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Strain differences in behaviour and immunity in aged mice: Relevance to Autism

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ABSTRACT

The BTBR mouse model has been shown to be associated with deficits in social interaction and a pronounced engagement in repetitive behaviours. Autism spectrum disorder (ASD) is the most prevalent neurodevelopmental condition globally. Despite its ubiquity, most research into the disorder remains focused on childhood, with studies in adulthood and old age relatively rare. To this end, we explored the differences in behaviour and immune function in an aged BTBR T + Itpr3tf/J mouse model of the disease compared to a similarly aged C57bl/6 control. We show that while many of the alterations in behaviour that are observed in young animals are maintained (repetitive behaviours, antidepressant-sensitive behaviours, social deficits & cognition) there are more nuanced effects in terms of anxiety in older animals of the BTBR strain compared to C57bl/6 controls. Furthermore, BTBR animals also exhibit an activated T-cell system. As such, these results represent confirmation that ASD-associated behavioural deficits are maintained in ageing, and that that there may be need for differential interventional approaches to counter these impairments, potentially through targeting the immune system.

1. Introduction

The BTBR T + tf/J (Black and Tan Brachyury, BTBR) mouse is an inbred mouse strain which shows behavioural phenotypes comparable to the core symptoms of autism spectrum disorder (ASD). While not a model of an autism-associated genotype *per se*, the BTBR mouse is widely used given the pronounced deficits in social interaction and enhanced display of repetitive behaviours observed [1–5]. Much of the BTBR phenotype is driven by several genetic and epigenetic disruptions in multiple brain regions. These include disruptions in an enzyme regulating the metabolism of glutamate agonist kynurenic acid, leading to alterations in synaptic signalling [6]. Expression of the plasticity-related protein, Bdnf in the hippocampus is also down-regulated compared to C57BL/6 controls [7] in addition to alterations in serotonin [8] and cannabinoid receptors [9]. Structural alterations are present in numerous brain areas in these animals [10], with perhaps the most striking difference being the lack of a corpus callosum [11]. Furthermore, impaired neurogenesis, impaired axon guidance and an imbalance in neurotransmission [12] are observed in this strain. Along with changes in behaviour, physiology and brain structure the BTBR mouse has variations in specific bacterial taxa compared with the pro-social C57/Bl6 mouse [13,14], furthermore, BTBR mice display delayed intestinal transit and intestinal barrier dysfunction [3], symptoms similar to those seen in humans with ASD.

ASD is a life-long developmental disability characterized by social impairments, communication deficits, restricted interests and repetitive behaviours, as well as cognitive deficits, each of which are heterogeneously expressed throughout the disorder [15]. Estimates suggest that one in 132 individuals are affected by ASD [16]. The aetiology of ASD is not completely known but believed to involve both genetic and environmental factors. Genetic factors influencing development of the disorder include de-novo mutations, short nucleotide polymorphisms and common genetic variations that occur across its many incidents [17–19]. One aspect that has been suggested to play a role in the development of ASD is the immune system [20]. Prenatal immune activation has been shown to lead to an increase in autism-like symptoms both clinically [21] and in animals [22,23]. Postnatally, immune dysregulation and inflammation have been correlated with prevalence of ASD [24].

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Post-mortem tissue from ASD patients has shown both increases in activation of astrocytes and microglia, as well as increases in levels of proinflammatory cytokines in the prefrontal cortex [25]. Furthermore, genes linked to ASD that encode for immune system features are mutated in the disorder, leading to disruptions in structural and functional connectivity in areas of the brain key for socio-communicative function [26,27].

While almost all aspects of ASD have been studied in detail, an area that has not been the focus of much attention is the impact of the disorder in older individuals and in later life. With an ageing population, increasing numbers of ASD individuals are reaching old age. Provision of care for this population has been neglected and is only beginning to be addressed in a few small areas in Europe and the USA [28]. Of particular concern is the period during which individuals with ASD transition from parental care to a time during which this may no longer be possible, and the effects on cognition that may come with such a change [28]. While neuroimaging, pharmacological and pathological studies abound in individuals with ASD, to date they have been confined to children and adolescents (with a handful making assessments in middle age). As such, the impact of critical age-related physiological changes in the condition has been largely unstudied.

The United Nations defines 'older persons' as those over 60 years of age, corresponding to an age of approximately 20 months in a rodent model [29]. Behavioral alterations, as well as the prevalence of neurogenerative disorders are also known to be increased in an ageing population [30]. The underlying mechanisms including immune and inflammatory disturbances as well as metabolic alterations are only beginning to be understood and are explaining the age-related increases in the prevalence of metabolic and neurodegenerative disorders [31]. As changes in metabolism and cognitive alterations are commonplace in individuals with ASD there is a very strong case to be made to increase the knowledge base of the impact of ageing on these individuals.

The BTBR model is sensitive to environmental influences, such as dietary interventions [32,33]. Juvenile mice administered a ketogenic diet showed improvements in sociability, repetitive behaviour, and social behaviour [32]. Since this diet is known to modify gut microbiota composition in children [34] this highlights the potential importance of gut microbiota composition in expression of behavioural symptoms in this model [35], with the influence of the microbiota over behaviour linked to neuroimmune interactions [36]. To date, the behavioural and immune disruptions of the BTBR mouse has not been assessed in ageing. Thus, we aim to assess both of these factors and determine whether there is a greater level of 'inflammaging' (the heightened proinflammatory status and the decline in adaptive immunity progressively seen in older age [37]) compared to an age-matched C57 control group.

2. Materials and methods

2.1. Animals

All animal experiments were approved by the Animal Experimentation Ethics Committee in University college Cork (UCC) and by the Health Products Regulatory Authority (HPRA) of Ireland in accordance with EU directive 2010/63/EU. 8-week-old male BTBR and C57BL/6 mice were obtained from Harlan Laboratories, UK and housed in the animal unit until experiments were carried out at 19–21 months of age. Animals were kept under a strict 12:12-h dark-light cycle and temperature (20 ± 1 °C,55.5 % humidity), with food and water supplied *ad libitum*. Mice were group housed in 3–4 mice per cage.

2.2. Behavioural testing

2.2.1. Defensive marble burying

Defensive marble burying was performed as previously described [3]. Briefly, this test measures repetitive and anxious behaviours, with a greater number of marbles buried representing increasing levels of anxiety. Cleaned cages were lined with a 5-cm layer of chipped cedar-wood bedding. Twenty glass marbles were arranged in an equidistant manner in a 5 × 4 orientation on top of the bedding. Animals were allowed to habituate to the testing room for thirty minutes prior to testing. During the test phase, each mouse was placed in the test cage and allowed to explore for 30 min. At the end of the 30 min, animals were returned to their home cage and the number of marbles buried recorded and photographed. Any marble greater than two thirds covered in bedding was considered to be buried.

2.2.2. Elevated plus maze

The elevated plus maze (EPM) is a commonly-used behavioural test to screen for anxiety-like behaviours [38]. The apparatus is constructed from plexiglass and is arranged into a plus (+) shape with two open and two closed arms (arms are 50 cm in length, 5 cm wide and closed arms have a 15 cm wall surrounding). The apparatus is raised one metre above the ground to increase anxiety in the open arms. The apparatus is separated from the rest of the room using identical white curtains to mitigate for visual clues. The experiment also takes place under red light at defined light intensities. To start the test an animal is placed in the 'hub' at the centre of the maze facing one of the open arms and allowed to explore for five minutes. The apparatus is cleaned with 10 % ethanol after each subject to prevent olfactory clues from the previous mouse. The test is recorded using a video camera placed directly overhead. Scoring of the test assessed the total number of entries to open and closed arms as well as time spent in each. Entries to the open and closed arms were considered when mice place all four paws on the arm.

2.2.3. Three-chamber test

This test for sociability was performed as previously described [39]. The test is undertaken in a rectangular box divided into three chambers (left, right, and with a smaller centre chamber. Chambers are separated by partitions with a small semi-circular opening at the bottom, and the left and right chambers contained a wire mesh cage. The test consists of three ten-minute trials performed consecutively.

1. Habituation: animals can explore the three chambers for ten minutes with mesh cages in left and right chambers being left empty.

2. Sociability: an unfamiliar mouse is placed in one of the mesh cages with an object (plastic rubber duck) placed in the other – again, animals are allowed to explore for ten minutes.

3. Social novelty preference: the object is replaced with a novel animal, while the now familiar animal remains in position – exploration is undertaken for ten minutes.

All conspecific mice were age- and sex-matched, with each box cleaned and lined with fresh bedding between trials. For each of the three stages, behaviour was recorded with an overhead camera and interaction times in each chamber were measured.

2.2.4. Forced-Swim test

The forced-swim test serves as a measure of antidepressant-sensitive behaviours. The test was performed as previously described [40]. In this test mice were gently place in a cylinder containing water (23−25 °C) at a height of 17 cm. Animals were left in the water for 6 min with activity being recorded by a camera positioned overhead. Immobility time was scored for the last 4 of the 6 min. Following removal from the cylinder, animals were dried gently and placed in a separate cage for recovery.

2.2.5. Open field test

Animals are moved to the experimental room and allowed to acclimatize for one hour before behavioural analysis. Following this, animals are placed individually in the centre of an open field box (Perspex sides and base: 32.5cm × 42.7cm) and their spontaneous activity was recorded for five minutes using a camera placed overhead. Animals are returned to their home cage following the experiment, apparatus is cleaned with 10 % ethanol and allowed to dry between experiments. Videos were analysed using the Ethovision (Noldus, USA) software.

Total distance travelled, ambulatory activity, and time spent in the centre, were all measured and analysed. This test has previously been described [39]

2.2.6. Grooming test

The description of this test for repetitive behaviour has previously been described within our lab [3]. Briefly, animals were moved to the experimental room and allowed to acclimatize for one hour before behavioural analysis. Following this, animals are placed individually into clear Perspex cylinders (10 cm diameter and 20 cm high) with a thin layer of bedding in order to reduce neophobia but prevent digging, a potentially competing behaviour. Animals remained in the cylinders for ten minutes and were recorded with a camera placed horizontally level with the cylinders. Grooming time was scored manually by experimenters from watching video files.

2.2.7. Novel object recognition test

The novel object recognition test is a commonly used trial to assess hippocampal-dependent memory as described previously [41] and takes place over three trials on three consecutive days.

Day 1: Habituation – animals are habituated to a square open-field box (Perspex sides and base: 325cm × 42.7cm) in a dimly lit room by individually placing the mice to the apparatus for ten-minute habituation periods. This portion of the experiment also served as the basis for the generation of 'open field test' results.

Day 2: Two identical objects are positioned on adjacent corners approximately 5 cm from each wall of the open field, and each animal was introduced for a ten-minute exploration period. Animals were then placed directly back into their home cages.

Day 3: After a 24 -h inter-trial-interval, one familiar object was replaced with a novel object and each animal was introduced for a ten-minute exploration period.

On each day, animals are acclimatized to the testing room for approximately one hour before being placed in the box. Between trials, objects and testing arenas are cleaned with 70 % ethanol and rinsed with water before thorough drying.

Recordings were made with a camera placed above the apparatus and scoring was undertaken manually from these videos. Object exploration was defined as when the animal's nose comes within a 2 cm radius of the object.

2.3. Other physiological and post-mortem analyses

2.3.1. In-vivo Intestinal motility (carmine red test)

Mice were singly housed and habituated to new cages for three hours for acclimatization. Following acclimatization, mice received 200 u L oral gavage of Carmine (C1022; Sigma Aldrich) suspended in 0.5 % carboximethylcellulose (CMC) sodium salt (Sigma, St Louis, MO, USA). Time of the first red coloured bolus is recorded as previously performed [3]

2.3.2. Tissue collection for flow cytometry

Trunk blood was collected in 3 mL EDTA-containing tubes (Greiner bio-one, 454,086) and 100 µl was put in a separate Eppendorf for flow cytometry. Both tubes were centrifuged for 10 min at 3500 *g* at 4 °C. The remaining cell pellet of the Eppendorf containing 100 µl blood was stored on wet ice and subsequently used for flow cytometry. Mesenteric lymph nodes (MLNs) were extracted, fat tissue was removed and stored in RPMI-1640 medium with L-glutamine and sodium bicarbonate (R8758, Sigma), supplemented with 10 % FBS (F7524 l, Sigma) and 1% Pen/strep (P4333, Sigma) on wet ice for subsequent flow cytometry.

2.4. Flow cytometry

Blood and MLNs collected when animals were sacrificed were processed on the same day for flow cytometry similar to previously described [42,43]. Blood was resuspended in 10 mL home-made red blood cell lysis buffer (15.5 mM NH_4Cl, 1.2 mM $NaHCO_3$, 0.01 mM tetrasodium EDTA diluted in deionised water) for 3 min. Blood samples were subsequently centrifuged (1500 *g*, 5 min), split into 2 aliquots and resuspended in 45 µl staining buffer (autoMACS Rinsing Solution (Miltenyi, 130−091-222) supplemented with MACS BSA stock solution (Miltenyi, 130−091-376)) for the staining procedure. MLNs were poured over a 70 µm strainer and disassembled using the plunger of a 1 mL syringe. The strainer was subsequently washed with 10 mL media (RPMI-1640 medium with L-glutamine and sodium bicarbonate, supplemented with 10 % FBS and 1% Pen/strep), centrifuged and 2×10^6 cells were resuspended in 90 µl staining buffer and split into two aliquots for the staining procedure. For the staining procedure, 5 µl of FcR blocking reagent (Miltenyi, 130−092-575) was added to each sample. Samples were subsequently incubated with a mix of antibodies (Blood and MLNs aliquot 1; 1 µl CD4-FITC (ThermoFisher, 11−0042-82) and 1 µl CD25-PerCP-Cyanine5.5 (ThermoFisher, 45−0251-80); MLNs aliquot 2; 1 µl CD4-FITC (ThermoFisher, 11−0042-82) and 5 µl CD8a-PerCP-Vio700 (Miltenyi, 130−102-468); MLNs aliquot 3; 2 µl CD11c-PE (Miltenyi, 130−110-838) and 5 µl MHC-II-APC (Miltenyi, 130−102-139)) and incubated for 30 min on ice. Blood aliquot 1 was subsequently fixed in 4% PFA for 30 min on ice, whilst Blood aliquot 2 and MLNs underwent intracellular staining using the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (ThermoFisher, 00−5523-00), according to the manufacturers' instructions, using antibodies for intracellular staining (2 µl FoxP3-APC (ThermoFisher, 17−5773-82) and 5 µl Helios-PE (ThermoFisher, 12-9883–42)). Fixed samples were resuspended in staining buffer and analysed the subsequent day on the BD FACSCalibur flow cytometry machine. Data were analysed using FlowJo (version 10). Cell populations were selected as following: T helper cell: CD4+, Cytotoxic T cell: CD8a+, Treg cells: CD4+, CD25+, FoxP3+; Dendritic cells; MHC-II+, CD11c+. The investigated cell populations were normalised to PBMC levels. Gating strategies are depicted in **Supplementary Figures 1, 2, 3, 4, 5 and 6**.

2.5. Statistical analysis

Data distribution was checked by Kolmogorv-Smirnov test and variances were compared using Levene's test. For parametric data, a Paired Student *t*-test, a One-way ANOVA, Two-way ANOVA and two-way repeated measures ANOVA followed by Bonferroni post-hoc was applied accordingly to the protocol adopted. All statistical analyses were carried out using IBM SPSS Statistics 22.0 for Windows software package. Extreme outliers and technical outliers were excluded when values are *2 x Standard Deviation from the mean*. F values, P values are presented in the text of the results section.

3. Results

3.1. Behavioural results

3.1.1. Anxiety-like and repetitive behaviours

As a model of ASD, BTBR mice have demonstrated a robustly anxious phenotype as well as clear repetitive behaviour in various behavioural tests when assessed in early adulthood. Here we show that grooming behaviour remains significantly increased in ageing **(**Fig. 1**B)** ($T_{(20)} = 13.0$ $P < 0.001$)). However, in the marble burying test **(**Fig. 1**A)** ($T_{(20)} = 0.1137$, $P = 0.9106$) no observed difference in anxiety-like behaviour between the BTBR and C57 animals was observed. In a similar vein, in the elevated plus maze **(**Figs. 1**C-1E)** the time spent in the open arms **(**Fig. 1**D)** (T(20) = 0.191 %, P = 0.8501) and number of entries to the open arms **(**Fig. 1E**)**($T_{(20)} = 1.162$,$P = 0.2590$) were not found to be different between groups. The amount of time spent in the closed arm **(**Fig. 1**C)** ($T_{(20)} = 2.267$,$P < 0.05$) was however, significantly reduced in the BTBR group.

The open-field test is widely used in rodent models and it provides

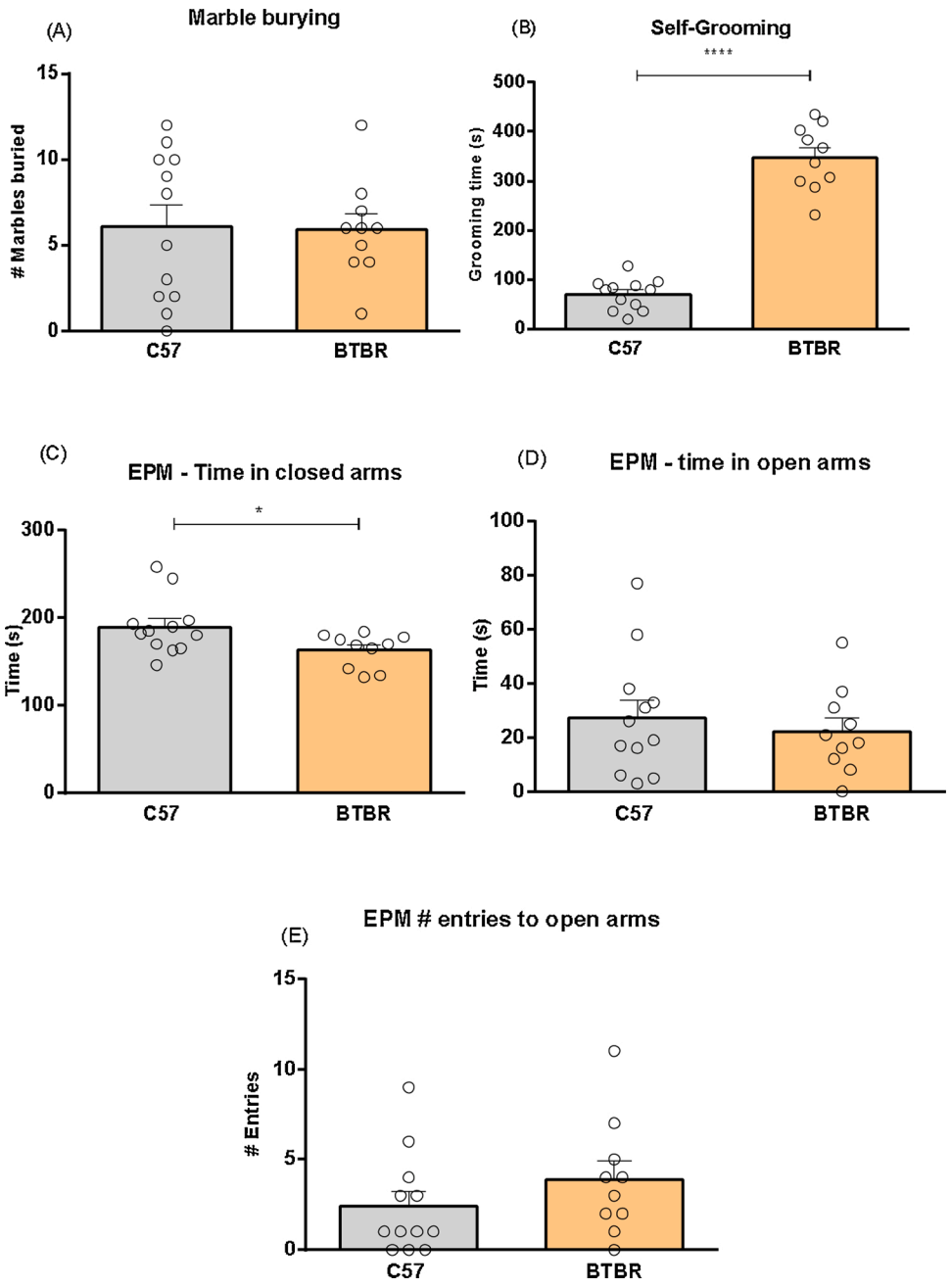

Fig. 1. Aged BTBR and C57 mice display variable levels of anxiety across a range of behavioural tests. (A) marble burying – no differences found in the number of marbles buried between the two strains. (B) grooming – BTBR mice spend significantly more time self-grooming than C57 (C-E) in the elevated plus maze BTBR mice spend less time in the closed arms of the maze, though there are no differences in the time spent in open arms, or the number of entries to the open arms. [C57 n = 12, BTBR n = 10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired *t*-test. *p < 0.05, ****p < 0.0001.

information regarding various aspects of emotionality in rodents [44]. In our test it was used as a measure of locomotor activity **(**Fig. 2**A)** and anxiety **(**Fig. 2**B)**. Aged BTBR animals display greater locomotion than their C57 counterparts **(**Fig. 2**A)**($T_{(20)} = 9.745, P < 0.0001$), however, the time spent in the centre of the arena is similar between the groups **(**Fig. 2**B)**($T_{(20)} = 0.0478, P = 0.9726$), indicating no difference in anxiety-like behaviour.

3.1.2. Antidepressant-sensitive behaviour

The forced-swim test is used as a measure of antidepressant-sensitive behaviour [45]. Aged BTBR mice had a reduced immobility time in the test, indicating a reduction in depressive-like behaviour **(**Fig. 3**)** ($T_{(20)} = 3.67$, $P < 0.005$).

3.1.3. Three-chamber test

In the three-chamber test the BTBR mice displayed a reduced preference for the novel mouse compared to the age-matched C57 control. When the interaction times were analysed it was found that both C57 ($F_{(22)} = 12.09, P < 0.0001$) and BTBR ($F_{(14)} = 14.475, P < 0.0005$) exhibited a significant preference for a mouse over an object **(**Fig. 4**A)**. However, while a preference for a novel over familiar mouse was observed in C57 mice ($t_{18} = 3.162, P < 0.005$), this was not seen in the BTBR animals ($t_{(14)} = 1.518, P = 0.1512$) **(**Fig. 4**B)**.

3.1.4. Novel object recognition

Aged animals in both strains display a preference for interaction with a novel compared to a familiar object, with both spending more time with the novel object **(**Fig. 5**B)** ($F_{(22)} = 5.282, P < 0.0001; F_{(18)} = 4.212$, $P = 0.0005$). When these results are expressed in terms of a discrimination index **(**Fig. 5**A)** ($F_{(20)} = 1.336, P = 0.1964$), no differences are observed between the groups.

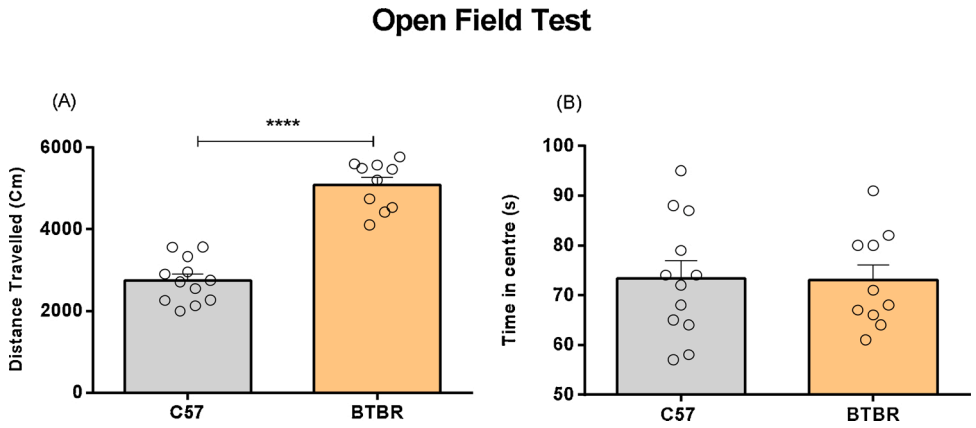

Fig. 2. Aged BTBR mice display increased locomotor activity in the open field test. (A) Aged BTBR animals travel a significantly greater distance in during the duration of the test than age-matched C57 controls. (B) There is no difference in the time spent in the centre of the arena between the groups. [C57 n = 12, BTBR n = 10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired *t*-test. *p < 0.05.

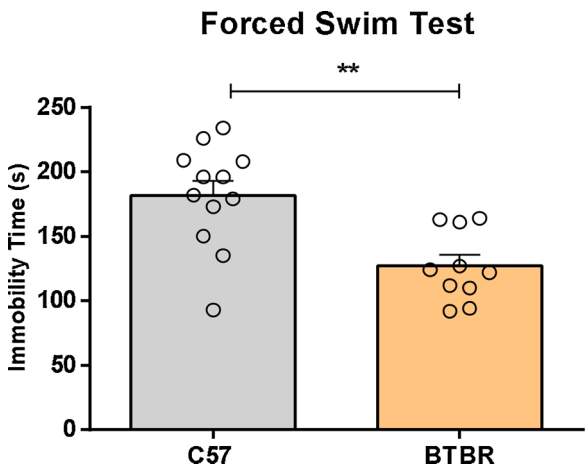

Fig. 3. Aged BTBR mice display less antidepressant-sensitive behaviour in the forced-swim test. BTBR mice spend significantly less time immobile in the FST than aged-matched C57 controls. [C57 n = 12, BTBR n = 10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired *t*-test. **p < 0.005.

3.2. Physiological data

Similar to previous reports in much younger mice [32], aged BTBR mice have a greater body weight compared to C57 mice (Fig. 6**A**) (F(20) = 8.716,P < 0.001). However, relative cecum weight is greater in the C57 animals (Fig. 6**B**) (F(19) = 3.734,P < 0.005). Aged BTBR mice also exhibit an increased intestinal transit (Fig. 6**D**) (F(20) = 3.346, P < 0.005) though it may also be linked to longer colon that was seen in these animals (Fig. 6**C**) (F(20) = 2.213,P < 0.05). Finally, no difference was seen in spleen weight between the two groups (Fig. 6**E**) (F(19) = 1.701,P = 0.1053).

3.3. Flow cytometry data

3.3.1. Aged BTBR mice display an altered T-cell repertoire

Aged BTBR mice show increases in (CD4+) T helper cells both in MLNs (Fig. 7**A**) (F(18) = 13.69,P < 0.0001) and the peripheral circulation (Fig. 7**D**) (F(18) = 5.908,P < 0.0001). In addition, there was a decreased prevalence of (CD8+) cytotoxic T cells in MLNs (Fig. 7**B**) (F(18) = 6.045,P < 0.0001), but not the circulation (Fig. 7**E**) (F(18) = 0.2661,P = 0.7932). Overall, this results in an increased CD4/CD8 ratio in both MLNs and blood (Fig. 7C**&F**) (F(18) = 23.90,P < 0.0001) & (F(18) = 6.081,P < 0.0001), which is often used as a marker of an activated adaptive immune system [46].

3.3.2. Aged BTBR mice express decreased MLN treg cells

Aged BTBR mice have decreased levels of MLN Treg cells (Fig. 8**A**) (F(17) = 3.120,P < 0.005), offering further indication of an inflammatory phenotype. No alterations were seen in circulating Treg cells, however (Fig. 8**B**) (F(16) = 1.423,P = 0.1738). Interestingly, differences in overall MLN Treg cell levels were explained by decreased levels of peripheral-derived Treg cells (pTreg) (Fig. 8C) F(17) = 8.527, P < 0.0001). This may be linked to the microbiota, as Treg

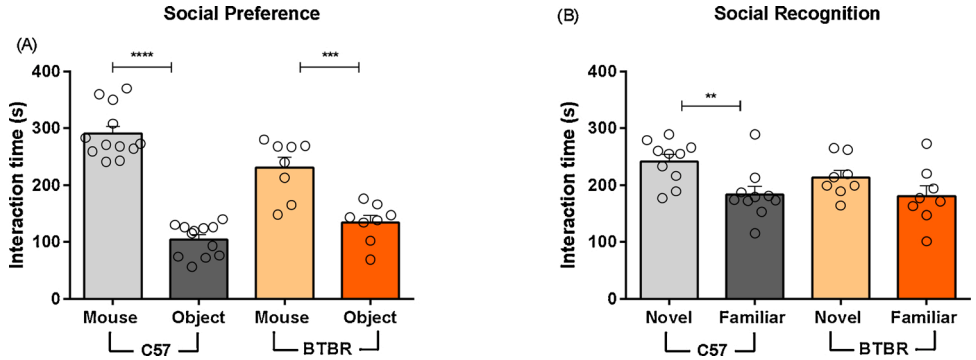

Fig. 4. Aged BTBR mice display impaired social behaviour compared to age-matched C57 mice in the three-chamber test. (A) Both C57 and BTBR exhibit a preference for a mouse over an object. (B) Only the C57 group display a preference for a novel over a familiar mouse. [C57 n = 12, BTBR n = 10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired *t*-test. *p < 0.05,**p < 0.005, ***p < 0.001,****p < 0.0001.

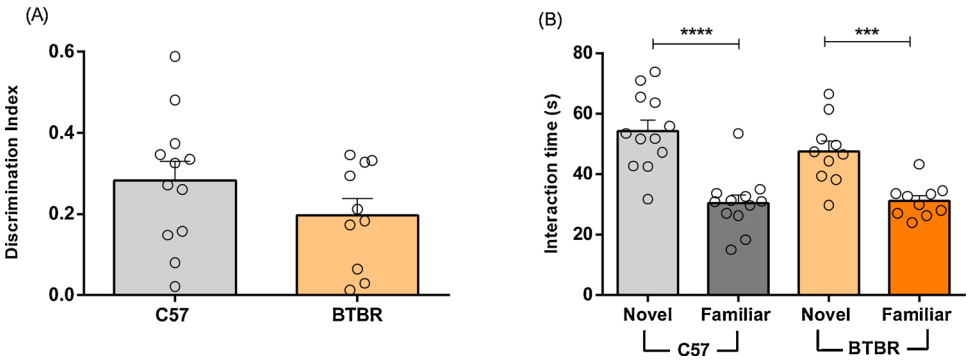

Fig. 5. Aged mice of both C57 and BTBR strains display a preference for a novel object in the novel-object recognition test with no difference in discrimination index between the groups. (A) No strain difference was observed in discrimination index in the novel object recognition test. (B) Both aged C57 and BTBR mice spend significantly more time interacting with a novel object that a familiar one. [C57 n = 12, BTBR n = 10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired *t*-test. ***p < 0.001, ****p < 0.0001.

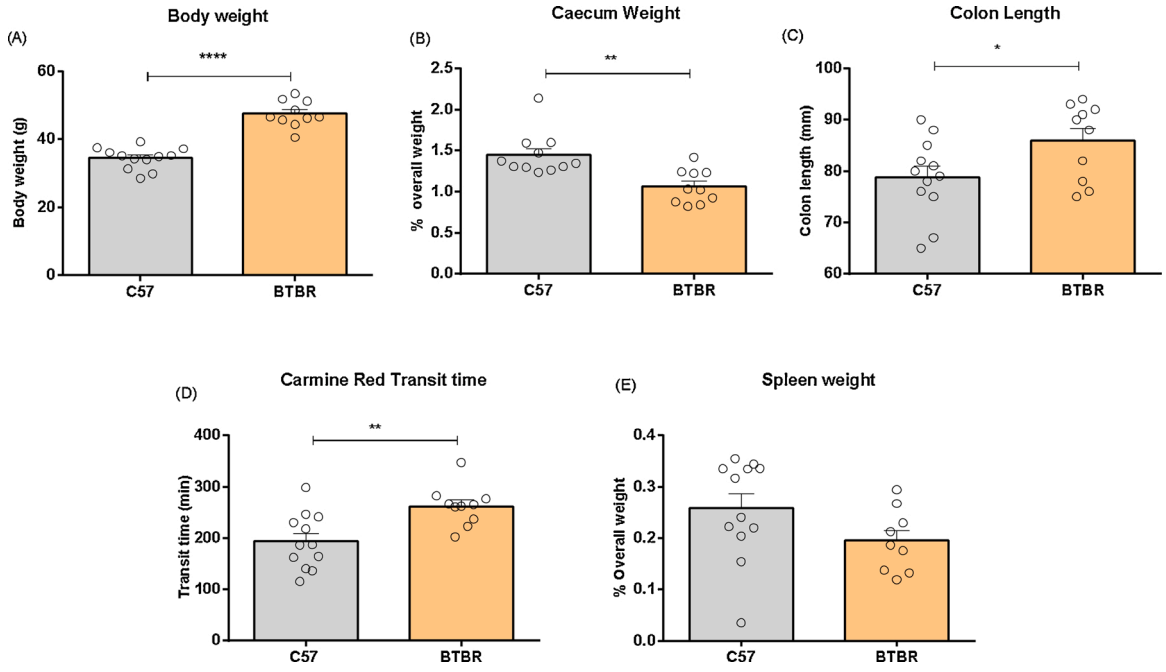

Fig. 6. Body tissue weight differences between the two strains (A) BTBR mice are larger than their age-matched C57 counterparts (B) C57 animals have a greater cecum weight relative to their body weight, however (C—D) both colon length and carmine red transit time were greater in BBTR animals (E) No differences were observed in spleen weight between the groups. [C57 n = 12, BTBR n = 10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired *t*-test. *p < 0.05.

differentiation can be induced by gut microbial metabolites in MLNs, which would be pTregs [47]. Thymus-derived Treg cells (tTreg) were increased **(**Fig. 8**D)** ($F(17) = 3.991, P < 0.001$).

3.3.3. *Dendritic cells are decreased in number in BTBR mice*

Dendritic cells are well-known inducers of Treg cell differentiation [47]. In line with the decrease in MLN Treg cell levels, BTBR mice showed decreased levels of MLN dendritic cells (Fig. 9**)** ($F(18) = 3.012$, $P < 0.005$).

4. Discussion

BTBR mice are a highly utilised, informative mouse model for many of the deficits seen in autism spectrum disorders [48]. Previous studies in young animals have shown that they exhibit several behavioural abnormalities, compared to control strains. Deficits have been observed for example, in sociability and social withdrawal [49], learning and attention [6], stress response [9], anxiety and depressive behaviours [3] as well as repetitive behaviours [50]. Here we characterise these parameters in older animals and show that many of the characteristic behaviours of the BTBR model during early-life and adult are maintained in the ageing animal, however, there are several notable changes in the older animals. We compared behavioural differences between these animals and a C57 aged control, also assessing immune system differences between the two strains.

The initial tests undertaken in the behavioural battery assessed levels of anxiety-like and repetitive behaviours in each of the strains. As a model of ASD, BTBR mice generally exhibit a higher level of repetitive and anxiety-like behaviours as has been demonstrated in the marble-burying test [3,51], the elevated-plus maze [3] and grooming behaviour [3]. We observed that this phenotype was still present in later age in the grooming test where BTBR mice spend significantly more time engaged in grooming behaviour than the C57 controls. As increased engagement in repetitive behaviour is among the most robust behavioural characteristics, this is an important result as it suggests that this core facet of the behavioural phenotype is maintained. A caveat to the increased levels of grooming in older mice is the observed hair loss in BTBR mice from excessive grooming [52], this was not observed in our mice. Excessive grooming may be both be a cause of this hair loss as well as exacerbated by it [52].

Other tests provided less of a robust anxious phenotype. In the marble-burying test, there was no difference in the number of marbles that were buried between strains. In the elevated plus maze, no differences observed in the number of entries to the open arms, or the amount

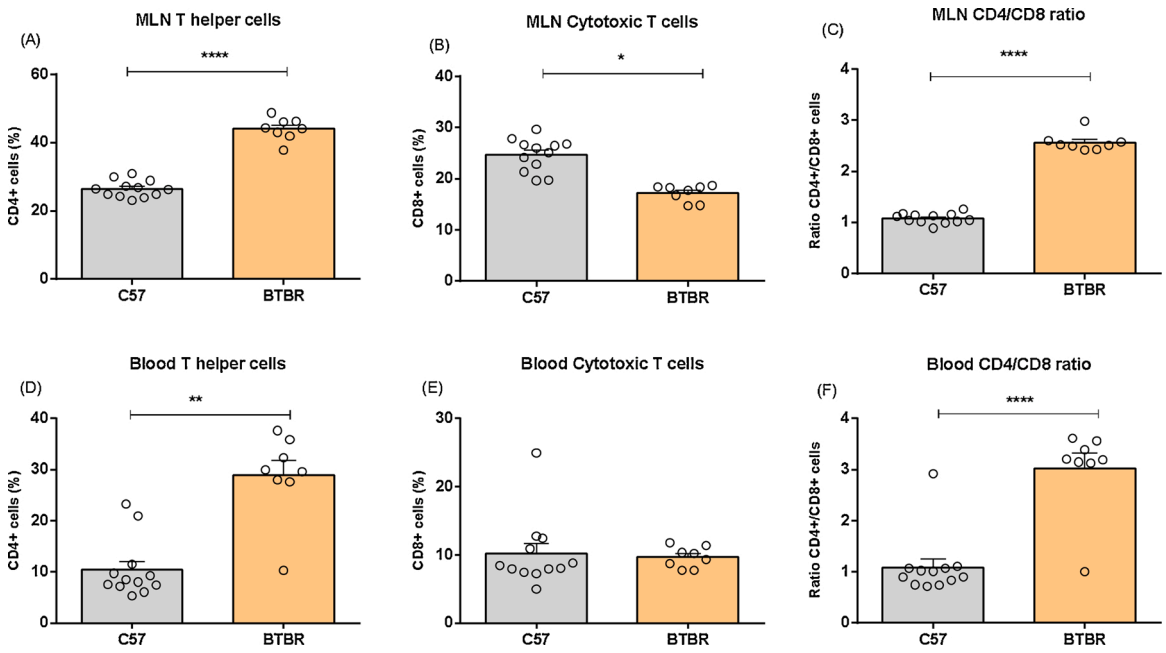

Fig. 7. BTBR mice display an altered T-cell repertoire. (A) BTBR mice have an increase in MLN helper T cells. (B) There is a lower level of cytotoxic T cells in the MLN, however (C) The overall effect is a significantly greater CD4/CD8 ratio in the BTBR animals (D) helper T cells are expressed in greater numbers in the blood in BTBR mice (E) No differences are observed in cytotoxic T cells in the blood (F) The CD4/CD8 ratio is significantly greater in the BTBRstrain. [C57 n = 12, BTBR n = 10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired *t*-test. *p < 0.05,**p < 0,005,****p < 0.0001.

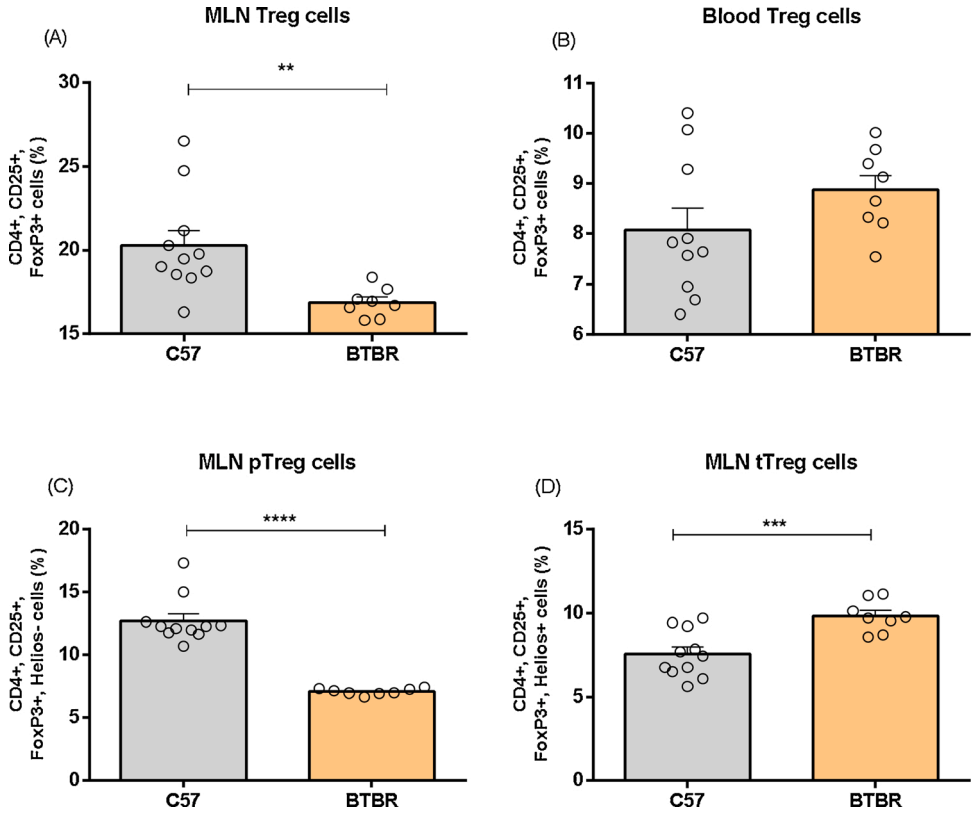

Fig. 8. Aged BTBR mice display altered MLN Treg cells. (A) Treg cells are expressed at a significantly greater level in C57 mice in the MLN (B) No differences are observed between the strains in Blood Treg levels (C) MLN tTreg cells are observed at a higher level in BTBR mice with the opposite being true of MLN pTreg cells (D) [C57 n = 12, BTBR n = 10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired *t*-test. **p < 0.005, ***p < 0,001,****p < 0.0001.

of time spent in the open arms; BTBR mice spent significantly less time in the closed arms. This test measures the conflict between the motivation of mice to explore a novel area, and their preference for a protected environment [53]. As such, a reduction in the time spent in the closed arms of the test is regarded as an anxiolytic behaviour, not what would be expected from a mouse model of ASD.

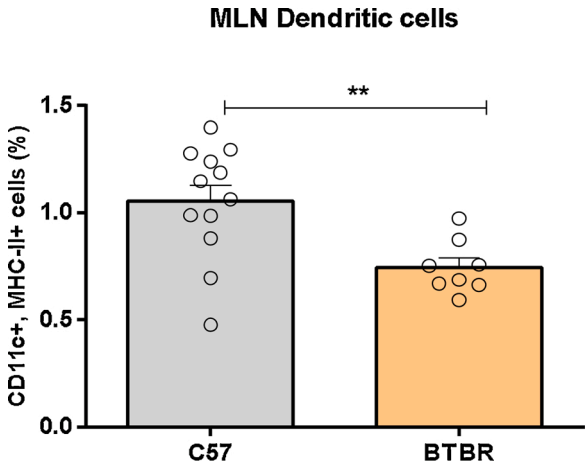

Fig. 9. BTBR mice display a decrease in the number of MLN dendritic cells. [C57 n = 12, BTBR n = 10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired *t*-test. **p < 0.00.

An alternative interpretation of the results takes into account the age of the animals. It has been established that aged C57 animals spend significantly less time in the open arms of the EPM [39], thereby increasing the amount of time in the closed arms. These results offer insight into the differential ageing trajectory between the two strains. The anxiety-like behaviour observed in this study is not as robust as what is seen in younger animals, with only the grooming test showing a much-increased anxious phenotype. This suggests that there may indeed be a lessening of anxiety-like behaviour in aged BTBR animals. At least in comparison to their C57, counterparts. Assessment against younger BTBR animals in a future study would provide empirical evidence of this.

Within the forced-swim test, a measure of antidepressant-sensitive behaviours, we observed that BTBR animals exhibit significantly less immobility time than C57 controls. This is consistent with what has been observed in younger animals [54]. Furthermore, it is unlikely to be an age-related effect of controls, as aged C57 animals do not display an alteration in the test compared to younger animals [39]. While these results are consistent with what has been observed in the past, there is also an inconsistency with what is seen clinically. Both individuals with ASD [55], and the elderly [56] are known to display elevated levels of depression. A further caveat is that the BTBR group exhibited increased locomotor activity in the open-field test, which suggests that the additional levels of activity may be due to a hyperactivity within this group that may mask differences in depressive-like behaviour. Hyperactivity is a factor that is known to be present in animal models of ASD [57], having also been observed in mice displaying autistic behaviour which lack the synaptic proteins proSAP1 and Shank2 [58].

Previous studies have reported social deficits in BTBR mice in young animals [59]. A greater preference was seen in a test for social recognition, where C57 animals exhibited a greater preference for the novel over the familiar mouse, a feature not observed in the BTBR mice who exhibited a similar preference for each. Considering that sociability is regarded as one of the most robust behavioural traits of the BTBR model, and has been demonstrated on numerous occasions [3,60], the maintenance of this phenotype into old age highlights its durability in the model.

Within ASD there exists a wide range of cognitive abilities, ranging from severe disability to high-functioning individuals [61]. We undertook the novel object recognition paradigm as our measure of cognitive function. Previous studies have demonstrated a reduced level of performance in BTBR animals in this test compared to other strains [3,62]. Tests of cognition, however are one of the most widely undertaken in studies of ageing and aged animals have been shown on many occasions to preform worse in these tests than younger animals [63,64]. In our experiment we saw that both strains of mouse exhibited a preference for a novel over a familiar object, and that there was no difference in discrimination index between the groups. It must be noted in this study, however, that there is no young control to which behaviour in the test can be compared. So while BTBR mice perform worse in the test compared to C57 controls in younger animals, it may be that the higher performing C57 groups have a bigger relative decline over their lifespan, explaining the similar performance of both groups in ageing.

Physiological measurements showed a number of differences between the strains of mouse. We observed that C57 mice exhibited a significantly greater cecum weight than their BTBR counterparts as a percentage of overall body weight, and this structure has a high density of bacteria that has been shown to have its own distinct composition [14,65]. Intestinal transit was observed to be delayed in BTBR mice compared to controls, though this also corresponds with an increase in colon length which may be a contributing factor. Previous studies have found corresponding results, however and suggested that the increases may be linked to a reduced intestinal availability of serotonin in these animals and a subsequent alteration in the ability of the neurotransmitter to act on NMDA receptors within the enteric nervous system [3,66]

In addition to behavioural and physiological differences between these two strains of animals we also performed flow cytometry analysis in order to determine whether any immune changes were present in these older animals. Here we show that aged BTBR mice display an altered T-cell repertoire to C57 animals. An increase in CD4 + T-helper cells was observed in BTBR mice in MLNs and the peripheral circulation, while CD8+ cytotoxic T cells were decreased in MLNs only. This resulted in an increase in the CD4/CD8 ratio, often associated with an activated immune system [67]. Indeed, it has previously been demonstrated that animal models of ASD [68], as well children with the disorder [69], show higher immune activation. Furthermore, BTBR mice had decreased levels of Treg cells in MLNs, further indicating an inflammatory phenotype [70]. This is in line with previous reports in adolescent BTBR mice [71]. Alterations in Treg cell subtypes in the MLN have been linked to inflammation in the gut [72], with children with ASD more likely to suffer from inflammatory disorders of the gut than neurotypical controls [73]. Our results also reveal that this inflammation may be linked to a deficit in non-thymic Treg production.

In line with this is a decreased prevalence of dendritic cells in the MLNs of BTBR mice, which are known to induce the Treg cell differentiation [74]. Even though increased levels of dendritic cells were observed in individuals with ASD [75]. Overall the immune data suggests that aged BTBR mice display a chronically activated T-cell system compared to control C57 animals, suggesting the involvement of autoimmunity in the differences observed between the strains. While there may be some alterations due to the natural disruptions to the immune system in advancing age, the data suggests that the observed increases are in autoimmune activation that has been observed in animal models of ASD [68], as well as in humans [69], and that this phenotype is maintained at a later age.

A caveat to any rodent study of ASD lies with the question of which model most accurately most completely represents both the genetic and behavioural aspects of the disorder. A recent review paper on the topic [76] highlights the difficulties that the multifaceted human genetic polymorphisms underlying the phenotypic diversity in ASD pose to the study of the disorder. As such, a wide array of preclinical models, both genetic and phenotypic models of ASD will bring greater clarity in uncovering the mysteries of the disorder. Furthermore, given the sex-differences in neurodevelopmental disorders [77] a beneficial addition to future studies in ageing would be to assess if there are differences in aged mice between male and female rodents. One caveat of our study is that the BTBR mouse line is not a model of an autism-associated genotype, nor is it a genotype associated with neurodevelopmental disorders [78]. Rather, it is proposed as a model of

social deficits, with putative face validity to autism based on mouse behavioural measures that do not represent the human ASD behavioural spectrum [79].

The results of this study yield information on a novel aspect of the study of ASD in mouse models. Most of the preclinical research in the area focuses on young animals where results will be translated clinically to young individuals where much of the focus in the disorder rests. Outside of these studies there are a handful of clinical studies in early middle age [28] though this is not the case preclinically. Our study assesses behaviour during this age in a preclinical cohort. We find that while many of the behavioural characteristics observed in early life are maintained at this later stage others are not, particularly in the case of anxiety-related behaviours. This study provides a platform for an interventional analysis targeting the gut-brain axis. Future studies must focus on interventional studies in this ageing model of altered gut-brain-axis function.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bbr.2020.113020.

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