Catarina Martins de Figueiredo

Licenciada em Biologia

The effect of excessive glutamate release on neuronal fitness, hyperactivity, and locomotor performance in a *Drosophila* model of Alzheimer's Disease

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientadora: Doutora Dina Coelho, Fundação Champalimaud

Co-orientadora: Professora Doutora Margarida Castro Caldas Braga, FCT-UNL

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Dezembro, 2020

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Abstract

Recent studies have shown that ectopic expression of the human Aβ42 (amyloid beta peptide) induces cell competition in the brain of a *Drosophila* model of Alzheimer's Disease. Neurons that are less fit upregulate low fitness markers such as *azot* and *Flower*^{LoseB}, activating pathways that culminate in their death, which has surprisingly positive effects. These unfit neurons correspond at least in part to Aβ42-induced hyperactive neurons which localize to brain areas exhibiting large amounts of glutamate, issues that are also common in the brains of patients but remain not well understood.

In this project, our aim was to uncover the relationship between glutamate accumulation and aberrant neuronal activity and to understand if neuronal silencing could rescue impaired locomotor activity seen in Aβ-expressing flies. We also aimed to understand if excess glutamate induces the upregulation of *azot* and *Flower*^{LoseB} in this context, since it is known that glutamate excitotoxicity contributes to neuronal death.

We could not find an effective strategy for neuronal silencing that improves the locomotor performance of flies, nor establish a reliable model for the relationship between hyperactivity and excessive glutamate. Silencing hyperactive neurons through different strategies did not result in changes in glutamate levels and downregulating glutamate signalling with different RNAi lines against DVGLUT showed signs of increased hyperactivity, which would go against what is predicted in literature, but was not a reliable outcome.

However, by treating AD model flies with memantine, a glutamate antagonist, we found an upregulation in *azot* and *Flower*^{LoseB} expression, which points to a role of excessive glutamate in modulating cell fitness in neurons and could provide further insights for the mechanisms of action behind the success of this drug in patients.

Keywords: Cell Competition, Alzheimer's Disease, Neuronal Hyperactivity, Glutamate, *Drosophila* melanogaster

Resumo

Estudos recentes demonstraram que a expressão ectópica de uma forma humana de Aβ42 (péptido beta-amiloide) induz competição celular no cérebro de um modelo da Doença de Alzheimer (DA) em *Drosophila*. Os neurónios menos "fit" aumentam a expressão dos marcadores de baixo fitness *azot* e $Flower^{LoseB}$, ativando vias que culminam na sua morte e traduzindo-se em efeitos surpreendentemente positivos na progressão da doença. Estes neurónios menos "fit", correspondem pelo menos em parte a neurónios que apresentam hiperatividade induzida por Aβ42 e estão localizados em regiões do cérebro que apresentam quantidades anormalmente elevadas de glutamato, fatores comuns nos cérebros de pacientes, mas que ainda não são completamente conhecidos.

Neste projeto, o nosso objetivo foi decifrar a relação entre acumulação excessiva de glutamato e hiperatividade neuronal, e compreender se o silenciamento neuronal poderia resgatar os defeitos na locomoção característicos de moscas que expressam Aβ42. Também pretendemos compreender se o excesso de glutamato induz o aumento da expressão de *azot* e $Flower^{LoseB}$ neste contexto, visto que já é conhecido que a excitotoxicicidade induzida pelo glutamato contribui para a morte neuronal.

Não conseguimos encontrar uma estratégia eficaz de silenciamento dos neurónios que resultasse numa melhoria na locomoção destas moscas, nem estabelecer um modelo consistente que explique a relação entre o excesso de glutamato e a hiperatividade neuronal. Silenciar os neurónios hiperativos através de diferentes estratégias não provocou mudanças nos níveis de glutamato no cérebro e reduzir a sinalização glutamatérgica através de diferentes linhas de RNAis contra DVGLUT pareceu promover o aumento de hiperatividade, o que poderia sugerir uma tendência para um papel inibitório do glutamato relativamente à hiperatividade neuronal, contrariando o que está descrito na literatura, contudo o ensaio não transmitiu a confiança necessária para chegarmos a essa conclusão.

Porém, o tratamento de moscas modelo para DA com memantina, um antagonista do glutamato, demonstrou um aumento na expressão de *azot* e $Flower^{Lose}$, apontando para um papel do excesso de glutamato na modulação do "fitness" celular, podendo resultar num melhor conhecimento dos mecanismos de ação responsáveis pelo sucesso deste medicamento.

Palavras-chave: Competição Celular, Doença de Alzheimer, Hiperatividade Neuronal, Glutamato, *Drosophila* melanogaster

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List of Abbreviations

AD	Alzheimer's Disease
AMPA	A-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
ANOVA	Analysis Of Variance
APOE4	Apolipoprotein E4
APP	Amyloid Precursor Protein
Aβ	Amyloid Beta-Protein
BACE1	Beta-Secretase 1
CCU	Champalimaud Centre For The Unknown
CNS	Central Nervous System
CO_2	Carbon Dioxide
CRISPR - Cas9	Clustered Regularly Interspaced Short Palindromic Repeats - CRISPR Associated Protein 9
DA	Doença De Alzheimer
DAPI	4′,6-Diamidino-2-Phenylindole
DCP1	*Drosophila* Caspase-1
DNA	Deoxyribonucleic Acid
DVGLUT	*Drosophila* Vesicular Glutamate Transporter
EAAT	Excitatory Amino-Acid Transporter
EDTA	Ethylenediaminetetraacetic Acid
EGFP	Enhanced Green Fluorescent Protein
Elav	Embryonic Lethal Abnormal Visual System
ER	Endoplasmic Reticulum
ERK/EGFR	Extracellular Signal-Regulated Kinases/ Epidermal Growth Factor Receptor
GFP	Green Fluorescent Protein
GMR	Glass Multiple Reporter
GS	Glutamine Synthetase
IL-7	Interleukin 7
JAK/STAT	Janus Kinases / Signal Transducer And Activator Of Transcription Proteins
KO	Knock-Out
LOP	Lexa Operator
LTD	Long Term Depression
LTP	Long Term Potentiation
MB	Mushroom Body
MCI	Mild-Cognitive Impairment

MDCK	Madin-Darby Canine Kidney
mfMRI	Muscle Functional Magnetic Resonance Imaging
NFAT	Nuclear Factor
NFT	Neurofibrilary Tangles
NMDAR	N-Methyl-D-Aspartate Receptor
OL	Optic Lobe
PBS	Phosphate-Buffered Saline
PBS-T	Phosphate-Buffered Saline - Triton
PCR	Polymerase Chain Reaction
RNAi	RNA Interference
SB	Squishing Buffer
ST	Speed Threshold
Ta	Annealing Temperature
UAS	Upstream Activating Sequence
VDRC	Vienna *Drosophila* Resource Center

1. Introduction

1.1. Alzheimer's Disease

With life expectancy consistently increasing in the western world and nativity levels decreasing, the global population is rapidly aging. One of the negative consequences of this sociological phenomenon is the ever-increasing frequency of neurodegenerative disorders, and the burden they represent on families and healthcare systems. The most prevalent neurodegenerative disorder is Alzheimer's disease (AD), a condition commonly associated with symptoms such as memory decline and loss of cognitive function. It is estimated that more than 100 000 people in Portugal are affected by this disorder, representing a considerable financial burden in our healthcare system (Santana et al., 2015).

1.1.1. Hallmarks of AD

Alzheimer's disease is characterized by 3 pathophysiological hallmarks: deposition of β-amyloid plaques, hyperphosphorylation, and aggregation of the tau protein and loss of brain weight, and decreased size due to increased neuronal death. These changes do not occur uniformly throughout the brain, but mainly affect the entorhinal area, the hippocampus, the neocortex, and the nucleus basalis (Kandel, 2013).

One of the first visually identified abnormalities in the brains of AD patients were amyloid senile plaques, extracellular structures that form from aggregates of misfolded β-amyloid peptides, which are more commonly 40 (Aβ40) or 42 amino acids long (Aβ42). These peptides are formed by the cleavage of the amyloid precursor protein (APP), a large transmembrane glycoprotein that is present in different types of cells but whose function is largely unknown, by enzymes such as β-secretases and the ɣ-secretase complex (Lane et al., 2018). Aβ peptides can aggregate into large insoluble protofibrils and amyloid fibrils or smaller, soluble, oligomers that can spread throughout the brain. It was initially thought that amyloid plaques and fibrils were the main culprits of AD pathogenesis, but recently it has been proposed that oligomers are more toxic to neurons. Unlike plaques, they can bind to different molecules, receptors such as the mGluR5 (metabotropic glutamate receptor type 5) and NMDAR (N-Methyl-D-aspartate) glutamate receptors, inducing calcium dysregulation, and cell membranes, which seems to, among other things, contribute to mitochondrial dysfunction and ER (Endoplasmic reticulum) stress (Chen et al., 2017).

Tau is a microtubule-binding protein that plays an important role in cytoskeletal transport, mainly in the axons of neurons. In the context of AD, this protein becomes hyperphosphorylated, forming aggregates that are toxic to the cell in addition to the inherent disabilities to axonal transport caused by its absence (Lane et al., 2018). Unlike APP, no disease-causing mutations in tau have been identified in familial forms of the disease. However, tau tangles are present in a considerable

number of neurodegenerative disorders, named tauopathies, and, in the case of AD, the degree of tangle burden seems to correlate well with cognitive decline. Over the years, there has been extensive debate on whether amyloid plaques or tau tangles were responsible for AD pathology, and although it remains unclear, most evidence points to a combined effect of these two dysfunctions (Busche & Hyman, 2020).

AD is undeniably an extraordinarily complex condition, due to all of the different aspects that seem to play a role in the course of its progression. Besides the 3 main hallmarks, impairments in many cellular and physiological functions such as oxidative stress, inflammation, metabolism, sleep deprivation, and even pathogens or microbiota have been shown to be associated with AD (Long & Holtzman, 2019). To further complicate this scenario, these impairments seem to not only involve neurons, but also different types of glial cells, and interactions between them can shape the course of disease progression (Henstridge et al., 2019).

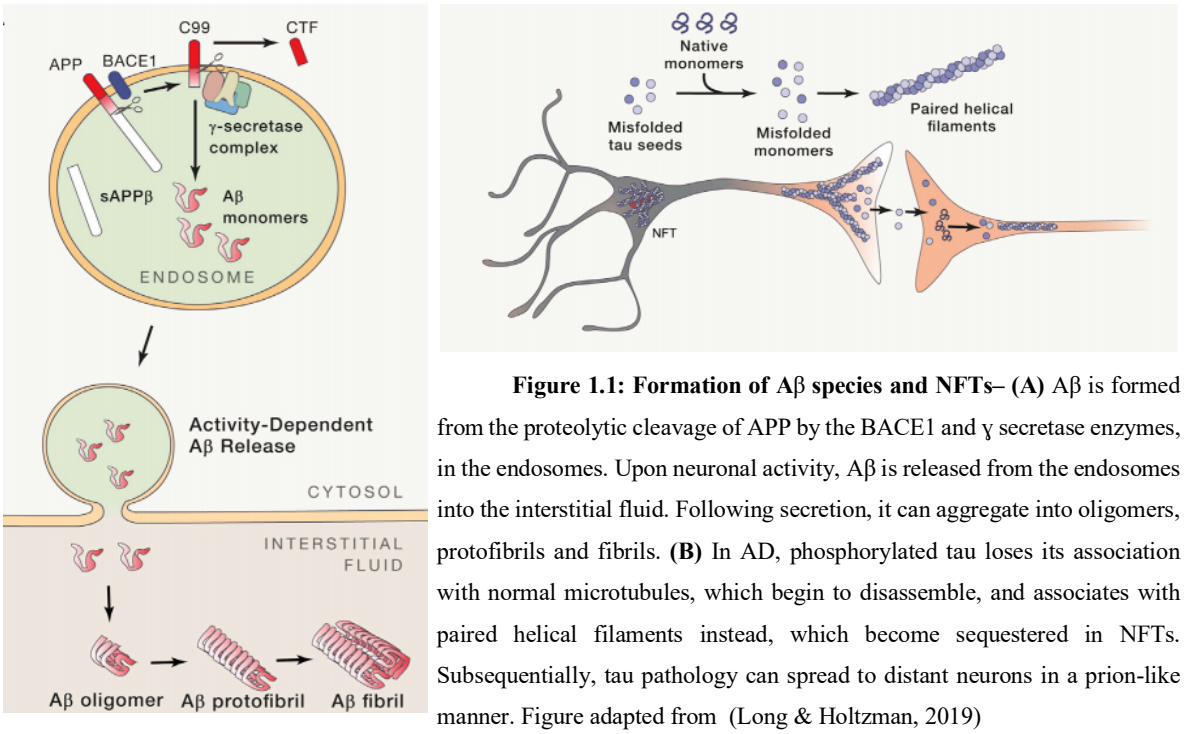


Figure 1.1: Formation of Aβ species and NFTs– (A) Aβ is formed from the proteolytic cleavage of APP by the BACE1 and γ secretase enzymes, in the endosomes. Upon neuronal activity, Aβ is released from the endosomes into the interstitial fluid. Following secretion, it can aggregate into oligomers, protofibrils and fibrils. **(B)** In AD, phosphorylated tau loses its association with normal microtubules, which begin to disassemble, and associates with paired helical filaments instead, which become sequestered in NFTs. Subsequentially, tau pathology can spread to distant neurons in a prion-like manner. Figure adapted from (Long & Holtzman, 2019)

1.1.2. Causes, risk factors, and treatments for AD

Aging is the primary risk factor for developing AD, however, a genetic risk factor has also been identified: individuals that carry the *APOE4* allele of the apolipoprotein E, a lipid carrier protein, are much more likely to develop AD and it is estimated that the allele is carried by 40-50% of AD patients. Although the relationship between this protein and AD is not well known, recent studies seem to correlate the APOE allele with defects in the blood-brain barrier (Montagne et al., 2020).

In recent years, many aspects of the pathophysiology of AD have been discovered, however, what is precisely causing the neuronal loss and synaptic dysfunction that characterize the brains of these patients remains unclear.

In a healthy brain, there is also production of Aβ peptides, where they seem to be required for synaptic plasticity and memory formation processes, but their production is counterbalanced by proteolytic degradation, cell-mediated clearance, active transport out of the brain, or deposition into insoluble aggregates. In AD patients, degradation pathways seem to be impaired and the concentration of Aβ becomes far greater (Chen et al., 2017).

There are rare familial forms of the disease, which affect younger individuals and are caused by mutations in the genes that encode APP or in the genes of enzymes that cleave the APP protein into smaller peptides, β, and ɣ-secretases. All of these mutations lead, in some way, to an increase in the production of β-amyloid peptides or their amyloidogenic propensity, exposing the brain to high levels of misfolded peptides that drive neurodegeneration and trigger the same symptoms shown in patients that have idiopathic AD. The fact that the genetic causes of hereditary AD all contribute exclusively to an increase in β-amyloid toxicity (Henstridge et al., 2019), is a strong argument in favour of the " amyloid cascade hypothesis", proposed by Hardy and Higgins in 1992. It states that β-amyloid driven toxicity is the primary cause of AD pathology, and is responsible for triggering the remaining physiological dysfunctions seen in patients' brains, including tau tangles and neuronal loss. Another factor that corroborates this idea is the fact that Aβ is the main component of senile plaques found in AD patients and that most animal models used to study AD were created by inducing human mutations that in some way increase Aβ burden. Most of these animals recapitulate some aspects of the neuropathology, cognitive decline, and other symptoms observed in patients, however, it is important to note that murine models that express Aβ do not display neuronal death or even tau tangles, so it can be argued that they should be considered mostly models of earlier stages of AD (Ashe & Zahs, 2010).

To counteract these arguments, amyloid targeting therapies remain unsuccessful in limiting or delaying disease progression in patients. Furthermore, there is minimal to no correlation between stages of amyloid deposition and progression of cognitive decline (Long & Holtzman, 2019), suggesting that Aβ alone is not responsible for sporadic AD. Currently, the cellular extension of the "amyloid cascade hypothesis" (De Strooper & Karran, 2016) is more widely accepted. It states that amyloid and tau pathology gradually accumulate in the brain during asymptomatic, often long, early stages of the disease. This "biochemical stage" is followed by a "cellular phase" when cell homeostatic mechanisms fail to tolerate the toxicity due to impaired clearance of proteinopathy debris. In this stage, besides the consequential synaptic failure and aberrant neural activity, neurovascular dysfunction, impaired astrocytes, and increased inflammation play a fundamental

role in disease progression, pointing towards the relevance of interactions between different cell types in AD pathology (Henstridge et al., 2019).

Despite years of extensive research, many clinical trials, and generous funding, there is still no cure for AD and a lot of its pathology remains unknown. Current treatments consist of cholinesterase inhibitors that counteract the loss of cholinergic input due to neuronal death and memantine, a glutamate antagonist, which might act by inhibiting glutamate-mediated excitotoxicity (Long & Holtzman, 2019). Both can only control patients' symptoms in the early stages of the disease and are mostly successful only in delaying its most aggressive onset.

1.1.3. Neuronal hyperactivity in AD

For a long time, it has been known that there is a correlation between the reduction in neuronal activity and excitatory synaptic transmission, and the memory and cognitive decline of AD patients. However, recent *in vivo* studies revealed a surprising aspect, and have found neuronal hyperactivity as a primary neuronal dysfunction in early stages of AD. Increase circuit activity has been described in different studies where mfMRIs were performed in brains of patients at risk to develop AD, for instance, people with MCI (mild cognitive impairment). Patients with AD have a higher probability of developing epileptic seizures, which has been associated with an earlier onset of cognitive decline, and patients with epilepsy have a higher risk of developing AD (Palop, 2009) (Vossel et al., 2013) (Busche & Konnerth, 2015). Furthermore, in mice models of AD, clusters of hyperactive neurons have been found in the vicinity of β-amyloid plaques by two-photon imaging (Busche et al., 2008).

Despite the apparent relevance of this dysfunction, there is still no consensus regarding what is causing hyperactivity in the context of AD. Most studies point to a synaptic origin of neuronal hyperactivity, involving the presence of amyloid species, a pro-inflammatory environment in the vicinity of plaques, and some suggest a pathological imbalance between excitatory and inhibitory synapses. Some evidence also points to a role of soluble Aβ42, rather than plaques in this process.

A recent study proposed that neuronal hyperactivity is triggered by excessive neurotransmitter release, mediated by malfunctioning intracellular calcium stores in pre-synaptic neurons (Lerdkrai et al., 2018). It has also been suggested that hyperactivity and β-amyloid mediated toxicity may contribute to the progression of AD in a vicious cycle since increased neuronal activity seems to trigger an increased release of Aβ42, which in turn leads to higher toxicity and even more hyperactivity, due to an increase in activity-dependent clathrin-mediated endocytosis which increases neuronal uptake of APP-containing vesicles by dendrites at synapses (Stargardt et al., 2015).

Regardless of its origin, such an overwhelming increase in neuronal activity and excessive action potential firing is bound to have consequences beyond the neuron level and, in fact, hyperactive neurons are not only detrimental for neural circuit function but have also been shown to be responsible for negative consequences in behaviour, mainly spatial navigation (Busche & Konnerth, 2015). Despite its key role in AD pathology, more information regarding neuronal hyperactivity is still necessary.

1.1.4. Glutamate excitotoxicity

Glutamate is an amino acid and likely the most important neurotransmitter in the human brain, where it is present in almost all of the excitatory neurons in the CNS and plays an important role in learning and memory formation. Glutamate is synthesized in pre-synaptic neurons from glutamine and transported to the presynaptic terminal where it is packaged into synaptic vesicles by VGLUTs (vesicular glutamate transporters) and released upon membrane depolarization. In the postsynaptic terminals, glutamate can bind to 3 different types of ionotropic receptors: NMDA, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor), and kainate (Purves, 2018). NMDA receptors are of particular importance in neurodegenerative disorders since their overactivation leads to a massive intake of calcium to the cytoplasm, where it acts as a second messenger, and in case of dysfunction, excessive calcium can lead to neuronal death by excitotoxicity (Findley et al., 2019).

Rapid glutamate clearance from the synaptic cleft is essential to prevent excitotoxicity and is performed by extra-synaptic receptors or by EAATs channels (Excitatory Amino-Acid Transporters) in astrocytes. In fact, most glutamatergic synapses are tripartite: constituted by a pre- and post-synaptic neuron and an astrocyte. Following reuptake, glutamate is transformed in glutamine by GS (Glutamine synthetase) in astrocytes, forming a cycle of glutamate production and recycling in these two cell types **(Figure 1.2)**.

Glutamatergic dysfunction has been linked to neuronal death in neurodegenerative disorders since the 20th century (Maragos et al., 1987). Specifically, in AD pathology, there is an association between excitotoxicity-derived neuronal death and deficits in memory and cognitive decline in patients, since glutamate plays a fundamental role in memory formation by synaptic plasticity through NMDA receptors, in processes named LTP (long-term potentiation) and LTD (long-term depression).

It has been shown that Aβ can interact and modulate the expression and activity of different glutamatergic receptors, mainly causing an excessive concentration of this neurotransmitter (Parameshwaran et al., 2008). Recently, several hypotheses have arisen to try to comprehend the dynamics of glutamatergic dysfunction in AD. The signal-to-noise hypothesis states that the Aβ -

mediated increase in glutamate concentration leads to more excitatory activity, which overstimulates receptors. To compensate for this chronic overstimulation in the course of disease progression, more excitatory input becomes necessary to activate these receptors, leading to a decrease in glutamate concentration and impairments in LTP in later stages of disease (Findley et al., 2019).

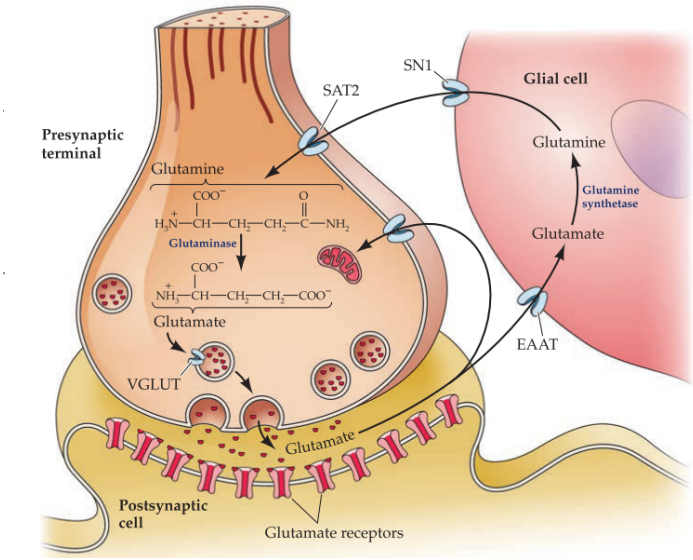


Figure 1.2: Overview of a glutamatergic synapse and the glutamate cycle – In the presynaptic neurons, glutamate is loaded into synaptic vesicles via vesicular glutamate transporters (VGLUTs), and released into the synaptic cleft upon membrane depolarization. The action of glutamate released into the synaptic cleft is terminated by uptake into surrounding astrocytes via excitatory amino acid transporters (EAATs). Within glial cells, glutamate is converted to glutamine by glutamine synthetase before being released back into the presynaptic neuron, where it is converted to glutamate by glutaminase. Adapted from (Purves, 2018)

1.2. Cell Competition

1.2.1. Overview of cell competition

The idea that Darwin's theory of natural selection could apply to the cells in our body, had been proposed before by scientists like Wihelm Roux and Ramon and Cajal, but it was only in the 20th century that evidence arose suggesting that competitive interactions could be important for cell quality control in the organism (Moreno & Rhiner, 2014). Competition between cells was described for the first time by Ginés Morata in 1975, when he discovered that wild-type cells and *Minute* mutants (cells lacking ribosomal genes) displayed competitive interactions in the *Drosophila* wing

disc (Morata & Ripoll, 1975). He coined cell competition as a non-autonomous cell behaviour characterized by the elimination of slow dividing cells when in contact with healthier or more successful neighbours, drawing a molecular parallelism to the ecological interaction between organisms. In recent years, the study of cell competition has grown exponentially, and it has emerged as a widespread phenomenon, not only being present across different organisms, ranging from *Drosophila* to mice and humans, but also having a fundamental role in different physiological contexts.

1.2.2. Biochemical pathways in cell competition

In the scenario in which Morata first described cell competition, clones of *Minute* +/- cells were eliminated when in contact with *wild-type* cells in the wing disc. Since then, other *in vitro* and *in vivo* scenarios have been used to study cell competition and uncover its key players. One of the most widely used scenarios is the induction of clones overexpressing the *Drosophila* orthologue of the *myc* oncogene, *dmyc,* in imaginal discs or the co-culture of cell lines expressing different levels of *myc*. In this case, cells carrying extra copies of *myc* can outperform *wild-type* cells with lower levels of *myc* and overproliferate at the expense of their apoptosis, in a processed deemed super-competition (Moreno & Basler, 2004). Competition scenarios can be achieved by inducing mutations in many other genes, such as *scribble* and other apico-polarity genes; *Dpp* or *Wnt,* and other genes important for development and pathways like *Hippo* and *JAK/STAT* (Lolo et al., 2013).

In all of these cases, surviving cells have an advantage in comparison to their neighbours that are eliminated, whether it be faster proliferation, higher resistance to mechanical stress or just being healthier after an insult, so we can say that their "fitness" is higher. The "fitness status" of a cell can change due to external aggressions or intrinsic factors (mutations) such as changes in growth and survival, proliferation, cell metabolism, exposure to toxicity, nutrient scarcity, or mechanical damage. However, a fundamental concept to retain is that cell competition depends on the differences in the fitness status of a cell in comparison to its neighbours, and not in its own intrinsic fitness, so, there is no standard definition of what characterizes an optimal fit cell, it always depends on the context the cell is in.

1.2.3. Types of cell competition

So far, three different types of cell competition have been described: competition for trophic factors, mechanical cell competition, and competition relying on the comparison of fitness fingerprints.

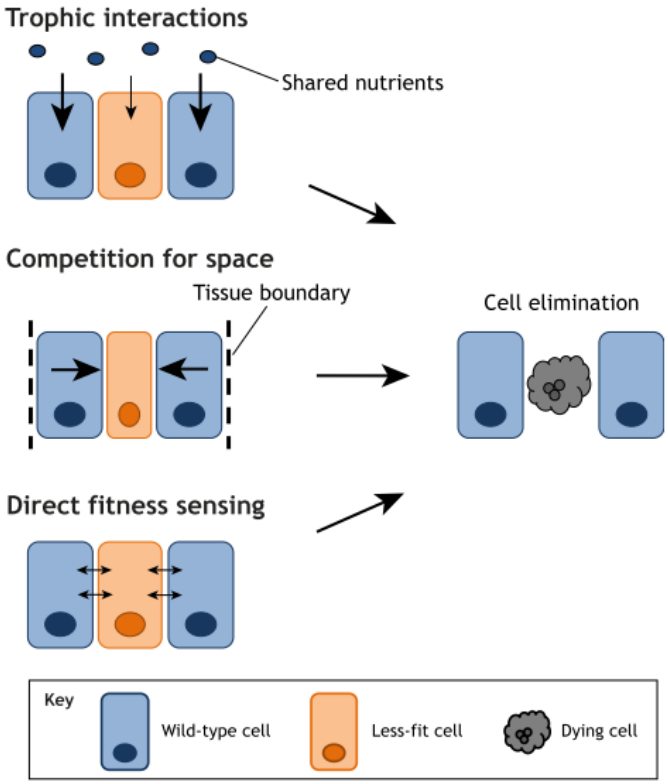


Figure 1.3: Types of Cell Competition – Illustrative scheme depicting the 3 different types of cell competition. Either by less success in capturing trophic factors, increased sensitivity to mechanical forces or by direct comparison of fitness fingerprints, less fit cells are eliminated from tissues. Adapted from (Bowling et al., 2019)

1.2.3.1. Cell competition for trophic factors

Competition for trophic factors is the type that most closely resembles the ecological phenomenon since it occurs when less fit cells show more difficulty in acquiring nutrients or different molecules necessary for their survival than healthier, more adequate cells, which results in their starvation and ultimately death. In the mouse, developing neurons compete for levels of nerve growth factor and only successful ones survive, a mechanism that is thought to ensure that the correct number of neurons enervate each cell and, in the thymus, T cell progenitors compete for IL-7 as a tumour suppressing mechanism (Martins et al., 2014). Unlike other types of competition, in this case, direct contact between cells is not required and rates of ligand capture and diffusion play an important role (Merino et al., 2016).

1.2.3.2. Mechanical Cell competition

Cells are subjected to mechanical forces in their environments, so they developed molecules that work as sensors that convert physical stimuli into biochemical signals in order to respond

accordingly, usually triggering signaling pathways that impact the cytoskeleton (Brás-Pereira & Moreno, 2018).

Mechanical cell competition has been described in the *Drosophila* pupal notum, a structure that gives rise to the adult thorax. There, cells converge to the midline, and compaction leads to the extrusion and elimination of cells through downregulation of ERK/EGFR and consequent activation of the pro-apoptotic gene hid (Moreno et al., 2019). This mechanism has been shown to play a role in zebrafish tail development, where tissue overcrowding in the fin leads to cell elimination, and it can be modulated in mammalian MDCK cell culture, where scribble knock-down cells are compressed and eliminated by *wild-type* ones.

Mechanical cell competition has a paradoxical nature in the sense that it can act as a tumour expansion or tumour suppressive mechanism: if tumour cells are more sensitive to compression, adjacent wild-type cells can trigger their elimination and prevent cancer development, however, if tumours acquire increased resistance to compression, they can promote wild-type cell elimination and expand further (Brás-Pereira & Moreno, 2018).

1.2.4. Cell competition relying on fitness fingerprints and its key players.

Competition also occurs in a manner that requires a direct comparison between cells' fitness statuses, which relies on differences in the expression of a set of specific molecules termed "fitness fingerprints" between more or less fit cells. We can view this type of cell competition as a sequential process that involves the sensing of relative fitness between cells, which can incite a pro-apoptotic signal in lesser fit cells and is followed by their removal from the tissue.

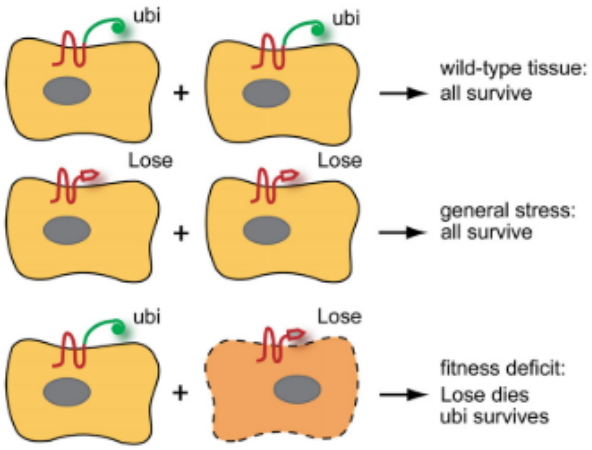


Figure 1.4: The "Flower Code" – Expression of different isoforms of the Flower protein determine the fate of competitive interactions between cells. Adapted from (Rhiner et al., 2010)

1.2.4.1. Flower

One of the key molecules of cell competition is a transmembrane protein named Flower, which works as a molecular sensor of fitness status. In *Drosophila*, the Flower protein has different isoforms that only differ in their extracellular C-terminal, Flower^{ubi} which is ubiquitously expressed in all cells, regardless of their fitness status, Flower^{LoseB} and Flower^{LoseA}, whose expression is upregulated in less fit cells (Rhiner et al., 2010).

The expression of different isoforms of Flower, depending on the fitness status of a cell, works as a "code" that signals a cell's fitness state to the extracellular environment. However, the molecular mechanisms that regulate the expression of the lose isoforms and those that allow for the detection of its expression in other cells' surfaces remain unclear.

It is important to note that the *flower* code is cell type specific, and, for example, in the brain, Flower^{LoseA} is expressed by default and only Flower^{LoseB} is a marker of low neuronal fitness.

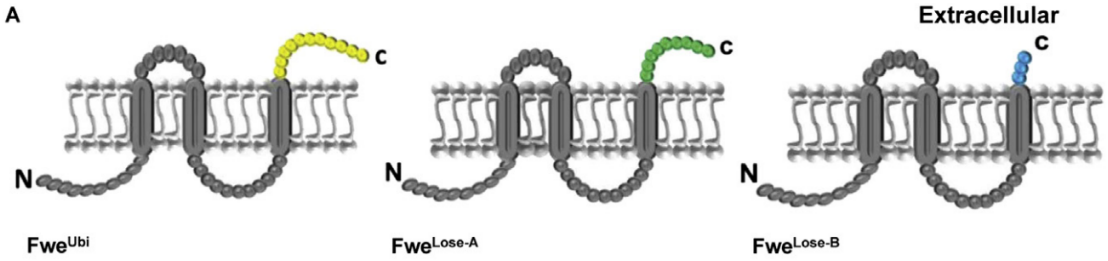


Figure 1.5: Different isoforms of Flower - The different isoforms of the Flower protein, Flower^{ubi}, Flower^{LoseB} and Flower^{LoseA} differ only in their extracellular C-terminal domain.Adapted from (Merino et al., 2013)

1.2.4.2. SPARC

Another important protein in this context is SPARC, a glycoprotein which, up to a certain point, can have a protective role against cell-cell differences in fitness. SPARC protects cells by possibly setting a higher threshold for caspase activation and, in competitive scenarios, it rescues cell elimination more successfully than caspase inhibitors (Portela et al., 2010). This seems to be the organism's strategy to allow cells to recover from transient stress and prevent unnecessary elimination of cells that are not damaging to the tissue in the long term.

1.2.4.3. Azot

These relative differences between the expression of *flower^{LoseB}* by the cell and by its surrounding neighbours, as well as its own endogenous expression of SPARC are integrated by the transcription factor Azot, which acts as a fitness checkpoint (Merino et al., 2015). *Azot* transcription is upstream of the activation pro-apoptotic genes in the less fit cells, promoting programmed cell death. The

pathways through which Azot integrates this information are not known yet, and neither are those responsible for triggering *flower*^{LoseB} upregulation.

1.2.5. Cell competition in different contexts

Cell competition has been shown to play important roles throughout different stages of life in an animal, ranging from development to maintenance of homeostasis in adult tissues and also in contexts of disease or damage.

1.2.5.1. Cell Competition in cancer development

A very relevant context in which cell competition has provided promising insights is tumor expansion and cancer development. In a super competition scenario, tumour cells can acquire mutations that make them "fitter" than their neighbours, which triggers their elimination by cell competition. This is only one way in which cancer cells can hijack cell competition mechanisms: tumour expansion can also benefit from the invasion triggered by cell mixing that potentiates winner-loser interactions (Levayer et al., 2015) and from the elimination of healthy tissue derived from mechanical tension that increases as the tumor grows. On the other hand, cell competition can also act as a tumour suppressive mechanism. For example, in the mammalian thymus, young T cells outcompete their old progenitors, preventing the development of acute lymphoblastic leukemia due to cumulative genetic insults (Martins et al., 2014).

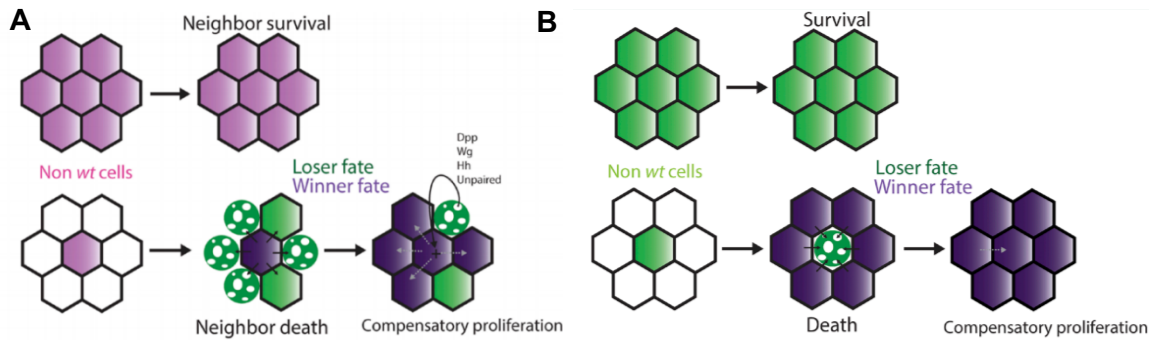


Figure 1.6: Cell Competition in a cancer context – A classical cell competition scenario works as a **(A)** tumour suppression mechanism, where mutant cells are eliminated when in contact with neighbouring *wild-type* cells, preventing their proliferation and consequent tumour expansion **(B)** In a super-competition scenario, tumour cells can highjack the cell competition machinery by outcompeting neighbouring *wild-type* cells and proliferating at their expanse. Adapted from (Levayer & Moreno, 2013)

1.2.5.2. Cell competition in the nervous system

As previously mentioned, cell competition occurs in different contexts and regions of an organism, and the brain is no exception. Most information about cell competition in the CNS is, so far, relative to *Drosophila melanogaster*. However, a reminiscent process of cell competition has also been

implicated in the brain development of mammals, where there is competition for neurotrophic factors to ensure optimal innervation. Also during development, in the *Drosophila* nervous system, surplus neurons forming incomplete ommatidia at the periphery of the retina upregulate *flower*^{LoseB} and are eliminated by cell competition (Merino et al., 2013).

In the adult fly, cell competition also plays a role in the brain's response to damage and has been determinant to growing knowledge about adult neurogenesis. In this case, damaged neurons overexpress *flower*^{LoseB} and are eliminated from the regenerating tissue by apoptosis. This comparison between the fitness levels of damaged and undamaged cells showed, for the first time, that there is negative neuronal selection in the adult brain (Moreno et al., 2015).

1.2.5.3. Cell competition in AD

Perhaps one of the most relevant areas of cell competition's influence in the *Drosophila* brain is in a disease context, mainly neurodegeneration. Recently, it has been shown that cell competition is particularly important for a puzzling phenomenon in AD: neuronal death.

In the brains of flies that ectopically overexpress human Aβ42 peptide, some neurons upregulate *flower*^{LoseB} and *azot.* These neurons are unfit in comparison to their neighbours that are less affected by the toxicity of the microenvironment in the vicinity of plaques. In these unfit neurons, activation of *azot* is involved in the expression of pro-apoptotic genes such as *hid* which prompt neuronal death (Coelho et al., 2018).

Azot and *flower* knock-out flies, where this fitness-based elimination is blocked, showed aggravated brain degeneration, shortened lifespan, and worsened cognitive and locomotor decline. Moreover, an extra copy of *azot* was shown to be sufficient to restore behavioural defects in Aβ42-expressing flies (Coelho et al., 2018), solidifying the importance of cell fitness fingerprints in these processes.

A recent study using Aβ42-expressing flies has shown that the neurons that upregulate *azot* and *flower* show sustained aberrant activity (Coelho & Moreno, 2020), connecting cell competition with neuronal hyperactivity, a prominent phenomenon in early stages of AD.

It is thought that early elimination of dysfunctional neurons is beneficial because it allows for the maintenance of healthier neuronal circuits, which in this way, are capable of rewiring and functioning better in the absence of neurons which would otherwise be damaging to the whole system (Coelho et al., 2018; Coelho & Moreno, 2019), however, this is yet to be confirmed. Fitness-based neuronal culling in AD might also explain how Aβ-related toxicity differentially affects brain regions, postulating that neuronal subpopulations with increased vulnerability might correspond to the ones with lower fitness (Coelho & Moreno, 2019).

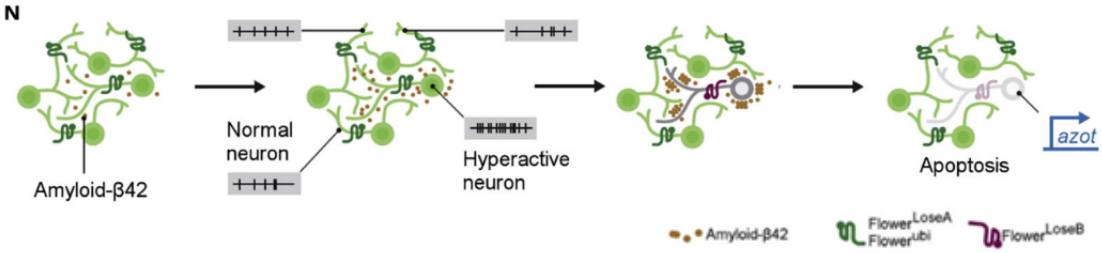


Figure 1.7: Cell competition in an AD *Drosophila* brain – Following Aβ-induced damage, some neurons display aberrant neuronal activity. Consequently, they lose fitness and upregulate *flower*^{LoseB} and *azot* and eventually die by apoptosis. This is beneficial for overall brain health as it allows for circuit rewiring. Adapted from (Coelho & Moreno, 2020)

1.3. *Drosophila melanogaster* as a model organism

The fruit fly *Drosophila melanogaster* has for long played a very important role in Biomedical Research and we owe this small invertebrate credit for major breakthroughs in our understanding of processes vital for the functioning of the human body, from development to genetics or disease. What apparently may distance us from this animal, its simplicity, is also its biggest advantage as an animal model, mainly in fundamental research.

1.3.1. Life cycle and development

Flies are holometabolous insects whose short life cycle has 4 main stages: eggs, larvae, pupae, and adults. Their life cycle is heavily connected with temperature and optimal growth occurs at 25ºC. At this temperature, 2 days after the mother lays her eggs on food, progeny grow into 1st instar larvae and progress to 2nd and 3rd instar larvae in the following days, as they feed in order to store energy for metamorphosis. During metamorphosis, pupa stay in their puparium (pupal case) for 4-5 days, during which their imaginal discs (tissue-specific progenitor cells) transform into adult structures like the wings, eyes, and antennae. Adults hatch from the puparium just 10 days after egg laying (Hales et al., 2015), and become sexually mature just 8 hours post-eclosion.

Manipulating the temperature they are exposed to can help either, accelerating development or delaying it when stocks are not being used. Typically, adults hatch in 7-9 days when crosses are left at 29ºC and about 20 days when they are left at 18ºC (Markstein, 2018).

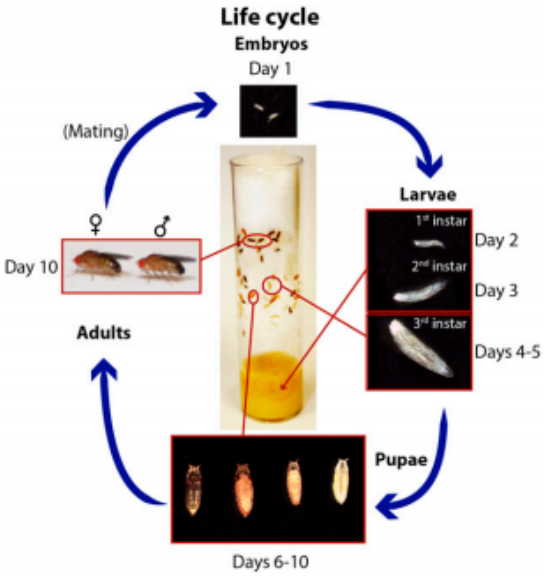


Figure 1.8: *Drosophila* life cycle – At 25ºC, 2 days after egg laying, larvae grow into 2nd and 3rd instar larvae as they climb the walls of the vials. After 6 days they pupate and undergo metamorphosis. Adults hatch after 10 days. (Hales et al., 2015)

1.3.2. *Drosophila* genetics

The study of *Drosophila* genetics was pioneered by Thomas Hunt Morgan in the late 19th century. He and his team were awarded a Nobel Prize for Physiology and Medicine for their work in successfully mapping the fly's entire genome, and developing several tools that facilitated work with this organism and led to the discovery of the function of many genes through forward and reverse genetic screens, techniques which they also perfected.

1.3.2.1. Karyotype, balancers, and genetic markers

This diploid organism has three pairs of autosomes: 2, 3, and 4 and one pair of heterosomes named 1, which can consist of two X chromosomes on females and one X in males. Due to their small size, chromosomes Y and 4 are typically excluded from written genotypes, since they carry very few genes. In these insects, sex determination occurs due to the ratio of X chromosomes and autosomes. This means that an XO fly is still a male and an XXY fly is still a female (Hales et al., 2015).

To track the inheritance of specific chromosomes or mutations in crosses, scientists use visual traits that are easy to detect in adult flies, called "genetic markers", which usually affect eye colour, wing

shape, or bristle length. The most commonly used genetic marker is white (w+), a dominant marker that produces a red eye colour and is used to track and detect the presence of transgenes in flies.

One of the most important tools in fly genetics is balancer chromosomes. Balancers are artificial versions of fly chromosomes that carry genetic markers, recessive lethal mutations, and have suffered several inversion events. As a result, their genes are arranged in a different order, which makes chromosomes derived from recombination events involving balancers unviable (Markstein, 2018). These features are helpful because, when crossing a fly carrying a gene of interest with another fly with a balancer version of the chromosome that gene is in, we know that the progeny of the cross will carry that gene of interest and the balancer, which is visible because of the genetic marker. In this way, we can ensure that the gene of interest will maintain its integrity since no progeny will have suffered crossing-over events.

1.3.2.2. Molecular tools

As the technology for molecular genetics evolved, so did the techniques used in fly genetics.

The discovery of P-elements, endogenous transposable elements in the fly genome, that were adapted as germline transformation vectors that allowed for target-specific mutagenesis in *Drosophila,* therefore creating transgenic flies, deeply influenced *Drosophila* genetics in the 21st century. Recently, gene-editing technologies such as the CRISPR/Cas9 system have provided an easier and more efficient way to generate complete loss-of-function mutants of most genes and also creating “knock-ins”.

1.3.2.3. Binary systems

Another big breakthrough came with the adaptation of binary systems from other organisms, allowing for more spatial-temporal control of gene expression. One of these systems is the yeast *Gal4/UAS* system, which has a role in regulating the organism’s galactose metabolism. The Gal4 protein is a transcription factor that binds to an upstream activating sequence (UAS) and activates the transcription of the gene following that sequence. *Drosophila* does not have endogenous UAS-linked genes, however, this system was adapted to work in its cells, and has been proven to be useful for different purposes, making it one of the most important tools in fly genetics. Nowadays, there are extensive libraries of different Gal4 driver lines, with specific expression patterns that can be crossed with different UAS-responsive lines, so that the progeny will only express the UAS-dependent gene in cells where the GAl4 driver is expressed. Gal4 driver lines can serve two different purposes: allow for the expression of genes of interest under the control of specific promoters, and in that case, the regulatory region is cloned upstream of Gal4, or to find new regulatory regions, using the “enhancer-trap” mechanism, in which Gal4 is randomly inserted in

the genome, and its expression pattern reveals the cells that are under the influence of the newly-found regulatory region (Caygill & Brand, 2016).

When experimental design requires a limited time window for Gal4 expression, the Gal80^{ts} (Gal80 Temperature-sensitive) system can be used. Gal80 is a dimer protein that binds to the C-terminal of Gal4, blocking its activation domain, and in consequence, blocking its ability to activate transcription through UAS binding. At 29ºC, Gal80^{ts} is unable to bind to Gal4, an ability that is restored under a permissive temperature of 18ºC (Caygill & Brand, 2016). So, through this system, maintaining flies at different temperatures works as a way to control the expression of genes of interest.

The *LexA/LexAoP* system works similarly to *Gal4/UAS* but it is based on a bacterial transcription factor, LexA, which binds to specific sequences called LexA operators (LexAOPs). This system has been optimized to work in *Drosophila* and it is particularly useful to use in conjugation with *Gal4/UAS*, as it allows for more precise control of gene expression and the creation of more complex genotypes (Yagi et al., 2010). For example, we can use the *Gal4/UAS* system to create a fly stock that works as a disease model and explore different aspects of it with the manipulation of different gene expression with the *LexA/LexAOP* system.

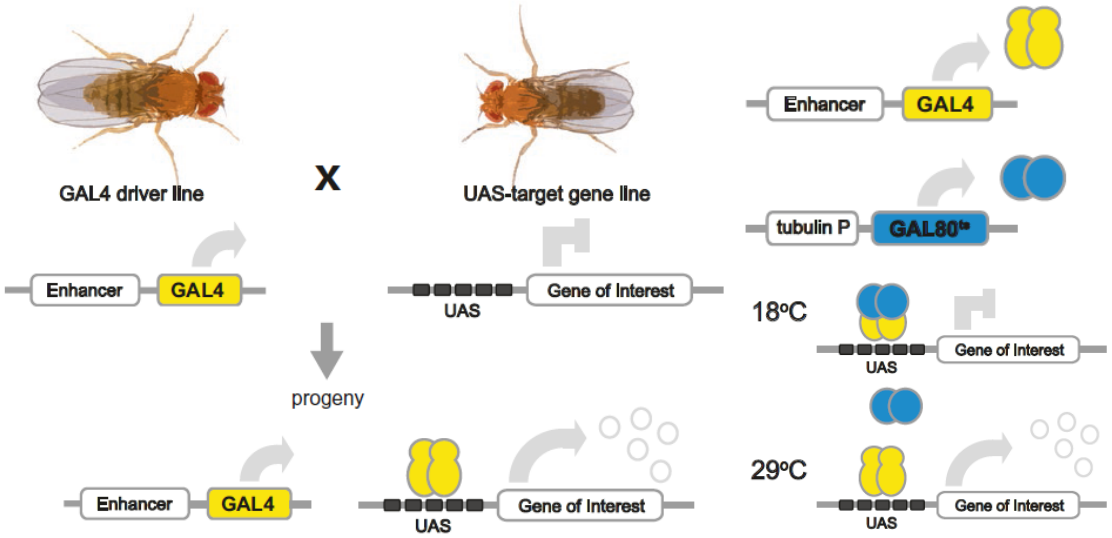


Figure 1.9: The Gal4/UAS system – To take advantage of the Gal4/UAS system, drosophilists cross a fly that is carrying a construct with the Gal4 gene expressed under the control of a driver of interest, with another fly carrying a construct where a UAS binding site is cloned upstream of the gene of interest. In the progeny, the Gal4 protein will be transcribed in the cells where the driver is active and drive the expression of the gene of interest in those cells. Gal80 is a dimer protein that binds to the C-terminal of Gal4, blocking its activation domain, blocking its ability to activate transcription through UAS binding. At 29ºC, Gal80^{ts} is unable to bind to Gal4, an ability that is restored under a permissive temperature of 18ºC. Adapted from (Caygill & Brand, 2016).

1.3.2.4. RNAi

Another very important tool in *Drosophila* Genetics is RNA-mediated gene interference (RNAi), a process in which RNA molecules recognize dsRNA and neutralize corresponding mRNAs, by inhibiting gene expression or translation. RNAi is an endogenous process, first discovered in plants and later described in *C. elegans* as a defense against virus that has been adapted and is now fit to use as a genetic tool, in different model organisms.

RNAi provides a different approach to gene "knock-downs" because it is not a permanent alteration in the genome but rather eliminates a gene's function by reducing mRNA levels. Currently, there are several large scale RNAi libraries with fly stocks available for drosophilists, expressing RNAis against many protein-coding genes, under the control of the Gal4/UAS system, enabling the conditional silencing of genes in a tissue-specific manner (Dietzl et al., 2007), which is not only useful for creating knock-downs of genes of interest, but also for genome-wide genetic screens.

1.3.3. *Drosophila* vs other animal models

Although they carry the disadvantage of not recapitulating as many aspects of disease pathogenesis as vertebrate models, flies are more complex than models such as yeast or *C.elegans*. However, they are much easier to care for and manipulate than vertebrates such as zebrafish or mice and they lack the ethical constraints associated with working with vertebrates.

Drosophila has a very well-curated, small genome, with an estimated 180 Mb length, corresponding to 13 920 protein-coding genes (Hales et al., 2015). This deep knowledge about the *Drosophila* genome, the fact that most human disease-associated genes have *Drosophila* orthologues, and all the molecular tools available give drosophilists the ability to ask fundamental questions about the role of different genes.

Despite being invertebrates, flies are commonly used as models in neurobiology, even in behaviour studies, as they exhibit a variety of complex behaviours regarding sexual behaviour, sensory biology, learning, memory, and more. Furthermore, given their extensive genetic toolkit, and their simplicity (they only have approximately 100000 neurons) they constitute a great system to study gene function alongside the development and function of neural networks.

Regarding human disease, *Drosophila* has been used as a model to study neurodegenerative disorders such as AD. An obvious advantage in comparison to vertebrate models is their short life cycle which enables researchers to have access to elderly flies in a span of weeks. Besides this, flies that ectopically express disease-related gene products, mainly Tau or Aβ42, show a phenotype that can be mildly comparable to that seen in patients. They show increased neuronal death, shorter lifespan, reduced locomotion, and memory decline, among others (Prüßing et al., 2013).

1.4. Goal of the Project

As the title of this work states, the aim of this project was to determine the causes and consequences of excessive glutamate in a *Drosophila* model of AD and characterize its impact on neuronal fitness status. To reach this goal, we asked 3 main questions:

1. **Is excessive glutamate release responsible for Aβ-induced neuronal aberrant activity?**

A high abundance of this neurotransmitter seems to co-localize with brain regions exhibiting neuronal hyperactivity in Aβ42-expressing flies. Therefore, in a first approach, we aimed to investigate a possible correlation between these two observations and determine if excessive glutamate is responsible for the neuronal hyperactivity or vice-versa.

2. Is excessive glutamate release driving the upregulation of cell fitness markers?

Aβ42-induced hyperactive neurons display low fitness markers and are eliminated by fitness comparison. So, we aimed to test if the excessive neurotransmitter release found in Aβ42-expressing brains is responsible for the upregulation of unfit markers such as $flower^{LoseB}$ and *azot,* and increased cell death.

3. **Can neuronal hyperactivity reduction improve Aβ-induced locomotor defects in *Drosophila*?**

Since glutamate is typically present in motor neurons, we hypothesized that it may play a role in the locomotor defects present in fly models of the disease and decided to test if silencing hyperactive neurons could rescue locomotor performance.

We expect that addressing these questions could provide novel insights into glutamate dysfunction in AD brains. We intend to elucidate if glutamate excess is the link between Aβ42-induced neuronal hyperactivity and the reported low fitness status of these neurons Mainly, we hope that possible improvements produced by modulating glutamatergic signalling in *Drosophila* models of AD, could increase the scarce knowledge regarding this aspect of AD pathology and provide a starting point for novel therapeutic strategies that improve the health of patients.

2. Material and methods

2.1. Materials

Table 2.1: Materials - Reagents and kits used in this work.

Name	Brand	Reference
DNA ladder III 1Kb	NzyTech	NZYDNA ladder III
DreamTaq Green PCR Master Mix (2X)	Thermo Scientific	K1081
Proteinase K	Nzytech	MB01901
Vectashield with DAPI	Vector_Baptista Marques	H1200
Vectashield	Vector_Baptista Marques	H-1000
Agarose	NzyTech	MB02703
GreenSafe Premium	NzyTech	MB13201
Memantine Hydrochloride	Supelco (Sigma)	PHR1886

Other chemicals and reagents were of the highest analytical grade and were purchased from local commercial sources

2.2. Fly stocks and maintenance

2.2.1. Stock maintenance and fly pushing

All flies used for experiments were kept at 25ºC, unless otherwise indicated, in a room with controlled humidity and phototaxis. Handling was done with a paintbrush while animals were placed in flypads where they were kept anesthetized with CO_2. Stocks were maintained in wide plastic vials with sponge plugs with food supplied by the Fly Platform of the Champalimaud Foundation **(Table 2.2)**.

Table 2.2: Vienna Fly Food recipe – Fly Food supplied by the Fly Platform at CCU

Ingredient	Quantity
Barley Malt Syrup (Próvida)	80g
Beetroot Syrup (Grafschafter)	22g
Agar (NZYTech)	8g
Biological Corn Flower (Próvida)	80g
Soya Flour (A.Centazzi)	10g
Instant Yeast (Sanf-Instant, Lesaffre)	18g
Propionic acid (Argos)	8ml
15% Niapigin (Tegosept, Dutscher UK) in 96% EtOH	12ml

Table 2.3: Fly stocks – Genotypes of the fly stocks used in this project are reported, along identification and source.

Collection Number	Genotype	Reference
-	*W; Lopkir2.1;+*	(Feng et al., 2014)
799	*w; if/CyO; Lopkir2.1EGFP/ TM6B*	(Prieto-Godino et al., 2012)
-	*W; UAS-Kir2.1/Cyo; TM6B/MKRS*	Vasconcelos Lab
892	*W; +; nSybGal4 (R57C10G4) /TM6B*	Janelia Farms
-	*W; UAS-Aβ42/CyO; nSybGal4 (R57C10G4)*	Dina Coelho
-	*W; UAS-Aβ42, tubGal80^{ts}/CyO; nSybGal4*	Dina Coelho
849	*w; If/CyO;MB247-Gal4*	This Work
-	*W; UAS-Aβ42 /CyO; MB247-Gal4*	Dina Coelho
157	*W; UAS-LacZ; +*	Moreno Lab
-	*W; UAS-LacZ/Cyo; nSybGal4/TM6B*	This work
814	*W; +; 26xLOP-GFP*	Terufumi Fujiwara
-	*W; LOP-p35/CyO; TM6B/MKRS*	(Ren et al., 2016)
-	*W; LOP-Reaper/CyO; TM6B/MKRS*	Florenci Serras
726	*W; azot{KO; KI-LexAp65}/CyO; MKRS/TM6B*	Moreno Lab
-	*W; azot{KO; KI-LexAp65}, LOP-Kir2.1/CyO; +*	This work
777	*ywF; GMR-GAL4,UAS-Aβ42/CyO; azot::mCherry/TM6B*	Dina Coelho
880	*W; UAS-Aβ42 (2x)/CyO*	From S. Casas-Tinto
725	*W;GMR-Gal4, UAS-Aβ42 /CyO*	From S. Casas-Tinto
850	*GMR-Gal4 ; Sco/CyO*	Moreno Lab
-	*EmptyLexA*	BL # 77691
-	*CaLexA*	(Masuyama et al., 2012)
-	*W; GMR-Gal4, UAS-Aβ42/ CyO; CaLexA/ TM6B*	This work
-	*W; UAS-Aβ42/ CyO; MB247-Gal4, CaLexA/ TM6B*	Dina Coelho
-	*w; Sco/CyO; nSybLexAp65*	BL #52817
-	*W; UAS-Aβ42, azotKO /CyO; nSybGal4 (R57C10G4)*	Dina Coelho
-	*ywF; GMR-GAL4,UAS-Aβ42/CyO; Flower^{LoseB}::mCherry/TM6B*	Dina Coelho
-	*W; +; UAS-RNAiDVGLUT/ TM3*	VDRC GD #2574
-	*W; UAS-RNAiDVGLUT;*	VDRC KK #104324
-	*W; +; UAS-RNAiDVGLUT;*	BL TRiP #27538
137	*W; UAS-yellow RNAi/CyO; +*	VDRC KK

All of the fly stocks used in this study are listed on **Table 2.3.**

2.2.2. Setting up crosses and balancing stocks

A fundamental aspect of any genetic-based research involving *Drosophila melanogaster* is crossing females and males with different genotypes, either to create a new line with the desired genotype or to perform experiments to analyze the progeny. In both cases, virgin female collection is necessary to make sure females have only mated with males of the desired genotype. Virgin female collection was performed approximately twice a day and females were selected based on the visual method which relies on the morphological features of newly hatched flies such as a white puffy appearance and the presence of the meconium, a very visible dark abdominal spot.

Before starting crosses, it is often necessary to balance stocks of original lines. Balancer chromosomes are essential tools in fly genetics as they allow for the maintenance of genotypes throughout generations by blocking recombination events and carrying genetic markers, visual traits used to track flies with different genotypes. To balance a fly stock, the typical procedure is to cross the desired stock with multibalancer flies, stocks of flies that only carry balancer chromosomes. Their genotype can be, for example, *w; if/CyO; TM6B/MKRS*, and in this case, the second and third chromosomes are fully balanced, each carrying a different "homolog" balancer. For example, if our gene of interest is in the second chromosome, we want to cross multibalancer flies with our own until the genotype of the second chromosome becomes *"gene of interest"/ CyO*, a balancer that is tracked by the presence of a curly wing phenotype.

2.2.3. Generating an *azot{KO;LexA},Lopkir2.1* recombinant

To generate this stock, *w; Lopkir2.1* virgin females were crossed with *w; azot{KO; LexA}/Cyo* males. Virgin females lacking a curly phenotype were selected from the progeny and crossed with *w; if/CyO; MKRS/TM6B* males. Females were specifically chosen to allow for a recombination event to take place between the *lopkir2.1* and *azot{KO; LexA}* genes in chromosome 2. From this second cross, males were selected from the progeny and individually crossed with w; *if/CyO; MKRS/TM6B* females to generate a balanced stock. The final step was to cross virgin females and males from the progeny of this third cross and obtain a balanced stock of possibly recombinant flies. Over 50 different candidate recombinant stocks were generated and to know if the recombination event had occurred, stocks were then tested for the presence of both genes, as described below.

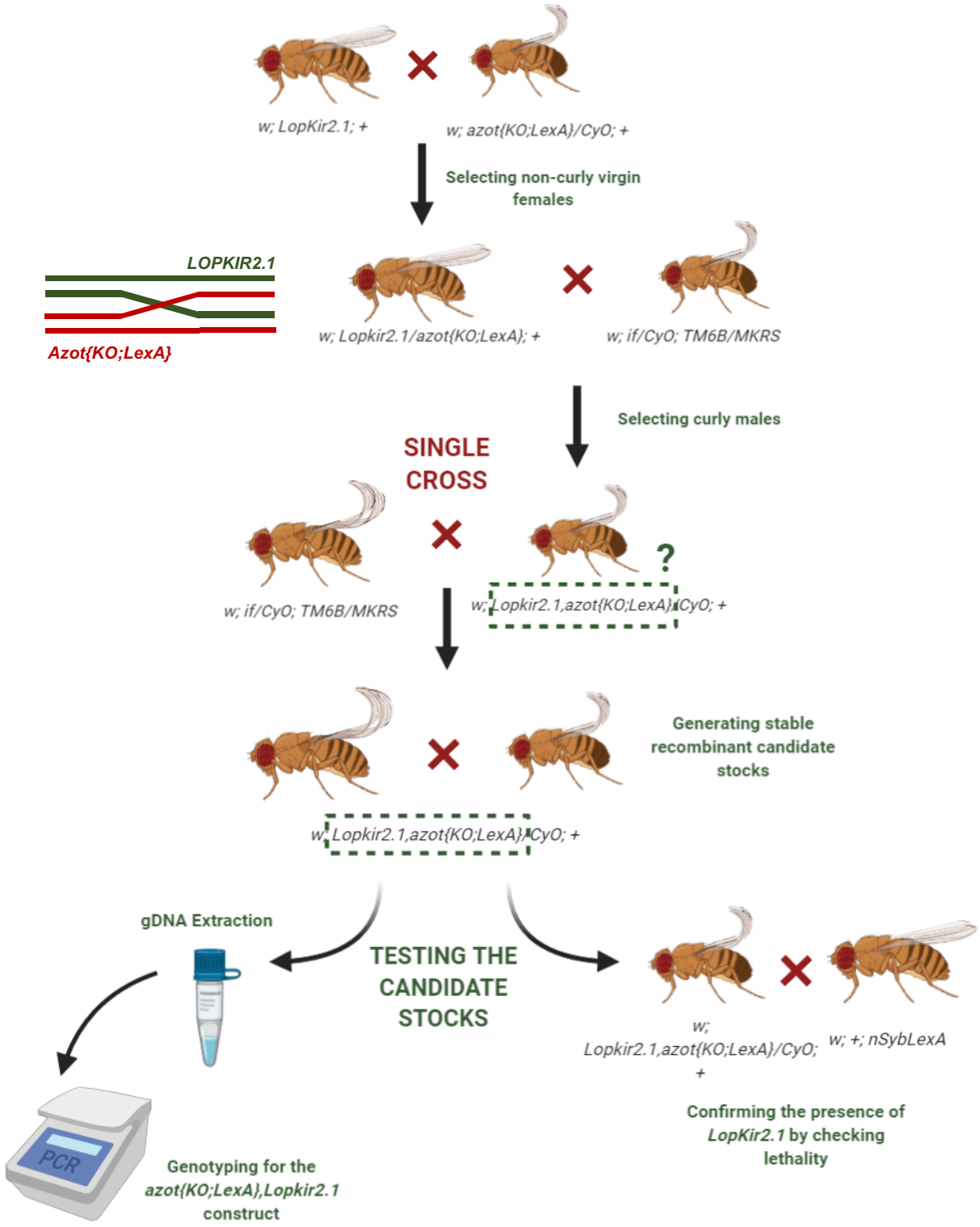


Figure 2.1: Steps to generate an *azot{KO;LexA},lopkir2.1* recombinant – Illustrative diagram depicting the crossing scheme to generate the desirable recombinant and the two steps to confirm the presence of both transgenes. *W; Lopkir2.1;+* females were crossed with *w; azot{KO;LexA}/CyO;+* males and progeny was sorted to select non-curly virgin females, which were crossed with multibalancer males to induce homologous recombination between the two constructs. Curly males from this cross were selected and individually crossed with multibalancer females to generate stable recombinant candidate lines. Candidates were tested for the presence of both constructs as described. Created with Biorender.

2.3. Molecular Biology procedures

2.3.1. *Drosophila* genomic DNA extraction.

Flies were individually placed in 1.5mL eppendorfs and frozen overnight at -20ºC. Later they were slightly crushed with the tip of a micropipette and placed in a solution of 1X SB (Squishing Buffer) (200mM Tris-HCl pH8, 20mM EDTA, 500mM NaCl), Proteinase K, and endonuclease-free H_2O. They were placed in a water bath at 37ºC for 30 min followed by 3 min at 95ºC.

2.3.2. DNA quantification

Genomic DNA quality and concentrations were accessed in a Nanodrop spectrophotometer (ThermoFisher), and only those between 100 ng/µL and 350 µg/ul were used for further experiments.

2.3.3. PCR

All PCRs were performed in a BIORAD T100 Thermal Cycler, following DreamTaq DNA polymerase protocol.

Reactions with a total volume of 20 µL were prepared, having a final concentration of 1x DreamTaq PCR Master Mix, 0,01x of each forward and reverse primers, and approximately 200 ng/µL DNA template.

The following program was used: an initial denaturation of 95ºC for 3 minutes, followed by 35 cycles of denaturation at 95ºC for 30 seconds, annealing at the annealing temperature of each pair of primers for 15 seconds, and elongation at 72ºC for 2 minutes and 30 seconds. The final elongation step lasted 5 minutes at 72ºC.

The following table summarizes all pairs of primers and respective annealing temperature for genotyping *azot{KO; LexA},* recombinant candidates.

Table 2.5: Set of Primers used for DNA Amplification – Name, sequence, amplified fragment length and annealing temperatures for the set of primers used to genotype *azot{KO;LexA}* recombinant candidates.

Set of Primers	Primer Name	Primer Sequence	Amplified Fragment Lenght	Ta (ºC)
1	pBH152	GTACCTACCTCGACTGCTACTC	3kB	52.1
	pBH157	TCCATTATGGCCCTCTAGCAC		

2.3.4. Sample analysis

To perform agarose gel electrophoresis, PCR products were loaded onto a 1% agarose gel, which was stained by supplementation with 2 µL of GreenSafe per 70 mL of gel. DNA ladder IV was also loaded to the gel to estimate the size of the DNA fragments. The gel was visualized in a transilluminator (BioRad).

2.4. Dissections and immunostaining

Flies were killed by anesthesia with CO_2 for approximately 2 minutes, after which they were placed in a small amount of vaseline and drowned in PBS 0,4% solution. Dissection was performed by placing the flies' heads in a drop of cold PBS 0,4%. After dissection, brains were transferred to an Eppendorf placed on ice, with a pipette with the tip cut off. Brains were later fixed for 30 minutes with fresh formaldehyde 4% (v/v in PBS) in agitation., followed by a 3x 20-minute wash in PBS 0,4% Triton solution.

Primary antibody incubation occurred overnight at 4ºC and in agitation, followed by 3x 20-minute wash in PBS 0,4% Triton solution and overnight incubation with a secondary antibody in the same conditions and following the wash protocol described above. Brains were then placed in wells in PBS through pipetting and, with forceps, transferred to a slide with a drop of Vectashield medium and placed between a bridge of two coverslips and aligned to the desired position. Finally, a coverslip was placed over the bridge and the preparation was sealed with nail polish and stored at 4ºC.

The antibodies used for immunostaining in brains are described in the table below.

Table 2.6: Antibodies used in Immunostaining – Concentration, dilution, and brand and catalog name are reported for both Primary and Secondary Antibodies

	Reagent	Initial Concentration	Dilution Used	Brand and Cathalog Number
Primary antibodies	Anti-GFP Chicken Polyclonal Antibody	10mg/mL	1:500	Abcam, ab13970
	Anti-L-glutamate Chicken polyclonal Antibody	1.2mg/mL	1:100	Abcam, ab62668
	Anti-cleaved Drosophila Dcp-1 (Asp216) Rabbit Polyclonal Antibody		1:100	Cell Signalling, 9578
	Anti-elav Rat Polyclonal Antibody	13.5ug/mL	1:25	DSHB, Rat-Elav-7E8A10 anti-elav
Secondary Antibodies	Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 488	2mg/mL	1:1000	Invitrogen, A-11039
	Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 647	2mg/mL	1:1000	Invitrogen, A-21449
	Alexa Fluor® 647 AffiniPure Donkey Anti-Rat IgG (H+L)	0.75mg/mL	1:250	Jackson, 712-605-153
	Donkey anti-Rabbit IgG (H+L) Secondary Antibody Alexa Fluor 488	1mg/mL	1:500	Invitrogen, A-21208

2.5. Confocal microscopy

All images were acquired in a Zeiss LSM 880, using a Zeiss Plan-Apochromat 40x/1.4 Oil DIC M27 objective.

2.6. Image analysis and quantifications

All image analysis was performed in Fiji software.

2.6.1. Neuronal hyperactivity quantifications

The first method used to quantify CaLexA positive neurons was to count them visually, using the counting tool on Fiji. Maximum intensity projections of Z-stacks of the optic lobes in the brain were screened for cells with high GFP content, where a higher green fluorescence co-localized with the neuron-specific α-Elav antibody, or with the nuclear stain, DAPI.

The second method to quantify CaLexA positive neurons relies on the Histogram tool of the software, which allows us to obtain the number of pixels that have a certain value of fluorescence. Values equal or bigger than 75% of the maximum fluorescence value for pixels in the green channel were considered to belong to a CaLexA positive neuron, so, the total number of these pixels was counted in each analyzed image. Images consisted of selections of halves of maximum intensity projections of z-stacks of brains, of optic lobes, or mushroom bodies.

2.6.2. Quantifying glutamate

To quantify glutamate, images of maximum intensity projections of z-stacks of the optic lobe region of brains stained with an α-L-glutamate antibody were analyzed. Median fluorescence values were measured using the "measure" tool to obtain the grey median value in Fiji. The area of measurement was selected based on the nuclei staining with DAPI.

2.6.3. Quantifying Flower, Azot, and DCP1-positive cells

The number of positive cells in the adult brain for DCP1, Flower^{LoseB}::mcherry and Azot::mcherry were counted on projections of the frontal side of the optic lobes. Noise signal was removed using a Gaussian blur filter (sigma=2). This number was divided by the area of each optic lobe, measured by delineating via DAPI staining.

2.7. Behavioral assays

2.7.1. Buridan assays

The Buridan assay is a locomotor performance assay based on Buridan's Paradigm as described by *Colombs et al (2012)*. At 15-days old, flies kept in 29ºC, had approximately ⅔ off their wings length cut off with surgical scissors, under CO_2 anesthesia, and were later placed in individual vials. The assay was performed 24 hours later and individually, flies were placed in the center of the arena,

and their movement was recorded for 5 minutes by a camera connected to a computer running the BuriTracker software. Before starting the assay, flies were transferred to empty vials, to minimize grooming as much as possible. Flies that displayed long periods of grooming behavior and those that jumped off the platform into the water more than 3 times, were excluded from the analysis. The analysis of parameters such as mean activity time and total distance walked were obtained with CeTran software (Colomb et al., 2012). The setup of the arena is described in **Figure 2.1**.

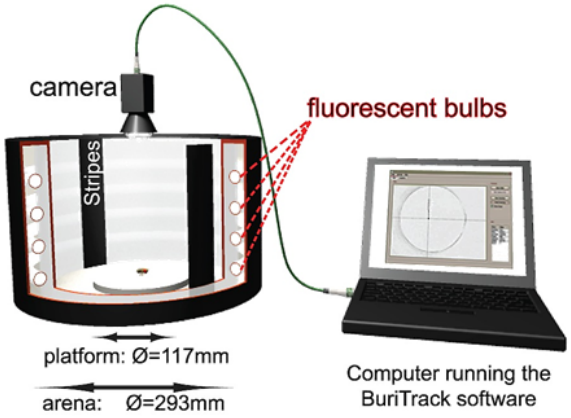


Figure 2.2: Buridan Assay Setup– Ilustrative scheme depicting the setup for Buridan assays. Individual flies are dropped on an arena, surrounded by water, in the bottom of a cylinder with light up walls and two vertical black stripes in each opposite direction. The fly is filmed by a camera that is placed above the arena and is connected to a computer that is running the BuriTrack software, that records the fly's trajectory in the arena for 5 minutes. Adapted from (Colomb et al., 2012)

2.7.2. Lifespan assays

Groups of 15-20 non-virgin females were collected after eclosion and aged at 29ºC. Vials were flipped 2-3 times a week and dead flies were counted.

2.8. Statistical analysis

Statistical analysis was performed with the software GraphPad Prism version 8.01 (GraphPad software). According to the results of a normality test, significance for comparison of parametric samples was determined using an unpaired t-test for 2 samples. For more than 2 parametric samples, ANOVA following, Dunnett's T3 test for multiple comparisons was performed. Statistical significance for comparison of two non-parametric, unpaired groups was determined using a two-tailed Mann-Whitney test, and for more than 3 groups, ANOVA was performed with a Kruskal-Wallis test for multiple comparisons. Significance was defined by a P-value < 0.05.

3. Results

3.1. Effect of silencing *azot*-expressing neurons with *kir2.1*

Recently, Coelho & Moreno,2020 described that a sustained increase in aberrant neuronal activity in the brain of a *Drosophila* model of AD leads to the upregulation of low fitness markers such as *flower*^{LoseB} and *azot,* which target unfit neurons to death by apoptosis. It had also previously been shown that an extra functional copy of *azot* significantly improved Aβ-related locomotor defects and overall brain health (Coelho et al., 2018), due to the elimination of less fit neurons. Since neuronal hyperactivity seems to be one of the factors responsible for poor neuronal fitness, we silenced *azot*-expressing neurons with the aim of testing if preventing hyperactivity also produced beneficial effects in locomotor performance.

Kir2.1 is an endogenous inward rectifying potassium channel expressed in different cells including cardiac muscle and neurons, that has been adapted to be commonly used as a tool in neuroscientific studies for silencing neuronal activity. When a *kir2.1* transgene is expressed in a neuron, there is an increase in inward K+ conductance which approximates resting membrane potential to the Equilibrium potential of K+, preventing action potential firing and therefore silencing the neuron (Okada & Matsuda, 2008) (Hibino et al., 2010) (Baines et al., 2001).

In this way, it is possible to silence *azot*-expressing neurons in individuals that carry both an *azot{KO; LexA}* and *LopKir2.1* constructs. Cells with this genotype do not express *azot* because the *azot c*oding region was replaced by a *LexA* which is controlled by the *azot p*romotor. Thus, in neurons where the *azot* promotor is active, LexA can bind to the Lop sequence in the *Lopkir2.1* construct to specifically induce expression of *kir2.1*, so that *azot*-expressing neurons are silenced by Kir2.1 overexpression.

3.1.1. Testing different kir2.1 lines

The first step to create a fly stock with this genotype was to test two different fly stocks carrying a copy of Lop*kir2.1* that were available in the lab: one on the second chromosome (from Barry Dickson Lab) (Feng et al., 2014) and one on the third, fused to an EGFP protein (a gift from Lucia Prieto).

An easy way to test if the *kir2.1* gene is working properly is to control its expression with a pan-neuronal driver, such as *nSybLexA*. It is expected that in individuals carrying both constructs, every neuron is silenced and development is not viable. So, it is possible to access the efficacy of *kir2.1.* by observing the lethality of a cross between individuals carrying each of the constructs. To test the quality of the two different *Lopkir2.1* stocks, virgins from each line were crossed with males with the following genotype: *w; Sco/CyO; nSybLexA*. Phenotypes of the progeny were analyzed to determine the efficacy of these stocks and specific constructs.

Table 3.1: Testing Lopkir2.1 stocks – Results obtained when crossing both Lopkir2.1 lines with w; Sco/ CyO; nSybLexAp65/ MKRS males. Expected ratios were obtained by calculating the probability of individuals inheriting both chromosomes carrying the genes of interest, instead of balancers, based on a Mendelian ratio. Observed proportions were calculated by sorting the progeny of the crosses by phenotype and dividing the number of individuals with the genotype of interest by the total number of progeny.

Cross	Genotype of the progeny of interest	Expected proportion	Observed proportion
w; if/CyO; Lopkir2.1EGFP/ TM6B ***x*** ***w; Sco/ CyO; nSybLexAp65/ MKRS***	*w; if/CyO; Lopkir2.1EGFP/nSybLexAp65* *or* *w;Sco/CyO; Lopkir2.1EGFP/nSybLexAp65*	25%	0%
W; Lopkir2.1; + ***x*** ***w; Sco/CyO; nSybLexAp65/ MKRS***	*w; Lopkir2.1; nSybLexAp65/+*	50%	5,7%

The progeny from the performed crosses show that both *Lopkir2.1* lines were functional as a very small percentage of flies expressing *kir2.1* under the control of *nSybLexA* ecloded, as shown in **Table 3.1**. Since pan-neuronal expression of the Kir2.1 channel is lethal, as expected, the constructs are properly working in both lines.

However, in the line expressing *Lopkir2.1EGFP* from Prieto, larvae exhibited fluorescence in the whole eye-antennal discs, not specific to the expression domain of this driver, demonstrating a non-specific response to the nSybLexA driver (data not shown).

It has been reported that some Lop lines show interference with Gal4 drivers and, since the disease model used in this project is *w; UAS-Aβ42; nSybGal4* (that will be referred to as *nSyb> Aβ42* from now on*)*, it was very important for further experiments that this was not occurring. To test this, *Lopkir2.1EGFP* flies were crossed with *w; +; nSybgal4,* and progeny were observed in a fluorescent scope and compared to control, wild-type Canton S. flies. If there is no interference, there should be no GFP visible in the eyes of either genotype. However, the intensity of fluorescence in the eyes of *w; Lopkir2.1EGFP; nSybGal4* adults is superior to that seen in control **(Figure 3.1A)**, suggesting that there may be interference between the Gal4 protein and the LOP sequence and further supporting the leaky nature of this line. For these reasons and based on poor performance in memory tests previously performed in the lab, the *Lopkir2.1EGFP* line was discarded from further experiments.

To further test the line from Barry Dickson and exclude a detrimental effect of the presence of the *Lopkir2.1* transgene in flies' locomotor performance, Buridan assays were performed on *Lopkir2.1;*

EmptyLexA and *Empty LexA* males. *EmptyLexA* is a plasmid containing all of the sequences present in a typical *LexA* vector except for the *LexA* gene.

In comparison to flies expressing only the *EmptyLexA* vector, there was no significant difference in the distance traveled and the mean activity time between both groups of flies **(Figure 3.1 B-C).** This assures that the presence of the *Lopkir2.1* construct is not detrimental to the locomotor behavior of flies and, therefore, this stock is fit to use in future behavioral experiments.

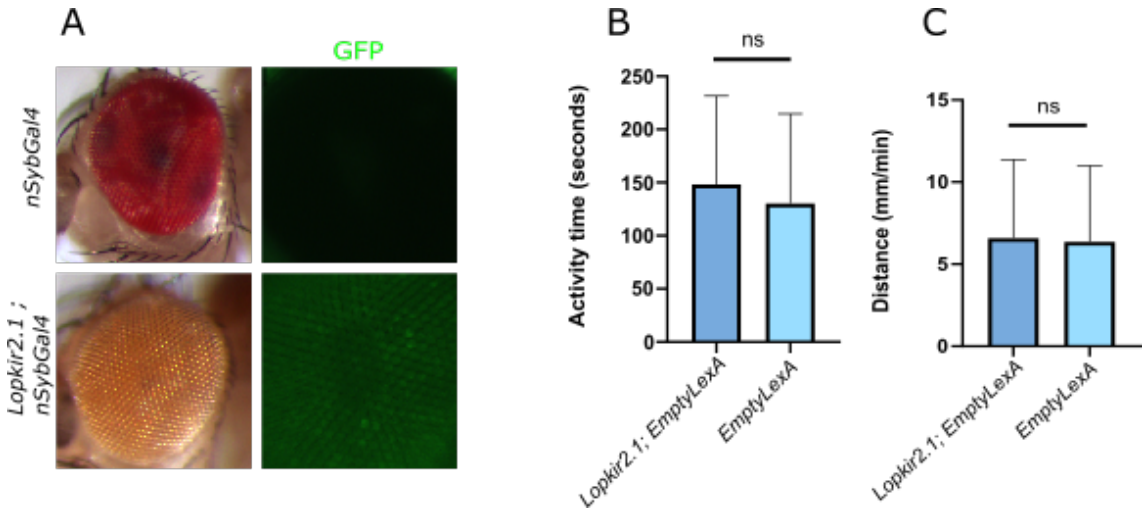


Figure 3.1: Testing different Lopkir2.1. lines – **(A)** Eyes of *Lopkir2.1EGFP; nSybGal4* flies and *nSybGal4* flies, in bright field and fluorescence. Green fluorescence from the EGFP attached to Lopkir2.1 can be seen in the eyes of *Lopkir2.1EGFP; nSybGal4* flies. Graphs display results of locomotor performance assays regarding **(B)** activity time in seconds (using a speed threshold [ST]) and **(C)** distance walked in millimetres per minute *of Lopkir2.1; EmptyLexA (n=51) and EmptyLexA* (n=29) males. Parameters were calculated from individual walks of flies for each genotype. Statistical analysis was performed using Mann-Whitney unpaired test. ns- Not significant ($p < 0.05$)

3.1.2. Generating an *lopkir2.1, azot{KO; LexA}* recombinant

Because the *Lopkir2.1EGFP* stock was discarded from the experiment, it became necessary to generate a new fly stock with the following genotype in the second chromosome *azot{KO; LexA}, Lopkir2.1/ Cyo* with the *Lopkir2.1* stock from Barry Dickson. Since both *azot{KO; LexA}* and *Lopkir2.1* are located in the second chromosome, it was necessary to promote a recombination event in this chromosome, so that both transgenes could be simultaneously in the same chromosome, enabling the generation of a stable genotype.

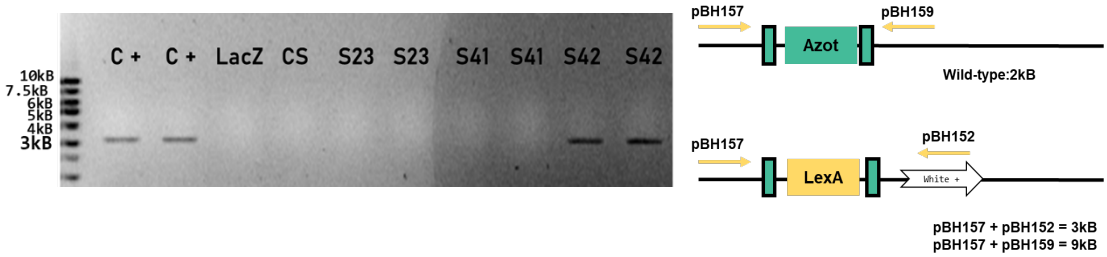


Figure 3.2: Screening for the presence of *azot{KO;LexA}* in recombinant candidates. (A) 1% Agarose gel after electrophoresis. Fragment size indicated by the presence of DNA ladder IV. DNA from PCR products for the positive control (C+): *azot{KO;LexA}* stock and two different negative controls: UAS-LacZ and Canton S. and 3 different candidates: S23, S41 and S42 is shown. Candidate S42 is positive as it presents a band in the region corresponding to a 3kB size, the size of the *azot{KO;LexA}* construct, amplified by the *pBH157* and *pBH152* primers. **(B)** Schematic representation of the *azot{KO;LexA}* construct , primer binding sites and fragment sizes.

After generating the possible recombinant candidates, as described above, the presence of both *Lopkir2.1* and *azot{KO; LexA}* constructs was tested. To test the presence of the *Lopkir2.1* construct, males from each recombinant candidate line were crossed with females carrying a *nSybLexA* driver. The presence of both of these transgenes is lethal in the progeny given that its expression implies that all neurons are silenced. To test the presence of the *azot{KO; LexA}* construct, we performed a PCR reaction, as described above, in all of the stocks that were positive for the presence of *Lopkir2.1*. Only candidates that showed positive results on both tests were considered recombinants with a *Lopkir2.1, azot{KO; LexA}* genotype. The presence of the *azot{KO; LexA}* construct was identified in one of the candidates **(Figure 3.2)** that had already tested positive for the presence of *Lopkir2.1*, by the presence of a band with 3kB.

3.1.3. Testing the effect of silencing *azot*-expressing neurons on walking behavior and life span

Previous studies have shown that ectopic expression of human *Aβ42* impairs walking behavior and lifespan of flies and that absence of *azot* further exacerbates this phenotype (Coelho et al., 2018). To study the effect of silencing *azot*-expressing neurons on walking behavior, Buridan assays were performed on 15-day old flies grown at 29ºC. These assays produce a variety of results for different parameters of walking behavior, including stripe deviation, velocity, and pause duration, among others. For the purpose of this work, we focused on two of the parameters in which differences between genotypes were observed, which appeared to be the most representative of a fly's locomotor performance and by proxy, overall health: total distance travelled (in millimetre by minute) and activity time (taking into consideration that each assay has a duration of 5 minutes).

w; UAS-Aβ42, azotKO / azot{KO;LexA}, Lopkir2.1; nSybGal4 flies were compared to 4 different controls: healthy flies that only express the *nSybGal4* driver (*w; Lop-GFP; nSybGal4*), flies that

express *Aβ42* in every neuron and carry a Lop construct without a *LexA* driver (*w; UAS-AB42/ Lop-GFP; nSybGal4* and *w; UAS-Aβ42/ Lop-kir2.1.; nSybGal4)* and flies that express *Aβ42* in every neuron and are homozygous for *azotKO*, but lack the *lopkir2.1* construct (*w; UAS-Aβ42, azotKO / azot{KO;LexA}; nSybGal4*).

As expected, there was a significant decrease in both distance and activity time in *nSyb>Aβ42* flies when comparing to wild-type flies expressing the *nSyb* driver, and an aggravation of the locomotor phenotype in the absence of *azot* function (*nSyb>Aβ42, azotKO/ azot{KO; LexA}* flies) **(Figure 3.3)**. However, in *nSyb>Aβ42 / azot{KO; LexA}, Lopkir2.1* flies, both distance and activity time values were significantly lower than those shown in healthy flies and also lower than those seen in *nSyb>Aβ42* flies. It is relevant to note that in comparison to *nSyb>Aβ42* flies only lacking Azot, those where *azot*-expressing neurons were silenced displayed higher activity times and distance values, even though this difference was not significant. So, we can propose that silencing of *azot*-expressing neurons is not sufficient for *azotKO-/-* flies to recover the locomotor performance of flies *wild-type* for *azot*.

A factor that could also explain this phenotype is the fact that silenced neurons, although no longer contributing to aberrant brain activity, still accumulate in the brain over time, and their presence might be more damaging for neuronal circuits than their eventual elimination if *azot* was still being expressed. In an attempt to clarify this hypothesis, the same experiment was repeated in 7-day old flies. At this age, the extent of damage caused by *Aβ42* expression is not yet visible in locomotor performance, as there is no longer a significant difference between *nSyb>* and *nSyb>Aβ42* flies **(Figure 3.4)**. However, 7-day old flies whose *azot*-expressing neurons were silenced by Kir2.1 display a more severe locomotor phenotype than *azotKO-/-* flies lacking Kir2.1, suggesting that accumulation of damaged neurons over time doesn't explain the inefficacy of this approach.

It is also relevant to note that *nSyb>Aβ42 /Lopkir2.1* individuals displayed a slightly better locomotor phenotype than *nSyb>Aβ42 /Lop-GFP.* Since this could raise concerns regarding the effect of the presence of *Lopkir2.1*, a possibility of leakiness in the construct, we performed Buridan assays to compare this genotype with flies carrying the same driver and a different Lop-construct (*Lop-GFP; nSybGal4)* and flies heterozygous for *Lopkir2.1 (w; Lopkir2.1).* Although *w; Lopkir2.1* performed worse than *Lop-GFP; nSybGal4* flies (**Figure 3.4C**), there was no difference between flies carrying *nSybGal4* and the different Lop constructs, suggesting that it is unlikely that Gal4 is promoting the expression of *kir2.1* by binding to Lop or that the genetic background of the *Lopkir2.1* stock is healthier or these flies naturally display a better locomotor performance. It should be noted that this particular experiment was performed with a low n, and in behavioral experiments, it is preferable to have a higher n to draw more reliable conclusions.

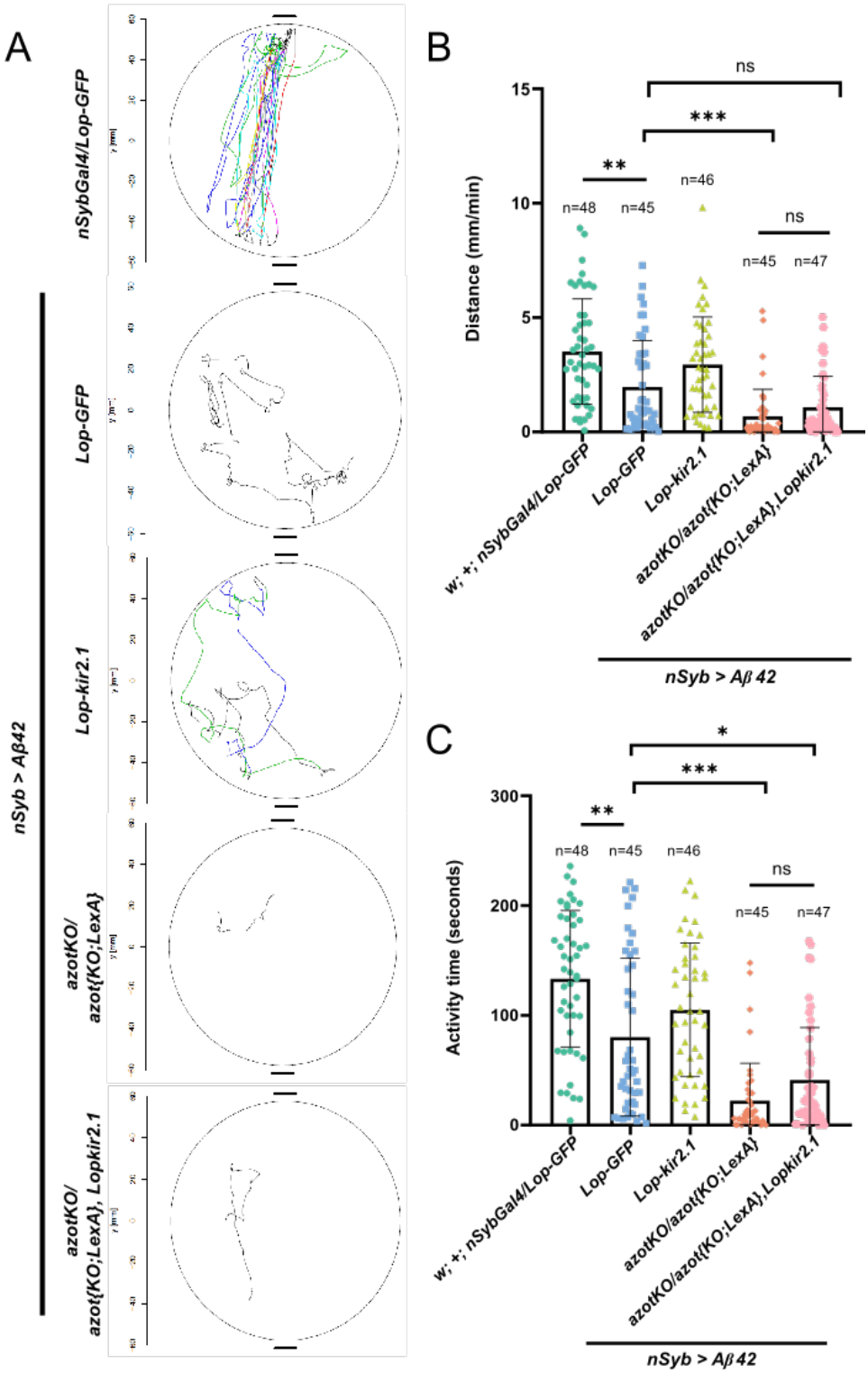


Figure 3.3: Effect of silencing azot-expressing neurons on the locomotor performance of 15-day old flies– **(A)** Individual trajectories representative of each indicated genotype. Graphs depict **(B)** distance walked in millimeters per minute and **(C)** activity time in seconds (using a speed threshold [ST]). Parameters were calculated from individual walks of 15-day old males for each genotype. Statistical significance was based on ANOVA and a Kruskal-Walis test for multiple comparisons. ** indicates $p<0.01$, *** $p<0.001$ and **** $p<0.0001$

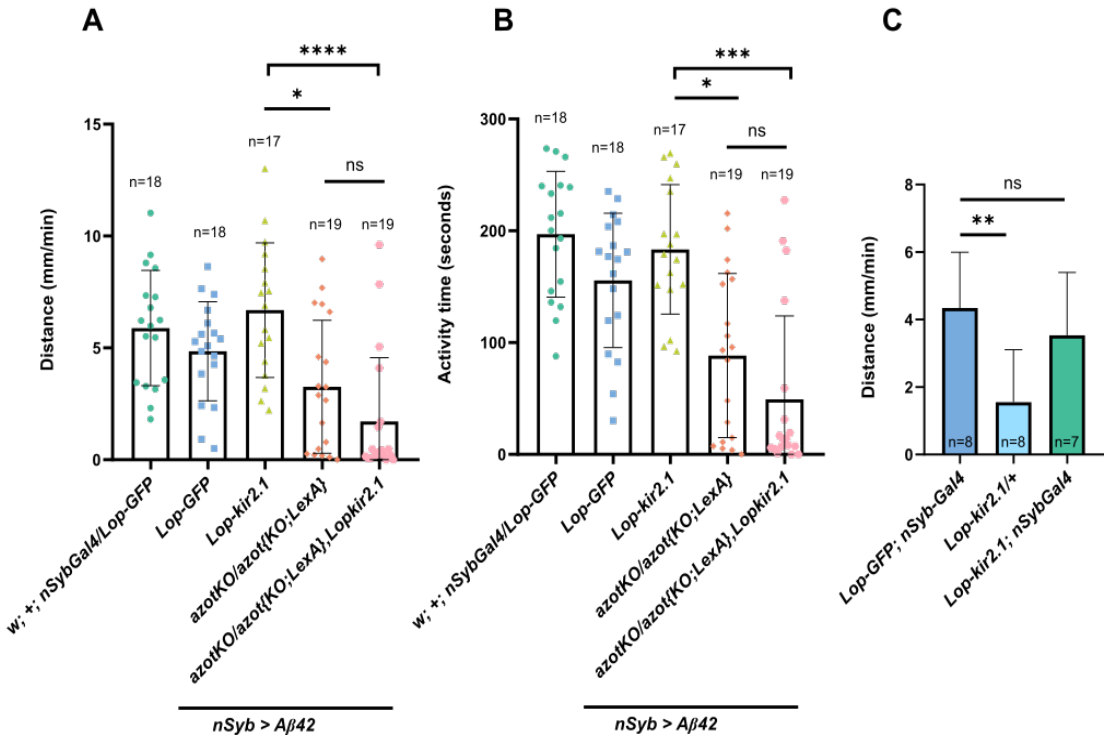
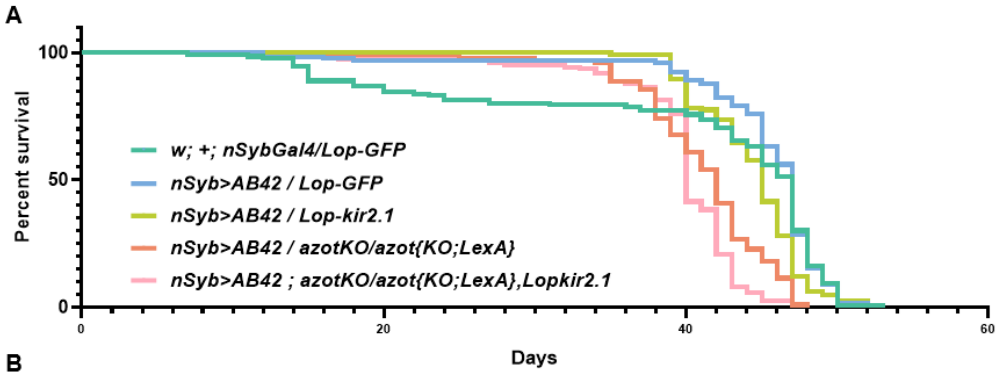


Figure 3.4 Exploring hypothesis for poor locomotor performance of *nSyb>Aβ42 / azot{KO;LexA},Lopkir2.1* flies - Graphs depict **(A** and **C)** distance walked in millimeters per minute and **(B)** activity time in seconds (using a speed threshold [ST]). Parameters were calculated from individual walks of 7-day old males for each genotype. Statistical significance was based on ANOVA and a Kruskal-Walis test for multiple comparisons. * indicates p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001

To further test the effects of silencing *azot*-expressing neurons in *nSyb>Aβ42* flies, a longevity assay was performed on non-virgin females with the same genotypes as the males tested for locomotor performance, grown at 29ºC. These results support the locomotor assays since, like *nSyb>Aβ42 ,azotKO-/-,* the *nSyb>Aβ42, azotKO / azot{KO; LexA}, Lopkir2.1* females showed a shorter life expectancy than *nSyb>Aβ42* **(Figure 3.5)**, further suggesting that silencing neurons with Kir2.1 cannot counterbalance the burden caused by lack of *azot.* In agreement with previous works, *nSyb>Aβ42; azot{KO; LexA}* females displayed a lower life expectancy than flies *wild-type* for *azot* ,but, *c*ontrary to what was expected, *nSyb>Aβ42* females lived, on average, for more days than healthy females.

A



B

	Mean Survival	% Mean Survival	Max Life span	% Max life span	Log Rank p
w; +; nSybGal4/Lop-GFP	40,5	-10,1	53	1,9	ns
nSyb>AB42/Lop-GFP	45,1	0	52	-	-
nSyb>AB42/Lop-kir2.1	44,4	-1,6	52	0	0,01
nSyb>AB42/azotKO/azot{KO;LexA}	41,0	-8,9	48	-7,7	<0,0001
nSyb>AB42 ; azotKO/azot{KO;LexA},Lopkir2.1	39,7	-11,96	47	-9,6	<0,0001

Figure 3.5 : Effect of silencing *azot*-expressing neurons on longevity – Lifespan curve **(A)** and table **(B)** depicting survival analysis for females of each of the represented genotypes, grown at 29ºC.

3.2. Effect of silencing and killing hyperactive neurons with *Kir2.1*. on locomotor behaviour

Since silencing *azot*-expressing neurons did not appear to be a successful strategy to improve locomotor performance in *nSyb>Aβ42 flies*, we proposed that a more downstream/specific approach to silencing hyperactive neurons, or effectively removing them from the brain by killing them, might be more effective in improving the health of these models.

It is possible to target hyperactive neurons more directly, by taking advantage of the CaLexA tool. CaLexa is an adaptation of the LexA driver fused with the NFAT (Nuclear Factor of Activated T Cells) calcium responsive transcription factor. In this way, LexA will only travel to the nucleus where it can activate the expression of the GFP gene that is downstream of a LOP sequence when there is a high influx of Ca^{2+} in the cell **(Figure 3.6)**. An additional advantage of the CaLexA system is that the sequence of the chimeric transcription factor mLexA-VP16-NFAT is downstream of UAS, so its expression is restricted to cells where the Gal4 protein is present.

CaLexa was developed as a tool to sense neuronal activity because a high influx of calcium is a consequence of sustained neuronal activity so, it is possible to visualize neurons where there is a higher influx of calcium since they display intense GFP fluorescence (Masuyama et al., 2012). However, it is also possible to "manipulate" this system and use this calcium-dependent LexA to drive the expression of other Lop-associated constructs, in this case, *kir2*.1, *reaper*, a pro-apoptotic gene, and *p35*, an inhibitor of caspases.

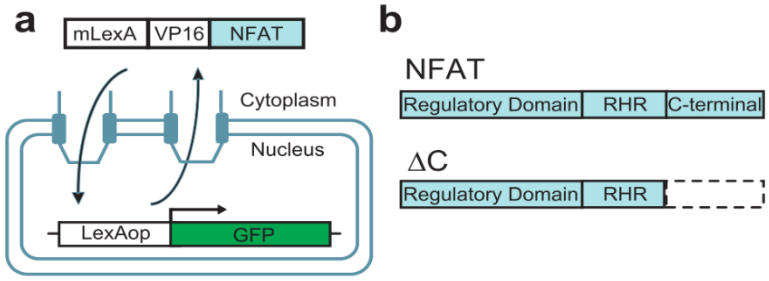


Figure 3.6: Schematic description of the CaLexA system – **(A)** The CaLexA system relies on the chimeric transcription factor mLexA-VP16-NFAT. When there is calcium accumulation in the cytoplasm, calcineurin is activated and dephosphorylates the NFAT, causing the chimeric transcription factor to shuttle into the nucleus. Once inside the nucleus, it induces expression of the GFP reporter gene, which is under the control of the LOP. **(B)** The regulatory domain of NFATc1 contains the nuclear localization signal (NLS), whose function is tightly controlled by the calcium/calmodulin-dependent phosphatase calcineurin but lacks its endogenous C-terminal domain. Adapted from (Masuyama et al., 2012)

Reaper is a pro-apoptotic protein in the *Drosophila* programmed cell death pathway, that is transcriptionally activated by different pro-apoptotic signals and triggers the expression of different effector caspases, by binding to and destroying DIAP1 *(Drosophila* inhibitor of apoptosis proteins) (Steller, 2008). By inducing *reaper* expression under the control of the chimeric LexA of the CalexA system, we expect that hyperactive neurons are killed and expect the contrary to occur when the expression of p35, an inhibitor of apoptosis is controlled by the same driver. Likewise, we expect that hyperactive neurons are silenced by *kir2.1* when this gene is expressed under the control of CaLexA.

We performed Buridan assays to compare the distance travelled and activity time of control AD flies (*w; UAS-Aβ42, tubGal80^{ts}; nSybGal4/CaLexA),* flies where hyperactive neurons are silenced *(w; UAS-Aβ42, tubGal80^{ts} /Lop-Kir2.1; nSybGal4/CaLexA),* flies where hyperactive neurons are protected from apoptosis by p35 *(w; UAS-Aβ42, tubGal80^{ts} /Lop-p35; nSybGal4/CaLexA),* and flies where hyperactive neurons are killed by reaper (*w; UAS-Aβ42, tubGal80^{ts} /Lop-reaper; nSybGal4/CaLexA)*. 15-day old males aged at 29ºC showed similar locomotor performance in all genotypes except for males where hyperactive neurons were silenced (*w; UAS-Aβ42, tubGal80^{ts} /Lop-Kir2.1)* which, on average, walked significantly less and for shorter periods than control flies **(Figure 3.7)**.

It is important to note that all these stocks carry a *tubGal80^{ts}* construct, which allows us to only induce the expression of the genes controlled by the *Gal4>UAS* system by maintaining flies at 29ºC. In this case, both the *Aβ42* and CaLexA expression are dependent on this system, so by performing the crosses to obtain the males used in this assay at 18ºC, a temperature at which the Gal80 protein is blocking the action of Gal4, we assure that in larvae, there is no expression of *Aβ42* nor of the genes that are being controlled by the chimeric LexA of the CaLexA system (reaper, p35 or kir2.1). There was a need to only induce the expression of these genes in adults because silencing and killing neurons with higher levels of calcium (which would be hyperactive) proved to be lethal during development, as no flies hatched.

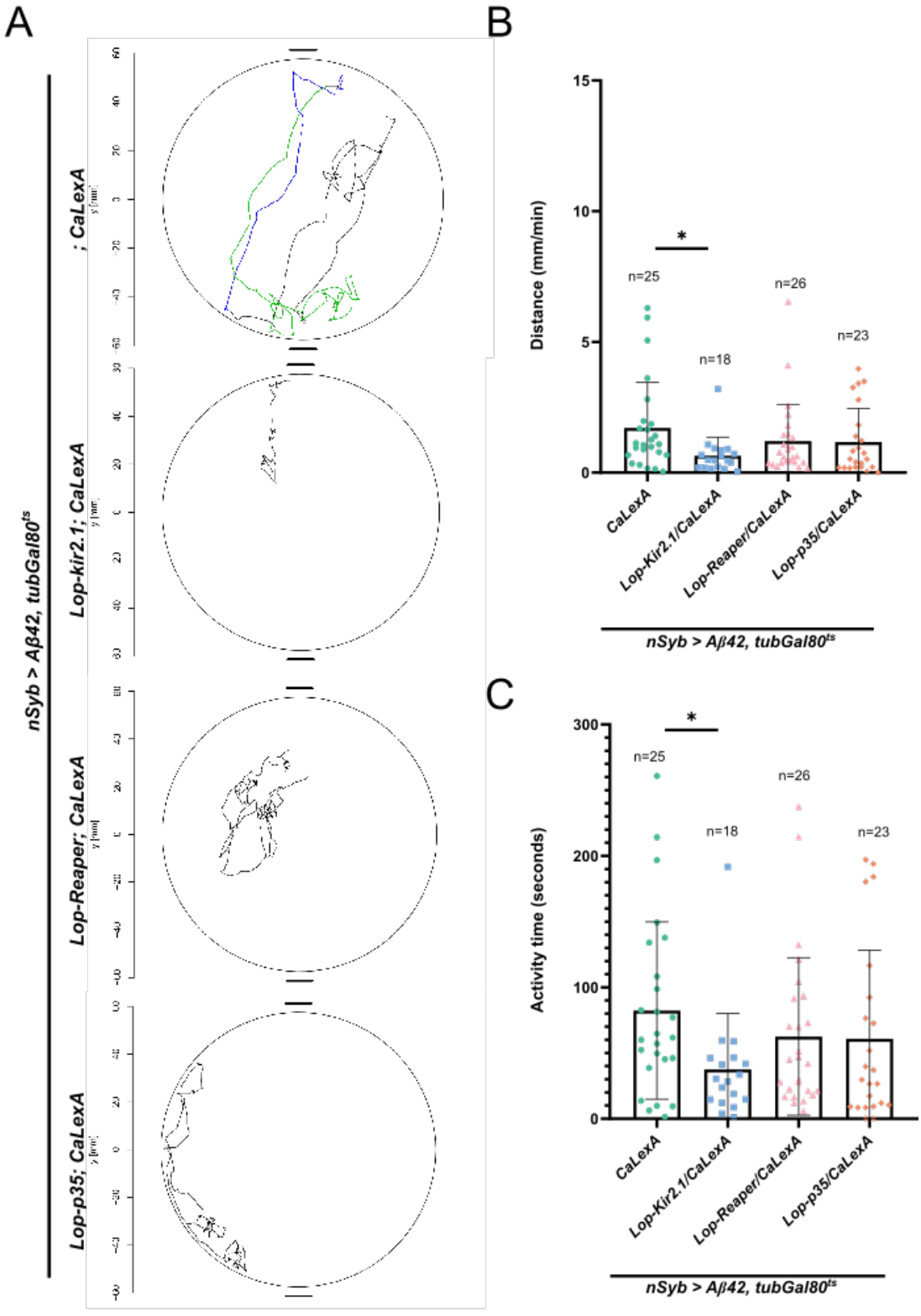


Figure 3.7: Effect of silencing and killing neurons on the locomotor performance of 15-day old flies - (A) Individual trajectories representative of each indicated genotype. Graphs depict **(B)** distance walked in millimeters per minute and **(C)** activity time in seconds (using a speed threshold [ST]). Parameters were calculated from individual walks of 15-day old males for each genotype. Statistical significance was based on ANOVA and a Kruskal-Walis test for multiple comparisons. * indicates $p<0.05$

3.3. Determining the role of glutamate on Aβ-induced neuronal hyperactivity

Ectopic expression of human Aβ42 in the optic lobes (OL) of fly brains has previously been shown to induce both neuronal hyperactivity and an increase in glutamate concentration in the OL (Coelho & Moreno 2020), reminiscent of the neuronal hyperactivity displayed by prodromal AD and the glutamate excitotoxicity that is associated with neuronal death in this condition. Since glutamate is an excitatory neurotransmitter in the human CNS, it is possible that a higher quantity of this neurotransmitter might be related to abnormal neuronal activity, either by stimulating neuronal activity or arising as a consequence of it.

3.3.1. Effects of downregulating glutamatergic signaling on neuronal activity

To test if glutamate is responsible for the increase in aberrant neuronal activity in an Aβ context, 3 different RNAi lines against DVGLUT were used. DVGLUT is the only *Drosophila* ortholog of the vesicular glutamate transporter human gene family. It is a transporter protein, responsible for packaging glutamate into synaptic vesicles (R. W. Daniels, 2004) that transport it through the axon until it reaches the synaptic cleft where it is released upon neuronal depolarization. So, by downregulating DVGLUT expression, glutamate is no longer packaged into vesicles (Daniels et al., 2006) in glutamatergic neurons and glutamate signaling becomes significantly reduced.

3.3.1.1. Hyperactivity in different regions of the brain

Neuronal hyperactivity had already been detected in previous studies of the lab, in the mushroom body of *MB>Aβ42* and the optic lobes of *GMR>Aβ42* flies. Aiming to identify in which regions of the fly brain hyperactivity is most prominent, the *nSybGal4* driver line was used to promote the expression of Aβ42 in every neuron. *nSyb>LacZ* and *nSyb>Aβ42* flies were crossed with *CaLexA* virgins and brains were stained with an antibody against GFP. High GFP fluorescence was identified, as expected, in the cytoplasm of neurons in the optic lobe and mushroom body regions, as well as in the antennal lobes **(Figure 3.8)**. Both visual quantification in the optic lobes and fluorescence-based quantification of high GFP in whole-mount brains showed at least a 50-fold increase in GFP in *nSyb>Aβ42* brains, in comparison to *nSyb>LacZ.* This solidifies the hypothesis that states that ectopic expression of Aβ42 is responsible for an increase in aberrant neuronal activity throughout the brain. It also suggests that expression of Aβ42 in every neuron of the fly brain produces more intense effects than when its expression is controlled by region-specific drivers such as GMR-Gal4 or MB-Gal4, however, this might also be due to an increased strength of the nSyb driver, in comparison to the others.

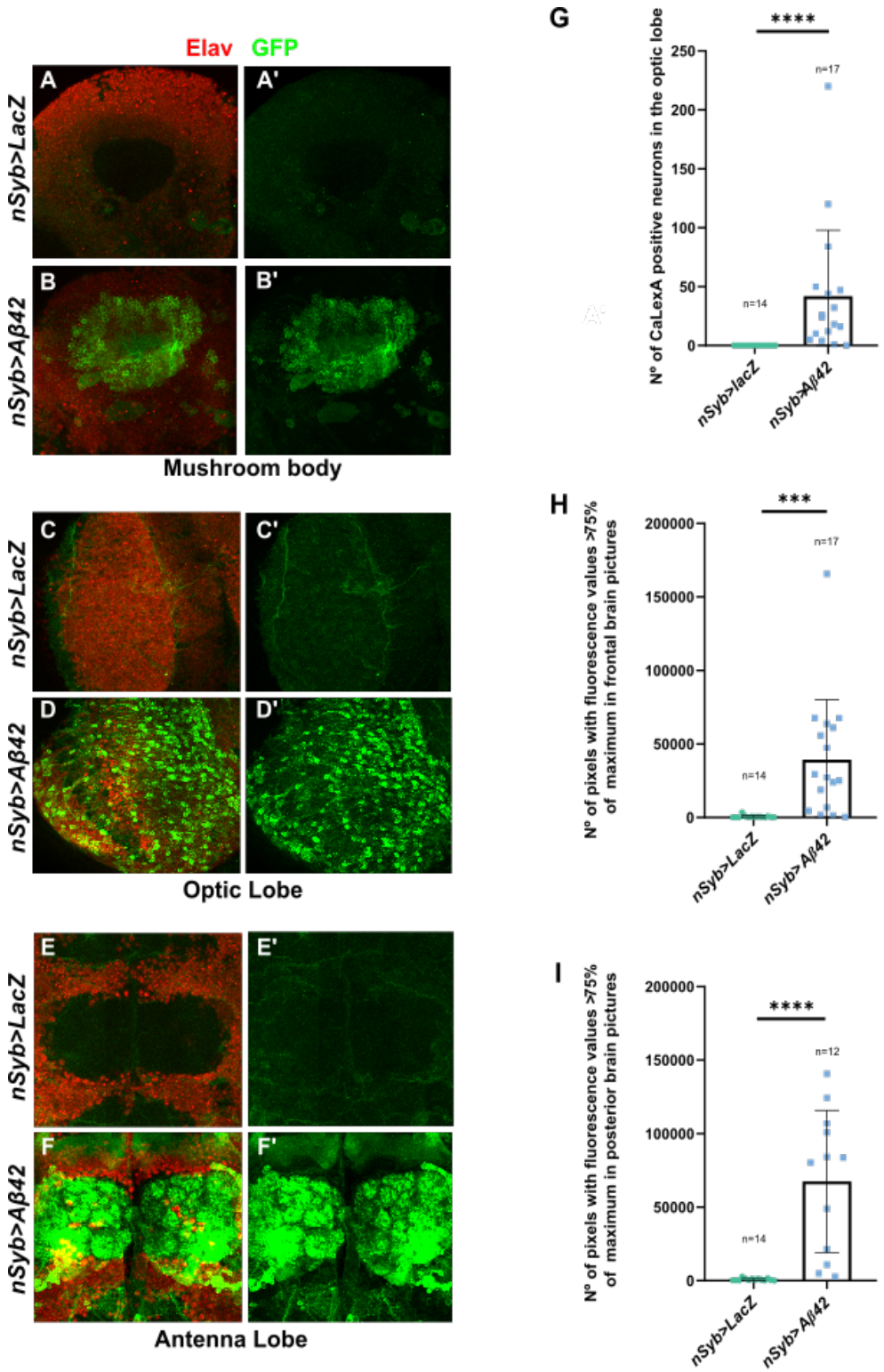


Figure 3.8: Amyloid-β-induced hyperactivity in different regions of the brain - Z projections of the **(A and B)** mushroom body, **(C and D)** optic lobes and **(E and F)** antenna lobes of *nSyb>AB42; CaLexA* and *nSyb>LacZ; CaLexA* brains, stained with an antibody against GFP (green)**.** Neurons are labeled in red by staining against Elav. **(G)** Quantification of neurons labeled by CaLexA in the optic lobes of *nSyb>AB42; CaLexA* and *nSyb>LacZ; CaLexA* in 10-day old flies and pixels with fluorescence values >75% of maximum in **(H)** frontal and **(I)** posterior brain pictures. Statistical significance was performed by Mann Whitney-U analysis. *** indicates $p<0.001$ and **** $p<0.0001$

3.3.2.2. Effect of downregulating DVGLUT in the mushroom body

The mushroom body is a relevant area of the brain in the context of AD because it plays a fundamental role in learning and olfactory memory in the fly (Heisenberg, 2003). However, the presence of glutamatergic neurons in this area has been controversial in the past. Some articles reported that glutamatergic markers were only present in Kenyon cells during development (Richard W. Daniels et al., 2008). Recently the presence of glutamatergic neurons in the MB has been confirmed by studies (Aso et al., 2014) that identified clusters of glutamatergic neurons close to the region of α and β lobes, especially glutamatergic output neurons.

Despite not being able to detect any excess in glutamate release on *MB>Aβ42* mushroom bodies by immunostaining (data not shown), it was still relevant to test if downregulating DVGLUT expression could lead to changes in neuronal activity. With this aim, *MB>Aβ42* females were crossed with males from the 3 different RNAi against DVGLUT lines (the TRiP line from the Bloomington stock center and the kk and GD lines from the VDRC) and an RNAi against *yellow* (an endogenous gene responsible for the color of the cuticle in *Drosophila*) as a control. The number of neurons with high GFP in each MB was quantified, both manually and by measuring the number of pixels with high fluorescence values in the green channel. Results from *MB>Aβ42; >RNAiDVGLUT* from the TRiP collection males show a more than 50% increase in the number of GFP positive neurons, compared to *MB>Aβ42; RNAiyellow* males **(Figure 3.9),** which might suggest that reducing glutamate concentration leads to an increase in neuronal activity. However, there is no increase in the number of GFP-positive neurons in the other RNAi lines.

To confirm that the presence of RNAi against yellow was not causing changes in neural activity, either by the absence of the gene or by changes in the endogenous RNAi pathway, an *MB>Aβ42 />LacZ* control was added and it displayed similar levels of GFP-positive neurons as the RNAi against *yellow*.

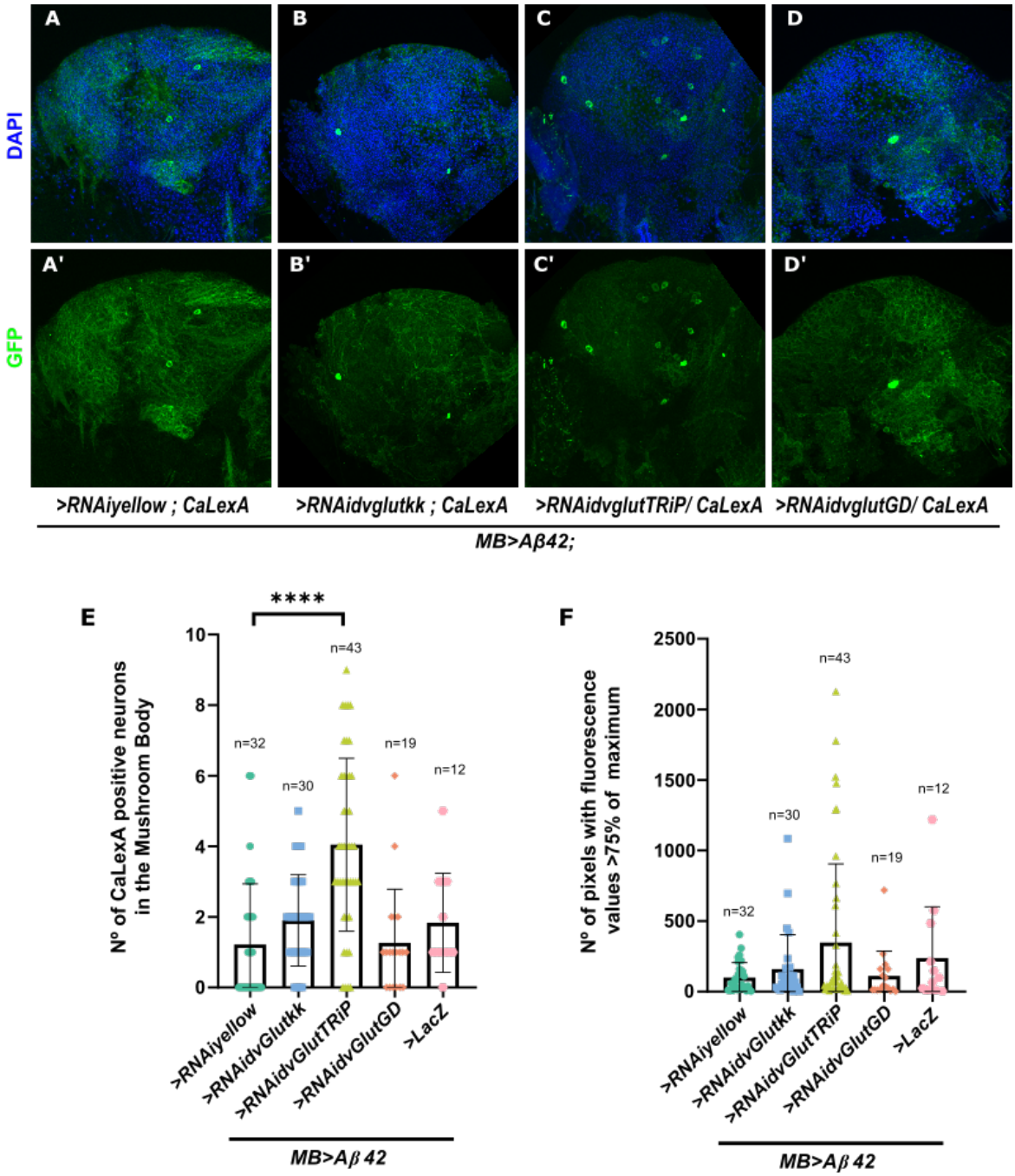


Figure 3.9: Effect of RNAi against dvglut on amyloid-β-induced hyperactivity in the MB: (A-D) Z projections of the mushroom body of brains of the indicated genotypes, stained with an antibody against GFP (green)**.** Nuclei are in blue. Quantification of **(E)** neurons labeled by CaLexA in 10 -day old flies and **(F)** pixels with fluorescence values >75% of maximum. Values are normalized for the mean of the control MB>Aβ42/ RNAiyellow. Statistical significance was based on ANOVA and a Kruskal-Walis test for multiple comparisons. **** indicates p<0.0001.

3.3.2.3. Effect of downregulating DVGLUT in the optic lobes

To perform the same experiment in a context in which excessive glutamate release had already been shown (Coelho & Moreno, 2020), we tested the effect of downregulating glutamatergic signaling with RNAi against DVGLUT on neuronal hyperactivity, in the OLs of *GMR>* and *GMR>Aβ42* flies. In this case, hyperactivity was only quantified by the number of pixels with higher GFP fluorescence, because GFP intensity was not clear enough to enable manual counting of single neurons.

In this case, fluorescence values were similar in all 4 genotypes, despite slight, non-significant, increases in the number of pixels with high fluorescence in the presence of RNAiDVGLUT kk and TRiP lines, which would suggest, once again, that glutamate is inhibiting neuronal hyperactivity, as results from the TRiP line in the mushroom body suggested.

However, unlike what occurred in the other experiments where both measures for quantifying CaLexA-positive neurons were used, values for pixels with fluorescence superior to 75% of the maximum were very low, despite clear GFP-positive staining in the axons and cell bodies. It is possible that GMR-Gal4 might be a weaker driver than MB-Gal4 and nSyb-Gal4, so it could be responsible for a smaller expression of the mLexA-VP16-NFAT transcription factor, which could, for the same amount of calcium, induce less production of the GFP protein. The smaller amount of GFP protein could be responsible for the deterioration of the reliability of the pixel measurements. They could also have been less effective due to the settings of image acquisition in the confocal not being accurately optimized.

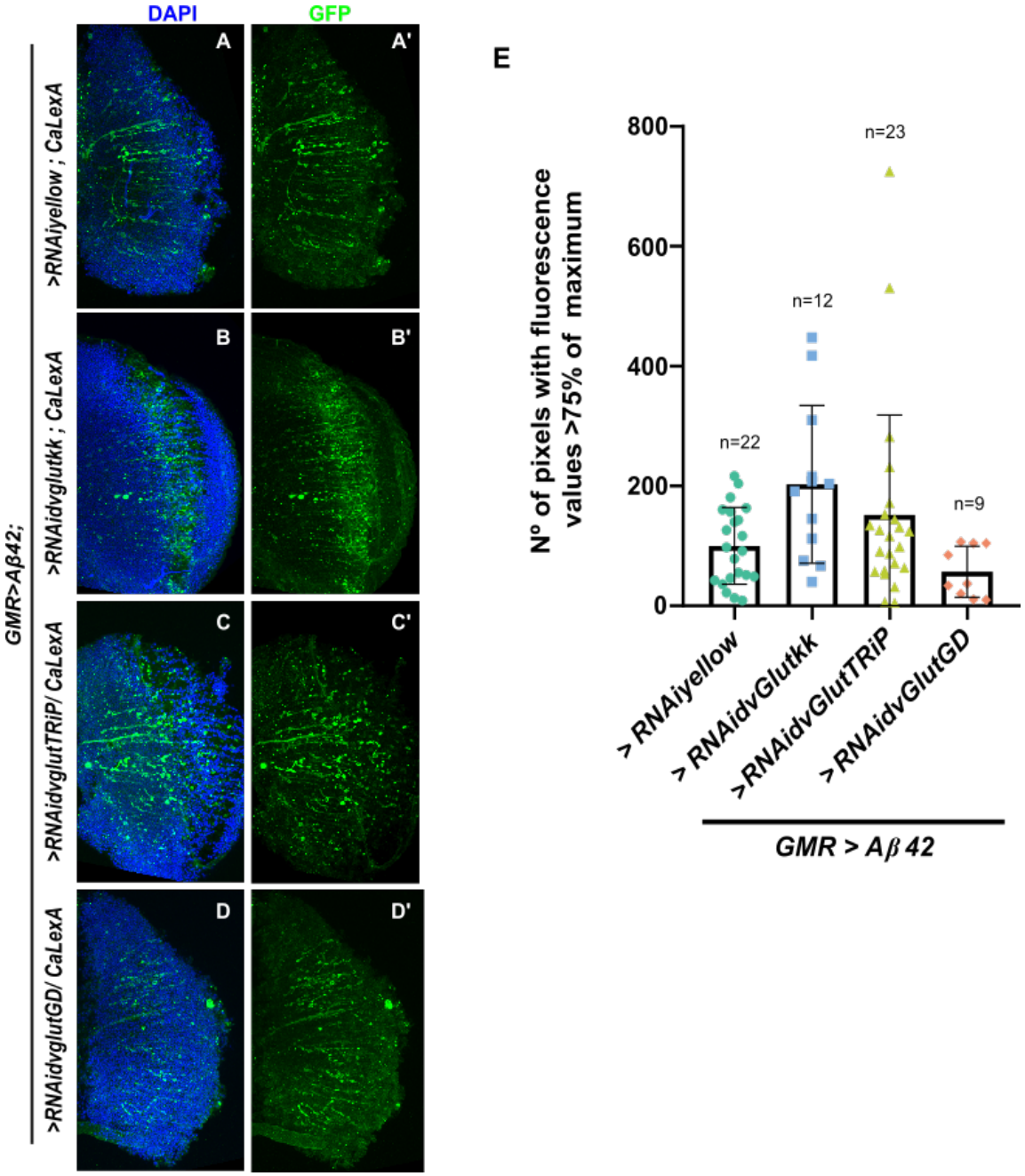


Figure 3.10: Effect of RNAi against DVGLUT on amyloid-β-induced hyperactivity in the OL - (A-D) Z projections of the optic lobes of brains of the indicated genotypes, stained with an antibody against GFP (green)**.** Nuclei are in blue. **(E)** Quantification of pixels with GFP fluorescence values >75% of maximum. Values are normalized for the mean of the control GMR>Aβ42/ RNAiyellow. Statistical significance was based on ANOVA and a Kruskal-Walis test for multiple comparisons.

3.3.2. Effect of neuronal silencing on glutamatergic release

Since there were no conclusive results showing a decrease in neuronal activity in the absence of glutamate, we decided to test the opposite hypothesis. In an attempt to decipher if neuronal hyperactivity is responsible for the increase of glutamate detected by the antibody in the optic lobes of *GMR>Aβ42* individuals, we silenced neuronal activity and monitored glutamate levels. In this case, 3 different approaches to induce neuronal silencing were tested: silencing *azot*-expressing and hyperactive neurons using the same strategies as in **3.1.** (with the w; *GMR, UAS-Aβ42/ azot{KO; LexA}, Lopkir2.1;+* stock, and by taking advantage of CaLexA, with the *w; GMR, UAS-Aβ42/ Lopkir2.1; CaLexa*, respectively) and silencing every neuron in the optic lobe of the adult brain (with a *w; GMR-Gal4, UAS-Aβ42/ tubGal80^{ts}; CaLexA* stock). Initially, different *UAS>kir2.1* stocks were crossed with *GMR>AB42* females, however, progeny from these crosses was either lethal in males or both males and females. This phenotype could be attributed to a damaging effect in silencing every neuron in the optic lobe during larval development, so a *w; GMR-Gal4, UAS-Aβ42/ tubGal80^{ts}, UAS-Kir2.1; CaLexA* stock was used so that neuronal silencing only occurred after pupal eclosion and didn't compromise development. In all genotypes, glutamate immunoreactivity was quantified in the optic lobes of 15-day old males grown at 29ºC, and values were compared to those of brains of individuals where no neuron was silenced.

GMR-Gal4, azot{KO; LexA}, Lopkir2.1 was used as a control where there is no ectopic expression of Aβ42, and, as expected, displayed a tendency for lower glutamate intensity in comparison to *GMR>Aβ42* individuals **(Figure 3.11.A-B, F)**, although lacking statistical significance. The 3 different genotypes where neurons were silenced **(Figure 3.11.C-E)**, displayed similar mean intensities of fluorescence for the α-L-Glutamate antibody as *GMR>Aβ42* flies where no neuron was silenced. The fact that there was no significant difference in glutamate immunoreactivity between genotypes, suggests that hyperactivity may not be responsible for excessive glutamate release.

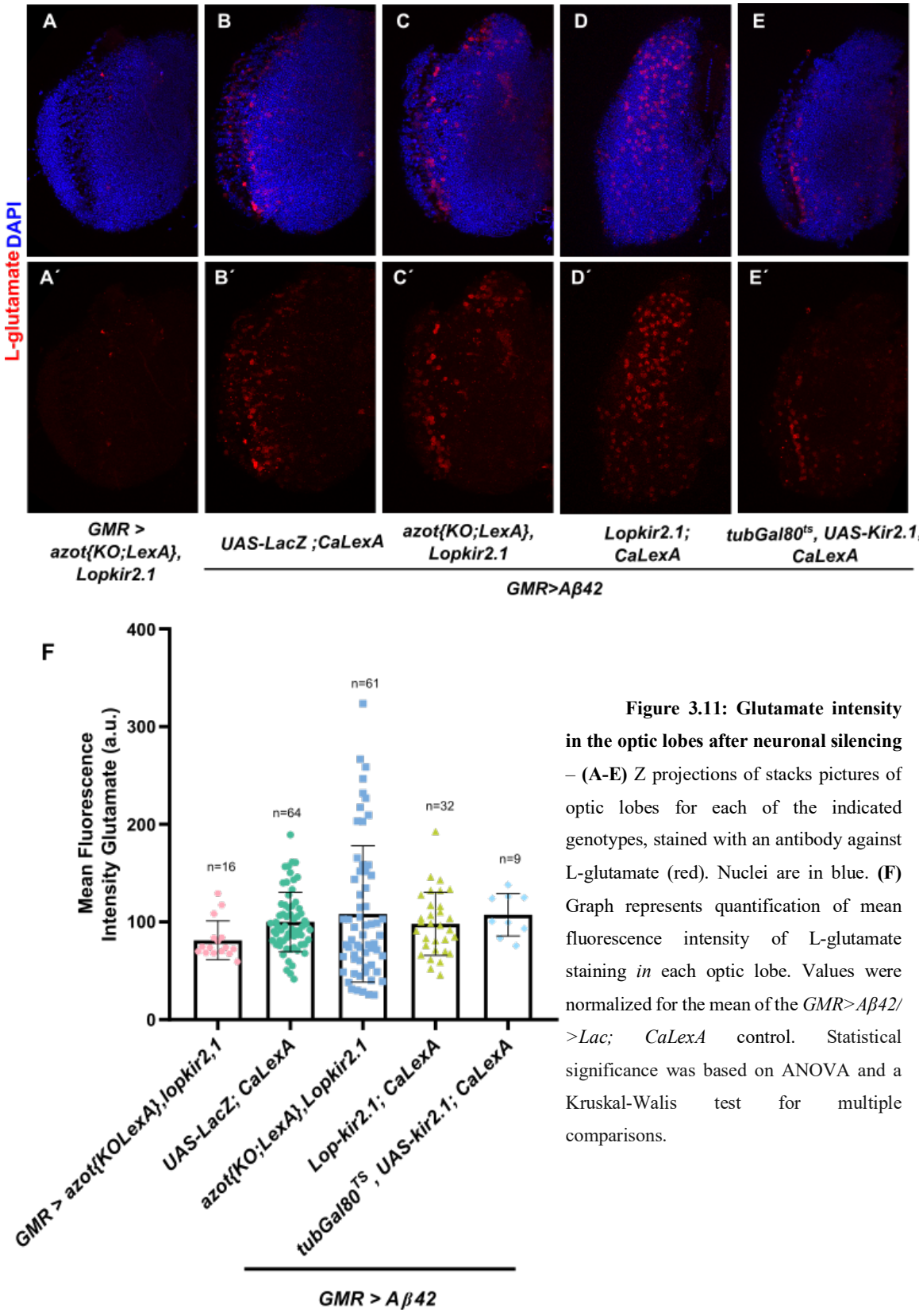


Figure 3.11: Glutamate intensity in the optic lobes after neuronal silencing – **(A-E)** Z projections of stacks pictures of optic lobes for each of the indicated genotypes, stained with an antibody against L-glutamate (red). Nuclei are in blue. **(F)** Graph represents quantification of mean fluorescence intensity of L-glutamate staining *in* each optic lobe. Values were normalized for the mean of the *GMR>Aβ42/ >Lac; CaLexA* control. Statistical significance was based on ANOVA and a Kruskal-Walis test for multiple comparisons.

3.4. Determining the role of glutamate in the upregulation of low fitness markers and neuronal death

Previous works reported that ectopic expression of human Aβ42 in the fly brain prompted upregulation of low fitness markers such as *Flower*^{LoseB} and *azot* (Coelho et al., 2018), demonstrating for the first time that there is cell competition in the context of AD and that this phenomenon is beneficial since it prompts removal of damaged neurons by apoptosis. Given that glutamate excitotoxicity is known to promote neurodegeneration and drive neuronal death, we sought to see if manipulating glutamate release interferes with the cell competition machinery and neuronal death.

3.4.1. Effect of downregulating DVGLUT on Flower^{LoseB}, Azot, and DCP1 expression

In a similar approach to the one in **3.2.2.** and **3.2.3,** 3 different RNAi lines against DVGLUT were used to downregulate glutamatergic signaling and check for changes in fitness markers in the optic lobes of *GMR>Aβ42* individuals. To check these changes in *flower*^{LoseB} and *azot* expression, flies carried *flower*^{LoseB}*::mcherry* and *azot::mcherry* reporters. The Azot reporter was originally created (Coelho et al., 2018) by adding an extra copy of *azot* to a different location in the genome, fused with a copy of the *mcherry* sequence. The Flower reporter is a knock-in of an mCherry in the *flower* locus, in frame with the coding sequence of the LoseB isoform. In both, mCherry expression is controlled by the Flower and Azot promoters.

At 15 days post-eclosion, there is no difference in Flower^{LoseB} expression between progeny from the cross between *GMR>Aβ42; flower*^{LoseB}*::mcherry* females and males carrying the different RNAi lines **(Figure 3.12.A-C, G)**. At this time point, there seems to be a significant reduction in *azot* expression in the DVGLUT TRiP RNAi line, but the DVGLUT kk RNAi line shows similar mcherry fluorescence as *yellow* RNAi.

To check for changes in neuronal death, the same brains were stained against DCP1 (*Drosophila* Cleaved Caspase 1), an important member of the apoptotic pathway in *Drosophila*, and DCP1-positive neurons were quantified. In this case, there was also a lower mean of DCP1-positive cells in the RNAi TRiP line but, the opposite could be seen in the kk line, suggesting that in one of the RNAi lines there is an increase in neuronal death, while in the other the opposite is happening. If we consider only the results from the TRiP line, we could suggest that excessive glutamate was inducing neuronal death, probably by cell competition, and that downregulating DVGLUT reduced neuronal death.

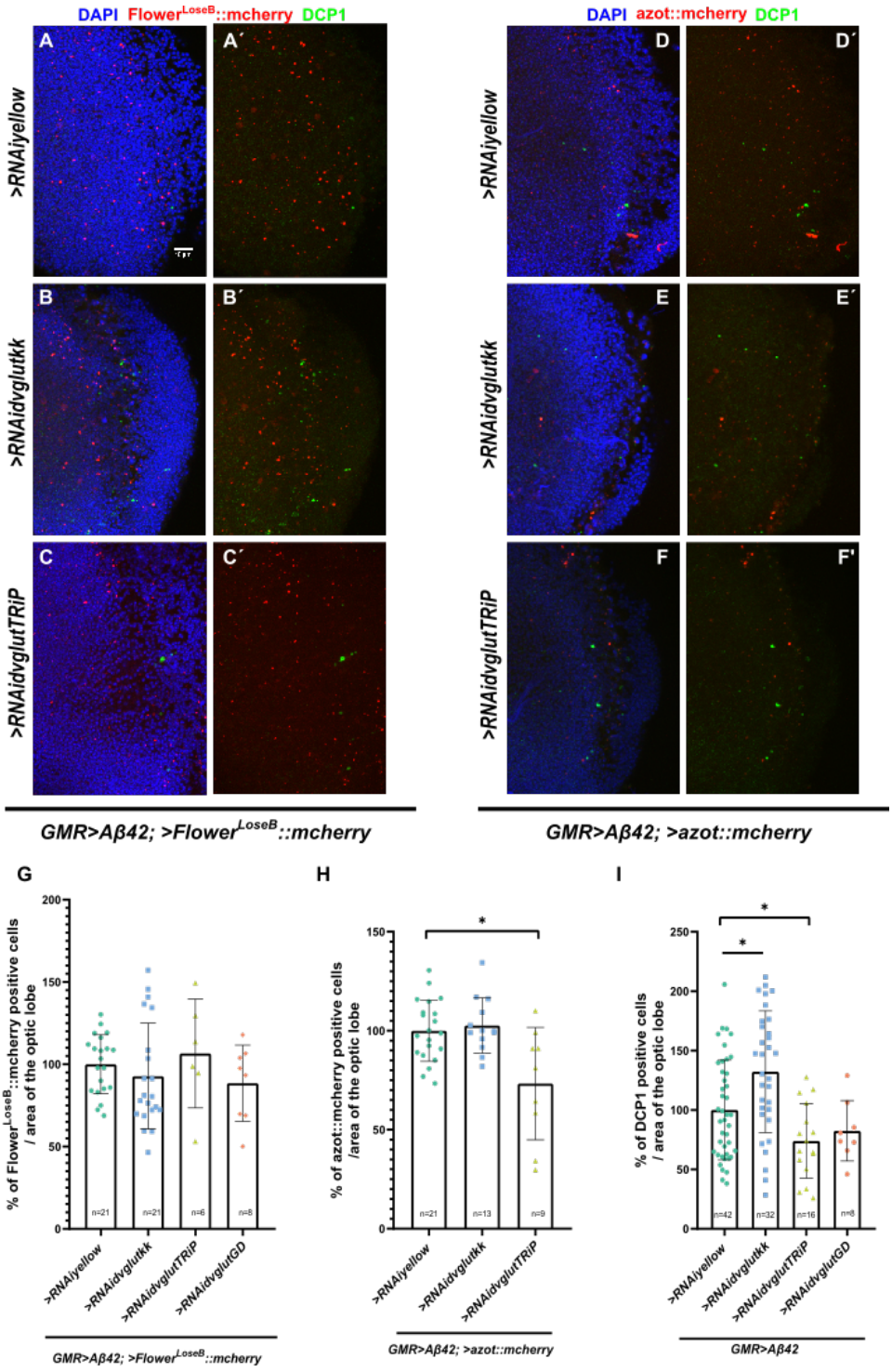


Figure 3.13: Changes in low fitness markers and cell death induced by DVGLUT downregulation - Expression of the **(A-C)** Flower^{LoseB}::mcherry, **(D-F)** azot::mcherry reporters and DCP1 in the optic lobe of adult flies, and quantification **(G)** of the % of Flower^{LoseB} -positive cells/ area of the optic lobe **(H)** % of azot -positive cells/ area of the optic lobe and **(I)** DCP1-positive cells / area of the optic lobe for the described genotypes. Values were normalized for the mean of the *GMR; >Aβ42/RNAiyellow; flower^{LoseB}::mcherry* control. Statistical significance was based on ANOVA and a Kruskal-Walis test for multiple comparisons. * indicates $p<0.05$

3.4.2. Effect of Memantine on Flower^{LoseB}, Azot expression, and neuronal death

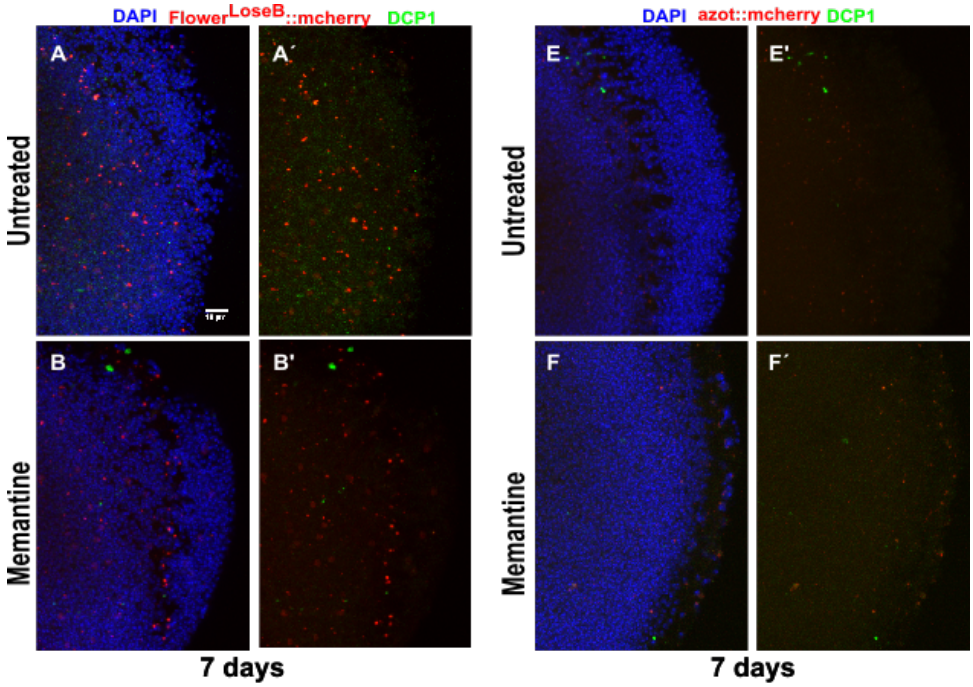
Memantine is a glutamate antagonist, that binds to glutamate receptors in post-synaptic neurons, blocking glutamate binding and the consequential depolarization and action potential firing. It is one of the few drugs that has been approved and commercialized for the managing of symptoms such as locomotor decline in AD patients. Due to its innate ability to downregulate glutamatergic signaling and its apparent success in reducing AD symptoms, treating flies with memantine was used as a different approach to test the effect of glutamate on cell fitness markers and cell death.

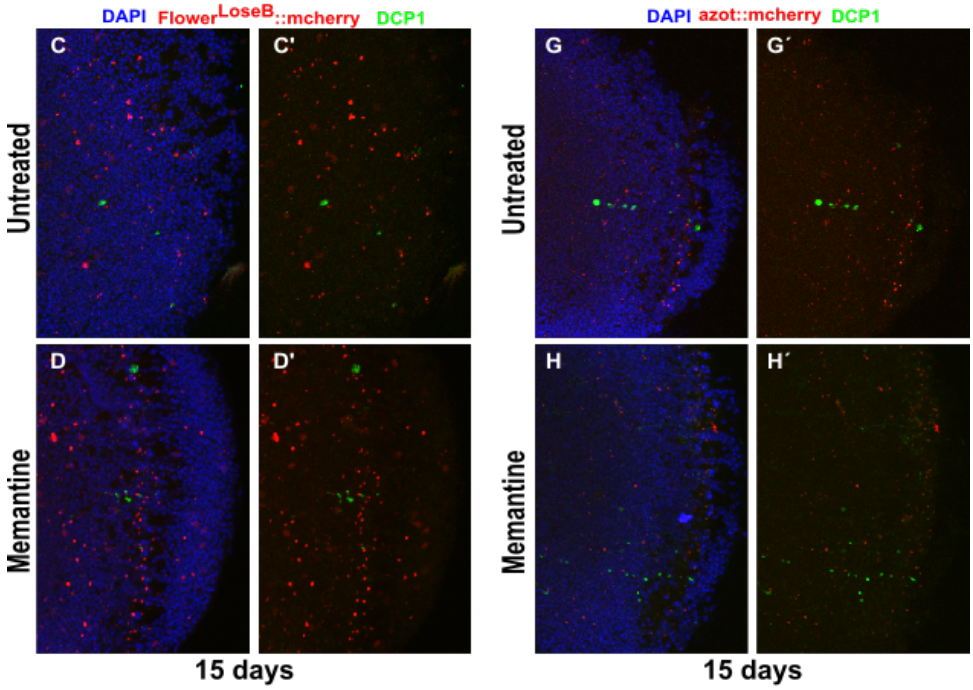
GMR>*Aβ42* flies were fed standard fly food supplemented with 10μM of Memantine Hydrochloride and Azot and Flower reporter and DCP1 expression were monitored at 3 different time points: 7, 10, and 15 days post-eclosion, on flies grown at 25ºC. There is an almost 50% increase in Flower^{LoseB}::mcherry expression in 7-day old flies and a 20% increase in 10-day old flies treated with Memantine **(Figure 3.13),** in comparison to untreated flies. This increase disappears in 15-day-old individuals.

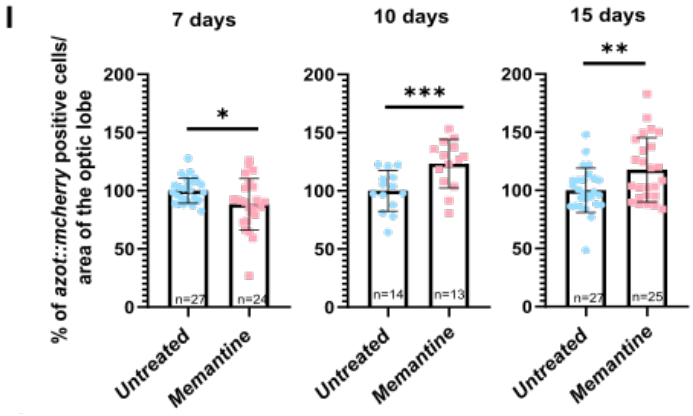
Regarding *azot* expression, the time course appears to be different. At 7 days old, flies treated with memantine display lower *azot* expression than untreated, however at 10 days old this tendency is inverted and *azot* expression becomes 25% greater than that of untreated flies, and at 15 days old this increase persists, although the difference becomes less significant.

DCP1 expression is not significantly different between treated and untreated 7- nor 15-day old flies, which suggests that memantine is not modulating cell death in this context, at these timepoints.

To understand if this effect of memantine in driving the upregulation of cell fitness markers is general or specific to the context of AD, and to rule out possible toxicity of the concentration of Memantine with which the flies were treated, *flower^{LoseB}* and *dcp1* expression were monitored in healthy, non-Aβ42 expressing flies (*GMR-GAL4; +; flower^{LoseB}::mcherry)* fed with fly food supplemented with Memantine or without. It is possible to observe that *flower^{LoseB}::mcherry* expression in treated flies was significantly lower, while DCP1 was slightly higher **(Figure 3.14)**, although non-significative. This is not indicative of a toxic effect of this dosage of Memantine in the *Drosophila* brain. To understand if memantine treatment was having an impact on the locomotor performance of flies, we performed Buridan assays between treated or untreated *nSyb>Aβ42*, 15-day old males and saw no significant difference in Distance or Activity Time.


DAPI FlowerLoseB::mcherry DCP1
DAPI azot::mcherry DCP1
A
A´
E
E'
B
B'
F
F´
Untreated
Memantine
7 days
7 days


DAPI FlowerLoseB::mcherry DCP1
DAPI azot::mcherry DCP1
C
C'
G
G´
D
D'
H
H´
Untreated
Memantine
15 days
15 days


I
7 days
10 days
15 days
% of azot::mcherry positive cells/ area of the optic lobe
200
150
100
50
0
*
***
**
n=27
n=24
n=14
n=13
n=27
n=25
Untreated
Memantine

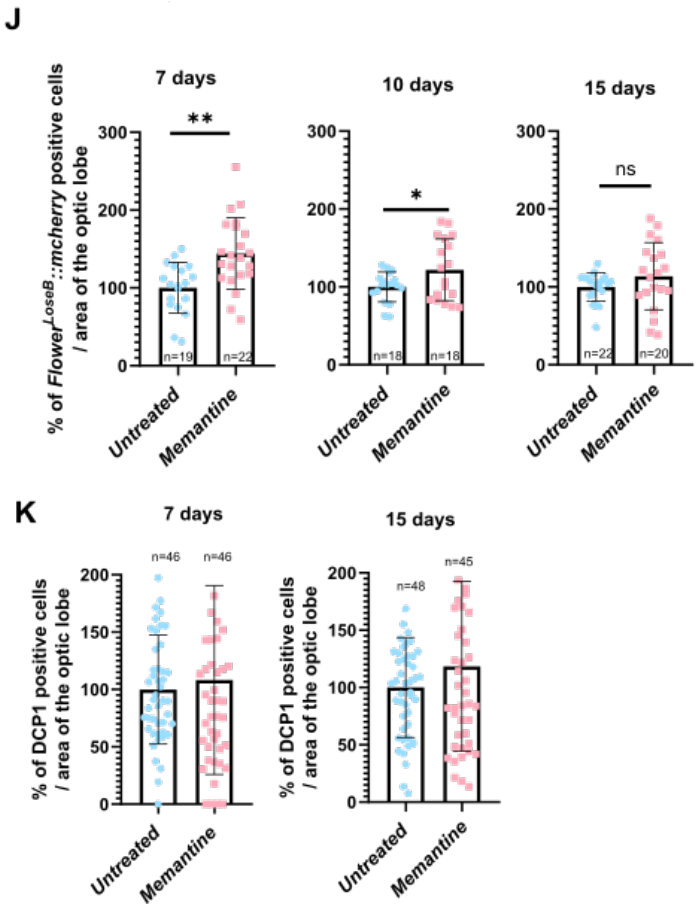


Figure 3.14: Changes in low fitness markers and cell death induced by Memantine Treatment - Expression of **(A-B)** Flower^{LoseB}::mcherry and **(E-F)** azot::mcherry reporters and DCP1 in the optic lobe of 7-day old and 15-day old **(D-E, G-H)** flies and quantification **(I)** of the % of Flower^{LoseB} -positive cells/ area of the optic lobe, **(J)** % of azot -positive cells/ area of the optic lobe and **(K)** % of DCP1-positive cells/ area of the optic lobe for the described genotypes. Values were normalized for the mean of the untreated controls. Statistical significance was accessed by unpaired t-tests. * indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$. Scale bar = 10µm

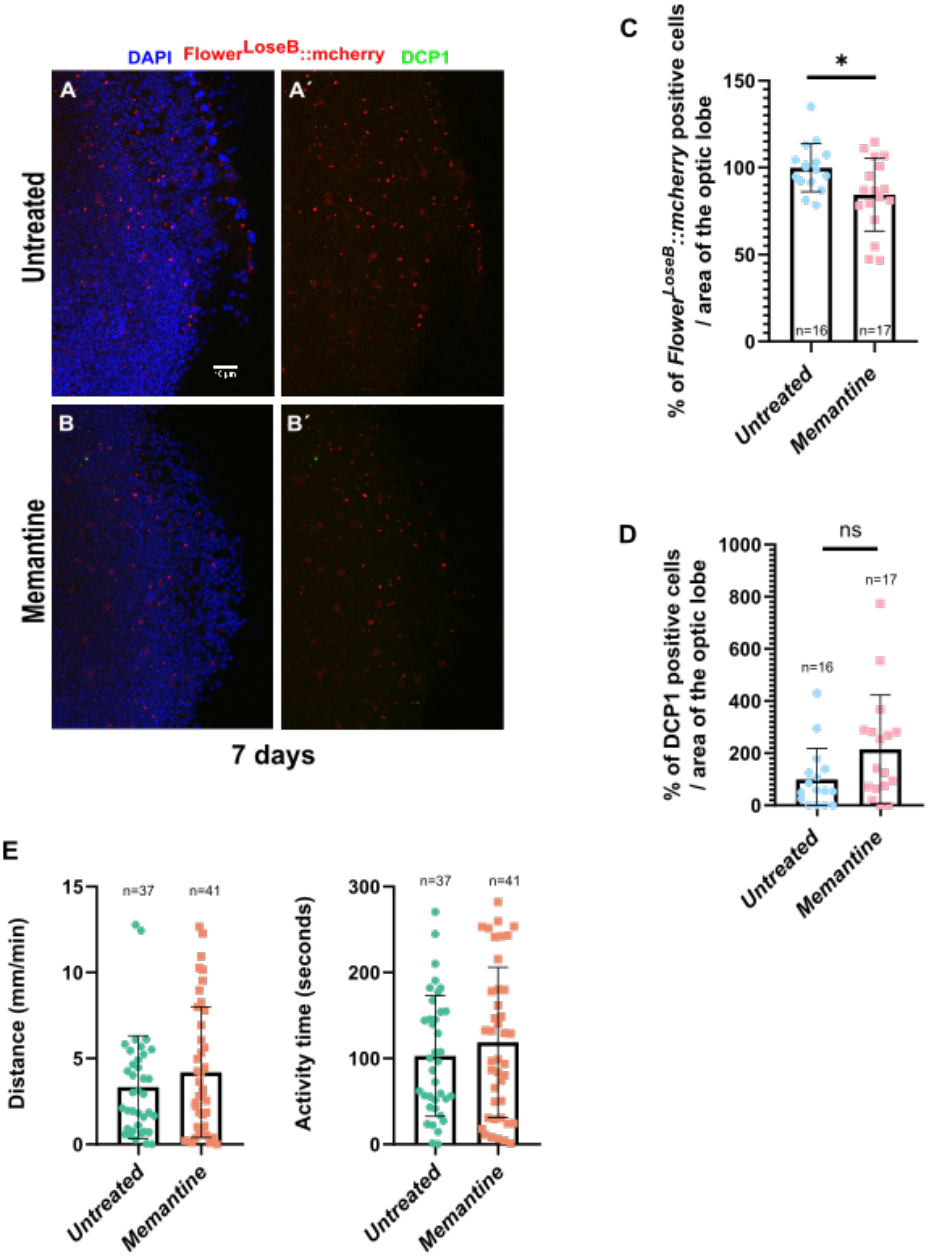


Figure 3.16: Effect of Memantine Treatment on the brain and in overall health of non-AD flies - Expression of **(A-B)** Flower^{LoseB}::mcherry in 7-day old *GMR-Gal4; +; Flower^{LoseB}::mcherry* male brains and quantification of the **(C)** % of Flower^{LoseB} -positive cells/ area of the optic lobe and % of DCP1-positive cells/ area of the optic lobe. . Values were normalized for the mean of the untreated controls. Statistical significance was accessed by unpaired t-tests. * indicates $p<0.05$. Scale bar = 10µm. **(E)** Graphics depicting the distance walked in millimeters per minute and activity time in seconds (using a speed threshold [ST]) of treated with Memantine and untreated 15-day old *nSyb>Aβ42* flies grown at 25ºC, on Buridan Assays.

4. Discussion

4.1. The role of excessive glutamate in a *Drosophila* model of AD

Glutamatergic dysfunction has long been known as a key player in AD pathology, however, its exact implications in disease progression are not yet fully understood. The current understanding of the role of glutamate in the pathology of AD follows a model that states that Aβ oligomers interfere with glutamatergic receptors, mainly NMDA, which causes their dysfunction and impairs processes of memory formation. This leads to their overstimulation in certain brain regions to compensate for memory loss, which leads to their overactivation, causing a glutamate spillover, that is exacerbated by problems in glutamate reuptake by astrocytes (Esposito et al., 2013). Over time, the excessive glutamate accumulation can contribute to neuronal death in AD by excitotoxicity.

In this project, we set out to better understand the role of this neurotransmitter in the brain of a *Drosophila* model of AD.

4.1.1. The relationship between glutamate and hyperactivity remains unclear

Excessive neurotransmitter release has been pointed to as a possible cause for neuronal hyperactivity due to impairments in excitation-inhibition balance in synapses (Lerdkrai et al., 2018). As the main excitatory neurotransmitter in the human CNS, glutamate has been the main candidate for this argument (Maragos et al., 1987) (Findley et al., 2019), primarily, amyloid-dependant reduced glutamate reuptake by astrocytes and enhanced presynaptic glutamate release have been proposed to be responsible for neuronal hyperactivity in the context of AD (Busche & Konnerth, 2015). This hypothesis for the role of glutamate on neuronal hyperactivity that the authors placed, has yet to be proven to be true in the brains of AD patients.

Since excessive glutamate release had been detected in the brains of Aβ42-expressing flies, in preliminary data from the lab, we proposed that it might be connected to the aberrant neuronal activity also displayed in their brains. The excessive glutamate may be causing hyperactivity by overstimulating synapses and increasing the number of action potential firings, but it might also be the case that the excess of released glutamate is a consequence of these excessive firings.

To uncover the mechanisms of this relationship, we decided to, in a first approach, downregulate glutamatergic signaling in the brain and check for changes in neuronal activity.

By using different lines of RNAi against DVGLUT in the mushroom body, we detected an increase in the number of CaLexA-positive neurons in only one of the 3 different RNAi lines used. If we take into account the results from the TRiP line, the only one that showed a different phenotype

than the control, they suggest that excessive glutamate is inhibiting Aβ-induced neuronal hyperactivity in the mushroom body, contradicting the hypothesis mentioned above.

The fact that the increase in neuronal hyperactivity was not consistent across the different RNAi lines, weakens the validity of the results we observed in the TRiP line. A possible explanation for this would be that the TRiP line is more effective in downregulating DVGLUT than the rest, so it would be important to test this in the future by comparing glutamate concentration in *GMR> Aβ42* flies expressing the 3 different RNAiDVGLUT lines or by comparing the levels of DVGLUT mRNA by qPCR. One especially important aspect to consider when analysing this experiment is that we could not find signs of excessive glutamate release in the mushroom body by immunostaining, unlike what was seen in the optic lobe. So, if there is no excessive glutamate in the mushroom body, it is possible that downregulating DVGLUT might be driving neuronal hyperactivity as a compensatory mechanism for the lack of basal glutamatergic signaling.

By looking at the same experiment in the optic lobe, a brain region where excessive glutamate was already documented, we saw no significant difference between the hyperactivity displayed in the 3 RNAiDVGLUT lines and the RNAiyellow control. Although manual counting was not possible, we think that in this case, differences in fluorescence values between optic lobes cannot be attributed to differences in hyperactivity levels because the number of saturated pixels was much lower than expected and does not seem to translate into differences in the numbers of neurons with higher GFP expression from what we could visually identify by looking at the pictures.

Based on these experiments, there is no evidence that excessive glutamate release is causing neuronal hyperactivity, but we also cannot exclude this scenario. An alternative and perhaps more promising strategy to test this hypothesis would be to perform the same experiments (using memantine and RNAiDVGLUT) in *nSyb>Aβ42* flies, since, as shown in the results, it is the model in which neuronal hyperactivity seems to reach higher values and in which quantification is simpler. It was not done in the scope of this work because it would require further stock construction and the timeframe did not allow it.

To test the alternative hypothesis that neuronal hyperactivity is responsible for the excessive glutamate accumulation, we silenced neurons with 3 different strategies and saw no difference in glutamate concentration in the optic lobes of flies in which *azot*-expressing neurons, neurons with high activity levels, or all the neurons in the adult brain were silenced.

A possible reason for not seeing effects in the glutamate levels is that the strategies to silence neurons were less efficient than ideal. In the case of the *W; GMR-Gal4, UAS-Aβ42/azot{KO; LexA}, Lopkir2.1.;+* genotype, the flies are heterozygous for the *azot*KO transgene, which means that they still carry a functional copy of *azot.*. The experiment would be cleaner if these flies were fully

azotKO-/-, because in this way neurons might still be expressing *azot*. In this genotype and also in the *W; GMR-Gal4, UAS-Aβ42/ LopKir2.1;CaLexa*, we did not confirm that Kir2.1 is effectively silencing the neurons under the control of the driver, so there is a possibility that it is not working properly. A possibility to test this would be to perform electrophysiology assays as they would allow us to record synaptic transmission (Bykhovskaia & Vasin, 2017) and, in this way, understand if there is still high neuronal activity in neurons where *kir2.1* expression is controlled by CaLexA.

Besides this, it is important to notice that there is an outstanding variability in the mean fluorescence of the antibody in all these genotypes since it varied from values close to zero to more than 3x the mean of control. Introducing an additional step of NGS blocking to the immunostaining protocol could contribute to reduce this variability, as it would decrease noise in the fluorescent signal by diminishing unspecific antibody binding. Few studies have yet quantified neurotransmitter release by immunostaining (Shin et al., 2018), so it would also be important to check if immunostaining can accurately portray the mean glutamate levels in a *Drosophila* brain over time, or if glutamate presence is too transient to do so.

Another factor that could be misleading for the interpretation of these results is that the controls used were not the most appropriate. In order to correctly compare the glutamate levels between *w; GMR, UAS-Aβ42/ azot{KO; LexA}, Lopkir2.1;+* flies and AD flies whose *azot*-expressing neurons are not silenced, we should have used flies with an identical genotype but missing the *Lopkir2.1* construct instead of the *w; GMR-Gal4, UAS-Aβ42/ UAS-LacZ; CaLexA,* stock which is only an appropriate control for the *GMR-Gal4, UAS-Aβ42/ Lopkir2.1; CaLexA* genotype. Ideally we should have also compared the flies in which every neuron in the adult optic lobe was silenced (*w; GMR-Gal4, UAS-Aβ42/ tubGal80^{ts}, UAS-Kir2.1; CaLexA)* with *w; GMR-Gal4, UAS-Aβ42/ tubGal80^{ts}; CaLexA* flies since comparing them with the *w; GMR-Gal4, UAS-Aβ42/ UAS-LacZ; CaLexA*, doesn't recapitulate the fact that with the *tubGal80^{ts}* construct, Aβ42 is only expressed in adult flies.

We can interpret these results by considering that silencing neurons does not affect glutamate concentrations in the optic lobe because neuronal hyperactivity is not being responsible for the excess of glutamate, which, despite the problems seen in the CaLexA and Azot approaches, is what the results of the *GMR-Gal4, UAS-Aβ42/ tubGal80^{ts}, UAS-kir2.1; CaLexA* flies suggest, because, regardless of not being compared with the most appropriate control, exhibit high levels of glutamate.

Based on both these experiments, we could not define a reliable model for the relationship between neuronal hyperactivity and excessive glutamate release in the brain of Aβ42-expressing flies. There is no evidence of hyperactivity being responsible for the excess in glutamate release, neither for the opposite being the case. In *Drosophila*, as well as in other insects, glutamate is an excitatory neurotransmitter in the neuro-muscular junction (NMJ), however, in the CNS, it can also act as

inhibitory in some neuronal circuits (Liu & Wilson, 2013). This further complicates interpretation of our results, as glutamate function might vary in different brain regions or neuronal networks. It is possible, for example, that by downregulating DVGLUT in the mushroom body, we are reducing inhibitory signalling, which could explain the increase in hyperactivity we observed in the TRiP line.

Zott et al recently proposed a model for a vicious cycle of neuronal hyperactivation triggered by Aβ42 that is dependent on disturbances on glutamatergic synapses. They showed that the suppression of glutamate reuptake is responsible for increases in neuronal activity in a mice model of AD (Zott et al., 2019), following a rationale stating that excessive glutamate plays a role in the increase in neuronal hyperactivity seen in the brains of AD patients. Our results do not appear to recapitulate this in a *Drosophila* model of AD, but further experiments are required to fully disregard this scenario.

4.1.2. Excessive glutamate might induce neuronal culling of unfit neurons

Glutamate excitotoxicity has for long been a known cause for neuronal death in the brains of AD patients. Recently, cell competition was described by Coelho et al. as a new mechanism that drives neuronal death in AD. These two mechanisms differ in a critical aspect, while excitotoxicity seems to greatly contribute to neurodegeneration and the progression of the disease, cell competition appears to be beneficial for brain health by removing damaged neurons before they further disturb neuronal circuits, in earlier stages of the disease. It is interesting to investigate if there is any connection between excessive glutamate and cell competition, as the consequences of excessive glutamate release might contribute to render some neurons less fit than their neighbours.

We tested two different approaches to downregulating glutamatergic signaling, RNAi against DVGLUT, and treatment with memantine, and looked for changes in the expression of the cell fitness markers Flower^{LoseB} and Azot and the neuronal death marker, DCP1.

By downregulating glutamatergic signaling with DVGLUT RNAi, we observed no difference in Flower^{LoseB} expression between the 3 RNAi lines in the optic lobes of 15-day old flies. Azot expression varied across RNAi lines, the kk line showed an increase in *azot* expression while the TRiP line displayed less Azot and, accordingly, a decrease in DCP1 expression.

This raised similar concerns regarding the RNAi lines that we had in previous experiments. The fact that the three different DVGLUT RNAi lines display opposite phenotypes regarding *azot* expression reduces the confidence in extrapolating conclusions from these results.

However, based on results from the TRiP line, there appears to be a tendency for reduced cell death and *azot* upregulation as a consequence of downregulating DVGLUT, which could indicate that excessive glutamate is contributing to the upregulation of unfit markers and for the death of less fit

neurons by cell competition in the brain of AD flies. This scenario is plausible because it is known that excessive glutamate can be very damaging to neurons and that it is plays a key role in neuronal death in AD. If this is the case, then it could indicate that cell competition might be playing a role in the death of neurons that are damaged by glutamate excitotoxicity. Perhaps it can even be a protective event that occurs as a result of earlier insults caused by glutamate dysfunction, prior to death by excitotoxicity, as an attempt to remove these neurons and maintain the homeostasis of neuronal circuits for longer.

4.1.3. Novel insights into the success of memantine as a treatment for AD

Memantine is commonly used in conjunction with acetylcholinesterase inhibitors as a treatment to improve symptoms of patients with moderate-to-severe AD. It is a low-to-moderate affinity, uncompetitive antagonist to the NMDA glutamatergic channels, which blocks glutamate binding and therefore the depolarization of the postsynaptic neuron. Its success lies in the fact that it requires sustained receptor activation, as in excitotoxic conditions when there is an excess of glutamate in the synaptic cleft, to bind to the receptor, thus not interfering with glutamatergic signaling in normal brain activity (Esposito et al., 2013). As a treatment, memantine appears to be successful in allowing patients to remain independent for longer by improving several aspects of their behaviour (such as reducing agitation or aggression) and the ability to perform everyday tasks (Wilkinson, 2012).

Due to its role as an antagonist to glutamatergic synapses, we treated *GMR>Aβ42* flies with memantine as a different approach to testing the relationship between excessive glutamate release and cell competition in the context of AD. In this case, we decided to look for *flower*^{LoseB}, *azot,* and *dcp1* expression at 3 different time points: after 7, 10, and 15-days of treatment, in an attempt to reach a more complete outlook of the progression of brain pathology after treatment.

At 7-days old, there is a big increase in *flower*^{LoseB} expression in flies treated with memantine, while there is a small decrease in *azot* expression. At 10 days old there is an increase in both *flower*^{LoseB} and *azot* expression, while at 15 days post-treatment there is no longer an increase in *flower*^{LoseB} expression but *azot* expression remains higher in memantine treated flies.

In a first look, this might seem contradictory to what would be expected if we consider that memantine plays a beneficial role in delaying disease progression and improving symptoms in patients since we would expect that there would be a decrease in the number of less fit neurons that upregulate these markers. However, we can also look at these results in a different way and consider that, since cell competition is advantageous for brain health in the early stages of AD, the upregulation of Flower^{LoseB} and Azot might be connected to the success of memantine as a treatment for AD, by somehow stimulating the elimination of unfit cells at a faster rate.

So, there are these two paradoxical ways to interpret these results: one explanation for the higher levels of Flower and Azot could be intrinsic toxicity of the dosage of Memantine that was mixed in the food, which could be damaging to neurons. However, results from Buridan Assays of *nSyb>Aβ42* flies treated and untreated with memantine did not reveal any differences, suggesting that, at least on the surface, we cannot observe any physical changes that suggest that treated flies are less healthy than untreated ones. And, on the contrary, the expression of Flower^{LoseB} in 7-day old healthy, non-Aβ expressing, treated flies is lower than that seen in untreated ones. So, this concentration of Memantine in the food does not seem to be negative for fly health and although there is an increase in cell death in the brains of healthy treated flies, further experiments are necessary to confirm that it contributes to toxicity in the brain. Ideally, it would be interesting to repeat these experiments with different concentrations of Memantine in the food, so that we could achieve an optimal concentration.

DCP1 expression remained equal in treated and untreated flies in the three timepoints, which suggests that memantine treatment does not affect neuronal death at these timepoints. Knowing if unfit neurons are actually dying and being eliminated faster than they would in a normal AD setting, is crucial to understand if overexpression of Flower^{LoseB} and Azot in the brains of flies treated with memantine is beneficial. DCP1 is one of the effector caspases in the *Drosophila* cell death pathway (Song et al., 1997), so its presence should be an accurate indicator of apoptosis, however, not seeing differences in DCP1 doesn't mean necessarily that there is no cell death occurring. It is possible that complementing DCP1 staining with different approaches such as TUNEL assays could give us a better understanding of the progression of neuronal death in this context. To better comprehend how neuronal fitness changes over time in the brains of flies treated with memantine, and if the neurons that upregulate Azot are being eliminated from the brain, it would be interesting to monitor *azot* expression with the *azot{KO; GFP}* reporter line at several time points, that allows the visualization of all neurons that activate the *azot* promotor by GFP expression, since they accumulate in the tissue and are not eliminated.

By analysing results from the two different strategies used for downregulating glutamatergic signalling, they appear to show opposing effects, since one of the RNAi lines against DVGLUT appeared to lead to a reduction of the expression of Azot, and treatment with memantine resulted in the contrary. At a first glance, this could indicate that one of these approaches might not be working correctly, however, it is important to note that they might not be comparable. RNAi against DVGLUT is expressed in larvae, so it should be reducing glutamatergic signalling during larval development, yet flies were only fed with memantine-enriched food post-eclosion. Besides this, the efficacy of each of these approaches to downregulating glutamatergic signalling is expected to be different, since they work in very different ways. Decreasing levels of DVGLUT reduces the release

of glutamate from presynaptic neurons, while memantine acts by blocking the binding of glutamate that is already at the synaptic cleft, to glutamate receptors in the post-synaptic neuron.

So, we could speculate that while the downregulation of DVGLUT expression might be effective in preventing glutamate-induced damage in neurons, memantine might be acting in a different scenario in which some neurons in the brain are already further damaged and promoting cell competition would be protective to neuronal circuits and improve brain health in this way.

4.2. Different strategies to reduce neuronal hyperactivity failed to improve Aβ-induced poor locomotor performance

Recent studies showed that ectopic expression of Aβ42 decreases locomotor performance in *Drosophila* models of AD, recapitulating what occurs as a consequence of aging in these animals, and even in *C.elegans* models of AD (Machino et al., 2014).

To uncover if neuronal hyperactivity played a role in the motor difficulties displayed in these flies, we tested 2 different approaches for silencing hyperactive neurons and checked for improvements in Buridan Assays.

In a first approach, we attempted silencing *azot*-expressing neurons, since previous works have shown that CaLexA-positive neurons co-localize with Azot::mcherry expression (Coelho & Moreno, 2020). We generated an *azot{KO; LexA}, LopKir2.1* recombinant stock so that *kir2.1* was expressed and silencing only *azot*-expressing neurons and observed that it could not restore the impairments in activity time and distance traveled seen in *nSyb>Aβ42* flies, despite a slight, non-significant improvement in comparison to *w; UAS-Aβ42, azotKO/ azot{KO; LexA}; nSybGal4* flies, that might suggest that kir2.1 has a beneficial effect in an *azotKO-/-* background.

To further investigate if silencing azot-expressing neurons could improve overall health in these flies we performed lifespan assays with females with the same genotypes. Results corroborated what we saw in locomotor assays since *w; UAS-Aβ42, azotKO/ azot{KO; LexA}, LopKir2.1; nSybGal4* females lived shorter lives than *nSyb>Aβ42,* further suggesting that silencing *azot*-expressing neurons doesn't improve overall health in these flies.

The first and perhaps most direct conclusion to infer from results regarding this experiment is that silencing *azot*-expressing neurons is not an effective way to revert the locomotor defects caused by the consequences of Aβ42 expression in the *Drosophila* brain. It also does not seem to be a good strategy to improve the overall health of AD flies as results from the lifespan assay suggest.

Our first instinct, when interpreting these results was to attribute the poor success of this strategy to the possible accumulation of unfit neurons in the brain. In a scenario in which *azot* would be normally expressed, these neurons would eventually be eliminated and, although that would

undoubtedly have negative consequences for the brain, it is possible that the permanence of these damaged neurons can be even more detrimental due to the continuous impairment of neuronal circuits. However, when we repeated the same experiment in 7-day old flies, where locomotor differences between healthy and AD flies were not noticeable yet, *nSyb>Aβ42; azot{KO; LexA}, Lopkir2.1* still displayed a much worse phenotype than any of the genotypes in which Azot is present, which goes against our hypothesis.

Previous studies have shown that otherwise healthy, *azotKO-/-* flies, display developmental malformations such as damaged wings (Merino et al., 2015). Besides, in our own experiment, it is possible to see that *w; UAS-Aβ42, azotKO / azot{KO; LexA}; nSybGal4*, perform significantly worse than just *nSyb>Aβ42* flies. Cell competition has been described as a mechanism that contributes to the maintenance of homeostasis in different contexts so, one can assume that by disturbing the cell competition machinery with the removal of one of its key players, Azot, regular organism functions might be disturbed. In addition, without Azot, there is accumulation of unfit cells that would otherwise be eliminated. So, perhaps the biggest fault in this approach is not silencing neurons itself, but the absence of Azot, and it might not be appropriate to compare *azotKO-/-* flies to flies that are *wild type* for this locus. To understand if this is the case, it would be important to repeat this experiment with an added control of healthy flies in which *azot*-expressing neurons are also silenced by Kir2.1.

Another reason why this approach might have failed is that, although a high percentage of *azot*-expressing neurons seem to display neuronal hyperactivity, there is no evidence that all of them do. So, by silencing every *azot*-expressing neuron in the fly brain, we might be silencing a large number of neurons that were expressing *azot* due to other insults but are not hyperactive, and whose activity could be important for normal brain function. We believe that the failure of this approach cannot be attributed to the "technical problems" in the constructs, since the recombinant stock was thoroughly tested for the presence of both genes.

In the future, we hope to test if silencing *azot*-expressing neurons can improve the locomotor performance of AD model flies with a different genetic approach: using a *UAS>stop>kir2.1; azot{KO; LexA}/ UAS-Aβ42; LoP-Flipase/ nSybGal4* stock. In these flies, the expression of *kir2.1* in *azot*-expressing neurons is controlled by the action of a Flipase that removes a stop codon that is placed between the UAS sequence and the *kir2.1* gene.

Although it has been shown that there is co-localization between CaLexA-positive neurons and the upregulation of low fitness markers (Coelho & Moreno, 2020), it is not clear that all hyperactive neurons are unfit. So, by silencing only azot-expressing neurons there is a possibility that we might not be silencing a considerable number of hyperactive neurons. A different approach to try to improve the locomotor performance of these flies was to silence or induce apoptosis of hyperactive

neurons by taking advantage of the CaLexA system to effectively target neurons that have higher activity levels. We compared the locomotor performance of flies in which neurons with higher activity levels were silenced by Kir2.1, killed by the pro-apoptotic gene *reaper,* or protected from apoptosis by p35. The only difference between these 3 genotypes and flies carrying only the CaLexA construct was that Kir2.1 expressing flies performed worse in Buridan assays, by displaying shorter activity times and distance walked means. Consequently, this different approach to silencing hyperactive neurons also did not prove to be an effective strategy to improve the locomotor performance of Aβ42-expressing flies.

One aspect we think is worth noting is the fact that, contrary to what would be expected, there was no difference between the locomotor performance of flies with "opposite" phenotypes: those where hyperactive neurons were killed by Reaper and those in which apoptosis was blocked by p35. It might even be more surprising that there were no differences between any of the two genotypes and control flies, as it is highly unlikely that promoting the death of a large number of neurons in the fly brain, blocking it, or not interfering with the process in any way, doesn't translate in any visible differences in adult fly behaviour. This might raise concerns regarding the effectiveness of using CaLexA as a "driver" for the expression of genes in hyperactive neurons. The fact that flies carrying the CaLexA system also carry a copy of LOP-GFP in the third chromosome implies that only half of the LexA proteins that are produced in the neuron are binding to the LOP sequence of the gene of interest (in this case, *kir2.1*, *reaper* or *p35*) since the other half is promoting the expression of GFP. It is possible that there is not enough overexpression of *reaper*, *p35,* and *kir2.1* to produce visible effects in the neurons.

Other experiments performed in the lab, in which the expression of these same constructs (Lop-*reaper*, Lop-*p35,* and Lop-*kir2.1*) was controlled by $Gal80^{ts}$, showed that there were already differences between the behaviour of different genotypes before $Gal80^{ts}$ de-repression, suggesting that expression of these LOP-attached transgenes could be leaky. It is also important to keep in mind that overexpressing a K^+ channel, or a pro-apoptotic gene could disturb homeostatic mechanisms in a large number of neurons which could consequently impair regular brain function. So, there is also the possibility that this might overshadow a beneficial effect in silencing or killing hyperactive neurons. As an alternative to using *reaper* overexpression to promote neuronal death, we could have taken advantage of different pro-apoptotic genes, mainly *hid*, which could be more effective because it has been shown to act downstream of Azot to promote apoptosis in different scenarios of cell competition.

Overall, none of these different approaches was successful in improving the locomotor performance of AD flies, either by silencing hyperactive neurons in two distinct ways or by killing them, removing them from the brain entirely. However, the fact that silencing *azot*-expressing neurons

produced a small, although non-significant, improvement in the locomotor performance in AD flies with an *azotKO-/-* background, could be indicative of a promising effect of this strategy.

The relationship between hyperactivity and the symptoms of AD patients is not entirely understood, despite the success of levetiracetam in clinical studies, by reducing epileptic attacks in patients and improving the cognitive performance of animal models (Sanchez et al., 2012) by targeting network activity.

In the future, it would be interesting to perform a more precise version of this experiments: a screen that allowed us to silence excessive activity in different types or clusters of neurons would provide a more precise control of silencing, which is important since, although hyperactivity seems to be present in most regions of the brain, we do not know if it has ubiquitous consequences, or if an increase in activity in a specific region is most responsible for the locomotor consequences. Not many studies have attempted to address the locomotor impairments induced by AD in flies by manipulating neuronal hyperactivity. Ping and colleagues have been successful in improving the locomotor performance of *elav>Aβ42* flies in climbing assays by restoring the expression of an A-type potassium channel (Ping et al., 2015). They take a more upstream approach to the problem and target the hyperexcitability that seems to be causing hyperactivity by overexpressing this channel.

4.2.1. Limitations of Behavioral Assays in *Drosophila*

As it is true for most behavioural experiments, Buridan assays in *Drosophila* show remarkably high variability. It has been shown that individual flies with the same genotype display behavioural biases that can only be attributed to personality and not differences from the environment (Buchanan et al., 2015). Besides this, performing experiments using stocks with different genotypes vastly increases variability (Evangelou et al., 2019). A solution to this problem would be to isogenize the different genetic backgrounds of transgenic stocks, however, that is an exceptionally long and arduous process and is therefore impossible to achieve in the timespan of this thesis. It has also been shown that the circadian clock can influence levels of locomotor activity of flies throughout the day (Cascallares et al., 2018). We tried to overcome this variability by, whenever possible, performing the assays in the morning, the time of day in which flies are expected to be more active, however, since many individuals were tested, experiments often ended in the evening.

A specific disadvantage of Buridan Assays is the fact that they also depend on the vision of the flies, because they are expected to move back and forth in the arena between the two opposite stripes. Flower-dependant cell competition has been shown to be involved in the culling of unwanted neurons in the developing fly retina (Merino et al., 2013), so there is a possibility that problems in the visual system could be partially to blame for the poor locomotor performance displayed by the *azotKO-/-* flies that were tested in this project.

It would be interesting to complement the results of these Buridan Assays with different behaviour assays, such as climbing assays, that, although less reliable, carry the advantage of not requiring cutting the wings off, and therefore do not rely on the performance of injured flies. Accessing the effects of silencing hyperactive neurons on other symptoms of AD patients such as memory formation, either by odor assays or courtship-suppression, could also be promising. Despite these limitations, one particularly relevant advantage of *Drosophila* in comparison to most other animal models in behavioural assays is that one can quickly obtain a large number of individuals to perform the assays. A big N greatly reduces the effects of individual variability and increases the confidence of the results.

4.3. Conclusion

Results obtained from this work did not provide conclusive new insights into the role of glutamate in the brain of Aβ42-expressing flies and we could not establish a reliable model for the interaction between glutamate and neuronal hyperactivity. However, two strategies for silencing hyperactive neurons proved to be unsuccessful, which suggests that it might not be a good approach to improve the health of flies.

Treatment of flies with memantine, a glutamate antagonist resulted in upregulation of Flower^{LoseB} and Azot, showing for the first time that a neurotransmitter might be interfering with the regulation of cell competition in adult brains, regardless of its complicated interpretation in the context of AD. It could also be a starting point to the knowledge of new molecular mechanisms that contribute to the success of memantine as a treatment for AD patients.

It is important to consider that, although knowledge regarding cell competition has been rapidly evolving in recent years, many aspects of its mechanisms such as the biochemical pathways that underly the upregulation of unfit markers, and how it differs across organisms and even cell types, remain unknown. So, experiments that, like those in this work, manipulate the expression of key players in cell competition such as Azot, might be influenced by a lot of unknown factors, as we do not know, for example, if cell competition in AD occurs uniformly throughout all neuron types or if it also influences the function of glia .

This work further heightens the value of using *Drosophila* as a model to study very complex disorders like neurodegenerative diseases. It allowed us to quickly generate all the necessary genetic tools to test the different hypotheses to address our questions, while simultaneously exhibiting the complex phenotypes that characterize AD pathology.

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