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Age-related impact of social isolation in mice: Young vs middle-aged

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ABSTRACT

Social isolation is a chronic mild stressor and a significant risk factor for mental health disorders. Herein we explored the impact of social isolation on depression- and anxiety-like behaviours, as well as spatial memory impairments, in middle-aged male mice compared to post-weaning mice. We aimed to quantify and correlate social isolation-induced behaviour discrepancies with changes in hippocampal glial cell reactivity and pro-inflammatory cytokine levels.

Post-weaning and middle-aged C57BL7/J6 male mice were socially isolated for a 3-week period and behavioural tests were performed on the last five days of isolation. We found that 3 weeks of social isolation led to depressive-like behaviour in the forced swim test, anxiety-like behaviour in the open field test, and spatial memory impairment in the Morris water maze paradigm in middle-aged male mice. These behavioural alterations were not observed in male mice after post-weaning social isolation, indicating resilience to isolation-mediated stress.

Increased Iba-1 expression and NLRP3 priming were both observed in the hippocampus of socially isolated middle-aged mice, suggesting a role for microglia and NLRP3 pathway in the detrimental effects of social isolation on cognition and behaviour. Young socially isolated mice also demonstrated elevated NLRP3 priming compared to controls, but no differences in Iba-1 levels and no significant changes in behaviour. Ageing-induced microglia activation and enhancement of IL-1β, TNF-α and IL-6 proinflammatory cytokines, known signs of a chronic low-grade inflammatory state, were also detected.

Altogether, data suggest that social isolation, in addition to inflammaging, contributes to stress-related cognitive impairment in middle-aged mice.

1. Introduction

Long-term social isolation is considered a chronic mild stressor because humans, like many other animals, are social creatures preferring to live in small communities of trusted conspecifics (Cacioppo and Hawkley, 2009; Evans et al., 2020). The recent global pandemic caused by the SARS-CoV-2 viral outbreak has highlighted the potential detrimental effects of a protracted lack of social contact on mental health in different age groups (Manchia et al., 2022). Social isolation and loneliness are sometimes discussed interchangeably, but it is important to highlight the distinction between the two (Shankar et al., 2011). Loneliness is an emotional state of mind to perceived social disconnectedness, whereas social isolation is a more quantifiable lack of regular social interactions that may or may not lead to loneliness (Peplau and Perlman, 1982; Valtorta and Hanratty, 2012; Tanskanen and Anttila, 2016). Even so, isolation and a decrease in the regularity of social contact for prolonged times is thought to increase one's susceptibility to mental health problems (Weiss et al., 2004; Brenes et al., 2008; Fone and Porkess, 2008; Lukkes et al., 2009), such as cognitive decline, memory impairments, anxiety, depression, or even suicidal tendencies (Fratiglioni et al., 2004; Wilson et al., 2007; Umberson and Montez, 2010). By impacting cognitive function and flexibility (Bassuk et al., 1999; Quan et al., 2010) social isolation may, in turn, confer greater susceptibility to later-life dementia (Wilson et al., 2007; Ertel et al., 2008; Kuiper et al.,

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2015; Penninkilampi et al., 2018). Moreover, social isolation at different stages of life can often lead to a wide range of pathophysiological and maladaptive behavioural outcomes for isolated individuals (Suri et al., 2013; Hämmig, 2019).

When considering age-related susceptibility to social isolation, it is often assumed that it leads to worse health outcomes in the older adult population (>65 years) (Victor and Bowling, 2012. Likewise, studies have shown that middle-aged adults are often at greater risk of mortality due to social isolation (Holt-Lunstad et al., 2015) and in the United Kingdom and European Union, middle-aged men between the ages of 40 and 54 are most prone to commit suicide (NCISH, 2021). Isolation is also described as a significant risk factor for mental health issues in adolescents and young adults (NCISH, 2021). Though allowing better discrimination between isolation and loneliness, the high diversity of lifestyles and stressors in humans makes it relatively difficult to identify age-related differences in social isolation susceptibility. Animal models have different levels of complexity but allow comparisons in more standardized conditions. The main aim of this work was, therefore, to compare the susceptibility to social isolation of two distinct groups of mice, very young (with isolation starting immediately after weaning) and middle-aged mice. To do so, we focused on behavioural hallmarks (depression- and anxiety-like behaviours, as well as spatial memory) and some molecular markers related to neuroinflammation.

Neuroinflammation is defined as the immune response of the central nervous system against elements that interfere with its homeostasis and this response is involved in all neurological diseases, including developmental, traumatic, ischemic, metabolic, infectious, toxic, neoplastic, and neurodegenerative diseases. Neuroinflammatory processes are characterized by the activation of microglia and astrocytes – reactive gliosis – with overproduction of inflammatory mediators, such as cytokines, chemokines, reactive oxygen species and secondary messengers (Lucas et al., 2006), which contribute to modulation of the immune response (Ransohoff and Perry 2009; Hinwood et al., 2012). An increase in proinflammatory mediators in brain tissue and blood has been demonstrated after exposure to psychosocial stressors (Hodes et al., 2015; Wohleb et al., 2016), and neuroinflammation was associated with anxiety- and depressive-like behaviours in mice (Erta et al., 2015; Pan et al., 2014; Zhang et al., 2015; Wang et al., 2018b). It has also been shown that priming of microglial cells in key brain regions involved in learning and memory (hippocampus) and in the stress response (hypothalamus) can occur after short periods of social isolation (Vu et al., 2023). Some studies have assessed neuroinflammatory processes during adulthood (Wang et al., 2017; Alshammari et al., 2020; Niu et al., 2020; Wu et al., 2022), and the impact of post-weaning social isolation in the expression of inflammatory mediators in the hippocampus of young rats (Dunphy-Doherty et al., 2018). Additionally, no information addressing the expression of inflammatory mediators in the hippocampus of middle-aged animals has been revealed until now.

Recent studies have shown that a cytoplasmic multiprotein complex, the NLRP3 inflammasome (NLRP3, nucleotide-binding domain, LRR- and pyrin domain-containing protein 3), integrates stress-associated signals (Wohleb et al., 2016; Yue et al., 2017). This inflammasome comprises not only the sensor NLRP3, but also apoptosis-associated speck-like protein (ASC), and caspase-1 domains (Sheedy et al., 2013). Activation of NLRP3 requires a two-step signal. The priming signal triggers the transcription of NLRP3 protein and pro-interleukin-1β (IL-1β), while the activation signal promotes the oligomerization of the three domains (Li et al., 2021a). NLRP3 assembly promotes the auto-proteolytic cleavage of pro-caspase-1 into the catalytically active enzyme. In turn, active Caspase-1 promotes the secretion and maturation of the proinflammatory cytokines IL-1β and IL-18, which mediate immune responses (Latz et al., 2013; Bachiller et al., 2018; Wang et al., 2018b) IL-1β has a key role in anxiety- and depression-like behaviour (Zhang et al., 2015; Yue et al., 2017) and its protein levels increase after stress exposure in several brain regions, such as the hippocampus (Gadek-Michalska et al., 2013, 2016). Importantly, several lines of evidence imply that IL-1β is the first factor to mediate the proinflammatory response to psychosocial stress (Gadek-Michalska et al., 2013, 2015; Iwata et al., 2013) by promoting the release of other cytokines in the brain (Gadek-Michalska et al., 2017). NLRP3 was shown to be activated in depressed animals (Zhang et al., 2015; Yue et al., 2017), and social isolation in young mice was shown to cause increased levels of NLRP3 domain in the hippocampus, as well as higher levels of IL-1β and IL-18, in adulthood (Niu et al., 2020; Li et al., 2021b). Moreover, 10 weeks of isolation rearing causes a decrease in the anti-inflammatory cytokine IL-10 (Corsi-Zuelli et al., 2019).

Because it is not possible to quantify loneliness in rodent models, researchers have developed protocols to determine the physical, behavioural and cognitive effects of prolonged periods of 'living alone' in rats and mice, often termed 'isolation-rearing' when performed in young mice or 'social isolation' when adult and/or aged mice are involved (Fone and Porkess, 2008; Lukkes et al., 2009). Isolation-rearing involves housing newly weaned mice (21–25 days old), a critical period of brain maturation, in a small shoe box-sized cage, usually for periods of 1–8 weeks (Cassidy et al., 2010; Murphy et al., 2010). This often disrupts myelin formation in the frontal cortex and can lead to behavioural changes reminiscent of the negative symptoms of schizophrenia, or it can also leave mice more susceptible to developing addictive behavioural patterns when they reach early adulthood (approximately 80–90 days old) (Liu et al., 2012a; Whitaker et al., 2013).

Herein, we aimed to quantify and correlate social isolation-induced changes in hippocampal glial cell reactivity and pro-inflammatory cytokine levels with deficits in classic behavioural paradigms, including the Porsolt forced swim test, the open field arena, and the Morris water maze, which are often considered gold standard assessments of depression- and anxiety-like behaviours, and spatial memory formation in rodents, respectively (Porsolt et al., 1977; Morris 1984; Choleris et al., 2001). To our knowledge, there are relatively few studies that have investigated the effects of social isolation in middle-aged mice (9–14 months old) compared to the wealth of data published on young (3–12 weeks) or aged rodents (18–24 months) (Stepanichev et al., 2014; Benfato et al., 2022). We hypothesized that isolation would have a more negative impact on young male mice, since many studies have shown the importance of the 3–6 weeks critical period of brain maturation for cementing normal adult behaviours in rodents (Panksepp, 1981; Fone and Porkess, 2008; Jones et al., 2011). However, we found that young mice were more resilient to 3 weeks of social isolation than middle-aged mice (10–11 months old). We found minimal changes in the biochemistry of the young hippocampus and no changes in depression- or anxiety-like behaviours, or spatial memory scores, in young mice, but identified changes in inflammatory mediators and inflammaging signs, together with behavioural deficits, in middle-aged mice. Our results could help to explain why middle-age is such a vulnerable period for humans living in stressful health conditions (Han et al., 2020).

2. Methods

2.1. Ethics statement

All experiments involving animals and protocols used to obtain brain tissue were approved by the Animal Welfare and Ethical Review Body (AWERB committee) of the University of Brighton and were carried out under UK Home Office licence-approved protocols. This study was conducted in accordance with the principles of the Basel Declaration and adhered to the legislation detailed in the UK Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI, 2012/3039). All efforts were made to maximize animal welfare conditions and to reduce the number of animals used in accordance with the European Communities Council Directive of September 20th, 2010 (2010/63/EU).

2.2. Animals

This study was performed using male C57BL6/J mice (Charles River UK Ltd). All animals were housed under an artificial 12-h light-dark cycle at a controlled room temperature (19–21 °C) and humidity (40–60%). Food and water were available *ad libitum*. Two age groups were studied: young mice at 3–6 weeks old and middle-aged mice at 45–48 weeks old.

2.3. Experimental plan

2.3.1. Housing

Young and middle-aged mice were either group-housed (GH; 4 per standard size cage; dimensions (l x w x h) of 45 x 28 × 13 cm) or socially isolated (SI; one mouse per small cage; i.e., 33 x 15 × 13 cm) for a period of 3 weeks. Isolated animals were prevented from any form of contact with a conspecific at all times. However, mice could likely hear and smell their litter mates in neighbouring open-top cages in the animal room. Moreover, socially isolated mice were disturbed only for cleaning purposes (the cage bedding was changed every two weeks) and for behavioural testing. The forced swim test (FST), open field test (OFT), and Morris water maze (MWM) were performed during the final five days of the 3-week isolation protocol. All behavioural experiments were conducted between 10.00 a.m. and 4.00 p.m. under the same conditions. On the last day of behaviour, animals were sacrificed. The hippocampus was dissected from the brain and immediately snap frozen in liquid nitrogen and stored at −80 °C until required. A timeline of the experimental plan is shown (Fig. 1).

2.3.2. Forced swim test (FST)

Mice were subjected to the forced swim test (FST) on the 17th day of the experimental timeline (Fig. 1) to evaluate depression-like behaviour induced by social isolation (Porsolt et al., 1977). Mice were allowed to adapt to the experimental room for 30 min before the beginning of the task. Each mouse was placed into a clear glass cylinder (20 cm in height and 15 cm in diameter) filled with 1.4 L of water at a temperature of 25 ± 3 °C. The FST lasted 6 min and was recorded using a video camera. Only the last 5 min of the FST were evaluated, since the first minute was considered habituation time. Three main types of behaviour were analysed to assess the depressive-like phenotype of mice: 1) immobility time, defined as only minimal whole-body movements, with the exception of necessary movements to keep their heads above water; 2) escape behaviour, described as vigorous climbing movements executed with forepaws above water-level and along the cylinder wall, indicating an attempt to escape; and 3) latency to immobility, defined as the first time that immobility behaviour, lasting longer than 1 sec, began. The apparatus was cleaned and filled with fresh water between each animal. After the FST, each mouse was dried and returned to its home cage. All measurements were manually scored and expressed as percentage values (of the final 300 sec).

2.3.3. Open field test (OFT)

The open field test (OFT) was performed to evaluate anxiety-like behaviour and exploratory behaviour (Choleris et al., 2001). The OFT apparatus consisted of a white acrylic plastic box (60 × 60 × 30 cm) with the base divided into 12 equal-sized sections. The central square grid was considered the central zone of the apparatus (as represented in Fig. 1B). Again, before starting the test, mice were habituated to the

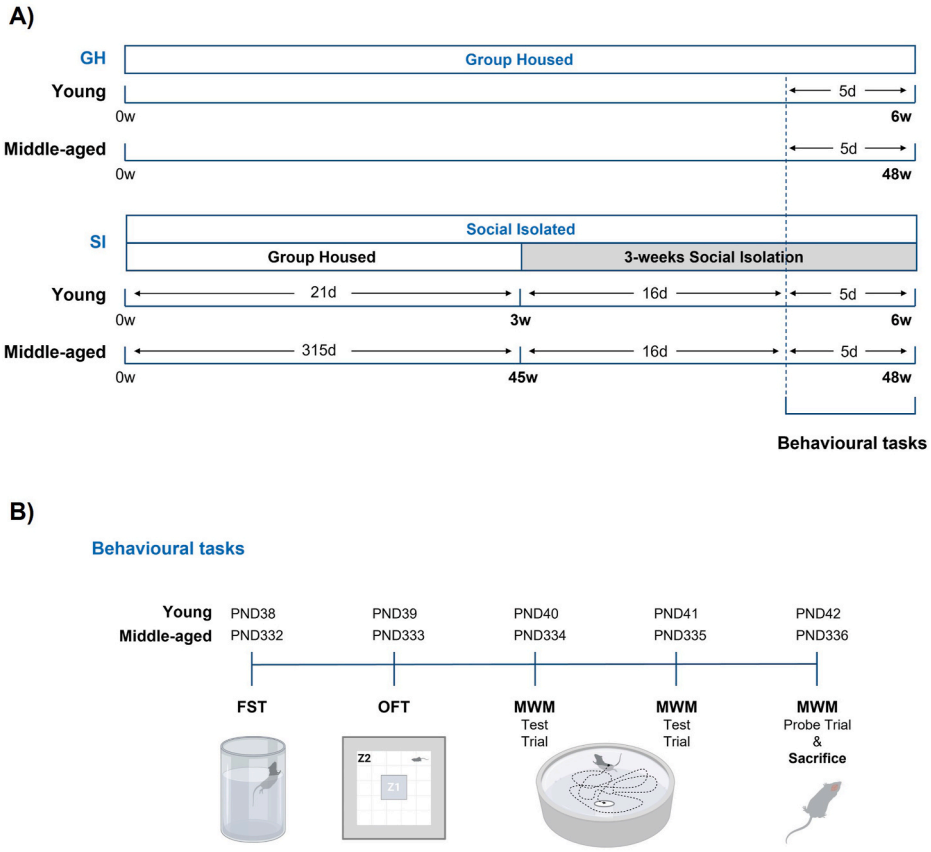

Fig. 1. Experimental methodology. Three-weeks old (Young) and forty-five-weeks old (Middle-aged) C57BL/6J male mice were used in this study. **A)** Mice were either group housed (GH) or subjected to a 3-weeks social isolation protocol (SI). Social isolation occurred from 3 to 6 weeks of age for Young mice and from 45 to 48 weeks of age for Middle-aged mice. **B)** Behavioural tasks started at PND38 and PND332 for Young mice and Middle-aged mice, respectively. Mice were sacrificed on the last day of behaviour. d, days; PND, postnatal days; w, weeks; FST, Forced Swim Test; OFT, Open Field Test; MWM, Morris Water Maze.

behavioural arena and placed in individual cages for 30 min. Mice were then placed individually in the central region of the arena and allowed to explore for 10 min while being recorded with a video camera. The apparatus was cleaned after each test. The ratio of time spent exploring the inner versus outer zone (Z1/Z2) and the immobility time were calculated to address anxiety-like behaviour. Locomotor and exploratory activities were also evaluated by calculating the distance travelled and average speed. The analyses were conducted offline using Any-Maze video tracking software (Stoelting Co, USA).

2.3.4. Morris Water Maze (MWM)

The Morris Water Maze task was performed, according to the experimental timeline represented in Fig. 1, to assess spatial learning and memory. The water maze apparatus consisted of a circular pool (50 cm in diameter and 16 cm in height) with water at a depth of 8.5 cm and a temperature of 25 ± 3 °C. A hidden circular platform (4 cm diameter) constructed from transparent polyvinyl plastic was placed 1.8 cm away from the outer wall in one of the four quadrants of the pool and it was submerged 0.2 cm below the water surface to hide it from the mouse's field of view. The hidden platform remained in the same position throughout the entire learning task. The spatial learning task consisted of two days of training, with 4 trials per animal each day, designed to allow the mice to learn the location of the hidden platform. Surrounding the pool, four different visual cues were placed to help guide the mice to the correct quadrant where the escape platform was located. Before starting each session, mice were placed inside the room in individual cages for 30 min to allow them to habituate to the surroundings. Mice were then lowered into the water facing the wall at one of three possible quadrant starting positions (the platform quadrant was excluded as a starting position), which were randomized in each trial. Each trial lasted a maximum of 90 sec, and the time required to find the hidden platform, the escape latency, was recorded. Mice that successfully found the escape platform were allowed to remain on it for 30 sec the first time and for 10 sec on the following successful trials. At the end of each trial, mice were dried and placed back into their trial cages during the intertrial interval of 15 min. In the case of an incomplete trial (when the mouse could not locate the platform within 90 sec), the mouse was guided to or placed on the platform for 30 sec to allow it to orientate itself. At the end of the session, mice were placed back into their home cages. Hippocampal-dependent spatial reference memory was assessed through a probe trial conducted the day after the training period. For the probe trial, the platform was removed from the pool and mice were placed into the water for 60 sec and recorded using a video camera. The videos were analysed offline using Any-Maze software. The percentage of time and the number of entries into the platform quadrant were calculated through Any-Maze video tracking software and used to evaluate the spatial memory of young and middle-aged mice, either group-housed or socially isolated.

2.3.5. Western Blot

The first step was the extraction and quantification of total protein from the hippocampus. Tissue homogenization was performed in 250 μL of RIPA buffer (RIPA: 1 M Tris pH 8.0, 5 M NaCl, 0.5 M ethylenediaminetetraacetic acid (EDTA, Sigma, Ronkonkoma, NY, USA), 1% nonyl phenoxypolyethoxylethanol (NP-40, Fluka Biochemika, Buchs, Switzerland) and 10% glycerol (Sigma), supplemented with protease inhibitors (Complete Mini-EDTA free, Sigma) and 1 mM phenylmethysulfonyl fluoride (PMSF, Sigma). The suspension was shaken for 15 min at 4 °C and the insoluble fraction was removed by centrifugation at 13,000 g for 10 min at 4 °C. The supernatant was collected and total protein was quantified using the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) in a 96-well flat bottom plate. Bovine serum albumin (BSA, NZYTech, Lisbon, Portugal) was used to prepare a calibration curve (ranging from 0 to 1 mg/mL). The absorbance was measured at 750 nm in a Microplate Reader TECAN Infinite M200 (TECAN Trading AG, Männedorf, Switzerland).

Samples (40 μg of total protein per lane) were denatured at 95 °C for 10 min. Samples and molecular weight markers (MWM, NZYColour Protein Marker II, NZYTech) were then loaded into a 12% SDS-PAGE gel, electrophoresed at 120 V and electrotransferred to PVDF membranes (Immuno-blot® PVDF Membranes for Protein Blotting, Bio-Rad) at a constant current of 350 mA for 150 min. Following blocking, 1 h with 3% BSA in Tris-Buffered Saline with Tween-20 (TBS-T, 200 mM Tris-HCL pH 7.6, 1.5 M NaCl, 0.1% Tween, Sigma), membranes were incubated with primary antibodies overnight at 4 °C: rabbit polyclonal antibody targeting ionized calcium binding adaptor molecule 1 (Iba-1, 1:500 dilution, Abcam ab108539), mouse antibody targeting Cluster of Differentiation 68 (CD68, 1:500, Abcam ab31630), rabbit polyclonal antibody targeting Glial Fibrillary Acidic Protein (GFAP, 1:5000, Sigma G9269), rabbit polyclonal antibody targeting NLRP3 (1:300, Abcam ab214185), rabbit antibody targeting apoptosis-associated Speck-like protein containing a CARD domain (ASC, 1:1000, Adipogen AG-25B-0006), and mouse antibody targeting Caspase-1 (1:500, Santa Cruz sc-56036). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was identified with a mouse monoclonal antibody (1:5000, Ambion AM4300) and used as the housekeeping gene. Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000, Bio-Rad) were incubated for 1 h at room temperature. Immunoreactions were visualized using an ECL Western blotting detection system (PerkinElmer, Waltham, MA, USA), and the chemiluminescence signal was captured on a ChemiDoc XRS^{+} system (Bio-Rad). The integrated intensity of each band was quantified using computer-assisted densitometry analysis with ImageJ software 1.44b. The protein band intensity was normalized to that of GAPDH.

2.3.6. ELISA

Hippocampal lysates were obtained and quantified as described above. Protein levels of pro-inflammatory cytokines, such as IL-1β, IL-6 and Tumour Necrosis Factor-alpha (TNF-α), were quantified by ELISA using selective antibodies and according to the manufacturer's suggested protocol (R&D Systems, Abingdon, UK). Absorbances were read at 405 nm. Values were calculated according to a standard curve generated during the experiment. The detection limit was <3 pg/ml.

2.3.7. Statistical analysis

All statistical analyses were determined using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA) and Excel (Microsoft). Data were expressed as the mean ± standard error of the mean (SEM) and normal distribution for every condition was examined using the Shapiro Wilk normality test. ROUT method with Q = 1% was used to identify outliers. Data were analysed using two-way analysis of variance (ANOVA), with the housing conditions (group housed or socially isolated) and ageing (young and middle-aged) as between-group variables. Šídák's was chosen as the multiple comparison test, comparing the data withing each age and between the same housing condition. Statistical significance was considered when the p *value* < 0.05.

3. Results

3.1. Social isolation increases depressive-like behaviour in middle-aged mice

Motivation and depression-like behaviour were evaluated through the FST. The total time each mouse spent immobile (not swimming or climbing) was calculated since it is thought to be a reliable measure of despair (Yankelevitch-Yahav et al., 2015). Young group-housed (GH) mice, young socially isolated (SI) mice, and middle-aged GH mice spent a similar portion of the total time immobile (Fig. 2A). On the other hand, middle-aged SI mice spent significantly more time immobile than middle-aged GH mice (middle-aged SI: 130.5 ± 6.8 vs middle-aged GH: 77.4 ± 9.3 s, $p < 0.05$) and young SI mice (56.1 ± 20.3, $p < 0.01$). This suggests that middle-aged socially isolated mice exhibited more

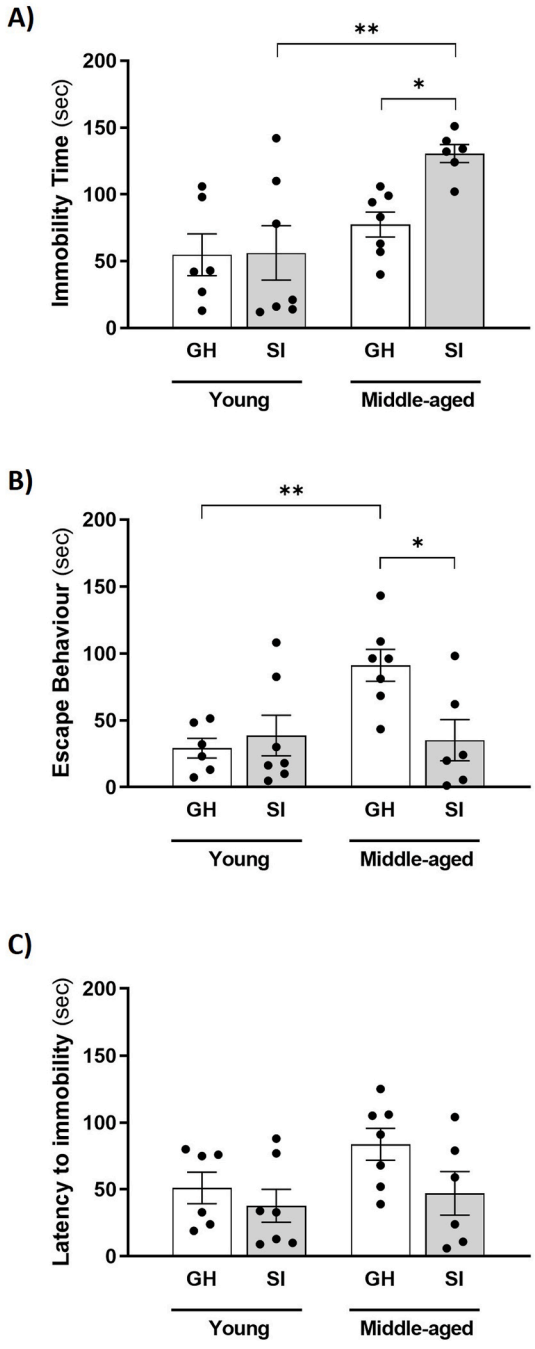

Fig. 2. Social isolation increases depressive-like behaviour in middle-aged mice. A) Immobility time, **B)** Escape behaviour and **C)** Latency to immobility in Forced Swim Test were evaluated to assess depressive-like behaviour in Young and Middle-aged C57BL/6J male mice, either group housed (GH) or socially isolated (SI). All values are mean ± SEM. N = 6–7 animals per group. Statistical tests were performed with two-way ANOVA, followed by Šídák's multiple comparison test, with *p < 0.05 and **p < 0.01. Statistical difference between groups is indicated by the connecting lines above the bars.

depressive-like behaviour than group-housed mice. Social isolation had no impact on the escape-like behaviour of young mice. Interestingly, middle-aged GH mice spent significantly more time climbing and attempting to escape the forced swim apparatus than young GH mice (middle-aged GH: 91.0 ± 11.9 vs young GH: 29.2 ± 7.4 s, p < 0.01, Fig. 2B), suggesting that middle-aged GH mice were more motivated to find an exit from the apparatus than young GH mice. The middle-aged SI mice, however, spent significantly less time on escape-like behaviour than their group-housed counterparts (middle-aged SI: 35.16 ± 15.3 s, p < 0.05), indicating a hopeless or depressive-like phenotype. There were no significant differences in the latency to immobility time between the four groups of mice (Fig. 2C).

3.2. Social isolation increases anxiety-like behaviour in middle-aged mice

The anxiety levels of mice were assessed using the open-field paradigm. We monitored how mice moved throughout the whole arena (Fig. 3A) and calculated the ratio of time spent by each mouse in the centre of the box (Zone 1) compared to the time spent along the edges and corners (Zone 2). No differences were obtained in the time young SI mice spent in the centre, compared to their GH counterparts. However, middle-aged SI mice spent significantly less time in the centre than the GH age-matched group (middle-aged GH: 0.124 ± 0.016 vs middle-aged SI: 0.065 ± 0.016, p < 0.05; Fig. 3B), suggesting an anxiety-like behaviour induced by social isolation. The young mice spent significantly less time immobile (Fig. 3C), travelled longer distances (Fig. 3D), walked at higher speeds (Fig. 3E), and reared on their hind legs more often (Fig. 3F) than the aged groups of mice. In mice of both ages, social isolation did not have a significant effect on time spent immobile, distance travelled or average speed. Similarly, social isolation did not impact rearing, nor the length of time mice spent grooming.

3.3. Social isolation impaired spatial learning in middle-aged mice

The day following the open field test, mice were trained in the Morris water maze spatial learning task. On day 2 of the water maze training, all four groups of mice learned the location of the hidden platform (Fig. 4A). Day 3 of the water maze task was the probe trial, and the platform was removed from the pool. Fig. 4B depicts representative swim paths of each group of mice in the probe trial. All four groups of mice spent significantly longer in the platform quadrant compared to the quadrant directly opposite (Fig. 4C). However, the middle-aged SI mice spent significantly less time in the platform quadrant than their GH counterparts (middle-aged SI: 30.5 ± 2.8 vs middle-aged GH: 41.9 ± 3.7 s, p < 0.05). This result is supported by the observation that middle-aged SI mice entered the platform quadrant significantly fewer times than aged GH mice in the probe trial (middle-aged SI: 7.7 ± 0.9 s vs middle-aged GH: 13.7 ± 1.3, p < 0.05) (Fig. 4D). Altogether, the results suggest that social isolation in middle-aged mice may impair spatial memory formation.

3.4. Ageing and social isolation upregulate microglia reactivity in the hippocampus

Two hours after the final probe trial in the Morris water maze task, mice were sacrificed, and the hippocampus was removed and snap-frozen. Protein lysates were used to address glial cell reactivity in the hippocampus. There were no changes in GFAP levels in response to ageing or social isolation (Fig. 5A). Similarly, ageing did not significantly increase the levels of Iba-1, a marker of microglia cells. Regarding the impact of social isolation, an increase in Iba-1 expression was observed in middle-aged SI mice compared with their GH counterparts (middle-aged SI: 0.52 ± 0.03 vs middle-aged GH: 0.29 ± 0.06, p < 0.01), but no significant difference was observed in young mice (Fig. 5B). The levels of CD68, expressed by reactive phagocytic microglia (Jurga et al., 2020), were higher in the hippocampus of middle-aged mice than in that of young mice (young GH: 0.60 ± 0.06 vs middle-aged GH: 1.13 ± 0.13, p < 0.01 and young SI: 0.58 ± 0.06 vs middle-aged SI: 0.97 ± 0.13, p < 0.05).

3.5. Social isolation increases NLRP3 inflammasome priming in the hippocampus

Next, we investigated the levels of NLRP3 domains in response to

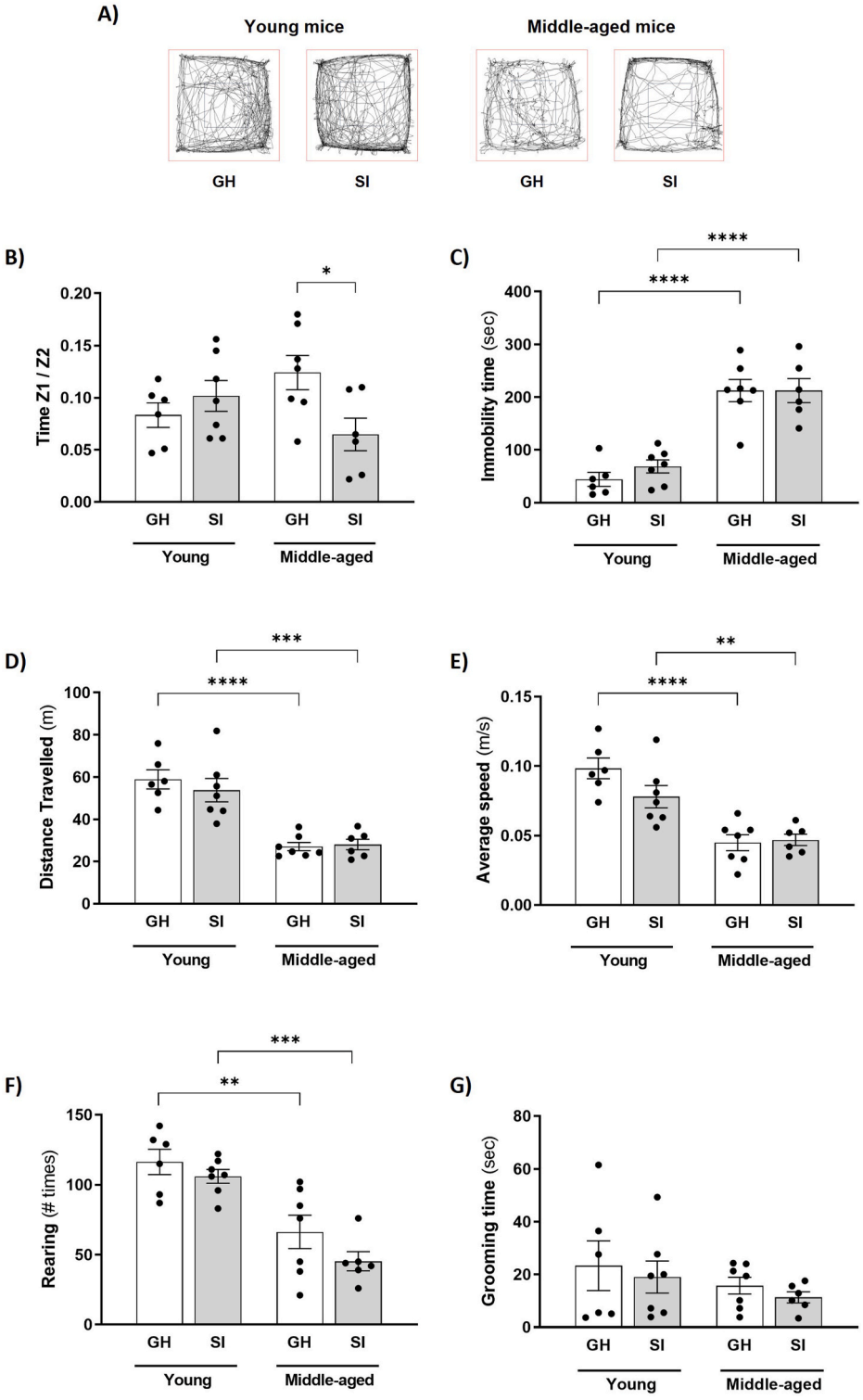

Fig. 3. Social isolation increases anxiety-like behaviour in middle-aged mice. Open Field Test was performed in Young and Middle-aged C57BL/6J male mice, either group housed (GH) or socially isolated (SI). **A)** Representative motion paths for each group of mice. The behavioural parameters used to address anxiety-like behaviour were **B)** Ratio of time spent in the centre zone (Z1) versus the periphery zone (Z2), **C)** Immobility time, **D)** Distance travelled and **E)** Average speed of mice. Other parameters, such as **F)** Rearing and **G)** Grooming were also assessed. All values are mean ± SEM. N = 6–7 animals per group. Statistical tests were performed with two-way ANOVA, followed by Šídák's multiple comparison test, with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$. Statistical difference between groups is indicated by the connecting lines above the bars.

ageing and social isolation in the hippocampus. Social isolation induced an increase in NLRP3 protein levels in both young (young GH: 0.44 ± 0.10 vs young SI: 0.65 ± 0.05, $p < 0.05$) and older animals (middle-aged GH: 0.30 ± 0.04 vs middle-aged SI: 0.52 ± 0.03, $p < 0.05$) (Fig. 6A). The findings indicate that in our model NLRP3 priming is related to social isolation-induced stress, rather than to age. There were no changes in the levels of the adapter protein ASC (Fig. 6B), nor in mature Caspase-1 (Fig. 6C). Given that mature Caspase-1 is the effector protein of NLRP3

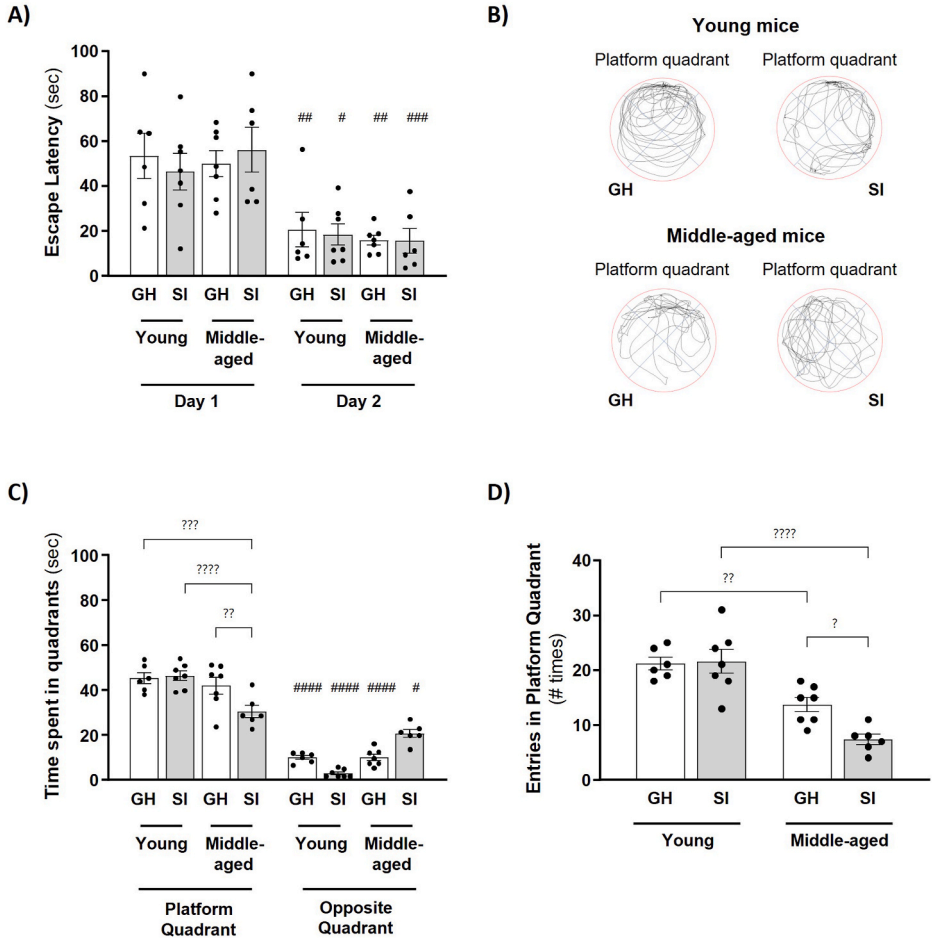

Fig. 4. Social isolation impairs spatial memory and learning in middle-aged mice. Morris Water Maze was performed in Young and Middle-aged C57BL/6J male mice, either group housed (GH) or socially isolated (SI). Four test trials were performed for 2 days. Probe trial was performed on the day following the test trial. **A)** Mean time to find the hidden platform calculated for each training session. **B)** Representative swim paths for each group of mice in the probe trial. **C)** Mean time spent in platform quadrant and opposite quadrant and **D)** Number of entries in platform quadrant calculated during the probe trial. All values are mean ± SEM. N = 6–7 animals per group. Statistical tests were performed with two-way ANOVA, followed by Šídák's multiple comparison test, with $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{***}p < 0.0001$ for comparisons between all four groups of animals (Young and Middle-aged, either group housed (GH) or socially isolated (SI), and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ and $^{\#\#\#\#}p < 0.001$ for comparisons between the two test days (Day 1 vs Day 2; Fig. 4A) or Quadrants (Platform Quadrant vs Opposite Quadrant; Fig. 4C) within the same group. Statistical difference between groups is indicated by the connecting lines above the bars.

activation, the results point to a limited activation of this inflammasome in response to ageing and social isolation-mediated cellular stress in the hippocampus.

3.6. Ageing, but not social isolation, increases the expression of proinflammatory cytokines in the hippocampus

Using ELISA, the levels of the main proinflammatory cytokines, namely IL-1β, TNF-α and IL-6, were measured in hippocampal tissue in response to ageing and social isolation. IL-1β protein levels in the hippocampus were significantly higher in aged mice (young GH: 1449.3 ± 445.7 vs middle-aged GH: 6199.7 ± 979.2 pg/mg total protein, $p < 0.0001$ and young SI: 2050.2 ± 249.8 vs middle-aged SI: 5195.78 ± 595.5 pg/mg, $p < 0.01$) (Fig. 7A). TNF-α levels were significantly elevated in older SI mice compared to young (middle-aged SI: 699.13 ± 98.0 vs young SI: 317.6 ± 68.5 pg/mg $p < 0.01$) (Fig. 7B). IL-6 concentration also showed an increase in middle-aged groups compared to young mice (young GH: 166.8 ± 27.4 vs middle-aged GH: 1070.4 ± 189.7 pg/mg, $p < 0.001$ and young SI: 214.4 ± 38.4 vs middle-aged SI: 757.8 ± 125.6 pg/mg, $p < 0.05$) (Fig. 7C). Taken together, the results suggest that ageing, but not social isolation, increases the levels of pro-inflammatory cytokines in the hippocampus, potentially reflecting a state of 'inflammaging' in older mice that impacts motivation, anxiety- and depressive-like behaviour.

4. Discussion

Using rodent models to decipher the detrimental effects of stress on behaviour, memory performance and cognitive flexibility is influenced by the nature of the stressor, its duration (acute, intermittent or chronic), and the age (and sex) of the animals subjected to a specific stress paradigm (Paulus et al., 2000; Arakawa, 2003; Lukkes et al., 2009; Stepanichev et al., 2014; Koert et al., 2021).

In this work we used social isolation, which is considered a chronic mild stressor, within a short period (3 weeks). Protocols in the literature usually last between 3 and 8 weeks (Walker et al., 2019; Bendersky et al., 2021), although shorter and longer durations have also been documented (Stepanichev et al., 2014; Wang et al., 2019; Panossian et al., 2020). Importantly, the nature of the stressor, the length of time the animal experiences the stressor, and the life-stage of the animal when the stress occurs can influence how the animal reacts. Acute (hours) stress experienced at a very young age (e.g. maternal separation), for example, can induce long-lasting detrimental effects on rodent behaviour in adulthood (Batalha et al., 2013; Sousa et al., 2014; Cheng

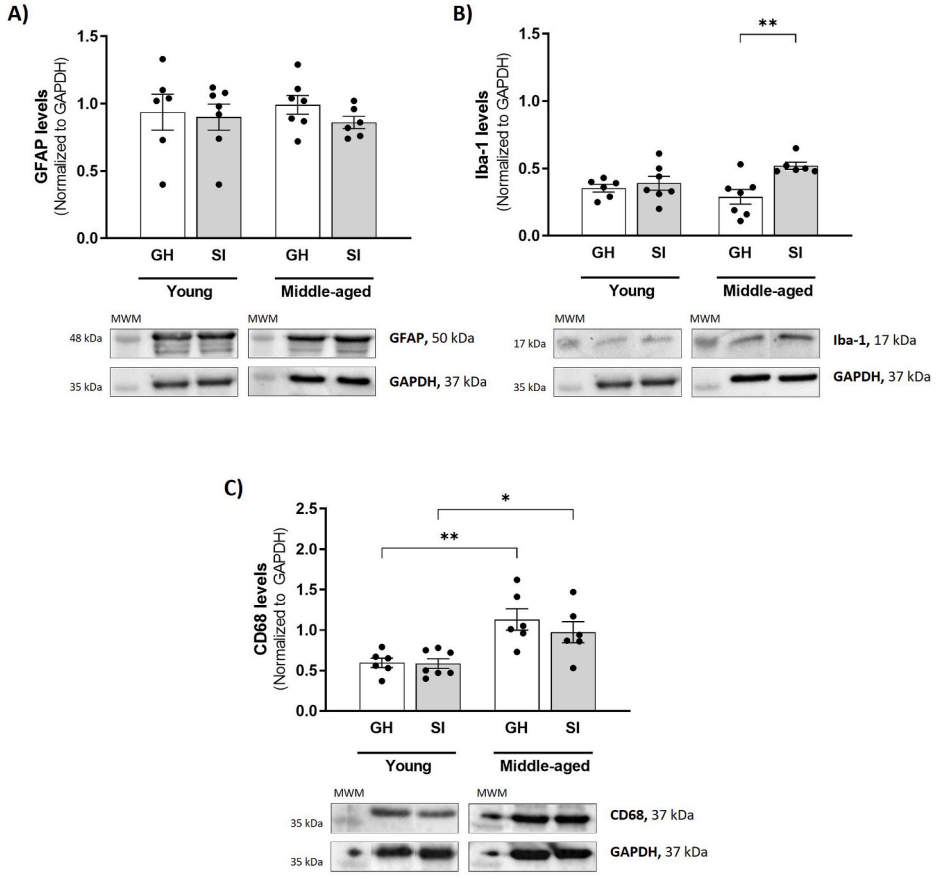

Fig. 5. Ageing and social isolation increase markers of reactive gliosis in the mouse hippocampus. Representative immunoblots and densitometry analysis of **A)** GFAP, **B)** Iba-1 and **C)** CD68 were assessed through Western Blot in hippocampal lysates obtained from Young and Middle-aged C57BL/6J male mice, either group housed (GH) or socially isolated (SI). All values are mean ± SEM. N = 6–8 animals per group. Statistical tests were performed with two-way ANOVA, followed by Šídák's multiple comparison test, with *p < 0.05 and **p < 0.01. Statistical difference between groups is indicated by the connecting lines above the bars.

et al., 2018). However, chronic restraint stress experienced at the same time of day, every day, for a long period, e.g. 4 weeks, can become predictable and almost routine and some resilient rodents can adapt to their stressful living conditions (Zhu et al., 2014). Therefore, the length of the social isolation protocol, as well as the life stage when the behaviour tests are performed, can significantly influence the behavioural responses of animals (Lu et al., 2003; Chen et al., 2016; Begni et al., 2020).

Indeed, when designing chronic stress paradigms for rodents, one must bear in mind that mice only live for approximately 2 years and that their perception of time may be quite different from humans, i.e. a 3-week period may seem much longer to a mouse than to a human (Flurkey et al., 2007; Burke et al., 2017). Different perception of time can also complicate within-species studies (such as the present study) that aim to investigate the impact of stress in "young versus middle-aged" individuals. Indeed, as children often perceive time differently from adults (Stojić et al., 2023), 3 weeks may be perceived as a longer period to young mice when compared to older mice.

We socially isolated young mice immediately after weaning and middle-aged male mice (45 weeks) for a 3-week period, starting the behavioural tests on the last five days of the isolation period (Fig. 1). Young mice were weaned on postnatal day 21 (equating to approx. 5 years of age in human development) and were culled at 6 weeks old (approx. 15 years of age in 'human' years), whereas 45-week-old mice (approx. 40 years of age in human years) were culled at 48 weeks of age. Rodents are known to go through an accelerated developmental period between 0 and 6 weeks of age and are considered middle-aged at approximately 10–12 months (Flurkey et al., 2007). If we assume that mice have a 2-year lifespan and that humans live to 80 years of age (i.e. 40 times longer), some researchers would argue that, in relative terms, a 3-week period of social isolation for middle-aged mice is equivalent to around 2½ years of isolation for a middle-aged human. It is also possible, therefore, that the young mice can adapt to the 3-week isolation better than middle-aged mice because the older mice had spent a higher proportion of their lives group-housed.

Studies have shown that 2–3 weeks of isolation can trigger behavioural patterns including increased anxiety-like symptoms (Oshima et al., 2018; Acero-Castillo et al., 2021) and development of depressive-like behaviour (Chan et al., 2017; Todorović and Filipović, 2017; Panossian et al., 2020; Stanisavljević et al., 2020), impairments in cognitive tasks (Famitafreshi et al., 2015; Chen et al., 2016; An et al., 2017), changes in social interaction (Lander et al., 2017; Bicks et al., 2020; Kinley et al., 2021), aggressive behaviour (An et al., 2017; Zelikowsky et al., 2018), alterations in neurotrophin levels (Han et al., 2011; Famitafreshi et al., 2015; Li et al., 2016), changes in neurotransmission systems (Heidbreder et al., 2000; Panossian et al., 2020), differences in neural cell adhesion molecules (Djordjevic et al., 2012), neurogenesis impairment (Famitafreshi et al., 2015; Chan et al., 2017; Cho et al., 2017), and hypomyelination (Liu et al., 2012a; Makinodan et al., 2012). Moreover, evidence suggests that stress during critical windows of brain development and maturation can have lasting effects on neuronal and glial cell structure and function, as well as behaviour in adulthood (Murphy et al., 2010; Cassidy et al., 2010; Liu et al., 2012b).

There is a large amount of literature studying the effects of different types of stressors in old age (Luine et al., 2007; Ricon et al., 2012; Buechel et al., 2014; Badowska-Szalewska et al., 2017; Hargis et al.,

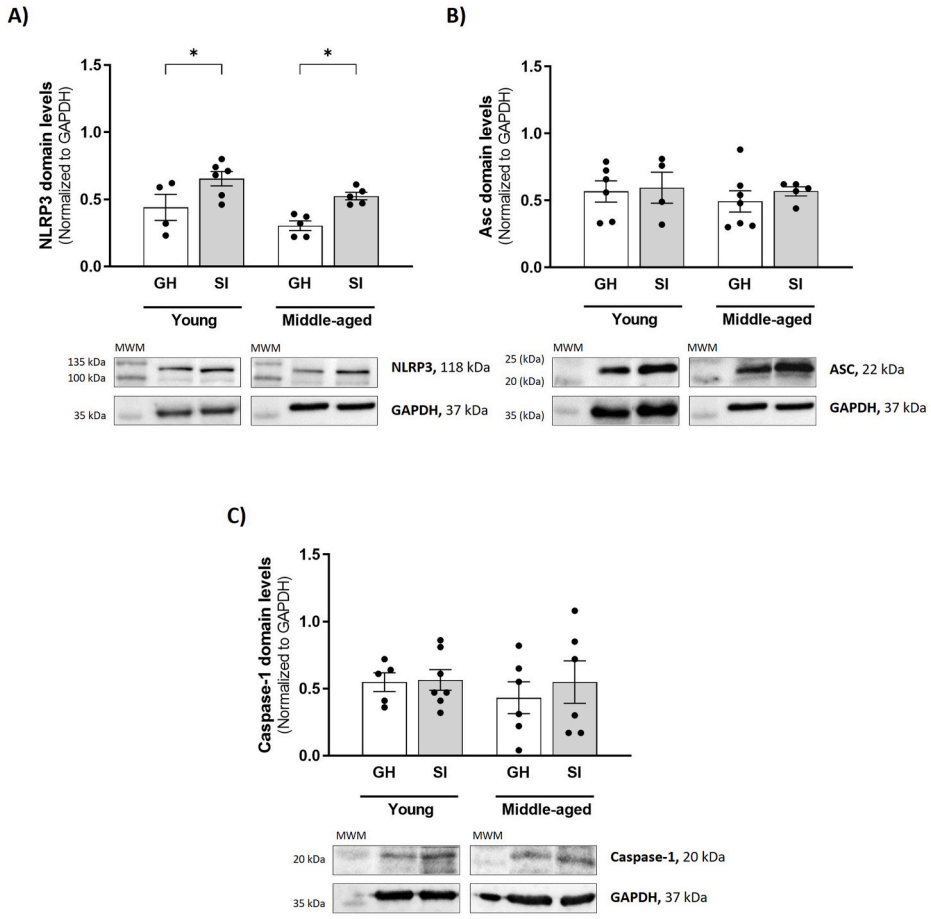

Fig. 6. Social isolation upregulates NLRP3 domain in the mouse hippocampus. The protein levels of NLRP3 inflammasome domains: **A)** NLRP3, **B)** ASC, and **C)** Caspase-1 were assessed through Western Blot in hippocampal lysates obtained from Young and Middle-aged C57BL/6J male mice, either group housed (GH) or socially isolated (SI). All values are mean ± SEM. N = 4–8 animals per group. Statistical tests were performed with two-way ANOVA, followed by Šídák's multiple comparison test, with *p < 0.05. Statistical difference between groups is indicated by the connecting lines above the bars.

2018; Lotan et al., 2018). Most of these studies are based on the hypothesis that, as we reach old age, our cognitive reserve declines, and we may be more susceptible to the damaging effects of chronic mild stress (Peavy et al., 2009; McEwen and Morrison, 2013; Liu et al., 2022). The impact of social isolation in middle-aged rodents has scarcely been investigated because of the general assumption that mature adults are the least susceptible age group to chronic mild stress. However, data on suicide rates in the UK and EU suggest that middle-aged males (45–49 years old) are more likely to take their own life than at any other stage in life (Snowdon 2021; NCISH, 2021). With these caveats in mind, the aim of this study was to compare the effects of social isolation in very young versus middle-aged male mice, focusing on behavioural readouts of anxiety- and depression-like behaviour, hippocampal-dependent memory performance, as well as some biomarkers of neuroinflammation in the hippocampus. The hippocampus is indeed one of the most stress-responsive brain areas (McEwen, 2008) and alterations in this region were suggested as a common feature of anxiety and depression (Cha et al., 2016; Chen et al., 2019).

Here, we report that 3 weeks of social isolation-mediated stress leads to depressive-like behaviour in the forced swim test (Fig. 2A), anxiety-like behaviour in the open field test (Fig. 3B), and spatial memory impairment in the Morris water maze paradigm (Fig. 4C) in middle-aged male mice. Young male mice appeared more resilient to the detrimental impact of chronic isolation-mediated stress. The observation that middle-aged male mice displayed more anxiety- and depressive-like behaviour than young male mice suggests that the fully mature adult brain may be more vulnerable to isolation-mediated stress than the young plastic brain which is still undergoing maturation processes (McEwen and Morrison, 2013; Zannas, 2018).

Another key aspect was the levels of neuroinflammatory mediators measured in the middle-aged hippocampus (Figs. 5–7) which may lead to a less resilient phenotype to chronic mild stressors (Calcia et al., 2016; Liu et al., 2017b; Fonken et al., 2018). As in middle-aged mice, young SI mice demonstrated elevated levels of the NLRP3 inflammasome domain compared to young GH controls, suggestive of NLRP3 priming (Fig. 6A), but to an extent that did not translate into significant changes in behaviour, at least in the tests performed. Since NLRP3 levels increase in both age groups, these results suggest a social isolation effect independent of age. Middle-aged GH mice also demonstrated higher hippocampal levels of CD68 protein (Fig. 5C) and higher concentrations of IL-1β and IL-6 pro-inflammatory cytokines than young GH mice (Fig. 7). No concomitant ageing-associated increases were observed in astrocyte reactivity, since the levels of GFAP remained constant across all groups of mice (Fig. 5A). Ageing-induced microglial activation and enhancement of proinflammatory cytokines are two well-studied phenomena considered to be key drivers of depression and stress-related cognitive impairment (Hinwood et al., 2012; Wohleb, 2016; Fulop et al., 2018; Lin et al., 2018; Wang et al., 2018a; Munshi et al., 2020; Barter et al., 2021; Li et al., 2022; Schramm and Waisman, 2022). Our data suggest that microglia priming in middle-age may be a susceptibility factor for social isolation-induced cognitive decline (Griffin et al., 2006; Buchanan et al., 2008; Niraula et al., 2017; Niu et al., 2020; Sanguino-Gómez et al., 2021). Interestingly, middle-aged mice did not display any problems in learning the location of the hidden platform in the water maze task, but

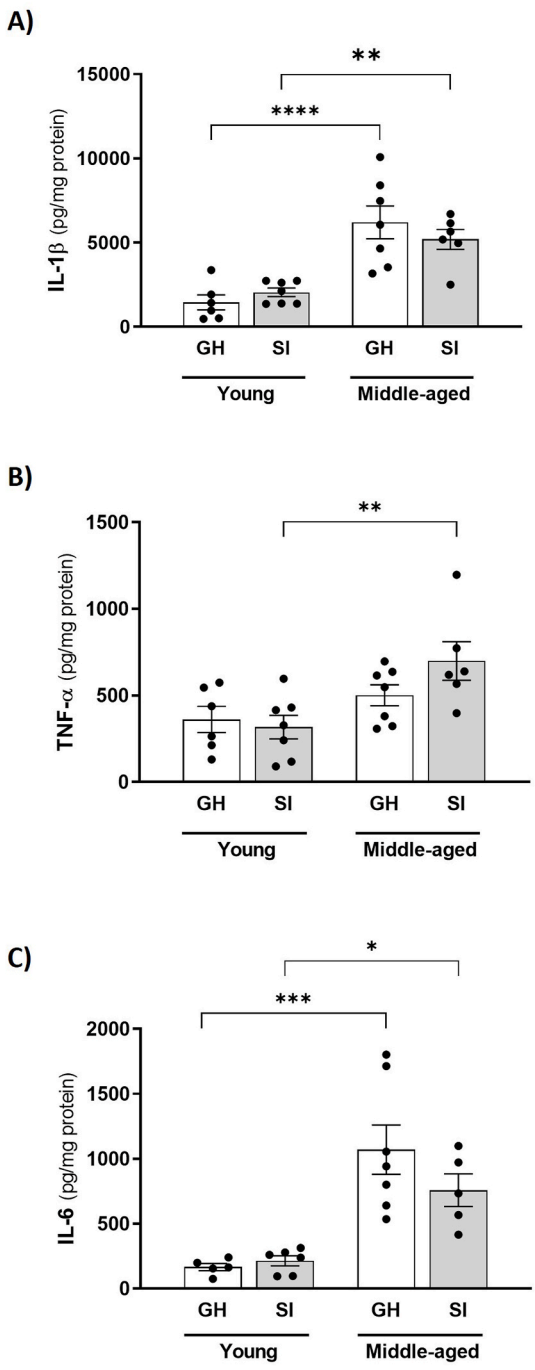

Fig. 7. Ageing increases the levels of pro-inflammatory cytokines in the mouse hippocampus. The protein levels of **A)** IL-1β, **B)** TNF-α and **C)** IL-6 were evaluated through ELISA in hippocampal tissue from Young and Middle-aged C57BL/6J male mice, either group housed (GH) or socially isolated (SI). All values are mean ± SEM. N = 5–8 animals per group. Statistical tests were performed with two-way ANOVA, followed by Šídák's multiple comparison test, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. Statistical difference between groups is indicated by the connecting lines above the bars.

did display problems with memory consolidation and memory recall in the probe trial (Fig. 4). There is a wealth of literature on the role of cytokines in learning and memory-related synaptic plasticity in the hippocampus (Levin and Godukhin, 2017; Bourgognon and Cavanagh, 2020) and, in general, high levels of IL-1β have been shown to inhibit long-term potentiation (Suri et al., 2000; Kelly et al., 2001; Liu et al., 2012a) and lead to poor memory recall in spatial learning tasks in rodents (Gahtan and Overmier, 2001; Goshen et al., 2007; Chen et al., 2008).

The more striking result from our data was that young 3-weeks-old male mice demonstrated stronger resilience to 3 weeks of social isolation-mediated stress than middle-aged male mice, which on average appeared more anxious, depressed and displayed memory deficits in response to isolation. This was somewhat surprising because the 3-week time-frame post-weaning has been well documented in the literature as a key period of young brain maturation and numerous studies have shown that different types of stressors endured during this 'critical window' of neuronal and glial cell maturation can lead to behavioural and cognitive deficits in adulthood (Tsoory et al., 2006; Pohl et al., 2007; Murphy et al., 2010; Wilkin et al., 2012; Stepanichev et al., 2014; Wang and Schmidt, 2016; Liu et al., 2017a). The key difference between our study and many others that investigate the effects of stress in young mice (Ibi et al., 2008; Hermes et al., 2011; Pisu et al., 2011; Evans et al., 2012; Chmelova et al., 2019; Kim et al., 2021) is that we did not wait for the mice to reach adulthood, but instead examined the behaviour of mice immediately after the isolation period at 5–6 weeks of age. It is possible that the impact of isolation on cognition, behaviour and neuroinflammatory mediators is delayed and may take several weeks to manifest in male mice, up to early adulthood at 9–12 weeks of age (Weintraub et al., 2010; Shao et al., 2013).

It is also important to state some limitations of this study. Although a growing body of evidence demonstrated that sex affects stress-related effects (Burke et al., 2016), we focused on male mice, as males seem to be more susceptible to social isolation outcomes (Ferdman et al., 2007; Ahern et al., 2016). Moreover, the oestrous cycle elicits alterations in the way females endure stress, which could be a variable in the study (Starkey et al., 2007).

Furthermore, this study evaluated alterations in the whole hippocampal region, but it is known that the ventral and dorsal regions of the hippocampus are ascribed to hold different functions. Ventral hippocampus is associated with emotions, while the dorsal one is responsible for cognitive functions (Fanselow and Dong, 2010). Thus, during a social-psychological stressor such as social isolation, the ventral hippocampus is responsible for mediating the behaviours assessed in FST and OFT. Interestingly, a decrease in hippocampal long-term potentiation within the ventral region has been shown (Almonte et al., 2017). On the other hand, the dorsal side could account for the social isolation-induced spatial memory impairment.

Also, a plethora of behaviour assessments are nowadays available. It is legitimate to say that other behaviour tests could have been used to evaluate anxiety- and depression-like behaviours, such as the elevated plus maze (Bailey and Crawley, 2009) or the sucrose test (Castagné et al., 2009). The inclusion of additional memory tests and social interaction tests could also have been interesting. But the behaviour tasks are stressful and could influence the study. Therefore, the number of behaviour tasks was rationalized, taking as much information as possible from each one, and all procedures were fully normalized among groups to counteract that possibility.

5. Conclusion

Our study highlights that social isolation, a chronic mild stressor, may exert detrimental effects on cognition and behaviour in middle-aged male mice through molecular mechanisms that involve microglia and neuroinflammatory signalling pathways, which will add to the impact of a systemic state of low-grade inflammation present in middle-age. This raises questions about whether more resources should be aimed at trying to prevent mental health problems arising in middle-aged men who are isolated or live alone. Further research is required to determine if this demographic range of the human population is particularly vulnerable to the maladaptive psychological and cognitive disturbances that can occur following prolonged periods of social withdrawal, such as the recent global COVID-19 pandemic.

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CRediT authorship contribution statement

Daniela M. Magalhães: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Myrthe Mampay:** Investigation. **Ana M. Sebastião:** Funding acquisition, Resources, Writing – review & editing. **Graham K. Sheridan:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. **Cláudia A. Valente:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare no conflicts of interest and no competing financial interests.

Data availability

Data will be made available on request.

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