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Gene Expression Analysis in Models of Cognitive Diseases

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Submitted by

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Declaration of independence

I hereby declare that the present doctoral thesis "*Gene Expression Analysis in Models of Cognitive Disease*" was writing on my own and independently without any other sources than indicated

Jonathan A. Cortés Silva
Göttingen, March 2023

"*Here's to the crazy ones, the misfits, the rebels, the troublemakers, the round pegs in the square holes… the ones who see things differently — they're not fond of rules… You can quote them, disagree with them, glorify or vilify them, but the only thing you can't do is ignore them because they change things… they push the human race forward, and while some may see them as the crazy ones, we see genius, because the ones who are crazy enough to think that they can change the world, are the ones who do"*

— Steve Jobs, 1997

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1.General introduction

1.1 Neuropsychiatric disorders and neurodegenerative diseases

1.1.1 Major Depression

Major depression disorder, commonly referred to as Major Depression (MD), is a prevalent neuropsychiatric disorder that affects millions of people worldwide. In fact, according to recent research by Norkeviciene et al. (2022), more than 300 million people are currently suffering from MD, making it a serious public health concern in our society. The impact of this disorder is further exacerbated by the lack of effective preventive measures and treatments, as MD is the leading cause of suicide (Beurel, Toups & Nemeroff, 2020). To tackle this issue, it is essential to gain a deep understanding of the mechanisms that drive the development of MD, particularly those that arise in childhood. As pointed out by Saleh et al. (2017), the events experienced in early life play a crucial role in the genesis of MD. Therefore, exploring these mechanisms could aid in the development of more targeted interventions for individuals with MD, ultimately helping to reduce the burden of this disorder on society.

MD can be defined in humans as a constant feeling of tiredness, low interest in recreational activities, predominance of dark mood, and a considerable reduction in the capacity of joy (American Psychiatric Association, DSM-V), the latter could also be defined as anhedonia, from the greek ἀν-: an- 'without' y ἡδονή: hedoné 'pleasure' (Martinotti et al. 2012). During the development of societies and the increase in the “speed of life”, MD became a major health problem in our societies (Hidaka, 2012). The constant competition, and struggles to obtain basic resources at the physical and psychological level, makes a perfect context for the genesis of depressive symptoms (Price et al. 1994). At the same time, the lack of resources and support networks for mental health in most of the countries around the world (Muñoz, Beardslee & Leykin, 2012), make the diagnosis and treatment of MD a very complex challenge. These challenges increase due the high heterogeneity of symptoms (Goldberg, 2011). Although the symptoms and treatment for Major depression (MD) have clear and

well-defined guidelines, the underlying molecular and genetic mechanisms are still not fully understood (Kamran et al. 2022).

1.1.2 Neurobiology of major depression

MD has been linked to the dysregulation and abnormal function of the dopaminergic system in the central nervous system (CNS) (Li et al. 2022). At the same time, brain areas involved in mood regulation like the amygdala, hypothalamus or hippocampus, known as the limbic system (Redlich et al. 2017), are involved in the neurobiology of MD. This is why considering an integrative approach (Foland-Ross & Gotlib, 2012) for the study of the disorder is very important. One of the most obvious reactions of our psychophysiological system to environmental stressors, or stimuli with a high emotional charge, is the activation of brain areas involved in affective processing and endocrine response (Berretz et al. 2021). Such brain areas have been identified by neuroimaging studies. Involvement of the Sublingual cingulate region, prefrontal cortex, amygdala, and Insula, are mentioned as key areas in the processing of MD (Castanheira et al. 2019). Probably one of the most accurate theories at the moment, considering the state of the art in the field, are those that consider MD as a disorder with clear traces of cellular inflammation (Gałecki & Talarowska, 2018) and Anterior Cingulate Cortex (ACC) activation (Pizzagalli et al. 2001.Yucel et al. 2008)

Due to the heterogeneity and interaction between genetic and environmental variables, it has been very difficult, if not impossible, to link a unique gene or genetic pathway to the genesis and development of MD. Nevertheless, associations with polymorphisms in the several genes such as the genes coding for nuclear receptor subfamily 3 group C member 1 gene (NR3C1), the glutamate metabotropic receptor 3 (GRM3) or glutamate receptor, ionotropic, kainate 4 (GRIK4) (Kupfer, Frank & Phillips, 2012), have been linked to MD. In addition genetic and non-genetic evidence has linked neurotrophic factors such as Brain-derived neurotrophic factor (BDNF), pro inflammatory cytokines or processes such as impaired regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis, are still taking the interest of the scientific community from different approaches (Kupfer, Frank & Phillips, 2012; Harsanyi et al., 2022).

1.1.3 Mouse models to study major depression

In addition to the complexity caused by the lack of understanding of the mechanisms that drive MD, one of the most difficult challenges in the field is its experimental study.

For obvious ethical reasons, we cannot deliberately depress a person, even if we had voluntary participants, which is why having access to the processes that cause depression from early stages in its development continues to be a constant challenge in the laboratory and the clinic (Sato & Lieh Yeh, 2013). To overcome this problem, during the last decades there has been great interest in developing an animal model (Czéh et al. 2016. Planchez et al. 2019), that allows us to study MD at the behavioral and molecular level, with the possibility to conduct mechanistic studies .

One of the most popular models to produce the depression phenotype in animals, are the paradigms applied to mice (Harro, 2019. Iñiguez et al. 2018). Early life stress or chronic exposure to stressors such as social defeat (SD), have shown to be very effective models to induce depressive symptoms in mice.

1.1.4 Early life stress

From a psychological perspective, the experiences to which we are exposed during our early childhood are extremely important in shaping our personality (Jäger, Han & Dingemanse, 2019) and the way we perceive and act against the stimuli we receive from our environment. A healthy attachment and free access to emotional support figures (Hong & Park, 2012), can make a clear difference between people with the same genetic background. Proof of this are the innumerable studies on homozygous twins, who, even having an identical DNA sequence, can develop totally different personalities (Tellegen et al. 1988. Torgersen & Janson, 2022), unquestionably marked by the environment in which they were raised.In 2020, Palma-Gudiel et al. conducted a comprehensive analysis indicating that the accrual of methylation variability over time may underlie the mechanisms contributing to elevated susceptibility to major depression among discordant twins (Palma-Gudiel et al., 2020).

It is because of the above, that being able to have access to subjects in their first years, even weeks of life, is a fundamental factor for the study of MD (Juruena et al 2021). This is the goal of the early life stress (ELS) paradigm (Smith & Pollak, 2020).

Producing an early and continuous separation from the fundamental attachment figure in the first weeks of life, the mother (Murthy & Gould, 2018), is one of the central points of the experimental procedures based on ELS. The standard protocol consists of separating the mouse pups from the mother, a week after they were born, for this a normal maintenance box is used, adapted with nesting material that allows the temperature of the pups to be maintained during the separation (Figure 1). Usually the separation is carried out for a period of 3 to 4 hours each day, for a total of 10 days.

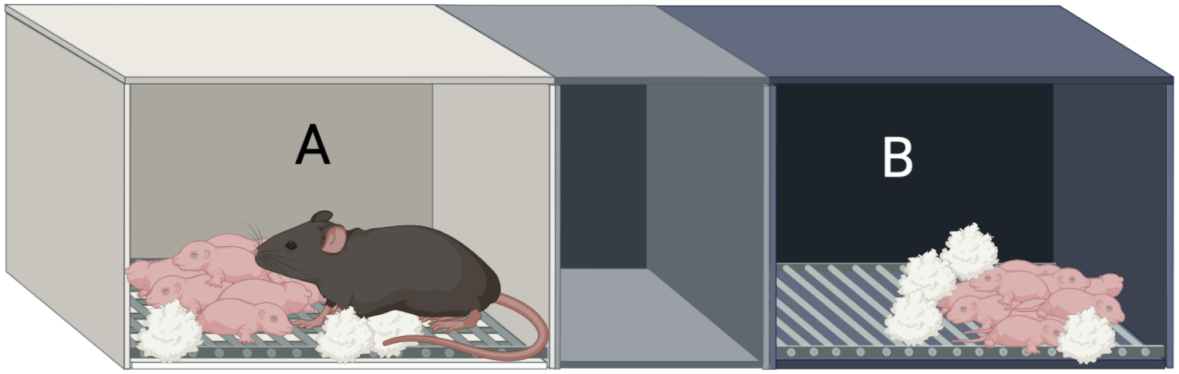

Figure 1: Scheme of an standar early life stress experimental design: A: pups are together with the mother during a prolonged period of time. **B**: In this set-up the pubs are separated from the mother during the period of time that the study required. Figures created with BioRender.com.

After the experimental period is over, and depending on the specific study objectives, the time in which the subjects will be exposed to different tests to assess cognitive function will vary.

1.1.5 Social defeat

Although the ELS paradigm allows researchers to access some of the aspects of depressive behavior, originally caused by maternal separation, the ELS procedure does not allow access to the adverse characteristics of a dysfunctional environment (Saveanu & Nemeroff, 2012), which have been shown to have a great impact on the origin and long- term maintenance of depression in humans. To compensate for the shortcomings of the ELS, there is the possibility of exposing mice of different ages to a highly stressful context in a controlled and effective manner. With the last we refer to the paradigm of social defeat (SD) (Bartolomucci et al. 2009. Golden et al. 2011.

Pagliusi & Sartori, 2019. Carnevali et al.2020. Van Doeselaar et al. 2021. Reguilón et al. 2022).

The main objective of the SD experiment is to reproduce sufficient and constant stress levels in the mice, so that they clearly show some of the symptoms associated with depression in humans. Decreased locomotor activity or exploratory behavior, lack of appetite and irritability, are some of the symptoms in humans that can be identified in mice after the exposure to SD (Krishnan & Nestler, 2011). Constant exposure to stress, and susceptibility to it, has been described as one of the most important factors in the origin of mood disorders (Iñiguez et al. 2014). It is for this reason that the exposure to stressors during early stages of life turns out to be a critical variable for the acquisition of depression symptoms in mice.

Exposure to a stressful environment, in which the stimuli received are of sufficient intensity to destabilize the affective state of the recipient, which can be accessed in the SD paradigm, through the physical attack of a dominant mouse on a subordinate (Figure 2 A), not only has it been shown to affect the affective-physical state perceptible through already defined signs in behavior (immobility, etc.), but there is also sufficient evidence that shows the impact of a stressful environment on cognition.

Figure 2: Scheme of an standard social defeat experimental design: A: a male dominant, usually a CD1, attacks a subordinate mice that is introduced in the home cage of a dominant **B:** After the exposure to the defeat (physical attack), the mice are maintain in the same cage, but now with a plastic separator that don't allow any physical contact between the mice during the next 24 hrs until next session. Figures created with BioRender.com.

1.1.6 Major depression and neurodegeneration

People who suffer from MD often show decreased attentional capacities and significant memory problems (Lohman, 2013). These signs have led researchers to consider that some neuropsychiatric disorders, in this case MD, can affect our cognitive abilities (Lam et al. 2014), and in some cases, permanently and irreversibly impact the CNS.

The possible relationship between MD and cognitive dysfunction has considerably increased research in this area during the last decade. In turn, this has allowed access to behavioral and molecular data which have been generated for this purpose (Upadhya et al. 2022), which allows more accurate interpretations during analysis. In this context, a reactive response of microglia, known as ""pro-inflammatory microglia", product of exposure to stimuli interpreted adversely for the organism has been identified (Rodríguez-Gómez et al. 2020). Activation of microglia stimulates the initiation of the inflammatory response, initially adaptive for the neuronal system, but nevertheless, in a prolonged and severe manner, it directly contributes to neuronal communication dysfunction. Part of this inflammatory response has also been observed in patients with Alzheimer disease (AD). In fact MD is an often observed comorbidity with AD and has also been described as an important risk factor (Sáiz-Vázquez et al. 2021). In the specific case of MD, there is evidence that could suggest the role of Interferon type I (IFN-I) in the excessive response of microglia (Roy & Cao, 2022), however, there is still a lack of experimental evidence to confirm this assumption.

1.2 Alzheimer's disease

In the context of neurodegenerative diseases, AD is the most common type of dementia in the elderly (Dening & Sandilyan, 2015. Duong, Patel & Chang, 2017. Arvanitakis, Shah & Bennett, 2019). Characterized by appearing mostly in old age, AD has as its first symptomatic signs, the loss of short-term memory, or anterograde memory, to later give way to a general deterioration of long-term memory, or retrograde memory, in advanced stages of the illness (Lyketsos et al. 2011. Atri, 2019).

At the molecular level, AD presents two biological hallmarks (Serrano-Pozo et al. 2011. DeTure & W.Dickson, 2019. Zhang et al. 2021) in the brain that have been intensively studied during the last century, the extracellular accumulation of amyloid plaques (Wildburger et al. 2018. Zaletel et al. 2021. Drummond et al. 2022), and the intracellular presence of tau protein (Lu & Wood, 1993. Gong, Grundke-Iqbal & Iqbal, 2010. Kolarova et al. 2012. Bhaskar et al. 2018). Both pathologies increase progressively during the progression of the disease; however, the description of their mechanisms of action in AD is still not clear.

1.2.1 Amyloid-beta pathology

The severe accumulation of amyloid plaques in the brain of AD patients has been shown to have a highly negative effect on memory, from short-term conditions to effects that impact long-range planning abilities. However, studies focused on the role of amyloid beta in the CNS have shown that its presence in small doses has a positive and even protective effect on memory processes (Morley & Farr, 2014). One of the theories about the accumulation of amyloid plaques in the context of AD is its role in inflammation processes (Meraz-Ríos, Toral-Ríos & Campos-Peña, 2013. Kinney, Bemiller & Lamb, 2018) and its effect on the immune system in the central nervous system. The exponential accumulation of amyloid in the hippocampus or cortex has been shown to generate an increase in the upregulation of genes involved in defense mechanisms. In turn, studies in the single cell field (Sala Frigerio et al. 2019), have been able to identify microglia as one of the protagonists in these mechanisms (Ismail et al. 2020).

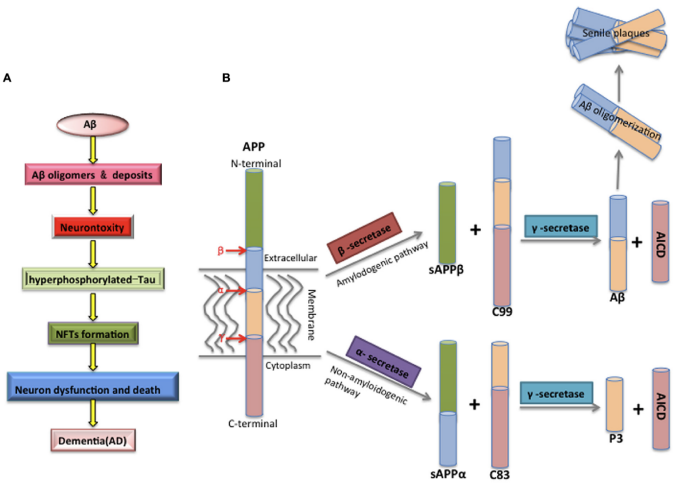

Figure 3. Scheme of the Amyloid beta toxicity from Sun, Chen & Wang (2015). A: The mechanism of Aβ toxicity. Accumulating Aβ will initially result in Aβ oligomerization, gradually depositing as the forms of fibrils and senile plaques. Furthermore, Aβ aggregation alters the kinase phosphatase activity that leads to the Tau protein hyperphosphorylated, which causes the formation of neurofibrillary tangles (NFTs), and eventual synaptic and neuronal dysfunction and AD. **B:** The proteolytic processing of the amyloid precursor protein (APP) and Aβ biogenesis (Sun, Chen & Wang. 2015).

1.2.3 Tau pathology

Tau protein belongs to the microtubule-associated proteins (MAPs), a group described as essential for the healthy formation of microtubule assembly (Pîrscovenau et al. 2017). These processes are one of the more affected during AD, the inability to produce effective microtubule formation (Cash et al. 2003. Drechsler et al. 2019. Kim et al. 2022) has a direct effect on the well-being of neurons and their ability to communicate with each other, in turn, the effect triggered by this signaling dysfunction product of tau hyperphosphorylation (Figure 4), begins again toxicity and its aggregation to form neurofibrillary tangles (NFTs), one of the primary biomarker of AD.

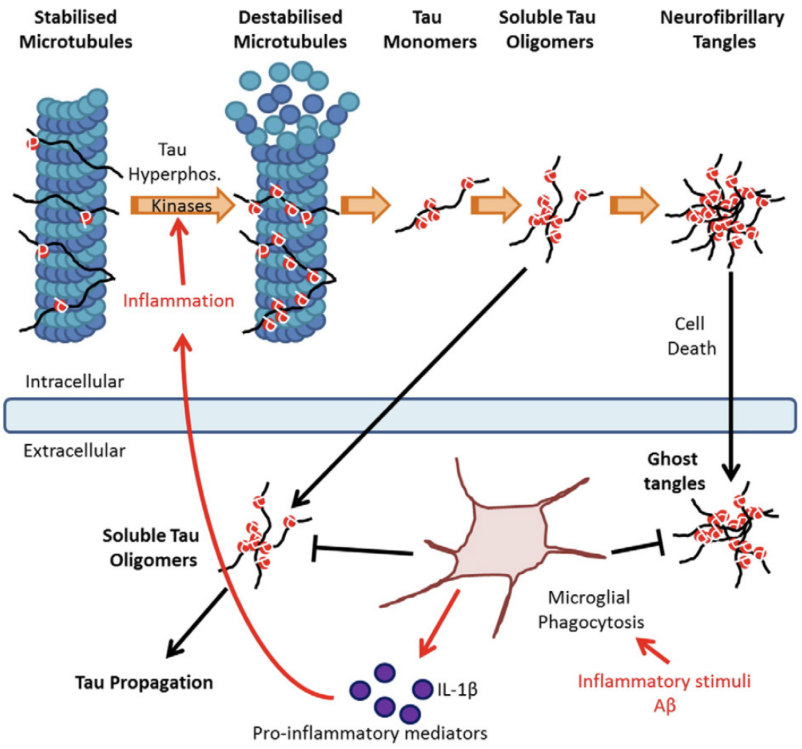

Figure 4. Scheme tau pathology progression from Barron et al. 2016. A: Inflammatory response, produced by microglia activation, stimulates up-regulation of kinases involved in Tau phosphorylation and microtubules destabilization, which in turn gives way to Tau monomers, soluble Tau Oligomers and Neurofibrillary tangles (Barron et al. 2017).

1.2.4 Risk factors for Alzheimer’s disease

One of the biggest challenges in AD, is the premature diagnosis of the disease (Chetelat & Baron, 2003. Bilgel et al., 2018. Pais et al., 2020. Ferrari & Sorbi, 2021), unfortunately, its diagnosis is usually in advanced stages of the disease, when on the one hand, the cognitive deterioration is quite severe, and on the other, the damage to the CNS is irreversible. Although a premature diagnosis will not prevent the progression of the disease and the cognitive deterioration will continue to be irreversible (Porsteinsson et al. 2021), it is possible to reduce the impact on the quality of life of the patients in a way that they can notice and experience in their daily routines.

An illustration of the latter category is the utilization of drugs such as Aducanumab, a monoclonal antibody directed towards amyloid beta (Dhillon, 2021), which has received approval for use in Alzheimer's patients. Aducanumab has been demonstrated to improve neuronal function that was impaired by the buildup of amyloid plaques in the central nervous system (Sevigny et al. 2016). On the other hand, there is Lecanemab, a humanized monoclonal antibody with a high affinity for soluble amyloid beta protofibrils (Van Dyck et al. 2023), which has been found to be one of the most toxic forms impacting neurons due to amyloid plaque accumulation. Both drugs have had positive effects on the clinical decline of Alzheimer's patients when used for a long-term period (Cummings et al. 2021.Dhadda et al. 2022), however, the question of whether this impact results in a noticeable improvement for patients remains a subject of debate.

As a result of the null existence of highly effective drugs for the treatment of AD, and as already mentioned, the difficulty in its diagnosis, in recent years the preventive approach to the disease has gained strength. Healthy lifestyles (Dhana et al. 2020. Mamalaki et al. 2022. Sabia & Singh-Manoux, 2023), such as balanced nutrition (Dominguez et al. 2021), constant physical activity (Gregory, Parker & Thompson, 2012. Mandolesi et al. 2018), and low stress levels (Johansson et al. 2010), have been shown to act as protective factors for the CNS, and in turn, for people's cognitive functions.

1.2.5 Mouse models for the study of Alzheimer's disease

With the intention of recapitulating part of the mechanisms that produce AD in humans, the scientific community has made a considerable effort to create effective animal models for the study of the disease. Currently, there are a couple of popular used mouse models for the experimental study of AD, which reproduce the accumulation of amyloid plaques, or tau protein as the most studied hallmarks in the context of AD. Although work has been done on the creation of a transgenic model that presents most of the biomarkers associated with AD, choosing between one or another model will be determined by the objectives of our study. Being more specific, AD is associated with a couple of known gene mutations (Bekris et al. 2010. Cacace, Sleegers & Broeckhoven, 2016). In the case of mutations in MAP, a gene that encodes the tau protein, have been associated with AD, due to being the protagonist in the formation of neurofibrillary tangles (NFTs), mainly as a player in the late onset of dementia, and the loss of microtubule-binding function (Ma et al. 2014). Also, the PS19 mouse model has been shown to overexpress human tau with the P310S mutation (Zampar & Wirths, 2021). At the same time it is possible to find the TauRDΔK280, Tg2576/Tau, hTau.P301S, JNPL3(P301L), Tau P301L and APP23.

In the other hand, amyloid plaques, APP, PSEN1 (PS1) and PSEN2 (PS2) genes, represent the processes that encode the exponential aggregation of plaques in the hippocampal or prefrontal cortex areas during AD (Götz, Bodea & Goedert, 2018). Some of the transgenic mouse models for amyloid production are: APP/PS1, 5xFAD (B6SJL), ARTE10, TgCRND8, APP(V717I) x PS1(A246E), 3xTg and rTg9191, however, the way in which the accumulation of the amyloid plaque present in this models, interacts with other characteristics of AD, the aforementioned tau pathology, is still unknown.

1.2.6 Cognitive impairment in the APP/PS1 mouse model

The APP/PS1 mouse model is widely used for the study of Alzheimer's Disease. It is a transgenic model that overexpresses amyloid beta and is frequently manipulated to examine the neuropathological mechanisms involved in the disease. The current study utilized the APP/PS1 model, which is known to exhibit a more aggressive pathology,

specifically, the transgenic mouse model with a C57BL/6J genetic background co-expressing mutated amyloid precursor protein (KM670/671NL) and mutated presenilin 1 (L166P) under the control of a neuron-specific Thy1 promoter element (APP/PS1 mice) (Radde et al. 2006). In 2016, Gertig et al. investigated the effects of the progressive accumulation of amyloid plaques in the central nervous system at specific time points, namely 2, 4, and 8 months. The study utilized the Morris Water Maze (MWM) and fear conditioning paradigm to test the animals. The findings indicated substantial disparities between the transgenic and wildtype models in MWM performance and fear conditioning from 4 months of age, becoming even more pronounced at 8 months (Gertig, 2016).

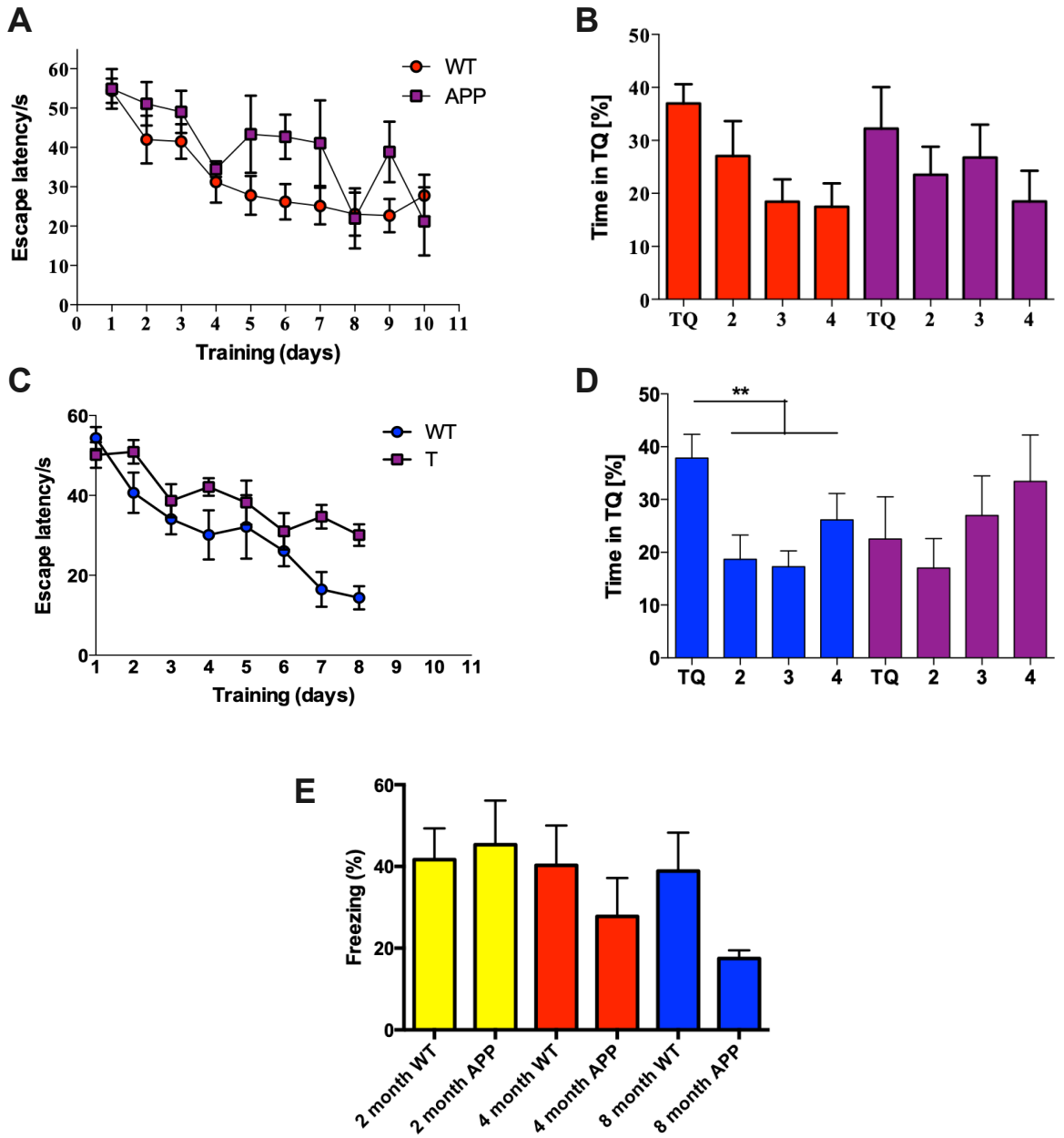

Figure 8 illustrates the results of the behavioral experiments indicating cognitive impairment in the APP/PS1 mouse model starting from month 4. The data includes the following: **A:** Bar plot presenting the escape latency results from a 4-month sample in the Morris Water Maze, revealing a significant difference between the transgenic (purple) and wildtype (red) mice. On average, the transgenic mice took longer to reach the platform during the training days. **B:** Bar plot from the probe test (day 8) showing that transgenic mice (purple) spent less time in the Target Quadrant (TQ) compared to the wildtype mice in the probe test of the Morris Water Maze at 4 months of age. **C:** Bar plot displaying the escape latency results from an 8-month sample in the Morris Water Maze, indicating a significant difference between the transgenic (purple) and wildtype (blue) mice. Transgenic mice took longer to reach the platform during the training days. **D:** Bar plot from the probe test (day 8) revealing that transgenic mice (purple) spent less time in the Target Quadrant (TQ) compared to the wildtype mice in the probe test of the Morris Water Maze at 8 months of age. **E:** Results from the fear conditioning procedure showing significant differences in the percentage of freezing between the wildtype and transgenic mice in month 8, which began to be noticeable from 4 months of age. Transgenic mice displayed a lower percentage of freezing in response to the fear conditioning procedure after 24 hours from first exposure compared to the wildtype mice.

1.3 Congenital diseases

Depression and Alzheimer's Disease are caused by a combination of genetic and environmental factors that appear at different stages in life. On the other hand, congenital disorders are present from birth (Corsello & Giuffrè, 2012), frequently, but not always, due to a genetic mutation. For genetic conditions such as Down syndrome or microcephaly, there is a significant amount of information available about the molecular basis and how certain mutations affect the central nervous system. However, there are some syndromes whose molecular mechanisms are still a mystery.

1.3.1 Genetics and epigenetics of congenital diseases

Congenital diseases, as previously noted by Corsello & Giuffrè (2012), can arise from variations within an individual's DNA sequence. However, they can also be caused by environmental factors, including epigenetic influences. Epigenetics refers to the external factors that affect genetic expression without changing the DNA sequence. By defining congenital diseases as those present at birth, we eliminate the possibility of their later origin from other sources. Nevertheless, recent research has shown that environmental stressors can modify the epigenome shortly after birth, causing changes in gene expression and phenotype (Kubota, 2008). This raises the possibility that epigenetic processes may play a role in not only an individual's neurological development, but also the development of certain diseases.

1.3.2 Floating Harbor Syndrome (FHS)

Sadly, some congenital disorders are characterized by a concerning lack of information (Patton et al. 1991), leaving patients and their families in a state of uncertainty. One such disease is Floating Harbor Syndrome, which has been extensively described in terms of the phenotypic features of those affected, such as short stature, ocular abnormalities (Asseidat & Kaufman, 2009), or a triangular-shaped face (Figure 4), but has very limited information regarding its molecular mechanisms, including its most prominent symptom of cognitive dysfunction, with less than five publications on the subject so far.

Figure 4. Facial features of Floating Harbor Syndrome patients. Adapted from Patton et al. 1991. A: The image shows different patients with a triangular-shaped face, one of the most common phenotypic characteristics of the Floating Harbor Syndrome, consequences of the SRCAP gene mutation.

1.3.3 SRCAP mutation in the Floating Harbor Syndrome

Although there has been limited research on the genetic basis of Floating Harbor Syndrome, with most studies focusing on phenotypic characteristics, evidence suggests that it is primarily caused by heterozygous mutations in the SRCAP gene. The SRCAP is a highly expressed ATP-dependent chromatin remodeler that incorporates the histone H2A.Z variant (Greenberg et al. 2019). Furthermore, the specific location of the mutation has been identified. Data from multiple patients suggest that the most frequent sites for the SRCAP mutation are the amino acids R2435 and R2444 (Figure 5 B).

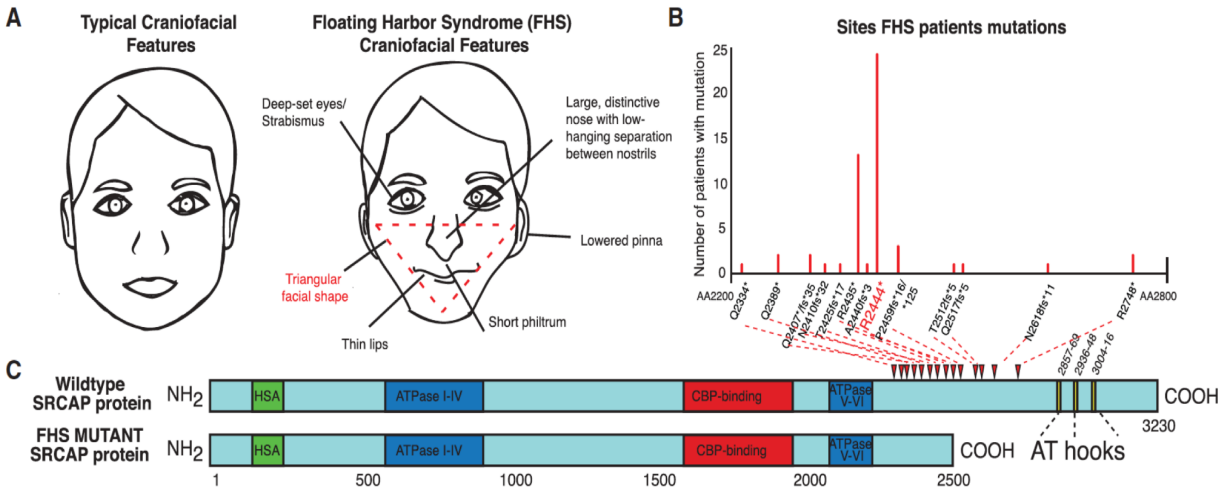

Figure 5. Floating harbor syndrome and SRCAP mutation representation. A: Comparison of the phenotypic characteristics of a normal person (left) and a patient with Floating Harbor Syndrome (FHS) (right). **B:** Graph indicating the number of most common mutations in patients with FHS, highlighting in red the location of the amino acid R2444, which is found in a greater number of patients. **C:** Scheme of the WT and truncated SRCAP protein in FHS. The critical location for the FHS truncating mutations is indicated by red arrowheads (adapted from Greenberg et al. 2019).

1.4 Gene expression control in the healthy and diseases brain

Thanks to advancements in Next Generation Sequencing (NGS) technologies (Thakur et al. 2018. Slatko, Gardner & Ausubel, 2018. Kumar, Cowley & Davis, 2019), there has been a significant improvement in the level of detail in which transcriptome plasticity can be analyzed in cellular models and tissues, including the brain (Behjati & Tarpey, 2013). This has greatly aided in understanding the composition of genetic

information in humans, animals, plants, etc. and describing how our bodies function in a state of health. At the same time, one of the most significant benefits of NGS technologies is the ability to study different diseases and disorders like never before (Precone et al. 2015. Kattoor et al, 2022. Jiang et al. 2022. Couce & González-Vioque, 2022).

To understand the brain's function in disease, it is crucial to study the brain in a healthy state, as this provides a reference point for comparison. Some genetic changes that occur over the course of our lives are not necessarily associated with disease states (Harada, Love & Triebel, 2013. Schott, 2017), and many of these mechanisms originate from normal aging processes. Therefore, it is essential to compare this information between conditions.

1.4.1 Gene-expression analysis in neuropsychiatric and degenerative diseases

A widely used method in the field of genome science for the study of neuropsychiatric and degenerative diseases, is the Differential Gene Expression Analysis (DGE) (Anders & Huber, 2010). Due to the large amount of information obtained after carrying out RNA-sequencing (RNA-seq) procedures, it is important to define our objectives before analyzing the data, this will allow us to find and use the computational resources that are most effective to reach the study aims. In this context, DGE contributes to the identification of gene expression levels that are significantly differentially expressed between two or more conditions (McDermaid et al. 2019).

Although it is possible to find a great variety of methods to perform DGE (Rajkumar et al. 2015), there is currently a consensus in the establishment of a standard structure to start with the analysis. Aligning reads to a genome of reference, producing a table of read counts, filtering, normalization and visualization (Chen et al. 2008) are some of the steps that are usually followed to perform DGE on RNAseq data. In this context, DGE is the main tool that will be used for the study of the models whose methods and results will be described below.

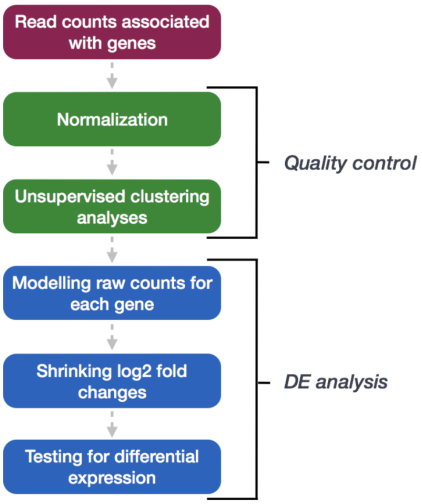

Figure 5. Standard procedures to perform Differential Gene Expression (DGE) analysis of RNAseq data. Schematic representation of the most common steps to perform DGE analysis, such as: association of read counts with genes, normalization and clustering, as part of quality control processes. Modeling raw counts for each gene, log2 fold changes and testing for differential expression of genes between two or more conditions, as the main objective of the DGE.

2. Objectives

The study of neuropsychiatric and neurodegenerative diseases has seen significant progress in recent years due to advancements in Next Generation Sequencing (NGS) techniques. These methods allow the genome-wide analysis of human and animal transcriptomes and epigenomes with the aim to identify novel pathomechanism and therapeutic approaches. In my PhD thesis I planned to study the transcriptome in relevant brain regions of 3 different model systems that represent neuropsychiatric diseases, neurodegenerative diseases and congenital cognitive diseases. More specifically I planned to analyze: 1) a mouse model for Major Depression (MD). Here, I decided to establish the Social Defeat (SD) paradigm, and identify dysregulated genes in the brains of mice with depressive and resilient phenotypes. 2) In the context of AD I planned to study an APP/PS1 mouse model as a reference model for Alzheimer's disease (AD)-like amyloid deposition with the aim to generate differential gene expression results for coding and NonCoding RNAs in different brain regions. 3) I aimed to study transcriptomics in a model based on human induced pluripotent stem cells (hIPSCs) and Bioengineered Neuronal Organoids (BENOS) to investigate the effects of the SRCAP mutation in Floating Harbor Syndrome.

CHAPTER 1

Gene Expression in a Mouse Model for Major Depression

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Abstract

Major depression (MD) is a prevalent neuropsychiatric disorder affecting millions of individuals worldwide. Despite an increasing understanding of the mechanisms that underlie MD's symptoms, this knowledge is far from being complete, hampering the development of effective therapies. The scarcity of reliable and controllable experimental models represents a significant challenge for MD research. In this study we aimed to identify differentially expressed genes in the brains of depressed and resilient mice of a 3-month-old mouse model that were subjected to the social defeat paradigm. Since depression is considered a risk factor of cognitive decline, we not only analyzed depressive-like phenotypes but also memory function. At 3 months of age, no impaired working memory was observed in mice subjected to social defeat stress, independent of the fact that mice developed depressive-like phenotypes or were resilients. No effect of long-term spatial reference memory was observed when analyzing mice subjected to social defeat stress at 3 months of age. At 12 months of age we observed that spatial reference memory was impaired in mice that show depressive-like phenotypes, while working memory was intact. At the transcriptome level we find several genes associated with these changes, among them Iigp1, Anpep and Foxd1, that hint to a key role of inflammatory processes..

Keywords: Major depression; cognitive dysfunction; social defeat; learning and memory; differential gene expression

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Introduction

Major depressive disorder, also known as Major Depression (MD), is a widespread neuropsychiatric disorder affecting over 300 million individuals globally (Pearce et al. 2022; Norkeviciene et al. 2022). Despite established protocols for evaluation and diagnosis, the molecular basis of MD remains largely elusive.

As defined in the DSM-V of the American Psychiatric Association, MD is characterized by persistent feelings of fatigue, decreased participation in leisure activities, and a predominant negative mood state, often referred to as anhedonia (Martinotti et al. 2012). This loss of pleasure experienced by individuals with MD can result in difficulties across a variety of life domains, including work, family, emotions, and recreation, making MD a complex mental illness to study.

One major impediment to the study of MD is the absence of suitable experimental models. This stems from the multifactorial nature of depression, which is often associated with environmental stressors such as childhood trauma, financial hardship, and interpersonal relationships, factors that are challenging to control in laboratory

settings and unethical to replicate in human subjects. Nevertheless, over the past few decades, various animal models have been developed to address this issue, including the Early Life Stress (ELS) and Social Defeat (SD) paradigms applied to mice.

The Social Defeat (SD) experiment entails exposing a novel mouse to a territorial and hostile resident mouse, leading to repeated aggressive interactions and the manifestation of submissive behavior and depression-like symptoms in the intruder mouse. Following the SD protocol, it is feasible to distinguish between depressive and resilient mice through a behavioral assay, known as the Forced Swim Test (FST).

In the current study, the Social Defeat (SD) paradigm was utilized to produce an animal model of Major Depression (MD) due to its convenience in examining various stages of development. Total RNA sequencing of the Anterior Cingulate Cortex (ACC) was performed to identify genes that were dysregulated in the brains of mice with depressive or resilient phenotypes.

Materials and Methods

Animals

During the experimental phase 45 wildtype B6J C57BL 3 month old, and 30 wildtype CD1 12 month old mice were used. From the 45 wildtype B6J C57BL, 30 were exposed to the social defeat procedure, and defined as the experimental condition, The other 15 mice were maintained as the control condition (no exposure to defeat). The 30 mice from the experimental condition were differentiated between depressive and resilient phenotype in base of the results of the forced swim test (immobility time). Animals were exposed to a 12 hour light dark cycle, a stable room temperature (RT) of 22 C and air humidity of 58%. Food and water were provided ad libitum. Housing, breeding and animal experiments were planned and conducted in full compliance with the German Federal Act on the Protection of Animals and were approved by the responsible ministry of Lower Saxony (”Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit”).

Brain dissection

The Anterior Cingulate Cortex (ACC) for RNA sequencing and analysis was dissected after sacrificing the mice by cervical dislocation. A first cut allows extracting the olfactory bulbs that are not of our interest. Subsequently, a cut of approximately 3 mm from the frontal part of the brain inwards, allows the separation of the ACC, characterized as a small darkened area in the shape of a triangle, identifiable in the dorsal region of the corpus callosum.

RNA isolation

Before RNA isolation the cryogenic tissue disruption and homogenization in lysis buffer under precooled conditions was performed. Proteins bound to RNA were removed by treating samples with a protease. The homogeniser was rinsed with a lysis buffer between samples to minimize cross-contamination. Finally, purified RNA was stored aliquoted in RNase-free water at -20°C or -80°C (under these conditions, no degradation of RNA is detectable after 1 year). Tubes were prepared and the centrifuge set to 4°C.

For RNA isolation the following protocol was followed: The samples were maintained on dry ice. 0.5ml TRI Reagent to the tissue (25-50 mg tissue/ 500µl & 50-100mg/ 1000µl TR Reagent was added. 3-5 beads to the tube and homogenize with Bioruptor:20 sec/ 1 Cycle/ 2.6 speed. Finished this process the incubation for 5 min was done at room temperature (RT). After 5 minutes, 0.1ml Chloroform was added (0.2ml Chloroform for 1ml TRI Reagent) and vortexed the samples. Incubate 3 min at RT. Centrifuge at 12000 x g, 4°C, 15 min. The aqueous phase was transferred into a new labeled tube. 1 Vol. (600µl) 100% EtOH and mix added. samples (max. 750µl each time) were transferred into a Zymo spin column and spin at 11000 x g, 4°C, 1 min. The flow through was discarded. 400µl RNA added, buffer washed and spin at 11000 x g, 4°C, 1 min. The flow through was discarded. DNA digestion prepared, mixed on ice per sample: 5µl. DNase I , 5µl DNA Digestion Buffer, 30µl DNase-& RNase-free water. 40µl of the DNase digestion mixed to the column was added, followed by a 15 minutes incubation at RT. Next, 400µl RNA is added to the Buffer prepared to the column and spin at 11000 x g, 4°C, 1 min. The flow through was discarded. 700µl RNA added, buffer washed and spin at 11000 x g, 4°C, 1min. The

flow through was discarded. 400µl RNA added, buffer washed and spin at 11000 x g, 4°C, 2min. To ensure complete removal of the washed buffer, the column was transferred carefully into an RNase-free tube. 22µl DNase-& RNase-free water added directly to the column matrix and spin 1 min. Finally RNA concentration was measured with NanoDrop and the quality checked with Bioanalyzer RNA Nano. RNA was stored at -80°C.

RNA sequencing

Library preparation for TotalRNA sequencing was performed according to Illumina stranded following the manufacturer's instructions. Briefly, libraries were prepared from 500 ug of input RNA.PCR: 12 cycles. After purification, the quality of the libraries was checked with the DNA 1000 Bioanalyzer (Agilent Technologies), and prepared libraries were quantified using a Qubit 3.0 Fluorometer (Life Technologies). A final library concentration of 2 nM was used for dual index single read run, 51 cycles + 10 cycles + 10 cycles on Illumina Nextseq 2000 platform. To reduce sequencing biases the samples were pooled.

Quantitative real time PCR

Quantitative real time PCR (qPCR) was used to quantify specific gene levels. Primers for qPCR were designed using the ”National Library of Medicine” (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Samples were mixed with Primers, LightCycler480 MasterMix and a universal probe library and run in a LightCycler 480 (Roche; Basel, Switzerland).

Behavioral procedures

Social defeat (SD)

During the social defeat (SD) protocol, an experimental mouse is introduced to the home cage of a larger, stronger, and more aggressive mouse (the resident) for 10 minutes. The resident mouse initiates an attack on the intruder. In cases of excessive aggression, the mice are separated for one minute before being reintroduced for the remainder of the 10 minutes. The intruder is then housed in the resident cage, separated by a high-density polyethylene wall for 24 hours. To ensure that physical

aggression did not occur while maintaining the required stress levels, the mice were able to see and smell each other while separated by the transparent barrier. The experimental mouse was placed with a new unknown resident every day to avoid habituation. This protocol was repeated for 10 consecutive days.

As an important objective of this study was to conduct the SD experiment without causing significant harm to the mice, a researcher was always present to observe the mice during the 10-minute interaction between the resident and the intruder. This ensured that rapid intervention could be employed when aggression levels were too high, thus preventing serious injuries in all subjects. Supervision is crucial because it reduces the risk of avoidable damage to the mice during handling, as well as prevents physical damage from impacting the sample's subsequent performance in motor activity-based procedures such as FST, MWM or Y-Maze. This is particularly important in avoiding irreversible damage to the mice's bodies.

Open Field (OF)

In this test, the general activity and exploratory behavior of the animals is measured for 5 minutes. Mice are placed in a 80 x 80 cm box and activity and location are recorded using special software. This test is essential to rule out any changes in behavior that could lead to misinterpretations in subsequent tests. The open field test examines the fearful behavior of animals. This test is an addition to any learning and memory test in order to eliminate possible differences in memory tests that can occur due to changes in the levels of stress.

Forced Swim Test (FST)

The forced swimming test is an established test for examining depressive behavior (Castagné et al. 2010). It can be assumed that the experiments we planned show differences in depressive behavior. The animals are placed in a tank filled with water for 5 minutes. The tank is made of transparent plexiglass, it is round (50 cm in diameter) and 70 cm high. The water has an ambient temperature of 22 °C. The activity of the animals is recorded using special software (TSE Systems). Activity serves as a measure of depressive behavior, as depressed animals are thought to be less motivated on this test when they show little activity. This test is also important in

order to correctly interpret the results of the water maze test. Unusual swimming behavior in the FST should be taken into account when evaluating the data. The FST does not require swimming with exhaustion as the end point.

Y-Maze

In this test the working memory of the animals is tested and the innate exploratory behavior of the mice is tracking enduring 5 minutes. The apparatus used for the Y-maze test consists of 3 identical arms that lie at an angle of 120° to each other and converge at a common center. Mice usually prefer to visit the arm they haven't been in the longest. This means that the animals keep walking with their arms one after the other and remember the order of the arms they visited and update this list continuously. A mouse with impaired working memory cannot remember which arm it last visited, resulting in fewer changes between arms (Holcomb et al., 1999) and which must be quantified here.

The Morris Water Maze (MWM)

This test evaluates the long-term spatial memory of rodents. Mice are placed in a pool (109 cm diameter) filled with water (25°C). The animals cannot see a platform directly below the surface of the water. The animals have 1 minute to find the platform. If the mice have not found the platform within the given time, the experimenter repeatedly places them on this platform. This training is repeated 4 times a day so that the mice learn the position of the platform. For this, various visual reference points are attached as spatial orientation aids, so that the animals can spatially orient themselves over time (on average 7 days) and swim to the platform in a targeted manner. The time it takes to reach the platform is recorded by a camera system and displayed as a spatial learning function. For a mouse with normal long-term memory, the time it takes to find the platform will decrease with each attempt after the learning phase. Conversely, in the case of a mouse that cannot correctly remember the position of the platform due to limited long-term memory, the time until found is reduced slightly or not at all. For the memory test (probe test), the platform is removed from the pool and the whereabouts of the animals are detected using the camera system. The time and distance the mouse spends in the region of the cluster the platform was in before provides information about the ability to learn spatially.

NGS data analysis

Sequencing reads obtained from biological samples were processed as FASTQ files, control and independent analysis for each sample confirmed the quality of the data. Mapping to the genome was performed using STAR software. Bam files obtained after genome mapping (mouse), were used to create the counts reads associated with genes. Read counts associated with genes, normalization and unsupervised clustering analysis were performed before differential gene expression analysis (DGE).

Differential gene expression

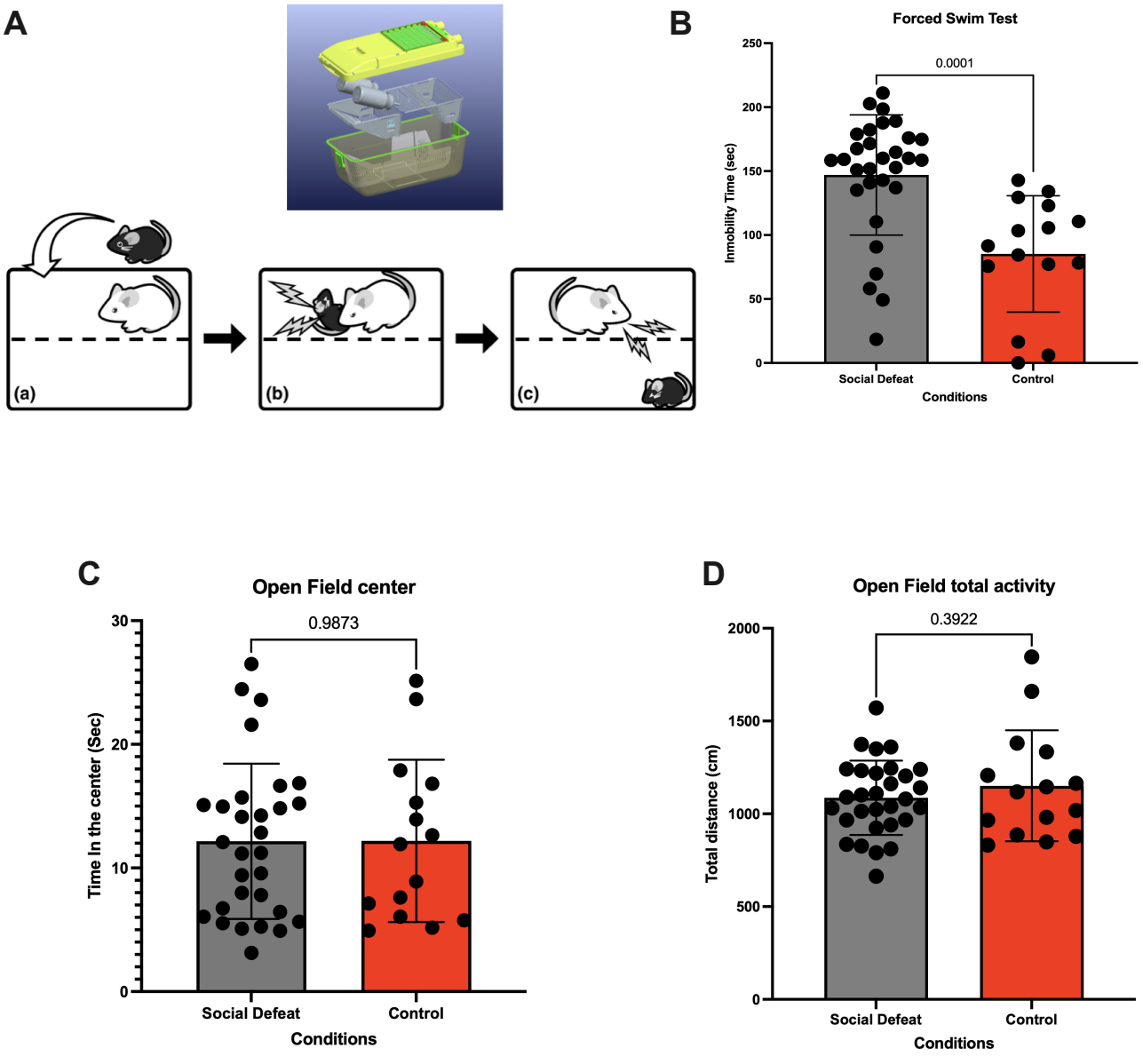
Counts from RNAseq were produced using FeaturesCount. DGE was carried out with the DESeq2 toolbox run in R. During DGE analysis read counts associated with genes, normalization and unsupervised clustering analysis were used as quality control procedures. In detail: Set-up variables and libraries of interest, associate text to genes. Load GTF annotation for later computing of Fragments per kilobase million (FPKM's). Next step, loading, curating, and organizing data considering the variable "coldata" as the main function to use the data counts. Visual diagnostic through PCA, normalization and filtering of counts. Computing FPKM's and extracting counts. Mapping ensemble and gene IDs in the order presented by DESeq results. Those genes with an adjusted p value <0.05 were considered significant differentially expressed genes. Additionally, the gene lists obtained by DESeq2 were filtered with a cutoff for log2 fold-changes of ± 0.3. Finally, plots of interest were produced, and results saved.

Results

Social defeat induces depressive-like behavior and impairs working memory in mice

To establish a model for depressive-like behavior, mice were subjected to a social defeat (SD) paradigm. During a time period of 10 days the mice from the experimental group were exposed to the social defeat procedure (as shown in Figure 1A). Mice that were housed in home cages served as control.

First, we subjected mice to the forced swim test (FST), with the aim of determining which mice developed depressive symptoms after 10 days of social defeat (SD). The results of the FST clearly demonstrated differences between the mice subjected to social defeat when compared to the control group (Figure 1 B, p value <0.0001), with the mice exposed to SD exhibiting longer immobility times compared to the control group indicative of depressive-like behavior. Based on these results, depressive (mean immobility time 180 seconds) and resilient (mean immobility time 145 seconds) conditions were established.



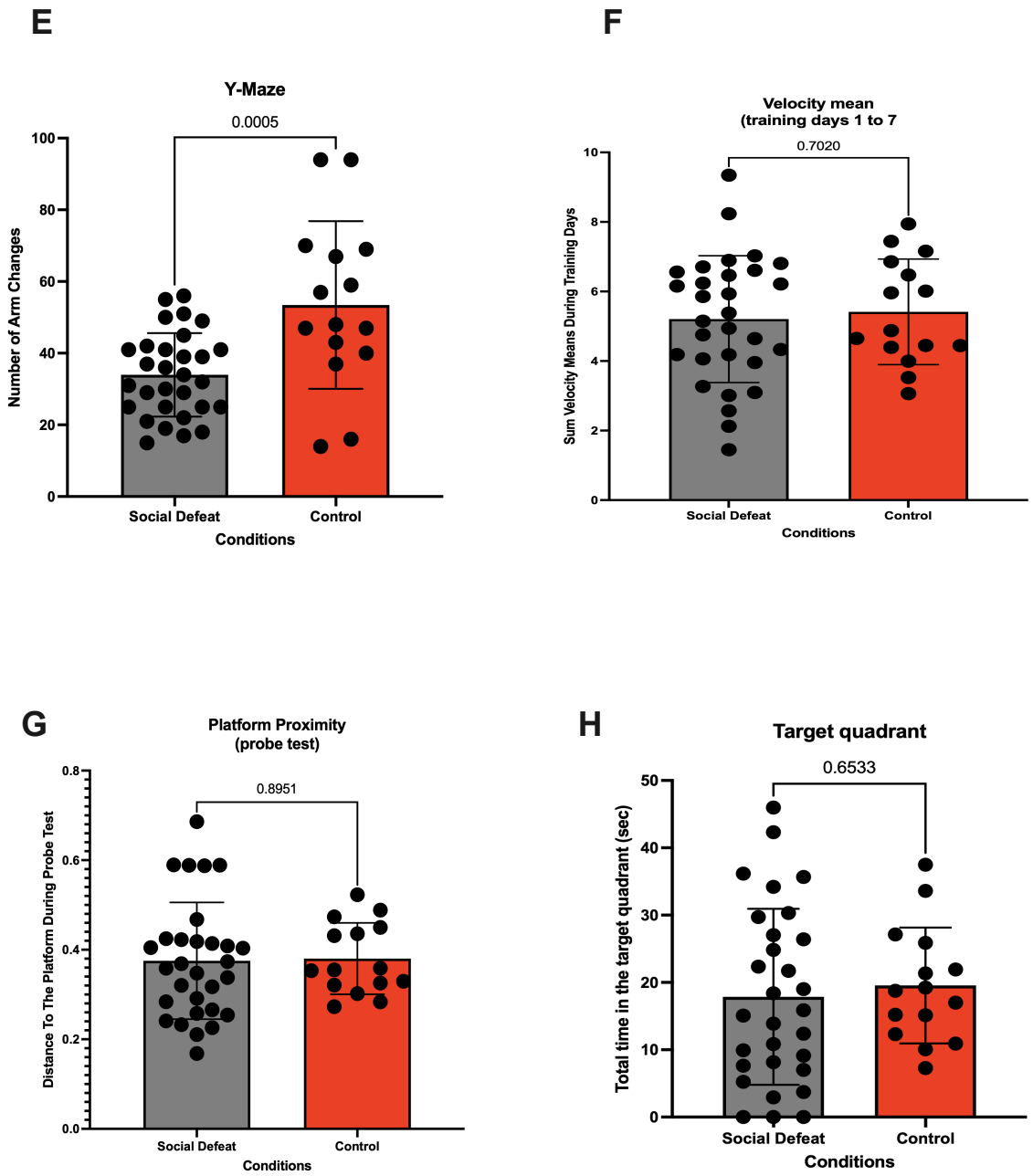


Figure 1 illustrates the effects of chronic social defeat on a 3-month-old mouse model. **A.** The experimental design involves a chronic social defeat procedure: (a) an intruder wildtype mouse is introduced into the cage of a territorial and aggressive resident CD1 mouse, (b) the resident initiates a physical attack against the intruder for 10 minutes, (c) the intruder and resident are kept in the same cage with a transparent plastic barrier to prevent physical contact for the next 24 hours. This procedure is repeated for 10 days. The results of the forced swim test (FST, **B**) showed significant statistical differences (t-test, p value = 0.0001) between the experimental and control groups, with the experimental group exhibiting longer immobility times in the FST, which was used to differentiate between depressive and resilient conditions. The results from the open field (OF) test (**C&D**) did not reveal significant differences (t-test, time in the center p value = 0.9 and total activity p value = 0.9) between the experimental (exposed to social defeat) and control group. The results of the Y-maze test (**E**) showed a significant difference between the social defeat and control conditions (t-test, p value = 0.0005). The results from the Morris water maze did not show significant differences between the social defeat and control conditions mean velocity (**F**), platform proximity (**G**) or target quadrant (**H**).

Next, we analyzed basal anxiety via the open-field (OF) test. This test provides a standard for evaluating anxiety-like behavior, the results from the OF test did not

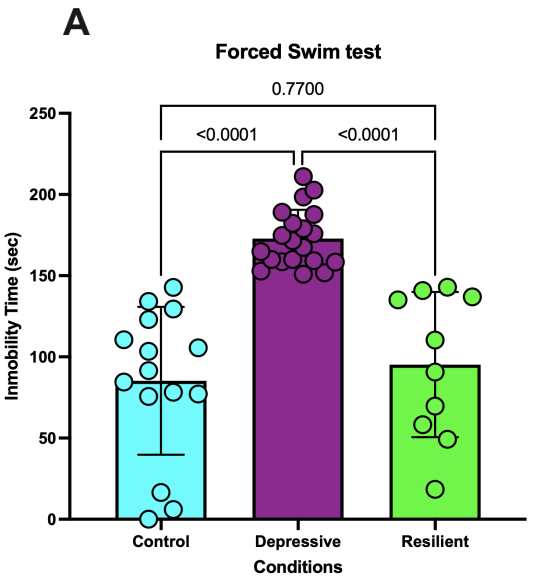
reveal any significant differences between the SD and control groups (Figure 1. C & D, p value = 0.9) suggesting that basal anxiety is not affected by SD stress in our experimental setting**.**

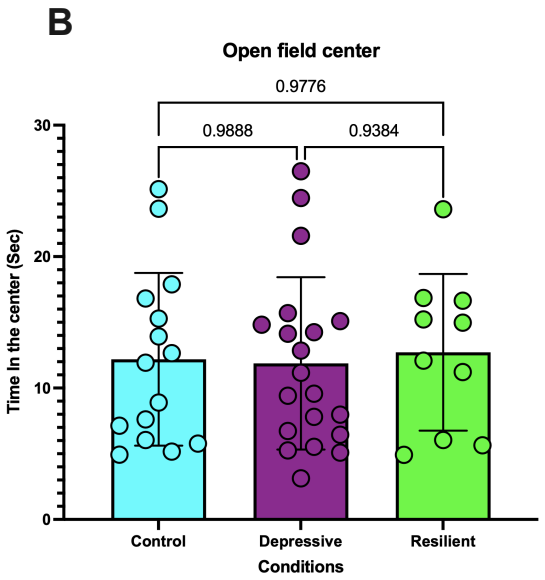
To test if mice that developed depressive-like behavior also exhibit cognitive impairments we first performed the Y-Maze test, a behavior test for working memory. Our data reveal that there was a significant difference between the group exposed to SD and the control group (Figure 1 E. p value: 0,0005). Mice in the control condition made on average more changes between arms inside the Y-maze compared to the SD condition, where the number of changes was lower. These data indicate that working memory was impaired in response to SD exposure.

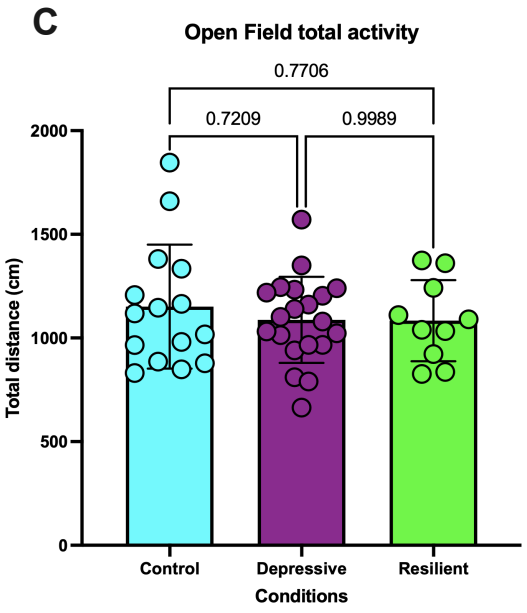
To test spatial reference memory mice were subjected to the MWM test. When analyzing the escape latency (time taken to reach the platform in each trial), the mean velocity (mean speed of mice during training days), and platform proximity (total distance to the platform during probe test), neither of these measures revealed any significant differences between the SD and control conditions (Figure 1 F,G & H). To validate these findings, we also calculated the total time that the mice spent in the target quadrant (TQ) during the probe test on day 8. Results showed no significant differences between the SD and control conditions (Figure 1 I; p value: 0.6). These findings suggest our SD paradigm did not affect the spatial-reference memory functions.

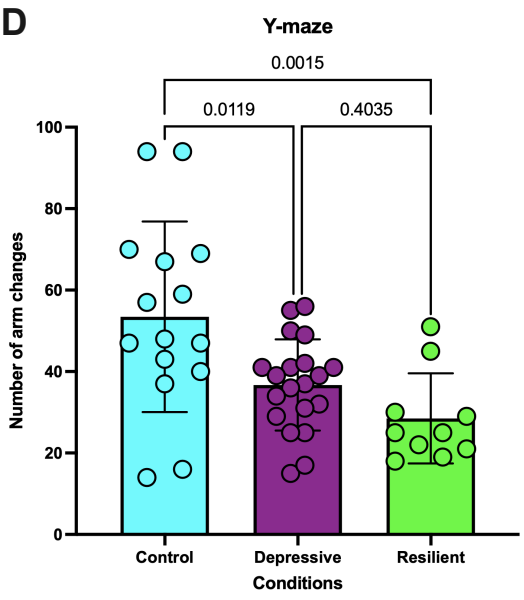
Impaired working memory is also observed in resilient mice

The mice do not respond equally to the SD exposure so that it is usually possible to further classify individuals into responders that exhibit depressive-like phenotypes and resilient mice that do not show depressive-like phenotypes (Alves-dos-Santos, Resende & Chiavegatto., 2020). As resilient, we defined mice that were exposed to SD but performed similar to the control group in the forced swim test. In our experimental setting animals exposed to SD that showed an immobility score below 145s were classified as “resilient” (Fig 2A).


A
Forced Swim test
0.7700
<0.0001
<0.0001
Inmobility Time (sec)
250
200
150
100
50
0
Control
Depressive
Resilient
Conditions


B
Open field center
0.9776
0.9888
0.9384
Time In the center (Sec)
30
20
10
0
Control
Depressive
Resilient
Conditions


C
Open Field total activity
0.7706
0.7209
0.9989
Total distance (cm)
2000
1500
1000
500
0
Control
Depressive
Resilient
Conditions


D
Y-maze
0.0015
0.0119
0.4035
Number of arm changes
100
80
60
40
20
0
Control
Depressive
Resilient
Conditions

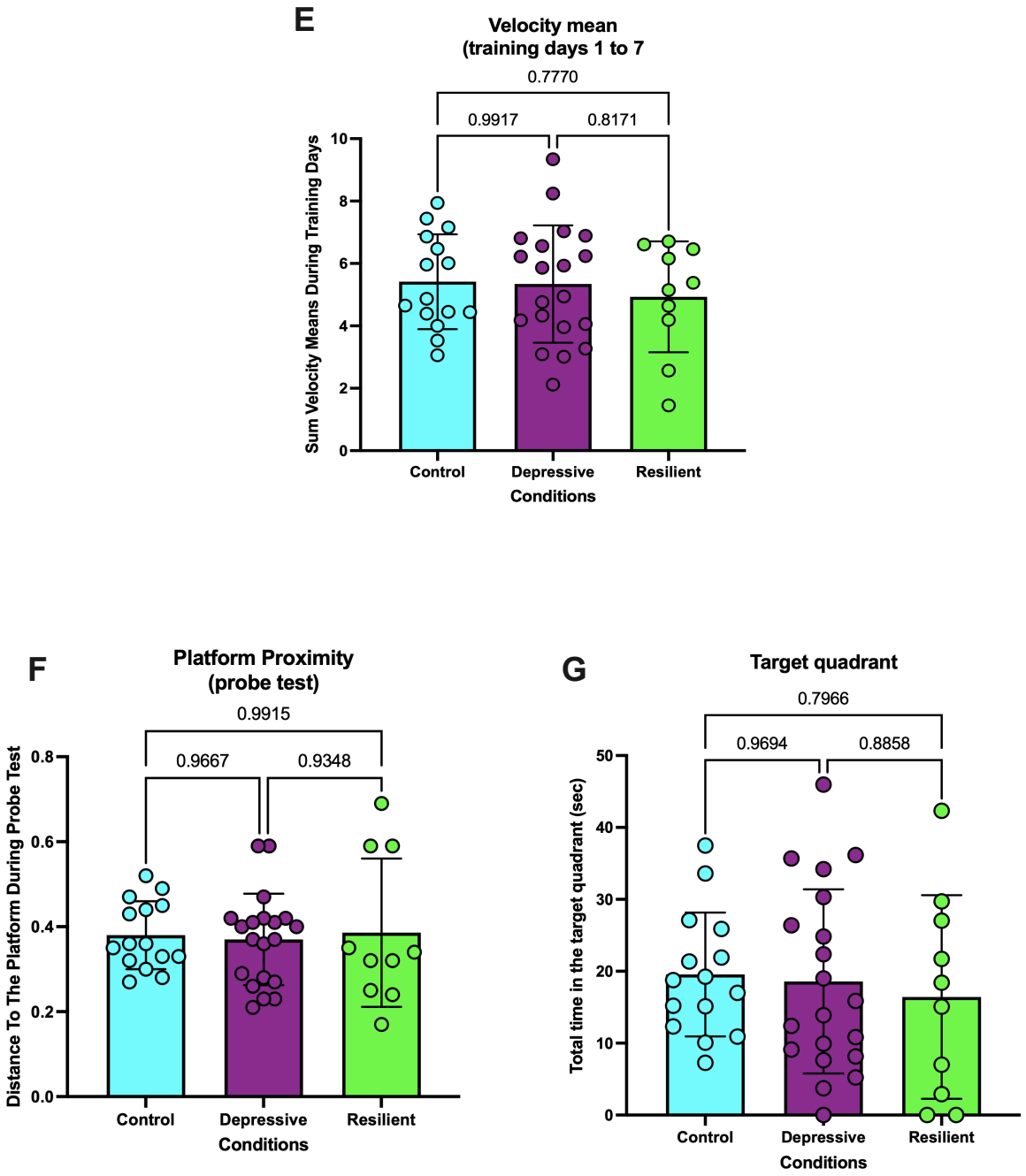
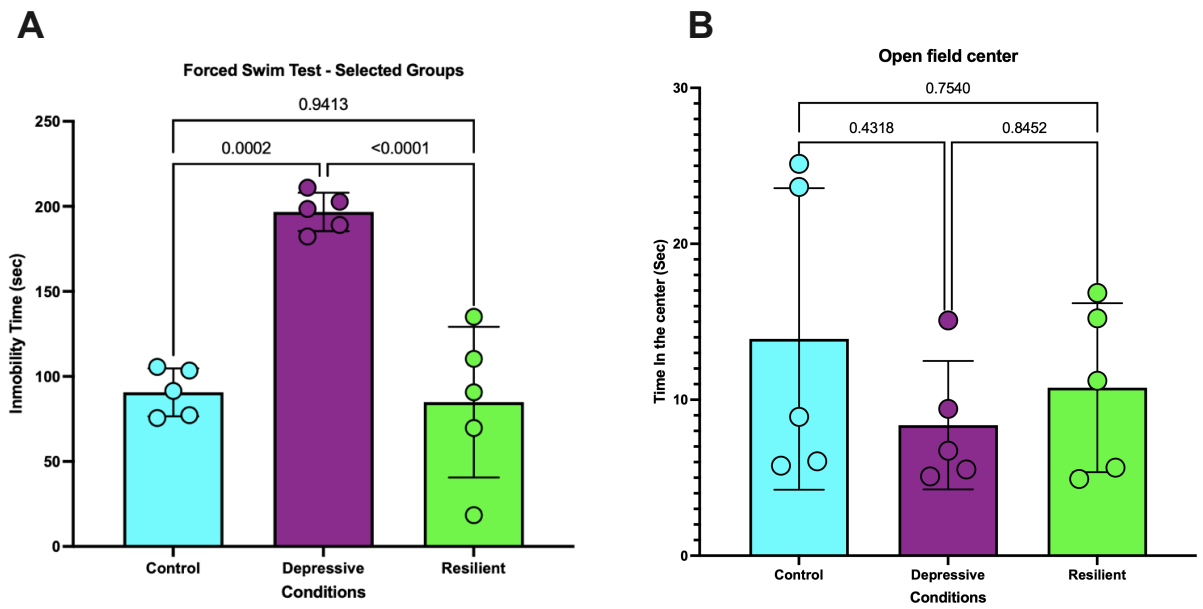


Figure 2 Impaired working memory is also observed in resilient mice. **A:** One Way Anova showing the results for the Forced swim test. Statistical significant differences in all the conditions, confirming the distinctions by phenotype. **B & C:** One way anova of the open field in the calculation of the training days. No significant statistical differences in the time that the mice spend on the center of the field, and the total activity between the conditions.**D:** One Way Anova showing the results from the Y-maze in the selected mice. Statistical significant differences in the conditions control vs depressive: p-value 0,01. and control vs resilient: p-value: 000,1). **E:** One way anova of the velocity means base in the calculation of the training days. No significant statistical differences between the conditions. **F:** One way anova of the platform proximity (distance to the platform) base in the calculation of the probe test (day 8). No significant statistical differences between the conditions. **G:** One way anova of the time that the mice expended in the target Quadrant (TQ), based on the calculation of the probe test (day 8). No significant statistical differences between the conditions.

When we specifically analyzed the performance in the open field test between control, depressed and resilient mice, there was no significant difference for the time spent in the center amongst groups, suggesting that basal anxiety levels did not differ between resilient and depressed animals (Fig 2B & 2C). Regarding the Y-maze, it was interesting to observe that working memory was impaired in depressed and resilient mice, when compared to the control group (Fig 2D). These data indicate that SD stress impairs working memory independent of the development of depressive-like phenotypes or resilience. Similarly, the performance in the MWM was similar amongst groups, showing that spatial reference memory was not affected by SD and that this was also independent of depressive phenotypes or resilience (Fig 2E, F & G).

Transcriptome analysis of the ACC in control, depressed and resilient mice

To analyze if SD, in relation to the development of resilience or depressive-like phenotypes would lead to gene-expression changes in the ACC, we selected 5 control animals, 5 resilient mice and 5 mice that developed depressive-like phenotypes as measured vai the forced swim test. We first confirmed that the selected animals resemble the same behavior phenotypes as seen in the whole group (Fig 3A).



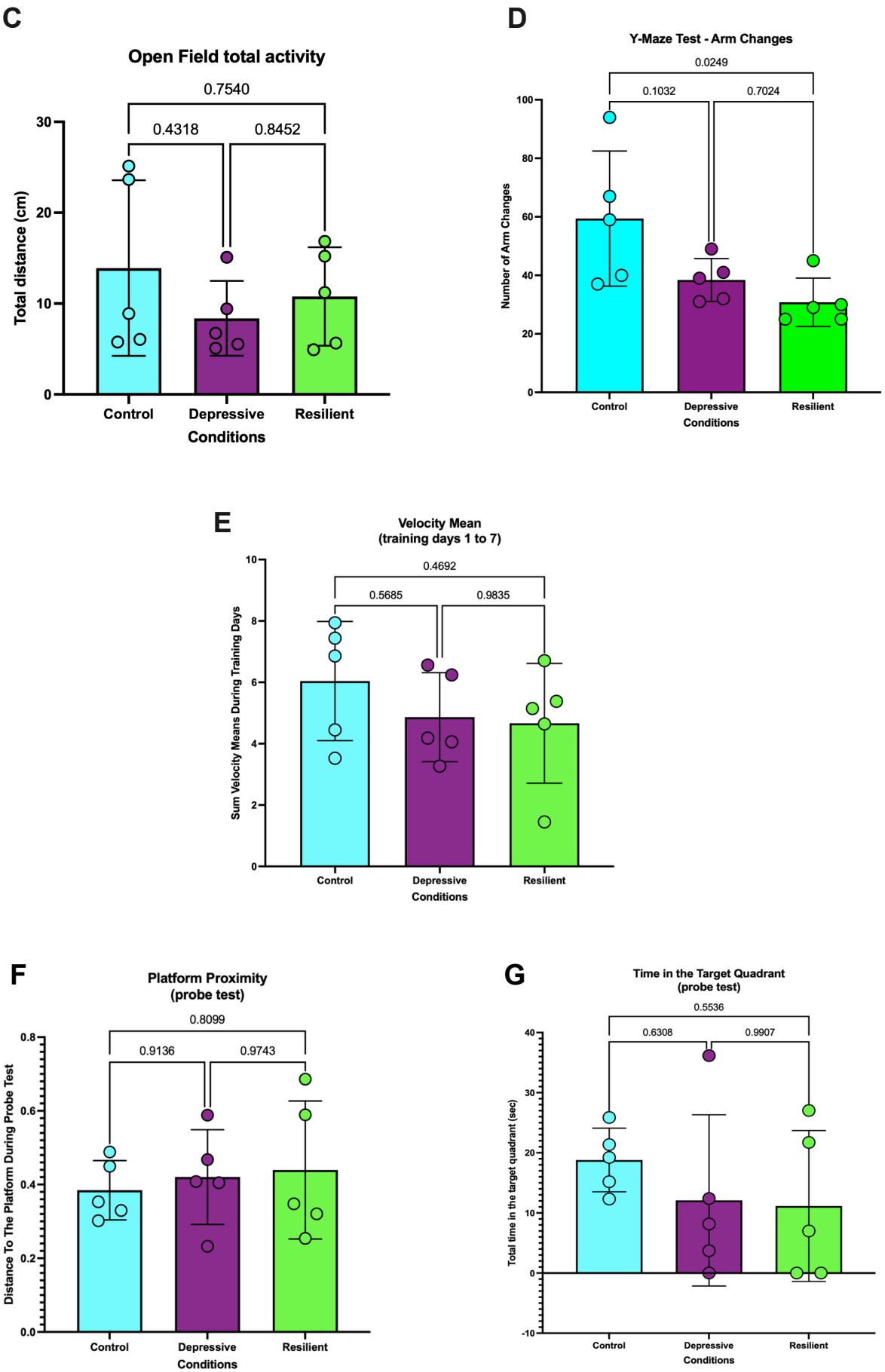

C
Open Field total activity
0.7540
0.4318
0.8452
Total distance (cm)
30
20
10
0
Control
Depressive
Resilient
Conditions
D
Y-Maze Test - Arm Changes
0.0249
0.1032
0.7024
Number of Arm Changes
100
80
60
40
20
0
Control
Depressive
Resilient
Conditions
E
Velocity Mean
(training days 1 to 7)
0.4692
0.5685
0.9835
Sum Velocity Means During Training Days
10
8
6
4
2
0
Control
Depressive
Resilient
Conditions
F
Platform Proximity
(probe test)
0.8099
0.9136
0.9743
Distance To The Platform During Probe Test
0.8
0.6
0.4
0.2
0.0
Control
Depressive
Resilient
Conditions
G
Time in the Target Quadrant
(probe test)
0.5536
0.6308
0.9907
Total time in the target quadrant (sec)
40
30
20
10
0
-10
Control
Depressive
Resilient
Conditions

Figure 3. Transcriptome analysis of the ACC in control, depressed and resilient mice . A: One Way Anova showing the results for the Forced swim test of the mice selected for sequencing. Statistical significant differences in all the conditions, confirming the distinctions by phenotype. **B & C:** One way anova of the open field in the calculation of the training days. No significant statistical differences in the time that the mice spend on the center of the field, and the total activity between the conditions.**D:** One Way Anova showing the results from the Y-maze in the selected mice. Statistical significant differences in the conditions control vs depressive: p-value 0,1. and control vs resilient: p-value: 00,2). **E:** One way anova of the velocity means base in the calculation of the training days. No significant statistical differences between the conditions. **F:** One way anova of the platform proximity (distance to the platform) base in the calculation of the probe test (day 8). No significant statistical differences between the conditions. **G:** One way anova of the time that the mice expended in the target Quadrant (TQ), based on the calculation of the probe test (day 8). No significant statistical differences between the conditions.

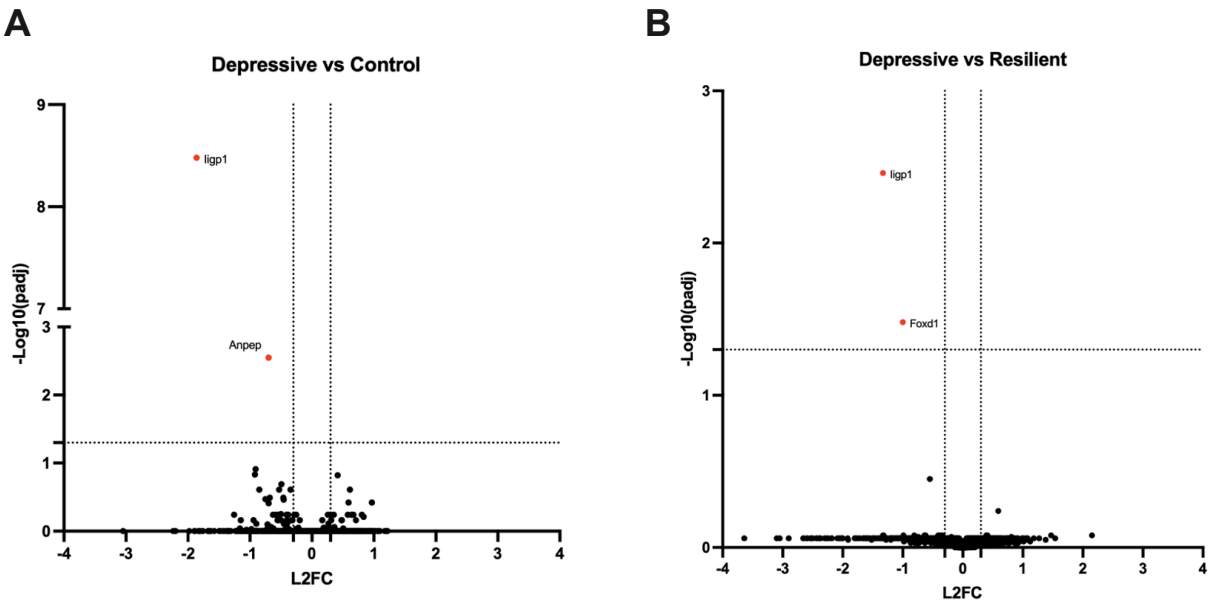
Even with the smaller groups size of n=5 we observed a significant impairment of working memory in the Y-maze test when comparing depressive mice to control and resilient mice to control (Fig 3D). This confirms that stress impairs working memory independent of the development of depressive-like phenotypes or resilience. Although the direct comparison of depressed vs resilient mice showed no significant difference (P = 0,7). There we conclude that the working memory defect is still observed in the groups selected for molecular analysis although it was only of borderline significance in the depressed mice.

In the MWM test, there were no significant differences in speed (velocity mean) between the conditions during the training days (Figure 3E), indicating the healthy physical condition of both groups despite the experimental group being exposed to constant physical attack during SD. These findings confirm the procedure's effectiveness in preventing serious physical harm to the experimental group (intruder). After completing the 7 days of training, a probe test was conducted on day 8 to evaluate platform proximity, which did not show significant differences between the conditions (Figure 3F; control vs. depressive = p value: 0.9; depressive vs. resilient = p value: 0.9; control vs. resilient = p value: 0.8). To further support these findings, the total time that the mice spent in the Target Quadrant (TQ), where the platform was located during the training days, was evaluated. However, this measure did not yield significant results for the established conditions (depressive, resilient, and control) (Figure 3G).

In summary these data confirm that we observe similar phenotypes in the mice selected for molecular analysis as in the entire group. Next we isolated the ACC and performed totalRNA-seq. Differential expression analysis revealed only minor changes in gene-expression.

Differential gene expression in the ACC of control, depressed and resilient mice. mouse model of depression and resilience

The total number of genes differentially expressed between the depressive vs control and depressive vs resilient conditions after applying the filters of adjusted p value <0.05 and a cutoff for log2 fold-changes of ±0.3, are 2 downregulated genes in depressive vs control (Figure 4 A), and 2 downregulated genes in depressive vs resilient condition (Figure 4 B). In the condition depressive vs control, we found the downregulation of the Interferon inducible GTPase 1 (ligp1) gene, and the Alanyl aminopeptidase membrane ANPEP. ANPEP is a protein coding gene involved in the processing of hormones. At the same time, it plays an important molecular function as developmental protein and is highly enriched in immune cells located in the cerebral cortex (Karlsson et al. 2021). What is interesting about these findings, is that ANPEP is considered playing a critical role in the processes that regulate mood, and have been identified in the context of Major Depressive Disorder (Li et al. 2012). On the other hand, we also found the gene ligp1 downregulated in the condition resilient vs depressive, and the Forkhead Box d1 (Foxd1). Foxd1 is described as a regulator of the inflammatory reactions and autoimmunity, its molecular function was defined as a DNA-binding and is highly expressed in microglia in the cerebral cortex (The Human Protein Atlas, 2022). A particularly interesting result from the DGE was the identification of the Interferon inducible GTPase 1 (ligp1) gene, which was found downregulated in both of the conditions analyzed,



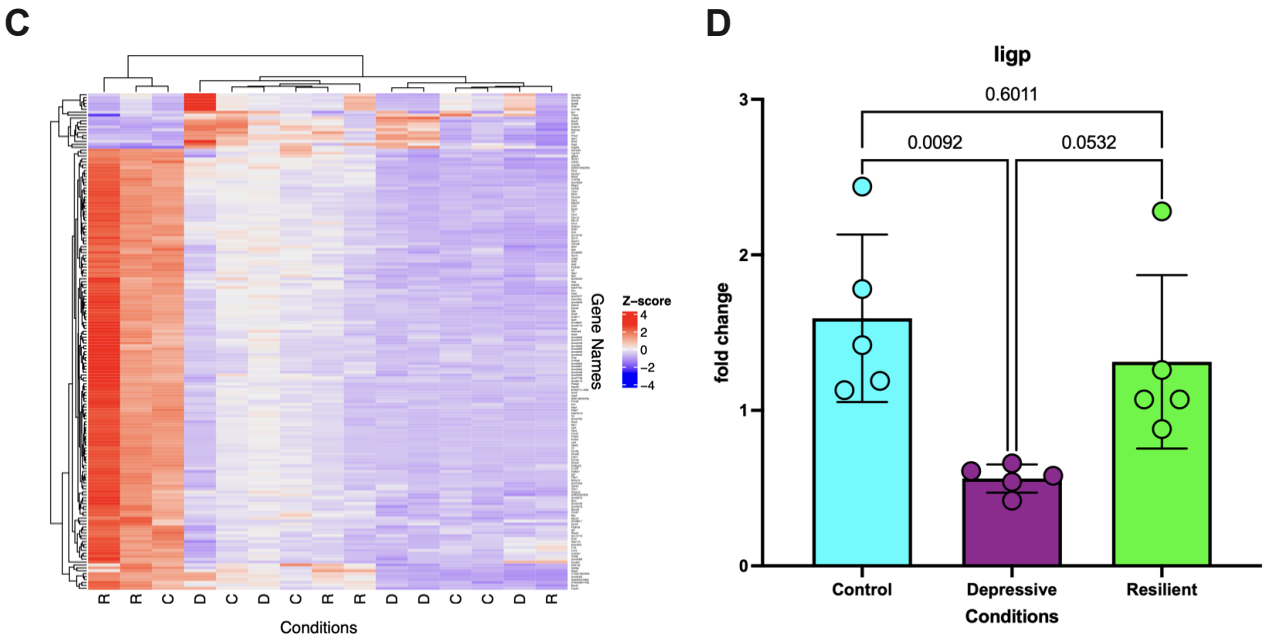


Figure 4. Exposure to chronic social defeat produces deregulation of interferon type 1 in a mouse model of major depression. A: Volcano plot of the gene expression changes between depressive and control group (L2FC > 0.50 difference and padj < = 0.05. Showing a clear downregulation of the ligp1 and Anpep genes. **B:** Volcano plot of the gene expression changes between resilient and depressive group (L2FC > 0.50 difference and padj < = 0.05. Showing a clear downregulation of the ligp1 and Foxd1 genes. **C:**Heatmap of conditions from the differential gene expression analysis. Letters: R(Resilient), C(Control), D(Depressive). **D:** One Way Anova from the results of the quantitative polymerase chain reaction (qPCR) of the gene ligp1, showing statistical differences between the selected conditions (control vs depressive: p-value 0,0092. Depressive vs resilient: p-value: 0,05).

One of the highlights of the present study was the deregulated presence of the ligp1 gene in the depressive and resilient conditions. ligp1 had already been mentioned before in a study by Stankiewicz (M. Stankiewicz et al. 2015), in a mouse model exposed to chronic stress. Stankiewicz found that ligp1 was downregulated in those mice that responded to stressful stimuli after a period of 13 days, which was in the opposite direction of the results obtained under an acute stress condition, where ligp1 was upregulated. However, our results would indicate that although it is possible to make distinctions based on phenotype after exposure to SD, this distinction would not be reproduced at the genetic level.

With respect to ligp1 it is important to note that the decreased expression was much more pronounced when comparing control vs depressed mice (p value 0,009) than in depressed vs. resilient mice (p value 0,05). These data may indicate that the

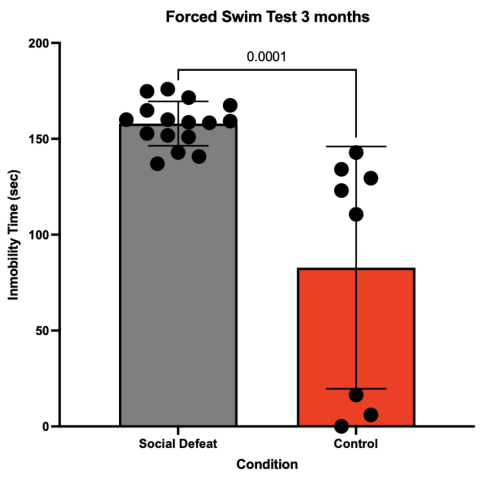
expression of Iigp1 could correlate with the occurrence of resilience or depression. To further test this hypothesis these results we performed a qPCR) (Figure 4 D). Significant differences were found in the control vs depressive and depressive vs resilient, which is in line with the hypothesis of deregulation levels of expression for Iigp1. On the contrary, the levels between the control vs resilient, do not indicate significant differences. It has to be mentioned however, that qPRC revealed a borderline significant p-value when comparing control to resilient mice and there was no significant difference when we compared Iigp1 levels in resilient vs depressed mice. In conclusion these data suggest that Iigp1 expression decreased in response to SD stress in the ACC of mice that develop depressive-like phenotypes or resilience, indicating that Iigp1 levels could be rather linked to stress induced working memory impairment rather than depressive phenotypes.

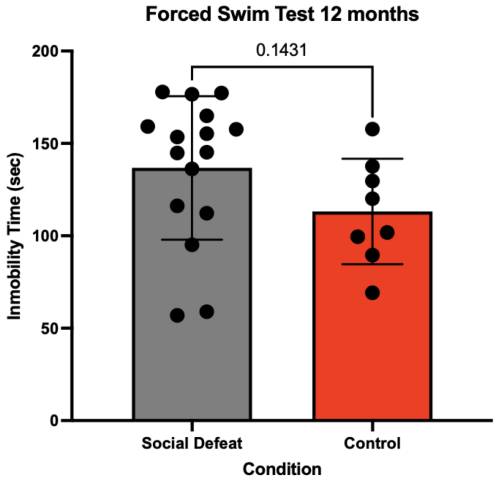
Cognitive performance in aged mice that were exposed to SD early in life

Since depression has been identified as a risk factor for age-associated memory impairment and AD, we decided to test mice that were exposed to SD at 3 months of age at 12 months of age.

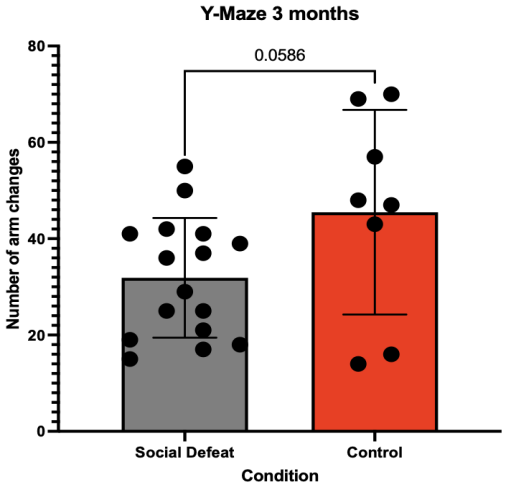
Mice that had been exposed to the SD paradigm at 3 months of age were tested in the forced swim test, the Y-maze and MWM tests. Analysis of the forced swim tests revealed that mice exposed to SD developed depressive-like phenotypes at 3 months (Fig. 5A, left panel). When we re-analyzed the same mice at 12 months of age, we didn't detect the depressive-like phenotypes in the SD group when compared to control (Fig 5A, right panel). In the Y-maze test, there was significant difference amongst groups when tested at 3 months of age (Fig 5B, left panel), however, this difference was lost at 12 months (Fig 5, right panel). The data at 3 months of age are in contrast to previous results, where SD-exposed animals exhibited impaired working memory in the long term, suggesting that the SD-induced impairment of working memory during early life is not a robust effect.

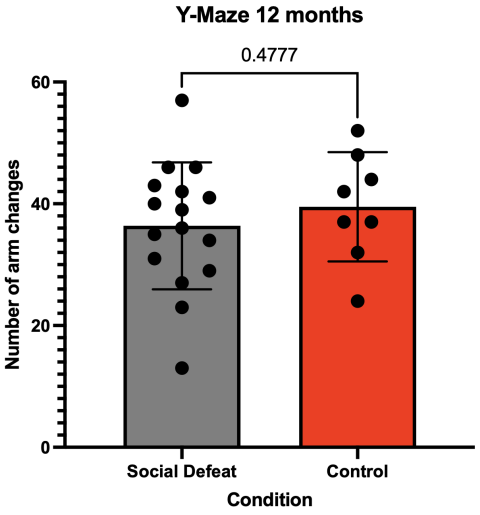
A


Forced Swim Test 3 months
0.0001
Inmobility Time (sec)
200
150
100
50
0
Social Defeat
Control
Condition

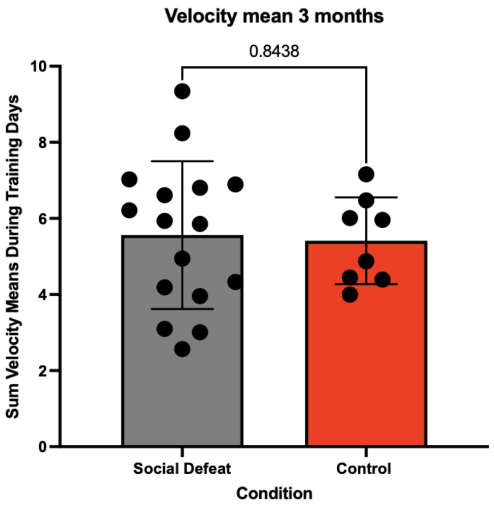

Forced Swim Test 12 months
0.1431
Inmobility Time (sec)
200
150
100
50
0
Social Defeat
Control
Condition

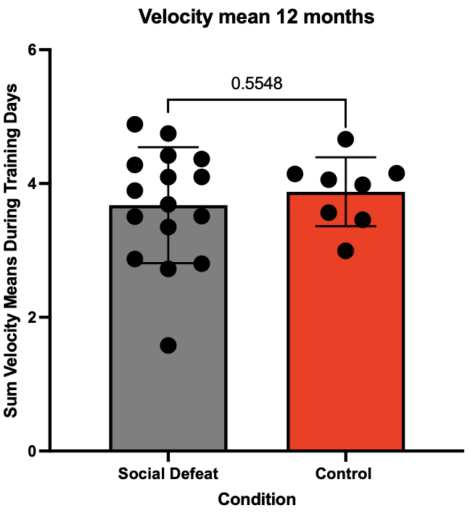
B


Y-Maze 3 months
0.0586
Number of arm changes
80
60
40
20
0
Social Defeat
Control
Condition


Y-Maze 12 months
0.4777
Number of arm changes
60
40
20
0
Social Defeat
Control
Condition

C


Velocity mean 3 months
0.8438
Sum Velocity Means During Training Days
10
8
6
4
2
0
Social Defeat
Control
Condition


Velocity mean 12 months
0.5548
Sum Velocity Means During Training Days
6
4
2
0
Social Defeat
Control
Condition

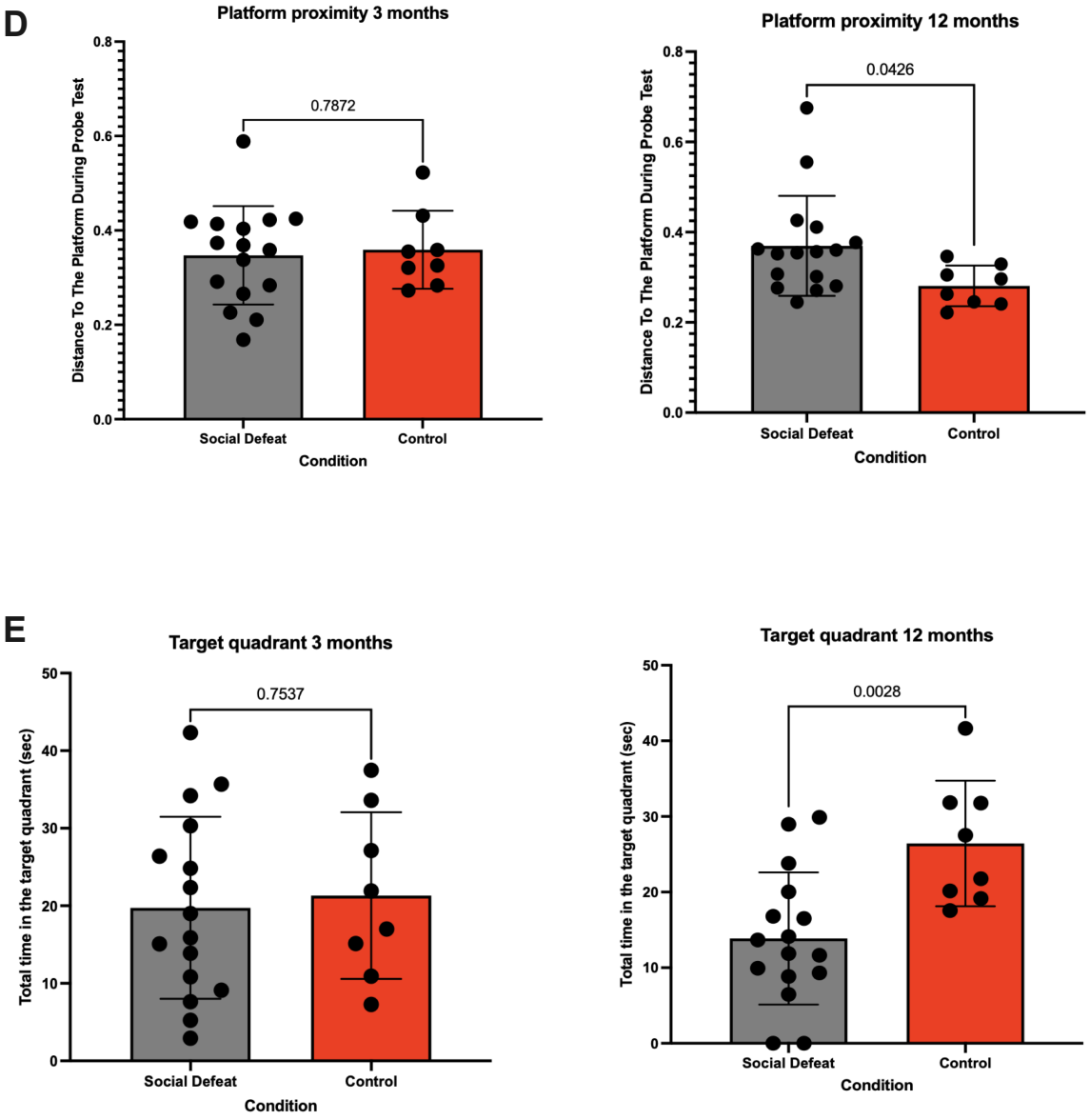


Figure 5. SD-exposure is linked to an accelerated age-associated memory decline in mice. A: Bar plot of the Forced swim test showing significant difference between social defeat and control condition in the selected sample at 3 months old (Panel A. t-test, p value: 0,0001), but not at 12 months old (Panel B. t-test, p value: 0,1). **B:** Bar plot of the Y-maze test showing significant difference between social defeat and control condition in the selected sample at 3 months old (Panel A. t-test, p value: 0,05), but not at 12 months old (Panel B. t-test, p value: 0,4). **C:** Bar plot of the velocity mean showing no differences between social defeat and control condition in the selected sample at 3 months old (Panel A. t-test, p value: 0,8), and at 12 months old (Panel B. t-test, p value: 0,5). **D:** Bar plot of the platform proximity showing no differences between social defeat and control condition in the selected sample at 3 months old (Panel A. t-test, p value: 0,7), but a significant difference at 12 months old (Panel B. t-test, p value: 0,04). **E:** Bar plot of the target quadrant showing no differences between social defeat and control condition in the selected sample at 3 months old (Panel A. t-test, p value: 0,7), but a significant difference at 12 months old (Panel B. t-test, p value: 0,002).

We also assessed memory in the MWM test in mice subjected to SD at 3 months of age. In agreement with our previous findings no difference in velocity mean, platform proximity and target quadrant was observed in 3 months old mice right after the

completion of the SD stress. However, when the same group of mice was analyzed at 12 months of age shows significant difference against the control group. SD-exposed mice showed impaired memory recall during the probe test as indicated by that fact that SD-exposed mice spent less time in the target quadrant (Fig 5E, right panel) and displayed a reduced proximity to the platform when compared to the control group (Fig 5D, right panel).

In conclusion, these data suggested that SD-exposure and the development of depressive-like phenotypes in 3- months old mice is linked to an accelerated age-associated memory decline in mice.

Discussion

We employed the SD-stress paradigm to induce depressive-like phenotypes in 3 month old mice to identify differentially expressed genes in the brains of depressive versus resilient mice. In agreement with previous data we could detect significant differences in the forced swim test, suggesting that SD-exposed mice develop depression phenotypes in response to SD (Can, T. Dao & D.Gould, 2011; Slattery & Cyrian, 2012; Yankelevitch-Yahav, Franko & Doron, 2015; Castagné et al. 2010; Zhao, Gao, & Huang, 2021; Mertens et al. 2019).

When we exposed the animals to the OF test we did not observe significant differences between the experimental and control group. The OF test is considered to measure basal exploratory behavior (Kalueff, Wheaton & Murphy, 2007). The fact that no difference was seen for the total activity as well as the center activity, which is considered a measure of basal anxiety, also confirms that the SD exposure did not cause any physical harm to the animals. It should be mentioned that stress exposure has been linked to increased anxiety levels in mice (Seibenhener & Wooten, 2015. Sturman, Germain & Bohacek, 2018). It is still possible that we might be able to detect altered anxiety levels if more sensitive tests such as the elevated plus maze would have been applied.

The mice were also exposed to different memory tests to assess cognitive performance (Sharma, Rakoczy & Brown-Borg, 2010; Willis, Bartlett & Vukovic, 2017). The first test used for this purpose was the Y-Maze, a procedure selected to measure

working memory (Hölter et al., 2015; Prieur & Jadavji, 2019). The results indicated significant differences between the performance of the experimental and control groups, which may be an indicator of the impact of SD on working memory in the mice of the experimental condition (Novick et al., 2013). While these data are in agreement with the notion that stress and depression have been linked to cognitive impairment (Yuen et al., 2012; Scott et al., 2015; Perini et al., 2019), we have to acknowledge that this phenotype was not very robust. As such, when we analyzed the subgroups selected for molecular analysis, or mice that were tested in the Y-maze at 3 and 12 months of age, no significant difference was observed between SD-exposed and control groups. Since different tests are available to assess working memory in mice, it would also be important to repeat the analysis of working memory in other paradigms.

Long-term spatial reference memory (Redish & Touretzky, 1998; Mulder et al., 2003; Vorhees & Williams, 2006; Nunez, 2008) was analyzed in all groups. We did not observe any differences amongst groups

These data suggest that exposure of mice to SD stress at 3 months of age induces robust depressive-like phenotypes but does not lead to conclusive short or long-term memory impairments when measured immediately after the stress exposure. These data are in agreement with previous studies in which SD exposure during adulthood, unlike during adolescence, may not have a significant impact on cognition (Vassilev et al., 2021). According to Gunnar et al. (2009), the alterations in the reactivity of the hypothalamic-pituitary-adrenal (HPA) axis during adolescence have been presented as evidence. This phenomenon may heighten the reaction to emotional stimuli, which may not be as pronounced during adulthood or at least to the same degree. However, some studies have also identified long-term effects to the exposure to SD (Buwalda et al., 2005; Kovalenko et al., 2014; Xu et al., 2020), which we have been able to partially observe when re-analyzing the sample at 12 months of age.

This evidence may be a foundation for the theories that would indicate that exposure to environments with high levels of stress would not necessarily have an automatic impact on the cognitive functions of the subjects, known as resilient behavior (Friedberg & Malefakis, 2018. Schafer, 2022). However, the results of the same sample at 1 year old, show that there was a long-term impact at the behavioral level.

This is interesting when reviewing the literature that indicates the effects produced by exposure to traumatic contexts in early stages of development, could impact cognitive function, and produce depression at times when biological maturity has already been consolidated (Lähdepuro et al. 2019. Smith & Pollak, 2020. Hanson et al 2021).

We closer examined the animals that were exposed to SD stress, we could distinguish the mice into mice that developed clear depressive-like behavior in the forced swim test and some that did not and were therefore referred to as resilient mice. To further elucidate the underlying mechanisms we selected control mice, depressed mice and resilient mice for gene-expression analysis.

Differential gene expression (DGE) analysis from the ACC revealed a very small number of differentially expressed genes that were all down-regulated in depressed animals. Thus, only two genes were significantly deregulated genes when we compared control to depressed mice. These were Anpep (depressive vs. control) and Iigp1.

Notably, the functional description (gene ontology) of both enzymes corresponds to what is observed in human patients with Major Depression (MD), as demonstrated in GWAS studies for ANPEP (Barnes, Mondelli & Pariante, 2017). ANPEP has previously been identified in GWAS studies as a potential factor in major depressive disorder (Verbeek et al., 2012), with its role primarily linked to inflammation and immune response processes (Song, Kim & Lee., 2013). Although there is evidence suggesting a connection between the development of neuropsychiatric diseases and inflammation/immune system mechanisms (Najjar et al., 2013; Bennett & Molofsky., 2019), how these factors interact in the context of MD remains unclear. Our findings provide further support for the association of ANPEP with MD, which has been found to be downregulated in metabolic stress conditions (Sevayan et al., 2013), such as aging.

Iigp1 (Interferon-inducible GTPase 1) is a gene coding for a protein that belongs to the large family of GTPases (guanosine triphosphatases) (Klamp et al., 2003) and is known to be induced by interferon signaling. This protein plays an important role in the innate immune response (Tian et al., 2020), as it is involved in the recognition and

clearance of intracellular pathogens, particularly viruses and bacteria. Its expression has been implicated in the regulation of inflammation and the activation of immune cells (Uthaiah et al., 2002) such as macrophages and dendritic cells. Additionally, Iigp1 has also been found to be involved in other cellular processes such as autophagy and mitophagy (Ye, Zhou & Zhang., 2018), which are important for maintaining cellular homeostasis. A previous study found Iigp1 to be differentially expressed in response to chronic stress exposure in mice (M. Stankiewicz et al. 2015). Stankiewicz found that Iigp1 was downregulated in those mice that responded to stressful stimuli after a period of 13 days.

Our findings, along with previously reviewed scientific literature (Irwin & Miller, 2007; Beurel, Toups & Nemeroff, 2020), suggest that MD is associated with immune and inflammation processes in the CNS. Chronic stress, a known destabilizer of hypothalamic-pituitary-adrenal (HPA) activity, has been shown to act as a stimulant for anxiety and depression (E.Leonard, 2010). While further research is needed to establish definitive conclusions, these results highlight the importance of investigating immune and inflammatory processes in the CNS in the context of MD.

When we compared gene-expression in resilient and depressed mice we also observed two down-regulated genes. These were Foxd1 and also Iigp1. Although the evidence for Foxd1 is less, its role in inflammatory processes and autoimmunity (Lin & Peng, 2006) raises questions about its potential role in mice that were unresponsive to the SD procedures (i.e., resilient). FOXD1 is a transcription factor-encoding gene that participates in the development of multiple tissues, including the brain. Although FOXD1 has been implicated in major depression (MD) by some studies, the underlying mechanisms and implications of this association remain elusive. Polter and colleagues have demonstrated that the Forkhead family may represent a transcriptional target for the treatment of mood and anxiety disorders (Polter et al., 2009), suggesting a potential connection between FOXD1 and MD

Despite these findings, further investigation is necessary to comprehensively understand the role of FOXD1 in depression and identify potential therapeutic targets. It is worth noting that MD is a multifaceted disorder influenced by a broad range of genetic, environmental, and social factors, and no single gene or pathway can fully

explain its pathogenesis. In contrast, the downregulation of Iigp1 in both conditions may be a direct indicator of the absence of a differentiating gene of interest between the groups. Although it was possible to differentiate the groups by phenotype after behavioral testing, specifically after FST exposure, this difference was not reflected in the genome.

Foxd1 may warrant further investigation in the context of resilience to SD stress, since it was specifically down-regulated in resilient mice but not in animals that developed depressive-like phenotypes. A compelling strategy for delving deeper into the role of FOXD1 in mood regulation involves generating a Knockout model that permits the evaluation of subjects under diverse controlled conditions. For instance, utilizing the Elevated plus maze to evaluate anxiety and stress, and the MWM to examine the enduring impact of FOXD1 on spatial and reference memory, can be an interesting design to implement.

The other gene that was downregulated in depressed mice when compared to resilient mice was Iigp1, a gene that was also decreased when comparing control mice to depressed mice. These data might suggest that Iigp1 levels could play an important role to decide if SD stress leads to depressive-like phenotypes or not. However, when we compared the expression of Iigp1 via qPCR amongst control, depressed and resilient mice, Iigp1 was similarly down-regulated in depressed and resilient mice, suggesting that Iigp1 levels may not be linked to the expression of depressive-like behaviors but may rather play a role in other stress-induced processes, potentially cognitive aspects. It is in this context interesting to note that exposure of mice to SD stress at 3 months of age leads to impaired long-term memory in the water maze test. This data is in agreement with the hypothesis that exposure to adverse contexts can lead to cognitive dysfunctions over time (Klier & Buratto, 2020; Reijmers et al., 2001; Peavy, Salmon & Galasko, 2009). It would be therefore interesting to test if Iigp1 would be still downregulated in the ACC of 12 months old mice and if in addition other inflammatory processes might become deregulated. Support for this hypothesis stems for example from data showing that in mice exposure to inflammatory insults early in life can induce impairments in memory function and synaptic plasticity later in life (Wendeln et al., 2018; Beyer et al., 2020).

In conclusion, we show that SD stress in mice leads to gene-expression changes in the ACC. While the identified genes are interesting and are associated with pathways that play a role in depression and cognitive impairment, the observed changes are rather mild. Thus, only two genes were showing decreased expression upon SD-exposure and only 1 gene was differentially expressed when comparing resilient to depressed animals. These data may indicate that altered gene-expression in the ACC may not play a key role in development of depressive-like phenotypes and resilience to SD-stress.

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Author contributions

The behavioral experiments and corresponding bioinformatics analysis were carried out by JC, with support from TP in the analysis. SB conducted the qPCR, RNA-Seq library preparations, and sequencing. AF provided valuable input, corrections, and final review of the manuscript.

Competing interests

The authors declare no competing financial interests.

Data and code availability

Upon acceptance of the manuscript, all RNA-Seq data and code will be made publicly available through deposition in GEO and github correspondently.

CHAPTER 2

A transcriptome analysis in aging APP/PS1-21 mice as a model for Alzheimer's disease - like amyloid pathology

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Abstract

Amyloid pathology is a key hallmark of Alzheimer's disease (AD). Several mouse models have been developed to mimic amyloid pathology with the aim to test therapeutic approaches and further elucidate the mechanisms by which amyloid pathology affects brain function. Amongst other approaches, transcriptomics is an established method to elucidate molecular processes affected in diseased tissues, including the brain. The APP/PS1-21 mice represent an early-onset model for AD with first amyloid plaques occuring in the cerebral cortex already at 4 months of age, while first memory impairments are detected between 5-6 months of age. This model has been used to study pathological processes and testing therapeutic approaches but a through transcriptome analysis is missing. In this study, we analyzed gene expression of APP/PS1-21 mice at different time points (1.5, 4, and 8 months of age) in three different brain regions, namely the Anterior Cingulate Cortex (ACC), the hippocampal cornu ammonis region (CA1), and Dentate Gyrus (DG), Our results reveal a significant inflammatory response in all brain regions analyzed, which was first observed in 4 months old mice and thus correlated with the onset of amyloid pathology In addition to the coding transcriptome, we also detected a number of lncRNAs to be differentially expressed. In summary, our data provide an important resource and provide further evidence for the impact of amyloid pathology on neuroinflammation.

Keywords: Alzheimer's disease; APP/PS1; amyloid; learning and memory; cognitive dysfunction; differential gene expression

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Introduction

Amyloid pathology is a key hallmark of Alzheimer's disease (AD). Amyloid beta (Aβ) is a peptide generated through the processing of amyloid precursor protein (APP) (Zhang et al., 2011). APP is a transmembrane protein that is expressed in different types of cells, including neurons in the brain. APP can circulate through two different pathways, the amyloidogenic and the non-amyloidogenic pathway. Through the first, APP is cleaved by an enzyme called alpha-secretase. This mechanism cuts the protein in the Aβ domain, which prevents the generation of Aβ (Al Khashali et al., 2022). On the contrary, if the process arises through the amyloidogenic pathway, APP is cleaved by beta-secretase, which stimulates the production of Aβ and its subsequent pathological accumulation (Zhao et al., 2020).

Several animal models for amyloid pathology have been generated and most of them express mutant APP or PS1 either alone or in variable combinations (Elder et al., 2010; Sasaguri et al., 2017). These models have been instrumental to understand the

cellular changes and pathological consequences mediated by amyloid pathology. The APP/PS1 mice generated by Radde et al. over-express mutated amyloid precursor protein (KM670/671NL) and mutated presenilin 1 (L166P) under the control of a neuron-specific Thy1 promoter element and are referred to as APP/PS1-21 mice (Radde et al. 2006). The APPPS1-21 mice show first signs of hippocampal amyloid plaque pathology already at 3 months of age (Radde et al., 2006). Depending on the employed paradigm, first memory impairments have been observed at 5 - 6 months of age (Radde et al., 2006; Govindarajan et al., 2011; Van den Broeck et al., 2019). The APPPS1-21 model has also been successfully used to study different therapeutic approaches (Govindarajan et al., 2011; Kim et al., 2011; Lo et al., 2013; Ulrich et al., 2014; Jay et al., 2017; Dodiya et al., 2020). Surprisingly, although RNA-sequencing (RNAseq) data has recently been provided for the dorsal cerebral cortex of 9 week old mice in the context of a manipulated gut microbiome (Dodiya et al., 2021), a thorough transcriptome analysis of this mouse model is still lacking.

Therefore we performed RNAseq in 3 different brain areas relevant to AD pathogenesis, namely the Anterior Cingulate Cortex (ACC), as well as the hippocampal cornu ammonis region 1 (CA1) and the Dentate Gyrus (DG). Furthermore, we performed a 3 different time points to reflect a very early (1.5 months of age), middle (4 months of age) and advanced (8 months) stage of pathology. Our data reveals a marked and forceful upregulation across all the cerebral regions examined, displaying an exponential escalation that becomes prominently evident after the fourth month. Our functional analysis of the physiological mechanisms triggered by the deposition of amyloid plaque evinces a preponderance of immune system, inflammatory and defense response biological processes.

Materials and Methods

Animals

For this study, mice were selected from the APPPS1-21 mouse strain (Waldron et al., 2016) B6-Tg(Thy1-APPswe; Thy1-PS1 L166P) which included both wildtype and transgenic mice heterozygous positive for human APP with the Swedish mutation (Sturchler-Pierrat et al., 1997) and human PS1 with a point mutation at position 166

(leucine to proline) (Moehlmann et al., 2002). The mice were categorized into three age groups: 1.5 months (young and healthy), 4 months (mid-age with early AD pathology), and 8 months (old with late AD pathology) for the experiments.

All mice were raised with their respective littermates in groups and housed in individually ventilated cages (365x207x140mm) unless specified. They were kept in a 12 hour light/dark cycle with a consistent room temperature of 22oC and air humidity of 58%. Food and water were provided ad libitum. The housing, breeding, and animal experiments were planned and conducted in strict accordance with the German Federal Act on the Protection of Animals and were approved by the responsible ministry of Lower Saxony (”Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit”).

Genotyping

To extract DNA from mouse tail biopsies, 200µl of DirectPCR tail-lysis buffer (Viagen Biotech; Los Angeles, USA) and 40µg of Proteinase K (7528.2 from Carl Roth GmbH + Co. KG; Karlsruhe, Germany) were added to the samples, which were then incubated in a thermoshaker at 1400rpm and 55oC for 3 hours followed by 85oC for 45 minutes. PCR was performed using DreamTaq polymerase (Thermo Fisher Scientific; Waltham, USA) with primers for both human APP (3’: GAATTCCGACATGACTCAGG, 5’: GTTCTGCTGCATCTTGGACA, amplicon size: 264bp) and human PS1 (5’: AATAGAGAACGGCAGGAGCA, 3’:GCCATGAGGGCACTAATCAT, amplicon size: 608bp). The resulting DNA samples were analyzed using gel electrophoresis on a 1.5% agarose gel containing 0.1% ethidium bromide.

Brain dissection

To obtain mouse brains for dissection, cervical dislocation and decapitation were performed. The scalp was carefully opened to expose the skull and the posterior and lateral muscles were cut to facilitate brain isolation. The occipital, temporal, and parietal bones were removed after a precise cut that fractured the interparietal bone. A

transverse incision of the frontal bone allowed for the removal of the brain, which was immediately placed on a sterile metal plate on ice for further dissection.
The olfactory bulbs and the most anterior 0.5mm of the forebrain were excised using a scalpel. A second coronal cut, 3 mm posterior to the previous one, was made to obtain a brain slice containing the anterior cingulate cortex (ACC), a darker triangular region located dorsally to the corpus callosum. A superficial cut along the midline of the forebrain was then performed, followed by dissection of the forebrain hemispheres and separation from the thalamus using a surgical scoop. The hippocampus was then isolated by removing the vessels and meninges, and the dentate gyrus (DG) was dissected from the hippocampus.

RNA isolation

In order to avoid contamination and RNA degradation, all equipment used for RNA isolation was washed with 70% ethanol and RNAseZap, and the RNA isolation was performed in a sterile fume hood. Brain samples were kept on dry ice until processing, and all steps were performed on ice to prevent degradation. The protocol used for RNA isolation was adapted from the TRIzol reagent protocol provided by Thermo Fisher Scientific.

For each sample, 500µl of TRIzol-reagent and 10 ceramic beads were added before homogenization in an Omni Bead-Ruptor 24 for 10 seconds at a relative speed of 2.6. The samples were then incubated at room temperature for 5 minutes to ensure proper lysis of the tissue. Chloroform was added, and the samples were manually mixed for 15 seconds before incubating for another 5 minutes at room temperature. After centrifugation at 12,000g for 15 minutes at 4°C, the transparent upper phase was transferred to a fresh tube. 250µl of isopropanol and 1µl of GlycoBlue were added to each sample before incubating overnight at -20°C to precipitate the RNA.

Afterwards, the samples were centrifuged at 12,000g for 30 minutes at 4°C and the supernatant was carefully discarded. The RNA pellets were washed by mixing with 300µl of 75% ethanol, vortexing, and centrifuging at 12,000g for 5 minutes at 4°C. This washing step was repeated once, and the tubes were briefly spun and freed from remaining liquid before air-drying the RNA pellets for 2 minutes to clean them from

ethanol. Finally, the RNA pellets were redissolved in sequencing graded water and incubated for 10 minutes at 42°C before proceeding with DNAse treatment.

RNA sequencing

The genome-wide gene expression analysis of messenger RNA from the ACC, CA1, and DG of both wildtype and transgenic APP/PS1 mice aged 1.5, 4, and 8 months (n=6) was conducted through sequencing using a HighSeq 2000 system (Illumina; San Diego, USA). For the CA1 region, paired-end RNA libraries were prepared, while single-end libraries were prepared for the ACC and DG regions using the TruSeq RNA Library Preparation Kit v2. Subsequently, the TruSeq PE Cluster Kit v3-cBot-HS or TruSeq SR Cluster Kit v3-cBot-HS were used, following the standard protocols from Illumina. The quality of RNA libraries was assessed using a Nanodrop 8000 (Thermo Fisher Scientific; Waltham, USA), Qubit 2.0 fluorometer (Life technologies; Carlsbad, USA), and RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, USA).

NGS data analysis

Sequencing reads obtained from biological samples were processed as FASTQ files, quality control and independent analysis for each sample confirmed the quality of the data. Mapping to the genome was performed using STAR software. Bam files obtained after genome mapping (mouse), were used to create the counts reads associated with genes. Read counts associated with genes, normalization and unsupervised clustering analysis were performed before differential gene expression analysis (DGE).

Differential gene expression

Counts from RNAseq were produced using FeaturesCount. DGE was carried out with the DESeq2 toolbox run in R. During DGE analysis read counts associated with genes, normalization and unsupervised clustering analysis were used as quality control procedures. In detail: Set-up variables and libraries of interest, associate text to genes. Load GTF annotation for later computing of Fragments per kilobase million (FPKM's). Next step, loading, curating, and organizing data considering the variable "coldata" as the main function to use the data counts. Visual diagnostic through PCA,

normalization and filtering of counts. Computing FPKM's and extracting counts. Mapping ensemble and gene IDs in the order presented by DESeq results. Those genes with an adjusted p value <0.05 were considered significant differentially expressed genes. Additionally, the gene lists obtained by DESeq2 were filtered with a cutoff for log2 fold-changes of ±0.26. Finally, plots of interest were produced, and results saved.

Results

Pathogenesis in APP/PS1-21 mice correlates with a massive upregulation of genes linked to immune-related processes

The ACC as well as the hippocampal CA1 and DG regions were collected from 1.5, 4 and 8 months old APPPS1-21 mice. The tissue was subjected to RNA isolation and RNAseq was performed. First we performed a differential gene-expression (DGE) analysis of the three brain regions (ACC, CA1, and DG) comparing APPPS1-21 to the corresponding wild type (WT) littermates that served as controls.

While in ACC 297 genes were significantly deregulated (230 genes were upregulated and 67 genes were downregulated) in 1.5 months old mice, the number of deregulated genes increased at 4 and 8 months of age (Fig 1A). Thus, at 4 months of age 643 genes were significantly deregulated (471 genes were upregulated and 172 genes were downregulated) while at 8 months of age already 1580 genes were significantly deregulated (1182 genes were upregulated and 398 genes were downregulated) (Fig 2A, left panel).

For CA1 10 genes were significantly deregulated (7 genes were upregulated and 3 genes were downregulated) in 1.5 months old mice, the number of deregulated genes as in ACC increased at 4 and 8 months of age (Fig 1B). 253 genes were significantly deregulated (238 genes were upregulated and 15 genes were downregulated) while at 8 months of age 1420 genes were significantly deregulated (1259 genes were upregulated and 161 genes were downregulated) (Fig 2A, center panel).

In DG 3 genes were significantly deregulated (1 genes were upregulated and 2 genes were downregulated) in 1.5 months old mice, the number of deregulated genes increased at 4 months, with a tendency to downregulated genes at 8 months of age (Fig 1C) . At 4 months of age 78 genes were significantly deregulated (77 genes were upregulated and 1 genes were downregulated) while at 8 months of age 1896 genes were significantly deregulated (774 genes were upregulated and 1122 genes were downregulated) (Fig 2A, right panel).

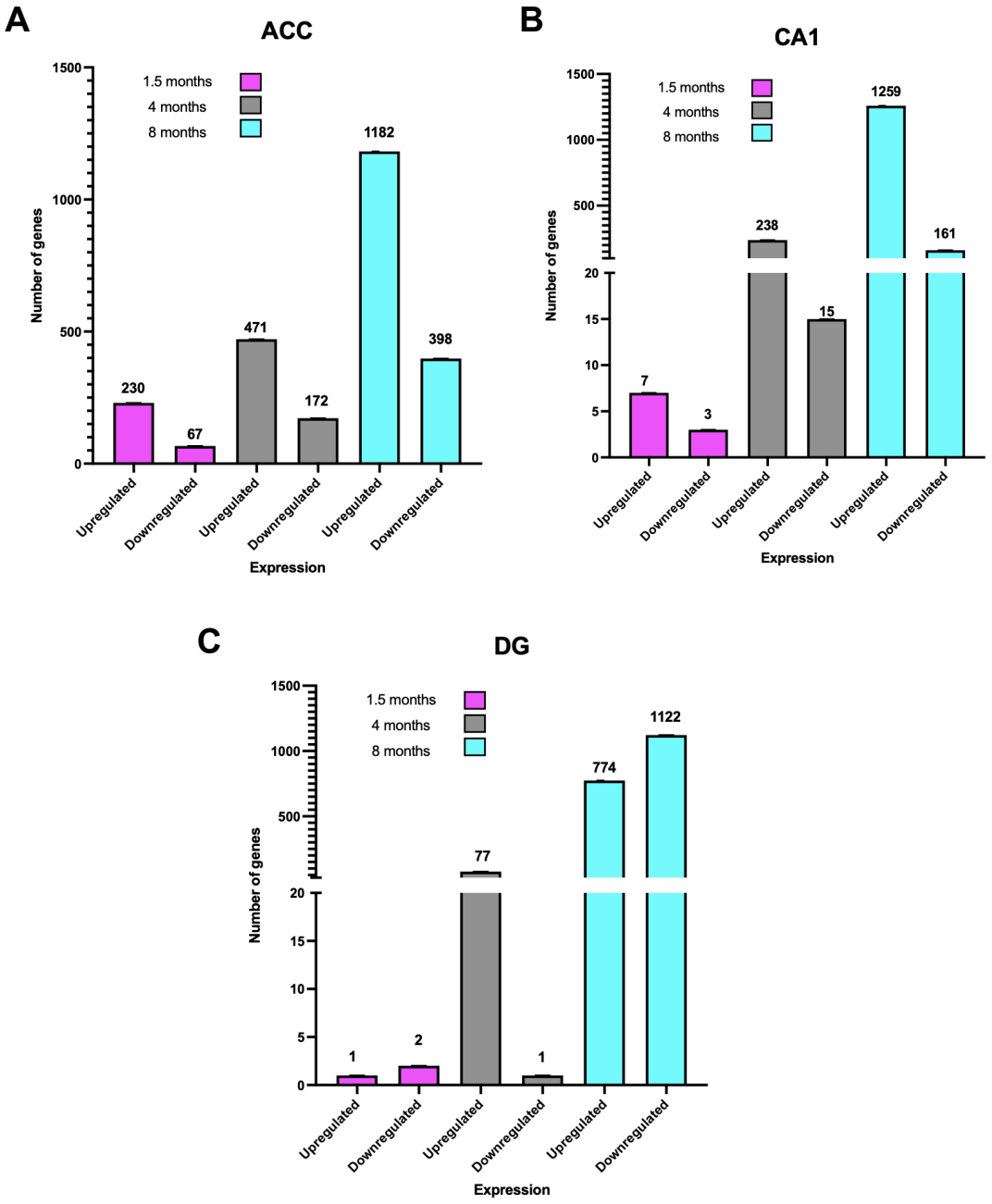


Figure 1. APP/PS1 mouse model shows exponential increase in gene upregulation during amyloid pathological progression. A: Bar plot showing up and downregulated genes in ACC. An exponential upregulation is clearly observed from

month 1.5 to month 8. **B:** Bar plot showing up and downregulated genes in CA1. An exponential upregulation is clearly observed from month 1.5 to month 8. **C:**Bar plot showing up and downregulated genes in DG. An exponential upregulation is clearly observed during month 4, with a tendency to downregulate genes at month 8.

The majority of the differentially expressed genes were up-regulated. When compared to downregulated genes it was also obvious that the degree of deregulation was more pronounced for up-regulated genes, when considering the fold change as well as the FDR. The only exception to this pattern was in DG at 8 months, where a greater downregulation of genes related to neuronal development processes was observed.

Next we subjected the lists of up- and downregulated genes to GO term analysis. While this approach did not yield and significantly enriched processes for 1.5 months old mice, a closer look at the deregulated transcripts identified interesting genes. Prr7, Schip1, and C1qtnf4 genes have been identified to undergo upregulation in the anterior cingulate cortex (ACC) at 1.5 months. Prr7 gene's activity is involved in apoptotic signaling (Hrdinka et al., 2011), while Schip1 is known to regulate axon development and the formation of neuronal circuits (Klinger et al., 2015). C1qtnf4, on the other hand, has been implicated in autoimmune disease (Vester et al., 2021). The upregulation of these genes could signify early genetic-level effects contributing to the pathological development of amyloid.

At 4 months, ACC showed 471 upregulated genes, with biological processes related to inflammasome complex assembly, regulation of defense response, and innate immune system as the most prominent categories (Figure 2B). Meanwhile, 172 downregulated genes were observed, highlighting central nervous system myelin maintenance and myelination categories (Figure 2C). At 8 months, 1182 genes were upregulated, with categories similar to those seen at 4 months (inflammatory response, defense response regulation, and immune system; Figure 2D). While 398 genes were downregulated at 8 months, with predominant presence in synaptic signaling categories (Figure 2E)

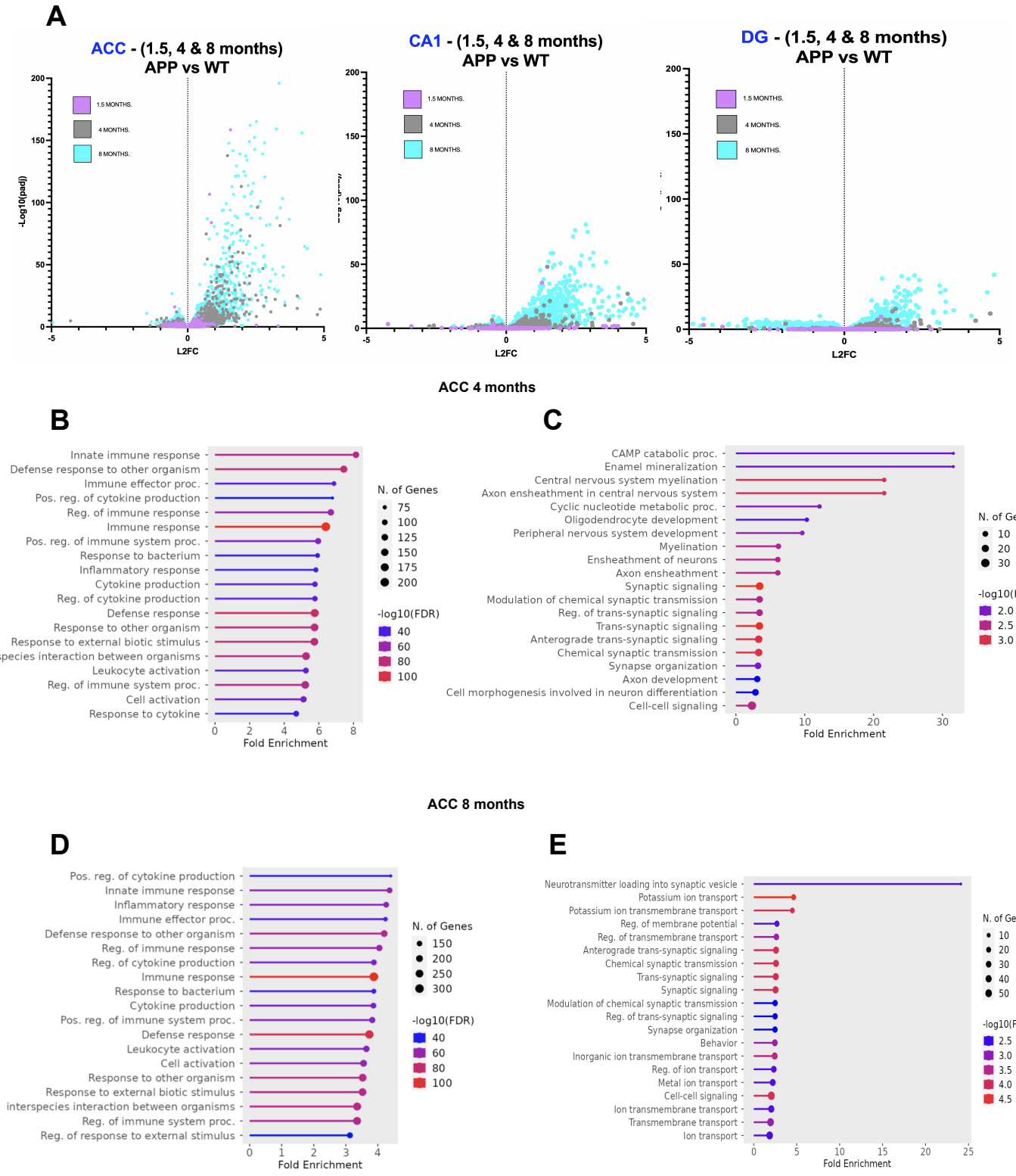
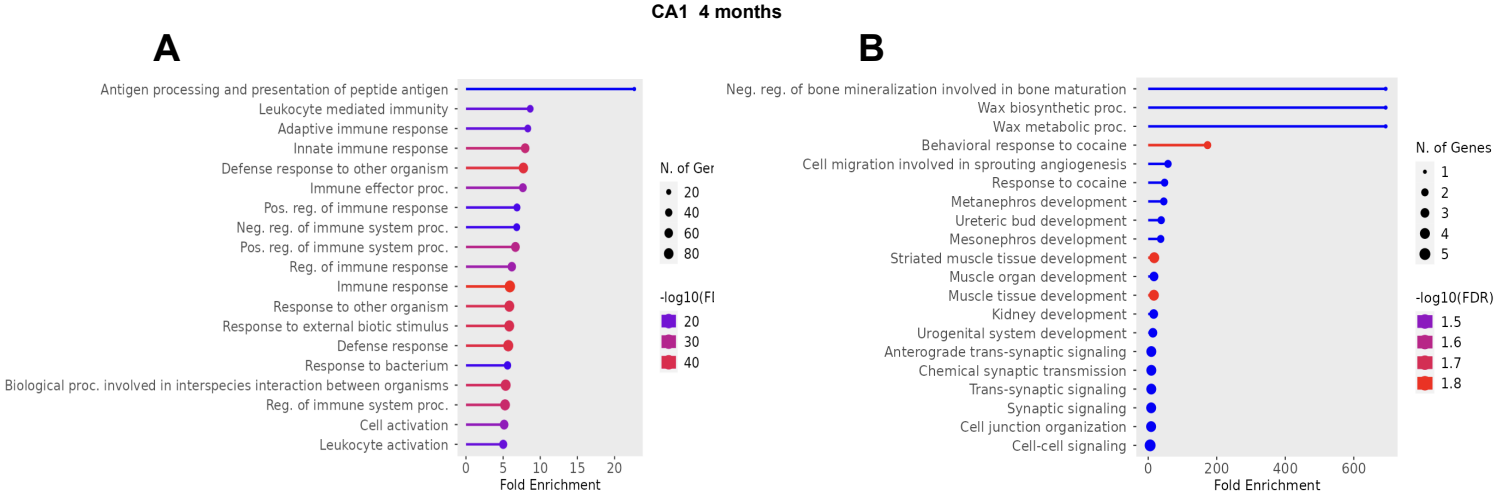


Figure 2. APP/PS1 mouse model shows a strong inflammatory response, during accumulation of amyloid plaques in Anterior Cingulate Cortex (ACC), CA1 and Dental Gyrus (DG). A: The study conducted volcano plots of the gene expression changes between wildtype and transgenic APP/PS1 in the three brain regions (ACC, CA1 & DG) at different time points (1.5, 4 and 8 months). Exponential upregulation in all conditions with a difference of 0.26 L2FC and padj < = 0.05. **B:** Gene ontology categories (biological function) from upregulated genes in the ACC at 4 months old. Predominance of inflammation, defense and immune response categories is observed. **C:** Gene ontology categories (biological function) from downregulated genes in the ACC. Uncovered deregulation of the nervous system myelination processes. **D:** Gene ontology categories (biological function) from upregulated genes in the ACC at 8 months old. Predominance of inflammation, defense and immune response categories is

observed. **E:** Gene ontology categories (biological function) from downregulated genes in the ACC at 8 months old. Predominance of synaptic signaling and cell communication categories is observed.

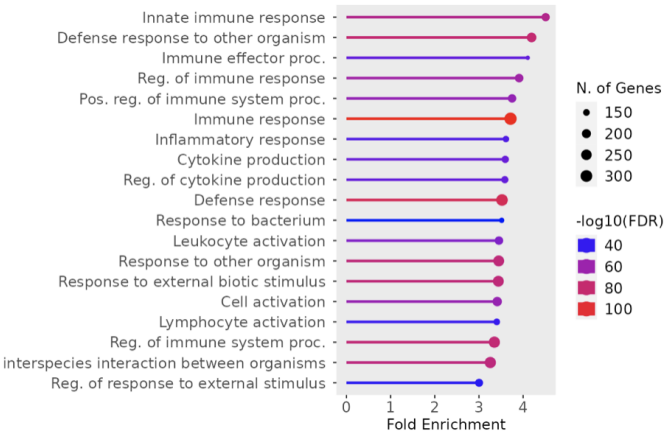
For CA1, 238 genes were upregulated at 4 months in the context of immune and defense response categories (Fig 3A), and 15 were downregulated with the highest number of genes expressed in synaptic signaling and cell-cell signaling processes (Figure 3B). At 8 months, 1259 upregulated genes were mostly involved in immunological and defense response processes (Figure 3C), while 161 downregulated genes affected synaptic communication at the level of biological processes (Figure 3D).

Dentate Gyrus had 77 upregulated genes at 4 months, with immune response categories being the most prominent (Figure 3E). And 1 gene was observed to be downregulated during this period after applying filters. At 8 months, 774 genes were upregulated in DG, with immune and defense response categories predominating (Figure 3F). Finally, 1122 genes were downregulated in DG at 8 months, with deregulation of synaptic signaling, neuronal development, and neurogenesis processes as the main categories (Figure 3G).



CA1 8 months

C



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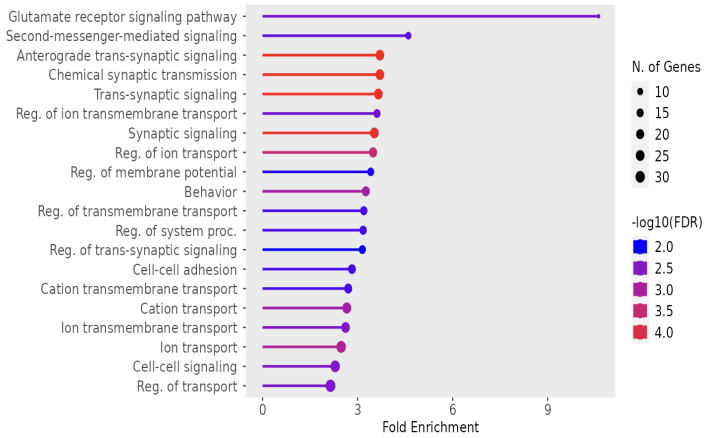
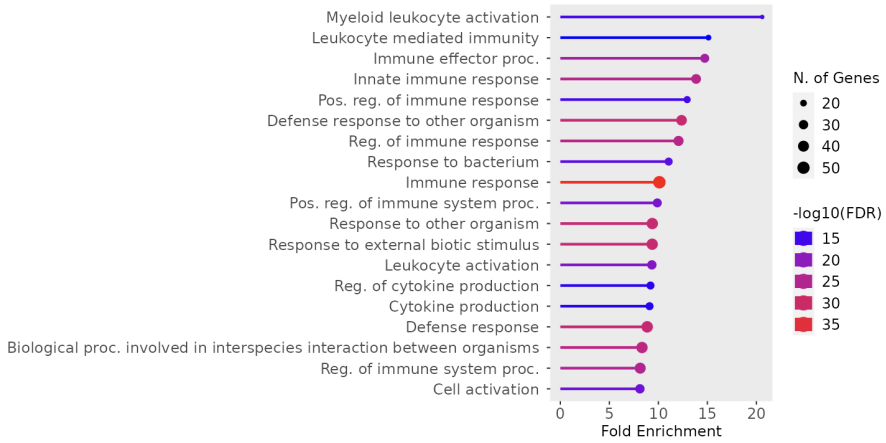


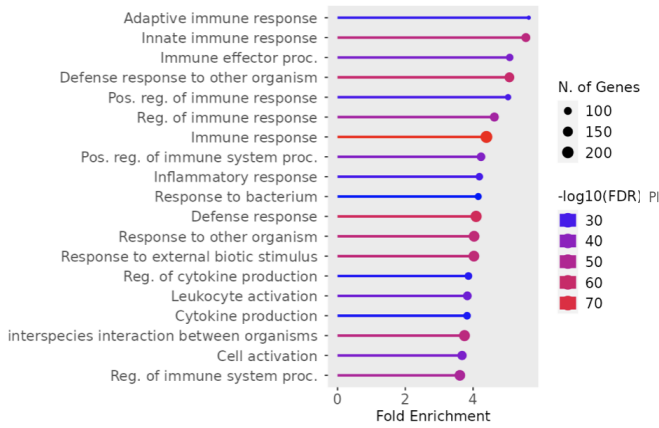
DG 4 months

E



DG 8 months

F



G

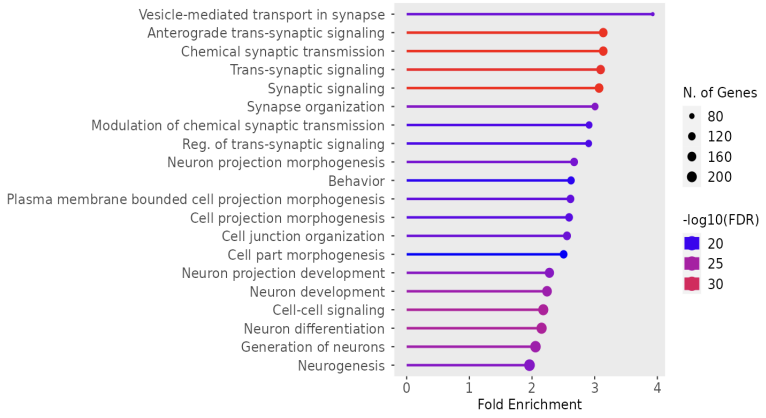


Figure 3. The APP/PS1 mouse model exhibits an upregulation of immune system, defense, and inflammatory response, as well as a downregulation of synaptic signaling in CA1 and DG at 4 and 8 months of age. A: CA1 at 4 months old, upregulated genes are primarily associated with immune system processes. **B:** Downregulated genes in the CA1 at 4 months are associated with deregulation of synaptic signaling categories. **C:** CA1 at 8 months old, upregulated genes as at month 4 are primarily associated with immune system processes. **D:** Downregulated genes in the CA1 at 4 months are associated with deregulation of synaptic signaling and cell communication categories. **E:** DG at 4 months old, upregulated genes are mainly related to immune response categories. **F:** DG at 8 months old, upregulated genes display a clear immune response of the system. **G:** downregulated genes at 8 months are associated with deregulation of synaptic signaling and neuronal development processes.

The APP/PS1 mouse model exhibits dysregulation of central nervous system myelination in the ACC, along with impacts on synaptic signaling and neuronal development in the CA1 and DG regions.

One important note to highlight is that the biological processes extracted from downregulated genes show different response mechanisms between the ACC and hippocampal regions. In the ACC, it is possible to observe deregulation of central nervous system myelin maintenance, central nervous system myelination, axon ensheathment and oligodendrocyte development (Figure 2C). Regarding the hippocampal regions, it appears that the synaptic signaling mechanisms are more affected (Figure 3B & D), as well as the cell communication process in CA1. The progression of amyloid plaques in hippocampal structures, specifically in DG, seems to affect neuron development, neuron differentiation, and neurogenesis processes (Figure 3G).

Results of gene expression analysis in an 8-month APP/PS1 mouse model indicate that there are common upregulated genes involved in immune system and inflammation response in the Anterior Cingulate Cortex, Hippocampus CA1, and DG

Using Venn diagrams for the gene lists of the DGE results at month 8 (Figure 4A), it was possible to identify 479 upregulated genes differentially expressed shared among the three sequenced brain regions (ACC-CA1 & DG), that are involved in the immune system, defense response and inflammatory processes (Figure 4B).

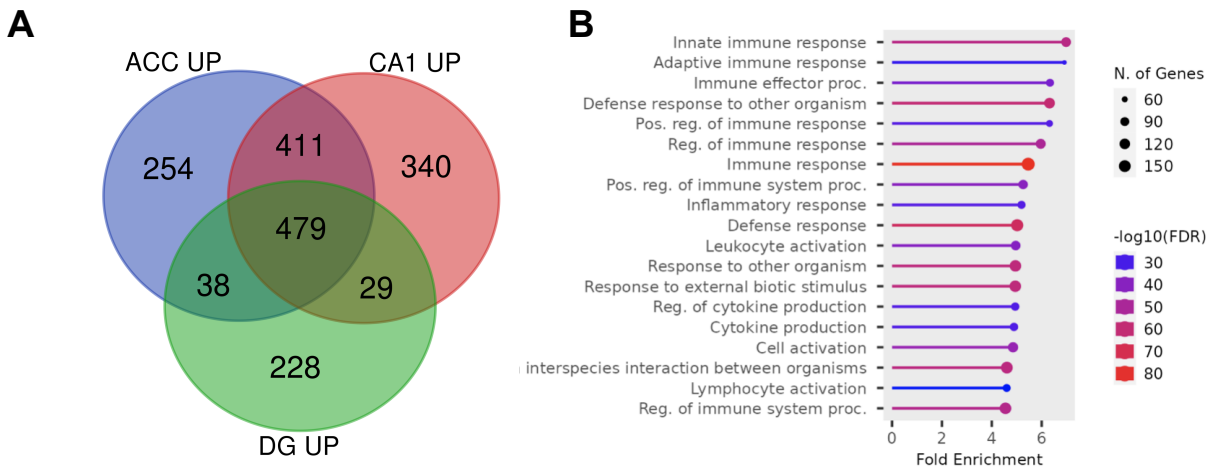


Figure 4. According to the gene expression analysis the Anterior Cingulate Cortex, Hippocampus CA1, and DG in an 8-month APP/PS1 mouse model share upregulated genes that are involved in immune response and inflammation. A: Venn diagram reveals 479 upregulated genes that intersect between the three brain regions (ACC-CA1-DG) at 8 months. **B:** Common upregulated genes between regions are associated with biological processes of the immune system, inflammatory and defense response.

Evidence from single cell data, shows that genes shared between cortex and hippocampus regions are highly expressed in microglia.

With the aim of reduce the list of gene of interest, seven of the preliminary 479 genes (Casp6, Eml3, Lpcat3, Acads, Slc29a1, Mif4gd, and Idua) were selected for further study due to their ontological relevance in the context of the APP/PS1 mouse model. Their expression levels were subsequently reviewed according to cell type. The use of Tabula Muris data (The Tabula Muris Consortium, 2018) allowed for the identification of higher expression levels of Cap6, which is involved in cell death, in endothelial cells. Lpcat3, whose primary function is to regulate stress response and inflammation, and Mif4gd, which is involved in RNA binding, also displayed significant expression levels in Oligodendrocytes (Figure 5).

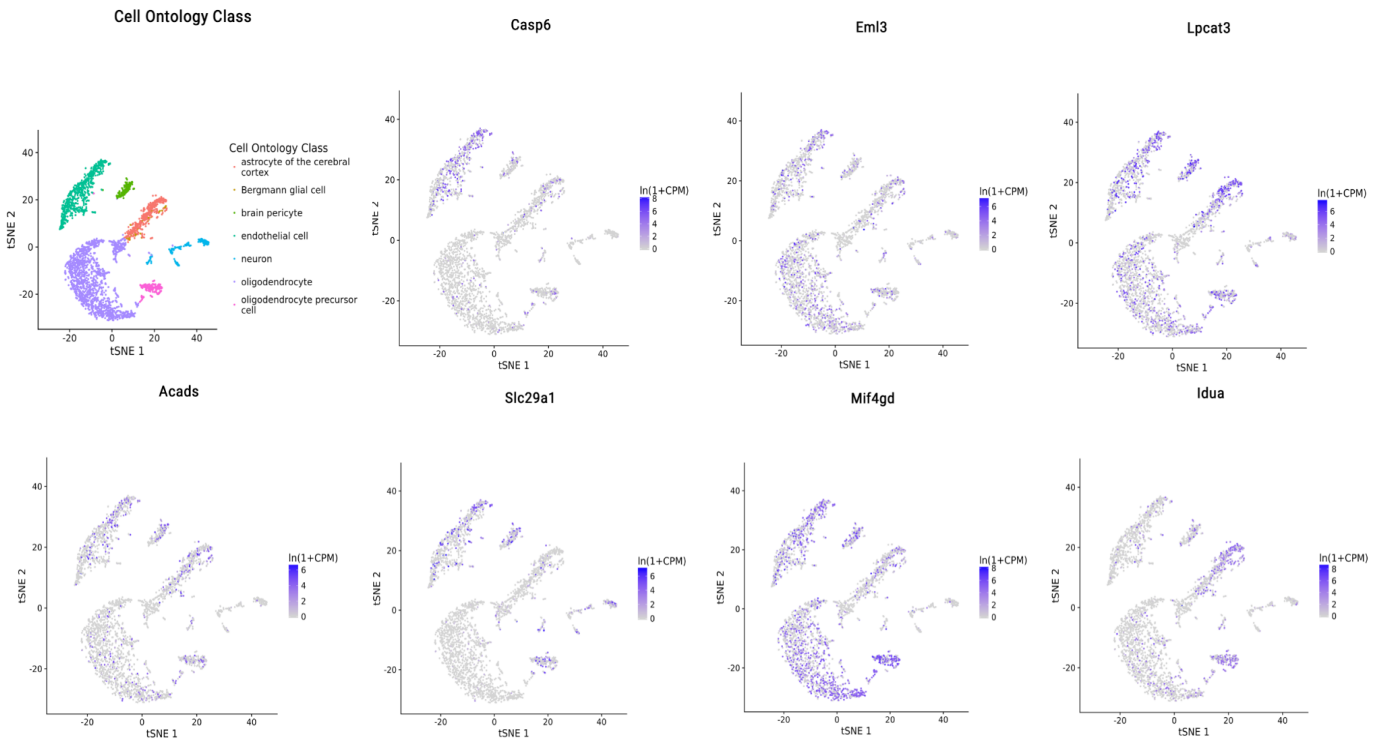


Figure 5: Single cell data from Tabula Muris shows endothelial cell and oligodendrocytes activation in Casp6, Lpcat3 and Milf4gd: UMAPs of single cell data showing different levels of expression between the 7 selected genes in the context of the APP/PS1 mouse model.

Following the evaluation of gene expression levels in endothelial and oligodendrocyte cells for Casp6, Lpcat3, and Milf4gd, the expression levels of these genes in microglia were assessed (Figure 6). Tabula Murris data revealed that all genes were highly expressed in microglia, with Lpcat3 and Slc29a1 showing the highest levels. These findings support the hypothesis of a significant inflammatory response in the APP/PS1 mouse model, potentially masking many of the underlying mechanisms related to AD.

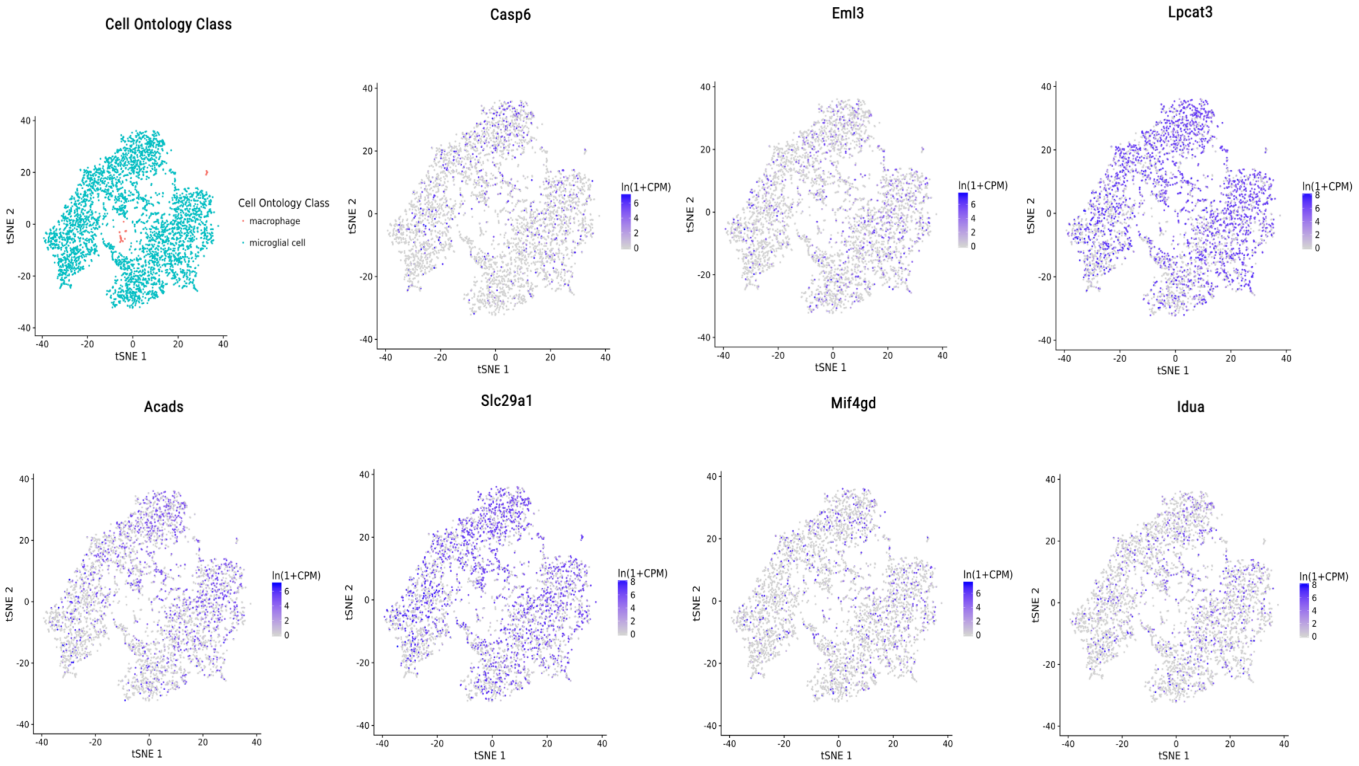


Figure 6: Single cell data from Tabula Murris shows predominance of microglia expression in all selected genes, with higher levels in Lpcat3 and Slc29a1: UMAPs of single cell data showing different levels of microglia expression between the 7 selected genes in the context of the APP/PS1 mouse model. Tabula Murris demonstrated that microglia showed greater expression of the selected genes compared to the expression levels observed in endothelial cells and oligodendrocytes (Figure 4).

Downregulated genes at 8 months in the ACC, CA1, and DG regions exhibit similar processes related to synaptic signaling, organization and transmission

Another crucial aspect of the DGE analysis was to define the genes that were downregulated in ACC, CA1, and DG which were common to all three regions at 8 months old. The Venn diagram presented in Figure 7A illustrates that 14 genes were identified as being downregulated in cortex and hippocampus. By performing functional analysis of these genes, we found that they are functionally associated with synaptic signaling, transmission, and organization (Figure 7B).

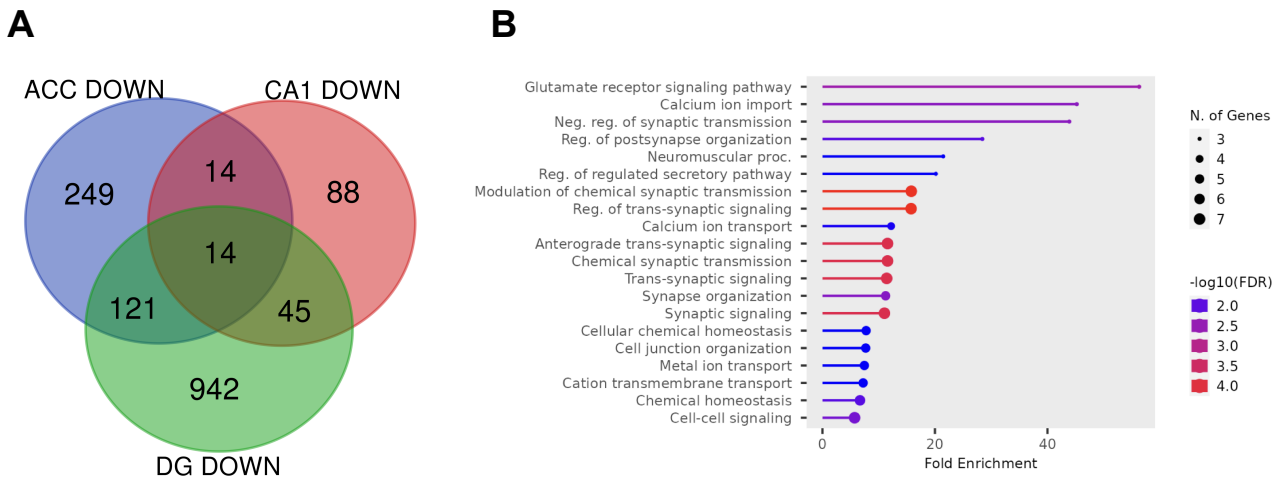


Figure 7. Downregulation of synaptic signaling, organization and transmission as common processes between ACC, CA1 and DG. A: Venn diagram reveals 14 downregulated genes that intersect between the three brain regions (ACC-CA1-DG) at 8 months. **B:** Common downregulated genes between regions are associated with synaptic signaling, organization and transmission biological processes.

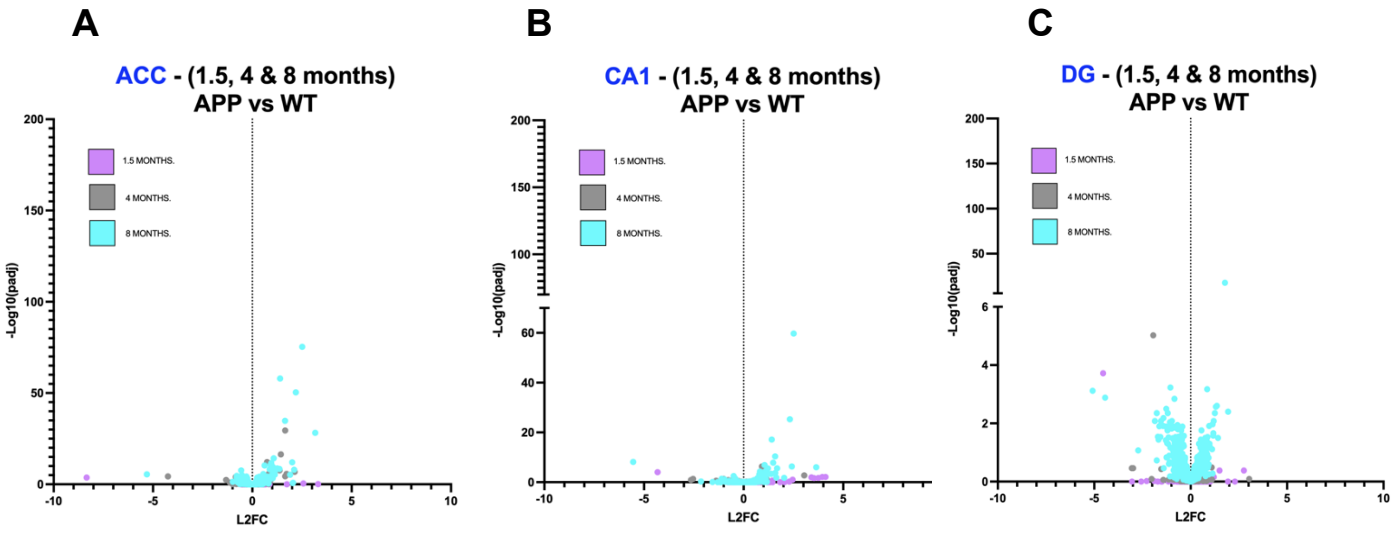
Long Non Coding RNAs in the APP/PS1 mouse model during AD progression

Following the completion of the DGE analysis on the totalRNAseq data, the focus shifted to performing a DGE analysis on noncoding RNAs (ncRNAs). While there have been recent studies on the impact of ncRNAs on neurodegeneration and neuronal inflammation processes (Idda et al. 2018; Ma et al. 2020; Zhang, He & Bian, 2021; Li et al. 2021; Dong et al. 2021; Lan et al. 2022; Hao et al. 2022; Varesi et al. 2022), the mechanisms by which ncRNAs, specifically lncRNAs, influence these processes remain unclear.

The expression pattern of the lncRNAs following the DGE analysis showed a similar exponential upregulation from month 1.5 to month 4 and 8 as observed in the totalRNAs (Figure 8A, B & C). This pattern was observed in both the ACC and CA1 regions, although in the DG region, there was a greater degree of down and upregulation of the lncRNAs at month 8 compared to the ACC and CA1 regions.

The analysis of long noncoding RNA (lncRNA) expression in the Anterior Cingulate Cortex, CA1, and Dental Gyrus of APP/PS1 mice revealed differential expression patterns associated with the regulation of nervous system homeostasis and apoptosis.

Following the DGE analysis of LncRNAs in the ACC region (Figure 8A), data was filtered by applying an adjusted p-value threshold of < 0.05 and a log2 fold-change cutoff of ±0.26. The results of this analysis were as follows: at month 1.5, 1 downregulated lncRNA, and two upregulated lncRNAs were identified, which have recently been described in post-transcriptional regulatory functions in primary neurons (Zhang et al., 2023) At month 4, 16 downregulated lncRNAs involved in the regulation of nervous system homeostasis were identified, alongside 16 upregulated lncRNAs involved in autoimmune disease, inflammatory response, and apoptosis. Finally, at 8 months, 32 downregulated lncRNAs previously associated with the inflammatory response and the regulation of natural killer cells (NKC) were identified, along with 53 upregulated lncRNAs that are highly expressed in microglia according to single cell data.



D

Ensemble ID Mouse	Mouse Symbol	Human Homolog	Human Symbol	ACC	Month	CA1	Month	DG	Month
ENSMUSG00000087197	**Gm13780**	ENSG00000255314	LOC101928894	Down	8	Down	8	Down	8
ENSMUSG00000097415	**AU020206**	ENSG00000259416	LINC02883			Up	4	Up	4
ENSMUSG00000085438	**Oip5os1**	ENSG00000247556	OIP5-AS1	Down	8			Down	8
ENSMUSG00000090063	**Dlx6os1**	ENSG00000231764	DLX6-AS1	Down	8	Down	8		
ENSMUSG00000100252	**Mir124-2hg**	ENSG00000254377	MIR124-2HG	Down	8			Down	8
ENSMUSG00000113121	**Gm35161**	ENSG00000223597	LINC02142	Down	8			Down	8

Figure 8: Long Non Coding RNAs in the APP/PS1 mouse model during AD development. A,B & C: Volcano plots of the gene expression changes for LncRNAs between wildtype and transgenic APP/PS1 in the three brain regions (ACC, CA1 & DG), at different time points (1.5, 4 and 8 months). L2FC 0.26 difference and padj < = 0.05. **D:** Table with significant LncRNAs shared between month 4 and 8 in ACC, CA1 and DG. Description of ensemble ID, gene symbol and gene expression (up or down) for mouse and human homolog.

The expression profile of long noncoding RNAs (LncRNAs) in the CA1 region of APP/PS1 mice was analyzed at different stages of pathological progression, as shown in Figure 8B. At month 1.5, no downregulated lncRNAs were found within a significant range, while five upregulated ncRNAs and lncRNAs were identified that participate in the regulation of homeostasis, the function of endothelial cells, and are expressed in astrocytes and natural killer cells based on previous studies. At month 4, one downregulated lncRNA was identified in single cell data that was highly expressed in microglia, and one upregulated lncRNA was found that is known for its role in regulating cell proliferation, apoptosis, and the cell cycle. Finally, at month 8, two downregulated lncRNAs were identified that regulate apoptosis and inflammation, while 15 upregulated lncRNAs were found that are involved in cellular signaling and have previously been described in the context of natural killer cells. Furthermore, evidence from single cell data suggests that these upregulated lncRNAs act as regulators of inflammatory processes due to their presence in microglia.

The differential gene expression (DGE) analysis of the Dentate Gyrus (DG) region (Figure 8C) revealed no significant up or downregulated long noncoding RNAs at 1.5 months. At 4 months, one downregulated lncRNA was found, which has been associated with the regulation of synaptic signaling processes and expressed in killer cells, and one upregulated lncRNA was identified, which is also expressed on natural killer cells. At 8 months, 39 downregulated lncRNAs were observed, which have been linked to immune system inflammation and have been shown to be expressed in

neurons and microglia. Some of these lncRNAs are also implicated in melanoma based on cancer studies. Furthermore, 20 upregulated lncRNAs were identified, primarily expressed in brain microglial cells, and have been associated with inflammatory processes.

The inflammatory response in the APP/PS1 mouse model appears to involve commonly expressed lncRNAs in the ACC, CA1, and DG regions during the 4th and 8th months.

Six lncRNAs (Gm13780, AU020206, Oip5os1, Dlx6os1, Mir124-2hg, Gm35161) were selected based on their expression in the ACC, CA1, and DG during month 4 and 8, and the possibility of finding homologues in humans. Figure 8D shows that, except for LINCO2883, which is homologous to AU020206 and upregulated in APP vs WT, the other five genes are downregulated in the same comparison. Although their functions and biological processes are not well-known, it was possible to associate these downregulated lncRNAs with immune response processes in the CNS.

Discussion

The present study aimed to investigate the differential gene expression patterns of coding and non-coding RNAs in the APP/PS1 mouse model of Alzheimer's disease (AD), with a focus on understanding cognitive dysfunction. To achieve this goal, we utilized sequencing data produced by Michael Gertig (Gertig, 2016), which provided access to the genetic expression of the APP/PS1 brain regions, including ACC, CA1, and DG. These regions were specifically chosen as reference areas for the study of different aspects of memory, with ACC being associated with remote memory processes, CA1 with memory formation, consolidation, and retrieval in the hippocampus, and DG with the initial processing of information.

Furthermore, the use of sequencing data from 1.5, 4, and 8 months provided a comprehensive understanding of the effects of amyloid accumulation in these brain regions over time. By analyzing the expression of coding and non-coding RNAs, we were able to gain insight into the molecular mechanisms underlying cognitive dysfunction in AD. Our findings shed light on the role of specific genes and lncRNAs in immune response processes in the CNS and their potential involvement in the

pathogenesis of AD. Overall, this study contributes to our understanding of the genetic mechanisms underlying cognitive impairment in AD and highlights potential targets for future therapeutic interventions.

Differential gene expression in the APP/PS1 mouse model during amyloid pathological progression

The results of differential gene expression (DGE) analysis for ACC, CA1, and DG in the time periods examined indicate a gradual increase in the upregulation of differentiated genes at 8 months (as described by Lukiw & Bazan, 2000, Heneka et al., 2015, and Li et al., 2022), as noted by Gengler, Hamilton & Hölscher (2010). Although DGE also yielded relevant results for month 4, the upregulation during this period was less noticeable when compared to month 1.5, as reported by Yan et al. (2009). To provide an accurate description of these findings, functional analysis of the gene lists for each condition was conducted. The gene ontology for all sequenced areas during months 4 and 8 revealed a high prevalence of categories related to inflammation, the immune system, and defense response (as described by Serpente et al., 2014), which is consistent with the known inflammatory response of the system due to the accumulation of amyloid plaques in the cortex and hippocampus (as reported by Meraz-Ríos et al., 2013, and López-González et al., 2015). Interestingly, in ACC, a downregulation of central nervous system myelination and oligodendrocyte development was observed, which may suggest that ACC plays a crucial role in regulating an appropriate neuronal communication system.

In contrast, CA1 and DG were involved in synaptic transmission and neuronal development. The roles of ACC, CA1, and DG are complementary, as highlighted by Lipton & Eichenbaum (2008), Schapiro et al. (2017), Cinalli et al. (2020), and Samborska et al. (2022). Decades of research in neuroscience have shown that making isolated interpretations for each brain structure in this context may be less interesting than describing their mechanisms in an integrative way within the brain's network.

The APP/PS1 mouse model demonstrates that there are shared genes implicated in the immune system and inflammation in both the cortex and hippocampus

The study conducted an integrative analysis of the results obtained after differential gene expression (DGE) analysis in the ACC, CA1, and DG regions, which led to the identification of 479 genes common to all three regions. The majority of these genes were upregulated and associated with programmed cell death, axonal degeneration, stress response, inflammation, and aging. This suggests a prevalence of immunity and defense processes, which is in line with what is expected in an APP/PS1 model. However, this raises the question of whether the APP/PS1 mouse model is an effective model for studying the neuropathology of Alzheimer's disease (AD). While the analysis allows us to identify the inflammation response resulting from the progressive accumulation of amyloid plaques, it is possible that other relevant processes of this disease may be overlooked. Overall, the findings suggest that the APP/PS1 mouse model can be a useful tool in understanding AD pathology, but caution should be exercised in interpreting the results, and further studies may be needed to fully capture the complex nature of the disease.

Genes from DGE are expressed in microglia, evidence from single cell data

Recent single-cell data suggests that microglia express highly shared genes between the cortex and hippocampus regions (Grubman et al., 2019). This finding emphasizes the important role of microglial cells in coordinating cellular activities and communication between different brain regions. Furthermore, the expression of these shared genes in microglia has implications for neuroinflammatory and neurodegenerative diseases, as well as for understanding the complex interactions between different brain regions (Grubman et al., 2019).

Differentially expressed upregulated genes in APP/PS1, which were found to be common to ACC, CA1, and DG, showed slightly higher expression in microglia (Ismail et al., 2020). This supports the notion that the inflammatory response observed in the APP/PS1 model may be an indicator of the role of amyloid plaques in Alzheimer's disease (AD) (Ismail et al., 2020). These plaques can disrupt neuronal field

homeostasis, impacting communication processes and neuronal signaling, which may contribute to the mechanisms of apoptosis in the CNS (Cai et al., 2022; Loo et al., 1993; Han et al., 2017).

Further studies are necessary to fully understand the exact mechanisms underlying the expression of these shared genes in microglia and their potential role in brain function and pathology. The Tabula Muris data can serve as a useful reference for single-cell analysis (Grubman et al., 2019).

Dentate gyrus regulates neuron development

The present study introduced a novel finding by identifying a greater number of downregulated genes in the dentate gyrus (DG) region, particularly at 8 months of age. Despite some overlapping deregulated mechanisms observed in all studied regions, such as inflammatory processes and immune response, the DG region displayed a greater degree of deregulation in neuronal development, differentiation, and neurogenesis. This discovery provides valuable insight into the distinct roles that different hippocampal regions may play in the pathogenesis and progression of AD.

LncRNAs and microglia expression at different time points in the APP/PS1 mouse model

The role of non-coding RNAs (ncRNAs) and long non-coding RNAs (lncRNAs) in neurodegenerative diseases, including Alzheimer's disease (AD), has garnered significant attention in the last decade (Idda et al. 2018; Ma et al. 2020; Zhang, He & Bian, 2021; Li et al. 2021; Dong et al. 2021; Lan et al. 2022; Hao et al. 2022; Varesi et al. 2022). In this context, the present study aimed to explore the differential expression of lncRNAs in the APP/PS1 model, with a focus on the mechanisms that regulate the immune system and inflammatory response.

Previous research has indicated the involvement of the immune system and inflammation in the pathogenesis of AD (Chen, Satpathy & Chang, 2017; Hadjicharalambous & Lindsay, 2019; Chen, Ao & Yang, 2019; Liu et al. 2022). The results of the present study support these findings, with lncRNAs mainly expressed in

mechanisms related to immune system regulation and inflammatory response in the ACC, CA1, and DG areas of the brain. Notably, the analysis revealed the involvement of microglia, astrocytes, and natural killer cells in the regulation of lncRNAs.

Despite the scarcity of functional evidence regarding lncRNAs, the advances in single cell studies and open-source platforms have allowed for a specific description of the differentially expressed lncRNAs in the APP/PS1 model. These findings suggest that the inflammatory response is a crucial aspect of AD pathology and may be masking other essential processes that contribute to the disease's progression.

Although the present study highlights the potential role of lncRNAs in the regulation of the immune system and inflammatory response in AD, providing insights for further investigation into the underlying mechanisms of the disease is required. At the same time, more functional evidence is necessary to confirm the involvement of these lncRNAs in AD pathology.

Downregulation of lncRNAs contribute to the inflammatory response in the APP/PS1 mouse model

The differential gene expression (DGE) analysis of long non-coding RNAs (lncRNAs) in the APP/PS1 model revealed an expression pattern similar to that of protein codings, with an upregulation of non-encoded proteins at month 8, indicating the presence of neuropathology resulting from the accumulation of APP. After filtering for differentially expressed genes and integrating the results, a set of lncRNAs shared by ACC, CA1, and DG between month 4 and 8 were identified. Among these, six lncRNAs with human homologs (Gm13780, AU020206, Oip5os1, Dlx6os1, Mir124-2hg, Gm35161) were selected for further functional characterization.

Interestingly, except for LINCO2883 homologous to AU020206, which was upregulated in CA1 and DG, the other five genes were found to be downregulated in the cortex and hippocampus, primarily during month 8. Although the functional characterization of these lncRNAs is still scarce, previous research has suggested their involvement in immune processes in the nervous system, with a high prevalence in cancer (Wu, Lin & Fu, 2021). These findings suggest that lncRNAs downregulation

may play a role in the inflammation processes that affect neurons during neurodegeneration in the APP/PS1 model, contributing to a smaller set of lncRNAs that require further investigation.

Cognitive dysfunction in the APP/PS1 mouse model at different stages of development

In this study, we were able to compare the results of the differential gene expression (DGE) analysis with the memory tests performed by Michael Gertig in 2016 (Gertig, 2016). Previous behavioral experiments conducted by Gertig showed significant differences between the WT and transgenic groups from 4 months onwards (Zhu et al. 2017). In line with this, the DGE analysis also revealed that no significant differences were observed between the two conditions during the early stages of development (1.5 months), but from month 4 onwards, an increase in the deregulation of coding genes was observed, with a trend towards upregulation which grew exponentially until month 8.

Furthermore, functional analysis of the differentially expressed genes between the WT and transgenic groups showed that from month 4 to month 8, processes related to inflammation, immune system and defense response were the main players, and this was consistent across all brain regions studied. This observation may explain the differences in memory test performance between the control and transgenic conditions, which were also affected from month 4 onwards. Similar to the DGE analysis of coding RNAs, the DGE of lncRNAs also showed a greater deregulation when comparing APP vs WT from month 4, although the impact was less than that observed in the coding RNAs. Moreover, the functional analysis of selected lncRNAs after applying filters for the 8-month condition showed a clear association with inflammatory response.

Conclusions

The aim of this study was to characterize gene expression in the APP/PS1 mouse model at 1.5, 4, and 8 months in the anterior cingulate cortex, CA1, and dentate gyrus brain regions. By studying a sample at different time points, we were able to assess

the impact of progressive amyloid plaque accumulation in the CNS with high descriptive power.

The results of the DGE during the first months of life of the APP/PS1, specifically at 1.5 months, did not show significant results that could have allowed functional and ontological analysis of gene expression. This is not surprising, given that little to no presence of plaques in the CNS is observed before 2 months of age. Therefore, we can conclude that in early stages of development, the accumulation of plaques does not significantly affect the brain areas sequenced in this study.

Continuing with the analyses, we observed a clear upregulation of differentially expressed genes from month 4. This coincides with the results of the Morris water maze and fear conditioning tests, where significant differences between the transgenic and control groups were observed during this period. The functional analysis of the DGE results at 4 months showed a predominance of biological processes linked to inflammation, the immune system, and defense response. This is in line with what we expected, given that amyloid plaque accumulation encourages high microglia activation.

During month 8, the results were quite similar to those observed in month 4, with the main difference being an increase in upregulated genes. These results also coincided with the performance of the mice in memory tests during the same period, where the difference with the control group became more evident. The biological processes characterized at 8 months also involved inflammation mechanisms, the immune system, and defense response in the ACC, CA1, and DG. This suggests that the aggressive inflammatory response observed since month 4 affects all the brain regions studied similarly.

In summary, our findings confirm a significant increase in inflammation in the hippocampus and cortex of the APP/PS1 mouse model during months 4 and 8, while the Anterior Cingulate Cortex experiences a decrease in myelination processes, CA1 show a reduction in synaptic signaling, and DG demonstrates impact on neural development processes. The results indicate that the APP/PS1 mouse model is a valuable tool for studying inflammatory processes in the central nervous system, but

other important mechanisms in Alzheimer's disease may be obscured by its effects on the brain.

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Author contributions

The behavioral experiments and corresponding bioinformatics analysis were carried out by MG and JC, with support from TP in the analysis. SB conducted the qPCR, RNA-Seq library preparations, and sequencing. AF provided valuable input, corrections, and final review of the manuscript.

Competing interests

The authors declare no competing financial interests.

Data and code availability

Upon acceptance of the manuscript, all RNA-Seq data and code will be made publicly available through deposition in GEO and github correspondently.

CHAPTER 3

A transcriptome analysis of cellular and organoid models for Floating Harbor Disease

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Abstract

The Floating Harbor Syndrome (FHS) is a rare congenital disease caused by a mutation in the Snf2-related CREBBP activator protein (SRCAP) gene. Despite the available literature describing the phenotype and symptoms of the disease, little is known about its cellular and molecular mechanisms. This study aimed to investigate the gene expression changes associated with FHS using two experimental models: Human Induced Pluripotent Stem Cells (hIPSC) and Bioengineered Neuronal Organoids (BENOS), both carrying a SRCAP mutation. RNA sequencing and differential gene expression analysis were performed. The results showed deregulation of synaptic signaling processes in early stages of cellular development, and the upregulation of Cathepsin F (CTSF) that has been linked to neurodegeneration. Overall, this study provides new insights into the molecular mechanisms underlying FHS and highlights the potential role of CTSF in the development of this disease.

Keywords: Floating Harbor Syndrome; SRCAP; cognitive dysfunction; differential gene expression

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Introduction

The Floating Harbor Syndrome (FHS) is a rare congenital disease, with approximately 100 cases reported worldwide (Turkunova et ., 2022; Bo et al. 2021). The underlying cause of FHS has been linked to the mutation of the Snf2 Related CREBBP Activator Protein (SRCAP) gene, which is involved in chromatin remodeling (Hood et al., 2012; Guo et al., 2014; Messina, Atterrato & Dimitri., 2016; Milani et al., 2017). The most common SCRCAP mutations occur for the amino acids R2435 and R2444 of, with R2444 accumulating the greatest number of cases reported in the literature (Greenberg et al. 2019). FHS patients present with delayed development milestones such as language delays (Amita et al. 2016; Bo et al. 2021; Nogueira et al., 2021).

The consequences of intellectual development problems can impede the individual's ability to adapt to the environment. In patients with FHS, mild to moderate learning disabilities have been reported (White et al., 2010; Seifert et al., 2014; Rots et al., 2021). Although the underlying mechanisms of these developmental difficulties remain elusive, there is a pressing need to investigate the possible genetic mechanisms that

contribute to cognitive dysfunction in FHS, as this represents a significant research challenge that requires further exploration.

FHS is also characterized by several physical features, including a triangular-shaped face, sunken eyes, wide mouth, short stature, and small finger size (Patton et al., 1991; Asseidat & Kaufman, 2008; Son et al., 2019). Recent literature has also identified nasolacrimal duct obstruction, dystrophic toenails, and preauricular skin tags as potential features of FHS (Ercoskun & Yuce-Kahraman., 2021). However, most scientific research on FHS has focused on the morphology of patients, limiting our understanding of the disease's origins and potential treatments for other aspects of the condition, such as cognitive dysfunction (Sankriti., 2021).

To gain a better understanding of the molecular processes that SRCAP mutations in the brain we decided to conduct a transcriptome analysis in iPSCs and iPSC-derived brain organoids that carry SRCAP mutations FHS. Our data suggest that the mutation of the SRCAP gene has disrupted the normal development of the nervous system in the mutant subjects. This supports the notion that this genetic alteration affects the brain during the early stages of cellular growth, before the cells have specialized into specific types. We also observed an increase in the expression of CTSF, a protein known to be involved in neurodegenerative diseases and cellular lipofuscinosis in neurons. This finding raises interesting questions about the potential role of CTSF in intellectual disability within the FHS population, and merits further investigation.

Materials and Methods

RNA isolation

Total RNA from WT and mutated hiPSCs and BENOS (day 15 and day 60 of differentiation) was prepared using TRIzol (Invitrogen, Cat# 15596-018) extraction following the manufacturer's instructions. Total RNA was treated with TURBO DNaseI (Ambion, Cat# AM1907) and purified using RNA Clean and Concentrator™ -5 Kit (ZYMO Research; Cat. No.: R1014) according to the manufacturer's instructions. The quality of the obtained purified RNA was checked on Agilent 2100 Bioanalyzer (Agilent

Technologies). Good quality RNA samples with RIN values above 8 and were further used for RNA-seq experiments.

RNA sequencing

Library preparation for TotalRNA sequencing was performed according to Illumina TruSeq Kit following the manufacturer's instructions. Briefly, libraries were prepared from 1 ug of input RNA. After purification, the quality of the libraries was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies), and prepared libraries were quantified using a Qubit 2.0 Fluorometer (Life Technologies). A final library concentration of 2 nM was used for 50 bp single-end sequencing on Illumina HiSeq 2000 platform. To reduce sequencing biases the samples were pooled.

NGS data analysis

Sequencing reads obtained from biological samples were processed as FASTQ files, quality control and independent analysis for each sample confirmed the quality of the data. Mapping to the genome was performed using STAR software. Bam files obtained after genome mapping (human), were used to create the counts reads associated with genes. Read counts associated with genes, normalization and unsupervised clustering analysis were performed before differential gene expression analysis (DGE).

Differential gene expression

Counts from RNAseq were produced using FeaturesCount. DGE was carried out with the DESeq2 toolbox run in R. During DGE analysis read counts associated with genes, normalization and unsupervised clustering analysis were used as quality control procedures. In detail: Set-up variables and libraries of interest, associate text to genes. Load GTF annotation for later computing of Fragments per kilobase million (FPKM's). Next step, loading, curating, and organizing data considering the variable "coldata" as the main function to use the data counts. Visual diagnostic through PCA, normalization and filtering of counts. Computing FPKM's and extracting counts. Mapping ensemble and gene IDs in the order presented by DESeq results. Those genes with an adjusted p value <0.05 were considered significant differentially expressed genes. Additionally, the gene lists obtained by DESeq2 were filtered with a

cutoff for log2 fold-changes of ±0.5. Finally, plots of interest were produced, and results saved.

Results

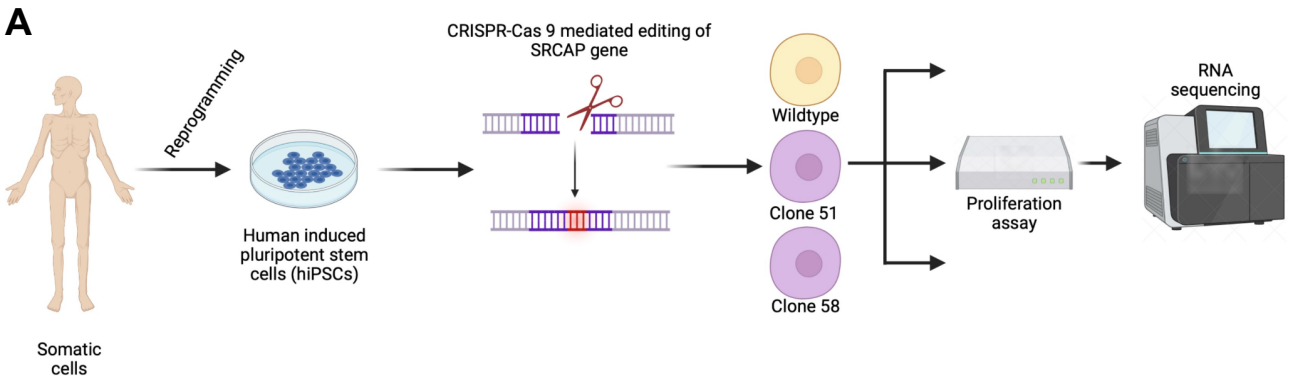
Differential gene-expression in hIPSC with SRCAP mutation

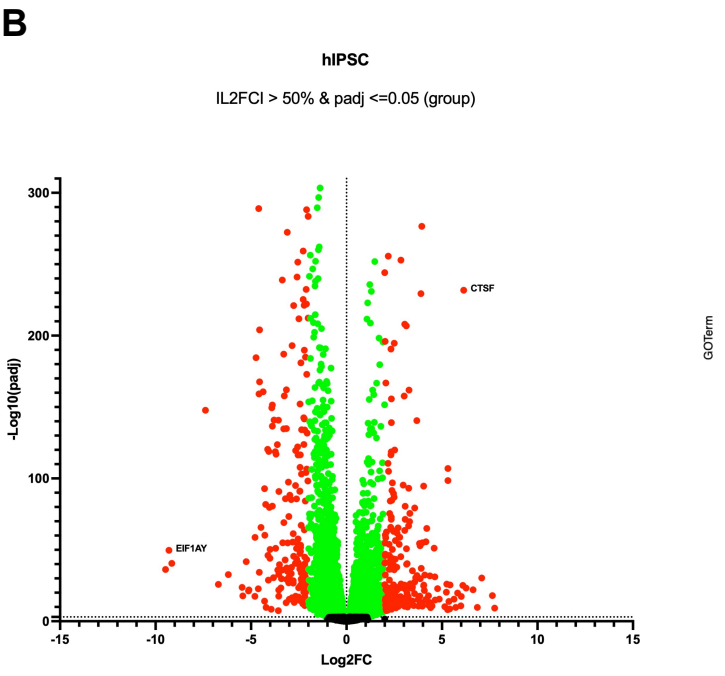
Using the CRISPR-Cas9 System we introduced the most common FHS-associated SRCAP mutant to hiPSCs, namely R2444 (Fig 1A). After we had confirmed the correct insertion of the mutation, we selected two clones, named clone 51 and clone 58 for further analysis. hiPSCs that were not mutated served as control (Fig 1A). First we analyzed gene-expression of undifferentiated hiPSCs (day 0) via RNAseq. For the analysis of differentially expressed genes (DEGs) we applied a *contrast function* to average the effects of applying the treatment of the SRCAP mutation to the two independent sets of clones. In other words, the contrast allowed to equally average the log 2-fold change (L2FC) estimated independently for each of the clones. This method offers, as an intrinsic advantage, a p-value for the null hypothesis that there is no differential expression across the average of the clones with respect to the control group. Further reference on contrasts can be found in texts Casella (2008).

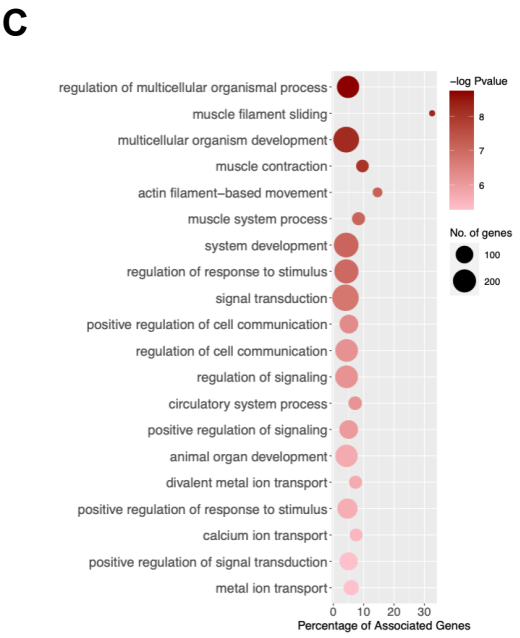
This approach revealed that 243 genes were consistently upregulated while 143 genes were downregulated (Fig 1B). To gain further insight to the biological processes affected by changes in gene-expression we performed a Gene Ontology (GO) term analysis. When analyzing the upregulated genes and number of highly significant GO terms linked to developmental processes such as multicellular organism development, animal organ development etc. were observed. Similarly, processes such as regulation of cell communication but also processes linked to ion transport were detected (Fig 1B). When analyzing the down-regulated gene similar processes related to development were affected. For example, multicellular organism development and animal organ development were also among the top GO terms detected when analyzing the downregulated genes. These data hint to a general deregulation of developmental processes which is in line with the described role of SRCAP mutants and FHS patients. Amongst the top GO terms detected for the downregulated genes

were also terms such as nervous system development (Fig. 1C). These data suggest that mutated SRCAP may directly affect processes related to brain development.

In summary, our data suggest that in hiPSCs mutated SRCAP effects processes of cellular communication and development, both of which play a crucial role for the integration of tissue and organs during normal cellular development. Moreover, especially the downregulated genes have an impact on the development of the central nervous system, affecting maturation, morphogenesis, segmentation, and neuronal differentiation. To test this hypothesis directly, we decided to study the role of SRCAP mutants in bioengineered human brain organoids (BENOs) (Zafeiriou et al., 2020).







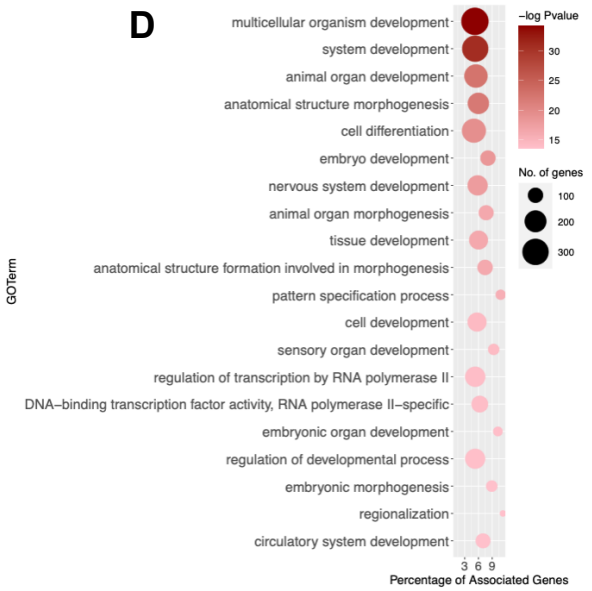


Figure 1. SRCAP mutation produces synaptic dysregulation from early stages of cell development. A: Experimental scheme of the reproduction of the mutation point present in the Floating Harbor Syndrome via CRISPR-Cas9 technology. Figures created with BioRender.com. **B:** Gene expression changes between wildtype and mutant SRCAP average clones (51-58). L2FC >50% difference and padj < = 0.05. Highlighted downregulation of "EIF1AY", protein coding gene involved in translation factors. And upregulation of "CTSF", a protein coding gene involved in neurodegeneration and neuronal ceroid lipofuscinosis. **C:** categories of gene ontology analysis for upregulated genes (n=243). Showing a predominance of regulation of multicellular organismal process and cellular development **D:** categories of gene ontology analysis for downregulated genes (n=143), showing a deregulation of nervous system development.

Transcriptome analysis of SCRAP mutants in Bioengineered Neuronal Organoids (BENOS)

BENOs represent a valuable and versatile tool for investigating various aspects of cortical development in vitro. The applications of BENOs are extensive, ranging from disease modeling to drug screening. This model enables researchers to study cell populations at different developmental stages, providing a unique opportunity to explore the effects of genetic mutations or pharmacological interventions on cortical development.

For instance, to investigate the effects of SRCAP mutation in the early stages of development, BENOs can be generated at day 15, exhibiting significant expression of neuronal progenitor cell (NPC) markers. Conversely, BENOs can be produced during periods of cell differentiation, specifically on day 60, where the presence of glia markers can be observed. During differentiation markers expressed by different types of neurons are present. Overall, BENOs provide a reliable platform for studying

cortical development in vitro, enabling researchers to investigate diverse cellular and molecular processes underlying brain development and disease. (Zafeiriou et al., 2020).

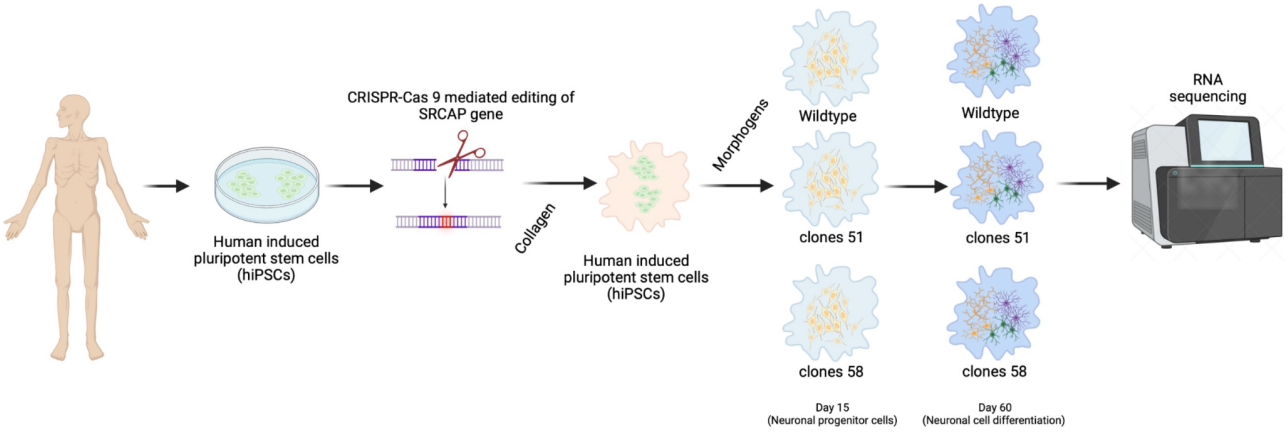
Therefore we decided to use the WT hiPSCs as well as the two SRCAP mutants to study BENOs at day 15 and 60, in order to capture the critical time window of neuronal progenitor cells enrichment (day15) and the completion of neuronal maturation (day 60). After collecting the tissue from day 15 and day 60 BENOs generated from wild type hiPSCs as well as SRCAP mutant clone 51 and clone 58, we isolated RNA and performed RNAseq (Fig. 2A). Again we applied the contrast allowed to equally average the log 2-fold change (L2FC) estimated independently for each of the clones.

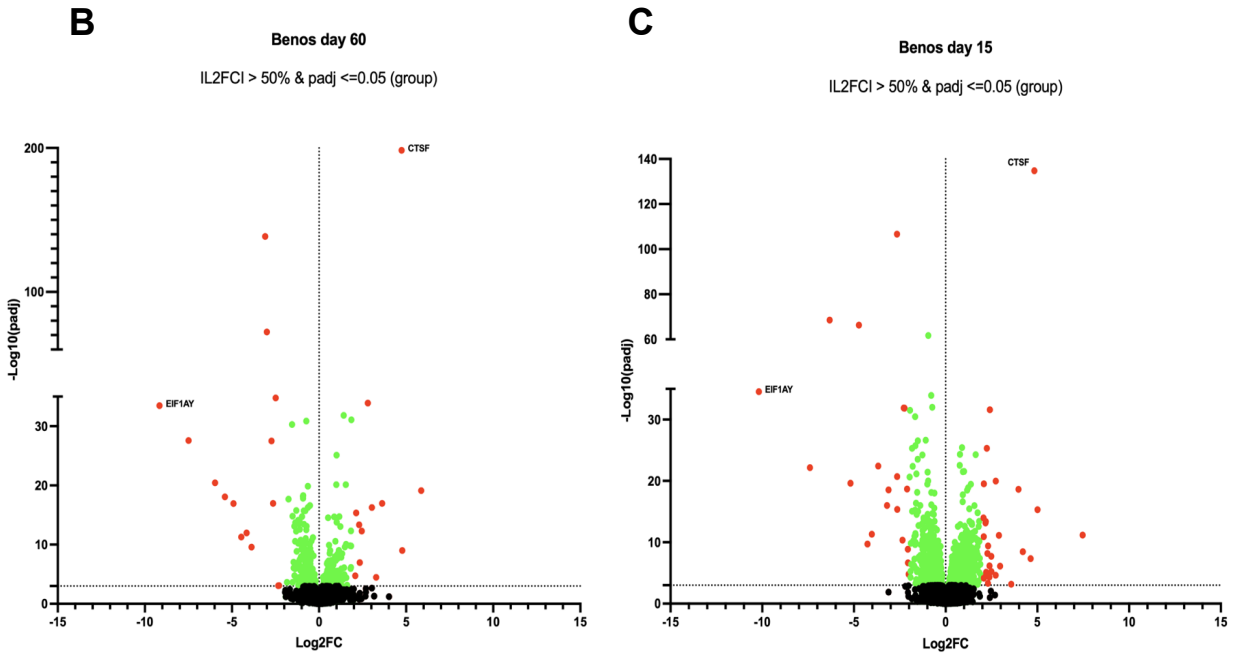
The analysis of differential gene expression revealed 267 genes that were upregulated and 253 genes that were downregulated at day 15 (Fig 2B). On day 60, 139 genes were upregulated and 143 genes were downregulated when comparing WT BENOs to SRCAP mutants.(Fig 2C) Functional GO term analysis of BENOs at day 15 showed upregulation of processes such as neuron differentiation, chemical synaptic transmission, neurogenesis, synaptic signaling, neuron projection morphogenesis and neuron development categories (Fig 2D). Downregulated genes were associated with vasculature development, anatomical structure formation, extracellular matrix organization, tube morphogenesis and regulation of angiogenesis (Fig 2 E).

At day 60, upregulated genes were associated with peptidase regulator activity, endopeptidase inhibitor activity, serine-type endopeptidase inhibitor activity, enzyme inhibitor activity, epithelial cell apoptotic process and negative regulation of cell apoptotic process (Fig 2F). Downregulated genes were associated with muscle filament sliding, skeletal muscle thin filament assembly, striated muscle cell differentiation, actomyosin structure organization and skeletal muscle tissue development (Fig 2G). The upregulation of genes intimately linked to neuronal processes in day 15 BENOs hint to a severe deregulation of neuronal differentiation processes, while the data obtained from day 60 BENOs may indicate that key cellular processes are altered as a consequence.

When comparing the data from the BENOs to the data obtained from the hiPSCs, two genes caught our attention. Eukaryotic translation initiation factor 1A Y-linked (EIF1AY) was severely down-regulated in hiPSCs and in day 15 and 60 BENOs, while the gene coding for a Cathepsin F (CTSF) was up-regualted in hIPSC and in day 15 and 60 BENOs (Fig 1 & 2). EIF1AY has a well-known role in RNA binding and translation processes, but the evidence around its involvement in the nervous system is limited so far (Yarahmadi et al., 2018). On the other hand, CTSF has been implicated in photolysis and is positioned in the extracellular space. Furthermore, it has been linked to neurodegeneration and Neuronal Ceroid Lipofuscinosis (NCL), a condition predominantly manifesting during childhood (Cotman et al., 2013; Simonati & Williams., 2022). Notably, this positions CTSF within the framework of FHS, a disorder primarily documented in the early stages of development. It should be mentioned that NCL is characterized by the accumulation of autofluorescent lipopigments in neurons and other cells (Kollmann et al., 2013), which has been associated with cognitive decline. CTSF plays an important role in intracellular and extracellular protein degradation, and its dysfunction has been implicated in early onset dementia (Van der Zee et al., 2016).

A

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C
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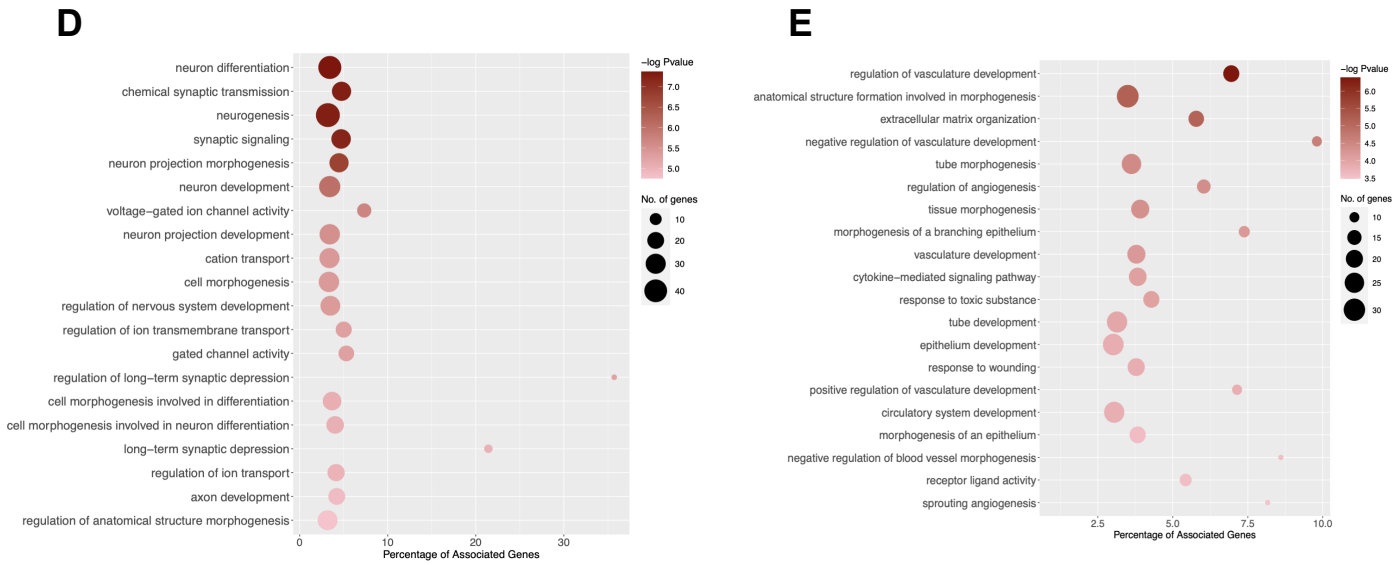
Log2FC
CTSF
EIF1AY

Day 15


D
neuron differentiation
chemical synaptic transmission
neurogenesis
synaptic signaling
neuron projection morphogenesis
neuron development
voltage–gated ion channel activity
neuron projection development
cation transport
cell morphogenesis
regulation of nervous system development
regulation of ion transmembrane transport
gated channel activity
regulation of long–term synaptic depression
cell morphogenesis involved in differentiation
cell morphogenesis involved in neuron differentiation
long–term synaptic depression
regulation of ion transport
axon development
regulation of anatomical structure morphogenesis
Percentage of Associated Genes
–log Pvalue
No. of genes
E
regulation of vasculature development
anatomical structure formation involved in morphogenesis
extracellular matrix organization
negative regulation of vasculature development
tube morphogenesis
regulation of angiogenesis
tissue morphogenesis
morphogenesis of a branching epithelium
vasculature development
cytokine–mediated signaling pathway
response to toxic substance
tube development
epithelium development
response to wounding
positive regulation of vasculature development
circulatory system development
morphogenesis of an epithelium
negative regulation of blood vessel morphogenesis
receptor ligand activity
sprouting angiogenesis
Percentage of Associated Genes
–log Pvalue
No. of genes

Day 60

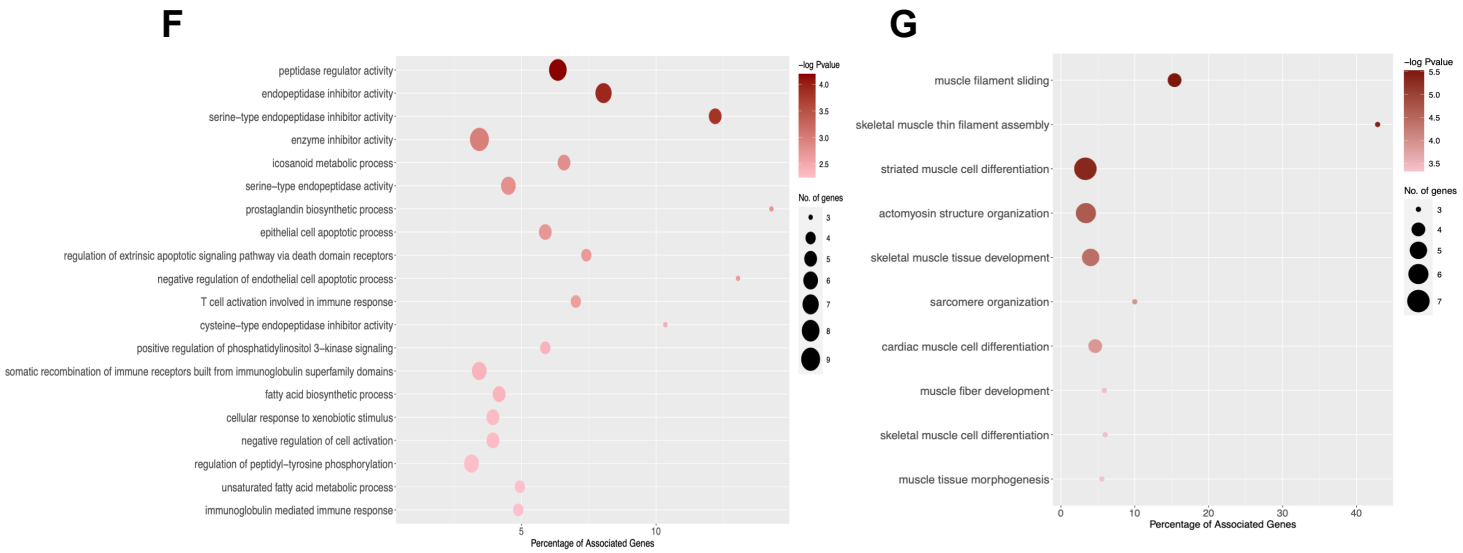


Figure 2. Early Defect in Neuronal Development Observed in Day 15 of Bioengineered Neuronal Organoids (BENOS) with SRCAP Mutation. A: Experimental scheme of BENOS with SRCAP mutation prepared at two time points (day 15 and day 60). Figures created with BioRender.com. **B**: Gene expression changes in mutant SRCAP clones (51-58) compared to wildtype on day 15, showing significant downregulation of "EIF1AY" and upregulation of "CTSF". **C**: Gene expression changes in mutant SRCAP clones (51-58) compared to wildtype on day 60, highlighting the same genes as in day 15. **D**: Gene ontology analysis of upregulated genes showing neuron differentiation, neurogenesis, and synaptic signaling categories. **E:** Gene ontology analysis of downregulated genes showing dysregulation of morphogenesis and anatomical structure formation processes. **F**: Gene ontology analysis of upregulated genes from BENOS day 60 showing dysregulation of endopeptidase inhibition and apoptotic processes. **G:** Gene ontology analysis of downregulated genes from BENOS day 60 showing dysregulation of skeletal muscle development and differentiation categories.

EIF1AY and CTSF gene expression in hIPSC and BENOS

Since the analysis of the expression levels of CTSF and EIF1AY in hIPSC and BENOs has revealed significant changes in their expression levels in comparison to control samples, we decided to have a closer look at these genes.

CTSF, a gene involved in neurodegeneration and neuronal ceroid lipofuscinosis, exhibited increased expression in both hIPSC and BENOS mutants (Figure 3A & B). CTSF has been linked to the degradation of proteins in the lysosome, a cellular organelle that breaks down cellular waste materials. Dysregulation of lysosomal function has been linked to several neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease. The high expression of CTSF in hIPSC and BENOS mutants may suggest that lysosomal dysfunction is a common feature in these diseases and may provide a potential target for therapeutic intervention.

On the other hand, the expression levels of EIF1AY were found to be very low, close to zero, in hIPSC and BENOS mutants compared to wildtype (Figure 3C & D). EIF1AY is a protein-coding gene involved in translation factors and plays a critical role in protein synthesis. A decrease in EIF1AY expression can affect the efficiency of protein synthesis, which can lead to various diseases, including cancer. The low expression of EIF1AY in the mutants may suggest that these models have reduced protein synthesis capabilities, which may contribute to the development or progression of the diseases associated with these cell types.

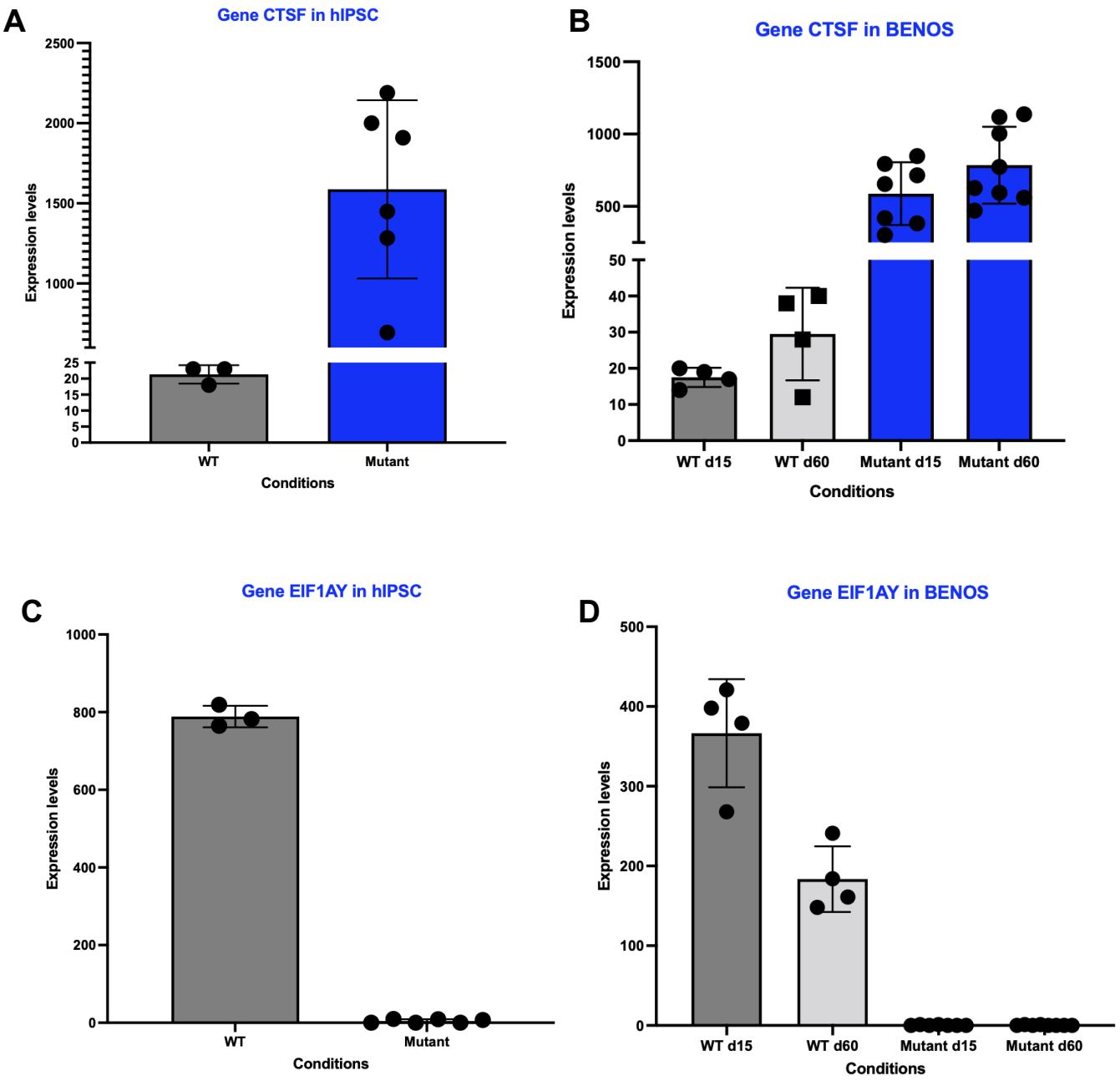


Figure 3. CTSF and EIF1AY expressions in SRCAP mutants. A: Gene expression levels from raw counts for CTSF in hIPSC, clearly showing that CTSF is highly expressed in hIPSC mutant. **B:** Gene expression levels from raw counts for CTSF in BENOS, clearly showing that CTSF is highly expressed in mutants at day 15 and 60 compared to wildtype **C:** Gene expression levels from raw counts for EIF1AY in hIPSC, clearly showing that EIF1AY is lower expressed in the mutant. **D:** Gene expression levels from raw counts for EIF1AY in BENOS, clearly showing that EIF1AY is lower expressed in mutants day 15 and 60 compared to wildtype.

Discussion

The primary aim of this STUDY was to characterize the impact of the SRCAP mutation on hIPSC and BENOS as models for analyzing gene expression in Floating Harbor Syndrome (FHS). At present there is a lack of evidence regarding the genetic mechanisms of FHS employing a DGE analysis; we obtained intriguing results as one of the first approaches to examining the effects of SRCAP mutation on the genomes of two FHS models at different stages of cellular development.

One of the key inquiries driving this study was to describe the effects of SRCAP on undifferentiated cells. The DGE analysis revealed that the truncation of the SRCAP gene resulted in a dysregulation of nervous system development in hIPSC, potentially confirming the hypothesis that this truncation affects the brain during the early stages of cellular development, that is, before cellular differentiation. With regards to the results of experiments conducted on BENOS, DGE analysis during day 15 revealed similar findings to those observed in hIPSCs, with an upregulation of synaptic signaling processes, which could also be associated with the dysregulation of crucial processes in the development of the central nervous system.

Given the differentiated state of cells during day 60, it is reasonable to be cautious and hypothesize that the observed dysregulation in peptidase activity, for instance, may be a secondary effect of the observations made during day 15. This can be substantiated by examining the results of the DGE analysis in hIPSC and BENOS mutants (day 15), which show the differentiated expression of EIF1AY and CTSF, whose molecular function is primarily associated with developmental disorders, including dementia, particularly in the case of CTSF (Wagner et al., 2021).

CTSF and EIF1AY downregulation in hIPSC and BENOS with SRCAP mutation

In this study, an interesting observation was made regarding the expression of EIF1AY and CTSF in hIPSC and BENOS experiments, where both genes showed a stable expression pattern in each of the contrasted conditions. Specifically, EIF1AY was found to be downregulated, while CTSF was upregulated. Previous research has suggested that EIF1AY plays a role in RNA and protein binding and enabling translation initiation factor activity (Yarahmadi et al., 2018), and has also been related to the apoptotic pathway in Synovial Fibroblast in the context of rheumatoid arthritis (Wang et al., 2020). It is worth noting that rheumatoid arthritis shares some common symptoms with the Floating Harbor Syndrome, such as muscle malfunction, skeletal abnormalities, and painful swelling (Menzies et al., 2020).

Moreover, the upregulation of CTSF observed in this study presents an intriguing avenue for further investigation. CTSF has been described in the literature in processes of neurodegeneration and neuronal celloid lipofuscinosis, specifically associated with type B Kufs, which is characterized by dementia with extrapyramidal motor symptoms (Smith et al., 2013). This is relevant considering that intellectual disability is one of the most evident symptoms in FHS (Nikkel et al., 2013; Budisteanu et al., 2018), which could be potentiated by mechanisms similar to those of dementia, specifically through processes of neurodegeneration and inflammation.

Conclusions

The study aimed to characterize the genetic expression produced by the mutation in the SRCAP gene, which serves as a model for the study of Floating Harbor Syndrome (FHS). FHS is a rare congenital disease characterized by a combination of symptoms, including intellectual disability, facial dysmorphism, and skeletal abnormalities. Despite the morphological description of the patients with FHS, the molecular and cellular mechanisms of the disease are still largely unknown. Therefore, the characterization of the effects of the SRCAP mutation is an important step towards increasing our knowledge of the disease.

The research conducted two experiments using hIPSC and BENOS with the SRCAP mutation. The results of the differential gene expression (DGE) analysis in hIPSC and BENOS at day 15 showed early effects on the developmental processes of the nervous system, reflected in the deregulation of biological processes linked to synaptic signaling. In contrast to previous studies that obtained an FHS-like phenotype on differentiated cells (Greenberg et al. 2019), our study suggests that changes occur before cellular differentiation. Deregulations in BENOS at day 60, such as peptidase regulator activity, negative regulation of cell apoptotic process (both upregulated), and processes of muscle cell differentiation and development, can be hypothesized as the product of changes that occurred during day 15.

One interesting finding of the study was the identification of the downregulation of EIF1AY in both hIPSC and BENOS mutants. EIF1AY is involved in RNA and protein binding and enables translation initiation factor activity. In addition, it has been related to the apoptotic pathway in synovial fibroblast in the context of rheumatoid arthritis. While the association of EIF1AY with FHS is still unclear, it is a potentially interesting avenue for further research.

Another finding of the study was the deregulation of CTSF in hIPSC and BENOS mutants. CTSF has been previously associated with processes of neurodegeneration and neuronal celloid lipofuscinosis, and is involved in type B Kufs disease, which has been partly defined as a disease with evident signs of intellectual disability. Since intellectual disability is a prominent feature of FHS, the deregulation of CTSF is of great interest. CTSF is expressed in oligodendrocytes, astrocytes, and microglia, indicating its involvement in the inflammatory process, which may also be related to FHS pathogenesis.

Several factors can influence gene expression levels, including genetic and environmental variables (Choi & Kim., 2007). The use of hIPSC and BENOS mutants provides a controlled experimental set-up that minimizes the impact of contextual perturbations on gene expression levels. The use of control samples also provides a baseline for comparison, which enables the identification of genes that are dysregulated in disease conditions.

Overall, the analysis of the expression levels of CTSF and EIF1AY in hIPSC and BENOS mutants has provided valuable insights into the molecular mechanisms underlying the development and progression of diseases associated with these enzymes. Further studies are necessary to validate these findings and to identify other genes that are dysregulated in these diseases. The identification of disease-specific biomarkers can facilitate early disease detection and intervention, which can significantly improve patient outcomes.

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Author contributions

CF conducted the experiments in hIPSC and BENOS, while JC performed the corresponding bioinformatics analysis, with assistance from TP. The qPCR, RNA-Seq library preparations, and sequencing were performed by SB. AF contributed with valuable feedback, edits, and final review of the manuscript.

Competing interests

The authors declare no competing financial interests.

Data and code availability

Upon acceptance of the manuscript, all RNA-Seq data and code will be made publicly available through deposition in GEO and github correspondently.

General Discussion

Neuropsychiatric disorders and neurodegenerative diseases have been a subject of significant interest in the scientific community for several reasons. A considerable portion of the population experiences discomfort due to the high prevalence of these disorders (Saarni et al., 2007). Despite a wealth of knowledge regarding the behavioral manifestations of these disorders, understanding the underlying cellular mechanisms remains a major challenge. Furthermore, research has revealed that these diseases are complex and heterogeneous (Wu & Yang, 2022 ; Young et al., 2018), with individuals who share similar diagnoses often exhibiting diverse responses to treatments and distinct cellular processes. Consequently, there is a pressing need to further investigate the cellular mechanisms that underlie neuropsychiatric and neurodegenerative diseases to develop more effective treatments for affected individuals.

As was described, Major depression (MD) is one of the most common neuropsychiatric disorders in contemporary society, affecting over 300 million individuals worldwide. The debilitating symptoms of MD have spurred significant scientific interest in the last decade, resulting in a deeper understanding of the disorder's complexity and comorbidity with other diseases (Li et al., 2020). Specifically, a hypothesis has emerged suggesting that MD may be a risk factor for the development of neurodegenerative diseases, such as Alzheimer's disease (AD), in adulthood (Agüera-Ortiz et al., 2021). This association is not surprising, given that MD and AD share some similar symptoms, including impairments in attention and memory (John et al., 2018). Investigations into the cellular mechanisms of MD have revealed an increase in immune system activity and pathways associated with inflammatory processes. However, the relationship between MD and AD is not yet fully understood, and the development of mouse models to study MD in the laboratory is crucial for advancing our knowledge.

In addition, there are diseases for which the scientific community knows the cause but still does not fully understand the underlying mechanisms. This is particularly true for monogenetic congenital diseases, which are present at birth and result from gene mutations. One such disease is the Floating Harbor Syndrome (FHS). This syndrome

is characterized by its significant impact on the cognitive abilities of affected individuals, and although the signs and symptoms of FHS have been well-described, little is known about the cellular mechanisms that underlie the disease. As a result, further research into the pathogenesis of FHS is crucial for developing effective treatment strategies and improving outcomes in the clinic.

In light of this context, the current study aimed to explore the genetic mechanisms underlying major depression (MD), Alzheimer's disease (AD), and Floating Harbor Syndrome (FHS) by investigating gene expression in corresponding models systems.

The research conducted in the context of MD aimed to find deregulated genes in depressive and resilient conditions after exposure to social defeat for a period of 10 days. The results showed little effect on the differential genetic expression of the experimental condition compared to the control group. These outcomes could be attributed to several variables, such as the sample size and age of the subjects. The literature on mouse models for the study of MD using the social defeat experiment, has demonstrated an impact on the cognitive abilities of subjects exposed to the adverse context for similar exposure periods (Huang et al., 2013; Vassilev et al., 2021). However, these studies typically used samples younger than two months old. In the present study, the sample was three months old, which corresponds to early adulthood. Evidence from psychology and psychiatry has suggested that adverse experiences tend to have a greater impact on subsequent development when they occur during critical periods of development, such as childhood or early adolescence. Thus, the impact of the social defeat paradigm may be less severe in subjects who have already achieved a level of biological maturity.

A later goal of the MD investigation was on analyzing the long-term effects of social defeat on memory abilities of mice (Koskinen et al., 2020). The memory tests conducted on the one-year-old mice, nine months after exposure to social defeat, showed no impairment in their working memory. However, the results of the Morris Water Maze, particularly the probe test, demonstrated significant differences between the experimental and control groups during this period of time. These findings support the hypothesis that adverse events experienced during early stages of development, such as the social defeat at three months of age, can have long-term effects on

referential and spatial memory (Lowry et al., 2021; Kalia, Knauft & Hayatbini., 2021; Brown et al., 2022; Lin et al., 2022). Although these behavioral differences were not observed in the initial procedures conducted at three months of age, they were evident when memory tests were repeated after nine months. To provide a more comprehensive understanding, it is necessary to analyze the sequencing data of this sample to confirm if differences in genetic expression are observed in the subjects.

The research conducted on the APP/PS1 mouse model highlights the significant inflammatory response of the CNS in specific regions of the brain, which is consistent with the current understanding of AD pathology. The differential gene expression analysis of both codingRNAs and lncRNAs demonstrated a similar response, with an increase in biological processes related to the immune and defense systems. This suggests that the accumulation of amyloid plaques in the CNS may play a role in the activation of inflammatory processes. The behavioral results of the study also align with previous studies, as lower performance in memory and fear conditioning tests was observed in the transgenic group compared to the control. This suggests that the accumulation of amyloid plaques in the CNS may have an impact on cognitive functions, particularly in advanced stages of amyloid pathology.

However, not all neurodegenerative processes are evidently expressed in advanced pathological states. The integration of the effects of SRCAP mutation as a model for the study of FHS has revealed that the impact of neurodegenerative processes may affect the CNS even before cell differentiation. The deregulation of CTSF in the hIPSC and BENOS model on day 15 further supports this notion. CTSF has been previously described for its involvement in neuronal ceroid lipofuscinosis processes, which is a group of neurodegenerative disorders. Being aware of the scope of these results, it is essential to promote subsequent analysis and studies that delve into the role that CTSF plays in cognitive abilities.

The study of neuropsychiatric disorders and neurodegenerative diseases poses a significant challenge due to their complex symptomatology and the heterogeneity of their underlying mechanisms. Investigating gene expression in affected individuals is one approach to understanding the pathophysiology of these conditions. Recent advancements in analysis methods and the increasing availability of information from

genome databases have enabled the identification of new processes and functions relevant to these disorders.

While it is not possible to directly correlate the results of studies on different diseases, there is evidence of shared mechanisms between neuropsychiatric, neurodegenerative, and congenital diseases. The presence of the immunomodulatory agent Interferon GTPase 1, the activation of the immune system, and the deregulation of CTSF have all been observed in the diseases described here. These shared mechanisms may impact biological processes that have been linked to cognitive abilities.

The social defeat experiment, in which mice are exposed to stressors, showed signs of cognitive dysfunction after a prolonged period. In the APP/PS1 model, a relationship between amyloid pathology and memory impairment was observed, while the effect of the SRCAP mutation in the hIPSC and BENOS, shows deregulation of biological processes linked to cognition, which can be observed in patients suffering from FHS. Although the relationship between these diseases is not direct, understanding the shared mechanisms that underlie different neuropsychiatric and neurodegenerative disorders is critical.

Finally, differential Gene Expression analysis has been shown to be a valuable method for studying these disorders.

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