of there being retention is that recall actually takes place. William James, 1872

Memory is a fundamental basis of an animal's behaviour, being the ability to retain and utilize information or knowledge acquired in the past. William James in 1890 assumed memory consists out of two systems: primary and secondary memory. The memory consolidation hypothesis was developed by Muller and Pilzecker ten years later, involving "trace hardening", whereby a physiological mechanism persists and renders the memory trace less resistant to interference induced by subsequent learning. (McGaugh, 2000). Memory formation is separated into two stages: acquisition/learning and consolidation (Fig. 1.7A). After one or more trials, when the individual is exposed to a context with several controlled variables (Fig.1.7A), disturbances can modify the original memory until the Recall,

which is defined as Working Memory (WM), Short Term Memory (STM), and Long-Term Memory (LTM) within a minimum period of 6 hours, more commonly used 24 hours. The consolidated memory can be altered, either diminishing with repetition without an adverse stimulus, resulting in extinction, or strengthened, requiring *de novo* protein synthesis, at least in the relevant brain areas, and only after the presentation of this memory reactivation session (Izquierdo I, 2000). Memories can be divided according to function, content, duration, nature, or motivation as illustrated in Fig.1.7B (Quillfeldt, 2015). It is important to avoid anthropomorphizing behavioural results since human memory possesses two non-translatable aspects (in comparison to animal models), symbolic language and conscience.

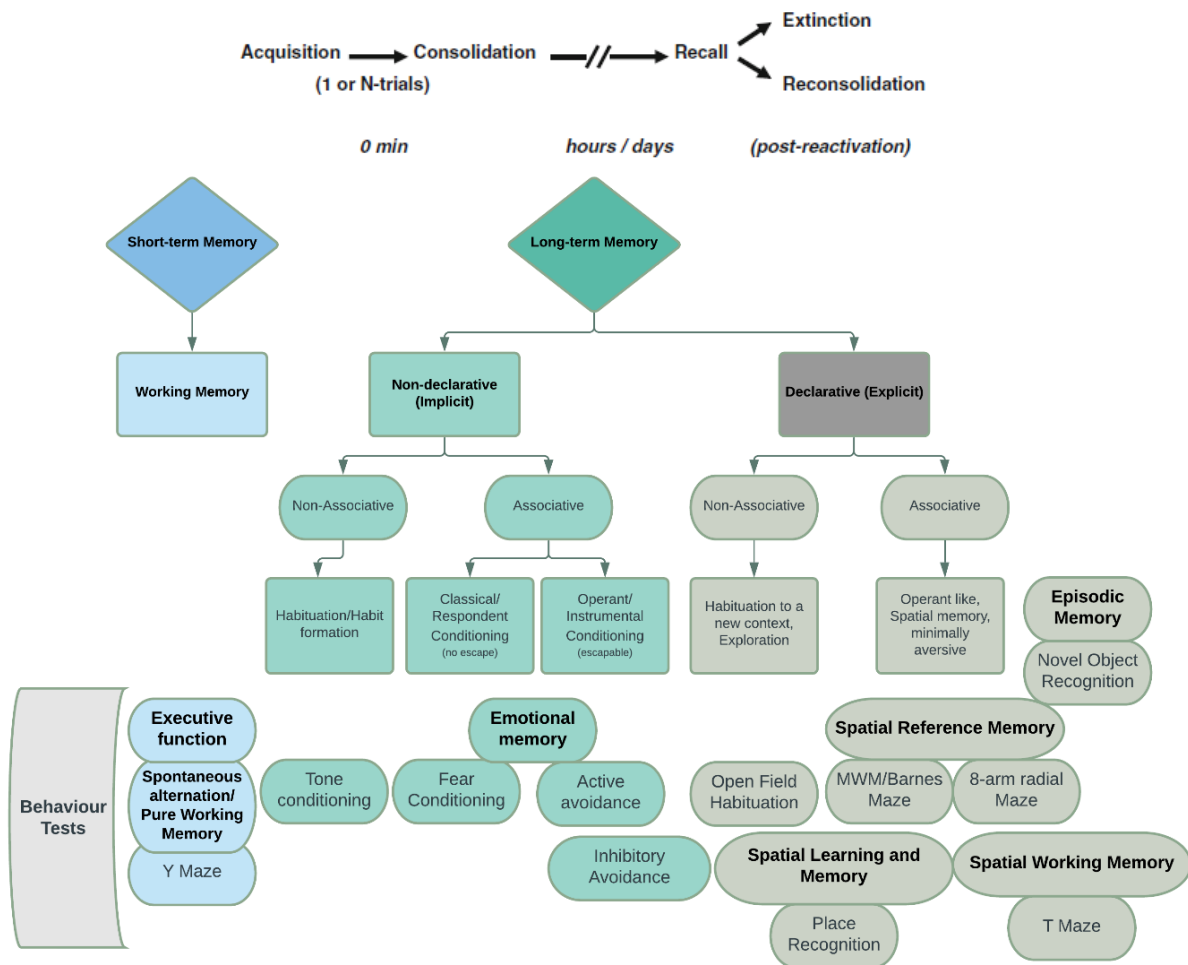


Figure 1.7 A) Phases of memory formation and B) Types of memory.

Acquisition, consolidation, retrieval (recall) , and the two possible outcomes, post-reactivation phases – extinction or reconsolidation (A). A taxonomy of memory. It is generally accepted that non-declarative (procedural/implicit) memory is independent of the hippocampus. Which aspects of declarative (explicit) memory are hippocampally-dependent is controversial. different types of memory and relevant behavioral paradigms for their assessment (B). (Adapted from Quillfeldt 2015).

Studies mainly tackle the performance in working memory and reference memory. Recognition memory is influenced by both hippocampal and cortical lesions, being dependent on the integrity of the parahippocampal medial temporal lobe, namely the perirhinal, entorhinal, and inferior temporal cortices (Aggleton *et al.*, 2010). Rodents with asymmetric disconnection lesions in the hippocampus and the prefrontal cortex (PFC) have shown impairments in these forms of recognition memory, which implicates the H-PFC pathway in recognition memory that involves contextual and temporal details for

where or when the stimulus occurred (Barker & Warburton, 2011). Memories are thought to be initially stored within the hippocampal–entorhinal cortex (HPC-EC) network (recent memory) and, over time, slowly consolidated within the neocortex for permanent storage (remote memory) and prolonged inhibition of neocortical networks during consolidation impairs remote memory formation (Goshen *et al.*, 2011; McClelland, 2013).

WM is a short-term memory, able to store and manipulate information to guide behaviour on a timescale of seconds, is a central cognitive function (Baddeley, 2011) where spatial information is encoded to influence consequent behaviour. In WM, the temporal enhancement does not reflect the consolidation of a memory trace, but rather the functionality of the underlying neuronal circuits and molecular signalling pathways. The hippocampus, septum, basal forebrain, and prefrontal cortex are involved in this task (Chudasama & Robbins, 2004). WM is tested if the information to be remembered on each trial is independent of the next and the response is contingent upon the information that was maintained during a specific delay.

Spatial memory storage and retrieval of information are needed both to plan a route to the desired location and to remember where an object is located or where an event occurred, being crucial to foraging for food, returning to sites of storage or safety, and avoiding dangerous places. Corresponding to the long-term acquisition of information that remains unchanged during trials and relies on the presence of a discriminative stimulus, a visual cue (Barker & Warburton, 2011).

1.11.1| Hippocampal-PFC communication

The hippocampus (HPC) is involved in long-term memory formation, contextual and spatial navigation. The dorsal hippocampus processes spatial information, whereas the ventral hippocampus is linked to emotion-related processes (Fanselow & Dong, 2010).

The prefrontal cortex (PFC) participates in cognitive functions regarding executive function, including working memory, temporal processing, decision making, flexibility, and goal-oriented behaviour. The HPC influences the activation of ensembles of neurons in the PFC during such behaviors, being the interplay expected to take place in tasks or behaviours that demand both navigational data and choice-related memory discrimination (Euston *et al.*, 2012).

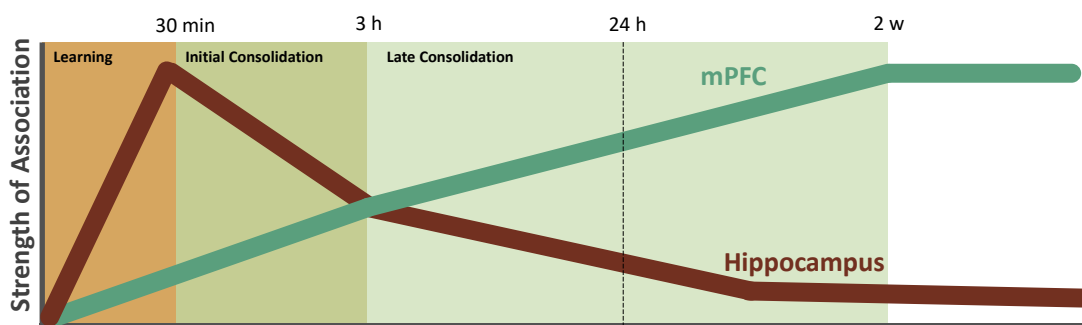


Figure 1.8 Timeline of mPFC and HPC role in memory.

During a phase of rapid consolidation occurring during the first few hours after learning, the hippocampus and mPFC replay the memories and, synapses supporting that memory are strengthened in mPFC while they degrade in hippocampus. There is also likely a transformation from a memory for specific episodes to a more schematic representation. Concurrently, episodic memory traces within hippocampus decay. This process continues during late consolidation, albeit at a slower rate (Adapted from Euston *et al.*, 2012).

The HPC-to-PFC pathway comprises the major efferent anatomical connection from hippocampal formation through monosynaptic unidirectional projection to PFC (Fig.1.9A). This pathway transmits information supporting WM and contextual information for recognition memory

It has been shown that inactivation of either the mPFC or dorsal HPC (dHPC) significantly reduces performance in delayed alternation tasks and impairs WM (arm choice accuracy) without altering reference memory OR arm choice response latencies. dHPC inactivation increases both reference memory errors and arm choice response latencies (T. Yoon *et al.*, 2008). These studies suggest a critical role of the HPC-mPFC pathway in the continuous updating of task-related spatial information during spatial working memory tasks. However, there is no direct connection between the mPFC and HPC.

The mediodorsal thalamus (MD), which is reciprocally connected with PFC, has increasingly been recognized as an active partner in cognition (Wolff & Vann, 2019). Specifically, indirect projections from the mPFC back to the HPC are also involved in WM. Anatomical findings point to the NRT as the likely candidate to mediate mPFC signals to the CA1 region (Fig. 1.9A) (Vertes *et al.*, 2007). For example, lesions or inactivation of the Nucleus reuniens (RE) cause deficits in tasks dependent on both the HPC and mPFC, being RE required for mPFC-HPC interactions in WM tasks (Hembrook *et al.*, 2012).

A

MEDIAL PREFRONTAL CORTEX

HIPPOCAMPUS (CA1, Subiculum)

THALAMIC RETICULAR NUCLEUS

NUCLEUS REUNIENS OF THE THALAMUS

PERI REUNIENS

PERIRHINAL CORTEX

ENTORHINAL CORTEX

direct HC to mPFC (no direct return)

B

Hippocampus

Mossy fibers

Interneurons

Dentate Gyrus

CA3

CA1

Schaffer collaterals

Perforant pathway

Entorhinal Cortex (I, II, III, IV, V)

Postrhinal Cortex

Perirhinal Cortex

Temporoammonic pathways



Figure 1.9 Schematic representation of the connectivity in the mPFC–RE– HC system (A) and Schematic representation of the intrinsic hippocampal circuitry.

The HPC projects directly to the mPFC, but there are no direct return projections. RE serves as the main link completing an HPC–mPFC loop as follows: HPC→mPFC→RE→ HPC (A). Information enters the hippocampus from layers of the entorhinal cortex and flows unidirectionally, to return predominantly to the deep layers of entorhinal cortex. Perirhinal and postrhinal cortices, which project to the EC, contribute to the perforant path projection. The EC’s major excitatory circuits terminate mainly in the dentate gyrus. Axons from layer II project to the granule cells of the DG and the pyramidal cells of CA3. Axons from layers III project to pyramidal cells of CA1 and the Subiculum. The dentate granule cells project to CA3 via the mossy fibers. CA3 cells send a collateral, the Schaffer collateral, to CA1. These receiving cells project to the subiculum. CA1 and the subiculum both project back to the far down layers of the EC, predominantly layer V (B) (Dolleman *et al.*, 2019, Adapted from Bimonte-Nelson 2015).

Bolkan *et al.*, 2017 showed that the mPFC activity led the MD activity during a choice period task. Accordingly, optogenetic inhibition of mPFC inputs to the MD during the choice period impaired Spatial Working Memory (SWM) performance. Complementing the roles for vHPC and MD inputs to the mPFC in spatial encoding and maintenance of SWM (Bolkan *et al.*, 2017; Spellman *et al.*, 2015), these findings suggest that signals from the mPFC to the MD help guide successful SWM retrieval or execution of actions (Fig. 1.10).

In conclusion, vHPC inputs to the mPFC support spatial encoding as previously shown, MD inputs to the mPFC support the maintenance of working memory by stabilizing prefrontal activity during the delay period, and that signals from the mPFC back to the MD guide successful memory retrieval and action execution, identifying the thalamus as a fundamental structure in memory circuits.

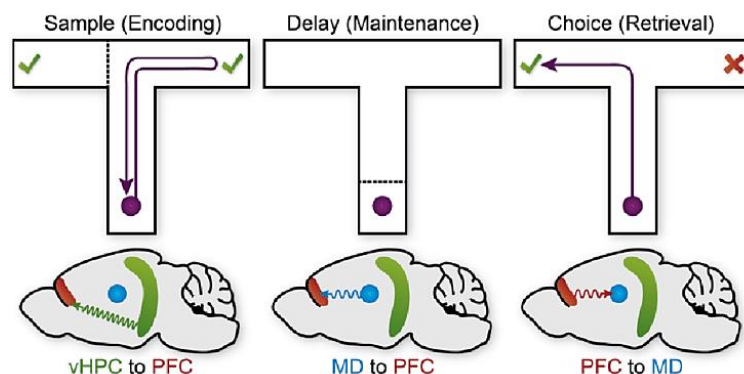


Figure 1.10 Neural pathway–specific roles in distinct periods of a delayed non-match-to-sample T-maze spatial working memory task.

MD inputs to the PFC sustain PFC representations across the delay period to support SWM maintenance. Performance on this task is impaired by optogenetic inhibition of ventral hippocampus (vHPC) inputs to the prefrontal cortex (PFC) during the sample period, mediodorsal thalamus (MD) inputs to the PFC during the delay period, and PFC inputs to the MD during the choice period (Bolkan *et al.*, 2017; Spellman *et al.*, 2015)

1.12| How to test memory and anxiety in rodents

As previously described in children with absence epilepsy, ASs animal models also show ASs associated cognitive comorbidities. Rats and mice animal models in behavioural neuroscience research are crucial to allow the relationship among molecular and cellular data with repercussions on the phenotypical behaviour of an individual. Rats entered research lab at the turn of the century. Willard Small was the first to use a rodent maze to study learning, examining “mental processes” in the rat,

thereby acknowledging that rats have a higher form of cognitive processing that can be measured. In 1901, he published the details of his new contraption (Bimonte-Nelson, 2015).

In 1950, Karl Lashley could refer to a “cognitive map”, looking for the brain location of memory. Hebb in 1942 then described synaptic plasticity, how brain cells adapt during the process of learning, and that repeated firing from a presynaptic cell strengthens the synapse with the postsynaptic cell, stating *“Let us assume that the persistence or repetition of a reverberatory activity (or ‘trace’) tends to induce lasting cellular changes that add to its stability... When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased.”* (Bimonte-Nelson, 2015). Milner’s in 1957 introduced what is known as a dissociation of memory systems, studying H.M. patient. Using mice and spatial mazes, Kandel evaluated CREB-dependent gene expression in the hippocampus. LTP was first described by Terje Lomo and Timothy Bliss in 1973 using the rabbit’s hippocampus, classifying it as the closest mechanism of learning and memory. Dostrovsky in 1977 surmise that deprivation of a hippocampal map in a rat would result in a lack of the spatial maze learning habit, describing place cells in the hippocampus. Ideas from 1978 from O’Keefe, Nadel, Bliss, and Lomo were fundamental for both Morris and Barnes to develop tasks that could be used to evaluate hippocampus-dependent behaviours (Bimonte-Nelson, 2015).

Since memory is required to witness acquired behaviour, the natural tendency is to make the opposite inference: if the behaviour is missing, we may deduce that the memory is not intact. In reality, there is not a clear and clean connection between memory and behaviour, indeed several factors may influence this directionality, such as the animal focus, the appropriateness of the behaviour in the context influence behaviour, inner state, personality, and motivation. Anhedonia, anxiety, and motor deficits may also influence the directionality of evidence. Most behavioural tests for rodents require some positive or negative emotion to motivate the animal's response.

Anxiety is considered a negative state of emotion, associated with a perception of a potential forthcoming threat (American Psychiatric Association, 2013). In rodents, there is a natural predisposition to explore novel environments but also a tendency to avoid aversive open and bright spaces, such as the center of an open field or the open arms in an elevated plus maze (Ennaceur, 2014). Anxiety-like behaviour tasks are assessed to evaluate the conflict between these competing drives.



Figure 1.11 Timeline of landmark discoveries from 1900 through the 1980s that set the foundation for current rodent research using mazes (Bimonte-Nelson 2015)

1.13| Astrocytes and memory

Astrocytes have been shown to play a role in synaptic remodeling and long-term memory development, representing potential active players in synaptic plasticity due to the ability to regulate synaptic neurotransmitter release in response to neuronal activity (Pitá et al., 2014). Optogenetic tools allowed the enhancement of memory acquisition by astrocytic activation alone, not attained when directly activating neuronal activity (Adamsky *et al.*, 2018). Recently, a KO mouse missing the IP3R2 receptor, disrupted astrocytic processes and impaired long-term memory consolidation (Pinto-Duarte *et al.*, 2019). A decrease colocalization between astrocytes and neurons during reactive states of astrocytes leads to memory impairments in 5XFAD mice (Choi *et al.*, 2021). Also, hippocampal LTP can be triggered by the coincidence of postsynaptic activity and astrocytic Ca^{2+} elevation, which stimulates glutamate release and the release of glutamate increases the probability of neurotransmitter release, mediated by NMDARs.

Vesicular release in astrocytes contributes to the maintenance of oscillations in gamma frequency (25-80 Hz), correlated with learning, memory storage and retrieval, and recognition memory, so fast neural circuit oscillations are tightly regulated by astrocytes, and disturbance of gamma oscillations provide evidence that they are vital for WM (Lee *et al.*, 2014), and when connexin 43 hemichannels are blocked during memory consolidation, LTM appeared impaired. In addition, disruption in the expression of astrocytic and neuronal lactate transporters blocks memory retention (Suzuki *et al.*, 2011). Impairment in memory may be due to activation of astrocytic CB1R and release of glutamate, which induces LTD by internalization of AMPA receptors (Han *et al.*, 2012). Astrocytes are essential for the induction of LTP, via the release of D-serine (Henneberger et al., 2010) and calcium signalling is the basis of astrocytic excitability, necessary for the release of D-serine or ATP, and consequently for synaptic plasticity. Also, inhibition of calcium signalling by astrocytes, also led to deficits in hippocampal dependent-memory (Tanaka *et al.*, 2013). These studies point towards the extremely complex yet distinct mechanisms between astrocytes and neural circuits to regulate different aspects of the same behaviour.

1.13.1| Memory and impaired GABAergic mechanism

The network oscillation and synaptic plasticity are regulated by GABAergic inhibition, by regulating the degree of local depolarization, and may be affected by changes in GABAergic inhibition during development (Meredith *et al.*, 2003). However, how GABA transporter activity changes affect these networks remain unclear. Impaired GABAergic transmission has also been linked with memory and LTP. Increased tonic inhibition in dentate gyrus suppresses LTP and results in memory deficits in 5XFAD animals (Wu *et al.*, 2014) and impairs memory performance in MWM (Gong *et al.*, 2009). The tonic activation of presynaptic GABA A receptors depolarizes hippocampal mossy fiber boutons and increases synaptic release probability, facilitating LTP induction by increasing presynaptic Ca^{2+} influx (Ruiz *et al.*, 2010). In addition, GABA elicits calcium transients in astrocytes culminating in the release of glutamate, activating NMDA extrasynaptic receptors which are important for LTP.

In the CA1 mouse hippocampus, pharmacological blockade or genetic deletion of GAT 1 impaired LTP induced by θ stimulation, reduced the frequency of hippocampal θ oscillation, and impaired hippocampus dependent-memory, passive avoidance and contextual fear conditioning tests (Gong *et al.*, 2009). The blocking of GAT 1 with tiagabine, impaired spatial learning in MWM (Schmitt & Hiemke, 2002), confirming that changes in GAT 1 activity affect hippocampal plasticity and GABA uptake may be fundamental in maintaining normal hippocampal θ activity, expanding the critical role of GABAergic inhibition for synaptic plasticity and learning.

1.14| BDNF, GAT1, and MEMORY

A possible target to CAE might be Brain-derived neurotrophic factor (BDNF). BDNF is a neurotrophin generally distributed in the mammalian brain. Among its major functions are the control of neuronal and glial development, neuroprotection, and modulation of both short- and long-lasting synaptic interactions, critical for cognition and memory (Kowiański *et al.*, 2018).

BDNF binds and activates, both pre- and postsynaptically, two different transmembrane receptor proteins: the tropomyosin related kinase receptor (TrkB) with high affinity, and the pan neurotrophin receptor (p75NTR) with low affinity. TrkB has two main isoforms, the full-length receptor, TrkB.FL, with a tyrosine kinase domain that autophosphorylates with BDNF binding, and a truncated receptor, TrkB.T1. TrkB-TC (truncated isoforms of TrkB receptors) act as a negative modulator of TrkB.FL, and the ratio of TrkB.FL:TrkB-TC may reflect a BDNF signaling dysfunction. TrkB.T1 is highly enriched in cortical astrocytes, particularly during the period of astrocyte morphological maturation, and BDNF/TrkB.T1 signaling represents a critical role in astrocyte morphogenesis (Holt *et al.*, 2019).

Indeed, BDNF was shown to induce suppression of GABAergic synaptic transmission, caused by acute downregulation of GABA_A receptors, its effects being mediated by its TrkB receptor and requiring PKC activation in the postsynaptic cell; the action of BDNF is predominantly postsynaptic (Henneberger *et al.*, 2002). In a short time frame, BDNF inhibits GABA_AR-mediated responses by down-regulation of surface expression, being this action synapse-specific towards inhibition of GABAergic transmission. In a long exposure to BDNF, the inhibitory action disappears, facilitating the action of GABA_AR (Brunig *et al.*, 2001).

Previous work revealed that BDNF increases GAT1-mediated GABA transport into astrocytes, leading to enhanced expression of GAT1 at the cell surface due to a reduced internalization (Vaz *et al.*, 2011). This facilitatory action of BDNF upon GAT1, modulating its activity might contribute to a decrease in tonic inhibition. Therefore, this molecule could be a possible target to CAE. This BDNF-

induced increase in GAT1- mediated GABA uptake involves the activity of at least two transduction pathways, namely the PLC and the ERK/MAPK pathways. BDNF also enhances synaptic transmission via the TrkB receptor in hippocampal neurons. TrkB mediates hippocampal plasticity via recruitment of PLC, and by subsequent phosphorylation of CaMKIV and CREB. In fact, learning increases BDNF expression in the hippocampus. TrkB inhibition is capable of blocking fear acquisition, which suggests that TrkB activation is necessary for the consolidation of fear memories. It has also been observed that conditional mutations of TrkB also result in deficits in hippocampal-dependent learning and memory tests (Andero *et al.*, 2014), so BDNF may modulate LTP through TrkB downstream signalling.

2| Aims

Children with ASs display adverse behavioural, psychiatric, and cognitive impairments, such as attentional difficulties, anxiety, depression, and reduction in learning and memory. Regarding animal models of AS, significant changes in seizure phenotypes have been detected between colonies, demonstrating that other characteristics also play a noteworthy role in determining the epileptic phenotype of GAERS. Similarly, discrepancies regarding anxiety-like behaviour have also been observed between colonies, as well a clear lack of studies regarding behaviour and cognition in GAERS is observed. Regarding stargazer mice, abnormal behaviour is extensively described, not anxiety-related. Moreover, cognition is poorly understood in stargazer mice.

Currently, it is not clear if whether existing antiepileptic drugs do affect cognitive deficits or if the systematic occurrence of seizures contributes to the psychiatric comorbidities observed. Due to the pharmacoresistance observed in ASs patients, there is an urgent need for new therapeutic targets that may modulate GAT1 transporters in astrocytes. Considering that BDNF was shown to increase GAT1-mediated GABA transport into astrocytes, increasing the expression of GAT1 at the astrocyte cell membrane might contribute to a decrease of tonic inhibition currents in ASs animal models, and thus, it was hypothesized that BDNF may ameliorate memory deficits and intrinsic cellular mechanisms related with behavioural or cognitive impairments in GAERS rats and STG mice. Accordingly, this work aimed to establish the anxiety-like behaviour, memory, and cognition of GAERS rats and STG mice and its modulation by BDNF.

This aim was achieved via the following objectives:

OBJ 1.| To identify the specific behaviour comorbidities of GAERS rats and STG mice:

- Evaluate anxiety-like behaviour
- Study of memory and cognitive impairments

This objective will be focused on testing Recognition Memory, Cross-Modal Object Recognition Memory, Working Memory, and Spatial Reference Memory.

OBJ 2. | To study hippocampal synaptic plasticity in ASs animal models

In this objective, the BDNF effect upon hippocampal long-term potentiation in GAERS rats will be evaluated.

OBJ 3.| To identify cellular morphological and molecular differences between GAERS, NEC, and Wistar animals.

This objective will be focused on the determination of GAT 1, GAT 3, and GFAP expression by western blot in brain regions implicated in ASs, and on the quantification of astrocytic morphological changes in brain areas involved in ASs.

3| Materials and Methods

3.1 | Animals and Ethical approval

Two models of CAE were used in this project. The ASs rat model GAERS, the experimental non-epileptic control NEC and Wistar as a second outbred control; in addition the ASs mice model, STG mice, and respective control group C57BL/6J wild-type mice.

GAERS and NEC rat were maintained in the FMUL (*Faculdade de Medicina da Universidade de Lisboa*) rodent facility. Wistar rats were ordered from Charles River Laboratories (Lyon, France) and arrived at least one week before the beginning of the experiments to acclimatize to laboratory conditions. All groups were housed in groups of 3- 4 animals (except in Experiment 1, groups of 2 for GAERS due to the separation of animals after weaning), in clear plastic cages, containing cardboard tubes, nests, aspen woodblocks, and bedding material. Animals were kept on a 12-hour light-dark cycle, 22° C temperature and 79% humidity, and given ad libitum access to both water and food (except in the experiments properly identified ahead). Only males were used.

STG and WT mice were maintained in the Rodent Facility of IMM (*Instituto de Medicina Molecular*). Mice were tagged and housed in groups of 3-5 animals, containing cardboard tubes, nests, and bedding material. Animals were maintained in a controlled environment on a 14-10-hour light-dark cycle, 22-24 °C temperature, and 45–65% humidity, with food and water provided ad libitum. Both sexes were used.

All experiments took place during the light phase of the cycle and in conformity with European Community Guidelines (Directive 2010/63/UE), and with the approval of the Committee for Ethics in Animal Research of the FMUL, as well as of the Portuguese Competent Authority for Animal Welfare. The number of animals and their suffering was minimized. Animals were handled for 5 min, 5 days consecutively before the beginning of the experiments.

3.2 | Behaviour Experiments

Given that experimental reproducibility can be challenging, being affected by the experimenter and animal-dependent factors; possible recognisable confounds were removed. For each behaviour task, the rats' testing order was randomized, as well as objects/maze position. Before each day of the experiment, animals were placed for 1h in the testing room for habituation. All equipment was cleaned with 30% ethanol between trials to erase olfactory cues. Trials were recorded using computerised tracking and video recording.

For behaviour tasks – Experiment 1, 2, and 4 - GAERS, NEC, and Wistar were aged 90-120 days, hereafter defined as young adults. For Experiment 3 animals were aged 180–190 days, hereafter defined as adults. For experiment 5, mice were aged 90–120 days.

3.2.1 | Anxiety -like behaviour tests

3.2.1.1 | Elevated Plus Maze

The Elevated Plus Maze (EPM, Pellow *et al.*, 1985) is the most common test to evaluate anxiety-like behaviour, postulating that under circumstances of elevation and open spaces, the natural exploratory drive of rats is suppressed. However, a natural preference for dark and protected spaces, allowing safety and security is also observed in animals. Indeed, a clear paradox emerges from this test, being that only the open and bright space is anxiogenic (aversive), nevertheless, rats are identifiable as anxious when naturally preferring protected and dark spaces (non-aversive).

The EPM was elevated 70 cm from the ground, consisting of two open arms crossing in the middle two arms enclosed by walls (Fig. 3.1) (Pellow *et al.*, 1985). Animals were placed on the center of the maze facing an open arm and allowed to explore the maze for 5 min. The total time spent in open arms and the total number of arm entries were taken as anxiety measures. An increase in open arm (OA) activity (duration and/or entries) reflects decreased anxiety-like behaviour (Hogg, 1996).



Figure 3.1. Structure diagram of the EPM.

The apparatus is composed of two open arms and two close arms. An entry was defined as moving all four paws into the arm.

3.2.1.2 | Emergence Test

Emergence Test (ET) is a less predominant task, used as a less anxiogenic adaptation of the open field test. ET allows the evaluation of neophobia and explorative behaviour. Neophobia can be characterized by an hesitancy to explore novel objects, places, or conspecific, highly correlated with stress vulnerability or anxiety-like behaviour (Paré, 2001). Testing neophobia in ASs animals is crucial to eliminate possible confounds of lack of interest/hesitancy in exploring novel objects, patterns, cues, and environments, critical to learning and memory tests assessed afterwards.

The testing arena of ET consisted of a 50 x 50 cm floor arena with high walls, black acrylic, and a square box [20x20cm] with a top cover and lateral escape hole (Fig. 3.2). Mild illumination was used. Rodents were placed in the box within the open field, for a unique trial of 10 min. The cover was closed and the latency for the rat to emerge completely from the box as well as the number of entries and average velocity as a locomotion parameter were recorded. Emergence latencies reflect anxiety levels, impulsivity, or loss of inhibition (Lalonde & Strazielle, 2009).

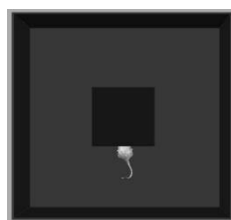


Figure 3.2 Structure diagram of the Emergence Test.

The apparatus is composed of the open field and a container (box 20 x 20 cm) inside the open field arena. An entry in the open field was defined as moving all four paws outside the box.

3.2.1.3 | Open Field Test

The open field test (OFT) is a broadly used test for anxiety-like behaviour, primarily developed to evaluate emotionality in animals, based on imperilling an animal to an unknown environment whose escape is prevented by surrounding walls. Rodents show distinct aversions to large, bright, and open environments (Choleris *et al.*, 2001), being phylogenetically conditioned to perceive these types of environments as risky. All of these aversions are combined in the OFT, forming the basis of the paradigm tested (Ramos, 2008). OFT also provides a qualitative and quantitative measurement of exploratory and locomotor activity in rodents, being useful to evaluate locomotion motivated by

exploration (Roth & Katz, 1979), fundamental to detect individual bias that could affect recognition or spatial memory performance.

An open field arena (50 x 50 x 60 cm) was used for 10 min trials, virtually divided into three different square zones- a peripheral zone, an intermediate zone, and a central zone (Fig. 3.3). Each rat was placed in the centre of the maze facing N, E, S, or W, randomly assigned. Percentage of time in the central zone, as well as entries in central zone, were used as an inverse indicator of anxiety levels. Fecal boli was a negatively related measure of emotionality in rats (Ramos, 2008), indicative of anxiety-like behaviour. Thigmotaxis, total distance covered as well as average velocity were analysed as general locomotor activity. A white open field was used for mice OFT, following the same protocol.

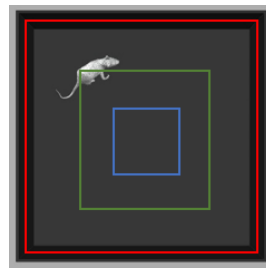


Figure 3.3. Schematic representation of the Open Field Test.

Virtually divided in three zones: peripheral (red), intermediate (green) and central zone (blue).

3.2.1.4 | Marble burying

Marble burying test (MBT) is used for the assessment of anxiety-like or repetitive-like behaviours in mice. It is accepted that mice bury marbles because the novelty of the marble elicits burying response, however it is equally assumed that burying associated to digging reflects a more obsessive and compulsive-like behaviour (Gyertyán, 1995, Deacon, 2006). Rodents MBT may be the by-product of a more adaptive, natural rodent behaviour – that of digging and burrowing in a new environment which naturally occurs into bedding material to find food, saves offspring from a predator, also for nesting (Deacon, 2006). MBT was interpreted as an anxiety-like behaviour task, as a more sensitive measure of this parameter than OFT. Test combinations result in fatigue, so the test must be done separately.

To MBT, bedding was added to a clean cage (26 x 48 x 20 cm) to a depth of 5 cm approximately. Twenty black marbles were placed on the surface of the bedding in 5 rows of 4 marbles (Fig. 3.4A). Mice were placed in a corner of the cage and allowed exploration for 30 min. If marbles represent an anxiety-provoking stimulus, it is expected that more susceptible mice would choose to bury. Marbles covered at least 2/3 by bedding (Fig. 3.4B) were scored as buried.

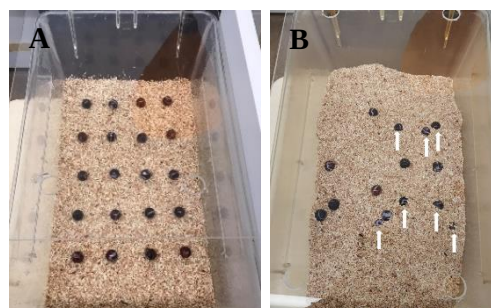


Figure 3.4. Representative presentation of the marble burying test.

5 rows of 4 marbles are arranged inside the cage (A). After 30 min trial, marbles buried more to two thirds are scored (B, white arrows). Burying positively correlates with anxiety.

3.2.2 | Learning and Memory Tests

Recognition Memory

3.2.2.1 | Novel Object Recognition Test

The Novel object recognition (NOR) test is used to study short-term memory, intermediate-term memory, and long-term memory, through manipulation of the retention interval. NOR test performance does not rely on spatial cues and does not require external motivation (eg. reward), assessing the innate natural preference for novel objects in rodents (Antunes & Biala, 2012)

To test recognition memory formation and retrieval abilities, the NOR test was conducted in the open field, being composed of a habituation period for 3 days, for 10 min, in the absence of any stimulus or object, to reduce anxiety and fear of the novel context, a learning trial, and a test trial. The familiar or novel objects were randomized and the position relative to the other object transposed. In learning trials, animals were placed in the open field with two familiar objects (yellow cylinders, Fig. 3.5A) and were allowed to freely explore the objects for 5 min. After a retention interval of 5 min (short-term memory) or 24 h (long-term memory), in the testing trial, animals were allowed to freely explore the open field containing the familiar object and a novel object (Fig. 3.5B). Exploratory behaviour was quantified as the time touching/focusing an object.

The difference between time spent exploring novel and familiar objects in the total exploration time was defined hereafter as Novelty index¹. An animal with intact recognition memory should spend more time investigating the novel object relative to the familiar one (Ennaceur, 2010, Antunes & Biala, 2012). Trials were rejected if the animal jumped the object or explored less than 10 s each object in the learning phase. Total time of exploration of each object and frequency of interaction, as well as the percentage of exploration of the novel object were assessed.



Figure 3.5. Schematic representation of Novel Object Recognition Test.

Learning phase with two familiar objects (A), Testing phase with a familiar and a novel object (B). Recognition memory is proportional with increased novelty index.

3.2.2.2 | Modified Novel Object Recognition Test

The modified novel object recognition test is similar to NOR described in 3.2.2.1. Nevertheless, an optimization was made by expanding the arena size (65 x 65 cm) and using a blue opaque open field, to avoid bright reflection of the animals or objects. Also, by increasing the complexity of the objects used (two bottles, distinguished by elongated or straight shape and different covers, Fig. 3.6A,B) and diminishing object size to avoid animals hiding behind them. This protocol was only assessed for long-term memory (24 h).

$$1 \text{ Novelty index} = \frac{\text{Time exploring the novel object} - \text{Time exploring the familiar object}}{\text{Total time exploration (s)}}$$

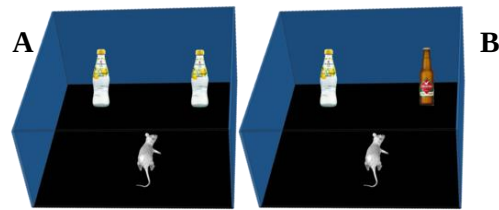


Figure 3.6. Schematic representation of modified Novel Object Recognition Test.
Learning phase with two familiar objects (A), Testing phase with a familiar and a novel object (B).

3.2.2.3 | Cross-Modal Object Recognition Memory

Cross-Modal object Recognition test (CMOR) was performed to understand if rats were capable of tactile-to-visual crossmodal object recognition, given that different cortical regions underlie different aspects of the object representation required for this cognitive function. This test was adapted from Winters & Reid, 2010.

A Y Maze was used as the testing apparatus and three distinct tests were assessed: tactile, visual, and crossmodal memory (Fig. 3.7A,B,C). Tactile exploration was conducted in red light illumination that prevented rats from seeing the objects, however, allowed the recording to posterior analysis. In visual exploration, under yellow light, transparent acrylic barriers were inserted in front of the objects to avoid tactile exploration. Visual objects were previously validated for rats. Firstly, 2 habituation in pairs and one individual habituation of 10 min occurred prior to testing. For half of each habituation session, white and red illumination were separately presented. The day after the last habituation session, testing began. Each test was composed of 3 min learning phase, 1 h delay, and 2 min test phase. During learning, the maze contained two identical objects at the end of each arm. In the test phase, the familiar object was paired with a novel object in the opposing arm. The order of testing was tactile (Fig. 3.7A), visual (Fig. 3.7B), and CMOR (Fig. 3.7C).

In this paradigm, visual recognition memory was described as more time spent observing a novel object that had only been seen (not touched) during the learning phase. Tactile recognition memory is referenced as increased tactile exploration of a novel object that had been previously touched (not seen) during the learning phase. Crossmodal recognition memory referred to a greater time spent looking at a novel object that had been touched, but not seen, beforehand. Total time of exploration in each trial, novelty index, and percentage of exploration of the novel object were analysed.

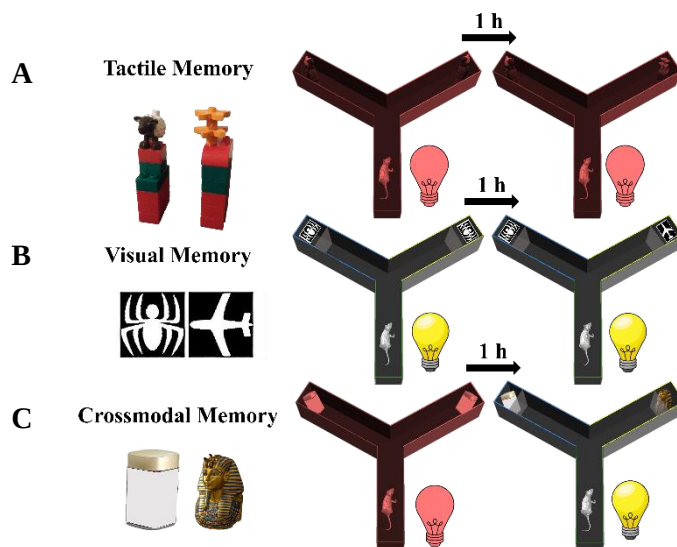


Figure 3.7. Schematic representation of the three crossmodal object recognition tests, including the tactile test (A), visual test (B) and crossmodal test (C).
Each variant contains a training trial with two identical objects and a test phase with one familiar object.

Working Memory

3.2.2.4 | Y-Maze

Working memory, specifically short-term spatial working memory was assessed using the spontaneous alternation Y-maze test. Rodents naturally tend to explore a new arm of the maze rather than returning to one that had been previously visited.

The maze was composed of three symmetric arms, 120° (Fig. 3.8). The rat was placed at the end of an arm randomly assigned and allowed 8 min to explore the apparatus. The series of arm entries (e.g ACBBACBAC) was recorded (Fig. 3.8). Percentage of correct alternations² was measured by summing the number of 3 arms entries of the maze in succession divided by the maximum number of possible alternations, being maximum alternations the total number of arm entries minus 2.

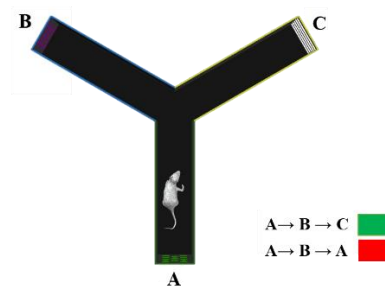


Figure 3.8. Schematic representation of Y-Maze Spontaneous Alternation Test.

Example of 3 arm sequence correct alternation (A→B→C), and incorrect alternation (A→B→A).

3.2.2.5 | T-Maze rat - Nonmatching to place task and delayed non-matching to place task

T-Maze protocol was assessed to evaluate the underlying mechanism that leads to spatial working memory in rats. The protocol was adapted from Hussein *et al.*, 2018, being the called spontaneous alternation tested in Y- maze reinforced by baiting the arms of the maze with food when animals are mildly food-deprived. On day 3 of handling, the protocol of food restriction began and body weight was recorded throughout the experiment (Fig. 3.9A). The animals were mildly food deprived to decrease the body weight to 85% of the initial free-feeding weight. Rats were familiarized with reward cookies during handling. On day 6, each rat was allowed two habituations to the T-maze in cage-mate pairs for 10 min, with treats all over the maze (approximately 1.5 treats per animal). One habituation in the morning and a second habituation in the afternoon were performed. At the end of the second habituation, rats were allowed pellets each, individually, depending on their body weight. On day 7, rats were allowed individual habituation for 5 min with treats only at the end of each goal arm in the morning. Another habituation took place in the afternoon, and treats were placed in the nests constructed with newspaper, so that animals were not able to see the reward. On days 8 and 9, Spontaneous rewarded alternation was tested, which comprised of 6 trials with intertrial interval of 10 min, composed of 5 alternations. Initially, 2 rewards were hidden at the end of each arm, and the rat was allowed to choose;

$$2 \text{ \% Correct Alternations} = \frac{\text{Number of correct alternating 3-choice sequences}}{\text{Total entries} - 2} \times 100$$

eats from one arm and was removed. Left and Right choices were scored (eg. LRLRRL→4/5 corrected alternations).

Spatial working memory test, specifically Nonmatching to place task (NMTP, Fig. 3.9B) was performed on days 10,11, and 12. A total of 6 trials, 3 right trials, and 3 left trials were accomplished. The rat was blocked for 10 sec, a forced trial began and the animal was forced to eat the reward from the open arm and removed. Immediately after, was placed in the blocked start for 10 sec, then released for the choice trial, with both arms opened and allowed to choose. A correct trial was scored if the rat visited the opposite arm to the previous choice. If correct, the animal was allowed 2 rewards at the end of the arm. If the choice was incorrect, the animal was blocked in the incorrect arm for 15 sec and was then removed from the maze. The delayed nonmatching place to task (DNMTP) occurred on day 13. A total of 6 trials, 3 right and 3 left were similarly performed, however with a 2 min delay.

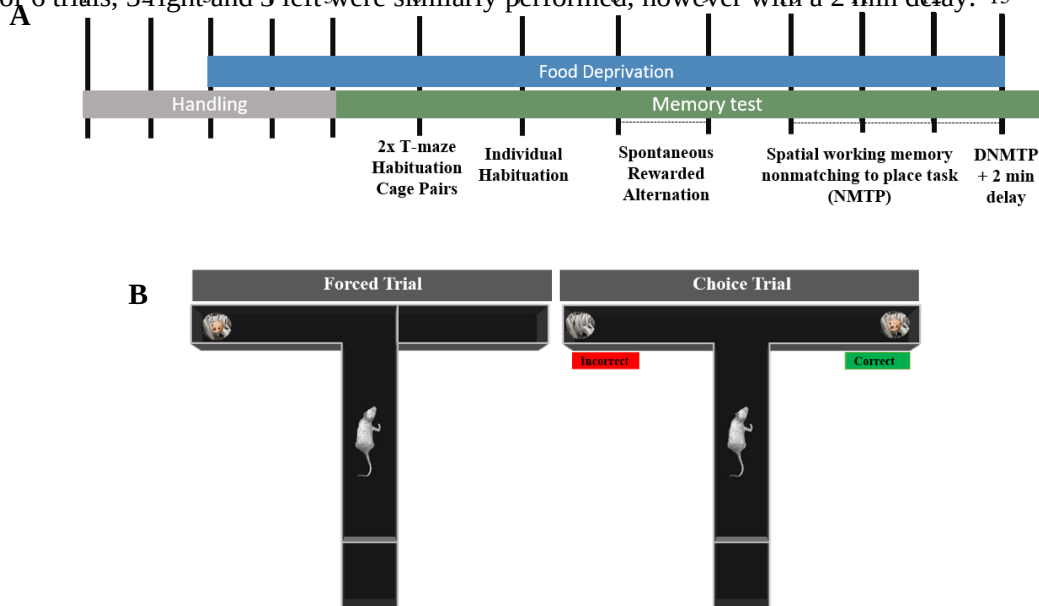


Figure 3.9. Schematic timeline and representation of the T-Maze test.

Food deprivation occurred from day 3 until the end of the testing protocol. T-Maze habituation occurred in day 6 and 7. In day 8 and 9, Spontaneous Rewarded Alternation with 10 min delay was tested. In day 10-12, Nonmatching to place task began with 10 sec of interval between forced and choice trials and the Delayed nonmatching to place task occurred in day 13 with a imposed delay of 2 min (A). Schematic representation of NMTP and DNMTP task with forced trial and choice trial with reward placed in the opposite arm of the forced trial (B).

3.2.2.6 | T-maze mice

For mice, the standard protocol of the T- maze was modified so that the task tested not only working memory and learning capacity but also decision making using spatial memory guided by intra maze cues.

During handling, mice received habituation to the diluted condensed milk solution, hereafter defined as a reward. Given the comorbidities identified in STG mice, no protocol of food deprivation was applied to these animals. Body weight was recorded throughout the experiment. All mice received 4 days of habituation to the maze, composed of 5 min each. On day 1 (Fig. 3.10A), T-Maze Spontaneous alternation was assessed for 8 minutes, with patterns (intramaze cues) at the end of each arm. After 2 h of water deprivation, habituation to reward started all over the maze (4 cups) along the floor of the arena to encourage exploratory activity and to habituate mice to the novel solution. On day 2, T maze

habituation was allowed with 4 cups with reward and introduction to 10 sec blockage door. On days 3 and 4, the cups of reward (100ul) were only presented at the end of each goal arm. Testing began at day 5, each mouse received 10 trials per day for 10 days, each trial of a maximum of 3 min and intertrial interval of approximately 8 minutes. Two intramaze cues were presented: black stripes and dark grey. If the entry arm was covered with stripes, the animal is expected to choose the goal arm covered with stripes and find a reward in the cup, being scored as a correct choice (Fig. 3.10B). On the other hand, if the arm was covered with stripes, and the animal chooses the grey arm, it will find a cup with reward, although covered with glue tape to enable the mouse to take the reward. It is then scored as an incorrect choice. To avoid several confounds such as getting the right choice from following the scent, glue tape was used; to avoid side preference or spontaneous alternation, not more than 2 sequence trials were assessed for the same pattern; to assure that animals were following the pattern to get the choice, not randomly fashioned, goal arm patterns changed each day, and in order to shatter the possibility of preference for a pattern, 5 trials were assessed with stripes and the other 5 with dark grey. Two measures emerged from this test: spontaneous T-Maze alternation and percentage of correct trials over the 10 days of testing. The 2 arms were visually different and lined with contrasting wallpaper

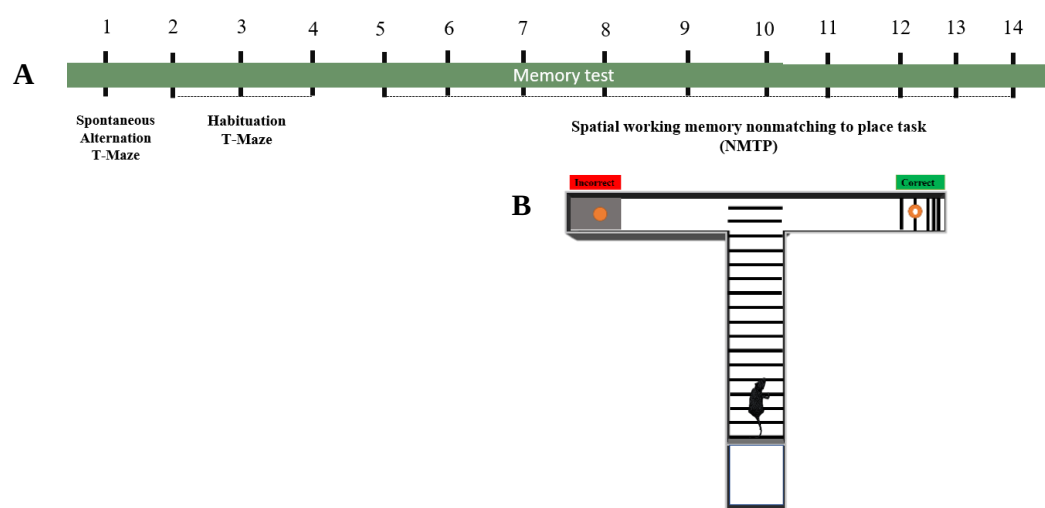


Figure 3.10. Schematic timeline and representation of T-maze test for mice.

Spontaneous Alternation occurred in Day 1. T-Maze habituation occurred in day 2,3 and 4. In day 5 to 14, Spatial working memory, nonmatching place to task using intramaze cues with intertrial interval of 8 min was performed (A). Schematic representation of NMTP, displaying the reward in the respective pattern assessed in entry arm (B).

Spatial Memory and Reference Memory

3.2.2.7 | Novel Object Placement Test

The Novel Object Placement (NOP) test allows the measurement of hippocampal-dependent spatial memory (Vogel & Wood, 2014). NOP relies on the rat's natural preference for novelty without additional external reinforcement (Denninger *et al.*, 2018).

In the learning phase, the rat was exposed to two similar objects (blue pyramids), placed in the adjacent corners of the open field (Fig. 3.11A), and allowed to explore both objects for 5 min. After a delay of 5 min or 24 h, the testing phase began in which one object was placed in diagonal (new location, Fig. 3.11B) with the familiar position object. Time of exploration of each object was recorded, as well as the frequency of interaction and percentage of novel object exploration. Rats with a good memory of the location of objects are expected to instinctively choose to explore more the changed object. To avoid

possible confounds (eg. light, shadows, intramaze cues preference), the position of the training and moved object was counterbalanced between rats.

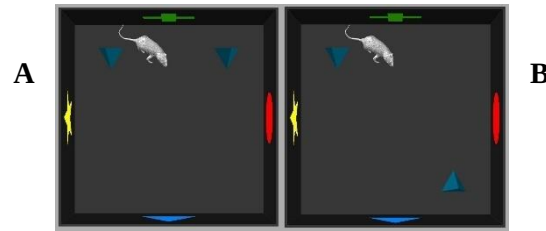


Figure 3.11. Schematic representation of Novel object placement test.

Learning phase with two familiar objects (A), Testing phase with a familiar object a novel location object (B) diagonally placed.

3.2.2.8 | Barnes Maze for rats

The circular platform maze, also known as Barnes Maze (BM), is a hippocampal-dependent spatial reference memory task, requiring rodents to use spatial cues to locate an escape hole. It was first developed to test rat's temporal lobe functioning to understand hippocampal function. The task is composed of a circular large platform placed under bright light with 20 holes in the perimeter. Perceptive that rodents find bright light aversive, a motivation to escape into the dark box is expected (Bimonte-Nelson, 2015). During training, the animal learns the spatial location of the target hole/box. Extra maze cues are placed as reference cues (Fig. 3.12A).

During habituation, under dim the rat was placed in the middle of the maze in a start box. After 10 s, the box was lifted, a strong light projector was turned on and the rat was trained to enter the escape box and remained there for 1 min. Then, testing began, the rat was placed in the start box, 10 s after the box lifted, and the light was turned on, allowing maze exploration for 3 min or until the rat entered the target hole. Immediately after entering the box, the light was turned off and the rat stayed inside for 1 min. An acquisition period occurred for 4 days (Fig. 3.12B), 4 trials per day, with an intertrial interval of approximately 15 min. After each trial, the maze was rotated to avoid intra maze cues such as animal's prior path or scratches and cleaned with 30% ethanol.

Several parameters were measured: latency to the target hole (time until finding the hole), distance travelled (path until reaching the hole), speed, errors, and search strategy. Errors were defined as nose pokes or head deflections over holes that were not the target hole. The search strategies were defined as (1) Direct/Spatial: rat moved from the centre of the maze directly toward the escape hole or initially moved away from the escape hole, but stopped after visiting one hole, and moved directly back to the escape hole; (2) Serial: rat moved around the periphery and making or not errors at adjacent holes from the escape hole; (3) Random: rat moved in an unsystematic manner, moving into the centre of the maze and visiting areas already visited. If the rat failed to enter the escape hole during the 3 min trial, it was considered random. Decreased latency and distance to the target hole as well as decreased number of errors indicates learning and allows the quantification of performance differences between groups (Barnes, 1979). Successful use of a direct strategy depends on the presence of many extra-maze spatial cues.

To avoid confounding factors (eg. If a rat learns the association between the spatial cue and target hole, but the number of errors and distance increase due to further exploration), the latency, path length, and the number of errors until finding the target hole for the first time were measured. These measures were named primary latency, primary path length, and primary errors (Harrison *et al.*, 2006).

The day after the acquisition phase, animals performed a probe trial for 90 s for short-retention memory. The box from the target hole was removed and rats were allowed to explore the maze and visits to the target hole, latency, distance, permanence close to the target hole, and strategy were recorded. On the 11th day, animals were tested once again in the probe trial for 90s to test long-term retention memory.

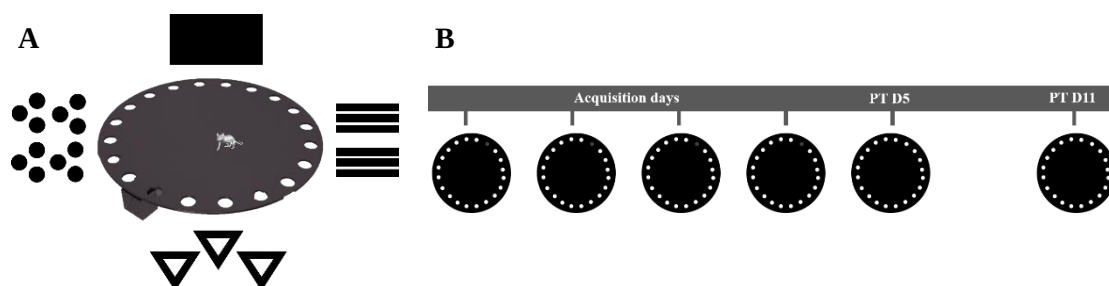


Figure 3.12. Schematic representation of the Barnes Maze test for rats.

Animals learn to locate an escape box using visual cues placed on the walls of the room (A). Protocol timeline comprising Acquisition days, probe test day 5 and probe test day 11 (B).

3.2.2.9 | Barnes Maze for mice

BM is widely held in rats; however, the task has been adapted to mice as well. Here, the protocol for mice was performed similarly to the description in 3.2.2.8. However, the apparatus changed in size and colour, to allow correct computerized tracking (Fig. 3.13A,B). On account of a protocol of 10 days in T-maze that had already been performed in these mice, as well as habituation to light, a more aversive BM protocol was assessed. This weak aversive stimulation was applied to increase the motivation to escape from the circular platform, comprising the introduction of not only light as an aversive stimulus but also the onset of a buzzer (71db) (Sunyer *et al.*, 2007).

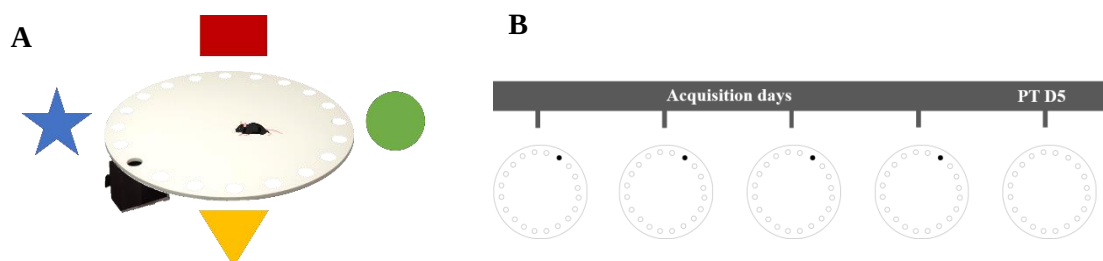


Figure 3.13. Schematic representation of the Barnes Maze test for mice.

Animals learn to locate the escape chamber using visual cues placed on the walls of the room (A). Protocol timeline comprising acquisition days and probe test D5 (B).

3.2.3 | Software of analysis SMART and Solomon Analysis

All trials were video-recorded using CamStudio and OBS studio (V. 27.1.3). Rats' behaviour was recorded using video-tracking software, PANLAB, (V. 2.5 and V3.0.06, Panlab, Harvard Apparatus, Barcelona, Spain). The reference point to determine the position of the animal was the centre of the rat dorsum. Post-analysis was performed using Solomon Coder (V. beta 19.08.02).

3.3 | Extracellular Recordings in Hippocampal Slices

After behaviour experiments assessment, animals were used for electrophysiological recordings, with ages ranging between 90-110 days. To evaluate synaptic plasticity, extracellular recordings of field excitatory potentials (fEPSPs) were performed in GAERS, NEC, and Wistar animals; STG, and WT animals.

3.3.1| Preparation of hippocampal slices

To obtain the hippocampal slices, animals were deeply anesthetized with isoflurane, and dissection of the two hippocampi was rapidly made onto ice-cold Krebs solution. Hippocampal slices (400 μ m thick) were cut perpendicularly to the long axis of the hippocampus using McIlwain tissue chopper and maintained at least 1h in a resting chamber at room temperature (RT), in oxygenated Krebs solution (5% CO₂, 95% O₂) to assure energetic and functional recovery of the tissue. After the recovery period, slices were placed in the recording chamber of 1ml capacity and continuously superfused with oxygenated Krebs solution at a constant flow of 3 ml/min and 32 °C.

To obtain the fEPSP recordings, as previously described (Silva-Cruz *et al.*, 2017), a microelectrode filled with Krebs solution (2–6 M Ω resistance), was placed in the stratum radiatum of CA1 region of the hippocampus, coupled to an Axoclamp 2B Amplifier (Axon Instruments) and digitized BNC-2110 (National Instruments). The stimulus was delivered (rectangular, 0.1 ms pulses every 20 seconds) through a concentric electrode placed on a Schaffer collateral-commissural fibers, in the *stratum radiatum*, near the CA3-CA1 border (P0) and near CA1 (P1). Stimulation alternately of two pathways of the Schaffer collateral-commissural fibers was performed, for P0 and P1. Individual responses were monitored, and averages of eight consecutive responses were continuously stored WinLTP Software.

3.3.2 | Protocol of LTP for Synaptic plasticity evaluation

To analyse synaptic plasticity, long-term potentiation was induced by θ -burst stimulation since this pattern of stimulation is similar to what occurs physiologically in the hippocampus during episodes of learning and memory in living animals (Albensi *et al.*, 2007). After a baseline of approximately 20 min, LTP was induced using TBS delivered at basal stimulus intensity, comprising of 5 bursts (200 ms interburst interval) at 5 Hz, with each burst composed of 5 pulses at 100Hz (Park *et al.*, 2016). fEPSPs slope was recorded for 60 min after LTP induction, and each slope value corresponding to an average of 8 responses of 20 s. For each slice, LTP was induced after obtaining a stable baseline for at least 30 min. An LTP control was induced through P0 electrode and after 60 min recording, BDNF (20ng/ml) was perfused in the bath for 30 minutes. After BDNF 30 min incubation LTP was induced in the second pathway (P1 electrode).

LTP was quantified as the percentage of change in the average slope of the fEPSP. The effect of BDNF upon LTP was estimated by comparison of the magnitude of LTP in the first pathway (control pathway) with the magnitude of LTP in the second pathway, in the presence of BDNF.

3.4| Immunohistochemistry

To study the cellular morphological differences between GAERS and non-epileptic animals, immunohistochemistry assays were assessed, using brains from Experiment 1, approximately 150-180 days rats. Slices were obtained from Motor Cortex and Somatosensory Cortex (Bregma 2.2 to Bregma -2.12), Thalamus and Hippocampus (Bregma -2.3 to Bregma -4.8), and Visual Cortex (Bregma -4.8 to -5.2).

3.4.1 | Slice's preparation

For slice preparation animals were deeply anesthetized with sodium pentobarbital, to perform transcardial perfusion via left ventricle, with Buffer (0.9% NaCl) solution followed by perfusion with fixative buffer (4% PFA). After perfusion, animals were decapitated, brains were removed and post-fixed by immersion in 4% PFA for 4h at 4° C. After a quick wash in PBS, brains were immersed in a 15% sucrose (in a 50 ml tube) solution at 4° C and stored for further use. The tissue was then sliced at 40 µm of thickness per slice, using a vibratome, and stored as free-floating slices for further use.

3.4.2 | Antibodies staining

For antibodies staining, free-floating slices were washed in PBS 1x (3x 10 min) and permeabilized for 30 min (5 % Triton X-100 in PBS). Slices were washed in PBS 1x (3x 10 min). Blocking was performed for 1h in 10% FBS and primary antibodies (Table: List of primary antibodies, ST 7.6) were incubated overnight at 4°C, diluted in the blocking solution (FBS 10%). The day after, slices were washed in PBS 1x (3x 10 min) and fluorescent-labeled secondary antibodies (Table: List of secondary antibodies, ST 7.7) were incubated for 1.30h, at RT, diluted in the blocking solution (FBS 10%). After antibody incubation, a wash in PBS (2 x 10 min) was performed and Hoechst 33342 (1:100, diluted in PBS) was added to slices for 15 min at RT. A quick wash in PBS 1x was made and preparations were mounted in Mowiol (non-absorbing compound without autofluorescence and light scattering). Three slices per slide were mounted.

3.4.3 | Visualization

Images were acquired on an inverted widefield fluorescence microscope (Zeiss Axiovert 200), using a monochrome digital camera and a 5x, 10x, and 40x objective.

3.4.4 | Astrocytic morphological analysis

Morphological analysis was performed in GFAP-stained astrocytes in each brain region identified according to rat brain atlas. GFAP, the intermediate filament protein, was used as a specific marker for astrocyte main processes, whose expression is strongly correlated with morphological alterations (Wilhelmsson *et al.*, 2006). The astrocytic structure of 40x magnification was analysed by performing reconstructions using the Simple Neurite Tracer plug-in from the Fiji software (Binley *et al.*, 2014; Tavares *et al.*, 2017). To select astrocytes for reconstruction, 5 images were obtained for each brain region, and astrocytes with clearly identifiable Hoechst-stained nucleus, with main structure devoid of truncated processes, per se, completed GFAP structures were chosen to further analysis. The centre of the Hoechst staining was always defined as the starting point

Total process length, number of processes, and Sholl analysis were appraised. The number of intersections at radius zero represents the number of main processes that leave the nuclei. Morphology complexity was predicted from the number of intersections at each radial distance from the starting point (Hoechst-stained nucleus), provided by the Sholl analysis.

3.5 | Western Blot

To study changes in the protein expression levels, Western blot assays were performed. Animals were anesthetized with isoflurane and decapitated. Brains were dissected and using a stereotactic mold (rat brain matrix, coronal sections, 1.0 mm) in ice and PBS 1x, slices of 2 mm were dissected. Slice 1, of 3 mm was discarded. Using a biopsy needle, regions of interest were isolated. In Slice 2, isolation of motor cortex; in slice 3, motor cortex and somatosensory cortex; in slice 4, somatosensory cortex, hippocampus, and thalamus; in slice 5, visual cortex and hippocampus and finally in slice 6, isolation of visual cortex was performed.

3.5.1 | Sample preparation

Tissue homogenates were prepared from the samples. Samples were homogenised through sonication in 150 μ L of RIPA (Ristocetin Induced Platelet Agglutination) buffer, 50 mM Tris, pH 8.0, 1 mM ethylenediamine tetraacetic acid [EDTA], 150 mM NaCl, 1% Nonidet P40 Substitute [NP40], 1% sodium dodecyl sulfate [SDS], 40% glycerol, protease inhibitors solution (25 x) and PMSF. Samples were centrifuged for 10 min, at 1300 rpm at 4°C, and supernatant was collected in ice.

3.5.2 | Protein Quantification

Sample protein quantification was performed using the DC protein assay, taking 10 μ L of bovine serum albumin (BSA) and samples. After quantification, samples were prepared by adding sample buffer, denatured for 30 min at 37 °C, and stored at -20 °C.

3.5.3 | Western Blot assays

Proteins were separated by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), in running buffer, at constant voltage (80-120V), with 1.5 mm thickness. NZYColour Protein Marker II (NZYTech, Lisbon, Portugal) was used as a protein molecular weight marker. Protein was then transferred to a Polyvinylidene Difluoride (PVDF) membrane (Millipore), previously soaked with methanol, in transfer buffer, at a constant voltage of 350 mA for 1h30.

Membranes were then blocked with 3% BSA in TBS-T (20 mM Tris base, 137 mM NaCl, and 0, 1% Tween-20) at RT for 1h. Subsequently, incubated with the primary antibodies (4 °C, overnight) and secondary antibody (RT, 1 h). Primary and secondary antibodies (Table 1 and 2 ; Fig ST 7.6, 7.7) were prepared in the blocking solution (BSA). Washes with TBS-T (3x 10 in) were used to remove previous antibodies. Stripping solution ((200 mM glycine, 0.1% SDS, 1% Tween 20, 50% acetic acid glacial, pH 2.2) was used for 20 min between incubations with different primary antibodies. Signal was developed using Western Lightning ECL and immunoreactivity was visualized with the ChemiDoc™ XRS with Image Lab software. Band intensities were quantified via image J, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) band intensities as a loading control. Data were normalized for the Wistar group and expressed as protein levels (fold-change for Wistar).

3.6 | Statistical Analysis

In this project, statistical significance was evaluated by the GraphPad Prism (V. 8) for Windows and Rstudio (V. 2021.09.0+351). Data were represented as Mean $\pm$ Standard Error of the Mean (SEM) of n independent observations in each experimental group. In behaviour analysis each rat performance corresponds to a n value. One slice or sample per animal was used for each fEPSP recording or western blot assay, thus n values will correspond to the number of slices or samples from different rats.

ROUT method was used for outlier identification. Unpaired two-tailed Student's t-test was used for independent samples, to perform two-sample comparisons. When normality was not met, data were analysed with Mann-Whitney U test. One-way ANOVA followed by Tukey correction was used for multiple comparisons to compare between more than two groups. Two-way ANOVA was used to determine how a response is affected by different groups followed by Tukey. Values of $p < 0.05$ were considered statistically significant.

4 | Results

4.1 | Behaviour Experiments

In this chapter, it was intended to address in a robust, specific and clarified manner, the phenotypical behavioural characterization of ASs rat animal model, GAERS, and respective controls NEC and Wistar rats (Experiment 1-4). The behaviour of ASs mice model, namely stargazer mice and respective control (Experiment 5) was also studied. Specifically, a descriptive anxiety-like behaviour, locomotion, and memory capacities of the ASs models colonies in Lisbon have been made.

4.1.1 | Experiment 1. GAERS: Anxiety-like behaviour, Locomotion, Recognition Memory and Working Memory in young adult rats

For this Experiment, 4 series of 6 animals per condition (GAERS, NEC and Wistar) were used; 2 series for 24h delay (n=12 per condition) and 2 series for 5 min delay (n=12 per condition), aged 90-120 days. The timeline for this set of experiments is shown in Fig. 4.1.

4.1.1.1 | Anxiety-like behaviour is decreased in young adult GAERS

Since the performance in memory tests can be affected by anxiety, multiple tests were assessed to strengthen the evaluation of the results.

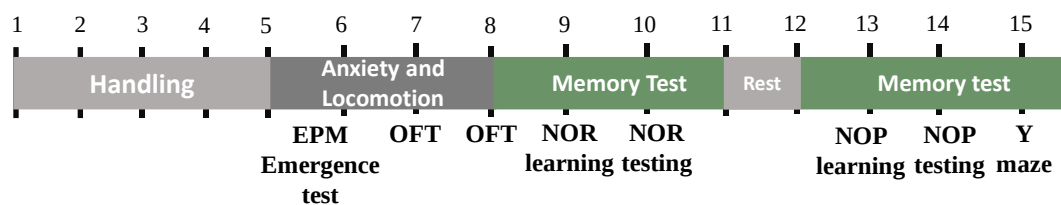


Figure 4.1. Timeline of Experiment 1.

4.1.1.1.1 | Elevated Plus Maze

To assess the anxiety-like behaviour EPM test was performed, and the percentage of time spent in the open arms of the maze, total entries and the number of open arm entries in EPM were compared. Between Wistar, NEC, and GAERS rats, it was observed a significant difference between groups ($F_{2,70}=8.51$, $p<0.001$), since NEC spent less time in open arms (NEC=11.9±1.90%) than Wistar (Wistar=23.8±2.73%; $p=0.009$; Fig. 4.2B) and GAERS (GAERS=26.8±3.19; $p<0.001$; Fig. 4.2B). For GAERS it is indicative of a decreased anxiety-like behaviour. Regarding the number of total arm entries, there was a significant effect of group, accounted by an increase in arm alternations in NEC (NEC=57.7±2.46) compared to both Wistar (Wistar=31.8±2.46; $p<0.001$; Fig.4.2C) and GAERS (GAERS=37.7±1.83; $p<0.001$; Fig. 4.2C), indicating an increase in locomotor activity. However, no differences between groups were observed for the number of open arm entries (Fig. 4.2D).

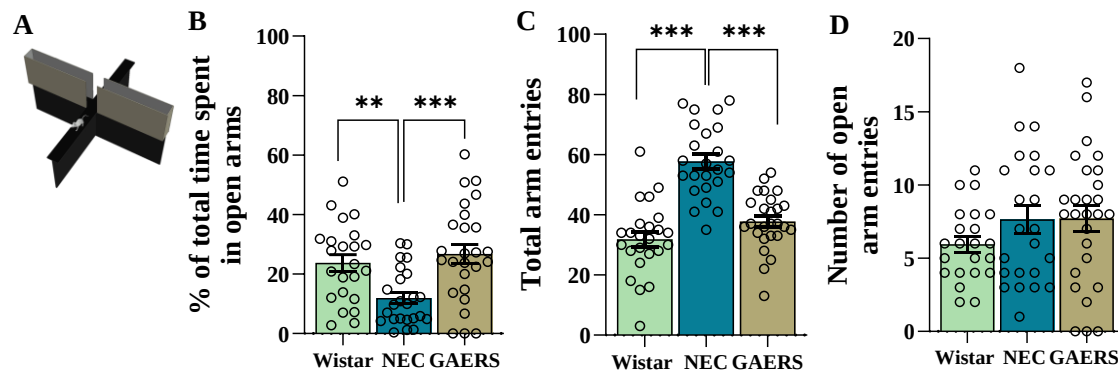


Figure 4.2. Young adult GAERS display decreased anxiety-like behaviour and NEC rats display anxiety-like behaviour in Elevated Plus Maze.

From the 5-min EPM trial (A), two measures of anxiety derived. Time spent in open arms as a percent of total testing time (B), the number of total arm entries (C) and the number of entries in open arms (D) in young adult male Wistar (n=24), NEC (n=24) and GAERS (n=26) rats. NEC spent significantly less time in open arms (**, *** p) and increased total arm entries (***p) compared to both controls. Data expressed as the mean $\pm$ SEM (n=23-26); *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA followed by Tukey pairwise multiple comparisons tests.

4.1.1.1.2 | Emergence Test

In order to evaluate neophobia and explorative behaviours in a less anxiogenic task, ET was performed measuring emergency latency time, the number of entries in the open field, and average speed between groups. In this test, there was a significant difference between groups ($F_{2,61}=7.02$, $p=0.002$); GAERS showed significant lower emergence latency (GAERS=9.50 $\pm$ 0.96 s) from the controls Wistar (Wistar=19.5 $\pm$ 3.04; $p=0.029$; Fig. 4.3B) and NEC (NEC=22.0 $\pm$ 3.37; $p=0.002$; Fig. 4.3B), indicating decreased anxiety-like behaviour. However, unexpectedly, regarding the number of entries in the open field/exits from the box, there was a significantly higher alternation in GAERS compared to NEC ($F_{2,64}=6.75$, $p=0.002$; GAERS=4.96 $\pm$ 0.47; $p=0.001$; Fig. 4.3C). Additionally, in terms of general locomotor activity, NEC display an increased average speed compared to the second control (NEC=5.17 $\pm$ 0.25, Wistar=3.76 $\pm$ 0.37; $p=0.001$; Fig. 4.3D), possible indicating hyperactivity-like behaviour.

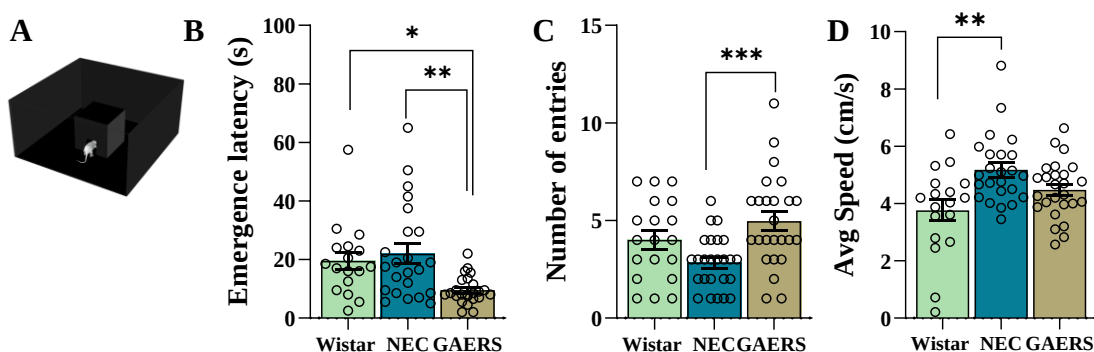


Figure 4.3. Young adult GAERS have reduced emergence latencies and increased entries to the open field.

From the 10 min ET trial (A), two measures derived. Emergence latency from the box (B), number of entries (C), and average speed (D) in young adult male Wistar (n=24), NEC (n=24) and GAERS (n=26) rats. GAERS significantly took less to exit the box (*, **p) and performed more alternations compared to NEC (***p). Data expressed as the mean $\pm$ SEM (n=17-26); *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA followed by Tukey pairwise multiple comparisons tests.

4.1.1.1.3 | Open Field Test

OFT was performed to measure spontaneous activity, as a complement of anxiety-like behaviour observed in EPM and specifically to detect any alteration in locomotor activity. In this test, there was a significant difference between groups ($F_{2,66} = 6.08$, $p=0.004$).

It was observed that GAERS spent more time in the central zone compared to Wistar (GAERS= 3.54 ± 0.52 , Wistar= 1.62 ± 0.25 ; $p=0.003$; Fig. 4.4B) and entered significantly more times in the central zone compared to Wistar (GAERS= 12.8 ± 1.30 , Wistar= 6.17 ± 0.88 ; $p<0.001$; Fig. 4.4C), which is indicative of reduced anxiety-like behaviour. Regarding fecal boli counts, no differences between groups were observed (Fig. 4.4D). Concerning alterations in locomotion and overall activity, no significant differences were observed between groups for average velocity (Fig. 4.4E) nor distance travelled (Fig. 4.4F). Schematic tracking is shown for each group (Fig. 4.4 G,H,I). Measurements descriptions for all zones are presented in Supplementary Data, ST7.1. GAERS displayed reduced permanence and covered distance in the peripheral zone and increased permanence and covered distance in the intermediate zone (statistics in Supplementary data: Table ST7.1).

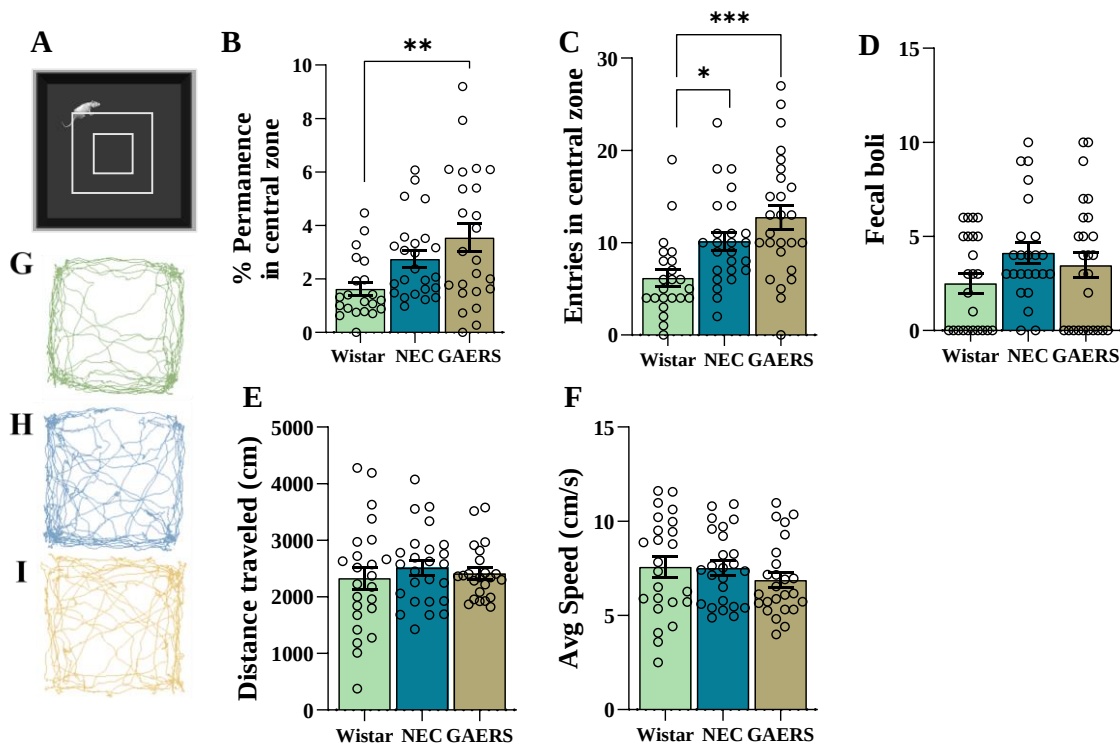


Figure 4.4. Young adult GAERS spent more time in central zone and performed more entries in central zone.

Behaviour on the 10 min OFT (A) was evaluated examining the permanence and entries in central zone (B, C, respectively), fecal boli counts (D), distance travelled (E) and average speed (F) in young adult male Wistar (n=24), NEC (n=24) and GAERS (n=26) rats. Representative movement tracks for Wistar (G), NEC (H) and GAERS (I). Anxiety-like measures show differences for ASs model (**, ***p). Data expressed as the mean $\pm$ SEM (n=21-24); * $p<0.05$, ** $p<0.01$, *** $p<0.001$ one-way ANOVA followed by Tukey pairwise multiple comparisons tests.

4.1.1.2 | Recognition memory and object placement is unimpaired in young adult GAERS, although presenting spatial working memory deficits.

4.1.1.2.1 | Novel Object Recognition Test

After 2 days of habituation to open field arena, NOR test was performed to investigate cognitive function in short and long-term memory (Fig. 4.5 A-C, D-F, respectively). Regarding short-term

memory, during the learning phase, GAERS displayed reduced total exploration of the objects compared to Wistar (GAERS=61.3±4.93, Wistar=82.7±8.30; $p=0.042$; Supplementary Data, Fig. SF 7.1A). In the test phase, after a short delay of 5 min, no differences were found for total time exploration (Fig. SF 7.1B). All groups showed a preference for the novel object-based on novelty index (Fig. 4.5B) and increased percentage of novel object exploration (> 50%, Fig. 4.5C). Also, the discriminate competence did not differ between groups.

To test Long-term memory, intertrial interval was increased to 24h between learning and testing. During the learning phase, GAERS also had a lower time of exploration of the objects compared to Wistar (GAERS=57.9±5.76 s, Wistar=77.6±4.84s; $p=0.020$; Fig.SF 7.1C). In the testing phase, epileptic animals also displayed lower interest in exploring objects (GAERS=56.9±7.42s, Wistar=79.7±5.11s; $p=0.018$; Fig SF 7.1D). Nevertheless, all groups showed a preference for the novel object (Fig. 4.5E, F). Therefore, both controls and GAERS were able to discriminate the objects after a delay of 5 min and 24h without significant differences in performance.

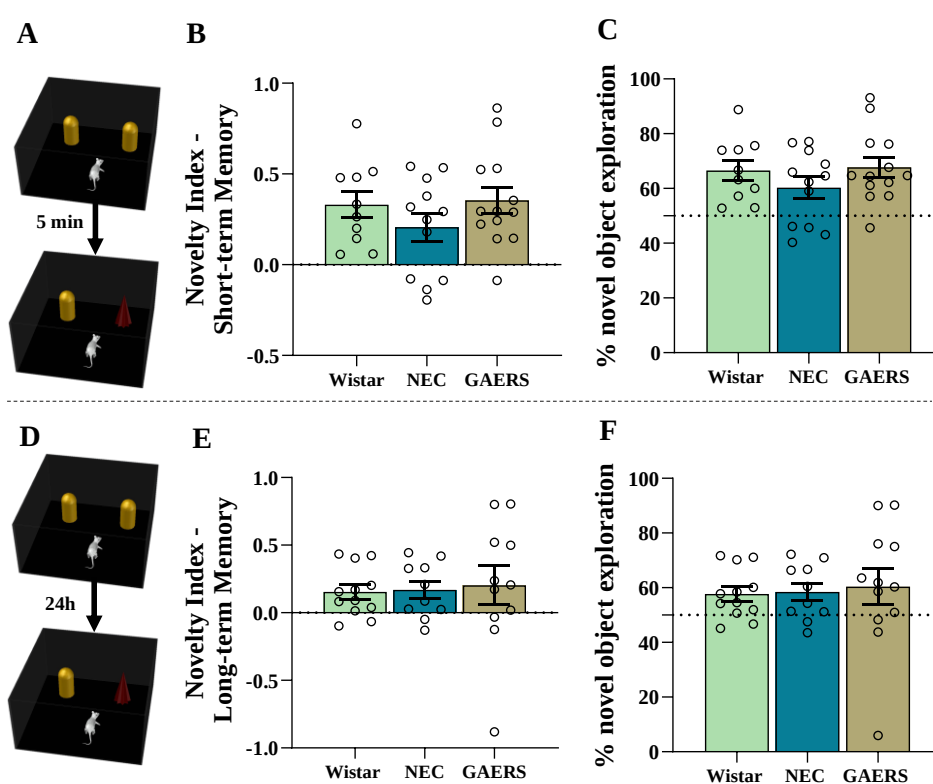


Figure 4.5. Young adult Wistar, NEC and GAERS have intact short and long-term recognition memory.

In 5 min trial, novelty index and percentage of novel object exploration were evaluated for short-term memory (A-C) and long-term memory (D-F) in young adult male Wistar (n=12), NEC (n=12) and GAERS (n=12) rats. No differences between groups were observed. Data expressed as the mean ± SEM (n=12); one-way ANOVA followed by Tukey pairwise multiple comparisons tests.

4.1.1.2.2 | Novel Object Placement Test

Following, NOP test was performed to evaluate spatial recognition memory at 5 min and 24h delay. Regarding short-term memory (Fig. 4.6A), during the learning phase, Wistar spent less time exploring the objects (Wistar=45.4±4.77, NEC=62.5±4.57; $p=0.040$; Fig. SF 7.2A), notwithstanding there was no significant difference in total exploration between NEC and GAERS (Fig. SF 7.2A). In the testing trial, all groups showed significant preference for the novel location object vs the familiar location object, showing similar novelty indexes and percentage of novel object exploration above 50%

(Fig. 4.6B,C). In the testing phase, no differences were observed for the total time of exploration (Fig. SF 7.2B).

After a 24h delay (Fig. 4.6D), during the learning phase, there were no significant differences in total exploration between groups (Fig. SF 7.2C). In the testing phase, after a 24h delay, exploration time did not differ between ASs model and controls (Fig. SF 7.2C). However, none of the groups were able to discriminate the novel location of the object, performing novelty index close to null (Fig. 4.6E,F).

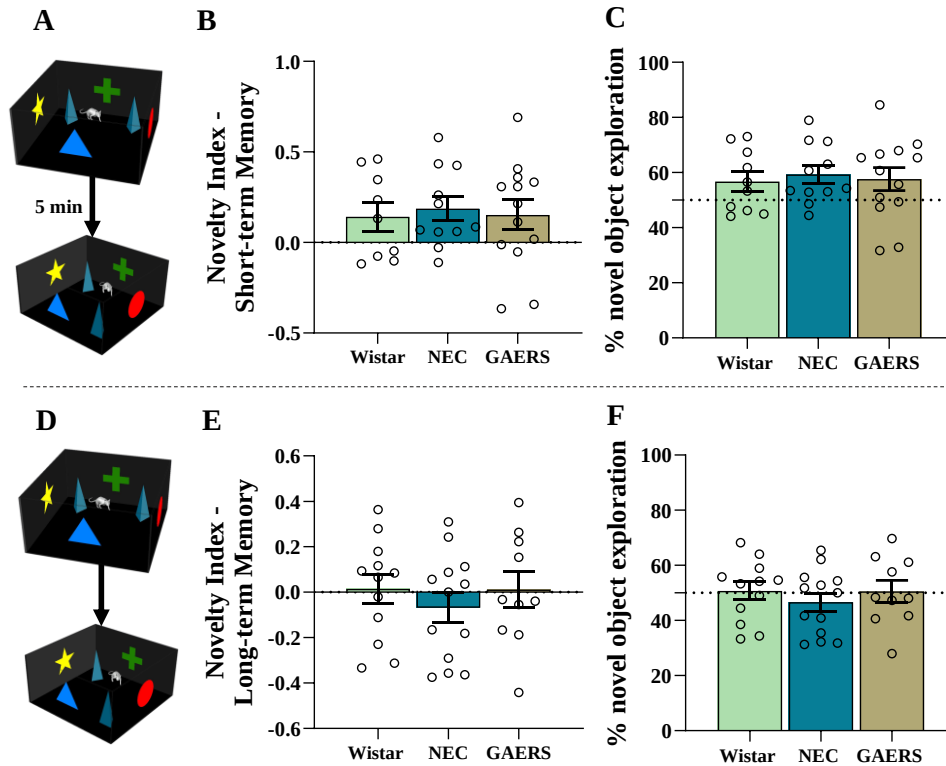


Figure 4.6. Young adult Wistar, NEC and GAERS have similar performance in short term identification of novel object location and fail to comply with the task in longer delays.

In 5 min trial, novelty index and percentage of novel object location exploration was evaluated for short-term memory (A-C) and long-term memory (D-F) in young adult male Wistar (n=12), NEC (n=12) and GAERS (n=12) rats. No differences between groups were observed. Data expressed as the mean $\pm$ SEM (n=10-12); one-way ANOVA followed by Tukey pairwise multiple comparisons tests.

4.1.1.2.3 | Y-Maze Spontaneous Alternation

To determine working memory capabilities, Y-maze test (Fig. 4.7A) was assessed on controls and GAERS to examine their spontaneous alternation. The total number of arm entries and the percentage of triples of arm entries in which the rat visited sequentially each possible arm without repetition were verified. No significant differences were observed for the total number of arm entries (Fig. 4.7B), suggesting no deficit in motor behaviour and exploration.

For spontaneous alternation, all groups had a percentage above chance level (>50%), however, there was a significant difference between groups ($F_{2,71}=4.69$, $p=0.012$). While comparing GAERS with both controls, there was a significant decrease in correct alternations (GAERS=65.8 $\pm$ 2.53%) as to Wistar (Wistar=75.5 $\pm$ 2.14%; $p=0.016$; Fig. 4.7C) or NEC (NEC=73.6 $\pm$ 2.41; $p=0.055$; Fig. 4.7C),

revealing a working memory impairment in epileptic animals. The difference between NEC and GAERS is a strong tendency, not being significantly different.

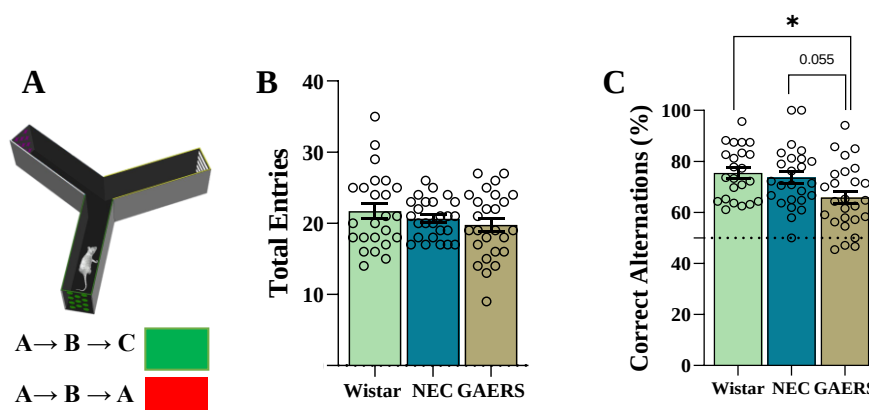


Figure 4.7. Young adult GAERS have spatial working memory impairment in Y-Maze test.

Performance in the 8 min Y-Maze paradigm (A), represented by total number of entries (B) and percentage of correct alternations, as a measure of retrograde working memory (C) in young adult male Wistar (n=23), NEC (n=24) and GAERS (n=26) rats. Percentage of correct alternation is decreased ASs model (*p). Data expressed as the mean $\pm$ SEM (n=23-26); one-way ANOVA followed by Tukey pairwise multiple comparisons tests.

In closure, results from Experiment 1 indicated that young adult male GAERS have normal locomotion parameters similar to controls, decreased anxiety-like behaviour, and non-existent indications of neophobia. Confirming that the memory results obtained were not biased by a decreased locomotion and/or increased anxiety-like behaviour, as suggested by previous studies concerning GAERS (Jones et al. 2008). Also, Wistar, NEC, and GAERS did not exhibit impairments in simple and direct object and spatial recognition memory, although GAERS displayed a general decreased exploration time of the objects. NOP at longer delays was not successful. However, regarding working memory there was a decrease in performance in GAERS.

4.1.2 | Experiment 2. GAERS: Spatial working memory NMTP and DNMTTP task in young adult rats.

For this Experiment, 2 series were conducted, allowing a total of 8 animals per condition (GAERS, NEC and Wistar), aged 90-120 days. The timeline for this experimental set is shown in Fig. 4.8.

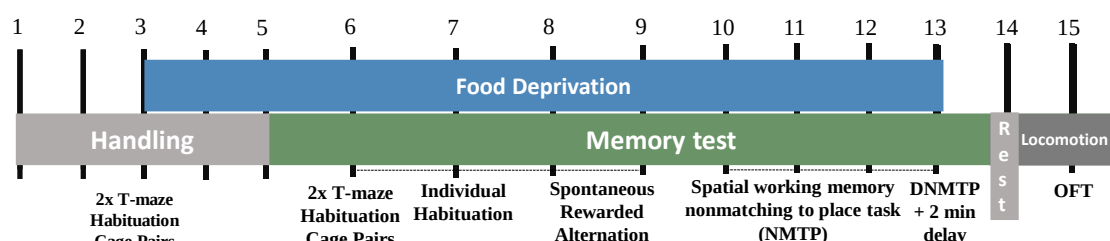


Figure 4.8. Timeline protocol of Experiment 2.

4.1.2.1 | Body weight monitorization

Rats body weight was monitored daily during all protocol to identify the decrease in body weight to 85% of initial free feeding weight, crucial to the beginning of testing phase. A significant difference

in weight between groups was observed; Wistar rats were heavier compared to both NEC ($p<0.001$) and GAERS ($p<0.001$). NEC exhibited increased weight compared to GAERS ($p<0.001$, Fig SF 7.3A,B).

4.1.2.2 | Normal working memory performance in the T-Maze non-delayed and delayed condition in young adult GAERS

4.1.2.2.1 | T- Maze NMTP task

Regarding spontaneous rewarded alternation, no differences were found between groups, comparing GAERS to NEC in both days ($p=0.1971$, Fig. 4.9B). Analysis of NMTP task indicates that GAERS initially alternated at the same rate as controls during the first 3 sessions of NMTP task (see detailed statistics in Table ST 7.2). Two-way ANOVA analysis of the effect of day and group on performance revealed no statistically significant interaction between the effects of days and groups ($F_{6,63}=1.18$; $p=0.33$, Fig. 4.9C). There was a statistically significant effect of day on the performance ($p=0.017$). Yet, the group did not have a significant effect on performance. When all animals have achieved a performance level above 70% of correct alternations, a retention interval of 2 min (day 4) was applied between sample and choice runs. During this retention, the performance of all groups declined and the rate of decline was comparable between groups. These results showed that GAERS were not impaired in the acquisition of the T-maze NMTP task and were as sensitive as both controls to the introduction of retention intervals between sample and choice runs. *Videlicet*, were as sensitive as controls to proactive interference generated by manipulating the ITI and increasing the working memory demands.

Regarding specific trials locomotion measurements, there was a general decrease in latencies and time in choice zone during NMTP task, whereas velocity generally increased. However, on day 3, during forced trials, NEC displayed increased latency to the choice zone, compared to both Wistar ($p=0.0365$, Fig. SF 7.4) and GAERS ($p=0.0472$, Fig. SF 7.4). On day 1, during choice trials, GAERS displayed an increase in latency to choice zone compared to Wistar ($p=0.0347$, Fig. SF 7.4) On day 2, in the choice zone, GAERS and NEC showed increased average speed compared to Wistar ($p=0.041$, $p=0.0499$, respectively, Fig. SF 7.4). An increase in average speed in the entry was observed for Wistar and NEC during the first 3 days (1.35 fold change (fc) and 1.39 fc, respectively) and a decrease of 1.01 fc was observed for GAERS.

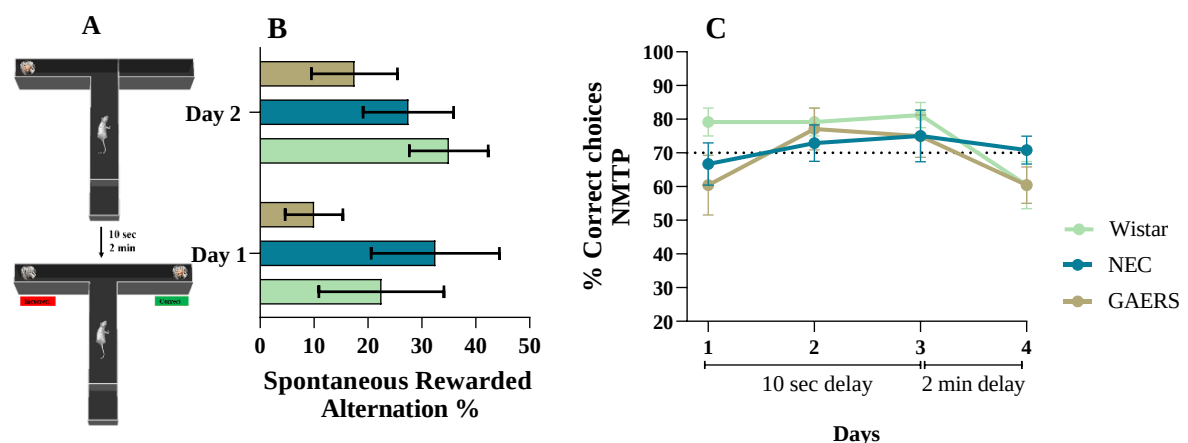


Figure 4.9. Young adult GAERS don't have impaired spatial working memory performance during NMTP task (A).

During spontaneous rewarded alternation, no significant differences were identified between groups (B). In NMTP and DNMTTP task, during the forced trial, rats were forced to enter either left or right goal arm and receive a reward. After a delay (10s or 2 min in the case of DNMTTP task), during the choice trial, rats are required to go to the previously unvisited arm for a second reward. All groups were able to achieve a performance level above 70% on the NMTP task. Mean percentage of trial with corrected choices (C). Data expressed as the mean $\pm$ SEM ($n=8$); two-way ANOVA followed by pairwise Tukey multiple comparisons test.

Ultimately, GAERS have normal working memory performance in the T-Maze NMTP and DNMTTP task. An important point to be made is that the second batch conducted, comprising all Wistar, 2 NEC and 2 GAERS, the behaviour of the non-epileptic controls and epileptic animals was inconsistent with the first batch, decreased learning, and animals were aggressive and stressed. This situation could be due to an emergence change of housing conditions due to lack of functioning of the extraction system and obligation to join our colony with females, other individuals, and new elements for the animals under testing, during the NMTP task.

4.1.2.3 | Open Field Test

To determine locomotor parameters on adult GAERS after a stimulation memory protocol, measures of locomotion were assessed in the OFT. Changes in distance travelled were observed; GAERS travelled reduced distances (GAERS=2893±190.1cm, NEC=4335±280.8cm; $t(10)=2.99$; $p = 0.01$; Fig. SF 7.5B). Nevertheless, there were no differences in average speed (Fig. SF 7.5A).

4.1.3 | Experiment 3. GAERS: Anxiety-like behaviour, Locomotion, demanding recognition memory in adult rats

Taking into account that memory data in Experiment 1 might be biased by a decreased exploration of the objects from the ASs model or feasible loss of interest, the current experiment was designed to minimize as many of those confounds as possible. 1) The decreased exploration might be due to a reduced size of open field arena; 2) higher proximity of the animals to the object and possibility of hiding behind the objects; 3) sides of the box were not opaque, built with mirrored acrylic; 4) All animals learned the NOR task, the objects yellow (cylinder) and red (pyramid) were too simple /obvious, easily recognisable. The difficulty of the test was increased by incrementing the complexity of the objects used- a *Pedras Salgadas* bottle and a *Cristal beer* bottle.

For this Experiment, 1 series of 8 animals per condition (GAERS and NEC) were used; aged 180-190 days. Firstly, it was imperative to understand if adult animals of the colony also displayed reduced anxiety-like behaviour and inexistent locomotion deficits. The timeline for this set of experiments is shown in Fig. 4.10.

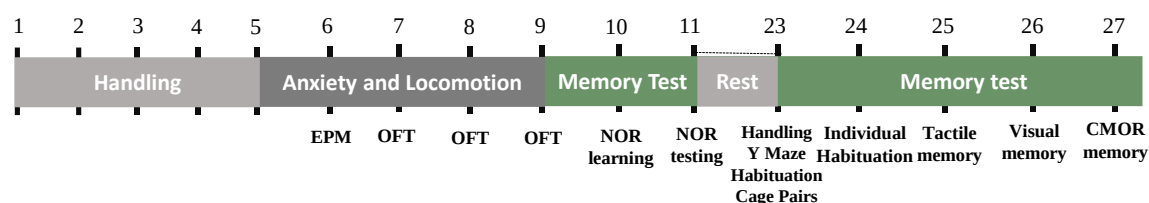


Figure 4.10. Timeline protocol of Experiment 3.

4.1.3.1 | Anxiety-like behaviour is decreased in adult GAERS

4.1.3.1.1 | EPM

To determine if the differences between the ages used in the first and second experiments reflected differences in the locomotor activity of the animals, EPM (Fig. SF 7.6A) was performed and scored similarly to that described in experiment 1. Anxiety-like parameters reveal that GAERS performance did not significantly differ from the respective control regarding the percentage of total time spent in the open arms (Fig. SF 7.6B) and the number of open arm entries (Fig. SF 7.6C). The total

number of entries (Fig. SF 7.6D) used as an index of locomotor activity also did not reveal differences between groups.

4.1.3.1.2 | OFT

As a complement measure of anxiety-like behaviour and general locomotion, OFT (Fig. SF 7.6E) was performed as described in Experiment 1. As expected, no differences were found regarding anxious behaviour, namely permanence, and entries in central zone, nor in fecal boli count (Fig. SF 7.6F-G). Locomotor parameters did not display differences as well in average speed (Fig. SF 7.6E,F), nor in specific zone measurements (Fig. ST 7.3). In GAERS, seizures may be a major cause of immobility. As observed in ST 7.3, a significant difference in total immobility time was observed; unexpectedly, NEC displayed an increase in immobility time compared to GAERS (GAERS=36.20±6.978, NEC=53.86±4.174; $t(14)=2.71$; $p=0.048$, Table ST 7.3).

4.1.3.2 | Demanding long-term recognition memory and crossmodal object recognition is impaired in adult GAERS

4.1.3.2.1 | Modified NOR – Higher object complexity

Given that the previous measures of locomotion indicated that adult animals of the colony have a similar general behaviour compared with young adults, memory tests in adult animals were progressed. Optimization of the NOR protocol was assessed, by augmenting the arena size of the open field, increasing the complexity of the objects used, and diminishing their size to avoid animals hiding behind them, so that the confound of low exploration time of GAERS could be overcome.

After 2 days of habitation to the new open-field arena, the learning phase occurred by presenting 2 familiar objects followed by 24h delay testing to assess long-term memory. In the learning phase, no differences between groups were observed regarding total object exploration time ($p=0.068$; Fig. SF 7.7A). In the testing phase, even though the complexity of the objects was advanced as well as the size of the arena, GAERS exhibited a lower total exploration time of the objects (GAERS=32.56±5.47, NEC=50.25±4.96; $t(14)=2.4$; $p=0.031$; Fig. SF 7.7B). Regarding the novel object recognition capacity, there was a significant difference between groups, with GAERS showing a lower novelty index (GAERS=0.0196±0.08, NEC=0.212±0.039; $t(13)=2.241$; $p=0.043$; Fig. 4.11B), not being able to discriminate the novel object from the familiar one. This result is corroborated by the decreased percentage of time exploring the novel object compared to NEC ($p=0.0241$, Fig. 4.11C).

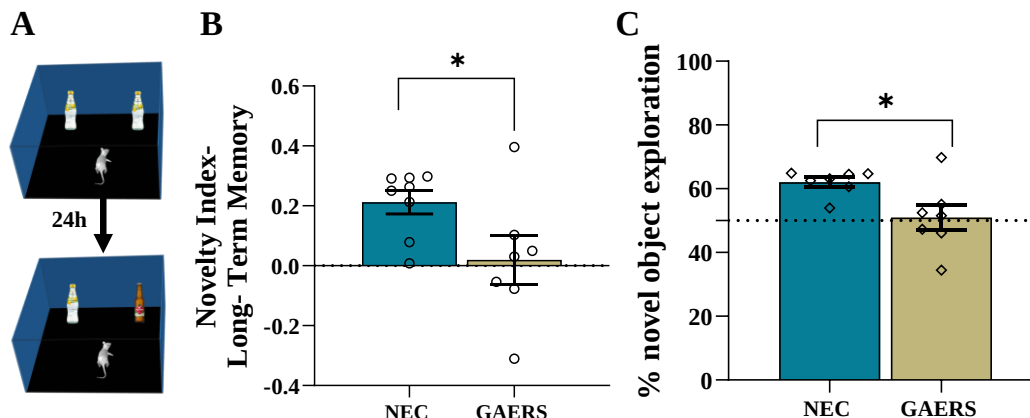


Figure 4.11- Adult GAERS have impairment in complex long-term memory NOR paradigm (A).

In 5 min trial, novelty index (B) and percentage of novel object exploration (C) was evaluated for long-term memory. Differences in performance between groups were observed, defined by GAERS lower exploration of the novel object (*p). Data expressed as the mean ± SEM (n=8); *p<0.05; independent Students t-test.

4.1.3.2 | CMOR

Even though cognition in GAERS is poorly known in the literature, in Saskatchewan colony, originally established in Canada, GAERS demonstrated recognition memory deficits in the visual and crossmodal variations of the CMOR task (Marks *et al.*, 2016). For CMOR performance /object information processing, the perirhinal cortex is crucial (Marks *et al.*, 2016). In order to understand cognitive similarities between colonies, CMOR test was assessed, allowing the dissociation into texture discrimination task and visual discrimination task followed by CMOR, a more challenging test, more sensitive for detection of recognition memory deficits. This test is an advantage to better identify and interpretate the deficits found in novel object recognition and increase the total time of exploration since the object exploration is restricted to two arms of the Y maze.

Analysis of total exploration time in the learning and testing phase (Table ST 7.4) revealed no significant differences in total exploration time in the sample phase. Nevertheless, in the visual sample phase, a reduced exploration time was observed compared to both tactile and crossmodal sample phases. Still, a significant group difference was found in the test phase exploration for the visual test (GAERS=24.13±3.17s, NEC=10.75±0.70s; $t(14)=4.12$; $p=0.001$, Table ST 7.4), with GAERS exploring objects more compared to NEC. Interestingly, a specific GAERS animal had multiple observable absence seizures during learning and testing, which is linked with decreased novelty indexes.

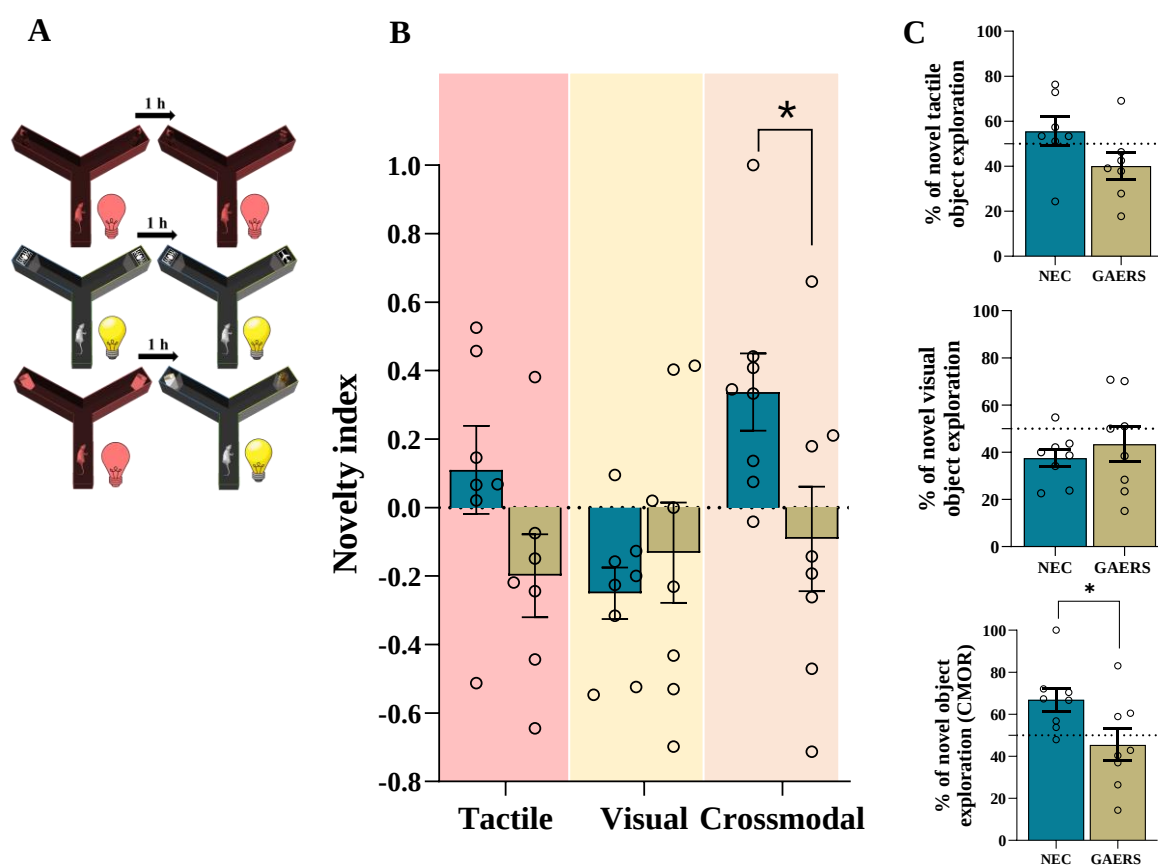


Figure 4.12. Adult GAERS have reduced crossmodal recognition memory.

Esquematised representation of the performance on Y -Maze distinct paradigm (A). Novelty index for 2 min testing trial (B), and percentage of novel tactile object, visual object and crossmodal exploration (C) for adult GAERS (n=8) and NEC (n=8). GAERS showed significantly reduced crossmodal recognition memory (*p). Data expressed as the mean ± SEM (n=8); *p<0.05; independent Students t-test.

Analysis of novelty index for the tactile, visual, and CMOR memory tests (Fig. 4.12), showed significant group differences in CMOR memory, designated by a negative novelty index for GAERS (GAERS=-0.091±0.152, NEC=0.338±0.11; t(14)=2.26; p=0.04). ASs model had reduced novel object exploration compared to NEC established by the reduced percentage of novel object exploration (GAERS=45.43±7.62 %, NEC=66.87±5.62; t(14)=2.26; p=0.04, Fig. 4.12). GAERS did not perform above chance (p=0.56). On the contrary, NEC showed a performance level above chance (p=0.009). No significant group differences were observed for tactile memory (p=0.10, Fig. 4.12B) or percentage of novel object exploration (Fig. 4.12C). For visual memory, although assessed with validated visual objects, none of the groups were able to distinguish between familiar and novel objects, with both displaying a negative novelty index (Fig. 4.12B) and percentage of novel object exploration lower than 50% (Fig. 4.12C).

4.1.4 | Experiment 4. GAERS: Final colony characterization. Anxiety-like behaviour, locomotion, demanding recognition memory, working and reference memory in young adult rats

For this Experiment, 1 series of 6 animals per condition (GAERS, NEC, and Wistar) was used; Animals with 90-100 days of age was used, to conclude the behavioural characterization of Lisbon colony and specifically to confirm recognition memory in young adult rats and test spatial memory. The timeline for this experimental set is shown in Fig. 4.13.

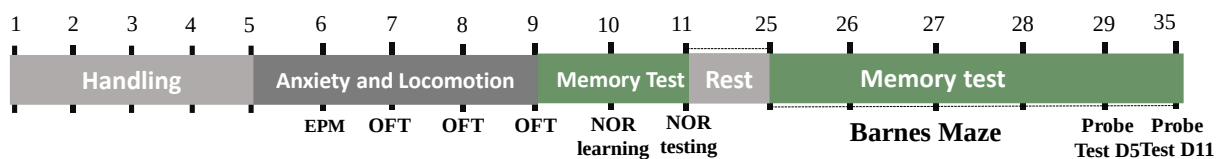


Figure 4.13. Timeline of Experiment 4.

4.1.4.1 | Anxiety-like behaviour is reduced in young adult GAERS

4.1.4.1.1 | EPM

The EPM was firstly performed to assess the same parameters as previously shown in Experiment 1 and 3 to guarantee no confound of locomotion and anxiety in the final tests. No differences between groups were found regarding the percentage of time in open arms (Fig. SF 7.8B) and the number of open arm entries (Fig. SF 7.8C). Conversely, as in experiment 3, comparison of total number of arm entries, found differences between groups ($F_{2,14}=7.79$, $p=0.005$); NEC showed general increased alternation among arms (NEC=57.8±3.65), compared to both Wistar (Wistar=43±3.08; $p=0.039$) and GAERS (GAERS=36.4±5.16; $p=0.005$; Fig SF 7.8C).

4.1.4.1.2 | OFT

OFT was assessed in this group similarly to experiment 1, to evaluate anxious-like behaviour, locomotion parameters and to complement EPM results. No differences between groups were found regarding the percentage of permanence in the central zone (Fig. SF 7.8F) and the number of entries in the central zone (Fig. SF 7.8G) nor the number of fecal boli (Fig. SF 7.8H). Instead, concerning locomotion parameters, NEC displayed increased distance covered compared to both controls ($F_{2,15}=6.86$; $p=0.008$; NEC=4087±214cm, Wistar=3193±200cm, GAERS=3179±181cm; $p=0.016$, $p=0.014$, respectively) and no differences in average speed. No alterations in immobility were observed.

4.1.4.2 | Demanding long-term recognition memory is impaired in young adult GAERS

4.1.4.2.1 | Modified NOR

Given that in 6 months animals deficits in object recognition, in texture and visual recognition memory were observed in experiment 3, and no differences were found in 12 weeks animals, however using a simpler protocol, this experiment aimed to understand if such deficits in more demanding tasks found in 6 months animals are shared by 12 weeks animals or is a feature of older animals.

The modified protocol of NOR was assessed similarly to experiment 3, testing long-term memory. In the learning and testing phase, no differences between groups were found regarding total object exploration time (Fig. SF 7.9). Yet, regarding the difference between time spent exploring novel and familiar objects, a difference between groups was observed $F_{2,14} = 5.91$; $p=0.014$). GAERS showed deficits in comparison with Wistar (GAERS= -0.0844 ± 0.06 , Wistar= 0.337 ± 0.110 ; $p=0.011$; Fig. 4.14B), in line with the results obtained from experiment 3 for animals with 6 months. This result is in accordance with the decreased percentage of exploration of the novel object of GAERS compared to Wistar (GAERS= $45.8 \pm 3.00\%$, Wistar= $66.9 \pm 5.51\%$; $p=0.011$; Fig. 4.14C).

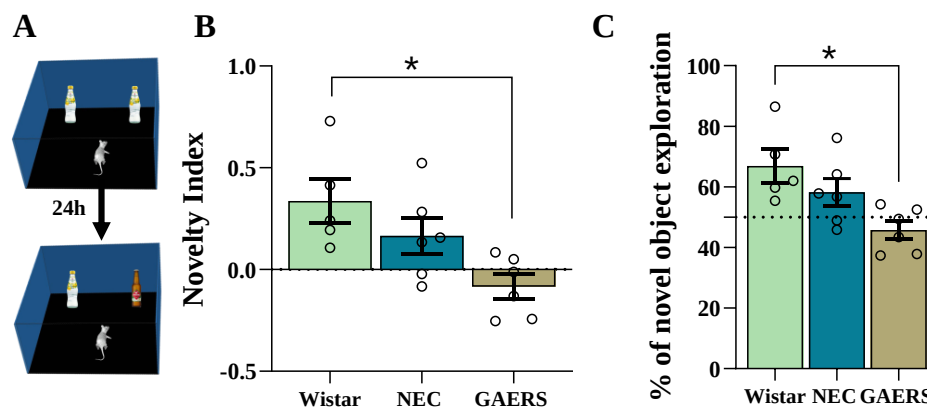


Figure 4.14. Young adult GAERS have impairment in complex long-term memory NOR paradigm (A).

In 5 min trial, novelty index (B) and percentage of novel object exploration (C) was evaluated for long-term memory. Differences in performance between groups were observed, described by GAERS lower exploration of the novel object (*p). Data expressed as the mean $\pm$ SEM (n=6); * $p<0.05$, one-way ANOVA followed by Tukey pairwise multiple comparisons tests.

4.1.4.3 | Short-term and long-term reference memory is impaired in young adult GAERS in the Barnes Maze

4.1.4.3.1 | Acquisition phase

During the training days, score, latency, distance travelled and the number of errors until entering the target hole were measured. The least three measures were applied to the first find of the hole (primary) and for entering the hole (total). Primary latency significantly decreased along the four training days in each group (54% decrease in GAERS, 81% decrease in Wistar, and 74% in NEC, Fig. 4.15B) marked for NEC ($p=0.022$, Fig. 4.15B), indicating a mean improvement of all groups. However, a tendency to performance decline for GAERS occurred in the last day ($p=0.0709$), not being significantly different from controls. For total latency, NEC exhibited the highest mean value compared to Wistar and GAERS in days 1, 2, and 4 (Table ST 7.5, Fig. 4.15C), associated with higher maze exploration. Nevertheless, a diminished effect in all groups was observed for total distance, not being so noticeable in the GAERS group (Fig. 4.15E).

Concerning distance travelled, primary path length did not differ between groups diminishing in all clusters (69% decrease in GAERS, 65% decrease in Wistar, and 76% decrease in NEC), decreasing significantly for NEC ($p=0.0173$). For total path length, NEC also exhibited a higher mean value compared to both Wistar and GAERS, due to increase exploration in days 1, 2, and 4 (Table ST 7.5). Wistar decreased significantly total distance travelled until the end of the training protocol ($p=0.024$).

In regard to primary errors, no group differences were found during the acquisition phase. NEC significantly decreased primary errors ($p<0.001$). However, in line with a decrease in performance observed for latency, on the fourth day of acquisition, GAERS also showed surprisingly an increase in the primary number of errors, detailing a worsened performance on the last day of acquisition, although no statistical significance was observed ($p=0.3374$). For the total number of errors, NEC committed more total errors, which is linked with higher hole exploration and Wistar significantly decreased the total number of errors ($p=0.027$). For primary and total measures, from Day 1 to 4, errors significantly decreased for NEC and Wistar indicating that rats learned to use reference points, and possibly follow spatial cues (72% decrease, $p=0.057$ for Wistar and $p<0.01$ for NEC). The same significant decrease was not observed for GAERS (45% decrease, $p=0.246$, Fig. 4.15A).

Considering the hole deviation score, the lower the score, the higher the proximity of the escape hole. For this measure, no differences were found between groups, and the scoring trend was to diminish during the acquisition phase in all groups (Fig. 4.15H).

Regarding escape strategies (Fig. 4.16), during D1 all groups used random, serial, and direct strategies to find the target hole. Wistar and NEC showed a decrement of random strategies throughout the training days (D4=100 % for Wistar, 86 % for NEC, and only 50% for GAERS). In contrast, direct strategies increased in those groups (180% for Wistar, 600% for NEC, and only 125% for GAERS). On day 4 of acquisition, Wistar and NEC showed more direct strategies compared to GAERS, indicating a clear preference for direct spatial strategies, using extra maze cues, over serial and random strategies.

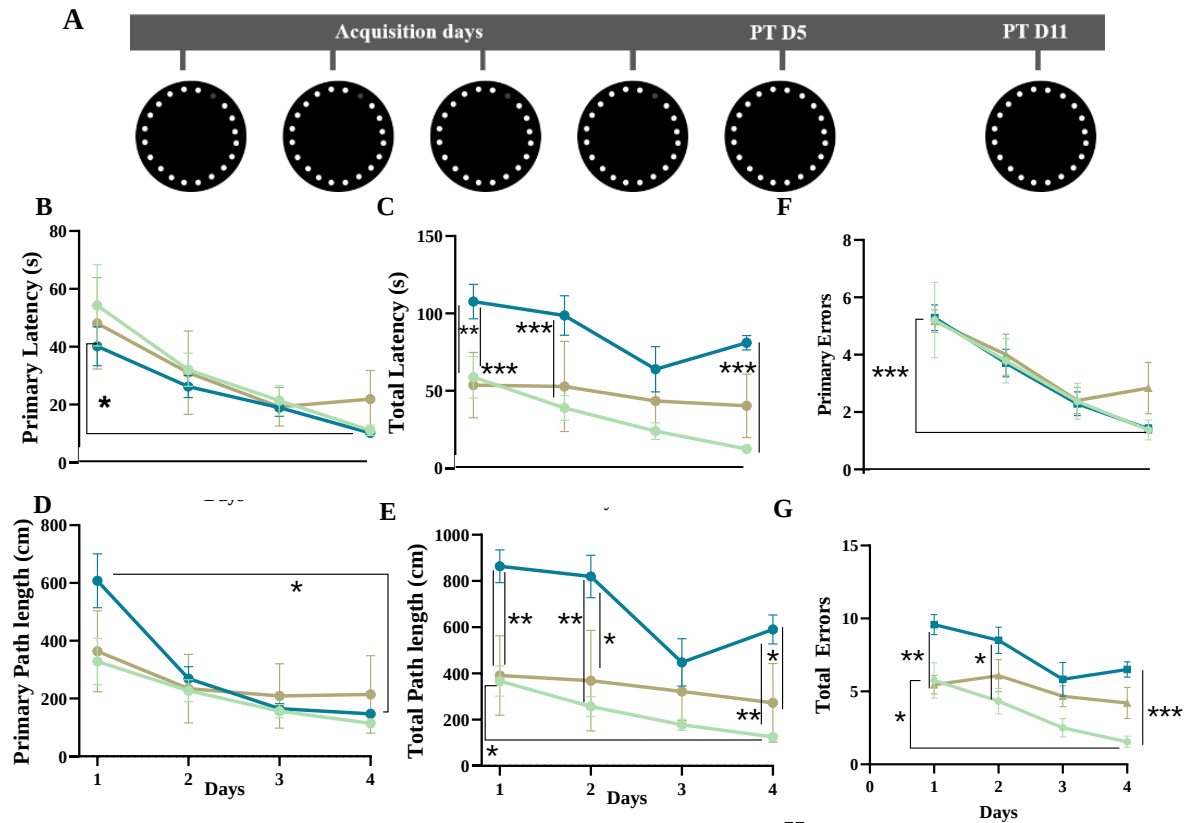


Figure 4.15. Young Adult GAERS did not show significant differences in acquisition phase.

Rats were trained in the Barnes Maze (A), Primary latency (B), total latency (C), primary path length (D) and total path length (E) were measured. Primary Errors (F), total number of errors (G) and Hole deviation Score (H) were analysed in young adult male Wistar (n=6), NEC (n=6) and GAERS (n=6) rats. Significant differences were only found in total measures, indicating a higher mean values of exploration for NEC compared with both GAERS and Wistar (*, **, ***p). Data expressed as the mean $\pm$ SEM (n=6); *p<0.05, **p < 0.01, *** p<0.001, Two-way ANOVA followed by Tukey pairwise multiple comparisons tests.

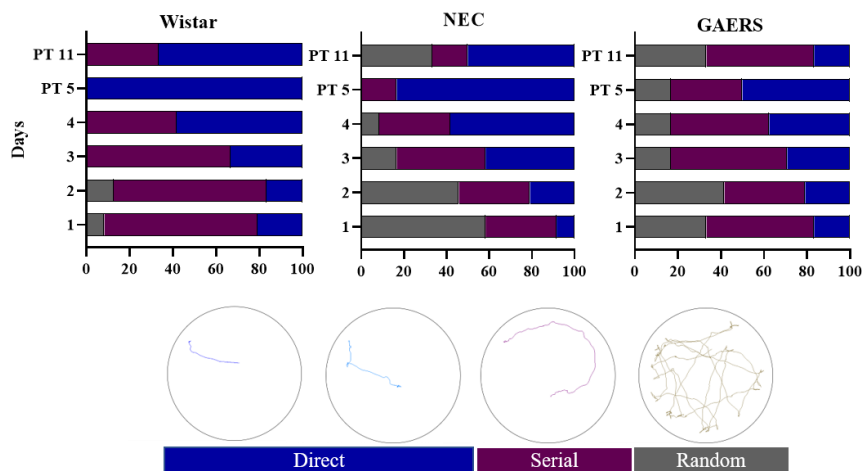


Figure 4.16 GAERS display decreased direct strategies during acquisition and probe trials.

Trials were described as Direct/Corrected (blue), Serial (purple) and Random (grey) for acquisition days (1-4) and probe test day 5 and day 11.

4.1.4.3.2 | Probe Tests

On probe test, day 5, namely the short-term retention phase, the number of visits to each hole was scored, as well as the primary latency, path length, permanence close target hole, primary and total errors, as well as the strategy used. Wistar and NEC performed significantly more visits to the target hole compared to GAERS ($p < 0.001$, Fig. 4.17A), and NEC explored more +1 hole compared to GAERS ($p = 0.034$, Fig. 4.17A). Primary latency to reach the target hole did not differ between groups, however, there was a strong tendency between NEC and GAERS ($p = 0.059$; Fig. 4.17B). Primary path length and primary errors did not show differences between groups (Fig. 4.17C,D), whereas the total number of errors was statistically higher in GAERS (GAERS = 12 ± 0.52) compared to Wistar (Wistar = 6.67 ± 1.43 ; $p = 0.002$) and NEC (NEC = 7.50 ± 0.428 ; $p = 0.009$; Fig. 4.17E), showing that GAERS performed worse compared to both controls. Even though during acquisition NEC always showed a propensity to higher search for holes, the contrary applies in the probe test, in which the behaviour of GAERS worsened, performing shoddier when the escape box was not in the hole. NEC appeared to remember where the box was, performing fewer errors compared to GAERS. The same applies to strategy, in which Wistar only performed direct strategy, NEC displayed both direct and serial strategy, whereas GAERS exhibited random strategies (Fig. 4.16).

On probe test day 11, i.e. long-term retention phase, the same parameters as in day 5 were measured. Regarding the number of visits to the target hole, there was no difference between groups, all visiting similarly the target hole. However, unexpectedly, GAERS displayed a preference for hole 5 ($p = 0.044$; Fig. 4.17F), the nearby hole of the experimenter exit door. GAERS took longer to reach the target hole compared to Wistar (GAERS = 28.2 ± 6.40 , Wistar = 5.40 ± 0.678 ; $p = 0.027$; Fig. 4.17G). Similarly, to the first probe test, no differences were found regarding primary path length (Fig. 4.17H). Apart from that, strong statistical significance was found for the primary and total number of errors, being increased in GAERS compared to both controls. ASs animals displayed increased number of primary errors compared to Wistar (GAERS = 6.17 ± 1.62 , Wistar = 0.200 ± 0.200 ; $p = 0.007$; Fig. 4.17I) and NEC (NEC = 1.20 ± 0.800 ; $p = 0.022$; Fig. 4.17I). For total number of errors, the same was observed (GAERS = 19.8 ± 0.735 , Wistar = 11 ± 1.63 , $p = 0.007$; Fig. 4.17J) and NEC (NEC = 11.30 ± 2.09 , $p = 0.009$; Fig. 4.17J). Regarding strategy, Wistar opted mainly for a direct strategy, whereas NEC and GAERS used the three possible strategies, with NEC bending to use a more direct strategy compared to GAERS (Fig. 4.16)

Overall, no major differences were observed during acquisition, however, differences in measurements in Barnes Maze indicated deficits in reference memory, more pronounced in longer retention interval probe test.

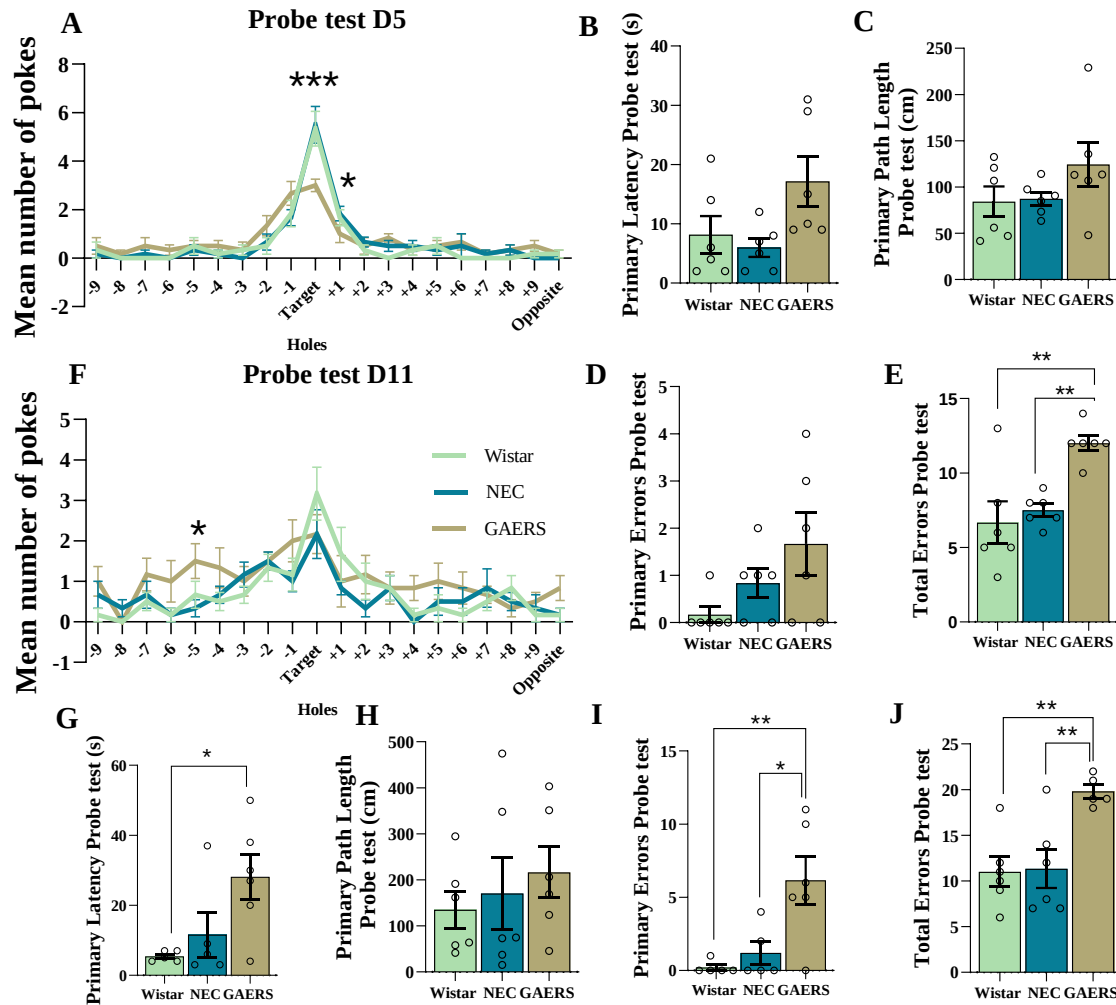


Figure 4.17. In the probe test, young Adult GAERS display fewer visits to the target hole and an increased number of errors.

The probe test was applied in day 5 (A-E) and day 11 (F-J). The mean number of pokes in holes (A, F), primary latency (B, G), primary path length (C, H), primary errors (D, I), and the total number of errors (E, J) were analysed. Reference memory parameters showed differences between groups for the mean number of pokes in the target hole, primary latency, and the number of errors (*, **, ***p). Data expressed as the mean $\pm$ SEM (n=6); *p<0.05, **p < 0.01, ***p < 0.001 one-way and Two-way ANOVA followed by Tukey pairwise multiple comparisons tests.

4.1.5 | GAERS phenotypical behavioural characterization

The results found in Experiment 1 to 4 were summarized in the table below (Table 4.1), distributed into anxiety-like behaviour, locomotion, and learning and memory performance.

Table 4.1 Summary table of Absence Seizures Rats Model phenotypical behavioural characterization. (GAERS compared to controls; =: GAERS equal behaviour; ↑: GAERS increased parameter; ↓ GAERS decreased parameter)

GAERS	
Anxiety-like behaviour	
Test	Phenotypic Observation
Elevated Plus Maze	↓ anxiety-like behaviour (90-120d) = anxiety-like behaviour (180 -190 d)
Emergence Test	↓ anxiety-like behaviour ↓ neophobia
Open Field Test	↓ anxiety-like behaviour (90-120d) =anxiety- like behaviour (180-190d)
Locomotion	
Open Field	= general locomotion
Learning and Memory Cognition	
Novel Object Recognition	= short-term recognition memory (5min delay, 90-120d) = long-term recognition memory (24h delay, 90-120d) ↓ object exploration time
Modified Complex Novel Object Recognition	↓ long-term recognition memory (90-120 d) ↓ long-term recognition memory (180-190 d) = object exploration time
Crossmodal Object Recognition Memory	↓ crossmodal recognition memory (1h delay, 180-190 d) ↓ texture memory ³ = object exploration time
Y-Maze	↓ spontaneous alternation (90-120d) ↓ spatial working memory
T-Maze	= spontaneous rewarded alternation (90-120d) = spatial working memory (NMTP and DNMTP task)
Novel Object Placement	= short-term memory (5 min delay, 90-120 d) ? long-term memory (24h delay) =object exploration time
Barnes Maze	= acquisition memory (90-120d) ↓ short-term retention (24h delay) ↓ number of visits to target hole ↑ total of errors ↓ direct strategy ↓ long-term retention (7 days delay) ↑ primary latency ↑ primary errors ↑ total errors ↓ direct strategy ↓ spatial working memory ↓ reference memory

³ not statistically significant

4.1.6 | Experiment 5: Stargazer mice: Anxiety-like behaviour, Locomotion, spatial working memory, spatial and reference memory

For this Experiment, 1 series of animals (n=4 STG, n=5 WT mice) with 90- 120 days old were used. The timeline for this experimental set is shown in Fig. 4.18.

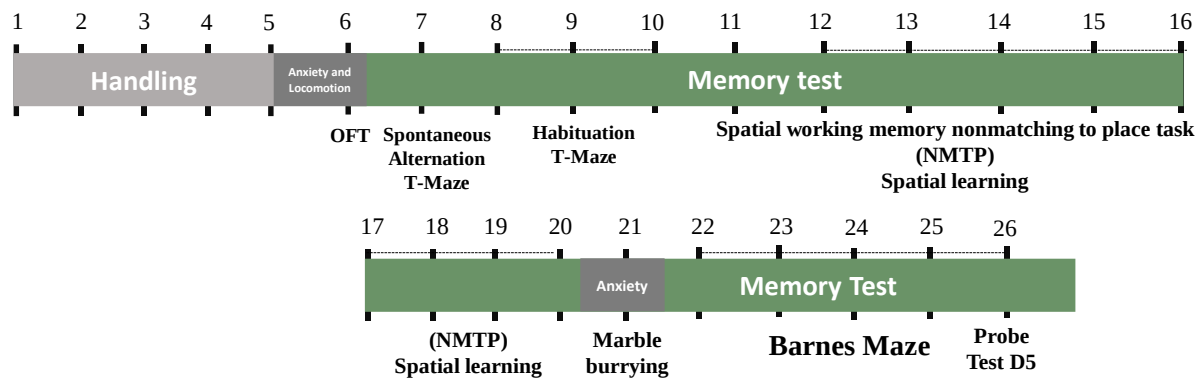


Figure 4.18. Timeline of Experiment 5.

4.1.6.1 | Anxiety-like behaviour is decreased in stargazer mice

4.1.6.1.1 | OFT

OFT was assessed for the identification of anxiety-like behaviour and specifically locomotor activity. Given that STG mice display ataxia, head tossing, and motor discoordination deficits, it is of major importance to understand if deficits would enable mice normal performance in memory tasks that involve locomotion. Regarding anxiety-like measures, no differences between control and STG were observed for the percentage of permanence in the central zone ($t(7)=1.23$; $p=0.258$; Fig. 4.19B) nor fecal boli (Fig. 4.19C). For locomotor parameters, no differences between groups were observed for distance travelled ($t(7)=2$; $p=0.072$; Fig. 4.19E) neither for average speed ($t(7)=2.078$; $p=0.076$; Fig. 4.19D). Although STG mice display motor deficiencies, OFT analysis indicated that they would be able to perform memory tasks that do not demand extensive locomotion tiredness.

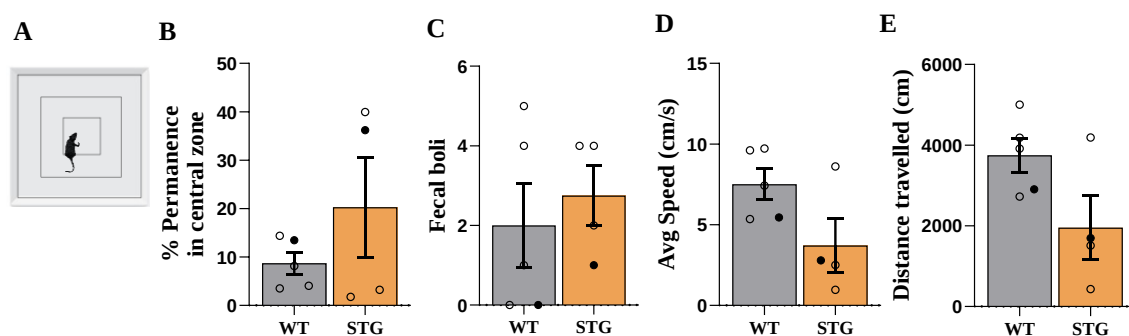


Figure 4.19. Stargazer mice displayed anxiety-like behaviour and locomotion parameters similar to WT mice. From the 10 min OFT trial (A), two measures of anxiety-like behaviour derived, % permanence in central zone (B) and fecal boli counts (C). No significant differences were observed between STG mice (orange) and WT (grey). Locomotion parameters, distance travelled (D) and average speed (E) did not show differences between groups. Data expressed as the mean $\pm$ SEM (n=5); independent Students t-test. Filled dot: male, open circles: females.

4.1.6.2 | Learning and Memory Assessment

4.1.6.2.1 | Spontaneous alternation is normal in STG mice in the T-Maze

To determine working memory capabilities, T-maze was assessed on controls and STG mice to examine their spontaneous alternation. The total number of arm entries and the percentage of triples of arm entries in which the mice visited sequentially each possible arm without repetition were verified. No significant differences were observed for the total number of arm entries (Fig. 4.20A), suggesting no deficit in exploration. Also, regarding the spontaneous percentage of correct alternations, no difference was found between groups ($t(6)=0.167$; $p=0.873$, Fig.4.20B).

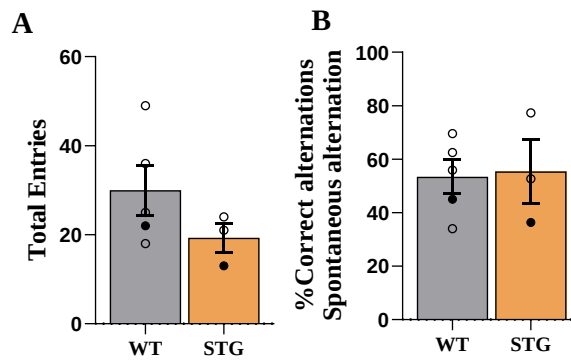


Figure 4.20. Stargazer mice did not display deficits in exploration nor in spontaneous alternation.

The total number of entries (A) and spontaneous alternation (B) were analysed. No differences between groups were observed, STG (orange) and WT (grey). Data expressed as the mean $\pm$ SEM ($n=4-5$); independent Students t-test. Filled dot: male, open circles: females

4.1.6.2.2 | Spatial learning was not achieved in the T-Maze

To test spatial learning with intra maze cues, a modified T-maze task was assessed for 10 days. Mice weight was daily recorded (Fig. 4.21B). The individual STG with decreased parameters measured in the OFT (Fig. 4.20) showed accentuated motor problems that were overcome after locomotion training in the T-Maze, being included only in the analysis from day 7 of acquisition. Unexpectedly, learning in this task was not achieved, after 10 days of acquisition neither WT nor STG mice were able to achieve at least 70% of correctness (Fig. 4.21C). Also, no differences between groups during the learning curve were observed. Mice may need more days of acquisition or were not able to distinguish between the two patterns given in the learning phase of the task, failing to recognize the existence of a reward, following a specific pattern.

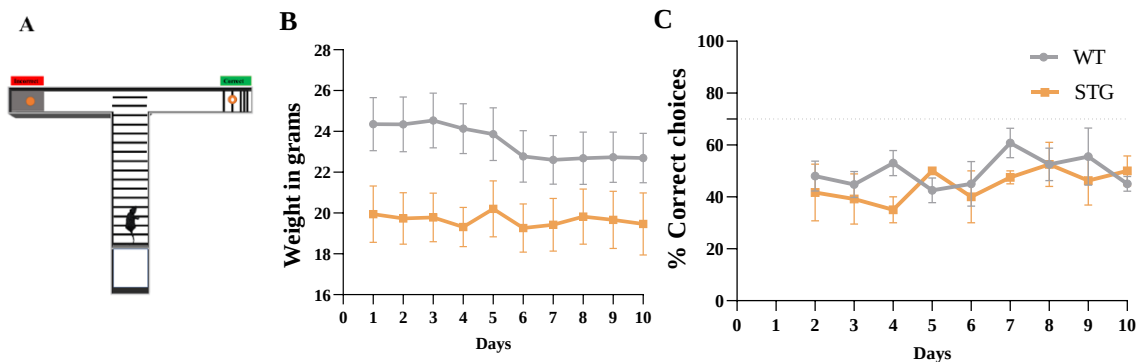


Figure 4.21. Stargazer mice spatial learning was not achieved in the T-maze (A).

Changes in animal body weight (B) and 10 days of Spatial learning (C) was assessed in STG mice ($n=4$) and WT mice ($n=5$). No differences were found between groups. Animals were not able to achieve 70 % of correct choices. Data expressed as the mean $\pm$ SEM ($n=5$); two-way ANOVA followed by Tukey pairwise multiple comparisons test.

4.1.6.2.3 | Working memory and reference memory deficits in stargazer mice in Barnes Maze

4.1.6.2.3.1 | Acquisition phase

During training days, score, latency, distance travelled and the number of errors to enter the target hole were measured. The least three measures were applied for the first visit to the target hole (primary) and for entering the hole (total).

Primary latency significantly decreased along the four training days in each group, indicating a mean improvement for all groups, however, a significant difference between days 1 and 2 of the acquisition was only observed in WT animals ($p < 0.001$; Fig. 4.22B), whereas STG only showed a significant difference at day 3 of acquisition ($p = 0.015$; Fig. 4.22B), from the beginning of acquisition. On days 2 and 3 there was a significant difference in primary latency between groups ($p = 0.012$ and 0.031 , respectively; Fig. 4.22B). On day 4 there was no significant difference between groups, STG would need more trials to successfully reach WT performance. For total latency, the significance between groups was observed in all acquisition days (D2, D3, and D4; $p < 0.001$; Fig. 4.22C). Performance from day 1 to 4 of WT was significantly better ($p < 0.001$; Fig. 4.22C) and not significantly different for STG mice.

For primary path length, a significant reduction was observed from day 1 to 4 on WT animals ($p = 0.0048$; Fig. 4.22D) and did not differ for STG animals. On day 3 of acquisition, there was a significant difference between groups ($p = 0.002$; Fig. 4.22D). For total path length, there was no significant difference between groups, however, a declining tendency was observed for WT whereas STG mice performance did not tend to diminish distance travelled along days. The highly discrepant difference from primary measures to total measures in STG may be linked to the reduced number of hole escapes during all protocols (Fig. 4.22J), being the percentage of latency to STG escape approximately 79%. STG mice displayed increased percentages of failure to reach the platform and used increased random and less directed strategies compared to control mice (79%). The results were not biased by motor dysfunctions since mice with mild phenotype were able to enter the hole.

Regarding primary errors, there was no significant difference (Fig. 4.22F). Though, regarding total errors, there was a significant reduction of WT errors ($p = 0.017$; Fig. 4.22G) on the last day of acquisition.

Apropos of the hole deviation score, significant differences were found between groups; control mice diminished the score from day 1 to 4 ($p = 0.033$), differing among groups for day 2 ($p = 0.018$), day 3 ($p = 0.003$), and day 4 ($p = 0.010$; Fig. 4.22 K), being higher for STG mice.

Regarding escape strategies, during d1 control mice used random, serial, and direct strategies to find the target hole, whereas STG mice only used random and serial strategies. WT mice showed a decrement of random strategies of 100%. In contrast, STG mice random strategies only decreased by 54%. On day 4 of acquisition, control mice showed more direct strategies compared to STG (50% vs 6.6%), indicating a clear preference for direct spatial strategies, using extra maze cues, over serial and random strategies.

During acquisition, in WT there was a linear strong positive correlation between latency and number of errors, whereas in STG mice there is no linear correlation regarding latency and number of errors, indicating once more the poor performance of the STG mice (Fig. SF 7.10).

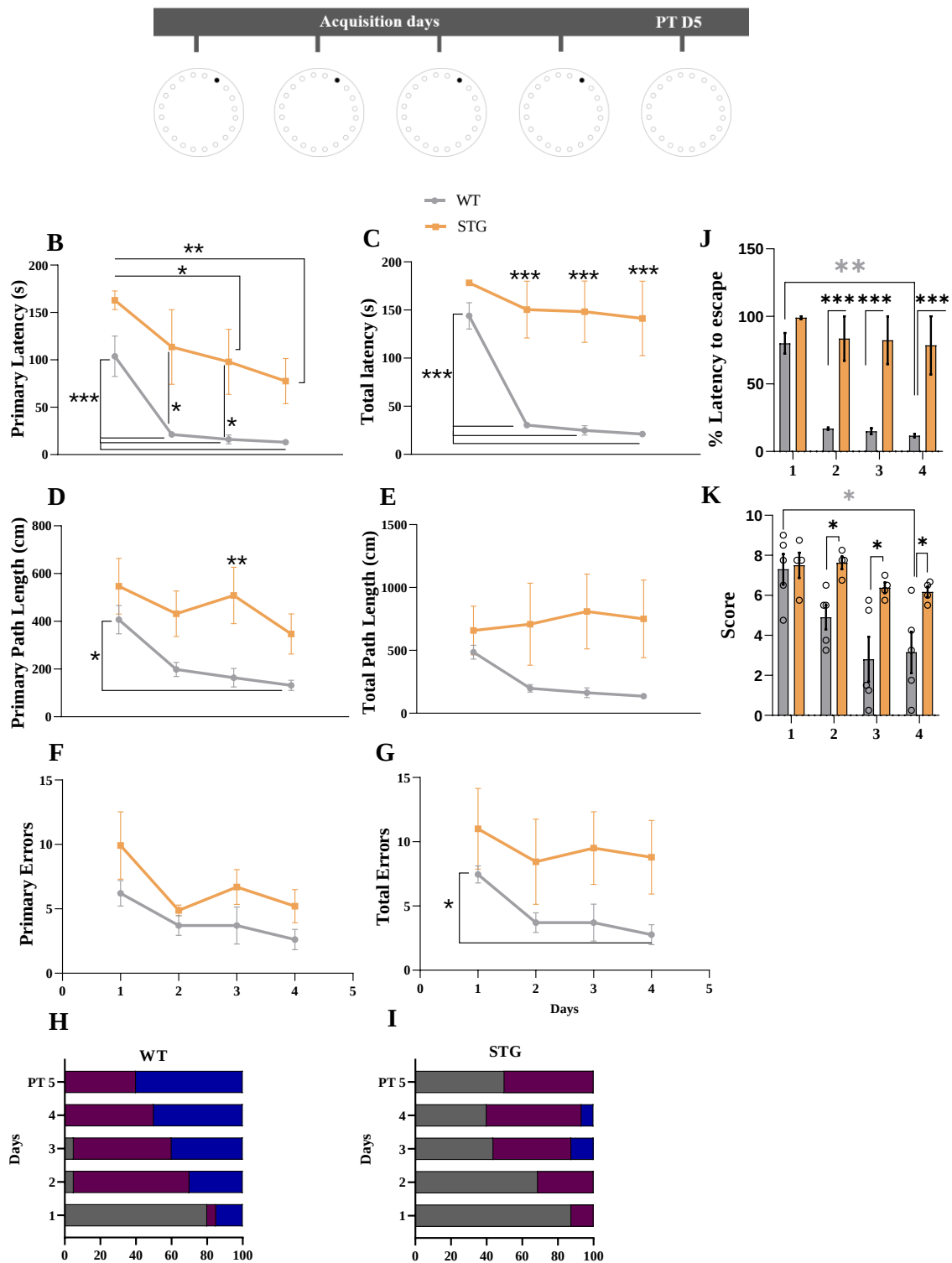


Figure 4.22. STG mice showed significant differences in acquisition phase, indicative of poor performance.

Mice were trained in the Barnes Maze (A), primary latency (B), total latency (C), primary path length (D) and total path length (E) were measured. Primary Errors (F), total number of errors (G), percentage of latency to escape (J) and hole deviation score (K) were measured. Trials were described as Direct/Corrected (blue), Serial (purple) and Random (grey) for acquisition days (1-4) and probe test day 5 (H,I). Data was analysed in adult STG (n=4) and WT (n=5) mice. Significant differences were found for all measures, except primary errors (*, **, ***p). Data expressed as the mean $\pm$ SEM (n=5); *p<0.05, **p < 0.01, *** p<0.001, Two-way ANOVA followed by Tukey pairwise multiple comparisons tests.

4.1.6.2.2.2 | Probe Test

On probe test, day 5, i.e. short-term retention phase, the number of visits to each hole was analyzed, as well as the primary latency, path length, permanence close target hole, primary, total errors, and strategy. WT animals showed significantly more visits to the target hole compared to STG ($p < 0.001$) and +1 hole ($p = 0.0061$, Fig. 4.23A). Primary latency to reach the target hole differ significantly between groups (STG = 61.50 ± 18.90 , WT = 13.38 ± 0.052 , $t(7) = 2.678$; $p = 0.03$; Fig. 4.23B), STG took longer to reach the target hole. Primary path length and % primary errors did not show differences between groups, whereas, permanence close to the target hole was statistically lower in STG mice compared to WT mice (STG = 1.675 ± 0.969 , WT = 19.66 ± 4.604 ; $p = 0.011$; Fig. 4.23C,D,E). percentage of the total number of errors was statistically higher in STG mice compared to controls ($p = 0.0067$, Fig. 4.23F), showing that STG performed worse compared to WT mice. The same applies to strategy, in which control mice only performed direct and serial strategies, whereas STG mice exhibited only random and serial strategies.

The number of errors was presented in percentage given that a decrease hole exploration has been observed during acquisition trials, so a mouse that does not explore, even if it doesn't reach the target hole, the number of errors would be decreased. To avoid this confound, errors were analysed as a percentage.

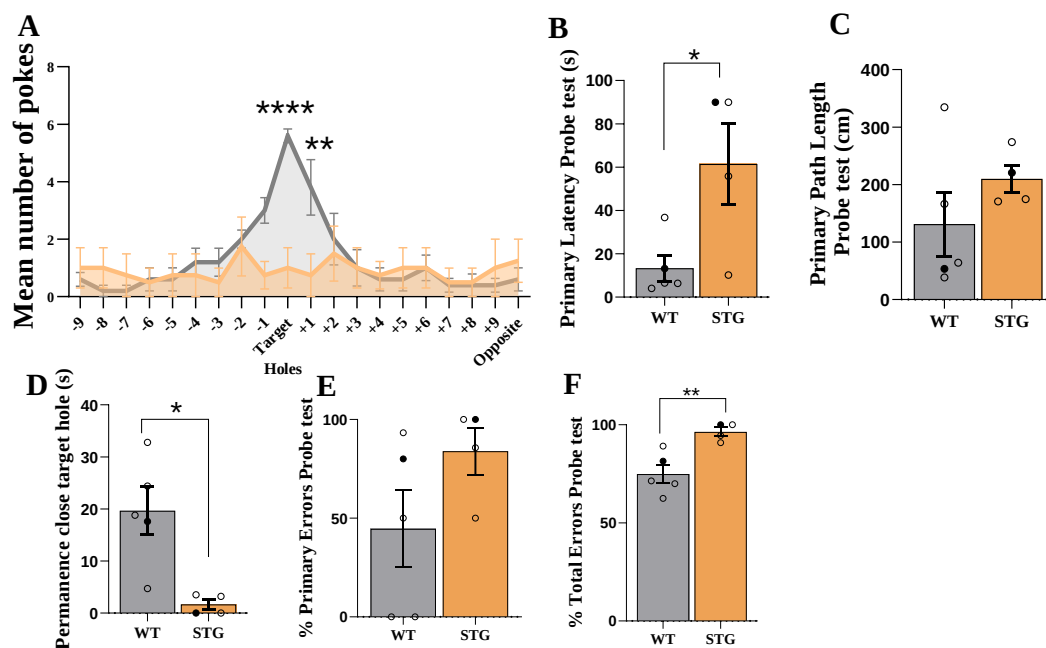


Figure 4.23. In the probe test, adult STG mice displayed less visits to target hole and increased number of errors.

The probe test was applied on day 5. Mean number of pokes in holes (A), primary latency (B), primary path length (C), permanence close target hole (D), percentage of primary and total errors (E, F) were analysed. Reference memory parameters showed differences between groups for mean number of pokes in the target hole, primary latency, permanence close target hole, percentage of total errors (*, **, ****p). Data expressed as the mean $\pm$ SEM ($n = 5$); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, independent Student's test and Two-way ANOVA followed by Tukey pairwise multiple comparisons tests.

4.1.6.3 | Anxiety-like behaviour and compulsive like behaviour is decreased in stargazer mice

4.1.6.3.1 | Marble burying

Given that MBT is a more sensitive assay of anxiety-like behaviour, this test was used as a complementary measure of the OFT, to analyse possible differences after several days of habituation to

different mazes. Analysis of the number of marbles buried during the 30-minute trial (Fig. 4.24A-C) showed no statistically significant difference between groups (WT=5.40±2.4, STG=1.25±1.25; p=0.200; Fig. 4.24D). This result was in line with OFT. However, unexpectedly, only one STG individual buried marbles. Trials were video-recorded during these experiments, allowing a clear observation of STG mice ignoring and surrounding the marbles, avoiding it during all trials, a specific unstated behaviour, not described in the literature, hereafter described as a “*pacman*”-like behaviour. This result was also intriguing given the motor deficiency in this ASs model.

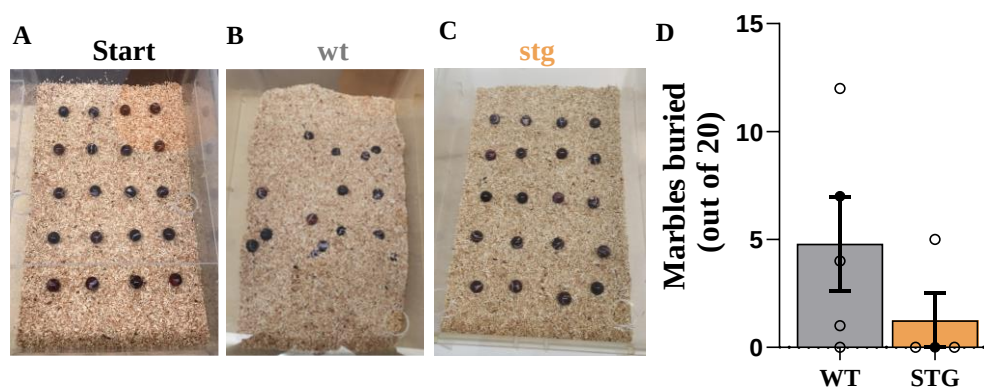


Figure 4.24. STG mice did not display anxiety-like behaviour in the MBT, although an uncommon behaviour was observed.

Representative photos of the beginning of each trial (A), end of 30-min trial for WT (B) and STG (C) mice. The number of marbles buried (D) was assessed for WT (n=5, grey) and STG mice (n=4, orange) and did not differ between groups. Data expressed as the mean ± SEM independent Student's test.

4.1.7 | Stargazer phenotypical behavioural characterization

The results found in Experiment 5 were summarized in the table below (Table 4.2), divided into anxiety-like behaviour, locomotion, and learning and memory performance.

Table 4.2 Summary table for Absence Seizures Summary table of Absence Seizures Mouse Model phenotypical behavioural characterization.

(STG compared to control mice; =: STG equal behaviour; ↑: STG increased parameter; ↓ STG mice decreased parameter)

Stargazer mice	
Anxiety-like behaviour	
Test	Phenotypic Observation
Open Field Test	=anxiety- like behaviour (90-120d)
Marble burying	= anxiety-like behaviour ↓ compulsive-like behaviour Abnormal undescribed behaviour (avoiding marbles)
Locomotion	
Open Field	= general locomotion = distance travelled = average speed

Learning and Memory Cognition	
T-Maze	= spontaneous alternation (90-120d) ? spatial learning ↓ acquisition memory (90-120d) ↑ primary latency ↑ total latency ↑ primary path length ↓ latency to escape ↑ score ↓ direct strategy
Barnes Maze	↓ short-term retention (24h delay) ↓ number of visits to target hole ↑ primary latency ↓ permanence close to target hole ↑ % total of errors ↓ direct strategy
	↓ spatial working memory ↓ reference memory

4.2 | Electrophysiological recordings in hippocampal slices

4.2.1 | Hippocampal Long-term Potentiation in GAERS

After Experiment 1, hippocampal slices from Wistar, NEC, and GAERS rats were used to evaluate hippocampal synaptic plasticity to assess the neuroanatomical basis of memory abilities. Long-term potentiation (LTP) experiments were achieved by recording field excitatory postsynaptic potentials (fEPSP). LTP is a form of synaptic plasticity, most closely linked to memory storage. Analogous to memory, LTP can be generated rapidly and is strengthened and prolonged by repetition (Citri & Malenka, 2008). LTP in hippocampal slices was induced by the application of a θ -burst stimulus in the CA1 region, which induced a robust increase in fEPSP slope that is maintained along time, and which, in the present work, were analysed in the Wistar, NEC, and GAERS rats.

In Wistar rats, LTP magnitude 1h after LTP induction was 162 ± 14.1 % compared to pre- θ -burst stimulation (Fig. 4.25 A, C). In NEC, similar results were observed, being the LTP magnitude after 1 h after stimulation of 143 ± 6.40 % (Fig. 4.25A,C) compared to pre- θ -burst stimulation. In GAERS, a significant decrease in fEPSP was observed, compared to Wistar, being $122 \pm 7.42\%$ (Fig. 4.25 A,C), 1h of stimulation. fEPSP slope of GAERS, 1h after stimulation, was the closest to pre- θ -burst stimulation compared with Wistar ($p=0.013$), indicating an impairment of LTP. No statistically significant differences for LTP magnitude were observed between the GAERS and non-epileptic control NEC (GAERS= 122 ± 7.42 , NEC= $143 \pm 6.40\%$; $p=0.216$; Fig. 4.25A,B).

Regarding PTP values, a short-term form of synaptic plasticity that is evaluated immediately after LTP induction, a decrease was observed in GAERS compared to Wistar, being significantly different (GAERS= $159 \pm 7.00\%$, Wistar = 221 ± 15.3 , $p=0.002$; Fig. 4.25 B). No differences were found between GAERS and NEC (GAERS= 159 ± 7.00 %, NEC= $185 \pm 13.7\%$; Fig.4.25 B).

4.2.1.1 | Long-term Potentiation in GAERS: BDNF effect

Next, BDNF (20 ng/ml) was applied exogenously, in perfusion to the hippocampal slices, to test its effect upon LTP. The θ -burst paradigm of stimulation was applied to the Schaffer-collaterals in CA1 region of the hippocampal slices of GAERS animals, inducing a robust LTP in GAERS in the presence

of BDNF. In hippocampal slices from GAERS perfused with BDNF, LTP magnitude was $162 \pm 14.5\%$ (Fig. 4.25 A,B), and PTP magnitude was $215 \pm 27.5\%$ (Fig. 4.25C). A significant increase was observed in LTP magnitude, comparing GAERS hippocampal slices perfused or not perfused with BDNF ($p=0.039$). Interestingly, regarding PTP values, the presence of BDNF also induced a significant increase in treated GAERS hippocampal slices ($p=0.010$), compared to LTP induced in GAERS untreated hippocampal slices.

According to these findings, GAERS-related *in vitro* tendency to synaptic dysfunction of hippocampal CA1 neurons can be linked to the *in vivo* memory impairments observed in tests conducted involving hippocampus efficiency. Interestingly, the preliminary data indicate that BDNF is capable to restore LTP to control levels in the hippocampus of GAERS rats. There is, nevertheless, the need to increase the number of experiments.

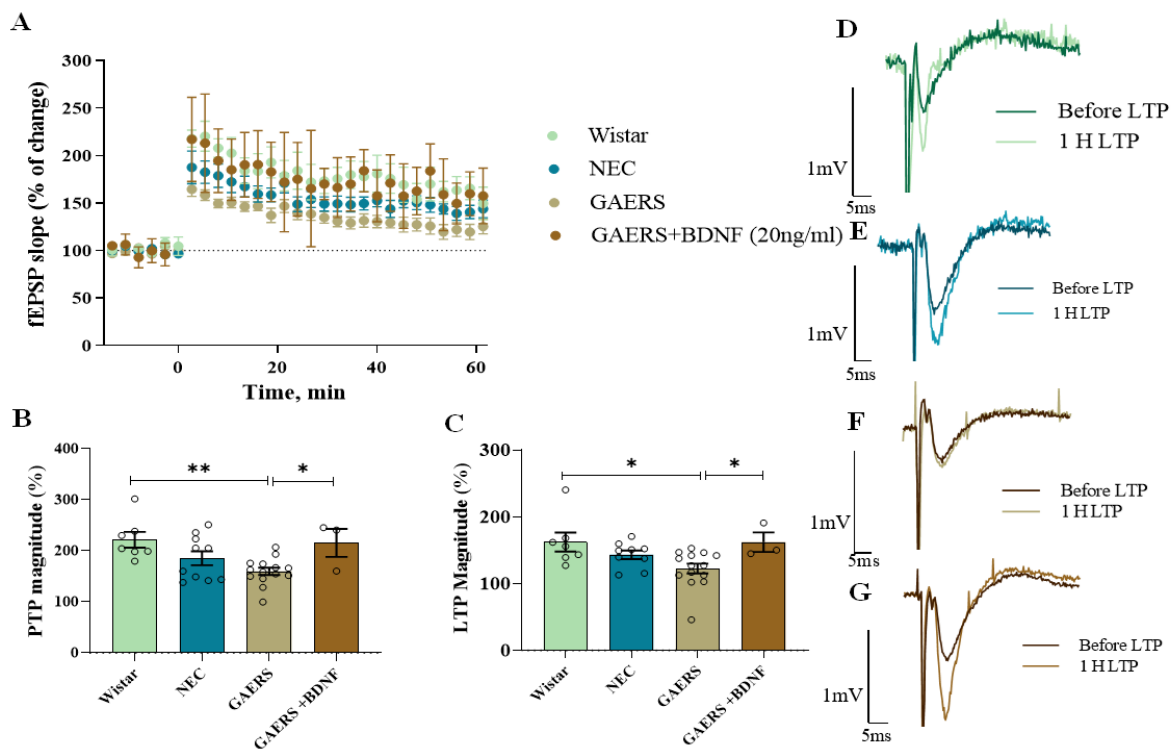


Figure 4.25 PTP and LTP is impaired in the GAERS hippocampal slices.

Time course of averaged normalized changes in fEPSP slope after delivery of a Θ -burst stimulus (5x5) in hippocampal slices from Wistar ($n=7$), NEC ($n=10$) and GAERS ($n=14$) (A) and in perfusion with BDNF (20 ng/ml, $n=3$). Individual comparison between control slices and in BDNF condition are shown for GAERS (A,B,C). Comparison of post-tetanic potentiation (PTP) (%) in both experimental conditions described (B). Comparison of magnitude of LTP (%) 60 minutes (C) following Θ -burst. Representative traces of fEPSP slope before LTP and after 1h of LTP for Wistar (D), NEC (E) and GAERS (F), and with BDNF (G). Data expressed as the mean $\pm$ SEM ($n=7-14$); * $p<0.05$, ** $p<0.01$, Oneway ANOVA followed by Tukey's multiple comparisons test and unpaired students T-test

4.2.2 | Long-term Potentiation in Stargazer Mice

In WT mice, LTP magnitude, 1h after LTP induction, was $224 \pm 13.62\%$ compared to pre- θ -burst stimulation (Fig. 4.26 A, C). In STG mice, a significant decrease in fEPSP was observed, compared to WT, being the LTP magnitude of $150 \pm 19.70\%$ (Fig. 4.26 A,C). These results show a significant impairment of LTP in STG compared with WT ($p=0.0347$). Regarding PTP values, no difference was observed in STG compared to WT ($p=0.156$; Fig. 4.26 B).

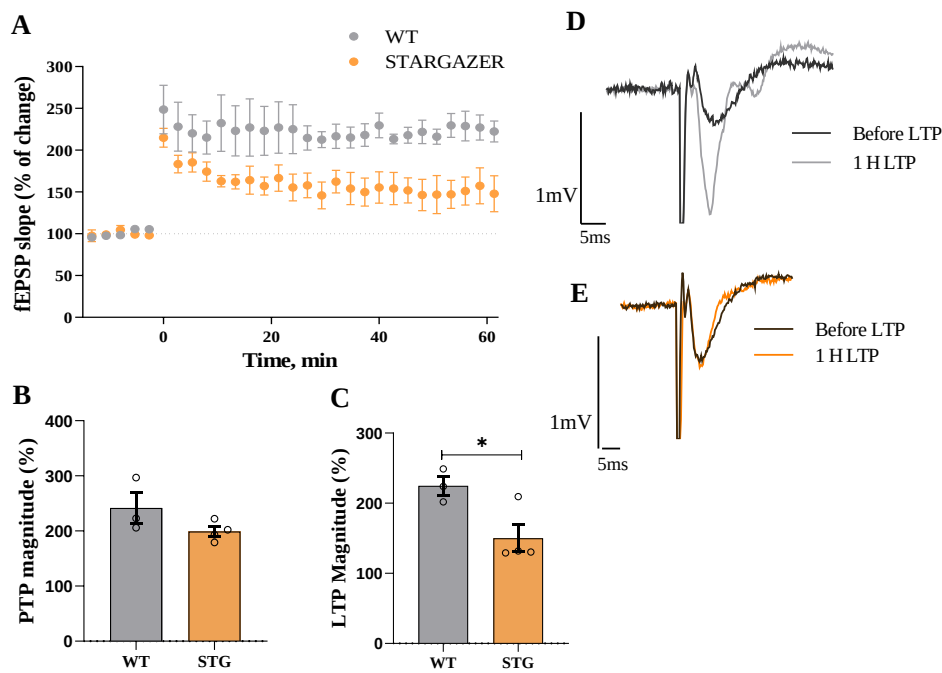


Figure 4.26 PTP and LTP in the Stargazer mice hippocampal slices.

Time course of averaged normalized changes in fEPSP slope after delivery of a Θ -burst stimulus (5x5) in hippocampal slices from WT ($n=3$), and STG ($n=4$)(A) Comparison of post-tetanic potentiation (PTP) (%) in the both experimental conditions described (B). Comparison of magnitude of LTP (%) 60 minutes (C) following θ -burst. Representative traces of fEPSP slope before LTP and after 1h of LTP for WT (D), and STG (E). Data expressed as the mean $\pm$ SEM ($n=3-4$); * $p<0.05$, unpaired students T-test

4.3 | Immunohistochemistry analysis

The results presented in this section were obtained using the Wistar, NEC, and GAERS rats that were subjected to Experiment 1. To correlate the behavioural results obtained in GAERS with astrocytic morphological alterations, a reconstruction of astrocytes was assessed from brain slices of rats' regions involved in seizures and also imperative in the processing of cognitive function.

4.3.1 | Astrocyte morphology in different brain regions of GAERS rats

To investigate the heterogeneity of astrocytes in the ASs animal, 2D morphology of astrocytes of the hippocampus, thalamus, motor cortex, somatosensory cortex, and visual cortex were examined. GFAP-labelled astrocytes were surveyed in each area, approximately 5 images were taken for each structure, to delineate key features of astroglial structure allowing anatomical reconstruction. The number of processes and total astrocyte length were compared between groups. Sholl analysis was performed since the complexity of astrocytes increases with radial distance from the soma, allowing a comparative analysis of key morphometric properties of astrocytes. GFAP served as a marker of astrocytes, being the mark intermediate filament protein in protoplasmic and specialized CNS astrocytes, labelling astrocyte main processes.

To analyse GFAP, NeuN, GABA transporters expression, as well as astrocytic morphology, in rat brain slices, an immunohistochemistry assay in adult brain slices was performed.

4.3.1.1 | GAERS display increased hippocampal astrocytic morphology complexity, with increased number of processes, and total length

As previously described for the hippocampus, it was observed that GAT1 was predominantly localized with astrocyte marker (GFAP) (Fig. 4.27 middle panel) and neuronal marker (NeuN, Fig. SF 7.11) indicative of GAT1 expression in neuronal presynaptic terminals and astrocytes. In the hippocampus there was an increased colocalization with GFAP in distal astrocytic processes, being most hippocampal astrocytes GFAP-positive. Modification of astrocytes processes can occur rapidly in tissue slices and *in vivo* in response to neuronal activity in the hippocampus and somatosensory cortex. No quantitative analysis was assessed for GAT1.

In the upper panel (Fig. 4.27), astrocytes labelled with GFAP (in red) were observed, predominately marked in GAERS compared to both Wistar and NEC. Interestingly, GAERS display increased morphological complexity and increased structure, reported by the Sholl analysis, showing that extent of process arborization, at a distance of $>11-29\ \mu\text{m}$ from the nucleus was greater for GAERS than for Wistar and NEC astrocytes ($p<0.001$). It was found that GAERS hippocampal astrocytes had a higher number of processes (GAERS= 47.2 ± 3.45) than Wistar (Wistar= 24.8 ± 1.68 , $p<0.001$) or NEC (NEC= 20.2 ± 1.30 ; $p<0.001$; Fig. 4.27). The total astrocyte length of GFAP positive processes was higher in GAERS (GAERS= 569 ± 38.6) than Wistar (356 ± 24.7 , $p<0.001$) or NEC (NEC= 345 ± 25.6 , $p<0.001$), revealing higher structural complexity in GAERS hippocampal astrocytes.

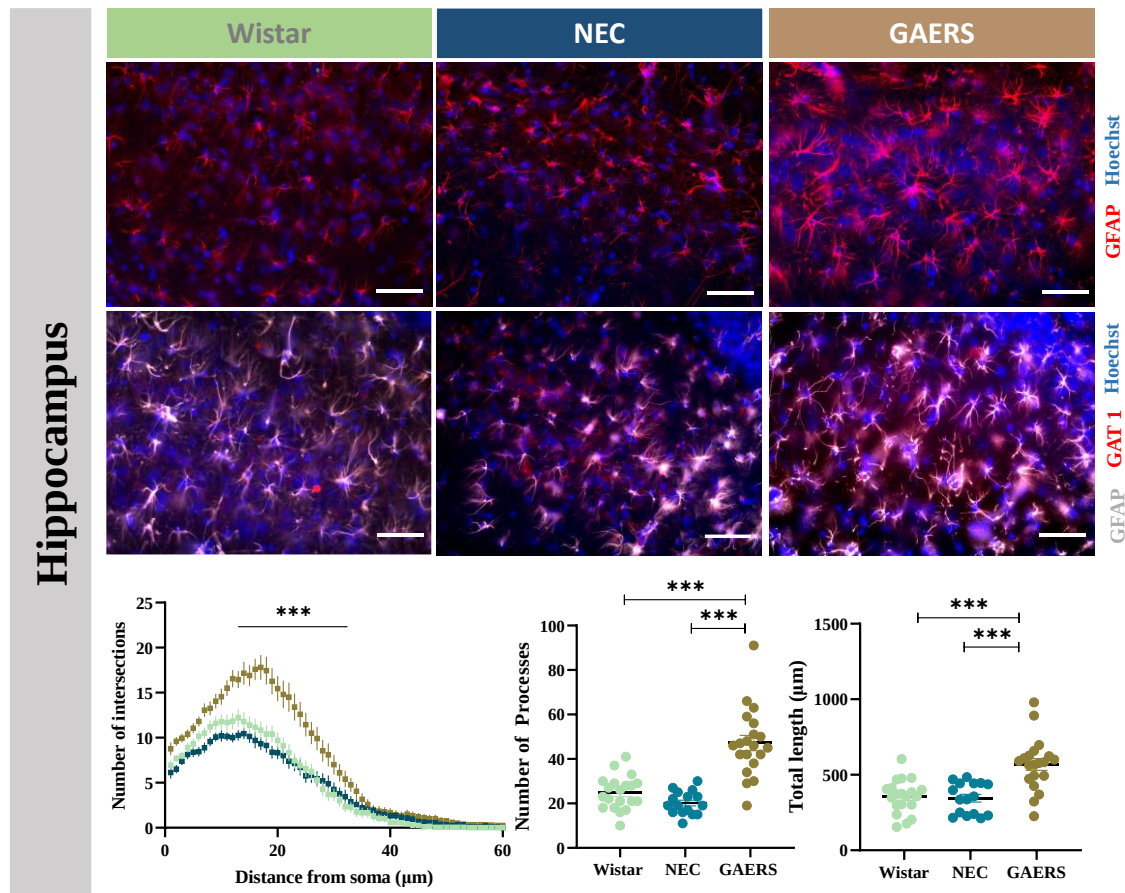


Figure 4.27. Hippocampus astrocytic morphology and GAT1 expression.

Double detection of GFAP and GAT1 in rat brain slices from Wistar, NEC, and GAERS (upper and middle panel). Nuclei were stained with Hoechst, GFAP stained astrocytes, and GAT1. Images were acquired with a 40x objective in a Zeiss Axiovert 200. Scale bar of 50 μm. Lower panel: Increased morphological parameters of hippocampal astrocytes structure, specifically number of intersections by Sholl analysis, number of processes, and total length. Data expressed as the mean ± SEM (n=17-20 astrocytes per group); ***p < 0.001 one-way and Two-way ANOVA followed by Tukey pairwise multiple comparisons test).

4.3.1.2 | GAERS display increased astrocyte complexity and number of processes in the thalamus

In the thalamus, images were obtained from the VB nucleus, from Bregma -2.3 to Bregma -4.8, from Wistar, NEC, and GAERS animals.

In the upper panel (Fig. 4.28), astrocytes labelled with GFAP (in red) were observed for the three groups analysed. It was observed an increase in complexity in GAERS, although few thalamic astrocytes are detected by GFAP immunostaining. In the middle panel, GAT1 was highly expressed in astrocytic processes, localized with astrocytic marker GFAP. The absence of colocalization with neurons was observed by labelling neurons with NeuN (Fig. SF7.11), indicative that thalamic GAT1 expression was exclusively observed in astrocytes, not being expressed in neurons. In the lower panel, remarkably, Sholl analysis showed increased process arbor complexity, indicated by a large number of intersections in GAERS at a radius of > 13- 21 μm compared to NEC (p<0.001). Thalamic astrocytes display increased number of processes in GAERS (GAERS=35.8±2.51) compared to NEC (NEC=23.9±1.59, p=0.001) or Wistar (Wistar=27.6±2.49; p=0.032; Fig. 4.28). Regarding the total length for all branches of the astrocyte, no differences were found between groups (Fig. 4.28).

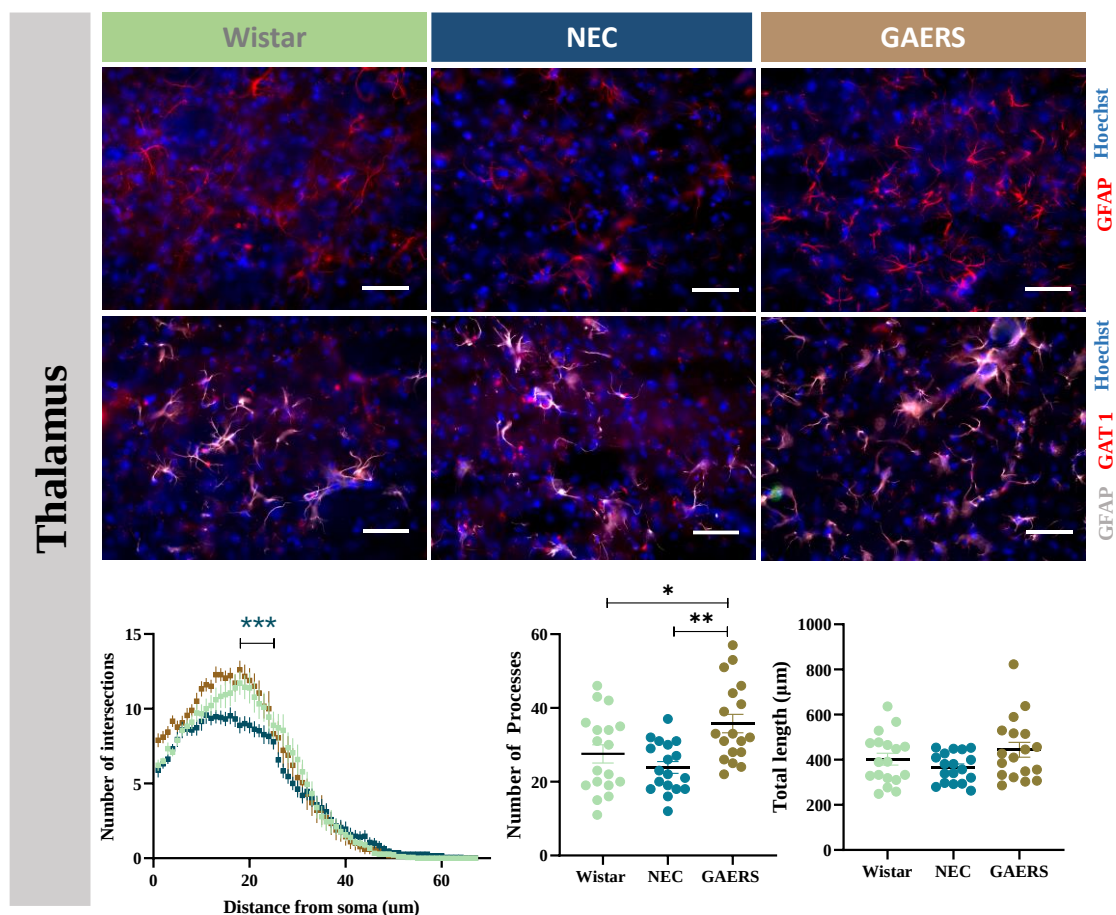


Figure 4.28. Thalamic astrocytic morphology and GAT1 expression.

Double detection of GFAP and GAT1 in rat brain slices from Wistar, NEC, and GAERS (upper and middle panel). Nuclei were stained with Hoechst, GFAP stained astrocytes, and GAT1. Images were acquired with a 40x objective in a Zeiss Axiovert 200. Scale bar of 50 μm. Lower panel: Increased morphological parameters of thalamic astrocytes structure, specifically number of intersection by Sholl analysis, number of processes, and total length. Data expressed as the mean ± SEM (n=18 astrocytes per group); *p<0.05, **p < 0.01, ***p < 0.001 one-way and Two-way ANOVA followed by Tukey pairwise multiple comparisons tests).

4.3.1.3 | GAERS displayed increased astrocyte complexity, number of processes, and total length in the motor cortex

Accounting for the motor cortex, images were obtained from Bregma 2.2 to Bregma -2.12, corresponding to Wistar, NEC, and GAERS brain slices. In the upper panel (Fig. 4.29), astrocytes were stained with GFAP marker. An increase in GFAP density was observed for GAERS in comparison to non-epileptic animals. In the middle panel, GAT1 expression was observed in astrocytes labelled with GFAP and in neurons labelled with NeuN (Fig. SF 7.11).

In the lower panel (Fig.4.29), vast differences in astrocytic complexity were observed between groups. GAERS displayed an increased number of intersections around the soma at a radius of > 15-29 μm compared to NEC ($p<0.001$) and 30-34 μm ($p=0.002$). However, differences were also observed between both controls, Wistar showing a higher number of intersections compared to NEC at a radius > 18- 27 μm ($p=0.006$). As expected from the Sholl analysis, an increased structural morphology was observed in motor cortex astrocytes number of processes from GAERS (GAERS= 33.5 ± 3.04) compared to Wistar (Wistar= 24.4 ± 2.48 , $p=0.020$) or NEC (19.1 ± 0.840 , $p<0.001$). As well as changes in total astrocytic length were observed for GAERS compared to NEC (GAERS= 422 ± 39.4 , NEC= 257 ± 20.3 , $p<0.001$). This morphological analysis indicated increased morphological complexity of GAERS astrocytes in the motor cortex.

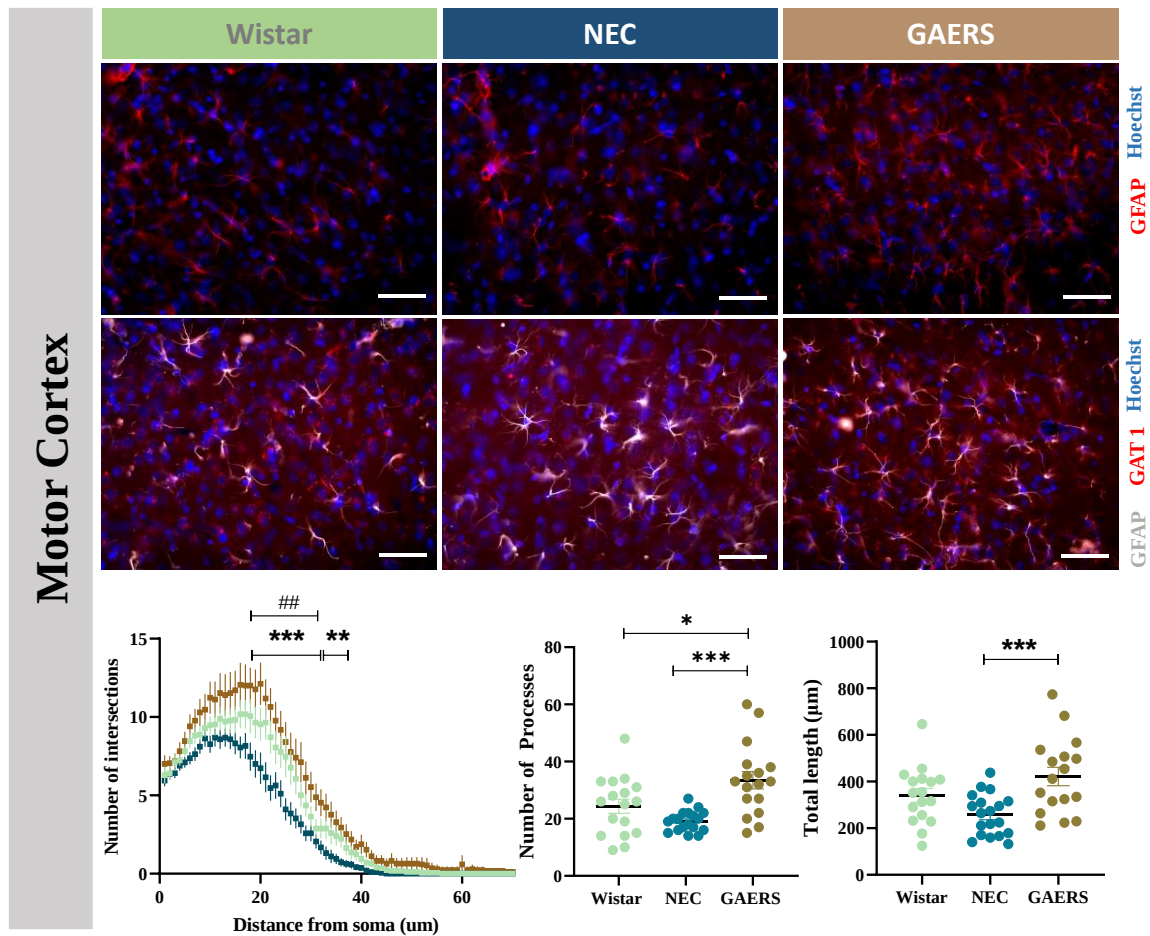


Figure 4.29. Motor Cortex astrocytic morphology and GAT 1 expression.

Double detection of GFAP and GAT1 in rat brain slices from Wistar, NEC, and GAERS (upper and middle panel). Nuclei were stained with Hoechst, GFAP stained astrocytes, and GAT1. Images were acquired with a 40x objective in a Zeiss Axiovert 200. Scale bar of 50 μm . Lower panel: Increased morphological parameters of motor cortex astrocyte's structure, specifically number of intersections by Sholl analysis, number of processes and total length. Data expressed as the mean $\pm$ SEM ($n=17-19$ astrocytes per group); * $p<0.05$, ## $p<0.01$ (Wistar vs NEC) ** $p<0.01$, *** $p<0.001$ one-way and Two-way ANOVA followed by Tukey pairwise multiple comparisons test).

4.3.1.4 | Different astrocytic morphological complexity in somatosensory cortex

In somatosensory cortex, images were obtained from the same slices as the motor cortex, from Bregma 2.2 to Bregma -2.12, corresponding to Wistar, NEC, and GAERS brain slices. In the upper panel (Fig. 4.30), staining with GFAP was observed in red. In this region, astrocytes morphology and density appear similar between GAERS and NEC. In the middle panel, GAT1 expression was observed in astrocytic processes as well as in neurons (Fig. SD 7.1). Strong modification of astrocytic processes was observed in somatosensory cortex in response to neural activity and indeed, fines astrocytic main processes were observed in these astrocytes.

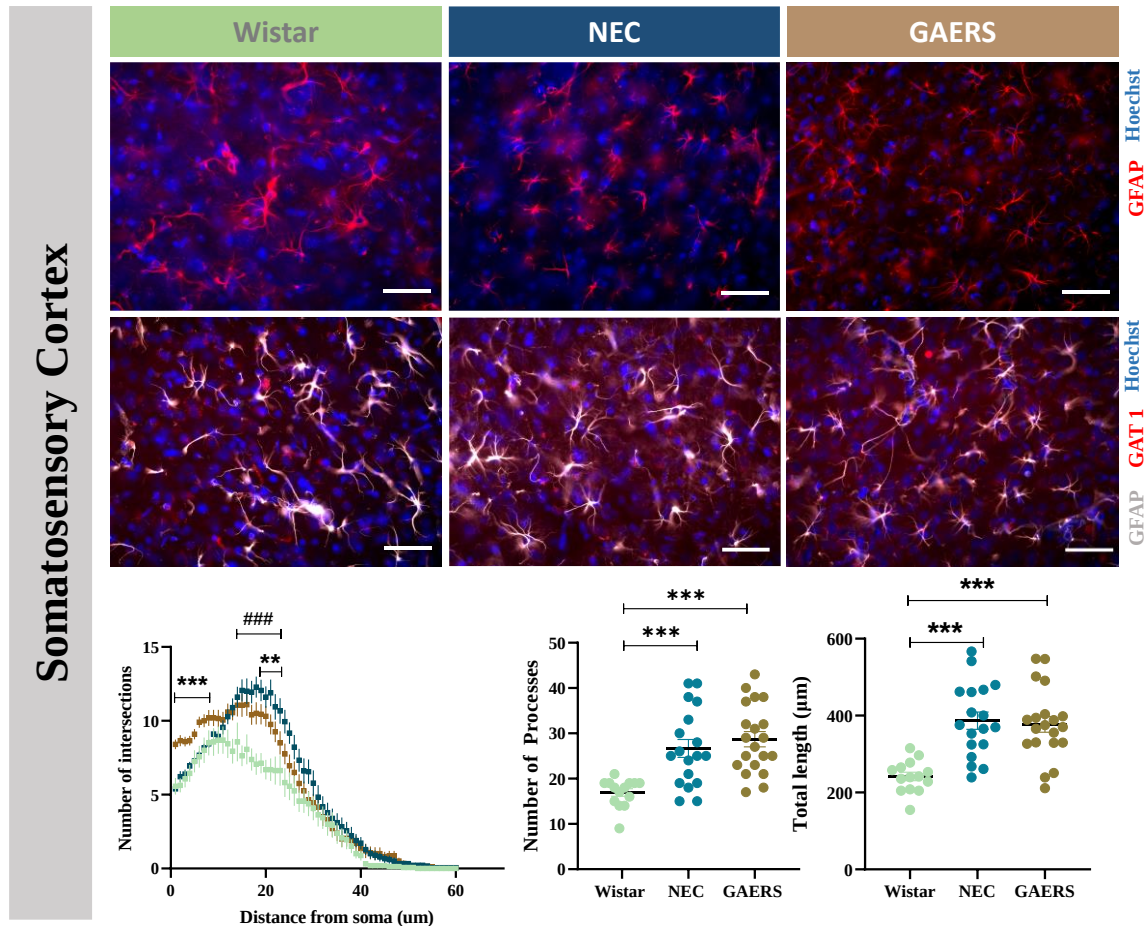


Figure 4.30 . Somatosensory Cortex astrocytic morphology and GAT1 expression.

Double detection of GFAP and GAT1 in rat brain slices from Wistar, NEC and GAERS (upper and middle panel). Nuclei were stained with Hoechst, GFAP stained astrocytes and GAT1. Images were acquired with a 40x objective in a Zeiss Axiovert 200. Scale bar of 50 μm. Lower panel: Distinct morphological parameters of motor cortex astrocytes structure, specifically number of intersection by sholl analysis, number of processes and total length. Data expressed as the mean ± SEM (n=18-20 astrocytes per group); ### p < 0.001 (Wistar vs NEC) **p < 0.01, ***p < 0.001 one-way and Two-way ANOVA followed by Tukey pairwise multiple comparisons test).

Interestingly, Sholl analysis revealed an increased number of intersections closer to the nucleus in GAERS compared to Wistar, at the radius of 1-5 μm (p<0.001). However, at the radius of 15-20 μm, NEC displayed increased complexity compared to GAERS (p=0.002) or Wistar, at a radius of 17 -21 μm (p<0.001). Regarding number of processes, NEC and GAERS displayed increased morphological complexity compared to Wistar (GAERS=28.7±1.69, Wistar=16.9 ±0.829, p<0.001), (NEC=26.7±1.99, p<0.001). Equally, the total length of astrocytes in the somatosensory cortex of GAERS and NEC was increased compared with the second control (GAERS=377±20.6, Wistar=242±11.1, p<0.001) and (NEC=387±22.4, p<0.001). Although a significant structural morphology was observed in the primary

processes of GAERS, no significant differences were observed for the nonepileptic control regarding all astrocytic length astrocytes.

4.3.1.5 | Different astrocyte morphological complexity in visual cortex

In the visual cortex, images were obtained from slices from Bregma -4.8 to -5.2, corresponding to Wistar, NEC, and GAERS animals. In the upper panel (Fig. 4.31), astrocytes-stained with GFAP (in red) were observed. Thin astrocytic processes were observed in GAERS slices as well as different orientations and structures. In the middle panel, astrocytic GAT1 expression was observed in all groups as well as in neuronal cells (Fig. SF 7.11). In the lower panel, Sholl analysis revealed no differences for this brain region between all groups. However, differences were found in morphology, regarding the number of processes, being increased in GAERS (GAERS=30±1.53) compared to Wistar (Wistar=20.7±1.39, $p<0.001$) or NEC (23.9±1.66, $p=0.018$). The total astrocyte length of GFAP positive processes was higher in GAERS (GAERS=395±17.2) compared to Wistar (Wistar=298±13.4, $p<0.001$) or NEC (NEC=330±18.2, $p=0.020$). The data suggests that although Sholl analysis revealed unaltered arbor complexity of ASs model as compared to control groups, an increase in processes and total length was observed for ASs model.

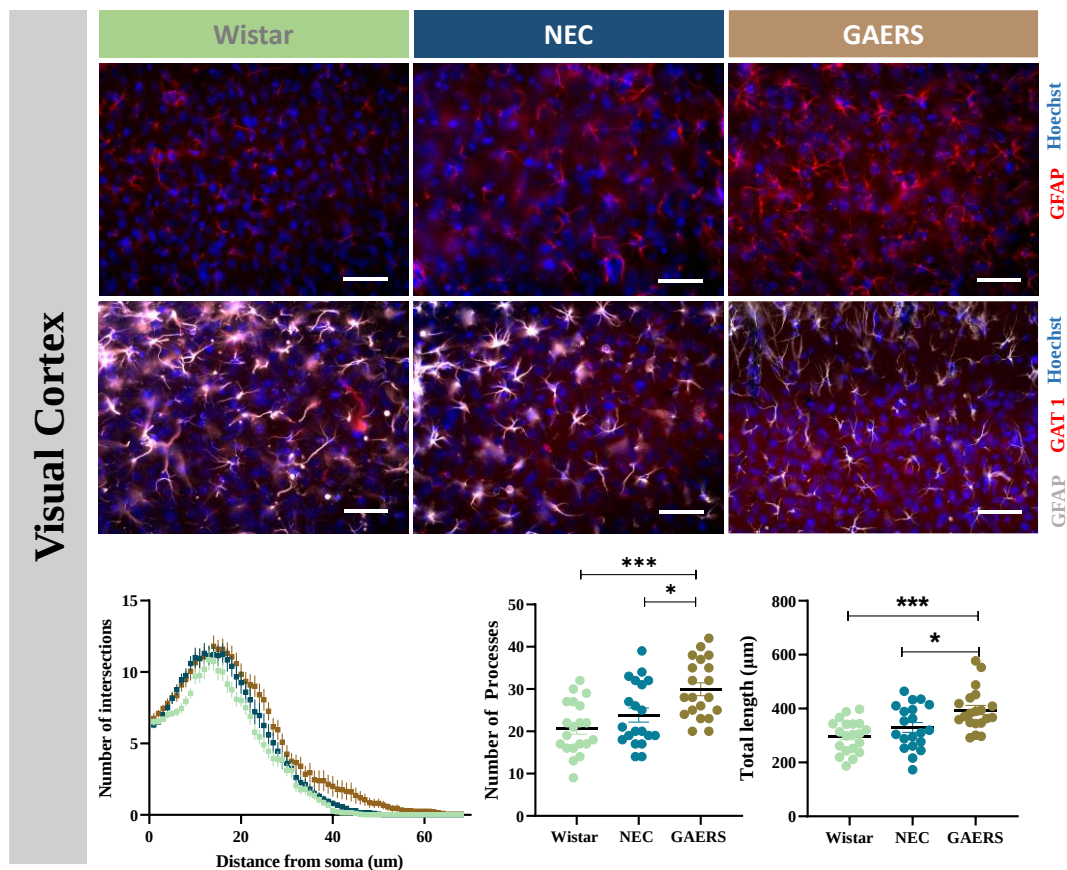


Figure 4.31. Visual Cortex astrocytic morphology and GAT1 expression.

Double detection of GFAP and GAT1 in rat brain slices from Wistar, NEC and GAERS (upper and middle panel). Nuclei were stained with Hoechst, GFAP stained astrocytes and GAT1. Images were acquired with a 40x objective in a Zeiss Axiovert 200. Scale bar of 50 μm. Lower panel: Increased morphological parameters of visual cortex astrocytes structure, specifically number of intersection by sholl analysis, number of processes and total length. Data expressed as the mean ± SEM (n=20 astrocytes per group); * $p<0.05$, ** $p<0.01$, *** $p<0.001$ one-way and Two-way ANOVA followed by Tukey pairwise multiple comparisons tests).

4.3.2 | GAT1 in the thalamus is exclusively expressed in astrocytes

The expression of GAT1 transporter was demonstrated in astrocytic cells in soma and cell processes of all brain regions of Wistar, NEC, and GAERS. It has been described that thalamic GAT1 is only expressed in astrocytes. In the thalamus (Fig. SF 7.11) a double detection of GFAP and GAT 1 was observed, in the prolongations of astrocytes. However, the double detection of NeuN and GAT 1 was not detected in the rat's thalamus. On the contrary, in the other brain regions studied this double detection of GABA transporter 1 was easily observable also in neurons (Fig. SF 7.11).

Table 4.3. Summary table of morphological parameters of GAERS hippocampal, thalamic, motor cortex, somatosensory cortex and visual cortex astrocytes compared to both controls. (=: GAERS equal parameter; ↑:GAERS increased parameter; ↓: GAERS decreased parameter compared to Wistar (W) or NEC (N))

Astrocyte Morphological Complexity			
	Sholl Analysis	Number of processes	Total length
Hippocampus	↑↑↑ N.W	↑↑↑ N.W	↑↑↑ N.W
Thalamus	↑↑↑ N	↑↑ N ↑ W	=
Motor Cortex	↑↑↑ N	↑↑↑ N ↑ W	↑↑↑ N
Somatosensory Cortex *	↑↑↑ W	↑↑↑ W	↑↑↑ W
Visual Cortex	=	↑↑↑ W ↑ N	↑↑↑ W ↑ N

4.4 | Brain weight and GAT1 differential expression in GAERS

4.4.1 | Brain weight is increased in GAERS from Brain/body weight ratio

In a final analysis, animals' brains from Experiment 2 were removed and brain weights were determined for animals from each group. Data were presented as brain weight normalized to body weight. As seen in Fig. 4.32, body weight differed significantly across groups ($F_{2, 21} = 24.8$, $p < 0.001$). ASs animals' weight was less compared to both controls (GAERS=331±9.94g, Wistar=404±7.49 g, NEC=393 ± 5.73 g, $p < 0.001$ for both, fig 4.32A). Normalized brain weight differed significantly across groups ($F_{2, 21} = 20.5$; $p < 0.001$; Fig 4.32B). Multiple comparisons indicated that brain-to-body weight ratio in GAERS (mean=0.0058) was significantly greater than in NEC (mean=0.0047; $p < 0.001$), and Wistar (mean=0.00487; $p < 0.001$).

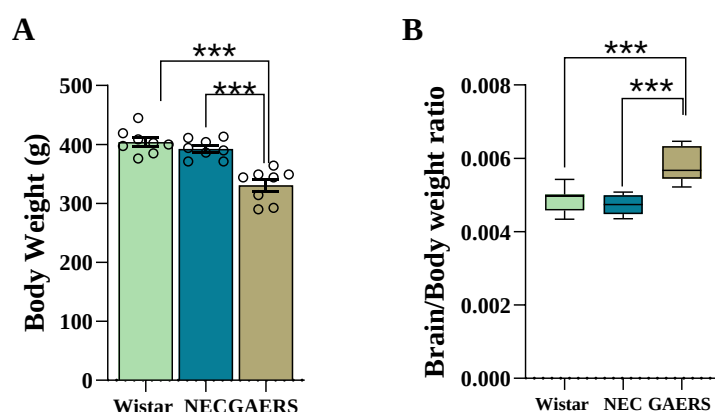


Figure 4.32. Body and brain weight differs between groups.

Body weight in markedly decreased in ASs animal model (A). Normalized brain weight, expressed as the brain/body weight ratio, of 8 individuals (open circles) from each group and means for each group (B). Data expressed as the mean $\pm$ SEM (n=8); *p<0.05, **p < 0.01, ***p < 0.001 one-way followed by Tukey pairwise multiple comparisons tests.

4.4.2 | GFAP expression in the motor cortex, thalamus, hippocampus, and visual cortex is increased, while levels of GAT1 did not changed

To confirm if the morphological and cellular density changes in immunohistochemistry were accompanied and translated in differential alterations in GFAP protein expression, the GFAP levels were quantified by WB in ASs and control animals. Given that GAT1 and GAT3 show particular cellular and sub-cellular distributions throughout the brain (Boisvert *et al.*, 2018), GAT1 being the most abundant transporter, expressed in GABAergic neurons and glia cells and GAT3 present in astrocytes, as well as in certain neuronal populations (Zhou *et al.*, 2013), protein expression levels were tested.

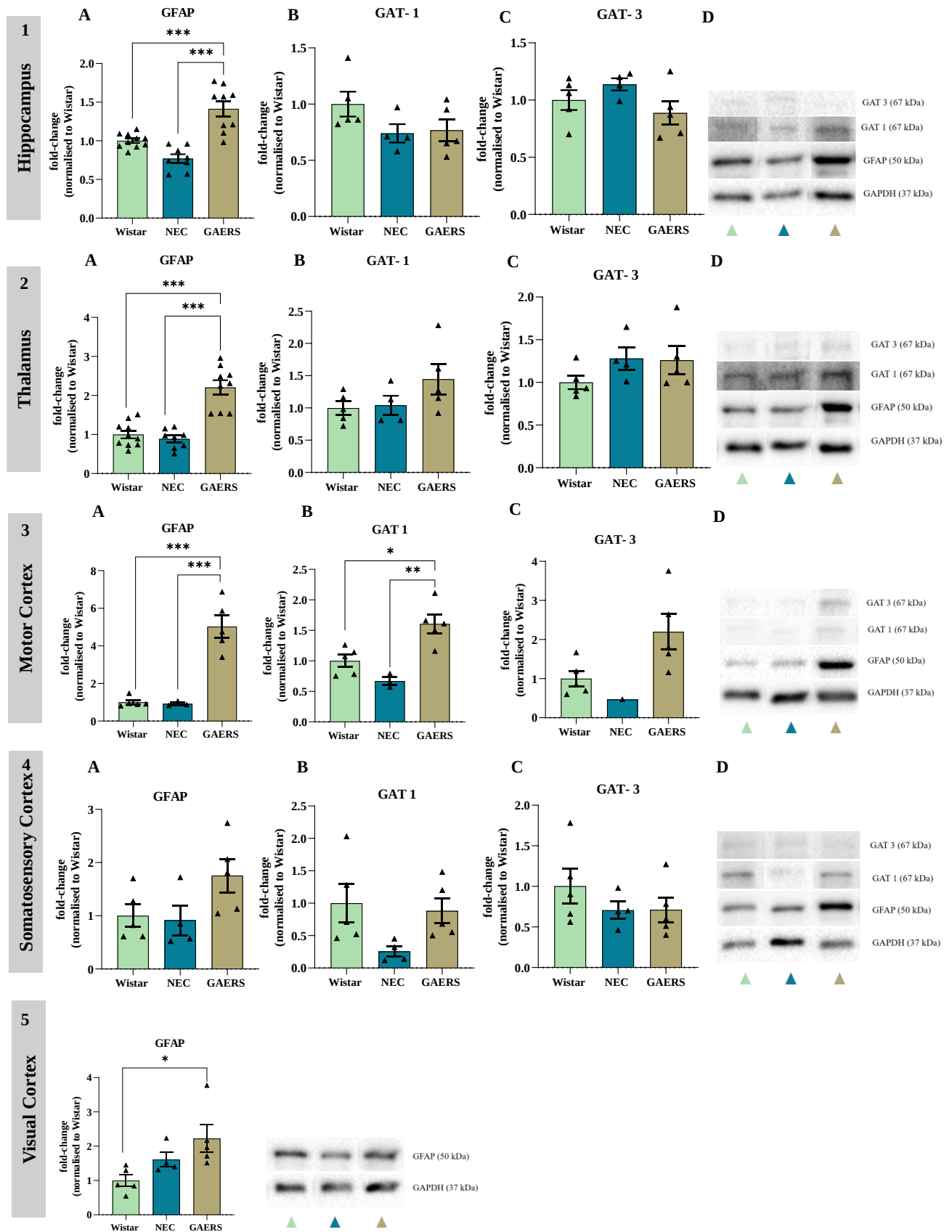
Weighted brains were used for quantification of protein levels of GFAP, GAT1, GAT3, and PSD95 in specific brain regions from Wistar, NEC, and GAERS by western blot (Fig. 4.33). The regions evaluated were hippocampus, thalamus, motor cortex, somatosensory cortex, and visual cortex, similar to what was done in the immunohistochemistry assay.

The GFAP protein expression levels showed differences in GAERS compared to both NEC and Wistar animals. WB results showed that GAERS had significantly high endogenous GFAP expression in the hippocampus (p<0.001, Fig. 4.33-1), thalamus (p<0.001, Fig. 4.33-2), and motor cortex (p<0.001, Fig. 4.33-3) compared to both Wistar and NEC. Also, increased GFAP expression in GAERS was found in the visual cortex, compared to Wistar (p=0.025, Fig. 4.33-5). In the somatosensory cortex, no significant differences were found for GFAP expression levels (Fig. 4.33-4).

Concerning GAT1 expression in different brain areas, congruently with the lack of apparent expression of GAT1 in microscopy images, no changes were found in either hippocampus, thalamus, or somatosensory cortex (Fig. 4.33). However, unexpectedly, GAT1 protein levels were increased in the motor cortex compared to Wistar (p=0.011) and NEC (p=0.002). A decrease in thalamic levels of GAT1 was expected, given its loss of function. However, given that in the thalamus, GAT1 is only expressed in astrocytes and there is an increase in the expression of GFAP in this cell type in GAERS, it's possible not to observe any differences regarding GAT1 expression. However, the number of experiments has to be increased.

Concerning GAT3 expression levels, no differences were found for the hippocampus, thalamus, motor cortex or somatosensory cortex (Fig. 4.33). In the visual cortex, protein expression of GAT1 and GAT3 was not achieved (Fig. 4.33). Postsynaptic density protein 95 (PSD95) expression levels were also evaluated given that PSD-95 deficiency can cause a significant decrease in AMPAR-mediated current, suggesting silent synapse formation causing an imbalance of NMDAR and AMPAR presence and function, which could lead to learning and memory deficits and impaired LTP (Nyffeler et al. 2007). In the expression of PSD95 compared to NEC ($p=0.15$, Fig. SF 7.12), no effect was observed between ASs animals and both control animals.

Thus, expression of GFAP and GAT1 protein increased significantly in the motor cortex of absence epileptic animals compared to both controls. The high GFAP expression was most distinct in the motor cortex, thalamus, and thereafter hippocampus, congruently with the increase in complexity morphology and GFAP apparent density in the GAERS brain regions described.



Protein expression				
	GAT-1	GAT-3	PSD95	GFAP
Hippocampus	=	=	=	↑↑↑ N.W
Thalamus	=	=	=	↑↑↑ N.W
Motor Cortex	↑W ↑↑ N	=	NA	↑↑↑ N.W
Somatosensory Cortex	=	=	NA	=
Visual Cortex	NA	NA	NA	↑ W

Figure 4.33. Analysis of protein levels of GFAP, GAT1 and GAT 3 in Hippocampus (1), Thalamus (2), Motor Cortex (3), Somatosensory Cortex (4) and Visual Cortex (5).Densiometric analysis for each brain region and representative immunoblot. GAPDH was used as internal control. There was an increase in the total expression of GAERS GFAP in Hippocampus, Thalamus, Motor cortex (***p) and Visual cortex (*p). GAT1 levels were increased GAERS motor cortex. Summary table indicating differences in protein expression of GAERS in each brain region compared to both controls (6). Data expressed as the mean $\pm$ SEM (n=3-10); one-way ANOVA followed by Tukey pairwise multiple comparisons test.

5 | Discussion and Future Perspectives

In this project, it was provided evidence for subtle memory impairments in Working Memory, and prominent deficits in Recognition Memory and Reference Memory in GAERS rats, and Spatial Reference Memory deficits in STG mice. LTP impairments were also described for both ASs animal models used, which was restored in GAERS by the perfusion with BDNF. In closure, increased GFAP expression in line with increased astrocytic morphological alterations were also observed in epileptic rats, not resulting in altered GAT1 expression.

Given that absence seizures are generalized seizures, with initiation at the somatosensorial cortex, it is reasonable to consider whether the focus and out-of-focus cortical areas undergo changes due to abundant seizures invading the thalamocortical circuit. On one hand, it is possible that SWDs are the link between seizures affecting consciousness and memory, and the mechanism which mediates the loss of consciousness may also contribute to the clear of stored memory or impair acquisition phase. On the other hand, astrocyte's sense and modify neuronal activity and changes in its protein content expression, morphology, and impaired functions may also contribute to memory impairments in ASs.

5.1 | Memory Impairments in GAERS rat and Stargazer mouse

In this project, definitive evidence was provided for impairments in Recognition memory, Cross-modal object recognition memory, Working memory, and Reference memory in an ASs rat model (GAERS) and impairments in Reference memory in mice model of ASs (STG), Fig. 5.

Anxiety-like behaviour results described are discrepant compared to the majority of anxiety assessments in worldwide colonies (Bouilleret et al., 2009; N. C. Jones et al., 2008; Marks, Cavanagh, et al., 2016; José Eduardo Marques-Carneiro et al., 2014; Powell et al., 2014). This may be due to the number of inbreeding generations in each colony or rodent facility housing. Indeed, that was one of the main reasons to use Wistar as a second control. Although presenting a reduced number of SWDs, Wistar rats are used as an outbred control to avoid confounds due to increased inbreeding in NEC. Interestingly, for GAERS, anxiety-like behaviour was discarded as contributing factor to memory deficits observed in the ASs animal.

Regarding memory impairments, deficits in WM, and detected in the Y Maze, were observed for GAERS compared to Wistar and NEC (C4, Experiment 1), congruent with acquisition trials of ASs animal observed in Barnes Maze. Since the hippocampus, the septum, the basal forebrain, and the prefrontal cortex are the main involved areas in this task (Chudasama & Robbins, 2004), these results highlight the involvement of cortical structures. However, no deficits were found regarding WM in the T-Maze (C4, Experiment 2), which may be due to the inefficient use of extra maze cues to guide behaviour. It is also important to note that the second batch of animals conducted in T-maze, due to lack of animals in the first batch, comprised all Wistar, 2 NEC, and 2 GAERS, the behaviour of controls and epileptic animals was inconsistent compared to the first batch, decreased learning, and animals were aggressive and stressed, possible due to changes in housing conditions during the protocol days. WM assessment tested different components, such as spontaneous alternations and the natural behaviour of spatially guidance in foraging food and escape from bright and noisy spaces in repeated trials, which may be differently affected in ASs animals.

Both visual and crossmodal variations of CMOR task rely on the perirhinal cortex. GAERS showed recognition memory deficits in crossmodal variation and texture recognition memory moderately decreased (C4, Experiment 3), relative to NEC. This deficit was observed for short-term memory (1h), which can be explained by the gain in the function of the Cav3.2 T-type calcium channels

in GAERS and dense expression of these channels in the perirhinal cortex (Marks *et al.*, 2016). These channels regulate synaptic NMDA receptor transmission and in cortical and NRT neurons are key to ASs expression. CMOR deficits are in line with the work from Marks *et al.*, 2016, although visual deficits were impossible to detect. This result highlights the critical role of T-type calcium channels in mediating cognitive deficits. However, for recognition memory relying on texture and visual (NOR) discrimination, no differences were found for short-term memory, only in long-term memory, not being explained by T-type calcium channels activity, not explaining this deficit.

It was important to confirm if GAERS can discriminate between textures when sensory integration is efficient. Despite the involvement of S1Bf, the somatosensory barrel cortex in SWDs, in previous studies (Studer *et al.*, 2019), sensory-information processing has been shown to be impaired in the epileptic barrel field primary somatosensory cortex of GAERS, although GAERS are able to discriminate textures, suggesting the existence of a compensatory mechanism within the epileptic neuronal network. This previous study (Studer *et al.*, 2019) confirms that deficits found in this project for cross-modal object recognition memory are due to impairments in the circuit and not in the inability of rodents to distinguish different textures. This way, GAERS deficits in texture memory found in our work may also be linked with a less efficient compensation mechanism. Undeniably, visual recognition memory was impossible to access, given that control rats were not able to distinguish between two visual objects. Possible because of low visual acuity of albino rats (Prusky *et al.*, 2002), impaired vision in bright and low light, few rods and photoreceptors (Lavado *et al.*, 2006), disorganized neural projections involved in vision, and retinal degeneration, contributing to visual deficits (Vinken *et al.*, 2021). However, in tests using visual cues, patterns contrasting in black and white were used to diminish this confound.

In the Barnes Maze (C4, Experiment 4), GAERS were able to achieve performance levels similar to Wistar and NEC although were not able to establish an operational reference memory and thereafter long-term reference memory. Thus, indicative of a weaker consolidation process and less efficient information sampling in the spatial environment. Impairment in working memory was also observed between acquisition trials, in the reference memory training, shown by the increased number of errors and less direct strategy. This impairment in WM would affect the maintenance of short-term memory and thereby make the transition to long-term memory difficult. The preferential use of random and serial strategies by GAERS could be compensation for difficulties in WM system. A similar interpretation was assessed for STG mice, showing increased major deficits in WM during acquisition for all parameters studied and in short reference memory deficits being mainly hippocampus-dependent. Impairments found for RM and WM are in line with previous studies from (J. E. Marques-Carneiro *et al.*, 2016) tested in Morris Water Maze.

Also, given that seizures only occur when animals are not excited (eg due to a new environment/maze test), it may occur that some animals had seizures during the trials. However, since tests are not of long duration and the animals are monitored by using video recording, the features observed do most probably not reflect a direct impact of seizures on the learning/unique trial.

Is the memory affected by structural brain damage which eventually causes deficits or is memory impairment a result of the SWDs? Given that SWDs occur in the Somatosensory Cortex and thalamocortical loop, not producing seizure activity in medial temporal lobe structures (hippocampus), deficits may be caused by disruption on cortical structures where SWDs are more frequent. Given that SWDs occur in the thalamocortical loop, the functional efficacy of this circuitry and distant structures could be impaired. Attention, which is severely impaired in children with ASs, is divided into different subtypes such as sustained attention, selective attention, generalized attention, alternate attention, spatial attention, and working memory (WM). In humans, it is associated with dorsal and ventral networks of

the thalamocortical loop, composed of regions involved in the selection of sensory information and the response to goal-directed stimuli, implying a voluntary allocation of attention, in the region involved in the thalamocortical network (Barone *et al.*, 2020).

The thalamocortical pathways promote behavioural flexibility, having a central role in cognition and functionally ranging from directing attention to solving cognitive challenges (Wolff & Van, 2019). Thalamic nuclei, as described in Chapter 1, plays a significant role in learning involving spatial memory and supporting upcoming choice in WM (Bolkan *et al.*, 2017). In GAERS, functional impairment in the midline thalamic nuclei, involved in the occurrence of SWDs could explain spatial memory deficits observed, as well as a functional impairment in connections involving PFC and MD thalamus. In previous studies it has been observed that damage to MD-PFC affected cognitive flexibility, compromised working memory, and resulted in the loss of recognition memory (Bolkan *et al.*, 2017, Aggleton & Brown, 1999), the thalamus being the regulator of the synchrony between neurons in the prefrontal cortex during information exchange related to cognitive control demands (Saalman, 2014) and specifically, the anterior thalamus interacting with hippocampus and cortex contributing to spatial navigation (Jankowski *et al.*, 2013).

5.2 | Synaptic plasticity is decreased in GAERS hippocampal slices

LTP is the cellular mechanism underlying the processes of memory formation, and alterations in LTP are believed to underlie behavioural deficits regarding memory and learning. In this work, synaptic plasticity was impaired in GAERS, 1 h after LTP induction, compared to Wistar rats. In addition, PTP values differ in GAERS compared to Wistar, being statistically decreased for GAERS (Fig. 5). However, alterations in LTP are more dependent on postsynaptic activity, whereas PTP values are related to the presynaptic activity (Citri & Malenka, 2008). Differences found following θ burst stimulation indicate compromised neuron excitability, indicative of a smaller postsynaptic activity, possibly deriving from a reduction in glutamate release or a decreased probability of postsynaptic neurons to generate action potentials, constituting the early phase of LTP. Hippocampal LTP can be triggered by the coincidence of postsynaptic activity and astrocytic calcium elevation, which stimulates glutamate release. Astrocyte release of glutamate increases the probability of transmitter release, mediated by NMDARs (De Pittà & Brunel, 2016; Navarrete *et al.*, 2012). However, different forms of synaptic plasticity involve different astrocytic signalling pathways, and some rely only on neuronal mechanisms. As described in Chapter 1, if tonic inhibition is also impaired in GAERS hippocampus, increased tonic inhibition might suppress LTP and result in memory deficits aligned with WM and reference memory, as observed in this project. If this tonic inhibition was indeed observed in this model, yet to clarify, a GAT1 loss of function could underlie an impairment in LTP, by establishing a new GABAergic tone impacting on LTP. Interestingly, in previous studies (Gong *et al.*, 2009), reducing the frequency of hippocampal θ oscillation, impaired hippocampus dependent memory, and GABA uptake may be fundamental in maintaining normal hippocampal θ activity.

The deficit in LTP for STG may be also linked with a deficiency in the calcium channel $\gamma 2$ -subunit gene *CACNG2*, reducing the expression of stargazin protein, being TARPS, a transmembrane AMPAR regulatory protein and phosphorylation of TARP- $\gamma 2$ essential for LTP, as well as to impairments in calcium signalling.

5.2.1 | BDNF effect on synaptic plasticity of GAERS

Interestingly, the results in this project show that BDNF can rescue LTP in the GAERS rat to control values (Fig. 5). Previous work revealed that BDNF increases GAT1-mediated GABA transport into astrocytes (Vaz *et al.*, 2011), leading to enhanced expression of GAT1 at the cell surface due to a reduced internalization. This facilitatory action of BDNF upon GAT1, by modulating its activity might

contribute to a decrease in tonic inhibition. Therefore, this molecule could be a possible target to CAE. This BDNF-induced increase in GAT1-mediated GABA uptake involves the activity of at least two transduction pathways, namely the PLC and the ERK/MAPK pathways. Moreover, exogenous BDNF is able to potentiate Schaffer collaterals – CA1 synapses, BDNF maturation is essential for LTP in the hippocampus and the application of exogenous BDNF is sufficient to potentiate LTP (Cunha *et al.*, 2010; B. Lu *et al.*, 2014)(Y. Lu *et al.*, 2008; Skaper, 2012). Although a significant effect was observed, the results regarding the induction of LTP by perfusion with BDNF are considered preliminary, and performing more experiments is necessary to gain solid knowledge.

The next step would be to confirm that the effect of BDNF is mediated by GAT1 activity modulation. For that purpose, a blocker of GAT1 will be used (NO711 or SKF89976A), before inducing the θ burst stimulation. Slices from GAERS, Wistar, and NEC will be perfused with NO711 or SKF89976A, NO711+BDNF or SKF89976A+BDNF, or only BDNF to confirm if indeed the effect of BDNF is mediated by GAT1.

Moreover, as future perspectives, it is intended to understand if BDNF may have an effect in memory impairments, by testing BDNF action in memory acquisition. To address this question, we have recently started a protocol, testing BDNF injection in the hippocampal CA1 area, before the learning phase of NOR followed by a long-term retention memory (24h), to understand if BDNF is capable of facilitating the memory acquisition in this protocol.

5.3 | Astrocytic morphological and expression changes in GAERS brain slices; GAT1 dysfunction is not due to reduced protein expression.

Astrocytes with disease-causing genetic mutations are unhealthy astrocytes that contribute to pathology and later became reactive, which is different from normal reactivity caused by external stimuli (Escartin *et al.*, 2021). There is a fundamental question, whether changes in astrocytes are caused by the SWDs or they contribute to its activity. In this project, different astrocytic morphologies were observed for the brain regions involved in SWDs: motor cortex, somatosensory cortex, visual cortex, and thalamus (Fig. 5). In the hippocampus, increased complexity in GAERS was observed compared to both Wistar and NEC, not directly linked to SWDs in thalamocortical circuits, although a link to memory circuits. GAT1 was observed in astrocytic processes in the membrane in astrocytes and neurons in all brain regions, except in the thalamus, which was only observed in astrocytes.

Interestingly, in previous studies in immature GAERS, the interaction between glutamatergic neurons and astrocytes appears normal, although an increase in astrocytic metabolism was observed (Margareta *et al.*, 2007). Astrocytic alterations are also a possible cause of seizures combined with increased GABAergic inhibition and GAT1 dysfunction. Indeed, GAT1 dysfunction could be due to several factors such as impaired translocation to the astrocytic plasma membrane, due to increased internalization, misplaced location around the synaptic cleft, abnormal phosphorylation, or aberrant intracellular transport (Crunelli *et al.*, 2020). However, a decrease in GAT1 expression was not observed in our project, indicating that the GAT1 loss of function is not due to a decreased protein expression, as the increase in GAT1 was only observed in the brain region where GFAP was heavily expressed, namely the motor cortex. Also, GAT1 expression was observed in the astrocytic membrane, indicative of non-impaired translocation to the astrocytic membrane. As expected, GAT3 expression was not altered between brain regions, confirming that GAT3 is not involved in this pathology. And since its expression is observed in astrocytes and neurons, an increase of expression was expected due to the increase in GFAP expression pattern.

Regarding general brain morphology in GAERS, our results show an increased brain/body weight ratio, which is in line with an increase in the whole volume, cortical volume, and somatosensory thickness as well as increased thalamic volume found in previous studies. Augmented brain weight found in this project may be linked to the thickening of brain regions such as the somatosensory cortex. An increase in GFAP protein expression was observed for all brain regions, except somatosensory cortex in GAERS animals. These quantitative changes appear in regions involved in SWDs, not being clear whether they are primary to epileptogenic processes or secondary to recurrent seizures, given that in previous studies an increase in mRNA and protein levels were observed before ASs, being possible an early age-related impairment. However, an increase in protein expression was also observed in regions not involved in seizures (eg hippocampus).

In this project, the increase in GFAP protein expression in several regions involved within the thalamocortical circuit can lead to astrocytes enwrapping synapses and augmenting extracellular space, following a compensatory mechanism to increase GFAP expression and therefore GAT1 to compensate its loss of function, leading to an increase in brain volume. In conclusion, an increase in brain volume, and a reduced number of neurons, and an increase in the number of terminals, can lead to a distinct neuronal cytoarchitecture.

Astrocytes play a role in synapse remodelling and long-term memory development. Astrocytes may have an essential role in WM by promoting cellular bistability via synaptic plasticity and contribute to delay activity, to changes in neural activity in the interval between a stimulus and the behavioral response in memory tasks, specifically WM (Pità *et al.*, 2014). Increased GFAP expression in temporal, parietal, and occipital cortices, in previous epidemiological studies, has been related to weak memory in healthy aging and disease (Bettcher *et al.*, 2021). Astrogliosis if sustained may be harmful, when activated, astrocytes release GFAP, having a role in astrocytic regulation of neurogenesis. Hippocampal astrogliosis may play a role in the development of memory deficits. In rats fed with a high-fat diet, an increase in GFAP positive cells was observed in the cortex, presenting also deficits in NOR (Bondan *et al.*, 2019). If astrocytes are pathological reactive, being astrocytic vesicular release is needed for cortical γ oscillations, and reduced γ oscillations impair NOR.

GFAP is the best marker to study astrocytic morphological changes, namely to identify if astrocytic alterations are observed before ASs phenotype or if the astrocytic changes are a result of ASs. However, changes in astrocytes have been observed only at the onset of SWDs, namely in the astrocytic reactivity (IL1 β), an inflammatory molecule, associated with SWDs in the somatosensory cortex but not in the cortical or extracellular areas adjacent (Akin *et al.*, 2011). Interestingly in this project, reduced changes were observed in somatosensory cortex astrocytes, being similar to NEC namely on GFAP expression and morphology. If the astrocytes are reactive, an increase in cell volume, dendritic thickness, and increase number of processes may contribute to a decrease in colocalization between astrocytes and neurons (as observed in 5XFAD mice (Choi *et al.*, 2021)), and a sufficient number of astrocytes to neurons connection is needed for LTM. The presence of reactive astrocytes may impair LTM formation.

Other markers could be used: S100 β could be used to the cortex and thalamus immunostaining, allowing an overall distribution and identification of changes in the number, staining nucleus, and part of cytoplasm processes; GFAP would be suitable to stain soma and processes in the hippocampus; connexin 43 only expressed in mature astrocytes; and vimentin, an intermediate filament, however also expressed in endothelial cells and immature astrocytes. To understand the astrocytic positioning in the synapse of GAERS, phalloidin marking neurons filaments and gephyrin an inhibitory marker for postsynaptic sites interacting postsynaptically with GABA_ARs, co-stained with GFAP and GAT1, also allowing GAT1 positioning at the synapse. Differences were found in astrocytes morphology, being important to understand if astrocytes with a malfunctioning receptor are by themselves responsible for

an alteration in astrocytic morphogenesis or if a deficient morphology contributes to this loss of function. For this, slices could be taken from animals PN 14, 16 and 18, to confirm the astrocytic and neuronal architecture of GAERS compared to NEC and Wistar, since SWDs occurs from day 19 to 30, when all animals have SWDs.

In closure, our results suggest memory deficits in GAERS, especially in spatial working memory, object recognition, long-term memory, and reference memory, in line with the reduced LTP magnitude, which can be rescued by BDNF. Since GAT1 is expressed in astrocytes and there is an increase in morphological complexity and GFAP expression in GAERS, the involvement of astrocytes in the pathology of ASs is reinforced. Opening a new door to a new therapeutic target, BDNF, by acting on GABAergic inhibition and GAT1 function, translation of its effect *in vitro* to *in vivo* is futured. Also, to understand the specific pathology underlying astrocytic modifications which may contribute to seizures or be aggravated by it, to study the circuit functionality as well as to confirm if BDNF can diminish absence seizures, morphological abnormalities, and behaviour impairments (Fig. 5) in GAERS and STG animals models is fundamental.

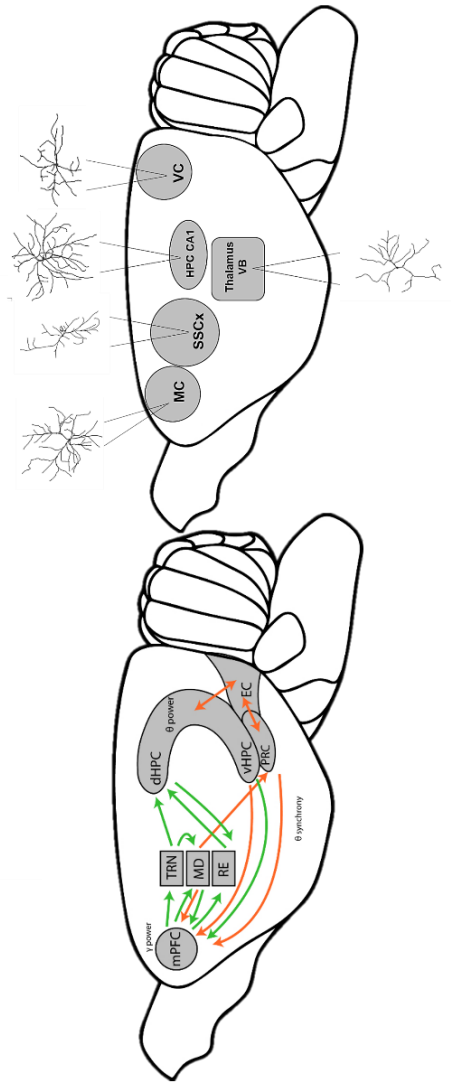
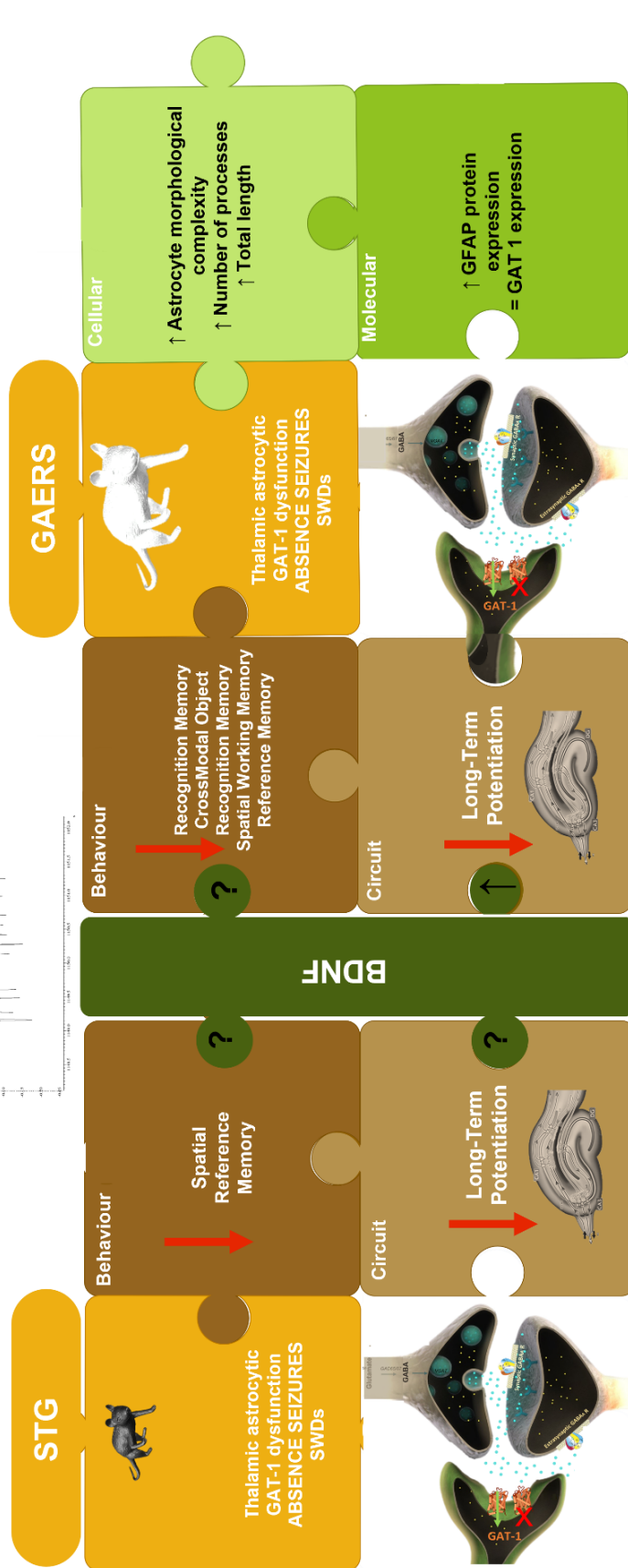
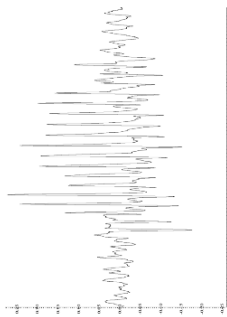


Figure 5.1. Scheme summarizing the results obtained in this work, divided into behaviour, circuits, cellular and molecular levels.

BDNF perfusion in hippocampal slices rescued LTP impairments in GAERS rats, and its effect will be tested in Long-term recognition memory deficits before acquisition memory occurs.

Conceptual model of circuit dynamics in Recognition Memory (orange) and Spatial and Working Memory (green). vHPC to mPFC encode spatial cues. TRN, MD, and RE modulate the functional connectivity between mPFC and HPC in working memory and spatial navigation. PRC has a mnemonic role and processes object information, relevant to visual recognition memory. (dHC) dorsal hippocampus, (mPFC) medial prefrontal cortex, (RE) nucleus reuniens of the thalamus, (MD) mediodorsal thalamus, (TRN) thalamic reticular nucleus, (vHC) ventral hippocampus, (PRC) Perirhinal Cortex, (EC) Entorhinal Cortex. **Representative astrocytic morphology obtained for each brain area .**

6 | References

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7| Supplementary Figures and Tables

Table 7.1. ST Results of OFT, % of permanence, average velocity and distance travelled in peripheral and intermediate zone.

	% Permanence			Average velocity			Distance travelled		
	Wistar	NEC	GAERS	Wistar	NEC	GAERS	Wistar	NEC	GAERS
Peripheral zone	92±0.867 **N,***G	81.4±2.12 *G	72.5±2.92	3.80±0.296	4.17±0.185	3.75±0.184	2027±156 *G	2019±72.8 *G	1583±72.8
	F (2, 70) = 18.5; p<0.001			F (2, 72) = 1.04; p=0.358			F (2, 72) = 5.39; p=0.007		
Intermediate zone	6.47±0.759 **N,***G	15.8±1.93 *G	22.8±2.22	8.99±0.576 **G	7.38±0.517	6.58±0.509	398±49.6 *N, ***G	597±45.5 *G	784±60.2
	F (2, 70) = 19.6; p<0.001			F (2, 72) = 5.25; p=0.007			F (2, 72) = 13.5; p<0.001		

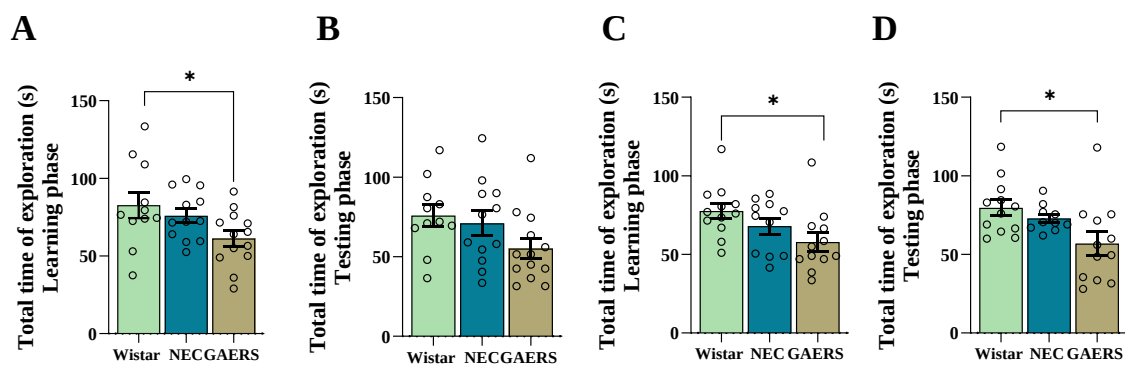


Figure 7.1 SF. Total time exploration of Novel Object Recognition for Short-term memory, learning phase (A) and Testing phase (B). Total time of exploration of Novel Object Recognition for Long-term memory, learning phase (C) and Testing phase (D).

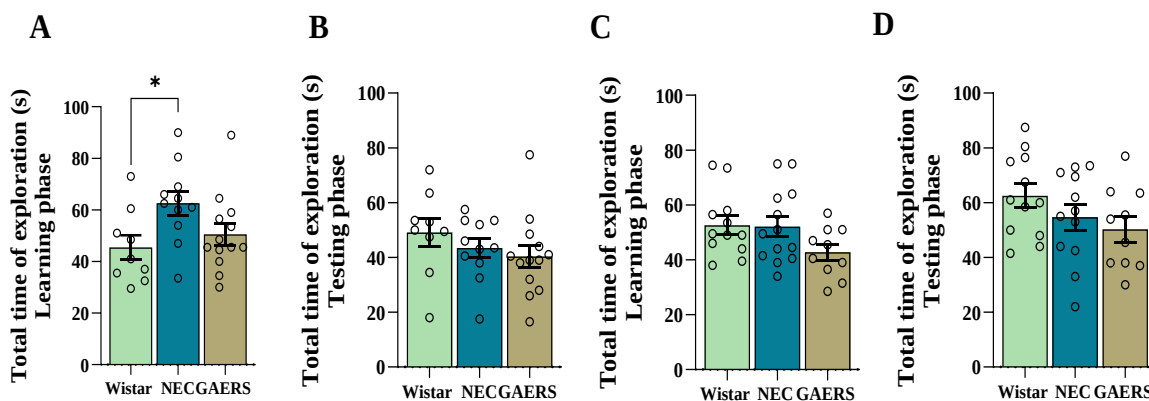


Figure 7.2 SF. Total time of exploration of Novel Object placement for Short-term memory, learning phase (A) and Testing phase (B). Total time of exploration of Novel Object Placement for Long-term memory, learning phase (C) and Testing phase (D).

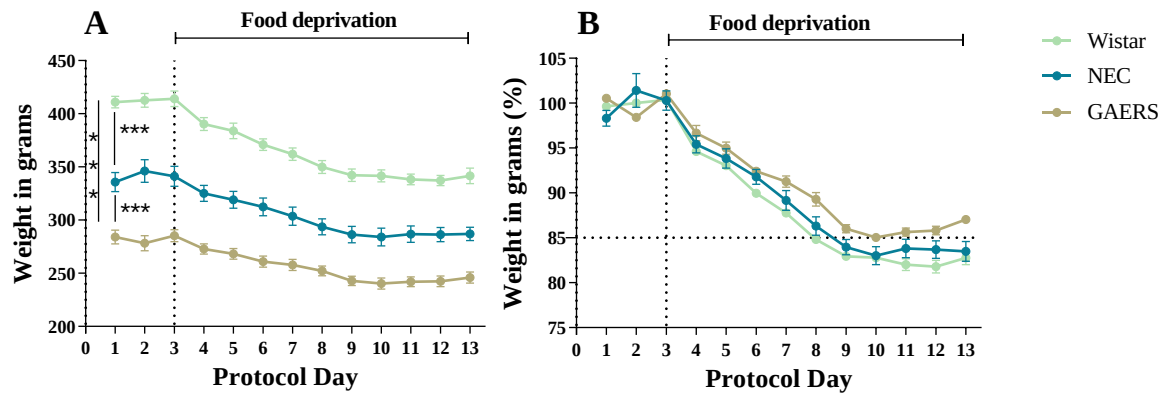


Figure 7.3 SF. Change in animal body weight over the 13 protocol days.

Body weight significantly differed between groups (***p, A). All animals reached 85 % of free feeding weight on day 10, when NMTP task began (B). Data expressed as the mean $\pm$ SEM (n=8); two-way ANOVA followed by Tukey's multiple comparisons test.

Table 7.2 ST. Results of NMTP task and DNMTP task. No significant differences were observed for multiple comparisons between groups during all protocol

Tukey's multiple comparisons test	Mean Diff,	Adjusted P Value
Day 1 Forced		
NEC vs. GAERS	6.250	0.836
NEC vs. Wistar	-12.50	0.261
GAERS vs. Wistar	-18.75	0.186
Day 2 Forced		
NEC vs. GAERS	-4.165	0.870
NEC vs. Wistar	-6.248	0.640
GAERS vs. Wistar	-2.083	0.959
Day 3 Forced		
NEC vs. GAERS	0.001667	0.999
NEC vs. Wistar	-6.249	0.753
GAERS vs. Wistar	-6.251	0.680
Day 4 Forced- 2 min		
NEC vs. GAERS	10.42	0.310
NEC vs. Wistar	10.42	0.434
GAERS vs. Wistar	-0.0004175	>0.999

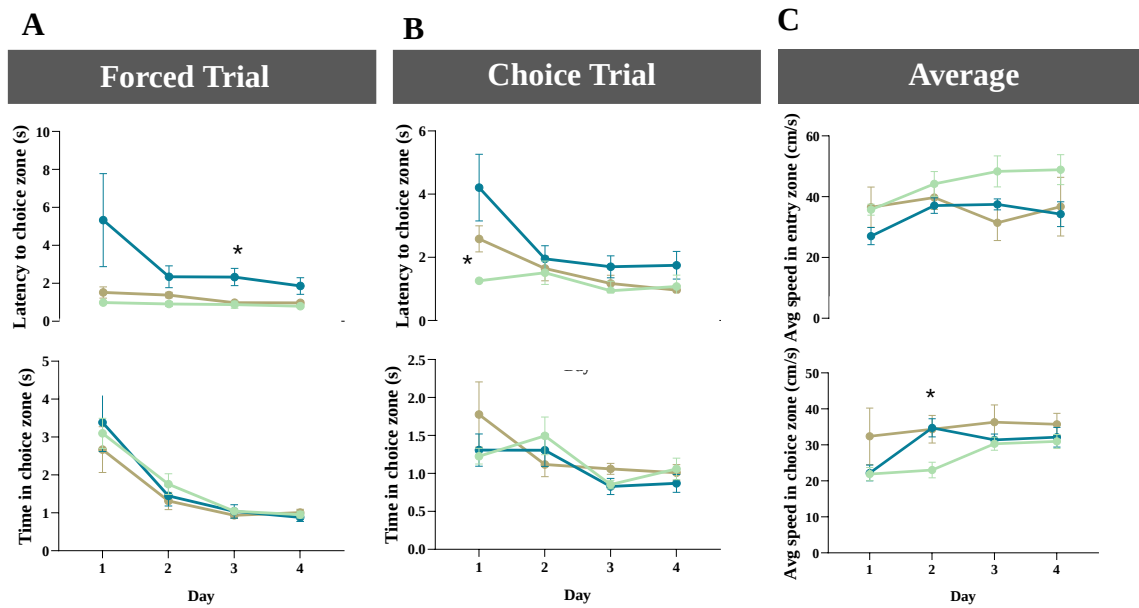


Figure 7.4 SF. Measurements of NMTP and DNMT task.

Latency to choice zone and time in choice zone in the forced trial (A), and during choice trial (B). Average speed in entry zone and in choice zone (C). Data expressed as the mean $\pm$ SEM (n=8); two-way ANOVA followed by Tukey's multiple comparisons test.

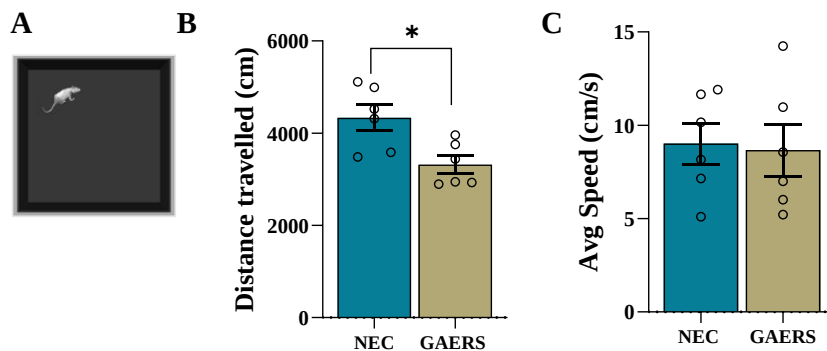


Figure 7.5 SF. Young adult GAERS travel reduced distances.

During the 10 min OFT trial (A), two measures derived: distance travelled (B), and average speed (C) for NEC (n=6) and GAERS (n=6). Differences were observed for distance travelled (*p). Data expressed as the mean $\pm$ SEM (n=6);

*p<0.5; unpaired Students t-test.

Table 7.3 ST. Results of OFT, Experiment 3, % of permanence, average velocity, distance traveled in peripheral and intermediate zone. Significant differences in total immobility time were observed (*p).

	% Permanence		Average Velocity (cm/s)		Distance travelled (cm)		Immobility (s)	
	NEC	GAERS	NEC	GAERS	NEC	GAERS	NEC	GAERS
Peripheral zone	81.56 ± 3.991	83.05 ± 3.270	6.866 ± 0.378	6.505 ± 0.189	1652 ± 144.9	2131 ± 207.4	53.86 ± 4.174	36.20 ± 6.978
	p=0.778		p=0.408		p=0.079			
Intermediate zone	16.76 ± 3.537	14.99 ± 2.906	8.029 ± 0.606	8.965 ± 0.516	571.0 ± 127.4	679.9 ± 123.2	p=0.048 (*)	
	p=0.704		p=0.259		p=0.5488			

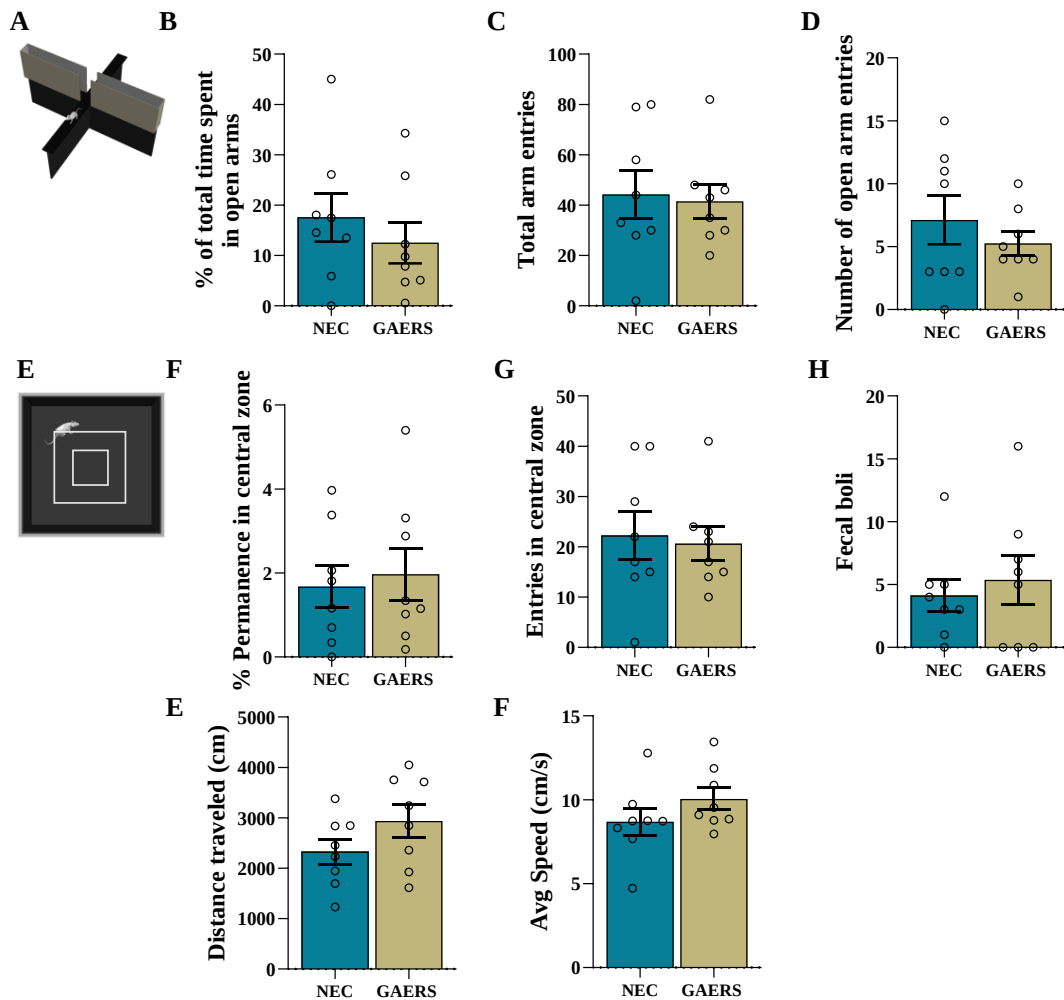


Figure 7.6 SF. Anxiety-like behaviour and locomotion of Adult GAERS similar to young adults.

Measures of anxiety derived from EPM 5 min trial (A), percentage of time spent in open arms (B), Total arm entries (C), and number of open arm entries (D). OFT 10 min trial (E) measures of anxiety, percentage of permanence in central zone (F), entries in central zone (G), fecal boli (H), and locomotion parameters, distance travelled (I) and average speed (J) in adult GAERS (n=8) and respective control NEC (n=8). No differences between groups were observed. Data expressed as the mean ± SEM (n=8); unpaired Students t-test

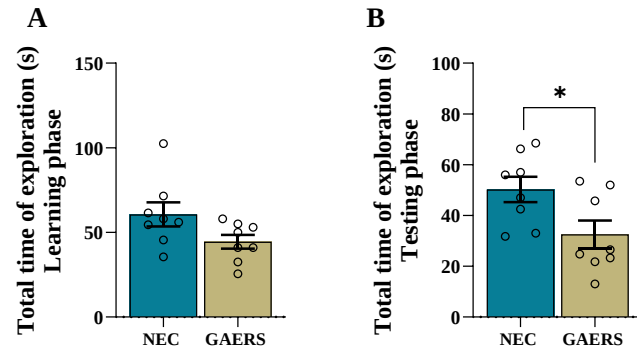


Figure 7.7 SF. Total time of exploration of Modified Novel Object Recognition in adult animals for Long-term memory, learning phase (A) and testing phase (B).

Table 7.4 ST. Total exploration time of the objects during the sample and test phase of the tactile, visual and crossmodal recognition memory tests. GAERS showed significantly increased exploration time in test phase of the visual memory test (**p)

Group	Testing	Tactile	Visual	Crossmodal
NEC	Sample	46.94±4.023	26.69±4.234	34.94±2.124
	Test	18.61±2.76	10.75±0.70	22.14±4.05
GAERS	Sample	53.44±2.107	24.38±4.209	36.94±4.399
	Test	23.43±3.06	24.13±3.17**	23.09±3.97

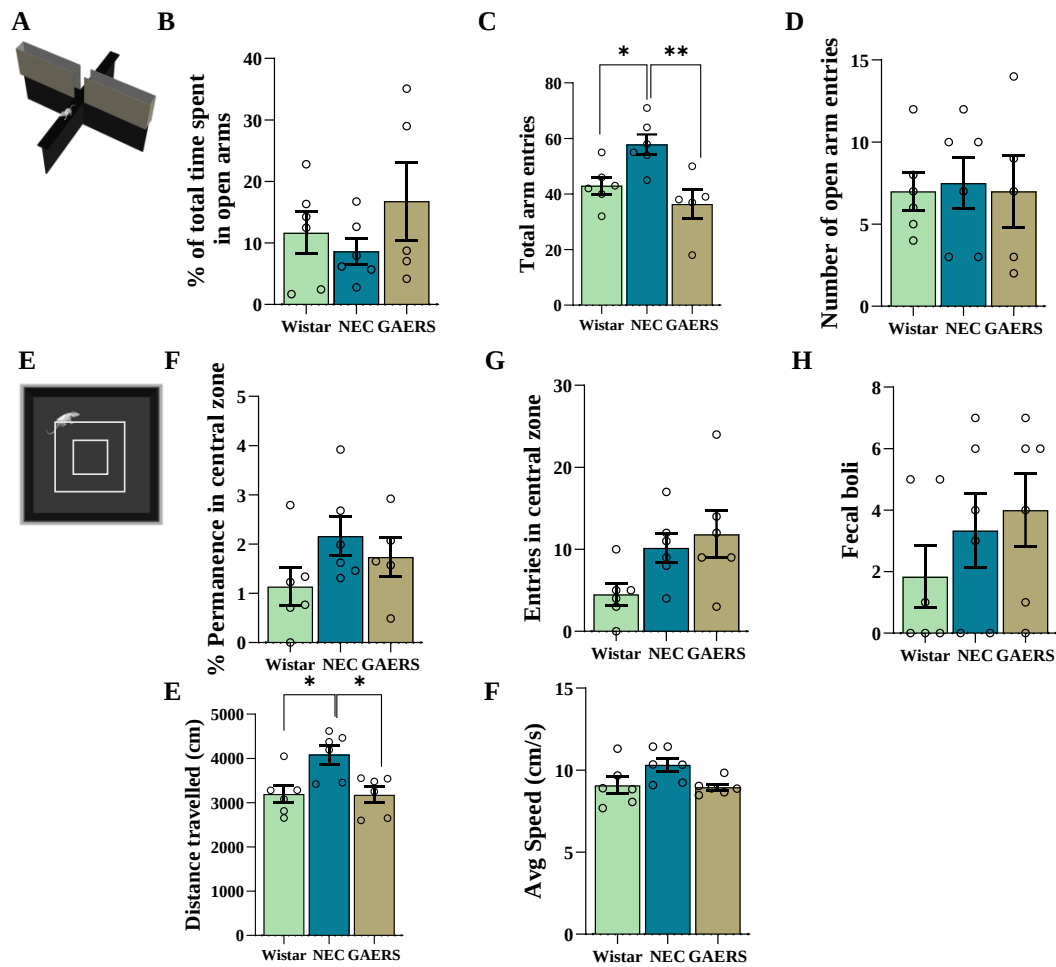


Figure 7.8 SF. Young adult GAERS do not display anxiety-like behaviour.

From the 5-min EPM trial (A), two measures of anxiety derived. Time spent in open arms as a percent of total testing time (B), the number of total arm entries (C) and the number of entries in open arms (D). NEC had increased total arm entries (*, **p) compared to both controls. Behaviour on the 10 min OFT (E) was evaluated examining the permanence and entries in central zone (F,G, respectively), fecal boli counts (H), distance travelled (I) and Average speed (J) in young adult male Wistar (n=6), NEC (n=6) and GAERS (n=6) rats. NEC travelled more distance (*p). Data expressed as the mean $\pm$ SEM (n=6); *p<0.5, **p<0.01, ordinary one-way ANOVA followed by Tukey's multiple comparisons tests.

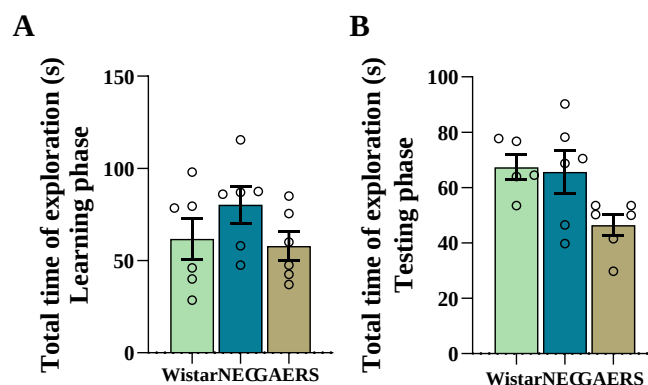


Figure 7.9. Total time of exploration of Modified Novel Object Recognition in adult animals for Long-term memory, learning phase (A) and testing phase (B).

Table 7.5. ST Total latency and path length variations between days for the three groups tested

Parameter	Day	Comparison	P value
Total Latency	1	Wistar vs. NEC	0.037
		NEC vs. GAERS	0.013
	2	Wistar vs. NEC	0.003
	4	Wistar vs. NEC	<0.001
Total path length	1	Wistar vs. NEC	0.001
		NEC vs. GAERS	0.002
	2	Wistar vs. NEC	0.002
		NEC vs. GAERS	0.014
	4	Wistar vs. NEC	0.001
		NEC vs. GAERS	0.017

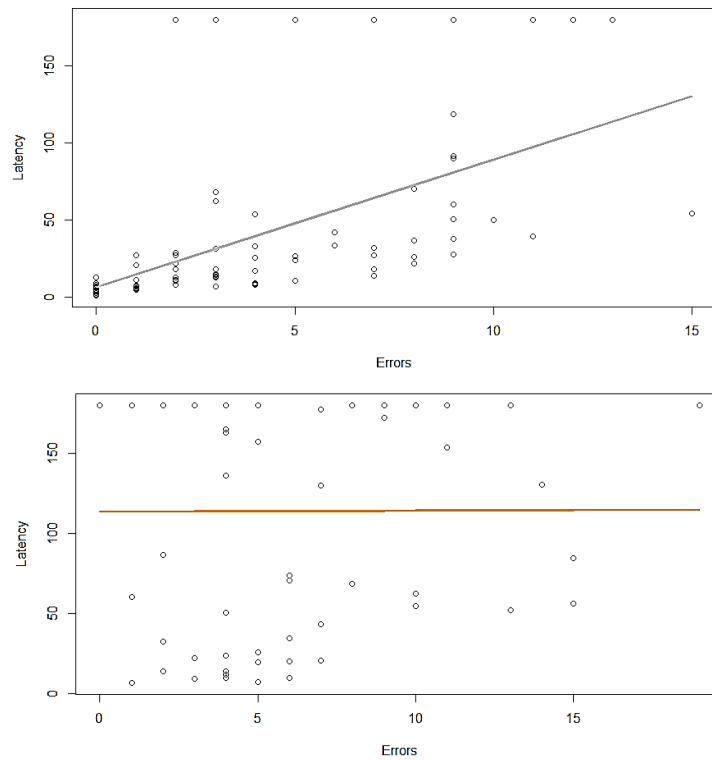


Figure 7.10 SF. Pearson correlation calculation of Number of errors and Latency for WT (upper panel) and STG mice (lower panel).

A powerful correlation was observed for WT mice ($r=0.56$) and 31% of the variance of latency can be explained by the variance in the number of errors. In WT mice there is no correlation was observed between number of errors and latency for STG mice ($r=-0.04$) and 0 % of the variance of latency can be explained by the variance in the number of errors

Table 7.6 ST. Primary Antibodies List

Antigen	Antibody	Host	Assay	Blocking	Dilution
S100B	Anti- S100B	rabbit	IHC	10%FBS in PBS 1x	1:500
Glial fibrillary acidic protein	Anti-GFAP	Mouse Monoclonal/rabbit polyclonal	IHC, WB	10%FBS in PBS 1x	1:750 1:500
GABA Transporter 1	Anti- GAT 1	rabbit polyclonal	IHC, WB	10%FBS in PBS 1x	1:750 1:500
GABA Transporter 3	Anti- GAT 3	rabbit polyclonal	IHC, WB	10%FBS in PBS 1x	1:750 1:500
NeuN	Anti –NeuN	Mouse monoclonal	IHC	10%FBS in PBS 1x	1:500
Glyceraldehyde-3-phosphate dehydrogenase	Anti-GAPDH	Mouse Monoclonal	WB		1:500
PSD95	Anti-PSD95	Mouse clone k28/43	WB		1:500

Table 7.7 ST. Secondary Antibodies List

Antibody	Assay	Dilution
Goat anti-mouse Alexa 568	IHC	1:600
Goat anti-rabbit Alexa 488	IHC	1:600
Goat anti-guinea pig 488	IHC	1:600
Goat anti-mouse 647	IHC	1:600
Goat anti-mouse HRP	WB	1:10000
Goat ant-rabbit - HRP	WB	1:10000
Goat anti-guineapig HRP	WB	1:10000

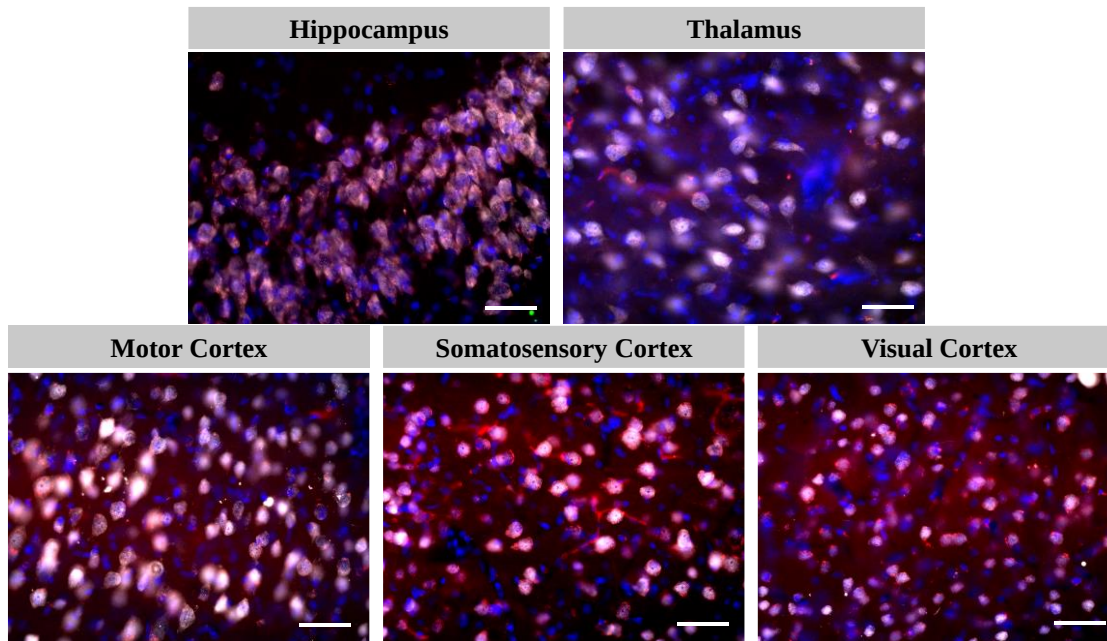


Figure 7.11 SF. Double detection of NeuN and GAT1 in rat brain slices.

Nuclei were stained with Hoechst, NeuN stained neurons and GAT1. Images were acquired with a 40x objective in a Zeiss Axiovert 200. Scale bar of 50 μ m Double detection was observed only in Hippocampus, motor cortex, somatosensory cortex and visual cortex. GAT1 colocalizations with neurons was not observed in thalamic neurons (NeuN:white, GFAP:red, Hoescht:blue).

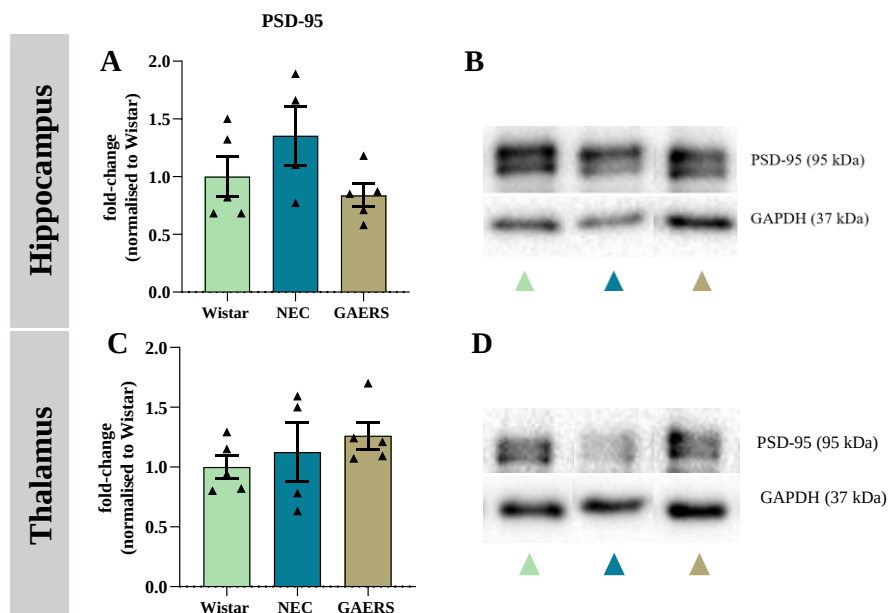


Figure 7.12 SF. Analysis of protein levels of PSD95 in Hippocampus (A,B) and Thalamus (C,D).

Densitometric analysis for each brain region and representative immunoblot (C,D). GAPDH is used as internal control. No significant differences between groups was observed.