The Effect of Neuronal Tumor Necrosis Factor Alpha Expression on Amyloid-Beta Based Alzheimer's Disease Symptoms in Mice

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ABSTRACT

Introduction Alzheimer's Disease is a progressive neurodegenerative disease, and the most common cause of dementia. It has been shown that TNF-α plays an important role in development and progression of the disease, but the exact role is not clearly understood. Previous research has focussed mainly on deletion of TNF-α or its receptors, which leaves a need for more research regarding the effects of elevated TNF-α levels in the brain and the dynamics of the TNF-receptors in an environment of chronically elevated TNF-α.

Objective The current study focusses on evaluating the effects of elevated TNF-α levels in certain brain regions on Aβ based Alzheimer's disease symptoms in mice. The primary outcome parameters of the study are cognitive phenotype, Aβ plaque development and neuroinflammation.

Methods Cognitive phenotype is assessed through several behavioural tests, including Morris Water Maze. Aβ plaque development is measured through a 6e10 staining. The number of Aβ plaques will be quantified as a percentage of the total area measured. Neuroinflammation is assessed through LCN2 expression, GFAP expression and microglia activation. A LCN2 and GFAP fluorescent co-staining pilot will be performed, in addition to an Iba1 staining to measure the amount of Iba1 positive cells which indicates the amount of activated microglia.

Results No difference in Morris Water Maze performance was observed in J20 versus MK41.3xJ20 ($p=0,9991$). MK41.3xJ20 showed a significantly lower percentage of 6e10 positive area in comparison to J20 ($p<0,0001$). In the Iba1 staining, the percentage of the Iba1 positive area was significantly higher in MK41.3xJ20 than in J20 ($p<0,0001$).

Conclusion From the current preliminary results it seems likely that an increase in TNF-α levels results in neuroprotective responses via the TNFR2 receptor. However, further results regarding neuroinflammatory responses and TNFR1 and TNFR2 dynamics are needed to draw a more complete conclusion. This could be investigated through the proposed LCN2 and GFAP stainings for neuroinflammation and through Western Blots for Caspase 3 and pAkt/Akt for TNFR1 and TNFR2 signaling, respectively.

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Preface

The purpose of this report is to show what I have accomplished during my research internship at GELIFES, in the lab of Dr. Ulrich L.M. Eisel under direct supervision of Yingying Wu.

During my internship, I was able to conduct research for Yingying's crossbreeding project. My objective was to perform research regarding Aβ plaque development and neuroinflammation in mice with AD symptoms and elevated TNF-α levels in certain brain regions.

You will find that, for some theoretics and methods that I will discuss, no results are shown. I chose to discuss theoretics and methods about several behavioural tests even though I was not involved in this part of the project. I feel that these tests are interesting to my part of the project. I included theoretics and methods for all behavioural tests that are included in the crossbreeding project. Thereby, results are shown from the Morris Water Maze.

Regarding the neuroinflammation, only the Iba1 staining is shown in the results section. Due to the extraordinary circumstances regarding the COVID-19 pandemic, I had to leave the lab sooner than expected. Therefore, I was unable to perform the LCN2 and GFAP stainings and Western Blots. I have mentioned the LCN2 and GFAP co-staining, which I was able to execute. However, no results are available to strengthen the conclusion of this project report.

Introduction

Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, first described by Alois Alzheimer in 1907. AD is the most common cause of dementia, accounting for 70% of all cases affecting 4% of individuals aged 65-74 and 50% of individuals that are older than 90 years of age (Kozlov et al., 2017; McAlpine & Tansey, 2008).

The neuropathological hallmarks of AD include accumulation of amyloid-beta (Aβ) plaques, presence of neurofibrillary tangles caused by hyperphosphorylation of the microtubule binding protein tau and chronic inflammation including microglia and astrocyte activation. The Aβ plaques and neurofibrillary tangles thereby induce neuronal loss (Dong et al., 2015; Kozlov et al., 2017; McAlpine & Tansey, 2008; Murphy & LeVine, 2010).

These hallmarks result in symptoms associated with AD: memory loss and cognitive decline. The loss of memory of events and people is most prominent, but patients thereby lose the ability to name objects or persons and the ability to perform skilled or learned tasks. The cognitive decline is shown as change of behaviour, including loss of inhibitions, agitation and aggressive behaviour, depression and sleeping disorders (McAlpine & Tansey, 2008).

All AD cases can be divided into two groups, which differ in the form of AD. The first form of AD is known as the hereditary form, or familial form of AD. This hereditary form is associated with mutations in the gene coding for the amyloid precursor protein and causes an early onset of AD. The second form is called sporadic AD or late-onset AD, which accounts for most AD cases. This form is characterized by a later onset and is not associated with any genetic mutations. Symptoms of both forms are similar and occur as mentioned above (Kozlov et al., 2017; McAlpine & Tansey, 2008).

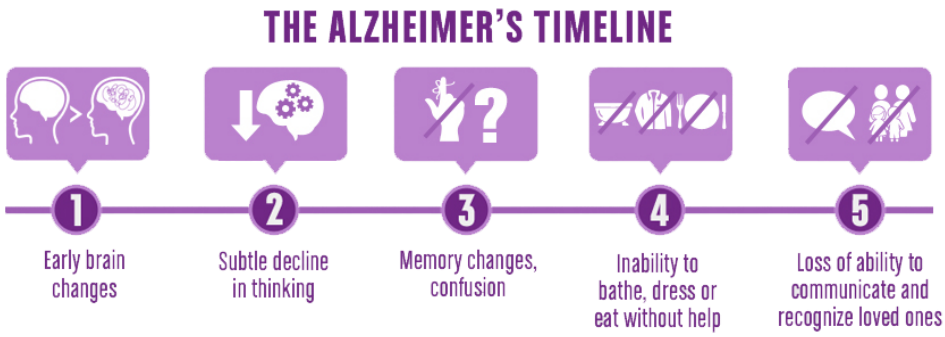

Figure 1: Timeline of Alzheimer's Disease illustrating the most common symptoms (The Alzheimer's timeline (n.d.), https://www.wesleylife.org/memory-care/get-the-facts/).

TNF-α

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine and is a master regulator of the innate and adaptive immune system. It is involved in various inflammatory diseases, including AD. TNF-α is synthesized and expressed as a 26 kDa type II monomeric transmembrane protein (tmTNF). It can be cleaved proteolytically by TNF-α converting enzyme (TACE/ADAM17) to form a 17 kDa soluble monomeric protein (solTNF). TNF-α interacts with two distinct transmembrane receptors, the 55 kDa TNF receptor I (TNRF1) and the 75 kDa TNF receptor II (TNFR2). TNFR1 is ubiquitously expressed in various cell types and tissues, and activation can be induced by both solTNF and tmTNF, but with a preference for solTNF. TNFR2 is predominately expressed in endothelial cells and immune cells and preferentially binds tmTNF. Interaction of TNF-α with its receptors leads to various cellular responses, with in general pro-inflammatory effects via TNFR1 and neuroprotective and promotion of tissue homeostasis and regeneration via TNFR2 (Dong et al., 2015; Dong et al., 2016; Orti-Casañ et al., 2019) (Fig. 2).

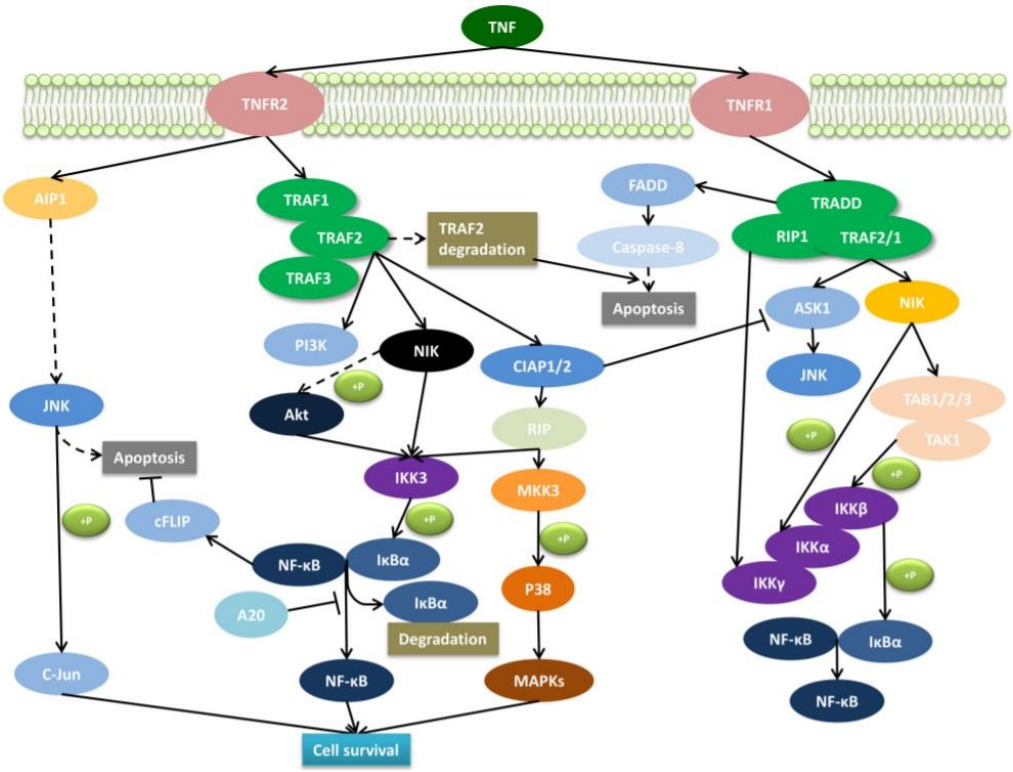

Figure 2 Signaling pathway of TNFR2 and TNFR1 (Orti-Casañ et al., 2019).

TNF-α in AD

Neuroinflammation is considered one of the hallmarks of AD, in which TNF-α is an important mediator. To gather more evidence about the involvement of TNF-α in AD, various methodological approaches have been used, including genetic, protein and histological approaches. On a genetic level, it has been shown that multiple polymorphisms in the TNF-α gene are associated with the risk of developing AD. One example is the TNF-α G308A polymorphism, which can cause higher TNF-α levels and has shown to increase the risk of AD. At the protein level, it has been shown that TNF-α protein levels were increased in plasma and serum as well as in brain tissue (Dong et al., 2016; Orti-Casañ et al., 2019). To prove the opposite, Paouri et al. (2017a) showed that deletion of TNF-α relieved AD pathology by showing an attenuation of Aβ production and a decrease in Aβ plaque formation in the 5XFAD mouse model of AD.

Since TNF-α clearly shows to have a role in AD, the function of its receptors, TNFR1 and TNFR2, are also of utmost importance. Previous studies assessing the role of TNFR1 and TNFR2 in AD using post-mortem AD brain tissue showed that there was an increase in TNFR1 levels and a decrease in TNFR2 levels (Cheng et al., 2010; Orti-Casañ et al., 2019). Cheng et al. (2010) thereby showed that TNF-α has an increased binding affinity to TNFR1 and a decreased binding affinity to TNFR1 in AD brains. As mentioned before, activation of TNFR1 is associated with pro-inflammatory responses and activation of TNFR2 is associated with neuroprotective responses. This statement was reinforced by both the fact that TNF-α increasingly binds to TNFR1 in AD and that TNFR2 deletion results in an exacerbation of AD pathology through TNFR1 and is reversed when TNFR2 is later overexpressed (Cheng et al., 2010; Jiang et al., 2014; Orti-Casañ et al., 2019). He et al. (2007) thereby showed that genetic deletion of TNFR1 in mice inhibits Aβ generation and reduces Aβ plaque formation leading to prevention of learning and memory deficits, which also shows a role of TNFR1 in neurodegeneration. And lastly, Steeland et al. (2018) found that abrogation of TNFR1 reduces brain inflammation. In the same mouse model they showed amelioration of amyloidosis with TNFR1 deficiency.

Current study

It is clear that the role of TNF-α in AD is thoroughly studied. However, most studies regarding TNF-α and AD address the effects of deletion of TNF-α or its receptors. Paouri et al. (2017b) studied the effects of an increase in TNF-α. This resulted in decreased amyloid plaque burden. Additionally, they showed that treatment with infliximab, which is a TNF-α blocker, restored the amyloid phenotype. However, this study was performed peripherally, and thus the effects of an increase of TNF-α in brain regions are still unknown. Thereby, the dynamics of both TNF-α receptors, TNFR1 and TNFR2, in an environment of chronically elevated TNF-α levels are unknown.

These fundamental questions regarding the opposite manipulation of TNF-α levels will lead to better understanding of the role of TNF-α in AD pathology. More specific, studying the role of increased TNF-α levels in Aβ pathology and TNFR1 and TNFR2 signalling will lead to better understanding of how AD development and progression is linked to neuroinflammation.

Thus, in the current study the aim is to evaluate the effects of elevated TNF-α in certain brain regions on Aβ based AD symptoms in mice. The primary outcome parameters of this study are cognitive phenotype, Aβ plaque development and neuroinflammation.

Expectations

Since TNF-α in AD brains has a higher binding affinity towards TNFR1 than to TNFR2 (Cheng et al., 2010), it is expected that TNFR2 will reach a higher activation level in a situation with increased TNF-α levels in certain brain regions in comparison to a situation with normal TNF-α levels. Since activation of TNFR2 has previously shown to result in neuroprotective responses, it is hypothesized that an AD mouse model with elevated TNF-α levels will show less AD symptoms than an AD mouse model without elevated TNF-α levels. This will result in an improved cognitive phenotype, less Aβ plaque development and decreased neuroinflammation, thus amelioration of AD symptoms.

Materials & methods

Mice

In this study, four experimental groups were included. The groups were characterized as follows:

1. Wildtype, n=12
2. J20, n=13
3. NR2B-TNF, n=17
4. J20 x NR2B-TNF, n=14

All mice were 8.5 months old at the start of this study. At the age of 8 to 9 months, Aβ pathology and thereby cognitive decline is detectable, but not fully developed. Therefore, at the age of 8.5 months, the J20 mice would reflect an early stage of human AD, which is currently believed to be the optimal timeframe for treatments strategies.
At the age of 8.5 months old, it was decided whether mice would be included in the study or not. Mice were removed from the study or not included at the start if blindness and/or other eye problems were observed and/or if movement problems, such as disability and stumps, occurred.

As mentioned above, at the age of 8.5 months the study started. Mice first underwent all behavioural tests. At the age of 10 months, mice were sacrificed and brain tissue was preserved for staining and western blot.
Mice were terminally anaesthetised before collection of blood. Blood was collected via cardiac puncture. After collection of blood and CSF, mice were perfused by cardiac perfusion using saline. Brains were then collected and frozen and later used for biochemical analysis.

J20

The J20 mouse model consists of mice overexpressing human amyloid precursor protein (APP) with familial AD mutations. J20 mice show some of the most important AD symptoms, including Aβ plaque development resulting in synaptic loss and cognitive impairment, which makes it a good model for AD. The model was developed by Mucke et al. using a transgene construct including two familial AD-linked mutations (Mucke et al., 2000; Tosh et al., 2018).

NR2B-TNF

The NR2B-TNF mouse model are transgenic mice with brain-specific TNF-α expression. Previous models designed and developed to assess TNF-α showed a widespread and high level of TNF-α expression, which is associated with several pathologies, and therefore cannot be used to specifically assess TNF-α expression in AD (Marchetti et al., 2004).
To avoid the previously shown widespread and high level of TNF-α expression, the NMDA receptor subunit NR2B gene was used. The NR2B subunit of the NMDA receptor is expressed in the forebrain, mainly in cortical and hippocampal regions. Therefore, when using the NMDA receptor subunit NR2B gene, mice were generated with strictly regional and moderate TNF-α expression (Marchetti et al., 2004).

J20 x NR2B-TNF

The J20 x NR2B-TNF mouse model is a model developed in the current study. The mouse model is created by crossing J20 mice with NR2B-TNF mice. The NR2B-TNF mice have specific TNF-α expression in forebrain regions and overexpression of human APP with familial AD mutations.

Collection of brain slices

As mentioned before, mice were sacrificed after behavioural tests and brains were then collected and frozen. Brains were sectioned by using a cryostat and cut into slices with a thickness of 20nm which were used for immunohistochemistry. Slices were collected in separate cups representing different brain regions.

Cognitive phenotype

In this study, mice were exposed to several behavioural tests. These tests were selected to cover AD relevant symptoms, several aspects of cognition or to control for baseline measures of fear and anxiety. The behavioural tests were carried out from least stressful to more stressful, in order to minimize the effects of stressful events on other tests. Thereby, before starting behavioural tests, mice were handled two minutes per day for five days to habituate them to being handled by the researcher and to the room in which behavioural tests were performed.

Home Cage Activity (HCA)

HCA measurements were started when mice were 8.5 months of age, after decision on inclusion. The activity of all mice was measured in their home cage, by using an infra-red sensor which was placed above the home cage for two weeks. Measuring home cage activity is useful since J20 mice generally show increased activity. Thereby, one of the characteristics of the AD phenotype in humans is a disturbed daily activity.

Open Field (OF)

In this test, mice were placed in an arena. For a maximum of ten minutes, movement was recorded with a video camera. The mice's location in the arena, and specifically the time spent in the centre zone of the arena was measured to indicate anxiety-like behaviour. The mice's movement speed and covered distance were analysed to assess locomotor activity.

Novel Location Recognition (NLR)

The NLR test provided information about the hippocampus dependent spatial memory. The test was performed as follows: mice were trained in an arena for ten minutes. Two objects were placed in this arena and one spatial que was placed on one wall. 24 hours after training, spatial memory was tested by changing the spatial location of one of the two objects. If spatial memory is intact, mice should have recognize the relocation of the one object.

Object Pattern Separation (OPS)

The OPS task was used to assess hippocampal dentate gyrus dependent memory. For this test, the same arena as the OF was used and it contained two objects and one spatial que on one wall. Mice were trained for four minutes. One hour after training, the hippocampal dentate gyrus dependent memory was tested by moving one of the objects to a new location. If the episodic memory were to be intact, the mice would have recognize the moved object.

Elevated Plus Maze (EPM)

The EPM consisted of a plus maze with two open and two closed arms and was, as the name suggests, elevated from the floor. Mice were placed in the middle and were able to move freely into both open and closed arms. For ten minutes, movement and location were measured. This provided information regarding anxiety-like behaviour, since a higher amount of time spent in the closed arms indicates anxiety.
Additionally, anxious mice generally perform worse in cognitive memory tests. Therefore, the EPM was used as a control for those tests.

Morris Water Maze (MWM)

The MWM was used as the primary spatial memory read-out. In the test, mice were placed in a circular water bath, which was placed in a room with visual cues on the wall. When placed in the bath, mice had to learn that they can escape by finding a platform that was placed under the water surface. For them,

the platform was invisible. Mice therefore had to learn and remember the spatial location of the platform by using the visual cues in the room.
The test was divided in three sessions: cued trials for two days, hidden trials for eight days and probe trials for two days. One trial had a maximum duration of 120 seconds after which the mice were allowed to dry and warm up.
During the learning phase (hidden trials), escape latency, swim speed and path length were measured each day. During probe trials, the number of platform crossings and the percentage of time spend in each quadrant of the water bath were measured, 24 hours and 48 hours after the last learning phase trial.

Aβ plaque development

Aβ plaques is one of the neuropathological hallmarks of AD. Aβ plaque formation, together with the formation of neurofibrillary tangles, leads to neuronal loss. Therefore, measuring intraneuronal Aβ plaque development will show the degree of AD pathological progression (Youmans et al., 2012). The number of Aβ plaques were quantified as percentage of the total area measured.
6e10 is a commonly used antibody to Aβ and was therefore used in this study to visualize Aβ plaques in mice brain sections (App. A).

Neuroinflammation

Lipocalin 2: fluorescent staining

Lipocalin 2 (LCN2) is an inflammatory factor involved in pathological processes in AD. It has previously been shown that LCN2 levels are elevated in human AD brains and in an AD mouse model (Dekens, 2019). Therefore, LCN2 can be seen as a biomarker for AD and was thus measured in this study by a fluorescent staining.

Astrocyte activation: GFAP fluorescent staining

In the case of damage to the central nervous system, for example during Alzheimer's disease development and progression, astrocytes can become reactive. This change of the astrocyte into a reactive state is called astrogliosis and is characterized by, among other things, an increase in glial fibrillary acidic protein (GFAP) expression (Kamphuis et al., 2014). Therefore, in this study, GFAP staining was used to visualize astrocyte activation in mouse brain tissue.
Apart from visualization of astrocytes, the GFAP staining was used to gather more information regarding the number of Aβ plaques present, since GFAP expression is highly association with plaque load due to surrounding of Aβ plaques by GFAP in early stages of AD (Kamphuis et al., 2014).

LCN2 and GFAP fluorescent co-staining

To assess whether LCN2 and GFAP could be successfully stained at once, a fluorescent co-staining pilot was performed. Several combinations of the primary antibodies Rat LCN2 and Mouse GFAP with secondary antibodies Goat Anti-Rat 555 and Donkey Anti-Mouse 488 were tested in order to properly assess the possible co-staining of LCN2 and GFAP (App. C).

Microglia activation: Iba1 staining

Iba1 is a cytoplasmic protein from which its expression is restricted to microglia (Hopperton et al., 2018). Thus, Iba1 staining was used in the current study to quantify the amount of Iba1 positive staining as a percentage of the total area measured (Dekens et al., 2018; Hovens et al., 2015) (App. D).

Results

Morris Water Maze

Learning phase

The learning phase was performed over the course of eight days. Every day, swim speed, path length and escape latency were measured for each mouse (Fig. 3).

Swim speed was reduced over eight days measurements (Wt p=0,008***; J20 p=0,4592; MK41.3 p=0,0002***, MK41.3xJ20 p=0,9891), as well as path length (Wt p=0,008***; J20 p=0,0403*; MK41.3 p<0,0001****; MK41.3xJ20 p=0,6836) and escape latency (Wt p=0,0011**; J20 p=0,2307; MK41.3 p=0,0007****; MK41.3xJ20 p=0,1774). The reduced swim speed could indicate a reduced stress level, whereas the reduced path length and escape latency indicate a learning effect.

No significant difference in performance over eight days is observed between genotypes.

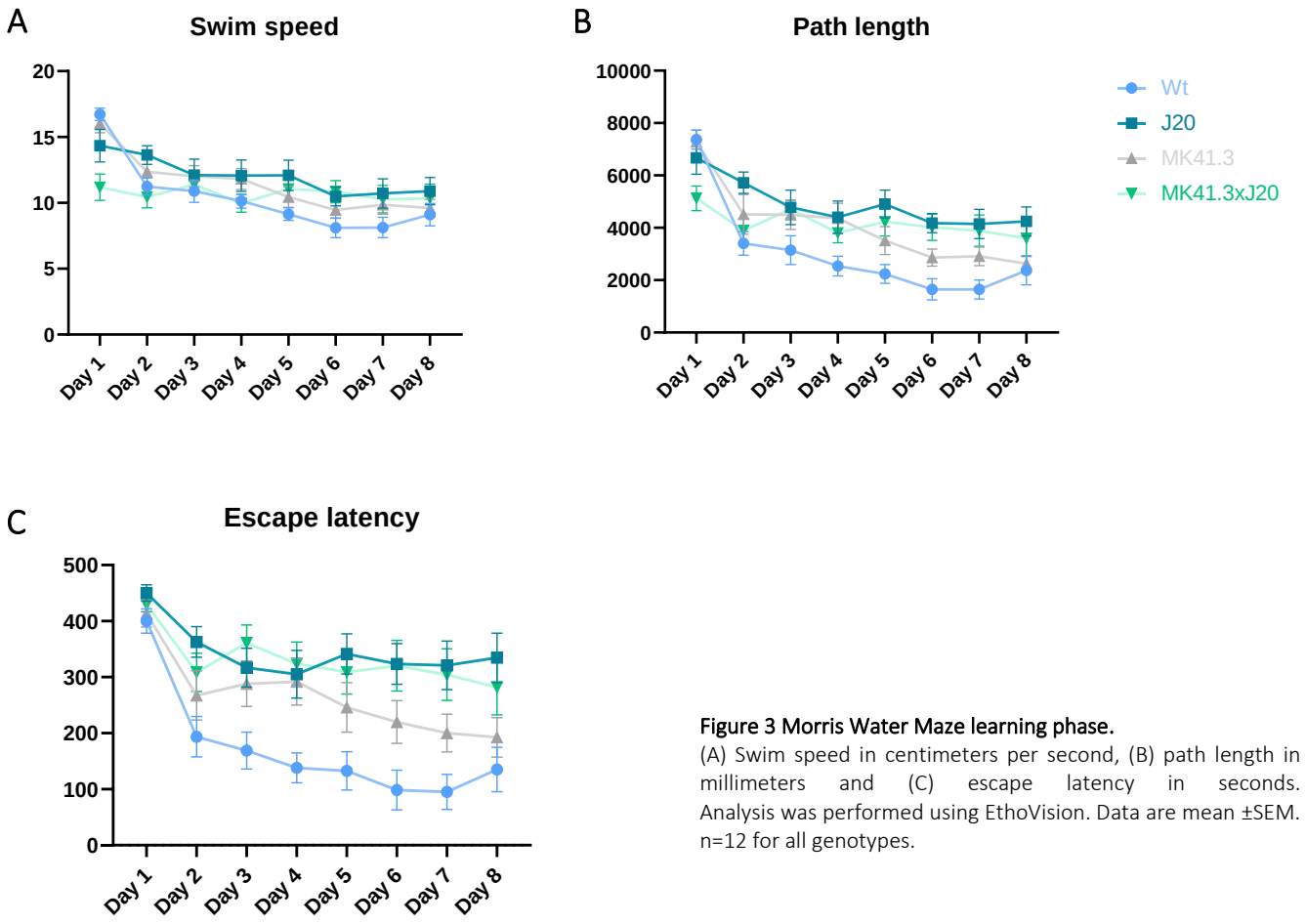

Figure 3 Morris Water Maze learning phase.
(A) Swim speed in centimeters per second, (B) path length in millimeters and (C) escape latency in seconds. Analysis was performed using EthoVision. Data are mean ±SEM. n=12 for all genotypes.

Probe trials

MWM probe trials were performed over the course of two days. The first trial was performed 24 hours after the last learning phase trial and the second probe trial was performed 48 hours after the last learning phase trial. During probe trial, both the number of platform crossings and the time spent in each quadrant was measured.

A significant difference in number of platform crossings during the first probe trial has been observed in Wildtype versus J20 (p=0,0316) and Wildtype versus MK41.3xJ20 (p=0,0224). A difference seems apparent between MK41.3 and MK41.3xJ20, albeit non-significant (p=0,6977). No difference can be observed between J20 and MK41.3xJ20 (p=0,9991) (Fig. 4A). In the second probe trial, again no difference is observed between J20 and MK41.3 (p=0,6651). Between other genotype groups differences are observed, but none are significant (Fig. 4B).

No significant difference in time spent in each quadrant has been observed between different genotype groups (Fig. 4C). At the second probe trial, the MK41.3xJ20 mice seem to spend more time in the target quadrant than J20. However, at the first probe trial the opposite is true (Fig. 4D).

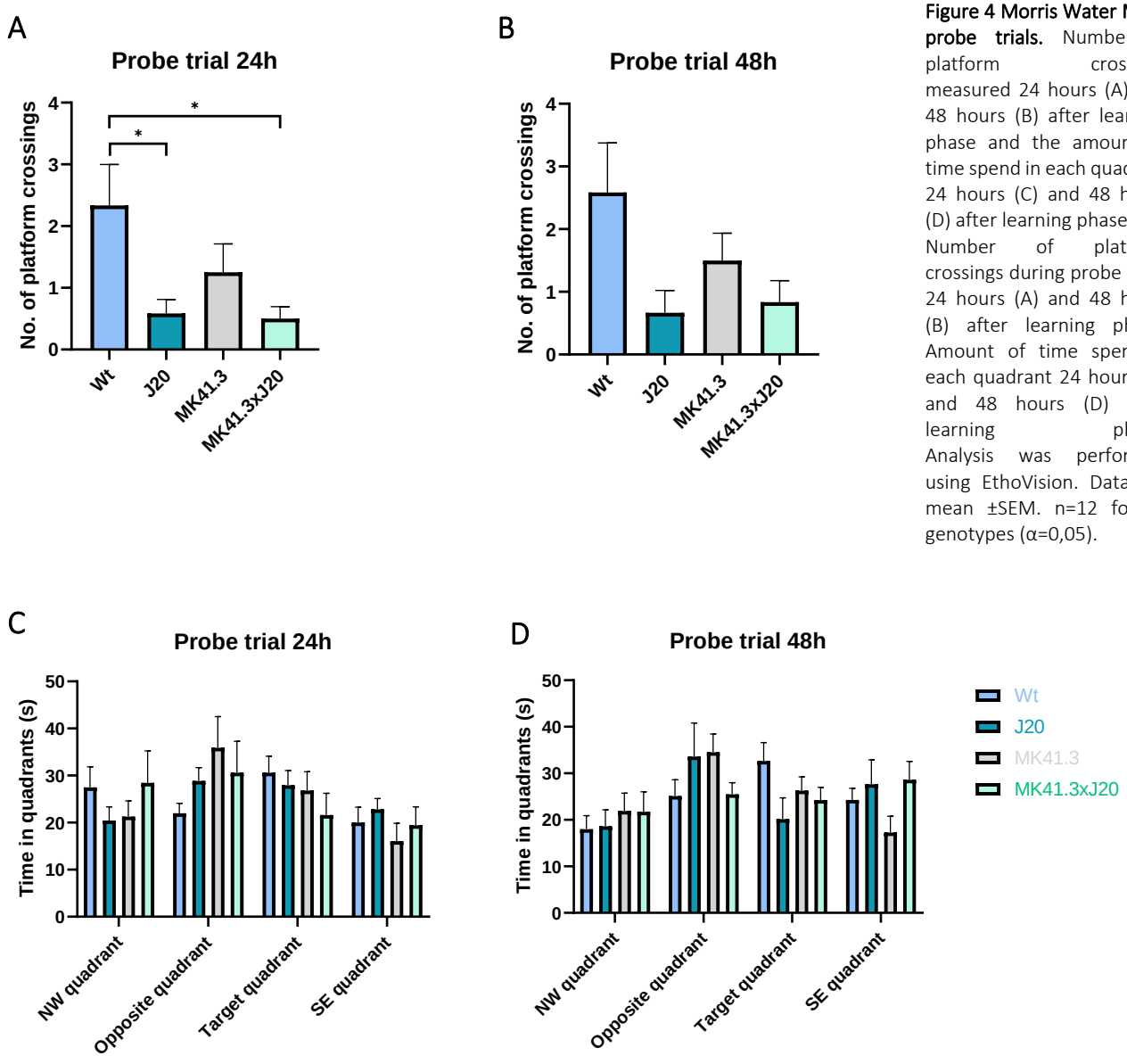

Figure 4 Morris Water Maze probe trials. Number of platform crossings measured 24 hours (A) and 48 hours (B) after learning phase and the amount of time spend in each quadrant 24 hours (C) and 48 hours (D) after learning phase. Number of platform crossings during probe trials 24 hours (A) and 48 hours (B) after learning phase. Amount of time spent in each quadrant 24 hours (C) and 48 hours (D) after learning phase. Analysis was performed using EthoVision. Data are mean ±SEM. n=12 for all genotypes (α=0,05).

Aβ plaque development

A 6e10 staining was used to visualize intraneuronal Aβ plaques. An analysis has been performed on the hippocampus area. The number of Aβ plaques are quantified as a percentage of the total area measured.

The 6e10 positive area is significantly higher in J20 than in MK41.3xJ20 ($p<0,0001$). Thereby, a significant difference is observed between Wt and J20 ($p<0,0001$), Wt and MK41.3xJ20 ($p<0,0001$), J20 and MK41.3 ($p<0,0001$) and between MK41.3 and MK41.3xJ20 ($p<0,0001$) (Fig. 5).

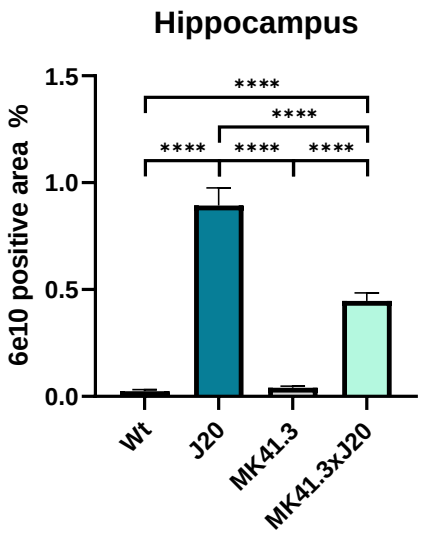

Figure 5 6e10 staining for Aβ.

Analysis was performed using ImageJ. Data are mean ±SEM. Wt n=6, J20 n=6, MK41.3 n=8, MK41.3xJ20 n=7 (α=0,05).

Neuroinflammation

Microglia activation

The Iba1 staining was used to quantify the amount of Iba1 positive cells, as a percentage of the total area measured.

A significantly higher percentage of the total area is positively stained in MK41.3xJ20 in comparison to J20 ($p<0,0001$). A significant difference is also observed between Wt and J20 ($p=0,0210$), Wt and MK41.3 ($p<0,0001$), Wt and MK41.3xJ20 ($p<0,0001$), J20 and MK41.3 ($p=0,0003$) and between MK41.3 and MK41.3xJ20 ($p<0,0001$) (Fig. 6A)

In all genotypes, the granular layer of the dentate gyrus shows the highest percentage of Iba1 positively stained cells. The CA1 region has the lowest percentage of Iba1 positively stained area (Fig. 6B-E).

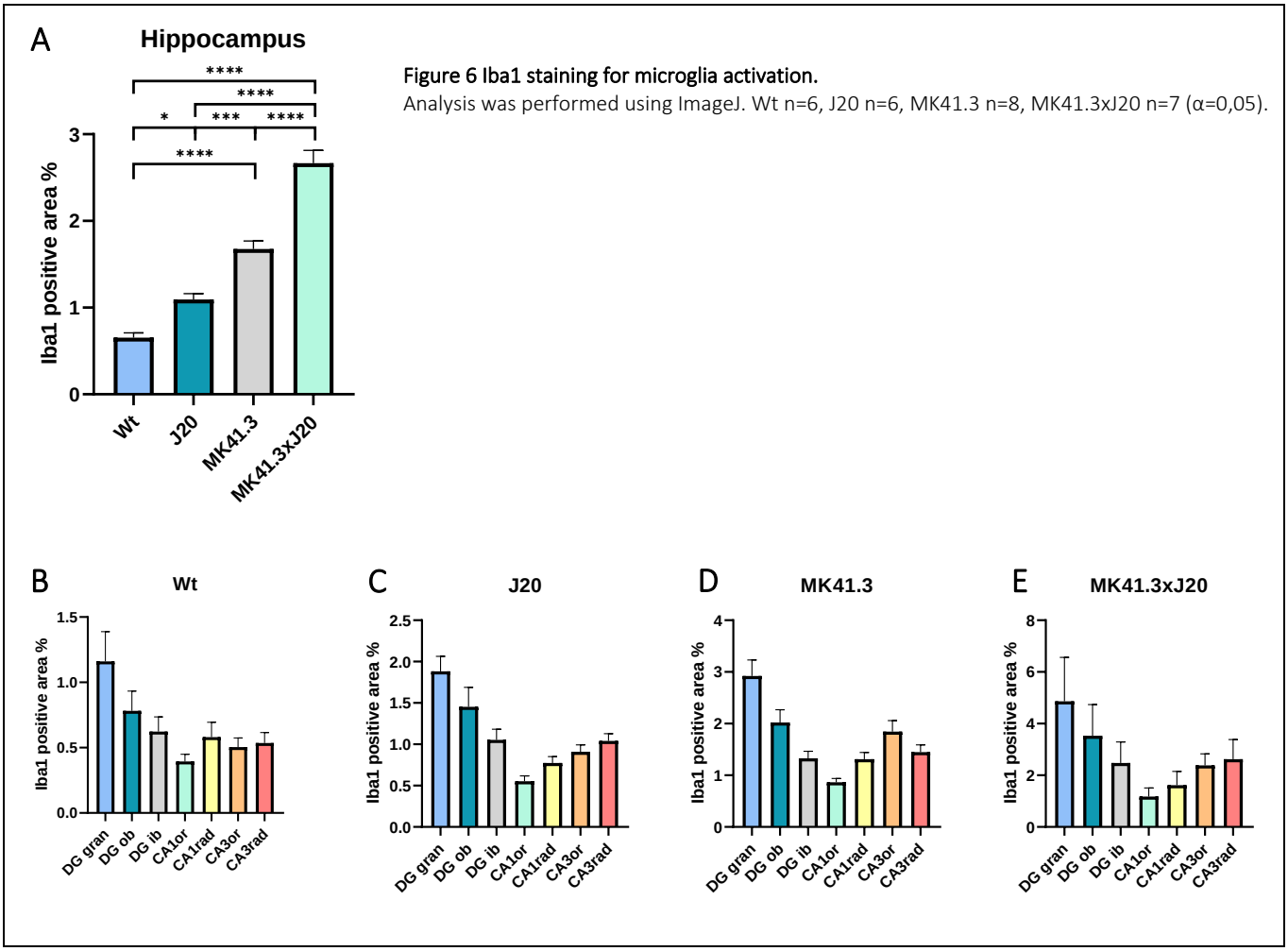

Figure 6 Iba1 staining for microglia activation.
Analysis was performed using ImageJ. Wt n=6, J20 n=6, MK41.3 n=8, MK41.3xJ20 n=7 (α=0,05).

LCN2 and GFAP fluorescent co-staining pilot

To assess whether LCN2 and GFAP can be successfully stained at once, a fluorescent co-staining pilot was performed (Fig. 7-11, App. C). Five different combinations of primary and secondary antibodies have been tested.

The first that was tested is the co-staining combination, with Rat LCN2 and Mouse GFAP primary antibodies and Goat Anti-Rat 555 and Donkey Anti-Mouse secondary antibodies (Fig. 7). Both the LCN2 and GFAP filtered pictures show lighter areas, which could indicate a successful staining.

To confirm this hypothesis, four other combinations were tested. In figure 8, the results of the Rat LCN2 primary antibody in combination with the Donkey Anti-Mouse 488 secondary antibody are shown. In both filters no areas appear lighter, which means this combination of primary and secondary antibody does not work, as was expected. The second combination is a control for the secondary Goat Anti-Rat 555 antibody binding to the Rat LCN2 primary antibody. No areas appear lighter in the LCN2 filtered pictures, which indicates that the secondary Goat Anti-Rat 555 antibody does not work (Fig. 9). In figure 10, the third combination of Mouse GFAP primary antibody and Donkey Anti-Mouse 488 secondary antibody is shown. Lighter areas appear in the GFAP filtered pictures, which is expected with this combination of primary and secondary antibodies. The last control combination is shown in figure 11, combining the Mouse GFAP primary antibody with the Goat Anti-Rat 555 secondary antibody. Some lighter areas appear in the LCN2 filtered pictures, which indicates nonspecific binding of the Mouse GFAP primary antibody.

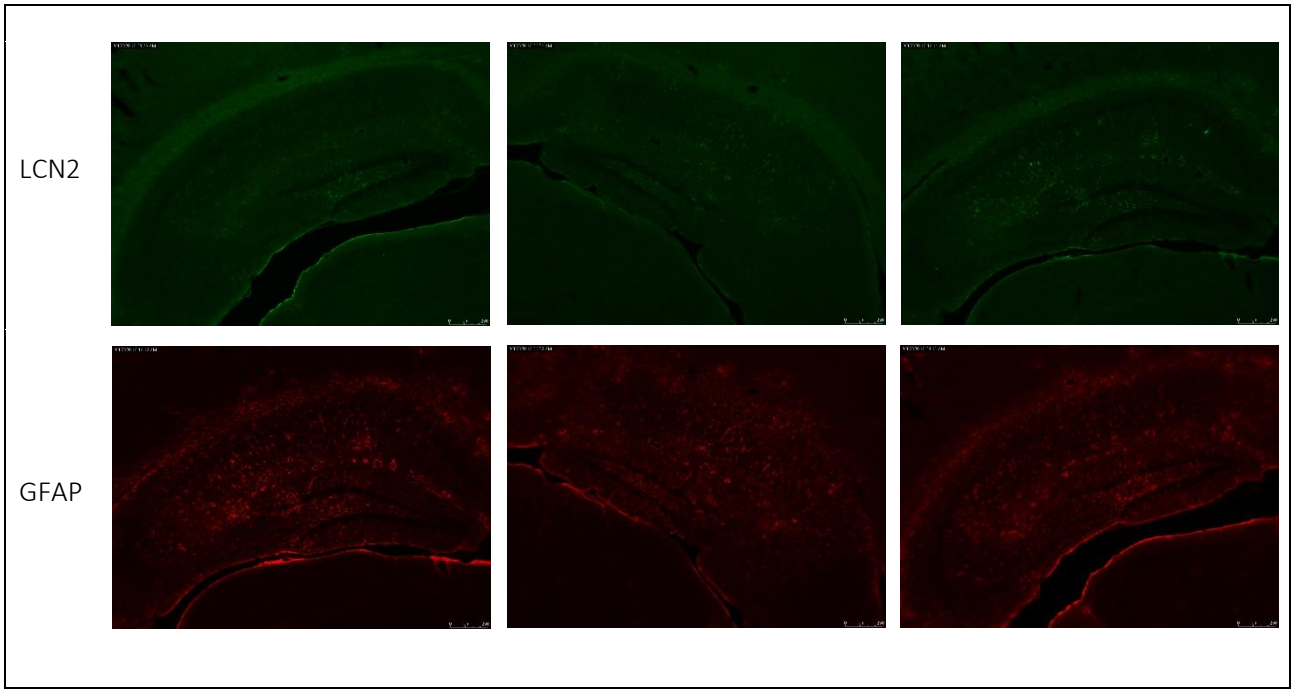

Figure 7 LCN2 and GFAP fluorescent co-staining pilot. The primary antibodies used are Rat LCN2 and Mouse GFAP. The secondary antibodies used are Goat Anti-Rat 555 and Donkey Anti-Mouse 488.

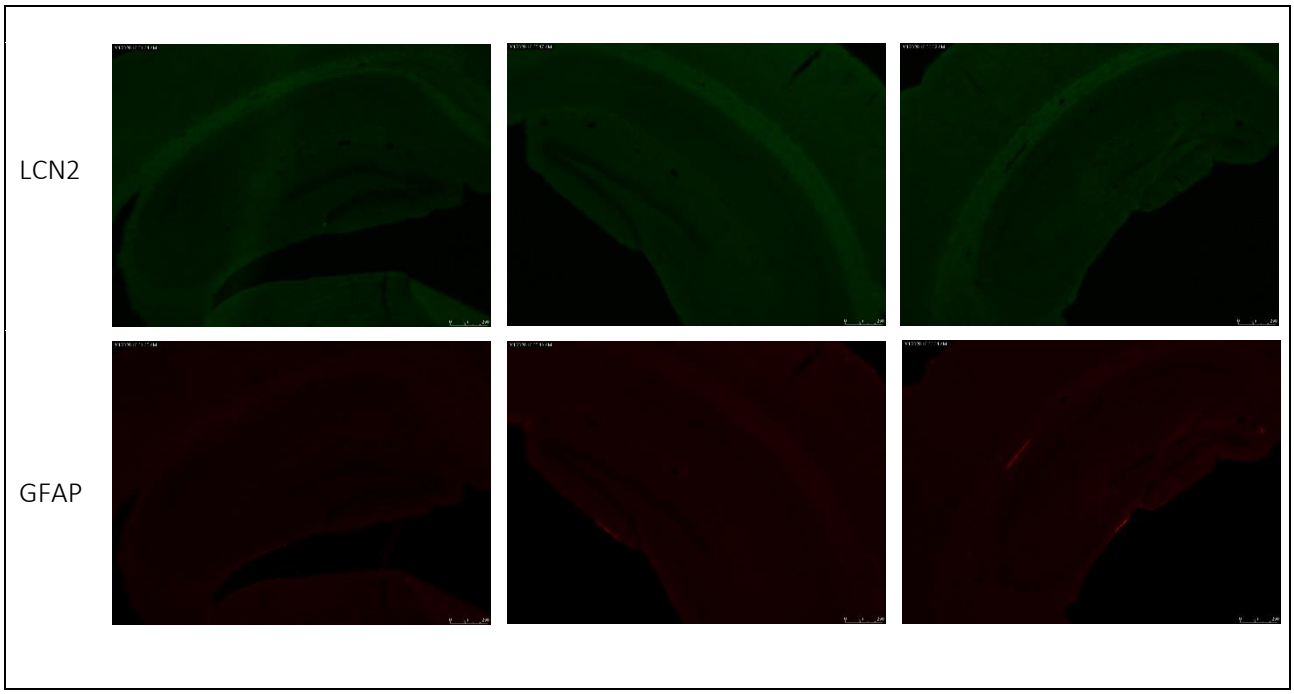

Figure 8 LCN2 and GFAP fluorescent co-staining pilot. The primary antibody used is Rat LCN2. The secondary antibody used is Donkey Anti-Mouse 488.

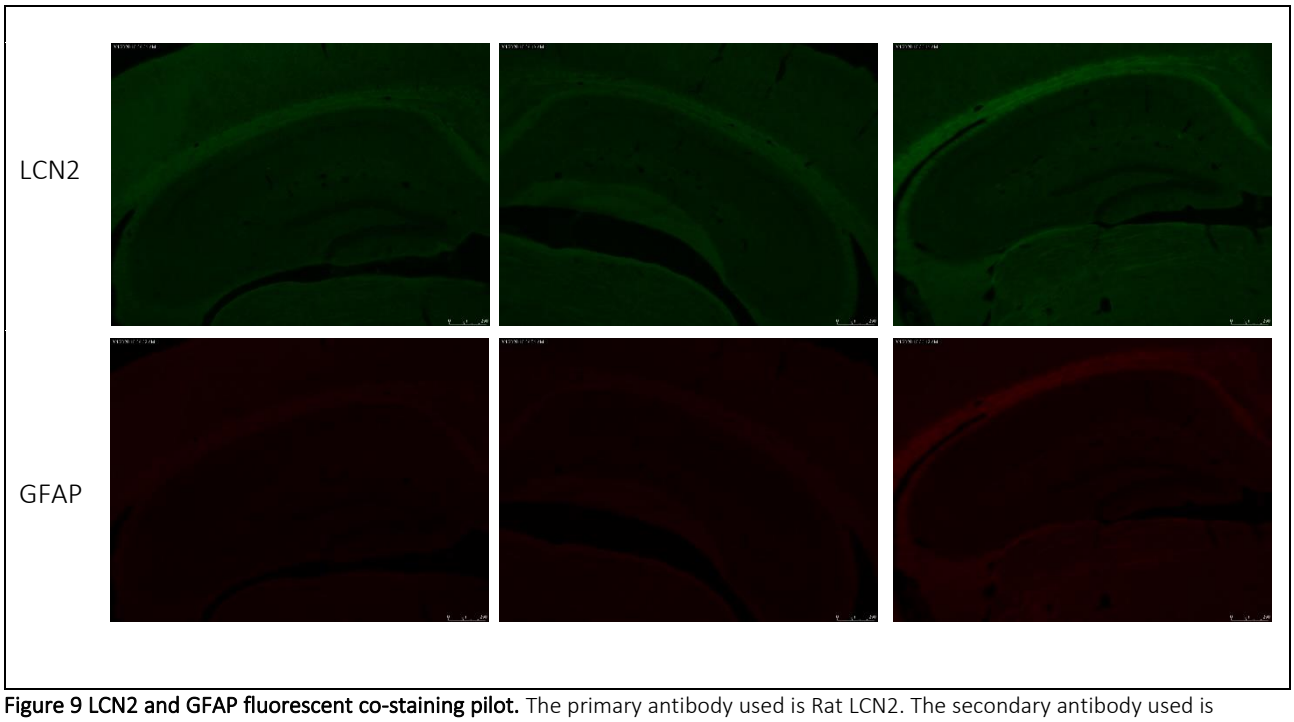

Figure 9 LCN2 and GFAP fluorescent co-staining pilot. The primary antibody used is Rat LCN2. The secondary antibody used is Goat Anti-Rat 555.

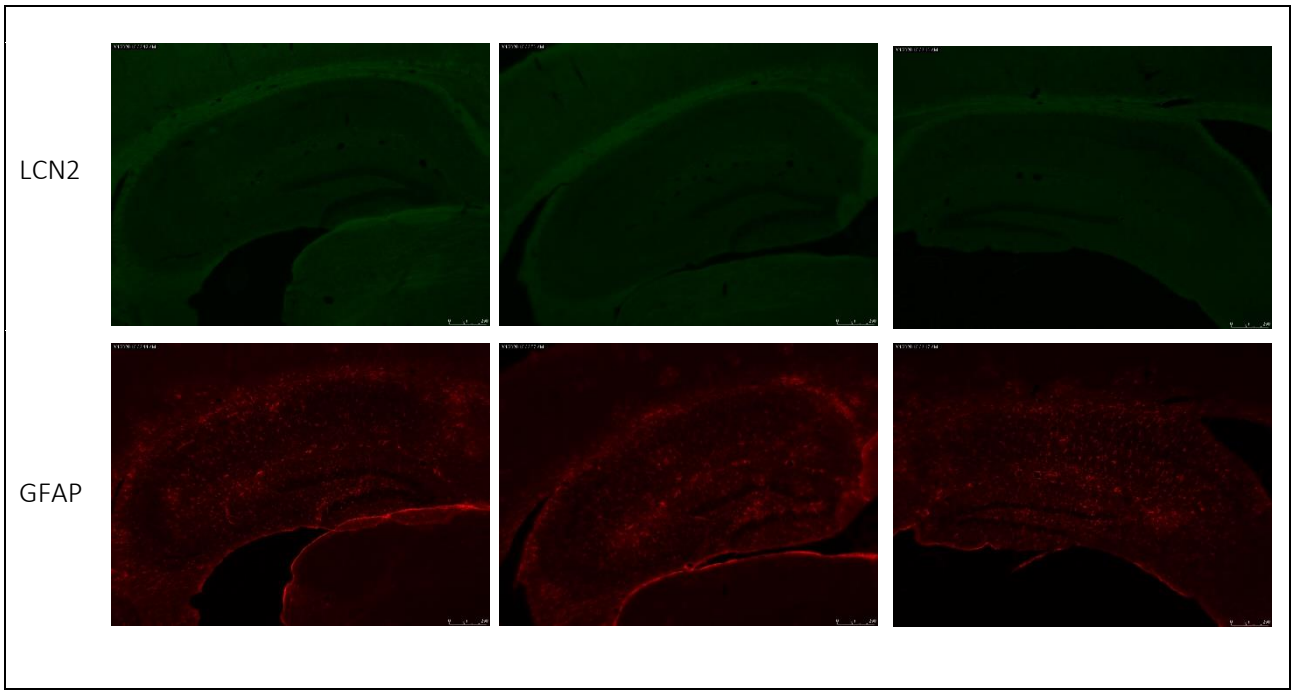

Figure 10 LCN2 and GFAP fluorescent co-staining pilot. The primary antibody used is Mouse GFAP. The secondary antibody used is Donkey Anti-Mouse 488.

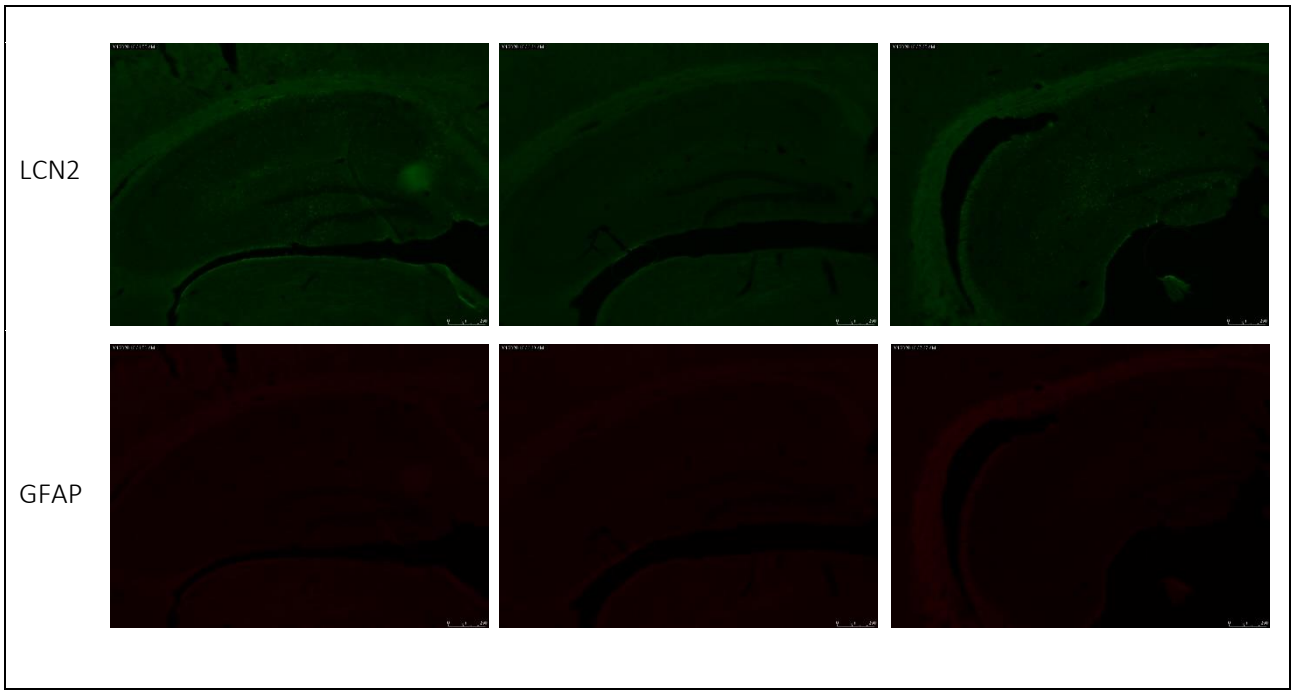

Figure 11 LCN2 and GFAP fluorescent co-staining pilot. The primary antibody used is Mouse GFAP. The secondary antibody used is Goat Anti-Rat 555.

Discussion

Morris Water Maze

The Morris Water Maze did not show any conclusive results: MK41.3xJ20 did not show any cognitive improvement in comparison to J20.

During the learning phase, no significant differences appeared between phenotypes. However, within several phenotypes, a significant difference was shown from day one to day eight for all parameters. The fact that swim speed decreased over the course of eight days for all phenotypes indicates a reduced stress level, as was assessed by Gehring et al. (2015) in a study where stressed and non-stressed animals were compared in Morris Water Maze behaviour. Thereby, the reduced path length and reduced escape latency shows the learning effect in this cognitive test (Morris, 1984; D'Hooge et al., 2001).

Probe trials were performed 24 hours and 48 hours after the learning phase. At the 24 hour probe trial, a significant difference in number of platform crossings was shown between Wt and J20 and Wt an MK41.3. Here, the J20 and MK41.3xJ20 groups show similar results. No (significant) differences were shown in number of platform crossings at the 48 hour probe trial.

At both 24 hours and 48 hours probe trial, the time spent in each quadrant was assessed for all phenotypes. No significant differences were shown between different genotypes. At 24 hours, J20 spent less time in the target quadrant than MK41.3xJ20, which could indicate a better performance within the group that had elevated TNF-α levels. However, at 48 hours, J20 performed better than MK41.3xJ20. The assumption that MK41.3xJ20 performed better than J20 cannot be rejected nor accepted, since mice could use different strategies in reaching the platform (Vorhees et al., 2006).

Aβ plaque development

The 6e10 staining showed interesting results indicating a lower number of Aβ plaques in an environment with elevated TNF-α. This is in line with the expectation that the MK41.3xJ20 model would show less Aβ plaque development compared to J20.

The Aβ plaque development was measured as the number of plaques in the hippocampus. This number was quantified as a percentage of the total area measured. Significant differences were shown between Wt and J20, Wt and MK41.3xJ20, J20 and MK41.3, MK41.3 and MK41.3xJ20 and, lastly, J20 and MK41.3xJ20 (each $p<0,0001$) (Fig. 5). The lower percentage of 6e10 positive area in MK41.3xJ20 in comparison with J20 shows that a higher level of TNF-α is associated with a decreased number of Aβ plaques. This could indicate a neuroprotective effect of the elevated TNF-α levels in this AD model (Kalovyrna et al., 2020).

Neuroinflammation

Microglia activation

To assess microglia activation, an Iba1 staining was performed. Microglia activation is shown as the number of Iba1 positive cells, which was quantified as the percentage of the total area measured. From the Iba1 staining can be appreciated that increased TNF-α levels result in a higher number of activated microglia, with significant differences between genotypes. Most interesting is the significant difference between J20 and MK41.3xJ20 ($p<0,0001$). MK41.3xJ20 show a significant higher percentage of Iba1 positive area, which indicates a higher number of activated microglia. The higher amount of activated microglia might indicate an increase in neuroinflammation. However, the elevated number of active microglia could also indicate that the neuroprotective effect of TNFR2 activation by increased TNF-α levels is achieved via enhanced microglia activation (Cai et al., 2020). It would be wise to assess this in further studies regarding TNFR2 dynamics.

LCN2 and GFAP fluorescent co-staining pilot

The LCN2 and GFAP fluorescent co-staining pilot was performed to assess whether LCN2 and GFAP can successfully be co-stained. Five combinations of primary and secondary antibodies were tested (App. C). The first combination with both Rat LCN2 and Mouse GFAP primary antibody and both Goat Anti-Rat 555 and Donkey Anti-Mouse secondary antibodies showed promising results in the form of highlighted areas in the LCN2 and GFAP filtered pictures (Fig. 7). However, other combinations of primary and secondary antibodies had to be tested to make sure the antibodies were working the way they should.
The first combination of primary Rat LCN2 and secondary Donkey Anti-Mouse 488 gave the expected results: no highlighted areas were shown in either of the filtered pictures (Fig. 8). In conclusion, the Donkey Anti-Mouse 488 secondary antibody does not show nonspecific binding to the primary Rat LCN2 antibody.
The second combination assesses the binding of the secondary Goat Anti-Rat 555 antibody to primary antibody Rat LCN2. In the pictures filtered for LCN2, no highlighted areas are shown. Since no lighter areas are shown in the GFAP filtered pictures either, it is concluded that the secondary Goat Anti-Rat 555 antibody does not bind, either specifically or non-specifically (Fig. 9).
The next combination is a control for correct binding of the secondary Donkey Anti-Mouse 488 antibody to the primary Mouse GFAP antibody. Lighter areas do appear in the GFAP filtered pictures and not in the LCN2 filtered pictures, which means the antibodies work according to expectations (Fig. 10).
Lastly, the Mouse GFAP primary antibody was combined with the Goat Anti-Rat 555 secondary antibody. This combination shows no highlighting in the GFAP filtered pictures, which is expected. However, some lighter areas appear in the LCN2 filtered pictures, which is unexpected and from which we can conclude that nonspecific binding occurs with the primary Mouse GFAP antibody.

Conclusion

The objective of this study was to evaluate the effects of elevated TNF-α levels in certain brain regions on Aβ based AD symptoms in mice. It was expected that elevated TNF-α levels would result in increased TNFR2 activation, leading to neuroprotective responses. It was hypothesized that the AD mouse model with elevated TNF-α levels, the MK41.3xJ20 model, would show less AD symptoms in comparison to the AD mouse model without elevated TNF-α levels, the J20 model. This would be shown in an improved cognitive phenotype, which was assessed through the Morris Water Maze, less Aβ plaque development, assessed through a 6e10 staining, and a decrease in neuroinflammation, which was assessed through an Iba1 staining.

In conclusion, from the current results it seems likely that an increase in TNF-α levels in certain brain regions results in a decrease in AD symptoms. However, results from both LCN2 and GFAP stainings should be included in order to confirm this.
Additionally, research regarding the TNFR1 and TNFR2 dynamics is needed to assess the role of both receptors in AD, including whether neuroprotection is achieved through TNFR2 activation. TNFR1 signaling could be assessed through a Western Blot for Caspase 3 and TNFR2 signaling could be assessed through a Western Blot for pAkt/Akt.

References

Cai, Z., Hussain, M.D., Yan, L.J. (2013). Microglia, neuroinflammation, and Aβ in Alzheimer's disease. International Journal of Neuroscience 124,.

Cheng, X., Yang, L., He, P., Li, R., Shen, Y. (2010). Differential Activation of Tumor Necrosis Factor Receptors Distinguishes between Brains from Alzheimer's Disease and Non-Demented Patients. Journal of Alzheimer's Disease 19, 621-630.

Dekens, D.W., Naudé, P.J.W., Keijser, J.N., Boerema, A.S., De Deyn, P.P., Eisel, U.L.M. (2018). Lipocalin 1 contributes to brain iron dysregulation but does not affect cognition, plaque load, and glial activation in the J20 Alzheimer mouse model. Journal of Neuroinflammation 15.

Dekens, D.W. (2019). Lipocalin 2 and the pathophysiology of Alzheimer's disease. Rijksuniversiteit Groningen.

D'Hooge, R., Deyn, P.D. (2001). Applications of the Morris water maze in the study of learning and memory. Brain Research Reviews 36, 60-90.

Dong, Y., Dekens, D. W., De Deyn, P. P., Naudé, P. J. W., Eisel, U. L. M. (2015). Targeting of Tumor Necrosis Factor Alpha Receptors as a Therapeutic Strategy for Neurodegenerative Disorders. Antibodies 4, 369-408.

Dong, Y., Fischer, R., Naudé P.J.W., Maier, O., Nyakas, C., Duffey, M., Van der Zee, E.A., Dekens, D., Douwenga, W., Herrmann, A., Guenzi, E., Kontermann, R.E., Pfixenmaier, K., Eisel, U.L.M. (2016). Essential protective role of tumor necrosis factor receptor 2 in neurodegeneration. Proc. Natl. Acad. Sci. U.S.A. 113(43), 12304-12309.

He, P., Zhong, Z., Lindholm, K., Berning, L., Lee, W., Lemere, C., Staufenbiel, M., Li, R. Shen, Y. (2007). Deletion of tumor necrosis factor death receptor inhibits amyloid β generation and prevents learning and memory deficits in Alzheimer's mice. The Journal of Cell Biology 178(5), 829-841.

Hopperton, K.E., Mohammad, D., Trépanier, M.O., Giuliano, V., Bazinet, R.P. Markers of microglia in post-mortem brain samples from patients with Alzheimer's disease: a systematic review. Molecular Psychiatry 23(2), 177-198.

Hovens, I.B., Van Leeuwen, B.L., Nyakas, C., Heineman, E., Van der Zee, E.A., Schoemaker, R.G. (2015). Postoperative cognitive dysfunction and microglial activation in associated brain regions in old rats. Neurobiology of Learning and Memory 118, 74-79.

Jiang, H., He, P., Xie, J., Staufenbiel, M., Li, R., Shen, Y. (2014). Genetic deletion of TNFRII gene enhances the Alzheimer-like pathology in an APP transgenic mouse model via reduction of phosphorylated IκBα. Human Molecular Genetics 23(18), 4906-4918.

Kalovyrna, N., Apokotou, O., Boulekou, S., Paouri, E., Boutou, A., Georgopoulos, S. (2020). A 3'UTR modification of the TNF-α mouse gene increases peripheral TNF-α and modulates the Alzheimer-like phenotype in 5XFAD mice. Nature Scientific Reports 10.

Kamphuis, W., Middeldorp, J., Kooijman, L., Sluijs, J.A., Kooi, E.J., Moeton, M., Freriks, M., Mizee, M.R., Hol, E.M. (2014). Glial fibrillary acidic protein isoform expression in plaque related astrogliosis in Alzheimer's disease. Neurobiology of Ageing 35, 492-510.

Kozlov, S., Afonin, A., Evsyukov, I., Bondarenko, A. (2017) Alzheimer's disease: as it was in the beginning. Rev. Neurosci. 28(8), 825-843.

Lane, C. A., Hardy, J., Schott, J. M. (2018). Alzheimer's disease. European Journal of Neurology 25, 59-70.

Marchetti, L., Klein, M., Schlett, K., Pfizenmaier, K., Eisel, U.L.M. (2004). Tumor Necrosis Factor (TNF)-mediated Neuroprotection against Glutamate-induced Excitotoxicity Is Enhanced by N-Methyl-D-Aspartate Receptor Activation. The Journal of Biological Chemistry 279(31), 32869-32881.

McAlpine, F.E. & Tansey, M.G. (2008). Neuroinflammation and tumor necrosis factor signaling in the pathophysiology of Alzheimer's disease. Journal of Inflammation Research 1, 29-39.

Morris, R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. Journal of Neuroscience Methods 11, 47-60.

Mucke, L., Masliah, E., Yu, G.Q., Mallory, M., Rockenstein, E.M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-Wood, K., McConlogue, L. (2000). High-Level Neuronal Expression of $A\beta_{1-42}$ in Wild-Type Human Amyloid Protein Precursor Transgenic Mice: Synaptotoxicity without plaque formation. The Journal of Neuroscience 20(11), 4050-4058.

Murphy, M.P. & LeVine, H. (2010). Alzheimer's Disease and the β-Amyloid Peptide. J. Alzheimers Dis. 19(1).

Orti-Casañ, N., Wu, Y., Naudé, P.J.W., De Deyn, P.P., Zuhorn, I.S., Eisel, U.L.M. (2019). Targeting TNFR2 as a Novel Therapeutic Strategy for Alzheimer's Disease. Frontiers in Neuroscience 13, article 49.

Paouri, E., Tzara, O., Kartalou, G.I., Zenelak, S., Georgopoulos S. (2017b). Peripheral Tumor Necrosis Factor-Alpha (TNF-α) Modulates Amyloid Pathology by Regulating Blood-Derived Immune Cells and Glial Response in the Brain of AD/TNF Transgenic Mice. The Journal of Neuroscience 37(20), 5155-5171.

Paouri, E., Tzara, O., Zenelak, S., Georgopoulos, S. (2017a). Genetic Deletion of Tumor Necrosis Factor-α Attenuates Amyloid-β Production and Decreases Amyloid Plaque Formation and Glial Response in the 5XFAD Model of Alzheimer's Disease. Journal of Alzheimer's Disease 60, 165-181.

Steeland, S., Gorlé, N., Vandendriessche, C., Balusu, S., Brkic, M., Van Cauwenberghe, C., Van Imschoot, G., Van Wonterghem, E., De Rycke, R., Kremer, A., Lippens, S., Stopa, E., Johanson, C.E., Libert, C., Vandenbroucke, R.E. (2018). Counteracting the effects of TNF receptor-1 has therapeutic potential in Alzheimer's disease. EMBO Molecular Medicine 10.

Tosh, J.L., Rickman, M., Rhymes, E., Norona, F.E., Clayton, E., Mucke, L., Isaacs, A.M., Fisher, E.M.C., Wiseman, F.K. (2018). The integration site of the APP transgene in the J20 mouse model of Alzheimer's disease. Wellcome Open Research 2(84).

Vorhees, C.V., Williams, M.T. (2006). Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nature Protocols 1(2), 848-858.

Youmans, K.L., Tai, L.M., Kanekiyo, T., Stine Jr, W.B., Michon, S.C., Nwabuisi-Heath, E., Manelli, A.M., Fu, Y., Riordan, S., Eimer, W.A., Binder, L., Bu, G., Yu, C., Hartley, D.M., LaDu, M.J. (2012). Intraneuronal Aβ detection in 5xFAD mice by a new Aβ-specific antibody. Molecular Neurodegeneration 7(8).

Appendix

A. 6e10 staining protocol

Day 1

1. 3 x 5 min washing with TBS.
2. 30 minute incubation with 0,3% H_2O_2 in TBS.

30 cups x 3 mL = 90 mL (100mL in total)		
30% H_2O_2	0,3%	1000 µL
TBS 0.01M		99000 µL

3. 3 x 5 min washing with TBS.
4. 1 hour (pre-)incubation with goat serum, room temperature.

30 cups x 1 mL = 30 mL		
TritonX 10%	0,1%	300 µL
Goat serum (NGS)	3%	900 µL
TBS 0.01M		28800 µL

5. 2 nights incubation with 1st antibody: 6e10, 4°C.

29 cups x 1 mL = 29 mL (2 DAB cups + 27 experimental)		
6e10	1:2000	14,5 µL
TritonX 10%	0,1%	290 µL
Goat serum (NGS)	3%	870 µL
TBS 0.01M		27825,5 µL

1 cup x 1mL = 1 mL: 1 negative		
TritonX 10%	0,1%	10 µL
Goat serum (NGS)	3%	30 µL
TBS 0.01M		960 µL

Day 3

1. 3 x 5 min washing with TBS.
2. 2 hours incubation with 2nd antibody: goat-anti-mouse, room temperature.

30 cups x 1 mL = 30 mL		
Goat-anti-mouse	1:400	75 µL
Goat serum (NGS)	1%	300 µL
TBS 0,01M		29625 µL

3. 6 x 5 min washing with TBS.
4. Prepare Avidin-Biotin solution (at least 30 minutes before use).

30 cups x 1 mL = 30 mL		
A	1:500	60 µL
B	1:500	60 µL
TBS 0.01M		29880 µL

5. 1,5 hours incubation with Avidin-Biotin solution, room temperature.

6. 3 x 5 min washing with TBS.
7. Incubate overnight at 4°C.

Day 4:

1. Dissolve DAB tablets (one tablet 15 mL in 15 mL mQ, no dilution). Protect from light.

30 cups x 3 mL = 90 mL + 5 mL (if 5mL DAB tablets are available)		
DAB tablet	1 tablet/15 mL + 1 tablet/5 mL	6 tablets + 1 tablet
Milli-Q		90 mL + 5 mL

2. 3 x 5 min washing with TBS.
3. Prepare H_2O_2 solution. Protect from light.

30 cups x 100 µL = 3000 µL -> prepare 4500 µL		
H_2O_2 30%	0,1%	10 µL -> 15 µL
Milli-Q		2990 µL -> 4485 µL

4. Add DAB to all cups. Wait 1 minute before adding H_2O_2.
5. DAB reaction is started when H_2O_2 is added. This is the START time.
6. 20 seconds circling the cup.
7. Wash quickly 3 x with PBS to stop reaction. This is the STOP time.
8. Wash 3 x 5 min with PBS.
9. Incubate at 4°C.

Notes:

- All incubation steps are done on orbital shaker.
- DAB = toxic. Wear gloves.
- DAB waste separate: add 3 drops of bleach and leave in fume hood.
- Clean everything with ethanol and change papers.

Where is it stored?

- DAB: left freezer (-20°C), top drawer. Always use the silver package.
- H_2O_2 30%: fridge.
- BSA: fridge.
- TritonX 10%: fridge.
- Goat serum (NGS): big -20°C freezer.
- Anti-Iba1:
- Goat-anti-rabbit: fridge. Box ‘Ate’.
- 6e10: right freezer (-20°C), top drawer. Box Yingying.
- Goat-anti-mouse: fridge. Box ‘Ate’.
- A-B kit: fridge.

B. Iba1 staining protocol

Day 1

1. 3 x 5 min washing with PBS.
2. 30 minute incubation with 0.3% H_2O_2 in PBS.

30 cups x 3 mL = 90 mL (100mL in total)

30% H_2O_2	0,3%	1000 µL
PBS 0.01M		99000 µL

3. 3 x 5 min washing with PBS.
4. 72 hours incubation with 1st antibody: rabbit anti-Iba1, 4°C. Prepare in 50 mL tube.

29 cups x 1 mL = 29 mL (2 DAB cups + 27 experimental)		
Anti-Iba1	1:2500	11,6 µL
BSA	1%	0,29 gr
TritonX 10%	0,1%	290 µL
PBS 0.01M		28698,4 µL

1 cup x 1 mL = 1 mL: 1 negative		
BSA	1%	0,01 gr
TritonX 10%	0,1%	10 µL
PBS 0.01M		990 µL

Day 4

1. Collect 1st antibody in 50 mL tube.
2. 3 x 5 min washing with PBS.
3. 2 hour incubation with 2nd antibody: biotin conjugated goat-anti-rabbit, room temperature. Prepare in 50 mL tube.

30 cups x 1 mL = 30 mL		
Goat-anti-rabbit	1:500	60 µL
PBS 0.01M		29940 µL

4. Collect 2nd antibody in 50 mL tube.
5. 6 x 5 min washing with PBS.
6. Incubate overnight at 4°C.

Day 5

1. Prepare Avidin-Biotin solution (at least 30 minutes before use).

30 cups x 1 mL = 30 mL		
A	1:500	60 µL
B	1:500	60 µL
PBS 0.01M		29880 µL

2. 1 x 5 min washing with PBS.
3. 1 hour incubation with Avidin-Biotin solution, room temperature.
4. 3 x 5 min washing with PBS.
5. Incubate overnight at 4°C.

Day 6

1. Dissolve DAB tablets (one tablet 15 mL in 30 mL mQ). Protect from light.

30 cups x 3 mL = 90 mL + 10 mL (if 5 mL DAB tablets are available)

DAB tablet	1 tablet/15 mL	3 tablets + 1 tablet
Milli-Q		90 mL + 10 mL

2. 1 x 5 min washing with TBS.
3. Prepare H_2O_2 solution. Protect from light.

30 cups x 100 µL = 3000 µL -> prepare 4500 µL		
H_2O_2 30%	0,1%	10 µL -> 15 µL
Milli-Q		2990 µL -> 4485 µL

4. Add DAB to all cups. Wait 1 minute before adding H_2O_2.
5. DAB reaction is started when H_2O_2 is added. This is the START time.
6. 20 seconds circling the cup.
7. Wash quickly 3 x with PBS to stop reaction. This is the STOP time.
8. Wash 3 x 5 min with PBS.
9. Incubate at 4°C.

Notes:

- All incubation steps are done on orbital shaker.
- DAB = toxic. Wear gloves.
- DAB waste separate: add 3 drops of bleach and leave in fume hood.
- Clean everything with ethanol and change papers.

Where is it stored?

- DAB: left freezer (-20°C), top drawer. Always use the silver package.
- H_2O_2 30%: fridge.
- BSA: fridge.
- TritonX 10%: fridge.
- Goat serum (NGS): big -20°C freezer. Box 'Normal antibodies'.
- Anti-Iba1:
- Goat-anti-rabbit: fridge. Box 'Ate'.
- 6e10: right freezer (-20°C), top drawer. Box Yingying.
- Goat-anti-mouse: fridge. Box 'Ate'.
- A-B kit: fridge.

C. LCN2 and GFAP fluorescent co-staining protocol

Cup 1:
Rat LCN2 primary antibody + mouse GFAP primary antibody.
Goat anti-rat 555 secondary antibody + donkey anti-mouse 488 secondary antibody.
Normal donkey serum.

Cup 2:
Rat LCN2 primary antibody.
Donkey anti-mouse 488 secondary antibody.
Normal goat serum.

Cup 3:
Rat LCN2 primary antibody.

Goat anti-rat 555 secondary antibody.
Normal donkey serum.

Cup 4:
Mouse GFAP primary antibody.
Donkey anti-mouse 488 secondary antibody.
Normal donkey serum.

Cup 5:
Mouse GFAP primary antibody.
Goat anti-rat 555 secondary antibody.
Normal goat serum.

Four ventral hippocampus slices in each cup.

Day 1

1. 3 x 5 min washing with PBS.
2. 40 min (pre-)incubation with donkey serum or goat serum, room temperature.

Cups 1, 3 & 5: 3 cups x 1 mL = 3 mL		
TritonX 10%	0.05%	15 µL
Goat serum (NGS)	3%	90 µL
PBS 0.01M		2895 µL

Cups 2 & 4: 2 cups x 1 mL = 2 mL		
TritonX 10%	0.05%	10 µL
Donkey serum (NDS)	3%	60 µL
PBS 0.01M		1930 µL

3. 1 night incubation with 1st antibody, 4°C.

Cup 1: 1 cup x 1 mL = 1 mL		
Goat serum (NGS)	3%	30 µL
Anti-LCN2	1:1000	1 µL
Anti-GFAP	1:10000	0.1 µL
PBS 0.01M		968,9 µL
Cup 2: 1 cup x 1 mL = 1 mL		
Donkey serum (NDS)	3%	30 µL
Anti-LCN2	1:1000	1 µL
PBS 0.01M		969 µL

Cup 3: 1 cup x 1 mL = 1 mL		
Goat serum (NGS)	3%	30 µL
Anti-LCN2	1:1000	1 µL
PBS 0.01M		969 µL

Cup 4: 1 cup x 1 mL = 1 mL		
Donkey serum (NDS)	3%	30 µL
Anti-GFAP	1:10000	0.1 µL
PBS 0.01M		969.9 µL

Cup 5: 1 cup x 1 mL = 1 mL		
Goat serum (NGS)	3%	30 µL
Anti-GFAP	1:10000	0.1 µL
PBS 0.01M		969.9 µL

Day 2

1. 4 x 10 min washing with PBS.
2. 1.5 hour incubation with 2nd antibody. Cover cups with aluminium foil!

Cup 1: 1 cup x 1 mL = 1 mL		
Goat serum (NGS)	3%	30 µL
Goat anti-rat 555	1:400	2.5 µL
Donkey anti-mouse 488	1:400	2.5 µL
PBS 0.01M		965 µL

Cup 2: 1 cup x 1 mL = 1 mL		
Donkey serum (NDS)	3%	30 µL
Donkey anti-mouse 488	1:400	2.5 µL
PBS 0.01M		967.5 µL

Cup 3: 1 cup x 1 mL = 1 mL		
Goat serum (NGS)	3%	30 µL
Goat anti-rat 555	1:400	2.5 µL
PBS 0.01M		967.5 µL

Cup 4: 1 cup x 1 mL = 1 mL		
Donkey serum (NDS)	3%	30 µL
Donkey anti-mouse 488	1:400	2.5 µL
PBS 0.01M		967.5 µL

Cup 5: 1 cup x 1 mL = 1 mL		
Goat serum (NGS)	3%	30 µL
Goat anti-rat 555	1:400	2.5 µL
PBS 0.01M		967.5 µL

3. 4 x 5 min washing with PBS. Cover cups with aluminium foil!
4. Wash overnight at 4°C. Cover cups with aluminium foil!

Day 3

1. Mount slices in PBS, in a dark room.
2. Cover slips with aluminium foil and leave to dry.
3. Cover slips using Mowiol.
4. Leave covered with aluminium in cold room, 4°C.

Notes:

- All incubation steps are done on orbital shaker.
- The fluorescent secondary antibodies are light sensitive and therefore have to be handled in the dark as much as possible to avoid fading/bleaching.

Where is it stored?

- TritonX 10%: fridge.
- Anti-LCN2: freezer. Box 'Yingying'.
- Anti-GFAP: freezer. Box 'Yingying'.
- Goat anti-rat 555: fridge. Box 'Ate'.
- Donkey anti-mouse 488: fridge. Box 'Ate'.
- Goat serum (NGS): big -20°C freezer.
- Donkey serum (NDS): big -20°C freezer.

D. CD11b and GFAP fluorescent co-staining pilot protocol

Cup 1:
Rabbit CD11b primary antibody (1:100) + mouse GFAP primary antibody.
Donkey anti-mouse 555 secondary antibody + donkey anti-rabbit 488 secondary antibody.
Normal donkey serum.

Cup 2:
Rabbit CD11b primary antibody (1:250) + mouse GFAP primary antibody.
Donkey anti-mouse 555 secondary antibody + donkey anti-rabbit 488 secondary antibody.
Normal donkey serum.

Cup 3:
Donkey anti-mouse 555 secondary antibody + donkey anti-rabbit 488 secondary antibody.

Four ventral hippocampus slices in each cup.

Day 1

4. 3 x 5 min washing with PBS.
5. 40 min (pre-)incubation with donkey serum or goat serum, room temperature.

3 cups x 1 mL = 3 mL		
TritonX 10%	0.05%	15 µL
Goat serum (NGS)	3%	90 µL
PBS 0.01M		2895 µL

6. 1 night incubation with 1st antibody, 4°C.

Cup 1: 1 cup x 1 mL = 1 mL		
Donkey serum (NDS)	3%	30 µL
Anti-CD11b	1:1000	1 µL
Anti-GFAP	1:100	10 µL
PBS 0.01M		959.9 µL

Cup 2: 1 cup x 1 mL = 1 mL		
Donkey serum (NDS)	3%	30 µL
Anti-CD11b	1:1000	1 µL
Anti-GFAP	1:250	4 µL
PBS 0.01M		965.9 µL

Cup 3: 1 cup x 1 mL = 1 mL		
Donkey serum (NDS)	3%	30 µL
PBS 0.01M		970 µL

Day 2

5. 4 x 10 min washing with PBS.
6. 1.5 hour incubation with 2nd antibody. Cover cups with aluminium foil!

3 cups x 1 mL = 3 mL		
Donkey serum (NDS)	3%	90 µL
Donkey anti-rabbit 488	1:400	7.5 µL
Donkey anti-mouse 555	1:400	7.5 µL
PBS 0.01M		2895 µL

7. 4 x 5 min washing with PBS. Cover cups with aluminium foil!
8. Wash overnight at 4°C. Cover cups with aluminium foil!

Day 3

5. Mount slices in PBS, in a dark room.
6. Cover slips with aluminium foil and leave to dry.
7. Cover slips using Mowiol.
8. Leave covered with aluminium in cold room, 4°C.

Notes:

- All incubation steps are done on orbital shaker.
- The fluorescent secondary antibodies are light sensitive and therefore have to be handled in the dark as much as possible to avoid fading/bleaching.

Where is it stored?

- TritonX 10%: fridge.
- Anti-LCN2: freezer. Box 'Yingying'.
- Anti-GFAP: freezer. Box 'Yingying'.
- Goat anti-rat 555: fridge. Box 'Ate'.
- Donkey anti-mouse 488: fridge. Box 'Ate'.
- Goat serum (NGS): big -20°C freezer.
- Donkey serum (NDS): big -20°C freezer.