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Sex-Specific Behavioural Deficits in Adulthood following Acute Activation of the $GABA_A$ Receptor in the Neonatal Mouse

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Abstract
Introduction: Sex differences exist in the prevalence of neurodevelopmental disorders (NDDs). Part of the aetiology of NDDs has been proposed to be alterations in the balance between excitatory and inhibitory neurotransmission, leading to the question of whether males and females respond differently to altered neurotransmitter balance. We investigated whether pharmacological alteration of $GABA_A$ signalling in early development results in sex-dependent changes in adult behaviours associated with NDDs. ***Methods:*** Male and female C57BL/6J mice received intraperitoneal injections of 0.5 mg/kg muscimol or saline on postnatal days (P) 3–5 and were subjected to behavioural testing, specifically open field, light/dark box, marble-burying, sucralose preference, social interaction, and olfactory habituation/dishabituation tests between P60 and P90. ***Results:*** Early postnatal administration of muscimol resulted in reduced anxiety in the light/dark box test in both male and female adult mice. Muscimol reduced sucralose preference in males, but not females, whereas female mice showed reduced social behaviours. Regional alterations in cortical thickness were observed in the weeks following $GABA_A$ receptor activation, pointing to an evolving structural difference in the brain underlying adult behaviour. ***Conclusions:*** We conclude that activation of the $GABA_A$ receptor in the first week of life resulted in long-lasting changes in a range of behaviours in adulthood following altered neurodevelopment. Sex of the individual affected the nature and severity of these abnormalities, explaining part of the varied pathophysiology and neurodevelopmental diagnosis that derive from excitatory/inhibitory imbalance.

Introduction

Neurodevelopmental disorders (NDDs) are a complex group of disorders with their origins in the first years of life and with consequences that span a lifetime. They are diagnosed on the basis of behavioural phenotypes (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition) due to the lack of reliable biomarkers [1] and collectively have a prevalence of at least 5%, though higher prevalences are reported in some countries [2].

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There are substantial population differences in the prevalence, onset, and symptom severity of many NDDs that also differ depending on the sex of the individual [3]. For instance, attention deficit hyperactivity disorder, autism spectrum disorder (ASD), and schizophrenia have a higher prevalence in males with a 3:1 male:female ratio [4], 4:1 ratio [5], and 1.4:1 ratio [6], respectively. By contrast, depression and anxiety disorder has a higher prevalence in females (1:2) [7].

There remains the question of what causes this behavioural diversity, and to what degree there is a commonality in the origins of these disorders. Interestingly, NDDs are often accompanied by an increase or decrease in the excitatory-inhibitory (E/I) ratio of neurons. For example, magneto-encephalographic and magnetic resonance spectroscopy (MRS) studies of autistic individuals have reported decreased gamma oscillation [8, 9] and reduced gamma-aminobutyric acid (GABA) concentrations [10, 11], respectively, when compared with control cohorts. Similarly, electro-encephalographic and magneto-encephalographic recordings from brains of schizophrenia patients showed decreased amplitude of gamma oscillations [12, 13]; MRS studies have shown that GABA concentration in patients with schizophrenia was reduced, whereas glutamate increased [14–16]. At a cellular level, postmortem studies in autistic subjects have identified increased spine densities in cortical pyramidal neurons [17] and a reduced parvalbumin-positive interneuron population in the prefrontal cortex (PFC) [18], while patients with schizophrenia showed decreased expression of glutamate decarboxylase 67 in parvalbumin-positive interneurons [19–21]. Additionally, increased E/I ratio has been identified with MRS due to either increased glutamate or decreased GABA levels in a variety of psychiatric conditions associated with NDD, including epilepsy [22], anxiety [23, 24], depression [25–27], and obsessive-compulsive disorder [28].

Disruption to the E/I balance has also been proposed in the aetiology of these disorders [29–36]. The role of the inhibitory neurotransmitter GABA in E/I imbalance is of particular interest, as GABA influences many developmental processes through trophic effects on migration, survival, neurite outgrowth, dendritic development, and synapse formation [37–40]. The $GABA_A$ receptor ($GABA_AR$) subunits display specific spatiotemporal expression patterns throughout cortical development [36, 41, 42], and the consequences of GABA receptor activation also change through development. GABA activity is excitatory early in development, until postnatal (P) 7–12 in rodents [43], before transition to the inhibitory function well described in adulthood. This transition is driven by a developmental change in the presence of Na-K-2Cl cotransporter 1 and the K-Cl cotransporter 2 (KCC2) that regulate the intracellular chloride concentration [30, 44, 45]. The change in the expression pattern of these cotransporters takes place around the time of birth [45]. Molecular cues such as oxytocin, oestradiol, and GABA influence the downregulation of Na-K-2Cl cotransporter 1 and upregulation of KCC2 [36, 46]. It is therefore possible that alterations in GABA can produce different short-term effects at a cellular level and long-term variation in behaviour depending on the age or brain region affected. Additionally, after birth KCC2 upregulation and presence of GABAergic hyperpolarising synapses in females precede males in rodents [47, 48]. Thus, during development GABA may act differently in male and female brains. In support of this hypothesis, increased E/I ratio in social-brain regions such as the medial PFC has been reported in autistic males but not in females [49].

Sexual dimorphism in NDDs suggests that distinct risk or resilience may be conferred by sex differences during brain development and hence similar insults might have different consequences depending on the sex of the individual. To determine whether behavioural differences associated with NDDs could result from sex differences in the neurological response to early-life E/I imbalance, we induced an acute pharmacological activation of $GABA_AR$ signalling with the agonist, muscimol in early postnatal life. We subsequently tested whether there was a long-lasting effect of this acute $GABA_AR$ activation across a range of behaviours relevant to, and associated with (e.g., anxiety), NDDs. Anhedonia-like behaviour was also assessed, as previous models using muscimol have suggested it may impact on behaviours relevant to depression [50]. Since males and females are exposed to slightly different internal environments, e.g., different hormones and concentrations, and their brain development follows dimorphic trajectories, we hypothesised that altering the E/I balance in early development when GABA is excitatory would elicit distinct long-term behavioural responses between adult male and female mice.

Material and Methods

Animals and Drug Administration

B6.Cg-Tg(Thy1-YFP)16Jrs/J transgenic mice (Jackson Laboratories, Stock No. 003782) were kept in 12-h light/dark (LD) cycles with lights on from 7:00 to 19:00 h (235lux) and were housed in individually ventilated cages with ad libitum access to food (PicoLab® Irradiated Rodent Diet 5053, LabDiet, USA) and tap water. All mice were housed in Tecniplast cages (32 cm × 16 cm ×

14 cm) with Lignocel wood fibre bedding (IPS Product Supplies Ltd, London, UK) and cage enrichment, consisting of nestlets and Bed-r'Nests (Datesand Ltd, Manchester, UK), a cardboard tunnel and shelter along with an aspen chew block extra small brick (LBS Biotech, Horley, UK). Breeding and experimental procedures were carried out in accordance with the UK Home Office (Scientific procedure) Act (1986), project licence number PDAD9E285, and project approval from the Royal Veterinary College Animal Welfare and Ethical Review Board (ref. 2018-0114N). All pregnant females had normal delivery, and the date of birth was considered as P0. No maternal behaviour differences were observed towards control or treated litters. ARRIVE guidelines were followed in the experimental conceptualisation and execution of these studies.

Mice were injected intraperitoneally with 5 μL of either vehicle (PBS) or 0.5 mg/kg muscimol for 3 consecutive days, on P3, P4, and P5 (as described in [50]), a period that corresponds to the third trimester of human pregnancy [51] and in which GABA activity is excitatory [43]. For the behavioural studies, at P21 litters were weaned from the mother and housed with same-sex litter counterparts, 2–4 mice per cage. Two males and/or females from each litter were used for behavioural testing as adults to avoid litter-specific effects on behaviour [52]. In total, there were 12 males and 11 females in the vehicle group and 10 males and 8 females in the muscimol-exposed group, originating from 15 independent litters.

Immunohistochemical analysis of the brain was performed to determine if there were any short-term responses to muscimol administration. For these, pups were killed by a schedule 1 method either 4 h after the first injection at P3 (n = 4 vehicle, n = 3 muscimol), 24 h after the last injections at P6 (n = 10 vehicle, n = 11 muscimol), or on P20 (n = 6 in both groups). At P3 and P6, brains were immediately dissected out and fixed by immersion in 4% paraformaldehyde (Sigma-Aldrich), while at P20 animals were perfusion fixed with 4% paraformaldehyde via cardiac puncture prior to dissection and further immersion fixation.

Mice were also killed by a schedule 1 method 4 h after the first vehicle or muscimol injection at P3 for analysis of cytokine expression in the brain tissue, n = 4 for vehicle group and n = 5 for muscimol group. Brain tissue was dissected, and cerebral hemisphere was placed directly in TRIzol prior to RNA extraction.

Compounds

Muscimol was purchased from Sigma-Aldrich (product No. M1523) and dissolved in 0.05 M HCl (20 mg/mL), as per manufacturer's instructions. Aliquots of 0.5 mg/mL muscimol were prepared in PBS for intraperitoneally injections.

Histology, Immunohistochemistry, and Light Microscopy

Brains were embedded in agarose (Sigma-Aldrich) and cut into 100-μm-thick coronal sections using a Leica Biosystems vibratome, with sections containing the somatosensory cortex collected for analysis. Brain sections were washed in PBS plus 0.1% Triton™ X-100 (PBST; Fisher BioReagents), and then those used for immunohistochemistry were incubated with protein-blocking solution (5% donkey serum in PBST) for 2 h at room temperature. Sections were then incubated overnight at 4°C in rabbit anti-iba1 antibody (1:200, Abcam) in PBST with 1% donkey serum, for the detection of microglia. Subsequently, sections were washed three times for 10 min each in PBST, followed by a 2-h room temperature incubation in donkey anti-rabbit Alexa Fluor® 546 (1:500; Life Technologies) in PBST with 1% donkey serum. Sections were washed again, and then all sections (for microglia and cortical layer analysis) were stained with DAPI (1:1,000; Sigma-Aldrich) in PBS for 5 min, followed by two washes with PBS for 10 min. Sections were mounted in SuperFrost™ Microscope Slides with ProLong™ Gold antifade mounting media (Invitrogen™).

Brain sections were imaged with an SP8 Leica confocal microscope using LAS X software. Images were acquired with a ×40 objective lens to frame the cortical somatosensory region. The sequential acquisition setting was 400 ms speed and 512 × 512 pixel resolution. Images were taken for cortical layer thickness measurements using the 405 λ channel for DAPI, with the laser power at 10% and intensity at 1 to visualise cell nuclei. The second set of images were with the 546 λ channel to detect microglia cells with the laser power at 10% and intensity at 1.5 and 405 λ channel for DAPI. Images were taken from each cerebral hemisphere, from at least 3 sections per brain (i.e., 6 technical replicates per brain), and were exported from the LAS X software as .tif files for microglia and cortical thickness analysis using the ImageJ (FIJI) program. Microglia were counted using the ImageJ cell counter plug-in and calculated as cells/mm^2 using area values provided by the measure tool. To measure cortical layer thickness, brightness and contrast of images were adjusted for optimal visualisation of cortical layer landmark cues. Landmark cues consisted of distinct pattern of nuclei density within the six cortical layers and the noticeable shape of a barrel between layers IV and III–II. The total cortical thickness and thickness of individual cortical layers were measured using the measure tool of ImageJ. Individual values for each brain were averaged from the 6 technical replicate images acquired.

Quantitative Real-Time Polymerase Chain Reaction

RNA was extracted from brain samples using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction was performed with 200 ng of RNA, using TaqMan™ RNA-to-CT™ 1-Step Kit (Thermo Fisher Scientific) in the StepOnePlus real-time PCR machine from Applied Biosystems. Cytokine gene expression assays were purchased from Applied Biosystems for detection of interleukin (IL)-1 beta (IL-1β), IL-6, IL-10, and interferon-gamma (IFN-γ). Relative RNA quantity was determined by the ΔΔCt method [53], and GAPDH was used for the internal control gene.

Behavioural Testing

Behavioural testing at P60–P90 was conducted over a 4-week period. Mice were transferred to a holding room adjacent to the testing room and housed individually, in individually ventilated cages in controlled conditions (20 ± 2°C, relative humidity 58 ± 10%, and 12-h light cycle), with a 5-day habituation period prior to behavioural testing. To minimise the potential effects of cage disturbance on the behaviour of the mice, cages were not cleaned the day before or on the day of testing, unless specified for particular tests. As previous studies have shown limited effect of oestrous cycle on variance in the behavioural performance of female mice [54], oestrous phase was not checked in this study. Similar to previous studies, there were no major differences in the variance observed in the behavioural measures between males and females.

The testing schedule was designed to limit stress from testing procedures and between test interferences, with at least 24 h scheduled between each test (see online suppl. Table S1 for

test schedule; for all online suppl. material see https://doi.org/10.1159/000536641). Weights of male mice ranged between 24 and 29 g and female weight between 18 and 22 g. Testing was carried out between 10:00 and 17:00 h, and light levels in the testing room were set to 10lux unless otherwise stated. For each test, the order of testing was assigned pseudo-randomly and the experimenter was blind to the drug group assignment at the time of testing and during data analysis. Behaviours were recorded with a Canon LEGRIA HF R706 Camcorder, except for the sucralose preference test where no visual analysis was performed. Behavioural parameters were recorded manually or using the contrast tracking software EthoVision (Noldus Information Technology by Wageningen, The Netherlands) from which the location and locomotor activity of the mice in each test arena were derived, as described below. After each trial, boli and urine were removed from the test arena which was then cleaned with 1% Anistel® solution (Tristel Solutions Ltd, UK). At the end of testing, mice were returned to their home cage which was returned to the housing room.

Social Interaction Test

The social interaction test was used to assess social behaviour [55, 56] and was conducted in clean housing cages. One hour prior to testing, mice were singly housed in clean and empty home cages with 1 cm thick layer of sawdust only, without access to water and food. After 1 h of habitation, a same-sex 5–6-week-old juvenile conspecific mouse was placed in the test mouse's cage (online suppl. Fig. S1a). A clear Perspex lid was placed on the cage, and a camera, located above, recorded the trial for 5 min. Duration (s) and frequency of social behaviours (following behaviour, anogenital sniffing and body sniffing), grooming, and aggressive behaviour initiated by the test mouse were hand-scored by the experimenter from the recording. The total time spent by the test mouse in following behaviour, body sniffing, and anogenital sniffing was defined collectively as social behaviour.

Olfactory Habituation/Dishabituation Test

The olfactory habituation/dishabituation test was conducted to assess the olfactory skill of the mouse, i.e., whether it could detect odours and differentiate between them [57]. Since mice rely heavily on olfaction for social interaction [58], this test was used to confirm that mice in this cohort could smell, and rule out a potential confounding factor for the social interaction test (i.e., inability to smell). The test was performed in the home cage, in the testing room, but bedding, water, and food were removed prior to testing. A cotton-tipped long wooden bud was inserted through the lid to a depth of 2.5 cm and clipped to the cage lid for a 10-min acclimation phase (see online suppl. Fig. S1b). After acclimation, the mouse was presented with a series of three odours (water, banana essence, and same-sex mouse odours), and each odour presented three consecutive times. Single odour trials lasted 2 min, with a 30-s intertrial interval, and there were a total of 9 trials. The odours were freshly prepared every day. The odour of water was prepared by pipetting 50 μL of distilled water onto a fresh cotton bud for each trial. The banana odour was prepared by diluting a banana essence (Uncle Roy's natural banana essence, Scotland, UK) 1:100 in distilled water and pipetting 50 μL of the solution onto a fresh cotton bud for each trial. The social odour was collected by swiping the bottom of unfamiliar, but same-sex, cages in a zig-zag fashion with clean cotton buds. All cotton buds were stored in sealable bags to avoid spreading the odours in the testing room. The entire duration of the test was video-recorded. The analysis was performed based on the video recordings, using a stopwatch and a tally counter. Sniffs were counted if the mouse sniffed with a clear directionality towards the cotton bud and was approximately 1.5 cm from it.

Open Field Test

The open field test was used to assess locomotor and anxiety-like behaviour [59–61]. Individual mice were placed in a circular arena of white acrylic (diameter 40 cm, 40 cm height, online suppl. Fig. S1c), with light level at the centre of the arena at 10lux (white light) for 10 min. A 20-cm-diameter circle at the centre of the open field arena was designated the centre zone, a ring 20 cm thick around the perimeter of the arena designated as the outer zone. Avoidance of the centre zone was used as a measure of anxiety, and total distance moved (cm) and velocity (cm/s) in the outer zone were used as measures of locomotor activity.

LD Box Test

The LD box was used to assess anxiety-like behaviour [62, 63]. The LD box arena was made of white acrylic (44 cm long × 21 cm wide × 21 cm height), unequally divided into two chambers by a high white acrylic separator (21 × 50 cm). The dark zone chamber was 1/3 of the arena and had low lighting (10lux), whereas the adjacent chamber was brightly lit, at 100lux, and was defined as the light zone. A small opening (5 × 7 cm) in the separator allowed mice to move freely between chambers (online suppl. Fig. S1d). Individual mice were placed in the dark zone of the arena facing the end wall (opposite to the separator) for the start of the trial. Mice were allowed to freely explore the arena for 5 min, avoidance of the light chamber was used as a measure of anxiety, and total distance moved (cm) and velocity (cm/s) in the dark zone were used as measures of locomotor activity.

Marble-Burying Test

The marble-burying test was used to assess repetitive behaviour [64, 65]. Mice were individually placed in empty home cages (39.6 cm wide × 21.5 cm depth × 17.2 cm height) containing 12 glass marbles, in 4 × 3 arrangement with equal distances between marbles, on a 5 cm thick layer of sawdust (online suppl. Fig. S1e). Marbles were placed occupying ¾ of the cage. For the beginning of the trial, mice were placed in the marble-free zones and allowed to freely explore the arena for 30 min. The number of buried marbles was scored manually at 10, 20, and 30 min, and total distance moved (cm) and velocity (cm/s) in the empty (marble-free) zone were used as measures of locomotor activity for the entire 30-min test period.

Sucralose Preference Test

The sucralose preference test was carried out in home cages based on the two-bottle paradigm (online suppl. Fig. S1f) [66] and was used to assess anhedonia [67, 68]. On each test day, measurements of both bottles of water, food, and mouse body weight were taken. On day 1 of testing, mice were exposed for 24 h to two bottles both containing drinking water. On day 2, mice were given free access to one bottle containing a 0.5% sucralose (Sigma-Aldrich) solution and another containing water. On day 3, the location of the water and sucralose bottles were switched between their left and right positions, to counteract any potential side

preference, with final measurements of water bottles, sucralose bottles, food, and mouse body weight recorded on day 4. Sucralose solution was freshly made every morning. Six empty "spill" cages were placed on the housing racks to control for normal cage handling spillage. Side preference was computed by calculating the percentage of consumption of water placed on the left side out of the total (left and right side) fluid intake ([left side intake/total intake] × 100). Sucralose preference was determined by calculating the percentage of sucralose intake out of the total (sucralose and water) fluid consumption ([sucralose intake/total intake] × 100) over 48 h.

Statistics

Statistical analyses were conducted using GraphPad Prism 9 and Statistica (version 5.5, StatSoft, Inc., Tulsa, OK, USA). All data were tested for normality using the Shapiro-Wilk test. Data did not deviate from normality, which was set at $p = 0.1$. Data from the social interaction, open field, LD box, sucralose preference, and locomotor activity in the marble-burying tests were analysed using 2-way ANOVA (main independent factors of muscimol exposure and sex). Post hoc analysis was conducted with Sidak's multiple comparison test. The olfactory habituation/dishabituation test was assessed using a 3-way repeated measures ANOVA test where the repeated factor was time as odour stimuli were presented every 3 min, and sex and muscimol treatment were independent factors. For the marble-burying test, the number of buried marbles was assessed every 10 min, so a 3-way repeated measures ANOVA was used where 10-min time bins were the repeated factor, and sex and muscimol exposure were independent factors. Total cortical layer thickness and thickness of individual cortical layers were separately assessed using 2-way ANOVA where independent factors were muscimol exposure and sex. Sidak's multiple comparison test was used for post hoc comparisons. The number of cortical microglia (cells/mm^2) was compared between treatment groups using an unpaired Student's *t* test. Cytokine expression was statistically assessed using a 2-way ANOVA, with muscimol exposure and cytokine type as independent factors. Sidak's multiple comparison test was used for post hoc comparison. The effect size for biological relevance of the *p* value was estimated using partial eta square (η^2), where $\eta^2 = 0.01$ indicates a small effect, $\eta^2 = 0.06$ indicates a medium effect, and $\eta^2 = 0.14$ indicates a large effect. Data are presented as mean ± SEM, and the *p* value for statistical difference was set at $p < 0.05$. See online supplementary Table S2 for a complete record of data from statistical analysis.

Results

Muscimol Exposure Produces an Early Inflammatory Response in the Brain and Altered Cortical Layer Architecture at P6 and P20 in Treated Mice

Prior to analysis of adult behaviour, the short-term response of the brain to muscimol treatment was assessed. Cytokine analysis 4 h after a single exposure showed no overall effect of muscimol treatment on expression of inflammatory mediators (2-way ANOVA $F_{(1, 28)} = 3.858$, $p = 0.059$, $\eta^2 = 0.12$, Fig. 1a), but a specific increase in the expression of IFN-γ (1.6-fold, $p = 0.018$) following post hoc analysis. Microglia analysis performed at the same time point showed an average of 170 ± 37 cells/mm^2 in brains from vehicle-treated animals, which was not significantly different following muscimol treatment (192 ± 49 cells/mm^2, $p = 0.521$, Fig. 1b–d).

Muscimol administration from P3 to P5 resulted in short- and longer term alterations in cortical structure of the somatosensory cortex (Fig. 1e–h). Data from P6 showed that muscimol had a main treatment effect on total cortical thickness (2-way ANOVA $F_{(1, 17)} = 4.818$, $p = 0.042$, $\eta^2 = 0.22$, Fig. 1e). Sidak's multiple comparison test detected differences between vehicle and muscimol male groups where treated males displayed a statistically significant increase in total cortical thickness ($p = 0.011$). Average cortical thickness in male treated mice was 942.4 ± 24.1 μm, whereas the thickness of control mice was 784.5 ± 52.3 μm; an interaction between treatment and sex was also found (2-way ANOVA $F_{(1, 17)} = 5.634$, $p = 0.029$). When analysing cortical layers individually (Fig. 1f), a main effect of muscimol treatment was detected in layer VI thickness (2-way ANOVA $F_{(1, 17)} = 4.739$, $p = 0.043$, $\eta^2 = 0.21$), and in particular, layer thickness in the male muscimol group was increased (control 195.3 ± 21.2 μm, muscimol 249.3 ± 9.5 μm, $p = 0.029$). Additionally, an interaction between muscimol exposure and sex was detected in layer III–II (2-way ANOVA $F_{(1, 17)} = 5.843$, $p = 0.027$), suggesting that muscimol had different effects in male and female groups in layer III–II.

At P20, there were no differences in total cortical thickness (Fig. 1g) in male or female brains following muscimol treatment during P3–5. However, analysis of individual cortical layers (Fig. 1h) showed that muscimol had a main effect in layer VI in both male and female mice (2-way ANOVA $F_{(1, 8)} = 6.458$, $p = 0.034$, $\eta^2 = 0.44$), with a trend to increased layer thickness in treated mice. Specifically, treated females showed a statistically significant increment of layer VI thickness ($p = 0.048$). In layer VI, the average thickness was 267.4 ± 9.0 μm in the female muscimol group and 225.6 ± 5.2 μm in the female control group. In layers III–II, muscimol had a main treatment effect over the groups, with a trend towards muscimol increasing layer thickness in treated male and female (2-way ANOVA $F_{(1, 8)} = 6.037$, $p = 0.039$, $\eta^2 = 0.43$), although Sidak's post hoc comparison did not detect specific group differences.

Mice Respond to Early-Life Muscimol Exposure with Altered Social Interaction in Adulthood

There was a significant decrease in the frequency of adult social interaction in mice administered muscimol in early life compared with vehicle controls (2-way ANOVA

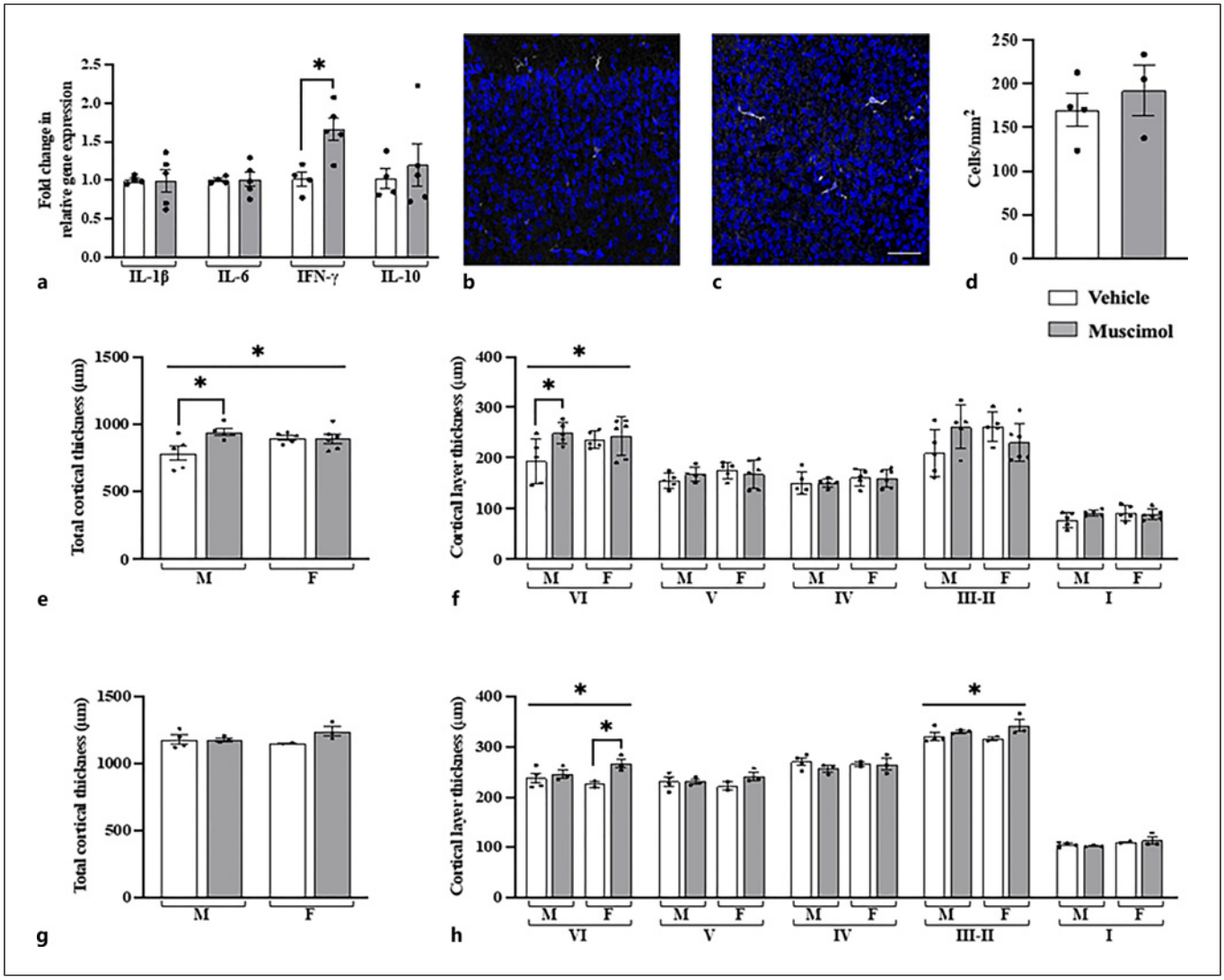

Fig. 1. Postnatal muscimol treatment is associated with an acute increase in IFN-γ and altered cortical development. **a** Cytokine expression analysis, using qRT-PCR in brain samples at P3, 4 h post-treatment, showed a significant increase in IFN-γ, but no change in other inflammatory mediators. Nor was there a change in the number of microglia within the cortex (**b–d**; white = microglia, blue = DAPI stained nuclei). **e** There was a main effect of muscimol on total cortical thickness of brains at P6. Particularly, total cortical thickness was increased in male muscimol group. **f** There was a main effect of muscimol treatment in layer VI thickness, where layer thickness was increased in the male muscimol group. **g** There were no differences in total cortical thickness following early muscimol treatment at P20. **h** There was a main effect of muscimol treatment in layer VI thickness, where layer thickness was increased in the female muscimol group, and in layers III–II with no specific group differences. Data are shown as mean ± SEM and were analysed using 2-way ANOVA. *p <0.05 (**a**, **e–h**), or Student's t test (**d**). Sample sizes are the following: P3 for PCR vehicle, n = 4, and muscimol, n = 3; P3 for histology vehicle, n = 4, and muscimol, n = 5; P6 vehicle, N = 5 males, N = 6 females; and muscimol, N = 5 males, N = 6 females; and P20 vehicle, N = 4 males, N = 2 females, and muscimol, N = 3 males, N = 3 females. Scale bar = 50 μm (**b**, **c**). M, male; F, female; VI, layer 6; V, layer 5; IV, layer 4; III–II, layers 3–2; I, layer 1; qRT-PCR, quantitative real-time polymerase chain reaction.

$F_{(1, 37)} = 7.474$, $p = 0.009$, $\eta^2 = 0.17$) but no change in the total time spent in social interaction (Fig. 2a, b). Following post hoc analysis, the difference remained significant for females (p = 0.006); there was a significant effect of sex (2-way ANOVA $F_{(1, 37)} = 6.403$, p = 0.015, $\eta^2 = 0.15$) as females performed more social interactions than males, and there was no significant interaction between treatment and sex. When the social behaviour was broken down into specific types of social interaction, data on anogenital sniffing showed that muscimol had a

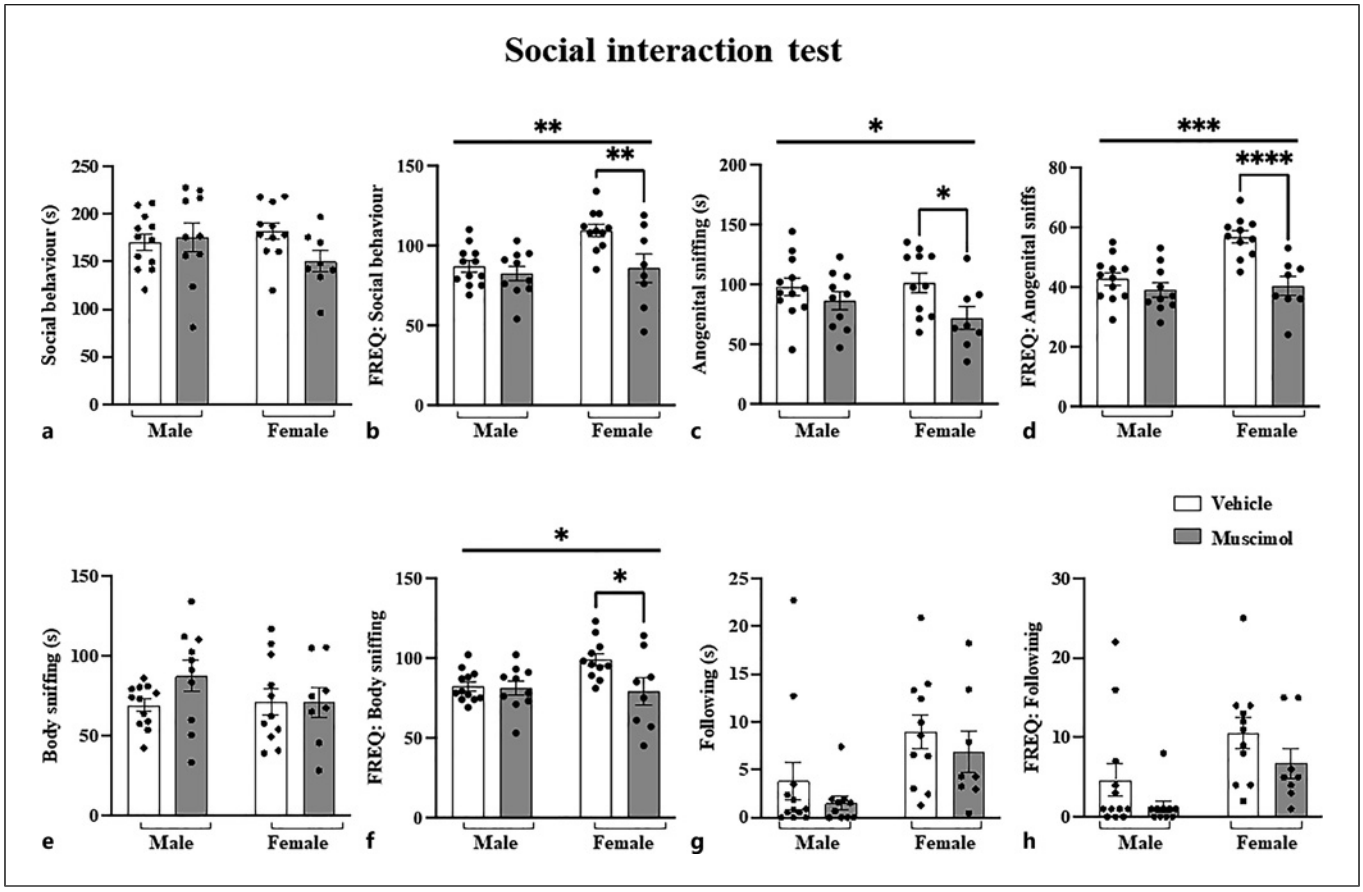

Fig. 2. Postnatal muscimol treatment induced social deficits in adult females. Cumulative time of social behaviours is shown in **a**, **c**, **e**, and **g**, and frequencies of respective behaviours in **b**, **d**, **f**, and **h**. Although total duration of social behaviour (**a**) was similar among groups, frequency of total social behaviour events (**b**) was decreased in muscimol-exposed females. Significantly reduced duration (**c**) and frequency (**d**) in anogenital sniffing were seen in the muscimol-exposed female group. There were also differences in frequency of body sniffing in muscimol-exposed females compared to control females (**f**), although duration of body sniffing was similar among groups (**e**). Duration of following (**g**) and frequency (**h**) were not different between muscimol-exposed and control animals. Data are shown as mean ± SEM and were analysed using 2-way ANOVA. *p <0.05, ***p <0.001, and ****p <0.0001. Sample sizes: male vehicle, N = 12, male muscimol, N = 10; female vehicle, N = 11, female muscimol, N = 8. FREQ, frequency.

main effect of reducing the duration (2-way ANOVA $F_{(1, 37)} = 6.215$, $p = 0.017$, $\eta^2 = 0.14$, Fig. 1c) and frequency (2-way ANOVA $F_{(1, 17)} = 17.350$, $p = 0.0002$, $\eta^2 = 0.32$, Fig. 2d) in both male and female groups, although this only remained significant for females following post hoc analysis (duration $p = 0.038$, frequency $p < 0.0001$). There was a significant effect of sex (2-way ANOVA $F_{(1, 37)} = 10.310$, $p = 0.002$, $\eta^2 = 0.21$) as females sniffed the anogenital area of the CM more than males, and there was also a significant interaction between treatment and sex ($F_{(1, 37)} = 6.967$, $p = 0.012$, $\eta^2 = 0.15$). In addition, muscimol had a main effect on body sniffing frequency (2-way ANOVA $F_{(1, 37)} = 4.695$, $p = 0.036$, $\eta^2 = 0.11$, Fig. 2f) as it decreased number of events in exposed females ($p = 0.016$). Following behaviour was not altered between control and treated groups (Fig. 2g, h), although females engaged more in following behaviour than males (duration of following: 2-way ANOVA $F_{(1, 37)} = 8.693$, $p = 0.005$, $\eta^2 = 0.19$, Fig. 1g; frequency of following events: 2-way ANOVA $F_{(1, 37)} = 9.789$, $p = 0.003$, $\eta^2 = 0.2$, Fig. 1h). No significant aggressive behaviour was observed among groups.

Since mice rely heavily on olfaction for social interaction [58], the olfactory habituation/dishabituation test (Fig. 3) was used to determine whether mice in this cohort could smell, in order to rule out a potential confounding factor for the social interaction test. This olfaction test confirmed that the olfactory skills of these mice were intact; hence, altered social interactions in muscimol-exposed female mice were likely due to specific effects of muscimol on social behaviour and not due to an

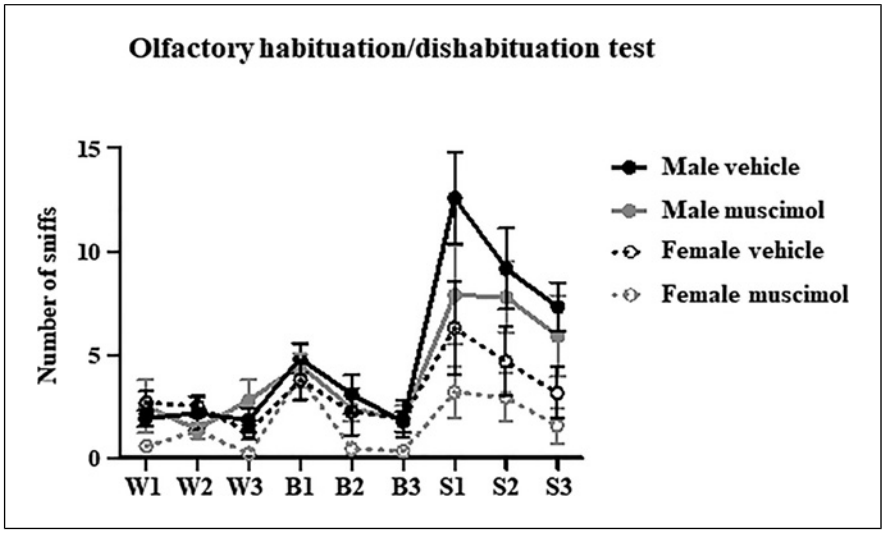

Fig. 3. Muscimol did not impair olfaction capacity in mice in the olfactory habituation/dishabituation test. There were no differences in number of sniffs between mice exposed to 0.5 mg/kg muscimol compared to controls for either male (solid lines) or female (dotted lines) groups. Mice were presented three odours three consecutive times as shown in the X-axis; these were water (W1–3), banana essence (B1–3), and social cues (odour from another same-sex mouse's cage, S1–3). Data are shown as mean ± SEM and were analysed using 3-way ANOVA. Sample sizes are the following: male vehicle, $N = 12$, male muscimol, $N = 10$; female vehicle, $N = 11$, female muscimol, $N = 8$.

inability of these mice to smell. However, there was a significant sex factor (3-way ANOVA $F_{(1,37)} = 11.845$, $p < 0.01$, $\eta^2 = 0.24$) as there was more sniffing in males versus females, this was expected as males are more driven to scent mark territory [69].

Male and Female Mice Exposed to Muscimol Showed Reduced Anxiety in the LD Box

Muscimol exposure during early postnatal life had no significant effect on adult locomotor behaviour and anxiety in the open field when compared with control groups (Fig. 4a–d), although all mice displayed a high baseline level of anxiety (spending on average <10% time in the centre). There was a significant sex factor in distance (2-way ANOVA $F_{(1,37)} = 5.339$, $p = 0.026$, $\eta^2 = 0.12$) and velocity in outer zone (2-way ANOVA $F_{(1,37)} = 4.284$, $p = 0.045$, $\eta^2 = 0.1$) as female mice covered more distance and moved faster than male mice. Subsequently, the LD test was used as this arena generates less anxiety in mice (Fig. 4e–h). There was no difference in the duration spent in the light zone (Fig. 4e), but there was a significant main effect of muscimol exposure in latency to first entry into the light zone (2-way ANOVA $F_{(1, 37)} = 12.75$, $p = 0.001$, $\eta^2 = 0.25$, Fig. 4f), with male ($p = 0.026$) and female ($p = 0.036$) groups displaying reduced latency suggesting a possible decrease in anxiety following muscimol administration. Consistent with data from the open field test, there were no significant differences in distance moved (Fig. 4g) or velocity (Fig. 4h) in the outer zone, suggesting no locomotor activity changes as a result of muscimol treatment. There was a significant sex factor in distance moved in the dark zone (2-way ANOVA $F_{(1,37)} = 13.23$, $p < 0.001$, $\eta^2 = 0.26$) as female mice moved more than male mice.

No Evidence of Repetitive Behaviour in the Marble-Burying Test following Early-Life Activation of $GABA_AR$

Repetitive behaviour, a common behavioural phenotype in NDDs [70], was assessed in the marble-burying test (Fig. 5a–c). Marbles were counted every 10 min and number of buried marbles increased over time, but there were no differences in the number of marbles buried between muscimol and control groups across the 30-min test (Fig. 5a). The only significant factor was time indicating that marbles were buried (3-way ANOVA $F_{(5, 185)} = 153.446$, $p < 0.001$, $\eta^2 = 0.8$). As with the previous tests, muscimol did not alter baseline locomotor activity in the empty (marble-free) zone (Fig. 5b, c).

Early-Life Muscimol Exposure May Have Reduced Sucralose Preference in Adult Males

To determine if early postnatal muscimol administration induced anhedonia, indicated by a decreased intake of a sweet (rewarding) substance, a sucralose preference test was performed. There was a main effect of muscimol which showed as a slight but significant decrease in the preference of mice for sucralose (2-way ANOVA $F_{(1, 37)} = 4.201$, $p = 0.047$, $\eta^2 = 0.1$, Figure 6), with post hoc comparison showing a significant subgroup difference in male mice ($p = 0.029$). However, the effect size was small and likely driven by an outlier in the male muscimol-treated group.

Discussion

In this study, mouse behaviour was examined to determine whether acute disruption of the E/I balance in early development could cause sex-specific long-term behavioural

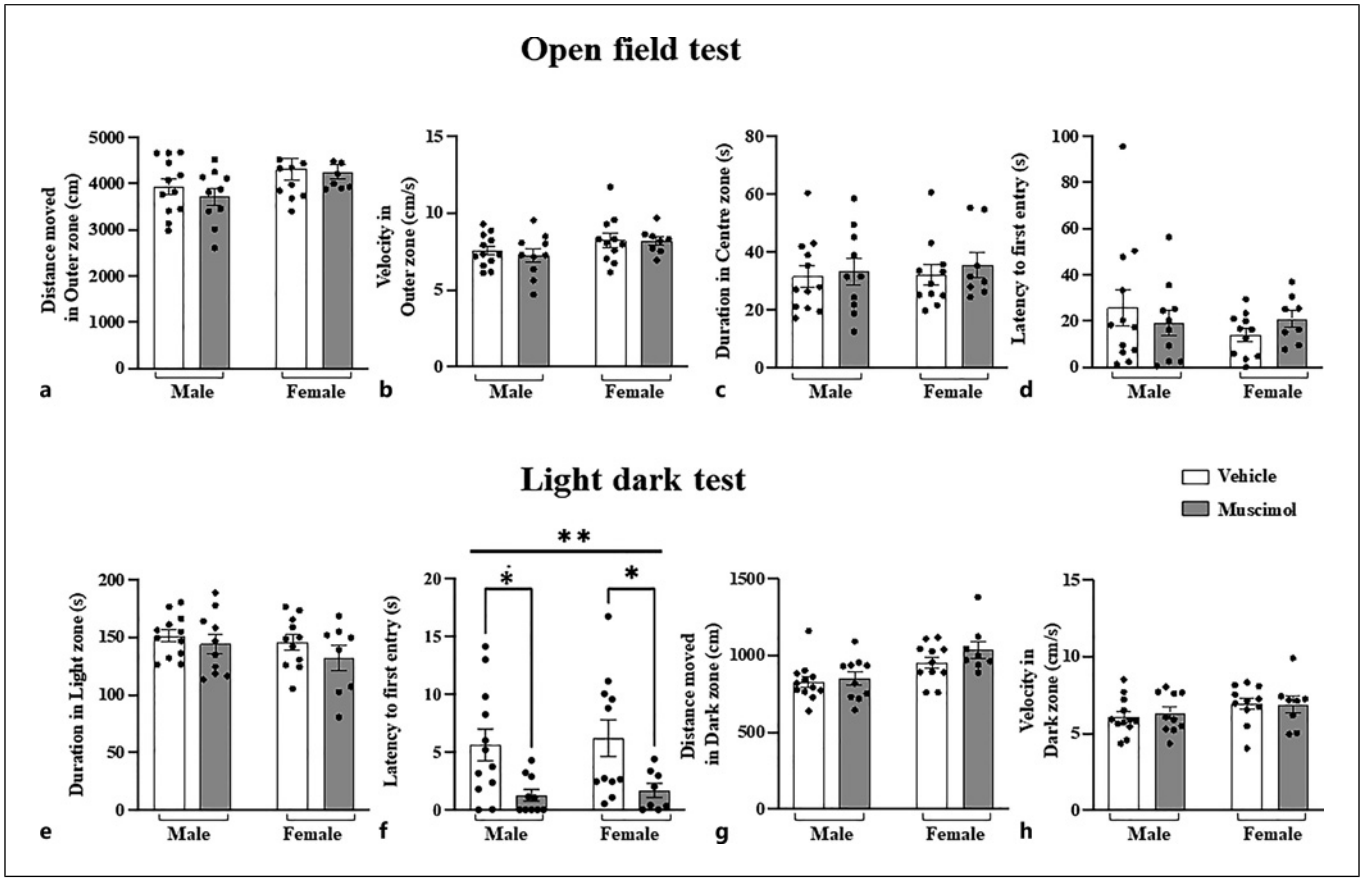

Fig. 4. Muscimol did not alter baseline locomotor activity or induce anxiety-like behaviour in the open field test, but it reduced anxiety-like behaviour in the LD box test. In the open field, there were no differences in locomotor activity between mice exposed to 0.5 mg/kg muscimol compared to vehicle-treated controls as shown in distance moved (**a**) or velocity (**b**) in the outer zone. Parameters of anxiety-like behaviour were not altered between groups, as measured by duration in centre zone (**c**) and latency to first entry into the centre zone (**d**). Duration in the centre zone was measured for the first 5 min and distance and velocity in the outer zone for 10 min. The duration in the light zone, a parameter of anxiety-like behaviour, was not altered between groups (**e**) but latency to first entry into the light zone was reduced in muscimol-exposed groups (**f**) suggesting muscimol reduced anxiety. In the LD box paradigm, there were no differences in locomotor activity between mice exposed to 0.5 mg/kg muscimol compared to controls as shown in distance moved (**g**) or velocity (**h**) in the dark zone. Data are shown as mean ± SEM and were analysed using 2-way ANOVA. *p <0.05 and **p <0.01. Sample sizes: male vehicle, N = 12, male muscimol, N = 10; female vehicle, N = 11, female muscimol, N = 8.

changes. These results show that acute activation of the $GABA_AR$ through muscimol treatment in early postnatal life led to significant changes in adult behaviours, supporting the link between E/I imbalance in early life and long-term changes in brain function. Moreover, there was a sex difference in the behavioural phenotypes observed, with muscimol exposure significantly altering social behaviour in female mice, but not in male mice exposed to muscimol in early postnatal life. Both male and female mice exposed to muscimol displayed reduced anxiety-like behaviour in adulthood. There was no confounding effect of muscimol on locomotor activity measures or on olfactory ability, suggesting specific effects of muscimol, and therefore $GABA_AR$ activity, on the developmental processes that define these behaviours. Furthermore, increased cortical thickness in some cortical layers was detected by P6 in male and by P20 in female mice treated with muscimol, suggesting that the actions of GABA on developmental events effecting cortical thickness may contribute to sex-specific differences in long-term behaviour.

The clearest sexually dimorphic change found in this study was for social behaviours; these behaviours clearly map to NDDs such as ASD. While ASD itself has a higher rate of diagnosis in males (4:1 incidence ratio [4, 71]),

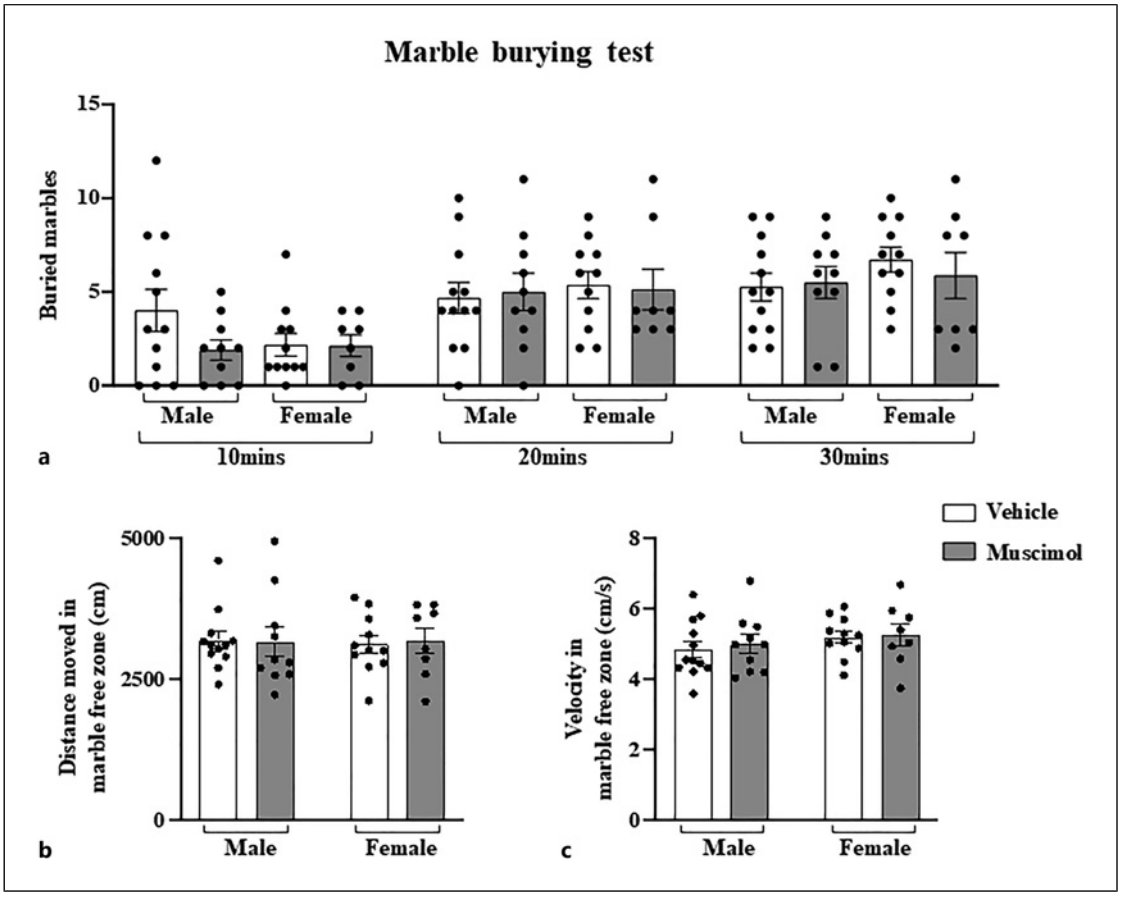

Fig. 5. Muscimol did not alter marble-burying behaviour. In the marble-burying test, there were no differences in number of buried marbles between mice exposed to 0.5 mg/kg muscimol compared to controls for either male or female (**a**) groups. Parameters of locomotor activity were also not altered between groups based on distance moved (**b**) and velocity (**c**) in the empty (marble-free) zone. Data are shown as mean ± SEM and were analysed using 3-way ANOVA for **a** and 2-way ANOVA for (**b**) and (**c**). Sample sizes are the following: male vehicle, $N = 12$, male muscimol, $N = 10$; female vehicle, $N = 11$, female muscimol, $N = 8$.

there is clear recognition of sex differences in the expression of behavioural phenotypes [3]. Social impairments have been observed in mouse models of ASD, such as the neuroligin 3- [72] or 4-deficient mouse [73] and the parvalbumin-deficient mouse [74]. Similarly, sex differences have been noted in clinical depression [75], although most rodent studies in this field are conducted in males. It is important to note that in the present study, while male mice had a small but significantly reduced sucralose preference compared to female mice, all mice still displayed a significant preference for sucralose. These findings are not, therefore, interpretable as anhedonia or a model of depression. They do still provide some evidence for a sex-specific effect of muscimol treatment on a range of behaviours and, together with the sex difference seen in social behaviour, show that findings from males cannot be directly translated to females.

The findings from this study are consistent with our growing understanding that the brain follows sexually dimorphic developmental trajectories. During brain development, gonadal steroid hormones, along with the interplay of glutamate and GABA neurotransmitters, exert long-term organisational effects that drive the establishment of sexually dimorphic neuronal architecture in the adult [76, 77]. One example of sexually dimorphic brain development is the evidence that there are sex differences in GABA concentrations as early as P1 in the rat [78], with higher levels of GABA and glutamate in the CA1 region of the hippocampus, and in the ventromedial nucleus and arcuate nucleus of the hypothalamus of males compared with females. In contrast, by P5, female rats had greater concentration of GABA in the arcuate nucleus. It is therefore plausible that a global increase in GABA activity, such as that produced in this study, would affect the

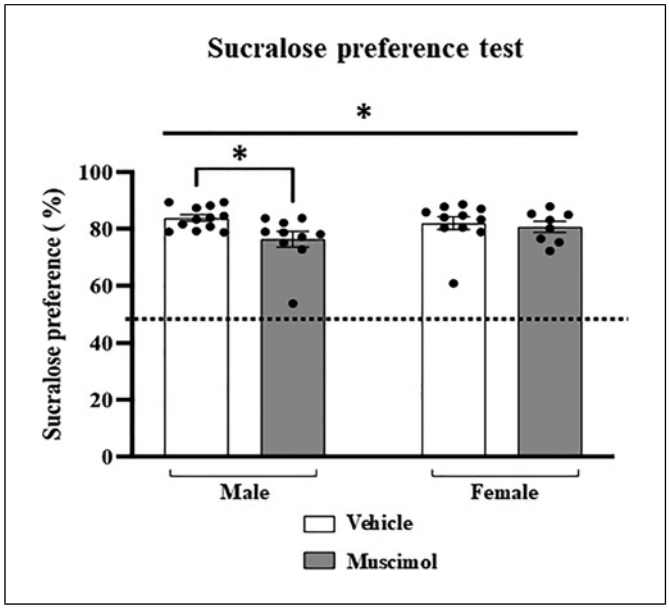

Fig. 6. Postnatal exposure to muscimol reduced sucralose preference in mice. A small but significant reduction in sucralose preference over drinking water was found in muscimol-exposed groups as adults. Post hoc tests showed a significant preference of vehicle-treated male mice for sucralose over drinking water compared to muscimol-exposed males. Data are shown as mean ± SEM and were analysed using 2-way ANOVA. *p <0.05. Sample sizes: male vehicle, N = 12, male muscimol, N = 10; female vehicle, N = 11, female muscimol, N = 8.

developmental trajectory of the brain differently between males and females, potentially in a regional specific manner. Similarly, the GABA switch occurs earlier in females [77] and $GABA_AR$ subunit expression follows a time and sex-dependent pattern [79], providing an additional potential mechanism for this process. Of note, while substantial developmental variation in $GABA_AR$ subunit expression has been reported, there are limited data on sex-dependent patterning. The data that exist show differential variation for α1, α2, and γ2 subunits from P15 onwards, depending on brain region [79, 80]. Further work is required to determine if earlier changes occur that may contribute to the events described here.

In the present study, muscimol was given systemically and therefore is likely to be acting on multiple brain regions which could explain the nature of behavioural changes observed. It has been suggested that the corticolimbic circuitry, including the medial PFC and the basolateral amygdala, is a key regulator of social behaviour [81, 82], and that normal GABAergic transmission is required for these behaviours [83]. Data supporting this idea come from studies where region-specific microinjections of bicuculline were used to decrease GABA function, increasing neural activity and generating E/I imbalance in adult rodents [83]. In social interaction and social preference tests, decreased duration and number of social interactions were observed upon bicuculline administration in either of these brain regions, suggesting that GABAergic function is implicated in social behaviour [83]. Previous rodent studies reported lower GABA levels, decreased number of $GABA_ARs$, reduced innervation and function of GABAergic axons, and reduced levels of glutamate decarboxylase 67, vesicular GABA transporter, and GABA transporter 3 in the PFC associated with anhedonia and other depression-like behaviours [84–89]. It could, therefore, be hypothesised that sex differences in depression and social behaviour may arise from developmental events during a period of sexual dimorphism that produce long-term, regionally specific reductions in GABA activity. More work is required to determine the veracity of this hypothesis, the age-dependent window of sensitivity, and the interaction between age and sex.

In addition to sexually dimorphic differences in social behaviour, a reduced latency for the first light zone entry in the LD test was found in male and female mice following postnatal administration of muscimol. This could be interpreted as a reduction in anxiety, or alternatively, this reduced latency behaviour could be indicative of decreased risk assessment or increased risk taking and impulsivity. These are behavioural features associated with adolescence, where the GABAergic system is still developing; its maturation has been proposed as a defining feature of adulthood [90]. Therefore, it may be that impulsive behaviour is indicatory of an immature GABAergic system, and that mice exposed to muscimol in the current study might possess an underdeveloped GABAergic system. Certainly, GABA, along with dopamine and serotonin, has been proposed to modulate impulsivity [90]. Spectroscopy studies in impulsive rats, confirmed by the 5-choice serial reaction time task, showed decreased levels of GABA, in the ventral striatum [91], in addition to reduced grey matter density and GAD65 and 67 expression in the nucleus accumbens [92]. This suggests that deficient GABAergic transmission could underlie the behavioural changes seen in the LD test. More specifically, impulsivity is a phenotype found in patients with ASD and attention deficit hyperactivity disorder, related to impaired executive function [93], and has been found in mouse models of NDDs, e.g., in Fmr1 knockout mouse model of Fragile X syndrome (reviewed by [94]). This study was not specifically designed to test impulsivity, and further work will be needed to understand the effects of early E/I imbalance in

this behavioural phenotype. Given the prevalence of anxiety as a comorbidity in NDDs [7] and previous research in this area, it had been hypothesised that early muscimol treatment would produce anxiety-like behaviours. A similarly designed study to the current one found opposing effects on anxiety, with NMRI (outbred) mice showing increased anxiety- (and depression-) like behaviours at P80–90, following subcutaneous administration of 0.5 mg/kg muscimol from P3 to P5 [50]. The study also reported anxiety-like behaviour in the open field test [50]. However, these studies were conducted under conditions that were more inherently anxiety-inducing, with test animals placed directly in the aversive area at the start of each trial and the open field arena directly and brightly illuminated [50].

Repetitive behaviour is a common feature of NDDs that has been recapitulated in some mouse models of ASD. In the present study, muscimol administered repeatedly at a critical developmental stage did not produce detectible repetitive behaviour in adulthood using the marble-burying test. There is evidence that acute systemic administration of muscimol (1 mg/kg), minutes before testing, can induce increases in repetitive marble-burying behaviour in 5-week-old male ICR (outbred) mice [95], presumably through increased inhibitory drive. In the current study, data suggest that any long-term changes of early-life muscimol exposure did not alter repetitive behaviour and, therefore, abnormal GABA function in the first postnatal week of mouse brain development may not be key to the establishment of these behaviours, at least not in mice with a pure genetic background.

Having determined that altered E/I balance in development can produce sex-dependent alterations in long-term behaviour, it is imperative that we understand the neurobiological changes that underlie these. As a starting point, increased cortical volume and social deficits are traits related to ASD [2]. Precise concentrations of GABA are required for normal brain development; therefore, excessive activation of the $GABA_AR$ might be interfering with aspects of normal cortical development such as cell proliferation or cell death, migration, neurite arborisation, or glia activation, and as a result, mice in this study developed thicker cortices. Interestingly, IFN-γ has been associated with acute increases in GABA release [96], inhibitory activity on layer V neurons [97], and neuronal differentiation and outgrowth [98]. An interplay between the administered $GABA_AR$ agonist and subsequent inflammatory signalling in the brain may, therefore, contribute to the increased cortical layer thickness observed in the present study. These findings are at least particularly supported by the work of Heck and colleagues [99], who reported severe cortical layering abnormalities following muscimol administration in newborn rats. Using a cortical surface ELVAX implant, local application of muscimol (25pmol/24-h release rate) during P0–2 generated abnormalities such as focal cortical dysplasia and heterotopia with atypical somatodendritic orientation of pyramidal cells in the outermost cortical layers and particularly ectopic cell cluster in layer I of P7 Wistar rats [99].

Microglia are activated by sex hormones [100] and have been found to be necessary for the sexually dimorphic brain development [101]. In addition to their roles responding to injury, microglia have been found to regulate dendritic pruning and remodelling of cortical neurons to eliminate weak synapses and increase efficiency and specialisation [102, 103]. They have also been linked to the pathogenesis of NDDs such as schizophrenia [104] or ASD [105]. No alteration in the microglia population was found 4 h following early-life muscimol exposure, despite the detectable increase in IFN-γ. Further analysis of microglial number, activity, and inflammatory response at later time points is warranted, along with other elements of cortical microstructure, to fully understand the cellular and molecular response of the brain to acute E/I imbalance.

Overall, the findings of this study showed that acute disruption to the E/I balance during a particular period of brain development resulted in a sexual dimorphic presentation of behavioural deficits in the adult mice. Reduced anxiety and social behaviour observed in this study could both result from decreased levels of GABA. The finding that females preferentially displayed social deficits is of particular interest as this paradigm could aid elucidating sex differences in GABAergic system development and in the pathophysiology underlying many NDDs. Further studies measuring regional and sex-specific changes in GABA levels and GABAergic transmission, and the impact on resting and stimulus-dependent activity over time would determine when and where muscimol may be exerting its effects. This would provide a deeper understanding of the circuitry of behavioural differences over critical periods of development which may be altered in NDDs.

Statement of Ethics

All animal work was carried out in accordance with the UK Animals (Scientific Procedures) 1986 Act, UK Home Office (Project Licence PDAD9E285), and project approval from the

Royal Veterinary College Animal Welfare and Ethical Review Board (#2018-0114N). ARRIVE guidelines were followed in the experimental conceptualisation and execution of these studies.

Conflict of Interest Statement

The authors have nothing to disclose.

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Author Contributions

Conceptualisation and investigation and formal analysis: H.B.S., A.G.-V., and C.F.; methods: A.G.-V. and C.F.; resources: C.T., H.B.S., and C.F.; writing – original draft preparation: A.G.-V. and H.B.S.; writing – review and editing: all authors; supervision: C.F., H.B.S., and M.S.C.T.; project administration: H.B.S.; funding acquisition: H.B.S., M.S.C.T., and C.T.

Data Availability Statement

All raw data are available upon request. All data generated or analysed during this study are included in this article and its online supplementary material. Further enquiries can be directed to the corresponding author.

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