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Behaviour testing in two different knockout mouse strains related to chronic inflammation and oxidative stress

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ABSTRACT

CD36, a fatty acid translocator and NRF2, a transcription factor, are two important players in inflammation and oxidative stress, including in the central nervous system. Both were associated with neurodegeneration as tilting arms of a balance: while activation of CD36 participates in neuroinflammation, activation of NRF2 seems to protect against oxidative stress and neuroinflammation. This study aimed to establish whether tilting the balance one way or the other, by knocking out either of them (NRF2-/- or CD36-/-), would show that one holds higher weight over the other in the cognitive behaviour of mice. We tested both young and old knockout animals in a long-term testing protocol (over one month), using the 8-arm radial maze. Young NRF2-/- mice exhibited a sustained anxious-like behaviour, which was not recapitulated in old mice nor CD36 -/- mice of either age. Neither knockout strain exhibited cognitive alterations, although CD36 -/- mice showed some improvement over WT littermates. In conclusion, NRF2-/- seems to affect behaviour of mice early in life, and could be considered a vulnerability factor for neurocognition, while CD36 impact on cognitive protection of the aging brain requires more investigation.

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Introduction

Inflammation and oxidative stress have been proposed as key pathophysiological mechanisms involved in neurodegenerative processes and cognitive impairment. Key players in the oxidative stress and inflammatory cascades are the transcription factor Nrf2 and membrane fatty acid translocator CD36. Nrf2 (NF-E2-related-factor 2) also known as a stress-responsive transcription factor induces an antioxidative stress response, reduction of the proinflammatory milieu, suppression of the reactive astrocytosis and therefore limiting the harmful effects of oxidative stress (1). Activation of NRF2 was reported to mediate improvement of cognitive behaviour in mice (2,3), while NRF2-/- knockout is not enough to induce an AD-like pathology by itself (4,5).

CD36/SCARB-2, a microglial scavenger receptor for amyloid B, is also involved in the mediation of proinflammatory states. A CD36 signalling pathway is activated following either Aβ binding or activation of microglia and subsequently increased synthesis of pro-inflammatory cytokines (6). Activation of CD36 has been reported as beneficial in AD mice models (1,7). On the other hand, knocking out CD36 protects against macrophage-induced inflammation (8) and, as a consequence, there are studies targeting CD36 as a possible target for drug intervention, including for AD (9). However, there are no reports to state a direct link between CD36 and cognitive impairment in mice. There is a single report showing that CD36 KO mice display anxious-like behaviour (10). Furthermore, one of the NRF2 targets is CD36 (11), partially regulating its expression and proposed "as a second important transcription factor involved in the induction of the scavenger receptor CD36" (12). Overall, data on the cognitive behaviour of mice, related to loss of CD36, is scarce and most of it was obtained using short-term testing methods, such as open field, novel object recognition or forced swimming test. Our article aimed to extend neurocognitive testing of two knockout mouse strains: Nrf2 -/- and CD36 -/-, using a long-term testing setup – the 8-arm radial maze. This would provide data regarding the adjustment of neurocognitive behaviour over time, by placing the animal in the same environment daily, for a month.

Materials and Methods

Animals

Two transgenic strains of mice CD36-/- (B6.129S1-Cd36tm1Mfe/J) and NRF2-/- were investigated at different ages for behavioural testing. C57BL/6 mouse colonies of the *Nrf2*-/- and *Nrf2*+/+ genotypes were established from animals kindly provided by Prof A. Cuadrado, Universidad Autonoma de Madrid and previously described in (13) and CD36 -/- were obtained from Jackson Laboratoires US. Adult animals were tested using open field and novel object recognition and old animals were tested for anxiety behaviour and memory impairment in 8-arm radial maze (animal ages and gender distribution are presented in Tables 1-3). Mice were group-housed in simple cages under a normal 12 h light/dark cycle, constant temperature and humidity, with ad libitum access to food and water. The studies were conducted according to the guidelines of

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Table 1. Age and gender of the experiment mice (open field testing).

Mouse strain	NRF2-/- (N=10)	CD36-/- (8)	WT(N=8)
Age (months)	4	4	4,5
Gender distribution	4F+4M	4F+4M	4F+4M

Table 2. Age and gender of the experiment mice (8-arm radial maze).

Mouse strain	NRF2-/- (N=6)	WT(N=8)
Age (months)	20.5±0.8165	18.6±6.948
Gender distribution	3F+3M	5F+3M

Table 3. Age and gender of the experiment mice (8-arm radial maze).

Mouse strain	CD 36-/- (N= 7)	WT(N= 6)
Age (months)	12.85±1.464	12.75±0
Gender distribution	7M	6M

the European Directive 2010/63/EU and approved by the Romanian National Authority for Veterinary Research, authorization No.588/13.01.2022 and No. 385/09.02.2018, respectively.

Open field protocol

All animals were acclimated for 30 minutes to the testing room in their cages to minimize stress. Between tests, the arena was cleaned with alcohol to remove odors. Each animal was placed in the middle of the arena and allowed to explore for 5 minutes. The trajectory in the central zone versus the periphery border was recorded using Smart 3.0 video-tracking software.

Radial maze testing protocol

The testing protocol consisted of three phases: 2-step habituation, 2-step training and a final testing phase. The first habituation step consisted in 5 minutes in the maze's central platform with all arms closed, for 5 successive days. In the second step of habituation (5 days), the mice were each, individually, placed in one arm and allowed to explore 4 random arms and the central platform. The second phase, of training, lasted for 21 days. During the first 7 days, each rodent was placed in the central platform with free access only to 3 baited arms, for 5 minutes. For the following 14 days, mice had full access to all 8-arms, out of which, the same 3 were baited with food. For the final testing phase (3 consecutive days) every rodent was placed in the central platform with all arms opened and animal behaviour was recorded using MazeSoft8, to assess memory errors and SMART 3.0 video-tracking software for pathway, speed and distance analysis.

Animals were food deprived for 12 hours before testing in order to increase motivation and performance. External visual cues or olfactory cues were suppressed.

Statistical Analysis

Statistical analyses were performed with Prism7 (GraphPad Software 9.1.0), using OneWay ANOVA and Student's *t*-test. Data are expressed as mean ± SD. Groups were considered significantly different when $P < 0.05$ ($^{*}P < 0.05$, $^{**}P < 0.01$).

Results

Loss of NRF2 was associated with early changes in mouse behavior in both short and long-term testing, while CD36-/- showed no significant change

First, we assessed the behavior of young mice in an open field, where mice were allowed to freely explore the environment for 5 min, while video-tracked. The open field was divided into 9 digital squares, using the Smart video-tracking software and the center green square was used to assess the time and distance (Fig.1). In addition, total distance and speed were recorded for each group. Of tested animals, NRF2-/- showed hyperactivity, quantified as total distance travelled and mean speed of movement.

The next question was whether this is a short-term or a sustained behavior, so we recorded the behavior of adult NRF2-/- mice, compared to WT, over a longer period (one month), using the 8-arm maze protocol. We noticed that consistently NRF2-/- mice are hyperactive when compared to WT. We also tested their ability to learn, by

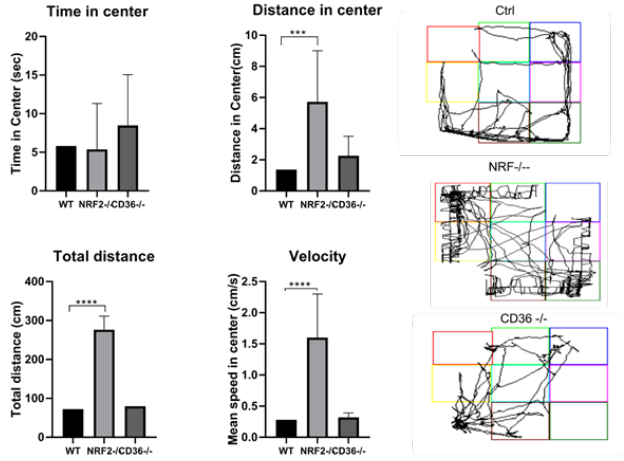

Figure 1. Open-field test of adult NRF2-/- and CD36-/- mice. Video tracking was used to record the pathway and speed of mice in an open-field arena for 5 minutes. A suggestive example of a pathway is shown in the right panels for each group. Bars represent the average per group (N=8)±S.D. and statistical significance was determined using the OneWay Anova test (***p<0.001, ****p<0,0001).

quantifying learning mistakes as entries in non-baited arms before entries in baited arms (attempts to find food in the wrong arms of the maze). We assessed both working memory errors (re-entries in the same wrong arm), for assessment of recent memory, as well as reference memory errors (entries in non-baited arms), for long-term memory. Although the NRF2-/- long-term memory seemed to perform better than the WT, it was not statistically significant in our experimental setup (Fig.2).

Loss of CD36 exerts some protection against aged-induced memory impairment, while NRF2-/- shows no alterations in cognitive behaviour, compared to controls

Finally, we asked the question of whether aging will make a difference in terms of behavior and learning for these two strains of KO mice. We tracked the distance and speed of aged mice, compared to WT. We also assessed both working memory errors (re-entries in the same arm), for assessment of recent memory, as well as reference memory errors (entries in non-baited arms), for long-term memory.

We observed that the exploratory behaviour of NRF2-/- diminished with age, the travelled distance was lower than WTs, while the maximum speed was comparable to control, but without indication of memory impairment. For CD36-/-, no significant differences were noticed compared to the control (Fig 3), although, in aged animals, loss of CD36 seemed to offer some protection against memory impairment.

Discussion

Neurodegeneration is a multifaceted pathological process, in which oxidative stress and inflammation are becoming increasingly investigated and targeted for novel therapeutics. Two important players in these processes are the transcription factor NRF2, which upregulates gene expression of antioxidant enzymes (14) and CD36, a scavenger receptor able to initiate inflammation responses following various ligands, such as oxidized LDL (oxLDL) (15), or advanced glycosylation end products (AGEs) (16). Loss of these proteins would have, in theory, opposite effects on behaviour of KO mice, especially with advanced age: NRF2-/- mice would experience increased oxidative stress and altered cognitive behaviour, whereas CD36-/- would be protected from cognitive alterations.

First, we were interested to see whether loss of either gene would impact on exploratory behaviour from early life. While CD36 was similar to controls in respect to behaviour in the open field, NRF2-/- showed early significant changes. Previous reports of NRF2-/- mice behavioural testing reported various impairments, from reduced immobility in forced swimming test (17), to reduced exploration in the water maze (18). By itself, NRF2 loss does not induce cognitive decline, but it has been reported to aggravate previous cognitive loss in various animal models of cognitive impairment (4,5). Aging induced a decrease in exploratory behaviour of NRF2-/- , which can be explained by somatic changes, as it has been previously

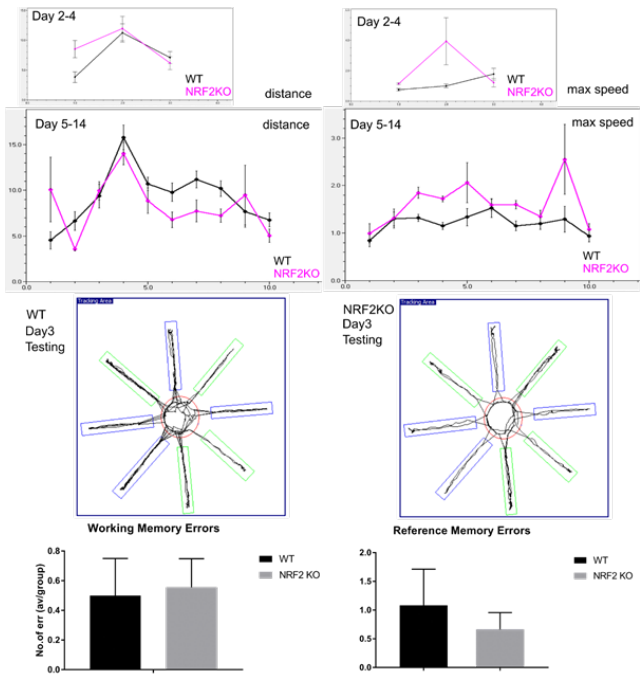

Figure 2. 8-arm radial maze testing of adult NRF2-/- and WT mice. Distance and maximum speed in the maze were assessed for early (days 2-4) and mid (days 5-14) term testing. The NRF2-/-mice consistently showed increased speed over WT littermates. Although animals of both groups made mistakes during the memory testing phase (middle panel, showing a representative pathway for each group, where all arm were visited before the end or the trial period), overall the NRF2 -/- group had fewer long-term memory errors.

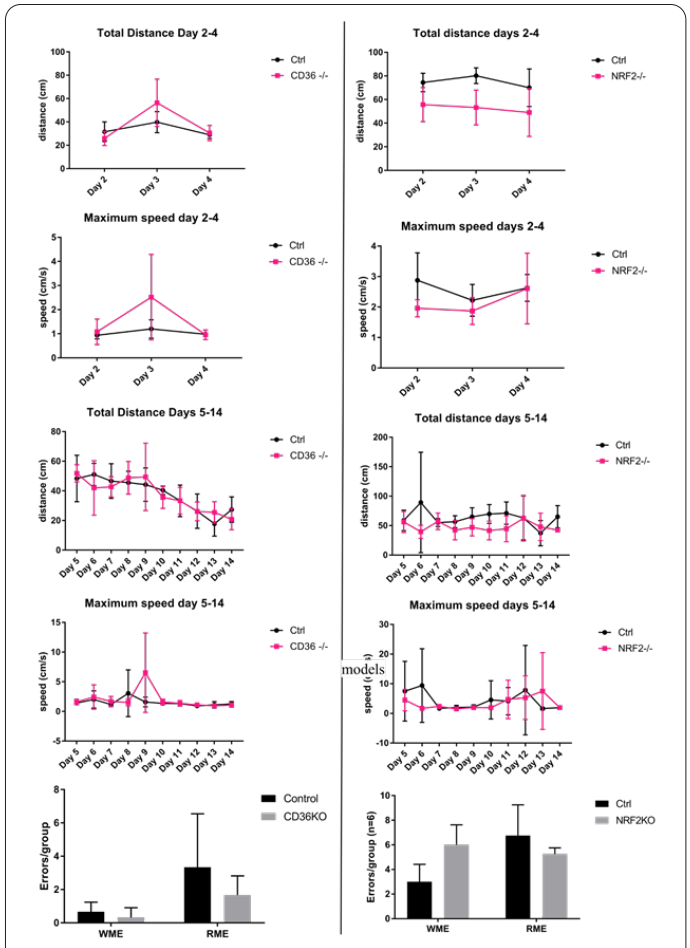

Figure 3. 8-arm radial maze testing of CD36-/- and NRF2-/- mice vs control. CD36 performed quite similarly to WT with respect to travelled distance and maximum speed, except for reference memory errors, where they performed slightly better. No significant differences were observed for NRF2-/- group either in terms of distance, maximum speed or learning errors

shown that Nrf2 deficiency caused declined physical function during aging, due to sarcopenia and increased frailty (19). The anxious-like behaviour could still persist, but it is masked by physical impairment. In contrast to most data in the literature, we started our testing with very young animals, which are not yet physically impaired. Also, we had an extended testing period (over 20 days) whereas most behaviour testing experiments last several days. Hence, we were able to detect a sustained anxious-like behaviour of young NRF2-/- over time.

In contrast, CD36-/- exploratory behaviour is similar to WT mice, with no significant changes with increased testing time or age. However, a decreased number of errors of CD36-/- mice was noted in aged animals, although it did not reach statistical significance. One possible reason is that our group consisted only of males, which are less sensitive to hormonal changes and hormonal impact on cognition with age, as compared to females, as well as CD36 distribution (20), although there are also reports showing no learning difference between aged males and females (21).

In conclusion, we were able to establish that losing protection against oxidative stress through NRF2-/- loss could be significant for anxious-like behaviour, possibly in a life-time long setup, but not significant for cognitive testing of aged mice. This highlights NRF2 as a vulnerability factor for the health of the central nervous system. In contrast, increasing protection against neuroinflammation by CD36 loss could show some benefit. However, as aging induces frailty in many aspects, from neurogenesis to skeletal muscle loss, one intervention is insufficient to offer protection against cognitive decline.

Conflict of interest

We have no conflicts of interest to disclose.

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Author contributions

AMD and GI performed the experiment, gathered and analysed data. AME and MEH designed the study, analysed the data. MEH acquired fundings. All authors wrote the manuscript.

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