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Lipocalin-2 as therapeutic target for Alzheimer's disease

Effect of Deferiprone in the cognition of J20 mice

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Abstract

Lipocalin-2 (Lcn-2), an acute phase protein associated with iron homeostasis, has been reported to be involved in various neurodegenerative conditions, including Alzheimer's Disease (AD). For instance, recent studies suggest that this protein, which is overexpressed in AD, may play a role in neuroinflammation and iron dysregulation, two hallmarks of the neuropathology of this disorder. Additionally, iron has been found to induce the up-regulation of Lcn-2. Here, we use the iron chelator Deferiprone to target Lcn-2. The main hypothesis is that Deferiprone might reduce iron accumulation in the brain of J20 mouse model of AD and, subsequently, prevent the up-regulation of the protein. For this, wild-type (WT) and J20 mice were given either Deferiprone (50 mg/kg) or water daily during 12 weeks. Concretely, this report will focus on the cognitive aspects, which were tested over a series of behavioural tasks that started after 5 weeks of treatment. While there are genotype differences between WT and J20 mice, the administration of Deferiprone did not improve the cognitive performance of the J20 mice. Since iron chelation therapies has shown encouraging results in the context of cognitive impairments in AD in the past, further research is needed to explain why in our study Deferiprone could not ameliorate the memory impairment in the J20 mice. At the same time, the neuropathological analysis of the brains might help to clarify the results obtained here, especially the stainings for iron and Lcn-2. Finally, although this study could not validate Lcn-2 as a therapeutic target, the role and relevance of this protein in AD should be studied more comprehensively.

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1. Introduction

Alzheimer's Disease (AD) is the fifth leading cause of deaths worldwide [1] and the most common form of dementia, accounting for around two-thirds of the total cases [2]. Being a progressive, neurodegenerative disorder, AD is characterized by a gradual decline in the cognitive functions, including impairments in the ability of thinking or reasoning, memory loss and language difficulties. As the disease advances, the severity of the symptoms will increase as well, leading ultimately to the death of the patient [3]. To this day, AD remains incurable, with the current treatments available offering only a short symptomatic relief.

One of the reasons that hinders the finding of a cure for AD is the multifactorial nature of this disorder, and the still uncertainty of its origin [4]. Indeed, AD pathology is described by a number of complex hallmarks, being the most distinctive the build-up of beta-amyloid (Aβ) plaques and neurofibrillary tangles (NFT). This contributes to the atrophy of the brain due to neuronal and synaptic loss [5], especially predominant in those areas of the brain responsible of memory and learning, such as the hippocampus, which is among the first regions affected by the accumulation of the protein aggregates.

In addition to the presence of misfolded proteins, two other mechanisms that are known to play a role in the pathogenesis of AD are neuroinflammation and iron dysregulation. Inflammation in the brain of AD patient is evidenced by the abnormal expression of pro-inflammatory mediators and the activation of immunocompetent cells in the brain, namely microglia and astrocytes. Although initially the conversion of these cells to an active state is intended to be a protective response towards the Aβ deposition, evidences show that it can also have detrimental consequences and contribute to the development of the disease and the cognitive malfunction [6]. Likewise, dysregulation of the brain iron homeostasis during AD has been found to be involved in the pathological progression of this neurodegenerative disorder [7]. Aberrant iron accumulation arises in areas of the brain that are particularly affected in this form of dementia (for instance, the cortex and the hippocampus), predominantly near Aβ plaques and NFT, as well as within various cells types, including neurons [7,8]. These deposits seem to contribute to Aβ and tau neurotoxicity [7,9,10] and promote oxidative stress by the abnormal generation of reactive oxygen species (ROS), which ultimately leads to oxidative damage and cell death, e.g. via ferroptosis [11]. Moreover, all of this induces in turn the activation of microglia, further enhancing inflammation and neurodegeneration in the affected regions.

Recent studies suggest that a protein that has been observed to be up-regulated in the brain of AD patients –called Lipocalin 2 (Lcn2) or Neutrophil Gelatinase-Associated Lipocalin (NGAL), alongside various other names–, may play a part in both neuroinflammation and iron dyshomeostasis, and ;thus, might be important in the pathogenesis of the disease [12–14]. Briefly, Lipocalin 2 is an acute-phase protein involved in iron homeostasis which is released as a response to inflammatory processes. As part of the innate immunity, and induced by various pro-inflammatory stimuli, Lcn2 is released to sequester microbial siderophores that iron-dependent bacteria produce to steal iron from the host [14]. Apart from this role in defence against bacterial infections, several other functions have been attributed over the past few years to this protein. For instance, it may have a protective effect during injury and inflammatory conditions [15–17], induce pro-apoptotic [18,19] or anti-apoptotic signalling pathways [20,21] and participate in other aspects of cell regulation, such as proliferation and migration [22,23]. Interestingly, brain cells such as microglia, astrocytes or cortical neurons are included among the various cell types that can be responsible for the secretion of Lcn2 [12,24], which supports the potential role of Lcn2 during neuroinflammation and brain-related disorders.

Indeed, there are various evidences that endorse the link between Lcn2 and AD. As it has been mentioned before, the expression of this protein has been found to be significantly higher in the brains of people with AD, notably in the regions commonly associated with this disease [12,25]. Furthermore, Lcn2 may promote reactive astrocytosis in an autocrine fashion [26,27], as well as induce the conversion of microglia to the activated M1 state [28]. At the same time, *in vitro* studies indicate that Lcn2 may sensitize neurons and astrocytes to the toxicity induced by Aβ plaques, and that Lcn2 expression itself is up-regulated in response to these Aβ peptides [12,29,30]. Moreover, high levels of Lcn2 in the brain seem to contribute to iron accumulation in a mouse model of AD, especially in the hippocampus [31]. Taken together, all these findings situate Lcn2 as a potential mediator in AD pathogenesis and opens the door to new therapeutic options that target this molecule.

Of relevance, it has been shown that iron may induce the expression and promote the neuropathologic effects of Lcn2 [32,33]. Additionally, iron chelators seem to counteract these effects in different *in vivo* models of various central nervous system (CNS) diseases that also display iron dyshomeostasis [32–34], supporting the connection between iron accumulation and Lcn2 up-regulation and proposing iron chelation therapy as a promising approach to target Lcn2 and iron dysregulation in neurodegenerative disorders.

In the context of AD, the addition of an iron chelator reduced the Aβ-induced Lcn2 production in cultured astrocytes [30]. *In vivo*, iron chelation has also been reported to improve cognitive performance and neuropathology in mouse models of AD [35–38]. Remarkably, a single trial was conducted in human patients of AD in the early 1990s using the iron chelator Deferoxamine (DFO), resulting in a slowdown in the development of the dementia associated with the disease [39].

Given all the positive outcomes of iron chelation therapy *in vivo*, and the decrease in Lcn2 overexpression associated with Aβ neuropathology achieved when using iron chelators *in vitro*, the aim of this project is to use the iron chelator Deferiprone to answer the following research question: Does the use of Deferiprone inhibit the overproduction of Lcn2 in a mouse model of AD and delay the progression of the symptoms? Based on the previously mentioned researches, we hypothesize that Lcn2 could potentially be a therapeutic target for AD and that Deferiprone may reduce the brain levels of Lcn2 *in vivo*, as well as decrease the severity of the iron dysregulation. Concretely, this report will focus on the behavioural aspects and, consequently, we expect a general improvement in the cognitive performance of the Deferiprone-treated mice when compared to the placebo group.

2. Materials and Methods

2.1. Animals

Male wild-type (WT) and J20 mice were used in this project. One Way Anova was performed to estimate the sample size. J20 mice are a mouse model of Alzheimer's Disease (AD) that express a mutant form of the human Amyloid Protein Precursor (APP) carrying the Swedish (K670N/M671L) and the Indiana (V717F) mutations, associated with the familial form of AD [40–42]. They were originally acquired from The Jackson Laboratory (MMRC Stock N.: 34836-JAX), and currently they are bred in the animal facilities of the University of Groningen (Zernike Campus). The genotype of the mice will be double-checked by Polymerase Chain Reaction (PCR) once the neuropathological features of the brains are analyzed.

Animals were housed in group cages until they reach the appropriate age for the start of the treatment (7,5 months old). This age was selected because it corresponds to the early stage of Alzheimer's disease, which is the optimal time for treatments. At this point, they were transferred to individual cages, located in the same room.

Mice were given standard food and kept under a 12:12 h light-dark cycles, with lights on at 7:00 and off at 19:00, and room temperature ~22°C. Cages of the group housed mice were cleaned once a week, while the individual cages were cleaned once every two weeks. Mice were checked every day to ensure they had enough food and water.

2.2. Treatment

The mice received as a treatment either Deferiprone (purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA) or placebo (water) during 12 weeks, after which they were sacrificed. The treatment was administered orally, via drinking water. Which mouse receives which treatment was randomized.

The desired dose of the treatment was 50 mg/kg/d. The choice of this dose was based on previous literature. To achieve it for each individual mouse, the water consumption was measured daily. Additionally, the body mass of the mice was also measured daily for one month. After that, it was measured twice a week until the mice were sacrificed. Using the average daily water consumption and the body mass, an individual mix of treatment was prepared and administered to each mouse every day.

Ultimately, four experimental groups were studied in this project: WT treated with Deferiprone, WT untreated, J20 treated with Deferiprone and J20 untreated.

2.3. Behavioural experiments

Behavioural experiments started after 5 weeks of treatment. In order to test different aspects of cognition and baseline behaviours such as anxiety, 5 different behavioural tests were performed. The tests performed were, in the following order: Home Cage Activity, Open Field, Novel Location Recognition, Elevated Plus Maze and Morris Water Maze. These were based on the protocols described by Dekens et al [31].

All the behavioural tests besides Home Cage Activity were carried out in a room different from where the animals were housed. The arenas for the experiments, except the maze from the Morris Water Maze, were cleaned using 30% ethanol in between trials, as all the mice were males, and 70% ethanol once the experiment was finished for the day. Prior to the start of the experiments, the mice were habituated to

the experimenters by being handled 2 minutes each day during a total of 5 days. After that, mice were also habituated to the arenas used for Novel Location Recognition for 5 minutes during 7 days.

The behavioural experiments were conducted during the light phase by the same experimenter blinded to the genotypes of the mice and to the treatments. Videos of the behavioural tests were recorded for further analysis using OBS Studio, except for Morris Water Maze and Elevated Plus Maze, which were recorded using Ethovision XT (Noldus).

Mice were studied in 3 cohorts. The number of mice per cohort was mainly dependent on their availability, taking into account that the treatment had to start at a certain age (Total number of mice studied=55; WT Water: n=15, WT Deferiprone: n=16, J20 Water: n=12, J20 Deferiprone: n=12).

2.3.1. Home Cage Activity

Home Cage Activity was recorded for 2 weeks immediately preceding the start of the Habituation, using passive infrared (PIR) sensors located above each individual cage. The times of the day during light phase (7am-7pm) when activity was unusually high (i.e. as a result of the movement of the cages while weighting the mice or cleaning the cages) were deleted from the final data as they do not reflect the real activity of the mice during their inactive phase.

2.3.2. Open Field

The first day of box habituation, the Open Field (OF) test was performed. Mice were placed in the centre of empty square arenas (50x50x36 cm, no ceiling, ~10 lux in the centre of the arena, and no intra-arena cue) and allowed to explore freely for 5 minutes, after which they were returned to their home cages. Videos were analyzed using a tracking software (Ethovision XT 11.5.1026, Noldus). In order to assess hyperactive and anxiety-like behaviour, the primary outcomes of this test were distance moved in the arena, speed and time spent in the inner core. To define this inner core, the software was used to divide the arena in 25 identical squares (5x5); the set of squares closer to the wall was defined as the outer zone, and the rest as the inner zone (Fig. S1 in Additional file).

2.3.3. Novel Location Recognition

The Novel Location Recognition (NLR) took place the day after the last day of box habituation. This task is widely used to assess hippocampus-dependent memory by testing the ability of the mice to recognize the location of the objects in the arena [43]. The NLR test consists of two different session separated by exactly 24 hours. During the first phase, the learning trial, mice were placed in the centre of the square arenas (the same used in the OF, ~10 lux in the centre of the arena), facing the wall opposite to an intra-arena cue (a sheet of paper with vertical white and black stripes stuck to one of the walls). The arenas contained a set of 2 identical objects (100 ml borosilicate glass 3.3 Erlenmeyer filled with light blue sand and a 50ml tube inserted on the opening to prevent the mice from sitting and staying on top of the object. Fig. S2) randomly located either by the wall opposite to the cue or by the wall with the cue (~12,5 cm distance from this and the lateral wall). Mice were allowed to explore the arena and the objects freely for 10 minutes before being returned to their home cages. After 24 hours, the test trial followed. Mice were placed in the arenas the same way as in the learning trial, but one of the objects was moved to a new spatial location relative to the cue, maintaining the same distance from the wall. The other object remained in the same position as during the learning trial. Which object was re-located was randomized

between mice. Mice were allowed to explore the arena and the objects freely for 10 minutes before being returned to their home cages. Before each trial, mice were placed in empty cages for 4 minutes to increase arousal.

The NLR was recorded with cameras above the arenas and the videos were analysed automatically using Ethovision (Ethovision XT 11.5.1026, Noldus). The criterion for exploratory behaviour applied was detecting the nose of the mouse within 2 cm of the object. For that, the nose-tracking feature of Ethovision was used and circles of 2 cm radius were drawn around the objects. Climbing, defined as having all the four paws on the object, was not considered exploratory behaviour. The primary outcomes scored for this test were the speed and distance moved from the mice, as a measure of hyperactive behaviour, and the discrimination index as a measure of memory.

The discrimination index was calculated as:

$$\frac{time\ spend\ exploring\ the\ relocated\ object\ -\ time\ spend\ exploring\ the\ non-relocated\ object}{total\ exploration\ time}$$

A discrimination index of 0 means that there is no preference over any of the objects (i.e. the mice explore both objects the same amount of time). Therefore, a discrimination index above 0 indicates preference over the relocated object, whereas a discrimination index below 0 indicates preference over the non-relocated object.

2.3.4. Elevated Plus Maze

For the Elevated Plus Maze (EPM), mice were placed in the central square of a grey plus-shaped maze (closed arms of 65,5 cm, open arms of 64,5 cm, 60 cm elevated above the ground. No ceiling. ~9 lux in the centre of the maze, ~12 lux on open arms). Mice were left to move freely around the arms and the centre for 8 minutes, after which they were returned to their home cages. This test allows to further study anxiety-like behaviours; the more time the mice spend in the closed arms (those enclosed by walls), the more anxious they are [43]. Therefore, the primary outcome of this test is the time spend on the open and closed arms. As noted above, the test was recorded with Ethovision XT, and, subsequently; the videos were analyzed using the same software (Ethovision XT 11.5.1026, Noldus). This software was used to track the centre-point and monitor the time the mice spent in each arm.

2.3.5. Morris Water Maze

The Morris Water Maze (MWM) test started the day after EPM. This test, considered the gold standard for measuring hippocampus-dependent spatial learning and memory [44], spanned 10 days, followed by one or two additional days for the cue trials in order to detect whether the mice have vision problems. The first 8 days of the test were the training phase. Each day, the mice were released into a circular maze filled with opaque water (140 cm of diameter, 35 cm high, water level ~21 cm, temperature of the water 23 ± 1°C, 44 lux in the centre of the maze), with a round platform (15 cm of diameter, ~19,5 cm high, ~1,5 cm below water surface) placed in one of the quadrants. Water was blended with non-toxic white paint (Dulux Roll-it Easy) to refrain the mice from seeing the platform, and the position of this remained the same throughout the whole training phase. In the same experimental room, around the maze, a set of extra-maze cues were arranged to serve as visual cues for the mice to find the platform. Mice were released at the edge of the pool, facing the wall, from 4 different entry points, one in each quadrant. Thus, in total every day each mouse entered the maze four times. Once they were inside the pool, the

mice were left to freely explore it and search for the platform for 120s or until they found the platform and stayed there for 20s. If they could not find the platform, the mice were guided by the experimenter to the platform and let them stay there for 20s. Afterwards, mice were returned to their home cages and placed under heating lamps to help them dry and warm up. The following 2 days correspond to the trial phase. For this stage, the platform was removed from the maze and the mice only enter the water once per day, being released from the point opposite to the quadrant where the hidden platform was during the learning phase (i.e. target quadrant). The mice were left to swim freely for 100s, after which they were returned to their home cages and placed under the heating lamps. Like the EPM, the trials of the MWM test were recorded using Ethovision XT, and the videos analyzed with the same tracking software (Ethovision XT 11.5.1026, Noldus). For the learning trials, the primary outcomes were the escape latency (time it took for the mice to find the platform), swimming speed and distance. By contrast, the primary outcomes of the probe trials were the time spent in each quadrant and the number of crossings over the place where the platform was during the learning phase.

For the cue trials, an object (50 ml tube filled with green sand) was placed on top of the platform to help the mice find it. The platform was semi-randomly positioned in all the four quadrants and mice entered the maze from one entry point. If the mice have no problems with their vision, they should be able to see and move towards the object, allowing them to find the platform easily.

2.4. Histochemistry for detection of iron

Once the behavioural tests were finished, the mice (~10,5 months old) were terminally anesthetised by an intraperitoneal injection of sodium pentobarbital and perfused with 4% paraformaldehyde (PFA) and saline (0,9% NaCl + Heparin) in order to collect the brains. These were placed in cups filled with 4% PFA and post-fixated 24h later by washing 7 times (one every hour) with 0,01M PB. Then, the brains were submerged in 30% sucrose solution and left at room temperature overnight for dehydration. Finally, the brains were frozen with liquid nitrogen and kept at -80°C until they were cut in 20 µm thick slices on a cryostat (Leica CM3050, Leica CM1860). Each region of the brain was collected in different cups filled with phosphate buffer (0,01M PBS + 0,1% Sodium Azide, pH 7,4) and they were stored at ~4°C.

Given the link between Deferiprone, iron and Lcn2, we were interested in studying the presence of iron in the brain of the different experimental groups, specifically in the hippocampus. Several pilots were conducted in order to optimize the protocol described by Dekens et al [31]. Briefly, the 3,3'-diaminobenzidine (DAB)-enhanced Perls' iron stain was applied for the detection of non-heme iron: free-floating hippocampal slices were washed 3x15 min. in 0,01M PBS (pH 7,4), incubated in 0,3% H_2O_2 (Hydrogen peroxidase 30%, Merk, Casnr 7722-84-1) in PBS during 30 min. to block the endogenous peroxidases, washed again 4x15 min. in 0,01M PBS and incubated for 50 min. in Perls Solution, consisting of 5% potassium ferrocyanide in milliQ water (Potassium hexacyanoferrate (II) trihydrate, Sigma-Aldrich, CASnr 14459-95-1) and 5% HCl in milliQ water (Hydrochloric acid fuming 37%, Merk, CASnr 7647-01-0). Afterwards, the slices were washed 3x15 min. or 9x5 min. with milliQ water. Finally, the DAB step was performed, enhanced by the addition of Nickel (di-ammonium nickel (II) sulphate hexahydrate, BDH Chemicals Ltd., Prod. 27189) to the DAB solution (SigmaFAST 3,3'-Diaminobenzidine tablets, Sigma-Aldrich). The reaction was started with 0,1% H_2O_2 in milliQ water and stopped by washing 3x quickly with 0,01M PBS, followed by 3x5 min. washes in 0,01M PBS. The slices were incubated in 0,01M PBS overnight at 4°C before being mounted with 1% gelatin in Menzel Superfrost slides (ThermoFisher scientific). Once the mounted slices were dry, they were dehydrated through EtOH → Xylol baths (2x5 min. 100% EtOH,

1x5 min. 70% EtOH/30% Xylol, 1x5 min. 30% EtOH/70% Xylol, 3x5 min. 100% Xylol), and covered with a glass coverslip using DPX (Sigma-Aldrich). Images of the stained slices were acquired through an Olympus BH2 microscope using Leica Application Suite.

2.5. Statistical Analysis

All statistical analysis were performed using GraphPad Prism 6 for Windows (version 6.01). Normality was tested using D'Agostino-Pearson omnibus normality test. The followings comparisons were performed with either unpaired t-test with Welch's correction or Mann-Whitney test, depending on whether the data were normally distributed or not, respectively:

- WT treated with water vs. WT treated with Deferiprone
- J20 treated with water vs. J20 treated with Deferiprone
- -WT treated with water vs. J20 treated with water
- -WT treated with Deferiprone vs. J20 treated with Deferiprone

For NLR and MWM, a one-sample t-test was additionally performed to test whether the results of the discrimination index and the percentage of time spent in each quadrant differ from chance level, respectively. MWM training phase was tested using two-way ANOVAs followed by Sidak's multiple comparisons post-hoc test. Data are expressed as the mean ± the standard error of the mean (SEM). $p<0.05$ was considered statistically significant.

3. Results

3.1. Both J20 water and J20 Deferiprone show hyperactive behaviour, but no clear differences can be observed upon the administration of Deferiprone within the same genotype

Both J20 water and J20 Deferiprone present hyperactive behaviour in the Open Field when compared to the WT groups ($p<0.001$ and $p<0.05$, respectively. Fig. 1A). This is endorsed by the average speed at which the mice moved during the test, being it significantly higher in the J20 groups than in the WT mice ($p<0.001$ for J20 water compared to WT water and $p<0.05$ for J20 Deferiprone compared to WT Deferiprone. Fig. 1B). However, no differences were detected between treated and untreated groups of the same genotype. This trend could also be observed during the Novel Location Recognition (Fig. 1), even though statistical significance was only achieved between J20 water and WT water ($p<0.01$ in the training phase and $p<0.05$ in the test phase. Fig 1C-F).

Home Cage Activity measurement further support the hyperactivity of J20 mice (Fig. 2). As expected, all the mice displayed the highest activity during the dark phase (i.e. when the lights were off). It is during this phase when changes in the behaviour are noticeable between J20 and WT groups, with the former exhibiting increased levels of activity than the latter ($p<0.0001$ between WT water and J20 water, $p<0.001$ between WT Deferiprone and J20 Deferiprone. Fig. 2B). Again, no statistical significance was obtained between water and Deferiprone treated mice of the same genotype. Thus, in general it appears that mice with a J20 genotype manifest a stronger hyperactive behaviour than their WT counterparts.

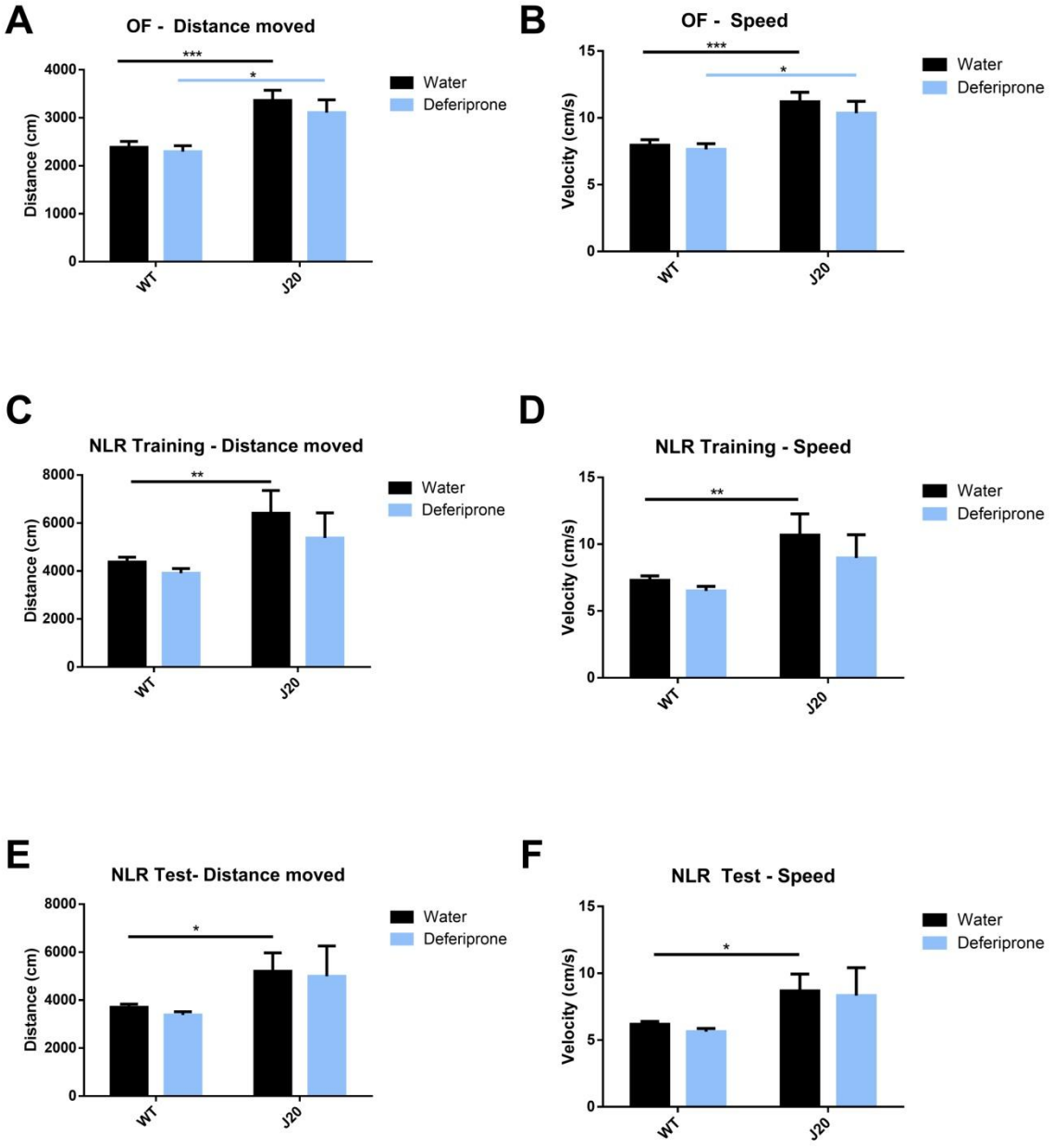

Fig. 1. Hyperactive behaviour in J20 mice indicated by the distance moved and speed during OF and NLR (both training and test sessions). (A) Distance traveled and (B) velocity of the mice in the OF (5 min). 4 mice were excluded from this analysis due to technical issues during the task (WT water n=14, WT Deferiprone n=14, J20 water n=11, J20 Deferiprone n=12). Mann-Whitney test was performed. Statistical significance was achieved between J20 and WT genotypes, but not between water and Deferiprone groups within the same genotype. (C) Distance traveled and (D) velocity of the mice in the training trial of the NLR (10 min). Statistical significance was achieved between WT and J20 water.This can also be seen in the total distance moved (E) and velocity (F) during the test trial of the NLR (10 min). For both trials of the NLR, n=55 (WT water n=15, WT Deferiprone n=16, J20 water n=12, J20 Deferiprone n=12) .Tested with Mann-Whitney. The asterisks indicate statistical significance (*p<0.05, **p<0.01, ***p<0.001).

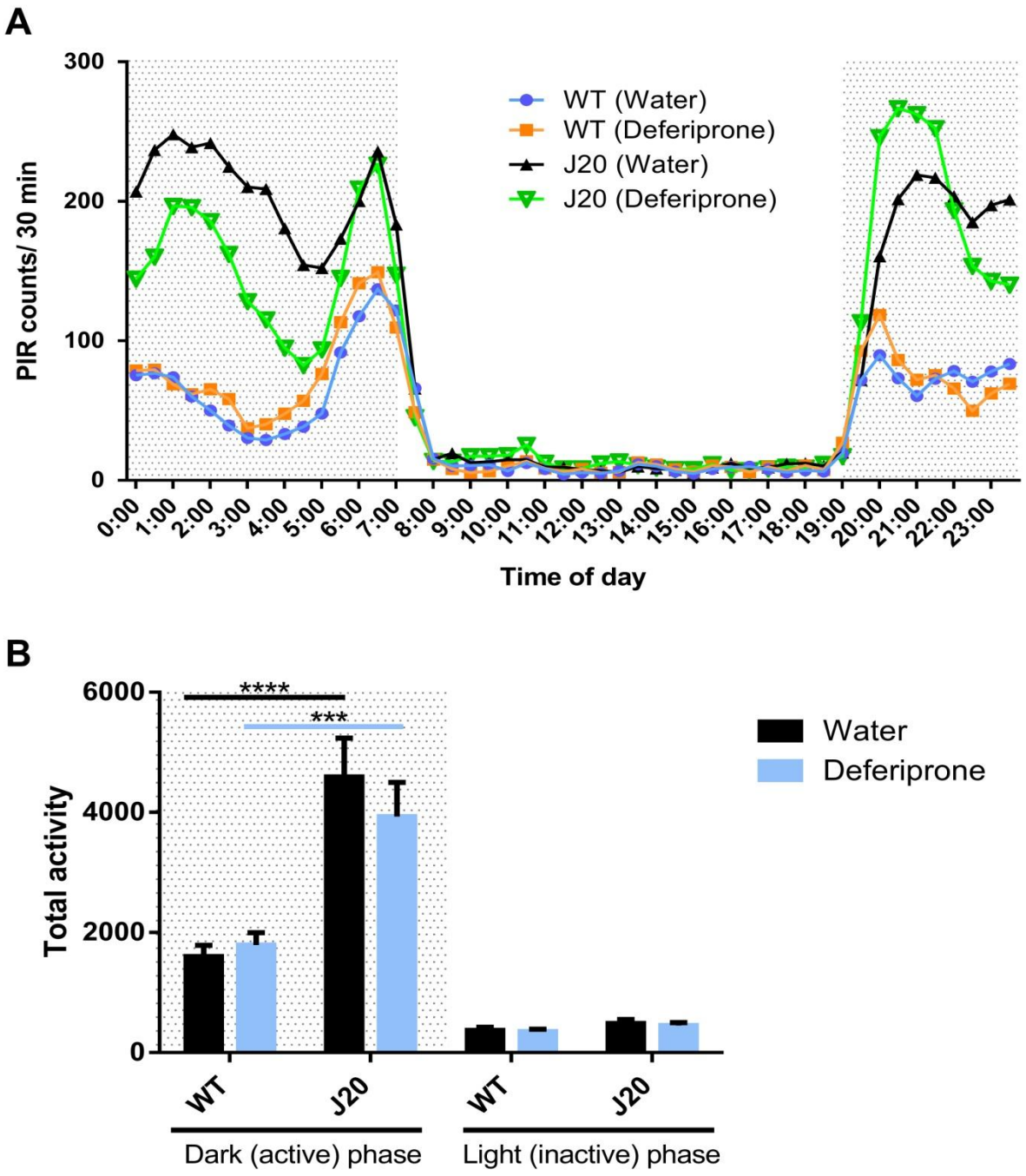

Fig. 2. Home Cage Activity results also show hyperactive behaviour in J20 mice. (A) Average activity per 30 minutes during the 14 days studied. Activity is plotted for each experimental group. Grey background in the figure defines the dark phase (7pm -7am). (B) Total activity estimated as the sum of the average activity per 30 minutes during the dark and light phase for each experimental group. Tested with Mann-Whitney. One mouse was excluded from the analysis due to problems with the sensor (WT water n=15, WT Deferiprone n=15, J20 water n=12, J20 Deferiprone n=12). *** indicates $p<0.001$, **** indicates $p<0.0001$. Significant differences were detected between J20 (both water and Deferiprone) and WT (water and Deferiprone) during the dark phase, but not between treatments groups within the same genotype. Significant differences between dark and light phase are not included as they are not considered relevant for this study.

3.2. No clear differences are observed in anxiety-like behaviour between either different genotypes or treatments

Anxiety-like behaviour was measured during Open Field and Elevated Plus Maze (Fig. 3). Even though J20 Deferiprone enters the inner zone of the OF more often than WT Deferiprone ($p<0.05$. Fig. 3B), this does not translate into the time spent in the inner zone (Fig. 3A), where no clear differences can be observed between the four groups. Similar results were obtained for the time spent on the open and closed arms of the EPM (Fig. 3C and 3D, respectively). Thus, overall it seems that there is no significant difference in the levels of anxiety in the experimental groups under study.

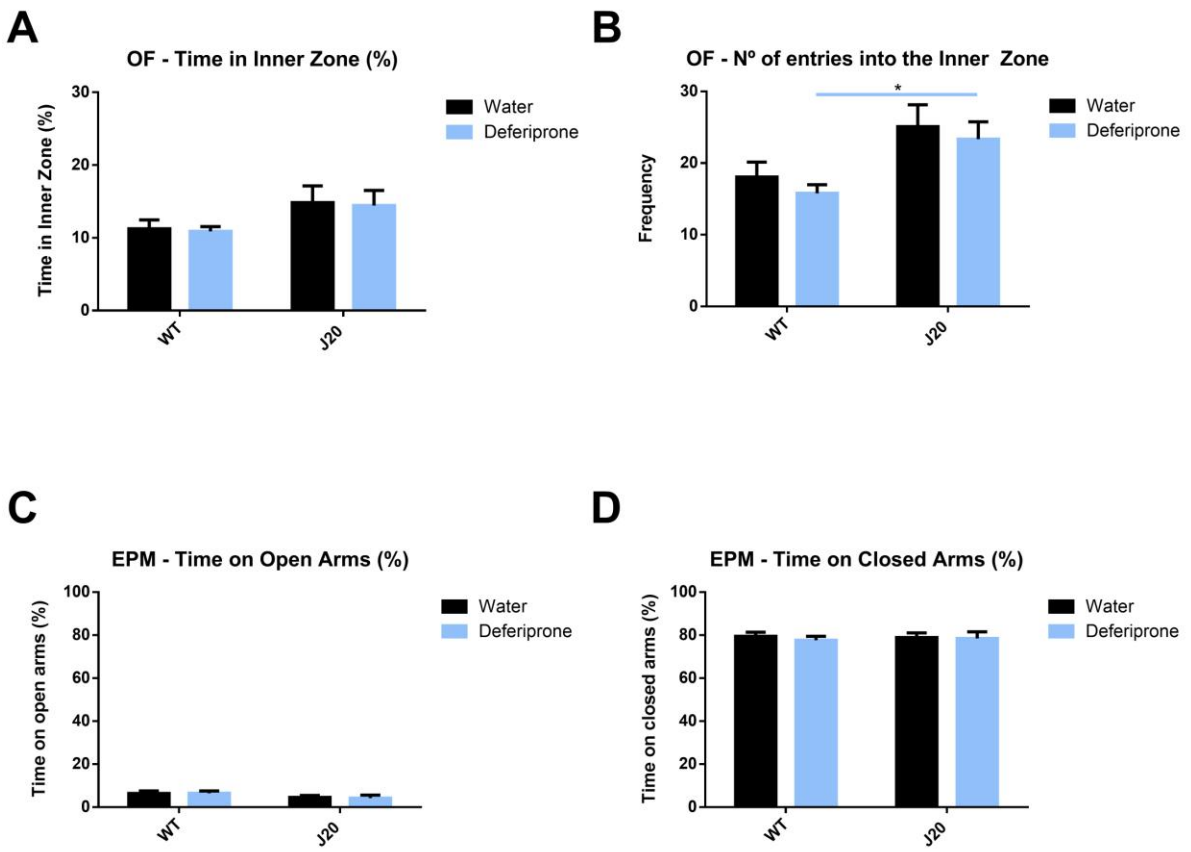

Fig. 3. No evident differences in anxiety-like behaviour can be observed between genotypes or treatment groups. (A) Time spent by the mice in the inner zone of the arena during the OF (expressed as % of the total time, 5 min). Tested with Mann-Whitney (B) Number of entries into the inner zone of the OF. Tested with unpaired t-test with Welch's correction. 4 mice were excluded from this analysis (A and B) due to technical issues during the test (WT water n=14, WT Deferiprone n=14, J20 water n=11, J20 Deferiprone n=12). The asterisk indicates statistical significance (*$p<0.05$). (C) Time spent by the mice on the open arms of the EPM (expressed as % of the total time, 10 min) (D) Time spent by the mice on the closed arms of the EPM (expressed as % of the total time, 10 min). Tested with Mann-Whitney. No statistical significance was achieved. For the EPM (C and D), n=55 (WT water n=15, WT Deferiprone n=16, J20 water n=12, J20 Deferiprone n=12).

3.3. Hippocampus-dependent memory could not be assessed by the Novel Location Recognition test

Results of the NLR test were not conclusive as the control group (WT water) failed to demonstrate novel location recognition (Fig 4). As it was expected, the mice did not show preference for any of the two

objects during the training session (Fig. 4A). However, values of the discrimination index during the test phase did not significantly differ from 0 for any of the experimental groups under study, nor were there any differences between them (Fig 4B).

To test whether this lack of positive results for the control group was due to loss of interest on the objects as a consequence of the long duration of the trial (10 min.), exploration was also scored during different time periods of the test (Fig. S3 in Additional file). Nonetheless, none of these time points could give any relevant outcome.

Interestingly, the mice did show exploratory behaviour, particularly J20 mice during the training session ($p<0.01$ for WT water and Deferiprone groups when compared to J20 water and Deferiprone, respectively. Fig. 4C). While this tendency could also be observed during the test session, the total exploration time was decreased and significance could only be achieved between J20 water and WT water ($p<0.01$, Fig. 4D). Thus, it appears that, even though they explore the objects, none of the groups show preference over the relocated object.

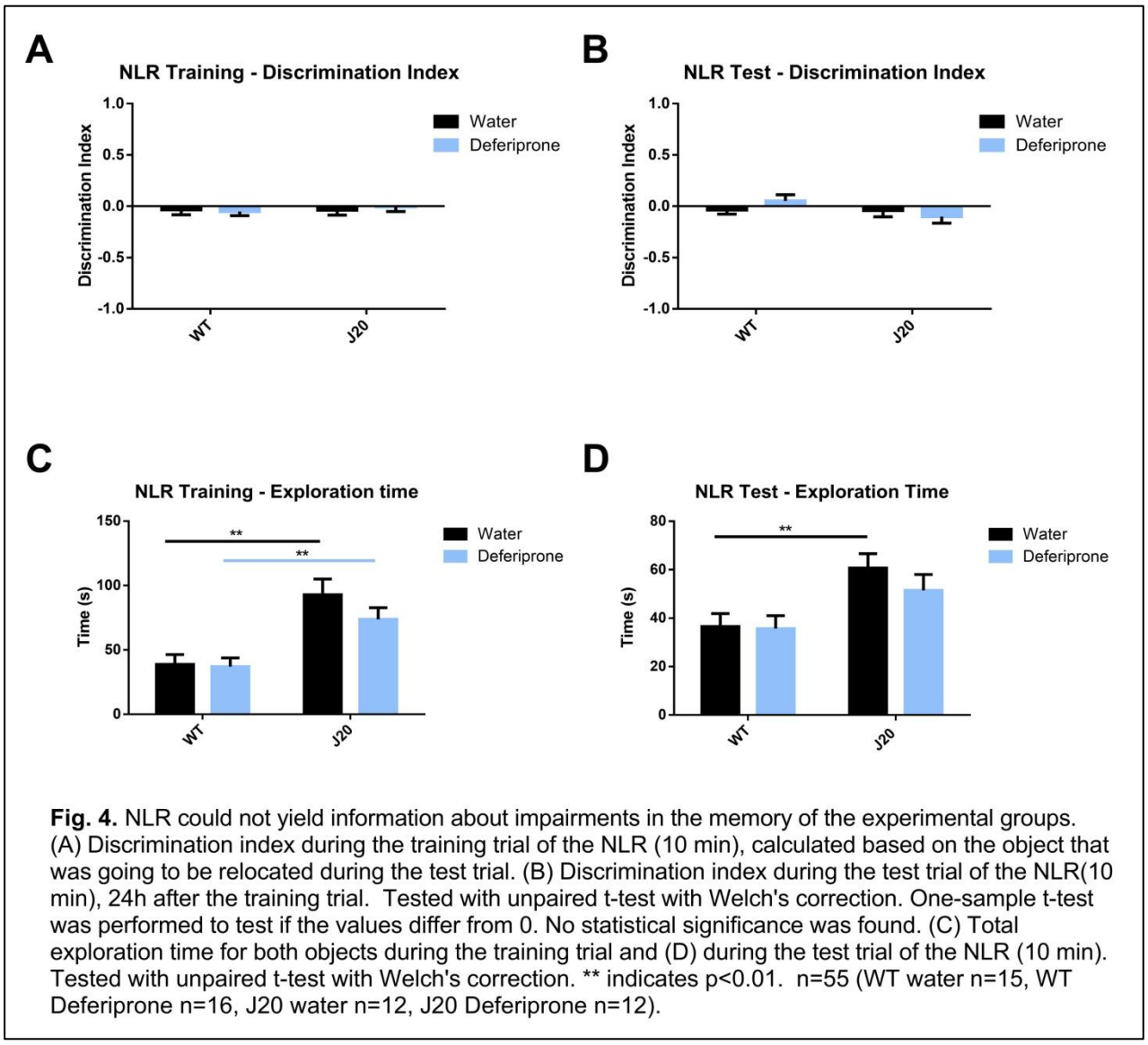

Fig. 4. NLR could not yield information about impairments in the memory of the experimental groups. (A) Discrimination index during the training trial of the NLR (10 min), calculated based on the object that was going to be relocated during the test trial. (B) Discrimination index during the test trial of the NLR(10 min), 24h after the training trial. Tested with unpaired t-test with Welch's correction. One-sample t-test was performed to test if the values differ from 0. No statistical significance was found. (C) Total exploration time for both objects during the training trial and (D) during the test trial of the NLR (10 min). Tested with unpaired t-test with Welch's correction. ** indicates $p<0.01$. n=55 (WT water n=15, WT Deferiprone n=16, J20 water n=12, J20 Deferiprone n=12).

3.4. J20 mice show impaired memory in the Morris Water Maze

Hippocampus-dependent memory was also tested in the MWM task. Contrary to the results obtained in the NLR, the MWM test shows differences in the spatial learning of WT and J20 mice, as illustrated by the decreased escape latencies of the former (Fig. 5A). Notably, both WT groups and J20 water performed similarly during the first days of the training phase, while J20 Deferiprone performed worse, as can be observed in the reduced slope and in the significance achieved on days 2 and 4 when compared to WT Deferiprone ($p<0.05$). However, from day 5 onwards both treatment groups of J20 started acting in the same manner, reaching a plateau with no further decrease in the escape latency. In contrast, WT mice did show a progressive decline in the time needed to find the platform over the 8 days of training (At day 8: $p<0.01$ between J20 water and WT water). Despite the statistical significances found between J20 and WT genotypes, no significance was found between treatments groups of the same genotype.

Unsurprisingly, the distance moved by the experimental groups corresponds largely to their escape latency for each training block (Fig. 5B), while the swim speed did not differ substantially between the experimental groups under study (Fig. 5C).

Probe trials in the absence of a platform were performed 24 and 48 hours after the last day of the training phase (day 9 and day 10, respectively). Despite showing a learning curve similar to the WT water group during the training sessions, WT mice treated with Deferiprone performed considerably worse during the 24h probe trial, with results comparable to those of J20 groups (Fig. 5D and 5E). Indeed, they spent a significantly higher percentage of time than chance level (25%) in the quadrant opposite to where the platform was instead of in the target quadrant ($p<0.05$). A similar trend was detected for the J20 mice both treated with Deferiprone or water. Meanwhile, for the WT mice treated with water it is the other way around, as they spent a significantly higher percentage of time than chance level in the target quadrant and lower in the opposite quadrant ($p<0.01$ for the target quadrant and $p<0.05$ for the opposite quadrant. Fig. 5D). Moreover, overall they spent significantly more time in the correct quadrant than WT Deferiprone and both groups of J20 mice ($p<0.01$. Fig. 5E). Nonetheless, WT mice treated with Deferiprone performed better than the J20 groups in remembering the platform position in view of the number of crossings, achieving significance when compared to J20 Deferiprone ($p<0.05$. Fig. 5F) and not differing significantly from WT water.

Of note, the behaviour of the groups during the probe trial 24h differ considerably (Fig. S4A). An appreciable number of J20 mice treated with Deferiprone present a clear thigmotaxis or even passivity, with some of them barely leaving the walls to explore the rest of the maze. While this kind of swim path can also be observed to less extent in mice of the other treatment groups, they more often show movement across the whole pool. Nevertheless, the only significant difference observed in the swim speed and distance moved during the probe trial 24h was between the WT water and J20 water groups (Fig. S4B and S4C).

At the 48h probe trial, the results of all the experimental groups tended to decline. WT water did not reach any significance for the time spent in the target quadrant (Fig. S5B) or for the number of crossing over the location of the platform (Fig. S5C) when compared to the other groups, and the percentage of time spent in each quadrant did not differ from chance level (Fig. S5A). Conversely, in the case of WT treated with Deferiprone the percentage of time spent in the opposite quadrant is even more accentuated than during the 24h probe trial, and they share this trend with both J20 groups.

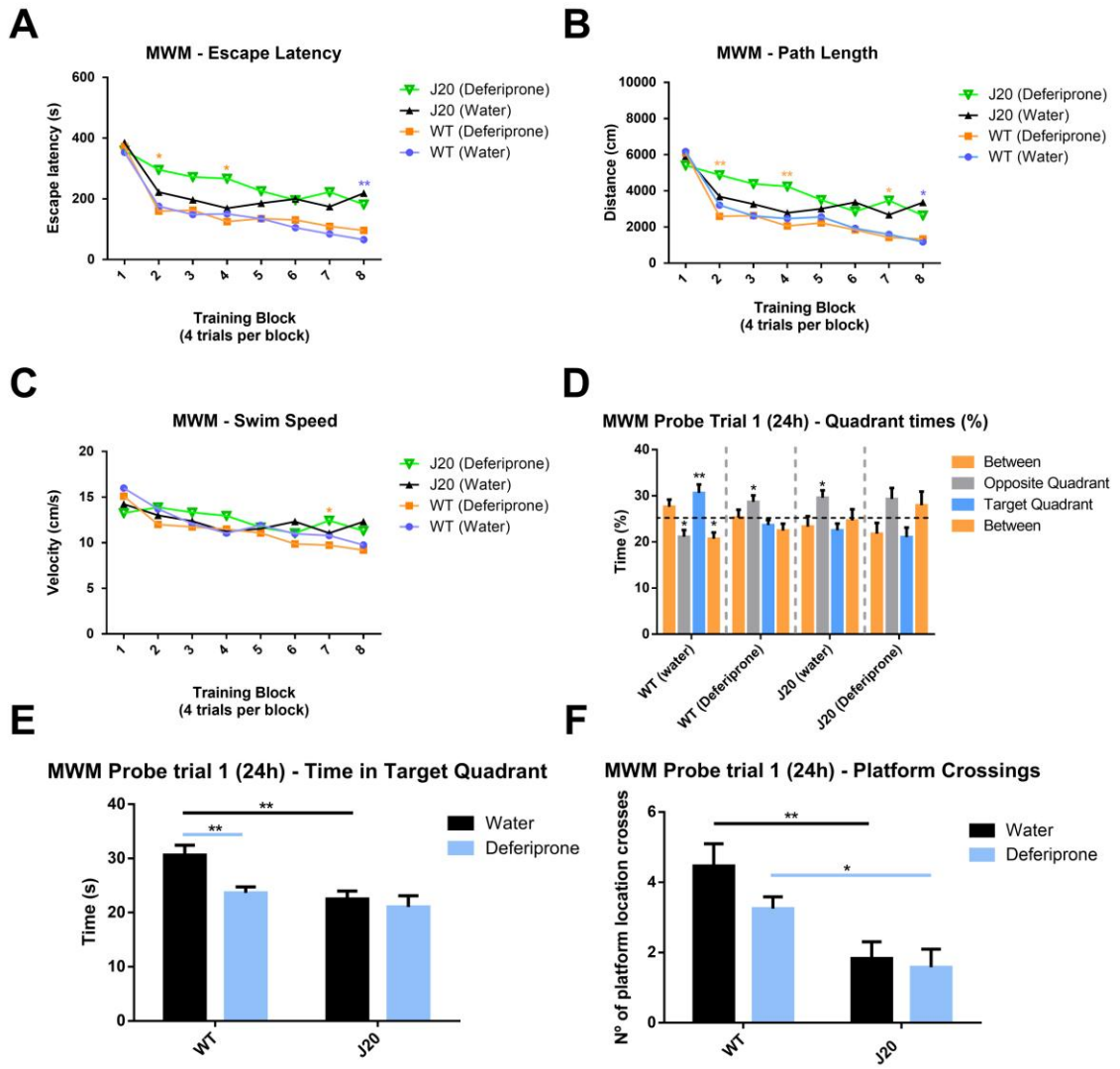

Fig. 5. J20 mice show impairment in hippocampus-dependent memory. (A) Escape latency during the 8 training days of the MWM. Both WT groups and J20 water learned the task at the same rate during the first days of the test. After day 4, the WT groups kept improving and decreasing their escape latency, while J20 water stopped learning. J20 Deferiprone presents a significantly reduced slope in the learning curve. (B) Path lenght and (C) swim speed in the training phase of the MWM. Tested with two-ways ANOVA followed by Sidak's multiple comparisons post-hoc. Significance was found between J20 Deferiprone and WT Deferiprone (orange asterisks) and between J20 water and WT water (blue asteriks). (D) Percentage of time spent in the quadrants during the 24h probe trial. Black dashed line indicates chance level (25%). One-sample t-test was performed to test if the values differ from chance level (significance represented by the asterisks). (E) Comparison of the time spent in the target quadrant by the four experimental groups. WT water spent significantly more time in the right quadrant than the other groups. Tested with Mann-Whitney. (F) Number of crosses during the probe trial 1 over the location where the platform was. WT water crossed significantly more times than J20 water and WT Deferiprone more than both J20 Deferiprone. No significant difference was achieved between both WT groups. Tested withunpaired t-test with Welch's correction. The asterisks indicate statistical significance (*p<0.05, **p<0.01). n=55 (WT water n=15, WT Deferiprone n=16, J20 water n=12, J20 Deferiprone n=12).

3.5. Optimization of the DAB-enhanced Perls' staining for the detection of iron

Several pilots were carried out to optimize the DAB-enhanced Perls' staining protocol. A troubleshooting guide with the problems we encountered and the way we dealt with them can be found as Table 1. Principally, we came upon two main problems: a background darker than it should be, which hinder the subsequent analysis (coverage and optical density) and a large amount of dirt that, on one hand, complicated the decision for the better DAB reaction time during the staining and, on the other, gave rise to false positives in the mounted slices when trying to analyse them under the microscope.

Problem	Possible reason(s)	Possible solution(s)	Conclusion
High Background/Poor Contrast	• Concentration of the Perls' solution • Age of chemicals • Inefficient washing	• Use of a lower concentration of Perls' solution • Use fresh chemicals • Increase washing steps	Reducing the concentration of the Perls' solution by half or more did not improve the background of the slices. Also, increasing the number of washes between Perls' incubation and DAB step did not show any major improvement. However, the use of fresh potassium ferrocyanide yielded a lighter background with nice contrast. Additionally, this contrast between the background and the iron spots can be enhanced using Leica Application Suite while acquiring the images (Fig. S6).
Dirt	• Impurity from Perls' solution or previous steps during staining • Too much tissue damage	• Filter the Perls' solution • Change the slices to new cups with fresh milliQ water prior to the DAB step • Avoid using brushes	Filtering the Perls' solution did not change the level of dirt accumulation in the buffer for the DAB step. While changing the slices to a new buffer and cup before the DAB step reduced slightly the dirt, the fact that there was still a considerable amount of dirt even when the buffer was fresh suggests that this dirt comes from the slices themselves. Indeed, reducing the use of brushes to move/flatten slices did improve this problem, so it seems that the dirt are small pieces of tissue that shed from the slices. Treating the slices gently during the staining helps in reducing tissue damage and, thus; dirt.

Table 1. Troubleshooting guide for the Perls' staining. The reason we mainly focused on the Perls' solution while trying to solve the problems faced was because, at first, we did not detect these problems in the other stainings performed as part of the whole project, and the only step unique from this staining is the one involving the Perls' solution.

4. Discussion

We report that, in this research, the administration of Deferiprone did not lead to any significant cognitive improvement in the J20 mouse model of AD. As expressed before, the main objective of this project was to target Lcn-2 with the use of the iron chelator Deferiprone. While it seems that Lcn-2 itself does not contribute to cognition –at least in the mouse model we are using– according to the cognition results from LCN2-deficient J20 mice [31], the benefits of an iron chelator therapy are well described by several other studies, which found an amelioration in the AD-like memory impairments upon administration of iron chelators [35–37]. Among all the different iron chelators available, Deferiprone was selected for this research because it has been proved to be safe for patients (indeed, it is already being used to treat iron overload in thalassemia syndromes), it can cross the blood-brain barrier (BBB) and it can be administered orally, as opposed to other chelators whose route of administration (i.e. intravenously or intranasal) are less convenient for the patients [45]. Deferiprone has shown to reduce the levels of Aβ in cultured astrocytes [30], and in an AD rabbit model [46], although this research does not test behavioural aspects. Furthermore, there is an ongoing clinical trial ("Deferiprone to Delay Dementia", also known as the "3D Study") that already seeks to test the efficacy of Deferiprone in AD patients [47,48]. They are currently in the phase 2 and Deferiprone or placebo is being administered orally twice a day to more than 150 participants, with the study estimated to finish in 2021.

Therefore, given all the previous and still growing positive outcomes of the use of iron chelators –including the one used in this research–, there are no reasons why we should not expect any changes in the cognitive behaviour of the treated J20 mice, even if it is not a direct consequence of a reduction in Lcn-2 overexpression as originally hypothesised. Thus, the question remains why our study did not yield conclusive results about any potential effect of Deferiprone in the context of cognition.

Firstly, it is hard to draw any conclusion when we do not have useful results from the Novel Location Recognition task. The reason why this test failed to show location recognition for the control group is not clear. Simply put, there are three main aspects that can affect the results: the mice, the scoring method and the task conditions.

The control group studied in this project were wild-type mice treated with water, which means that they should not have any memory impairment that could explain the lack of location recognition. Indeed, during the Morris Water Maze task these same mice learned and remembered the position of the platform, which supports that they possess an intact hippocampus-dependent memory functioning. While it is true that this failure could be attributed to high levels anxiety or stress in the mice [49], results from the OF and EPM are within the expectations and the mice were habituated to both the arenas and the experimenters precisely to reduce anxiety and avoid this kind of problem. Moreover, they do explore the objects, as illustrated by the exploration time, so they do not seem to fear or avoid them. Our original though that maybe the duration of the test was too long and the mice lost interest in exploring at some point also did not seem to be the reason, as observed while scoring shorter time periods of the test. We also tested whether mice undergoing the task in the same arena, and, therefore; with the same set of two objects, showed bias towards one of them, in case the objects were not as identical as we indented and they had an intrinsic preference, but this was also not the case (Fig. S7).

On another note, this test was scored automatically using Ethovision XT. This method can lead to overscoring, as the software will score exploration anytime the mouse is within 2 cm of the object, including when it is just passing by or standing near, but without actually facing or showing interest in the

object (i.e. the mouse is not actively exploring the object). This may derive in exploration times not being entirely precise for any of the two objects. However, manual scoring of the test session using The Observer XT (Noldus) gave comparable results (Fig. S8), ruling out the possibility of incorrect or inaccurate results because of the software system used.

One last option is that the conditions of the test were not optimal. While most of these are standardized as part of the protocol we followed, things like the objects used and their relative position from the walls of the arena and from the cue can influence they way the mice behave during this test. Nevertheless, at this point it is difficult to know whether any of these conditions were not adequate enough and contributed to the results obtained.

Having said all that, it is important to run pilots with control mice prior to the actual experiment in order to detect this kind of issues beforehand and have the option to amend them. It would also validate the selection of the arena and objects, as well as any other element that may influence the performance of the mice during the test. Increasing the number of days the mice were habituated to the arenas and the experimenter may also help to ensure that the mice are not too anxious and reduce any possible impact this may have on the results.

Nonetheless, because of the lack of results from the Novel Location Recognition test, the only information we have of the hippocampus-dependent memory comes from the Morris Water Maze task. Results of this test not only do not show any improvement in the treated J20 mice, but they seem to suggest that Deferiprone-treated mice, both wild-type and J20, perform worse than their water-treated counterparts. While no significance was achieved, it can be observed that during the first days of the training phase the Deferiprone-treated J20 mice learned much slower the task, as illustrated by the escape latency, although finally none of the J20 groups ended up mastering it. Together with the results of the probe trials, it is clear that J20 mice show memory impairment and that the administration of Deferiprone did not rescue it.

Interestingly, wild-type mice treated with Deferiprone showed no signs of memory problems during the training phase. On the contrary, they performed equally well as water-treated wild-type mice, demonstrating no impaired learning. However, during the probe trial their performance was closer to that of J20 mice rather than to the one expected and observed in the water-treated wild-type mice. What is more remarkable is that, even though they did not spend more percentage of time than chance level in the target quadrant, the number of crossings over the location of the platform is similar to the water-treated wild-type mice. Therefore, even though Deferiprone-treated wild-type mice spent the same amount of time as J20 mice in the target quadrant, which could be an indicative of some sort of memory impairment, once they were in the right quadrant it looks like they remembered and knew the location of the platform. It remains unclear why they did not search and spend more time in the target quadrant, then.

As the mice were released into the maze from the quadrant opposite to the target quadrant, it could be that they were reticent to move and explore and preferred to stay where they were released. This would explain why the percentage of time spent in the opposite quadrant is actually more than chance level. However, this kind of behaviour would be an indication of stressed or anxious animals, and based on the EPM and OF results all the four groups have the same levels of anxiety. Thus, if anxiety were the reason one would expect to have the same issue in the water-treated wild-type mice. Additionally, the mice did not show any problems during the 8 training days. In fact, they kept reducing their escape latency

progressively, so they were able to swim and find the platform irrespective of the entry point from which they were released and, in theory, they should know their way around the maze. The conditions of the probe trials are identical to the ones used during the training sessions, the only difference is the absence of the platform. Furthermore, the swim paths do not resemble those of stressed animals or animals that do not focus on the test, as behaviours such as thigmotaxis would suggest [50,51]. Instead, they seem to swim randomly across the whole maze. Stressed animals also tend to swim faster [49], but both the swim speed and distance moved did not differ between the wild-type groups. In sum, there is no apparent reason why Deferiprone-treated wild-type mice performed worse than water-treated ones in the percentage of time spent in the target quadrant during the probe trial, as both groups learned the task the same way and had the same levels of anxiety.

Despite the overall negative results obtained in the behavioural tests, whether Deferiprone has or not an effect in the studied mice cannot be fully determined until the neuropathological analysis of their brains is finished. As an iron chelator, Deferiprone is expected to reduce the excess of iron in the brain, especially since it has been found that the mouse model used here presents iron dysregulation in the hippocampus [31]. Thus, it would be interesting to see if the levels of iron are indeed decreased in the treated J20 group. Here, we show that for the Perls' staining to yield a good outcome in the detection of iron it is important to use fresh potassium ferrocyanide. Indeed, the relationship between the freshness of this chemical and the background has also been highlighted in the past by other study [52]. Equally important is to reduce the use of brushes or any other tool that may increase tissue damage, as this will results in an excess of dirt. Performing the washes gently and avoiding abrupt movements during the staining may also help in this matter. No improvement in the iron dysregulation of J20 mice treated with Deferiprone may explain why they did not perform better than water-treated J20 mice during the behavioural tests.

Measuring the levels of Lcn-2 in the brains would also help to determine whether the *in vitro* findings translates into *in vivo* work. Lcn-2 is up-regulated in the brain of AD patients [12,25] and its overexpression has being associated with a number of components of AD neuropathology [12,22–24,26,27,29,30,53]. Our original goal was to prevent this overexpression in the brain of J20 mice with the use of the iron chelator Deferiprone in order to delay the progression of the symptoms. While we did not achieve any decline in the cognitive symptoms of the disease, staining Lcn-2 in the brain will reveal if, indeed, the expression of this protein is reduced in the Deferiprone-treated mice.

As noted before, recent findings suggest that Lcn-2 may not be involved in the cognitive condition of AD, at least in J20 mice [31]. However, it has been found that wild-type mice deficient in this protein perform worse during the cognitive tests when compared to control wild-types mice [31,54]. It is true that the mice employed in the cited studies have an altered expression of Lcn-2 since birth, and here we are using wild-type mice with a normal development which do not start to receive the treatment until they are 7,5 months old, so they are not entirely comparable. Nonetheless, if differences regarding Lcn-2 levels are found in the brain of Deferiprone-treated wild-type mice, it could explain why they performed slightly worse during the probe trial of the MWM, acting as if they had some sort of mild memory impairment. Basal levels of Lcn-2 may not be high enough to detect them via staining [55], but it has been observed that this protein is up-regulated upon stress [56]. All the experimental groups showed the same levels of anxiety-like behaviour, so this should not be responsible for any major difference in their performance. However, it may be that this level of stress is enough to induce the expression of Lcn-2 in the hippocampus of the mice. It could be that Deferiprone is interfering with this physiological function of Lcn-2 in the wild-type mice. Still, further insights should be given into the possibility of an effect of

Deferiprone in healthy animals, especially since most studies about the use of iron chelators in AD models do not include treated wild-type mice as control.

In any case, given all the different and contrasting information on Lcn-2 available, the role of this protein in the pathogenesis of AD should be researched in more detail to clarify its actual function in neurodegeneration and its connection to the cognitive decline typical of this disorder. This would provide new evidences of whether Lcn-2 has real potential to become a therapeutic target or not.

Finally, it could be that the negative results obtained in this report are a consequence of the mouse model or the age selected. Maybe using different AD models with other degrees of neurodegeneration would lead to a different outcome. Indeed, studies showing positive results of the iron chelation therapy in AD used alternatives mouse models with other characteristics as the one used here (for instance, APP/PS1 mice or transgenic mice for tau protein). Similarly, it may be that the age selected for the start of the treatment and the behavioural testing was not adequate. Although this age was chosen because it corresponds to the early stage of AD, maybe it is still too soon for the iron dysregulation and Lcn-2 to be playing a substantial role in the progression of the disease, which would explain why targeting them did not lead to any changes in the condition. It would also be interesting to test different doses of Deferiprone, as maybe the one administered here was not appropriate and reduced the efficacy of the chelator.

In conclusion, we could not detect any improvement in the cognitive performance of J20 mice treated with Deferiprone. These results are conflicting with previous studies which use iron chelators in the context of AD and show an amelioration of the cognitive function of model mice. While the reason of this is still unclear, it should be mentioned that the NLR test could not provide any valuable information, so any conclusion about hippocampus-dependent memory impairments that could be drawn in this report is based merely on the results of the MWM task. The neuropathological analysis of the brains may shed some light on why the treated mice did not perform better during the tests, as well as reveal whether Deferiprone is, on one hand, alleviating the iron dysregulation accordingly to its function as an iron chelator, and, on the other, reducing Lcn-2 overexpression. Future effort should be focused on finding a mouse model that resemble more the human AD, as the ones primary used are mainly based on the accumulation of misfolded Aβ or tau proteins, mostly ignoring other hallmarks and aspects that may be crucial in the development of the disease. This hinder the validation of any potential new therapeutic target, as these could not have the same role and importance in mice as in humans. Nevertheless, given the complexity of this disease, it is true that such a model would be hard to find. Still, and despite our unfavourable results, iron chelation therapies should continue to be tested as a treatment for neurodegeneration, given the encouraging results obtained in alternative studies. Likewise, even though the role that Lcn-2 plays in AD is still for the most part uncertain, the possibility of this protein to be a therapeutic target for AD should be studied more in-depth, especially considering its connection with iron dysregulation and the relevance of this in the neuropathology of the disorder.

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Additional file

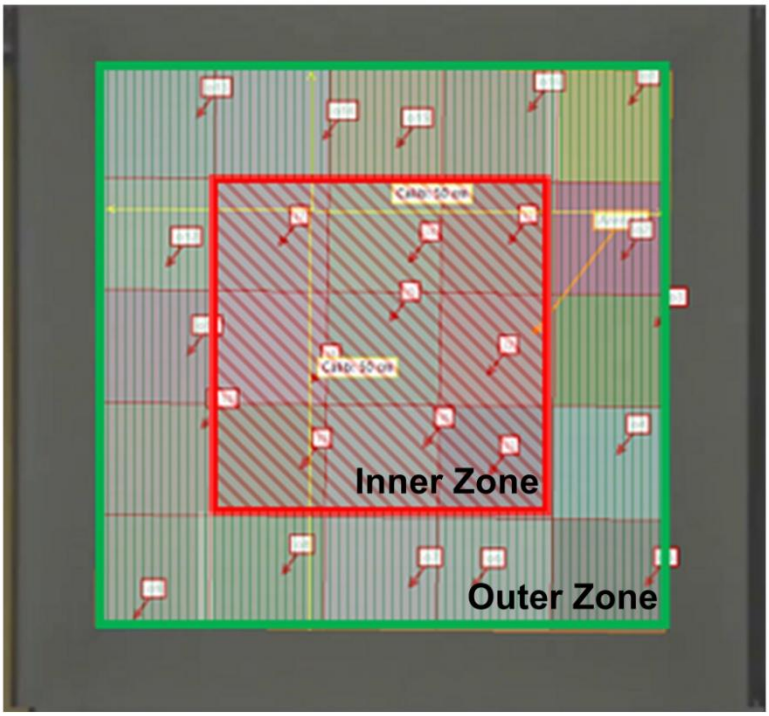
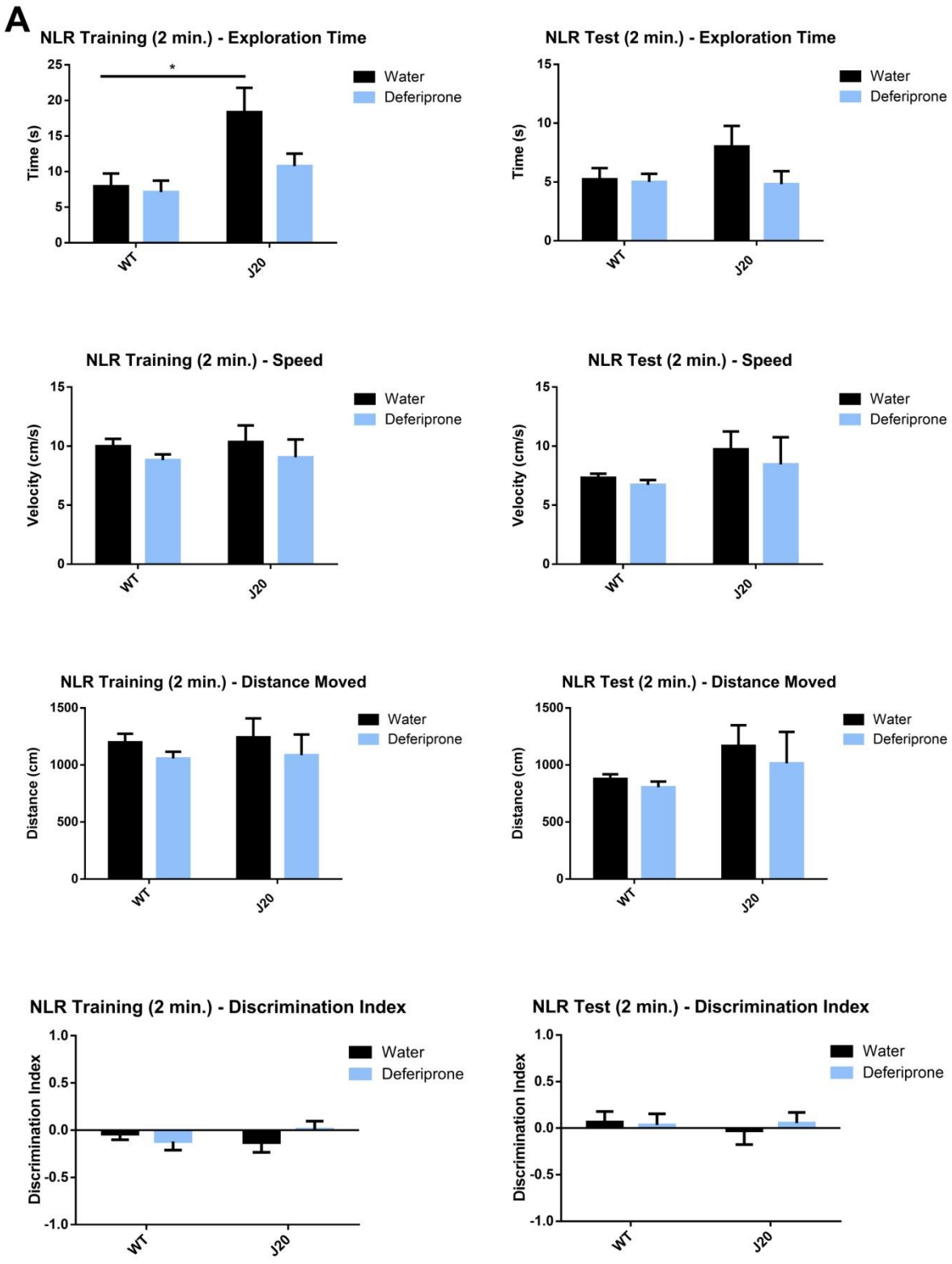


Fig. S1. Arena for the Open FIeld. Area under the red, diagonal lines constitutes the inner zone, the rest (under green, vertical lines) corresponds to the outer zone.The division of the arena in the two different zones was made using Ethovision XT.

Fig. S2. Object used for the Novel Location Recognition.



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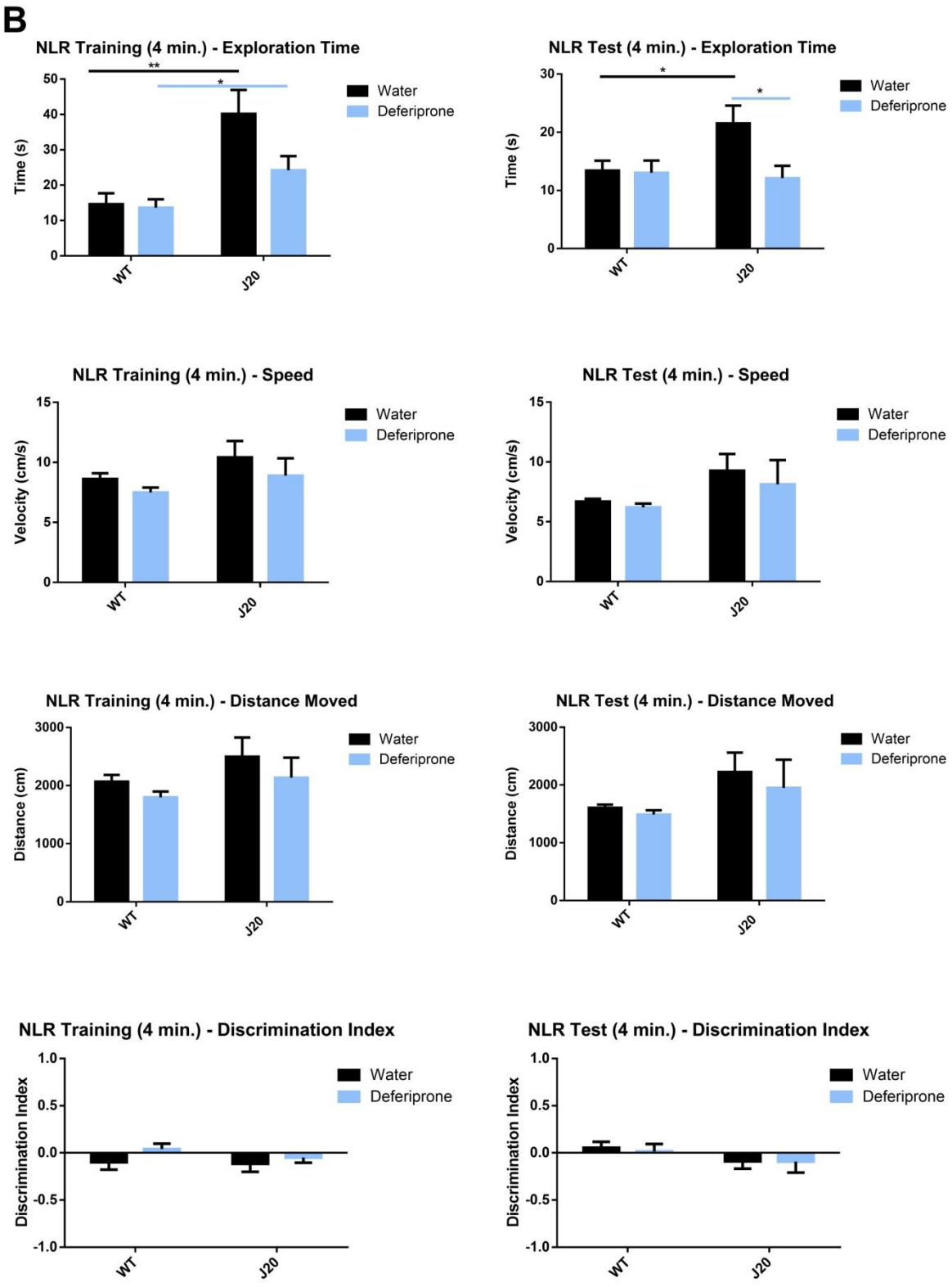

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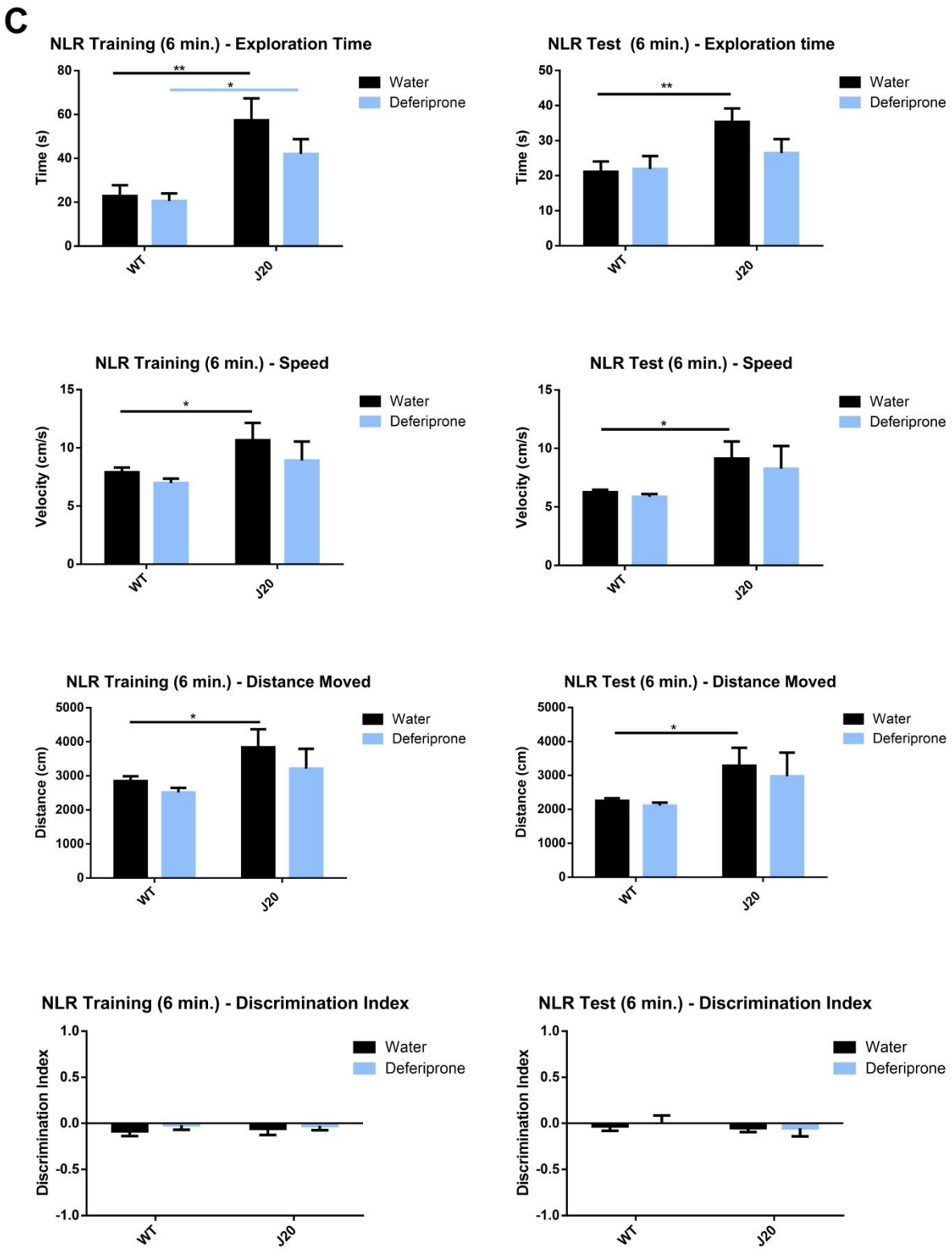

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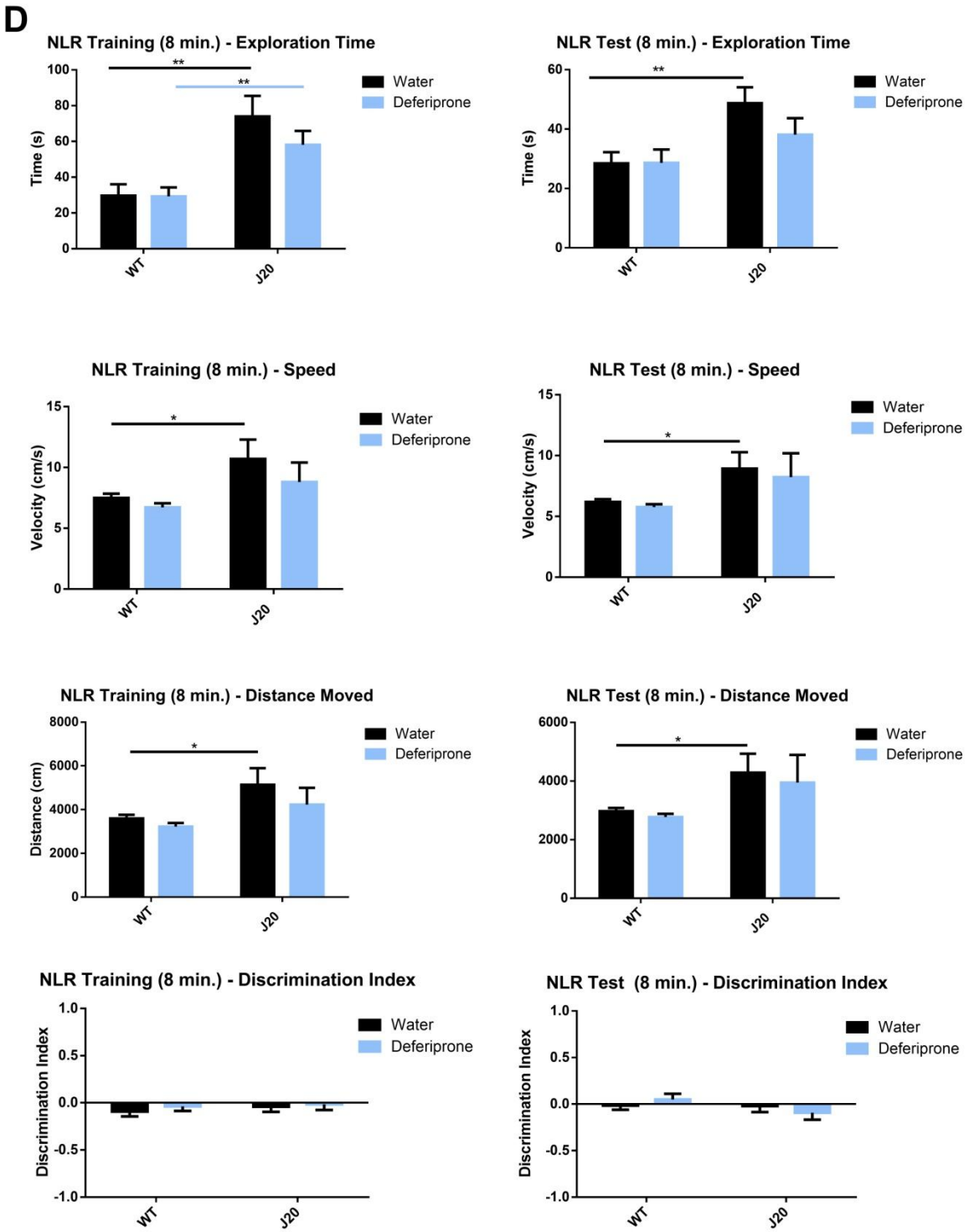

Fig. S3. Scoring the NLR at different times periods did also not yield any conclusive results. The time periods scored were 2 min. (A), 4 min. (B), 6 min. (C) and 8 min. (D). Results from both training (left) and test session (right) are included.Tested with Mann-Whitney (for Speed, Distance moved and Exploration time for the 6 min Test trial) or unpaired t-test with Welch's correction (for the Exploration time, expect for the 6 min Test trial, and Discrimination index) One-sample t-test was performed to test if the values of the discrimination index differ from 0. * indicates $p<0.05$ and **$p<0.01$. n=55 (WT water n=15, WT Deferiprone n=16, J20 water n=12, J20 Deferiprone n=12).

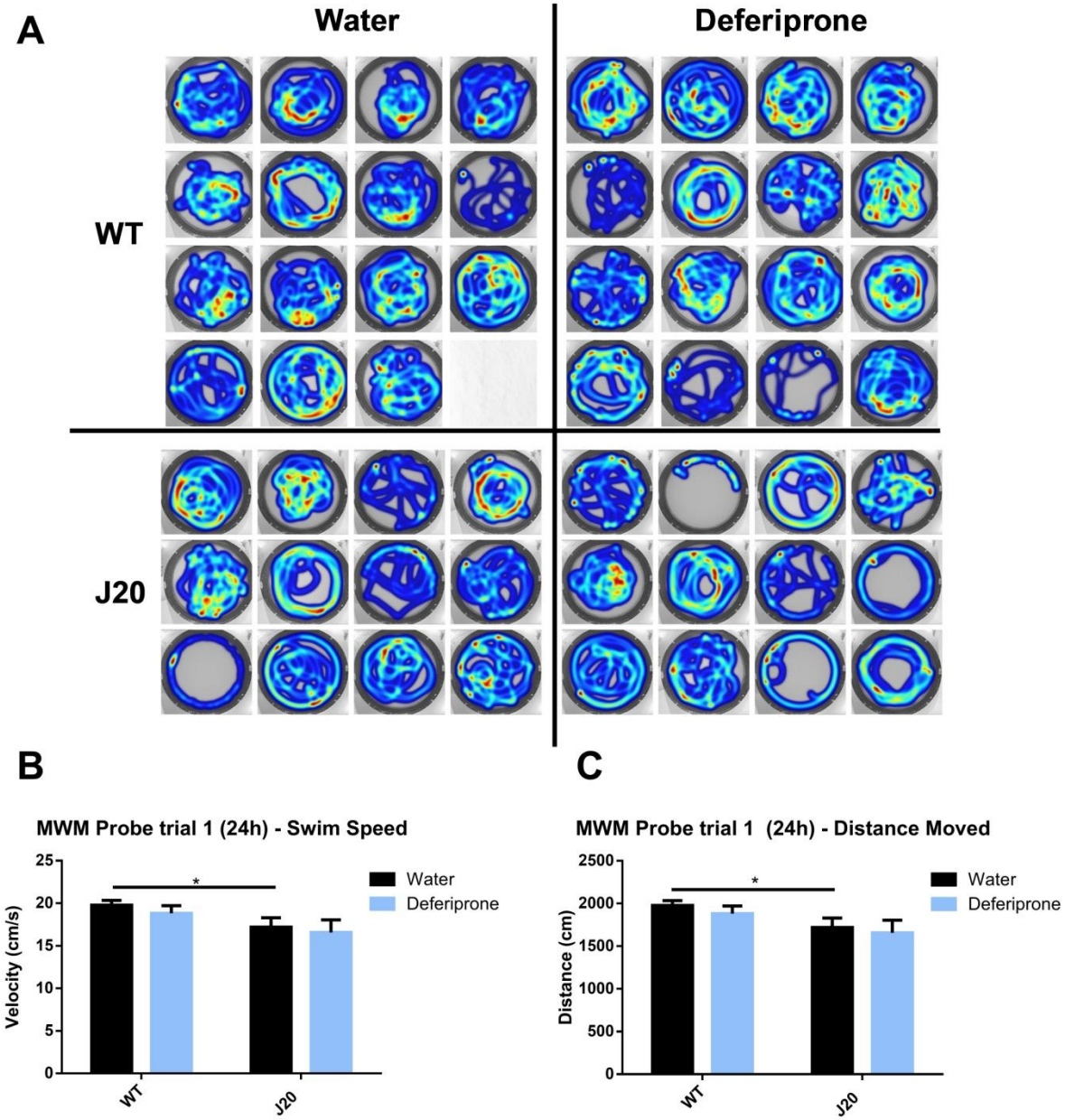

Fig. S4. Additional results of the probe trial performed 24h after the last training session. (A) Heatmaps with the swim path of the mice from each group.(B) Swim speed and (C) distance moved by the animals during this trial. Tested with Mann-Whitney. n=55 (WT water n=15, WT Deferiprone n=16, J20 water n=12, J20 Deferiprone n=12).

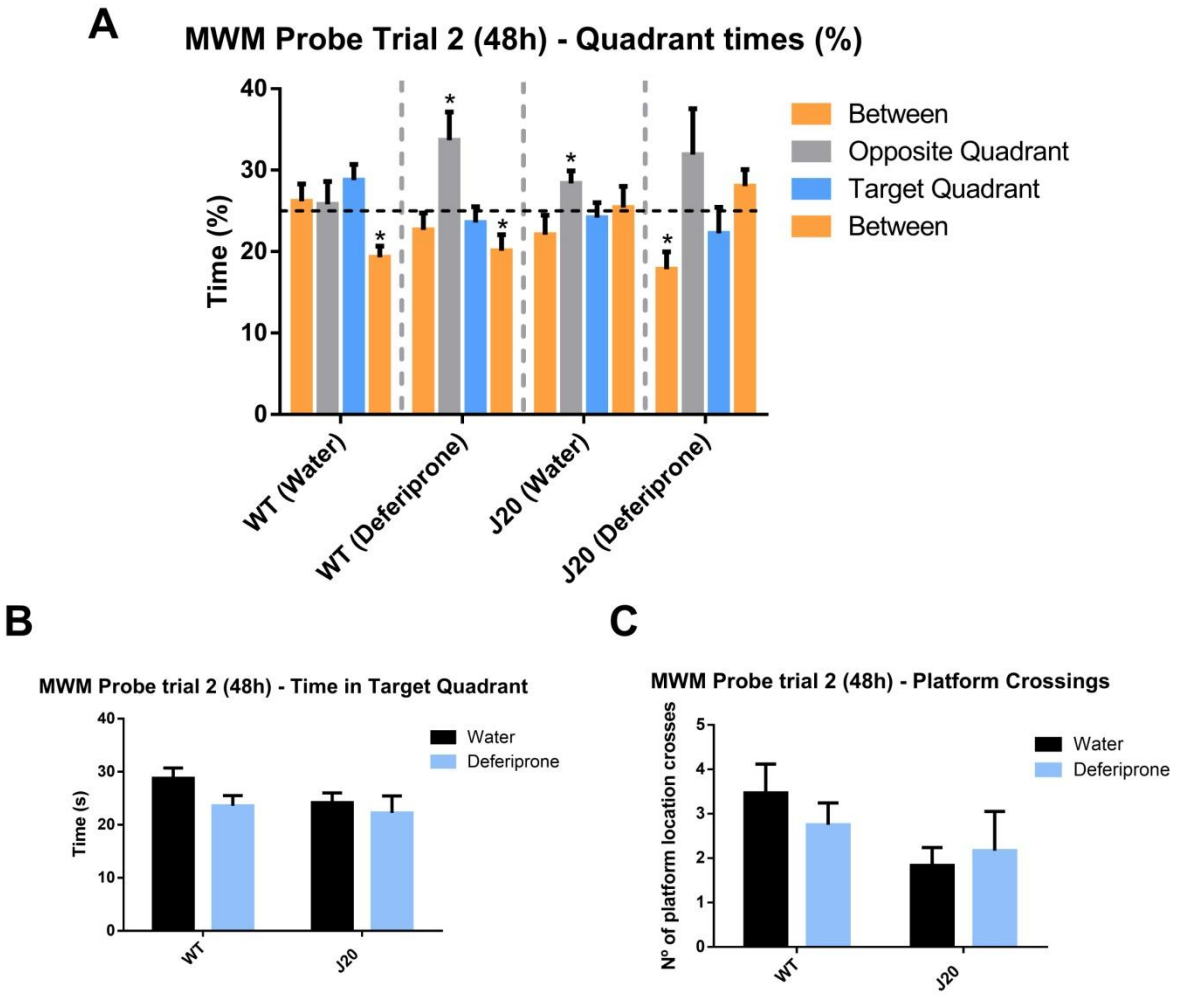

Fig. S5. Results of the 48h probe trial. In general, all the four experimental groups performed worse than in the 24h probe trial. (A) Percentage of time spent in the quadrants during the 48h probe trial. Black dashed line indicates chance level (25%). One-sample t-test was performed to test if the values differ from chance level (significance represented by the asterisks). (B) Comparison of the time spent in the target quadrant by the four experimental groups. Tested with unpaired t-test with Welch's correction. No significance was achieved. (C) Number of crosses during the probe trial 2 over the location where the platform was. Tested with Mann-Whitney. Again, no significance was achieved. n=55 (WT water n=15, WT Deferiprone n=16, J20 water n=12, J20 Deferiprone n=12).

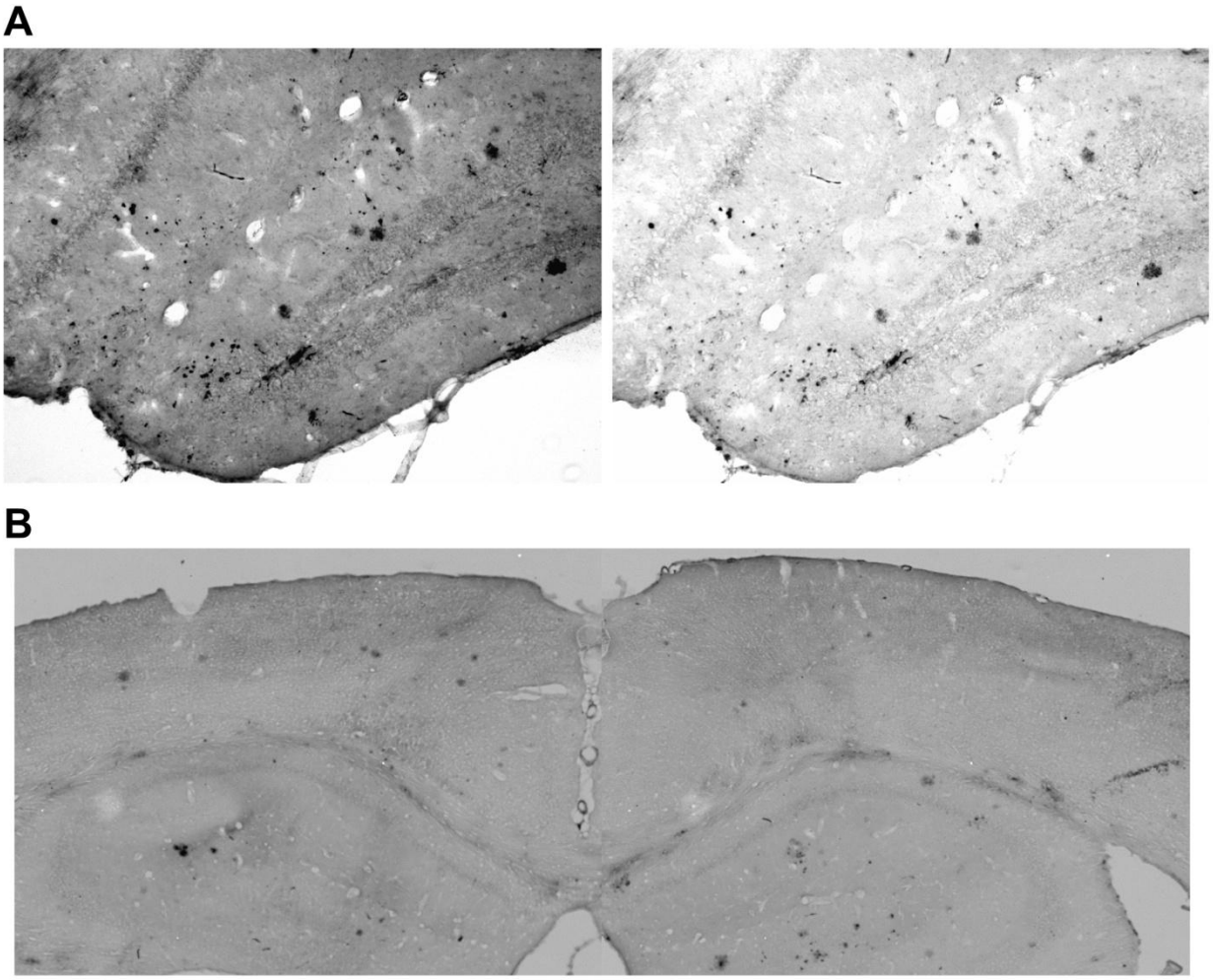

Fig. S6. Perls' staining. (A) Left, hippocampus with high background obtained in the first pilots prior using old potassium ferrocyanide (opened the 11/11/2017). Right, same image but with the contrast enhanced . While now the background is lighter and the dark, iron spots are more noticeable, the slice is not clear enough and false positive staining is present at different points of the hippocampus. (B) Hippocampus and upper cortex from a pilot using fresh potassium ferrocyanide (opened the 10/6/2019). Not only the background is lighter and no enhancing is needed, but in general the whole slice is clearer and no false positive staining is present. Images (A and B) were taken using an Olympus BH2 microscope on different days.

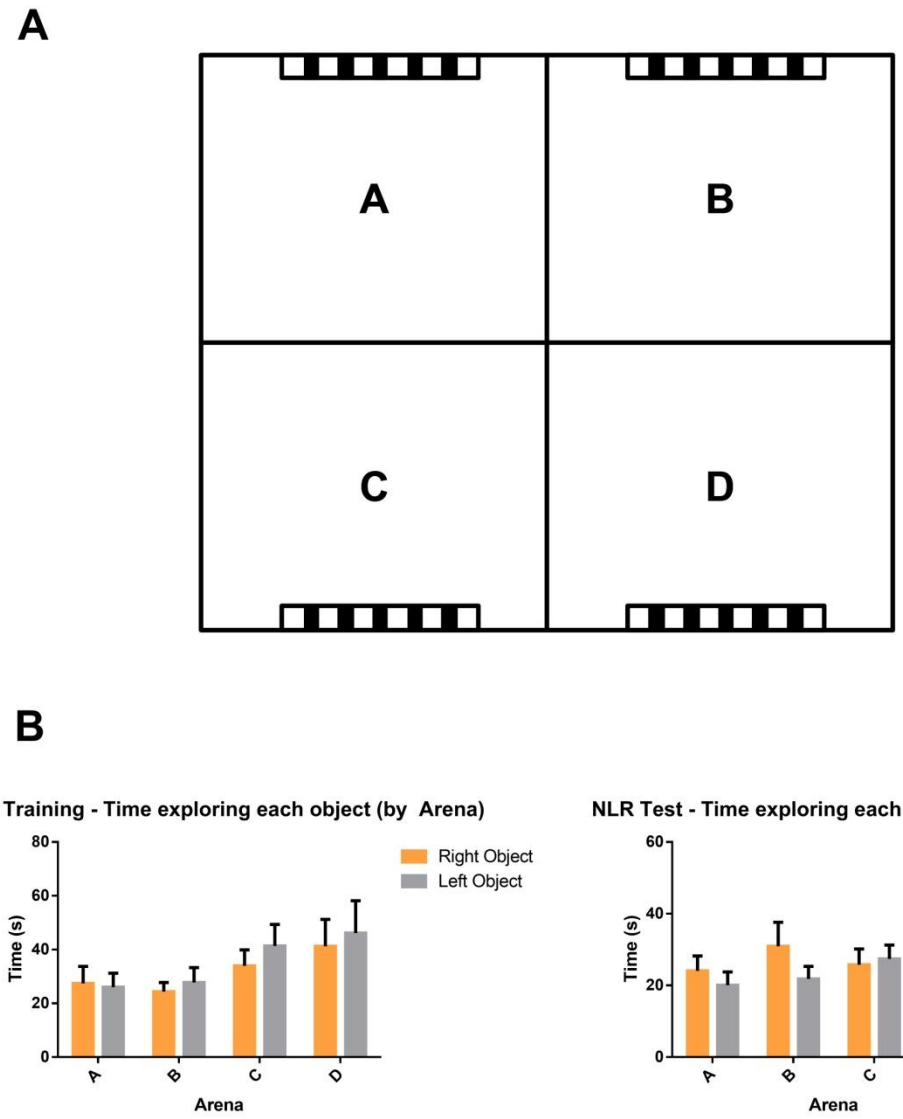

Fig. S7. No discernible preference for one of the object during the Novel Location Recognition (10 min.) can be observed (A) Distribution of the four arenas during the NLR. Each arena has its own set of two identical objects. (B) Time the mice performing the task in the same arena spent exploring each object during the training session (left) or the test session (right). Unpaired t-test with Welch's correction (Arenas A and C) and Mann-Whitney test (Arenas B and D) were performed to see if there were significant differences between the time exploring the right and the left object in each arena. No significance was found. n=55 (A n=15, B n=15, C n=13, D n=12. Note: mice from all the four experimental groups are included in each arena).

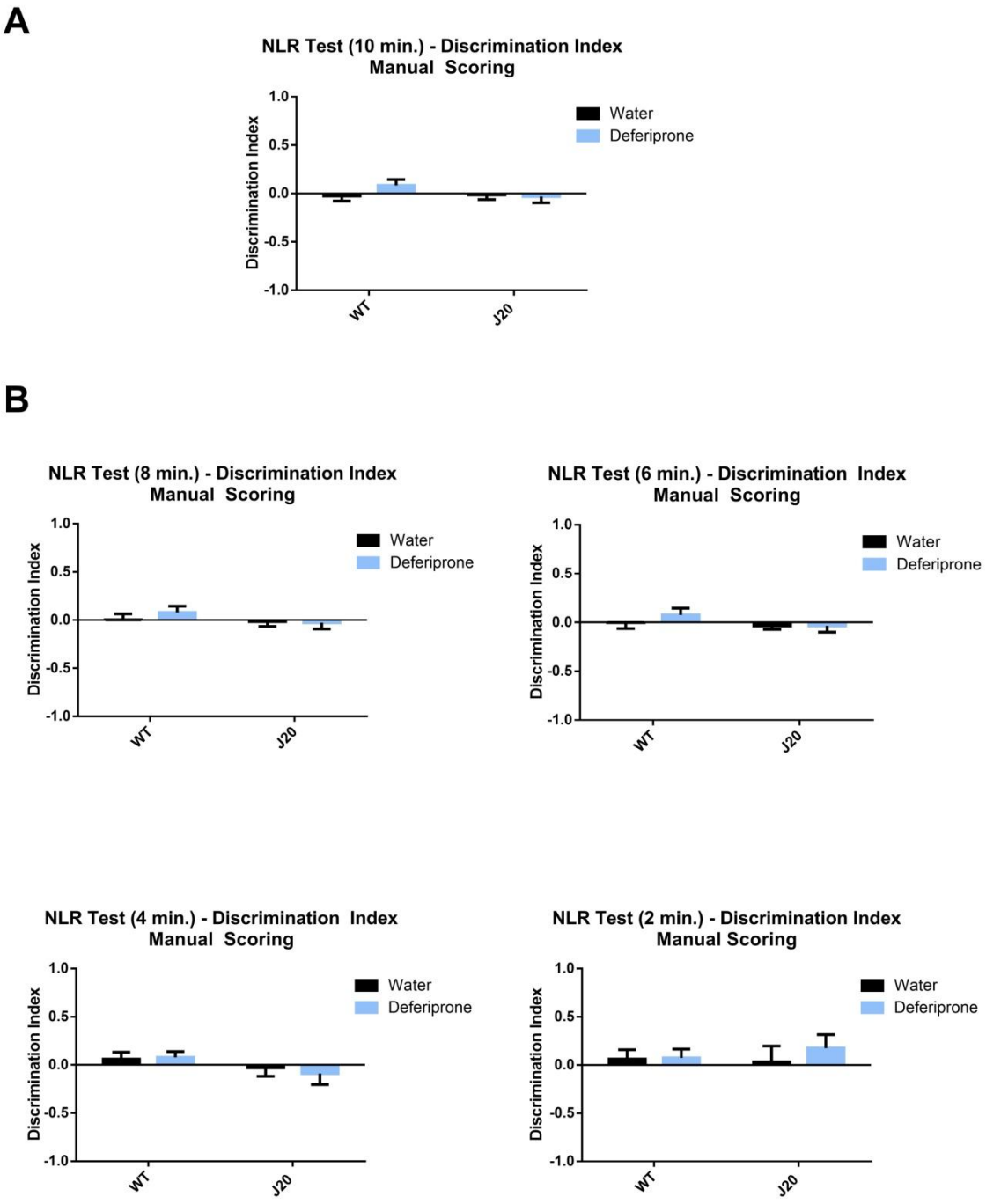

Fig. S8. Manual scoring of the test session of the Novel Location Recognition. Results of the discrimination index correspond greatly to the values obtained with the automatic scoring. (A) Discrimination index during the test trial of the NLR (10 min.). (B) Discrimination index during shorter periods of time of the test trial of the NLR. Tested with unpaired t-test with Welch's correction. One-sample t-test was performed to test if the values differ from 0. n=55 (WT water n=15, WT Deferiprone n=16, J20 water n=12, J20 Deferiprone n=12).