Original Paper

Acute treatment with 5-hydroxytryptophan increases social approach behaviour but does not activate serotonergic neurons in the dorsal raphe nucleus in juvenile male BALB/c mice: A model of human disorders with deficits of sociability

Journal of Psychopharmacology
2022, Vol. 36(7) 806–818

DOI: 10.1177/02698811221089039
journals.sagepub.com/home/jop

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Abstract

Background: The BALB/c mouse has been proposed as a model of human psychiatric disorders characterised by elevated anxiety and altered sociability. Juvenile BALB/c mice show decreased social exploratory behaviour, increased anxiety, and reduced brain serotonin synthesis compared to other strains including C57BL/6J mice.

Aim: To determine whether supplementation of brain serotonin synthesis alters social behaviour and activation of serotonergic neurons across subregions of the dorsal raphe nucleus (DR) in BALB/c mice.

Methods: Juvenile male BALB/c mice were assigned to one of four treatment conditions: vehicle/vehicle, carbidopa (25 mg/kg)/vehicle, vehicle/5-HTP (10 mg/kg), carbidopa (25 mg/kg)/5-HTP (10 mg/kg). Social behaviour was measured using the three-chamber social approach test, followed by immunohistochemical staining for TPH2 and c-Fos to measure activation of serotonergic neurons across subregions of the DR.

Results: Mice treated with carbidopa/5-HTP spent more time in the social cage zone and covered more distance in the social approach test compared to other treatment groups. There was no difference between treatment groups in the activation of serotonergic neurons across subregions of the DR. However, the DRD was associated with increased social approach behaviour in carbidopa/5-HTP treated animals.

Conclusions: Supplementation of serotonin synthesis can increase social approach behaviour in juvenile BALB/c mice. An increase in locomotor behaviour was also observed suggesting that increasing central serotonin synthesis may have led to a reduction in state anxiety, manifesting in increased exploratory behaviour. As no effect on serotonergic activation within the DR was found, alternative mechanisms are likely important for the effects of 5-HTP on social behaviour.

Keywords

Serotonin, 5-HTP, anxiety-like defensive behaviour, social behaviour, dorsal raphe nucleus, autism

Introduction

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder with an increasing prevalence rate (Onaolapo and Onaolapo, 2017) that currently affects one in 59 individuals (Baio et al., 2018). Apart from the core symptoms of ASD, which include persistent deficits in social functioning and restricted-repetitive patterns of behaviour (American Psychiatric Association, 2013), a recent review showed that up to 54% of individuals with ASD present with an anxiety disorder (Hossain et al., 2020). Furthermore, children with ASD present with higher anxiety levels compared to typically developing children (van Steensel and Heeman, 2017), highlighting the importance of understanding the neurobiological underpinnings of specific endophenotypes relevant to ASD including anxiety-like defensive behavioural responses and reduced sociability.

There is a growing body of evidence that suggests disruption of serotonin synthesis could play a role in endophenotypes relevant to ASD in humans. Variants in the *TPH2* gene, which encodes tryptophan hydroxylase 2, the rate-limiting enzyme for central serotonin synthesis, are associated with ASD risk (for review, see Ottenhof et al., 2018). In addition, positron emission tomography (PET) with the tracer, alpha[^{11}C]methyl-L-tryptophan, demonstrated that serotonin synthesis capacity is reduced in children with ASD compared to typically developing children

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(Chandana et al., 2005; Chugani et al., 1999). Interestingly, treatment with the selective serotonin reuptake inhibitor, fluvoxamine, which alters serotonin synthesis rates (Mück-Šeler et al., 2012) improves social relatedness in individuals with ASD (McDougle et al., 1996b). Surprisingly, depletion of tryptophan, an essential amino acid and precursor to serotonin, exacerbates repetitive stereotyped behaviours and anxiety, but does not alter social relatedness in individuals with ASD (McDougle et al., 1996a). However, supplementation using tryptophan in healthy individuals improves mood (Kikuchi et al., 2020). Together, this demonstrates that the role of serotonin in endophenotypes of ASD is complex and not well understood. In the biosynthesis of serotonin, tryptophan is oxidised to 5-hydroxytryptophan (5-HTP) which is then converted to serotonin by aromatic amino acid decarboxylase (AADC). Importantly, peripheral AADC can be inhibited using carbidopa, preventing the metabolism of 5-HTP in the periphery. This in turn increases the central availability of exogenous 5-HTP, and evidence has shown that in individuals diagnosed with an anxiety disorder, combining 5-HTP with carbidopa can be successfully used as a treatment for symptoms of anxiety (Kahn et al., 1987; Kahn and Westenberg, 1985). These results suggest that supplementing serotonin synthesis could offer potential therapeutic benefits for managing symptoms of anxiety and potentially aspects of social behaviour in the context of endophenotypes relevant to ASD.

Preclinically, supplementation of serotonin synthesis has been used to alter behaviour. For instance, in the BTBR mouse model of ASD, tryptophan supplementation increases social approach behaviour and whole brain serotonin turnover (Zhang et al., 2015). In a *Tph2* knock out mouse, restoration of brain serotonin synthesis via 5-HTP supplementation reduced marble burying and attenuated impulsive-aggressive behaviour (Angoa-Pérez et al., 2012). Importantly, while knockout (KO) models are crucial in elucidating the mechanisms involved in behaviours related to human psychiatric disorders, they do not accurately model human genetic variation and as such lack ecological validity (Donaldson and Hen, 2015). Furthermore, *Tph2* KO mice display a phenotype characterised by increased panic-like defensive behavioural responses (Waider et al., 2017), suggesting that to investigate anxiety-like defensive behaviour, an alternate option is necessary. The BALB/c mouse displays anxiety-like defensive behavioural responses that are sensitive to benzodiazepines (Griebel et al., 2000) suggesting increased conflict anxiety and/or altered approach/avoidance behaviour. The BALB/c mouse is thought to be a model of endophenotypes relevant to ASD, including anxiety-like defensive behaviour and reduced social behaviour (for review, see Brodkin, 2007). BALB/c mice have a single-nucleotide polymorphism (SNP) in their *Tph2* gene at C1473G, which results in a marked reduction in brain serotonin synthesis (Russo et al., 2019; Zhang et al., 2004). Furthermore, congenic mouse lines on a C57BL/6J and CC57BR/Mv background carrying the 1473G and 1473C alleles provide evidence for a role of the 1473G polymorphism in emotional behaviour (Bazhenova et al., 2017). Tryptophan enhancement in the BALB/c mouse has been shown to increase nesting behaviour (Browne et al., 2012), while depletion can have an anxiolytic effect in the light dark test (Biskup et al., 2012). Importantly, we have previously shown that chronic fluoxetine treatment increases social approach behaviour in male BALB/c mice, and that sociability in the three-chamber social approach test is correlated with serotonergic activation in the rostral part of the dorsal raphe nucleus (DR; Payet et al., 2018). The raphe nuclei are the principal site of brain serotonin synthesis (Gutknecht et al., 2009) and contain the largest proportion of forebrain projecting serotonergic neurons (Hale and Lowry, 2011). To our knowledge, no study has investigated the effects of 5-HTP supplementation in BALB/c mice on social approach behaviour, and activation of serotonergic neurons across subregions of the DR.

In the present study, we tested the hypothesis that reduced brain serotonin synthesis plays an important role in the modulation of anxiety-like defensive behaviour and social behaviour. Juvenile male BALB/c mice were first treated with either vehicle or carbidopa, then received a peripheral injection of either 5-HTP or vehicle. Pre-treatment with carbidopa prevents the peripheral metabolism of 5-HTP allowing exogenous 5-HTP to cross the blood brain barrier, and thereby increasing brain 5-HTP availability (Magnussen, 1984) and central serotonin synthesis. Following drug treatment, mice were then exposed to the three-chamber social approach test as an assay of social behaviour. Activation of serotonergic neurons across multiple subregions of the DR was then measured using immunohistochemistry to investigate the mechanism through which acute 5-HTP supplementation influences serotonergic functioning and potentially anxiety-like defensive behaviour and social behaviour.

Method

Animals

Thirty-two male BALB/c mice, including an additional eight male conspecific mice used as stimulus mice in the three-chamber social approach test (Nadler et al., 2004), were obtained from the Animal Research Centre (ARC, Western Australia). Animals were housed at the La Trobe Animal Research and Teaching Facility and experimental testing commenced following a 7-day acclimation period. All animals arrived at postnatal day 21 (PND 21). Adolescence in mice consists of early adolescence (prepubescent or juvenile, postnatal day (PND) 21–34), middle adolescence (periadolescent, PND 34–46), and late adolescence (PND 46–59) time periods (Spear, 2000). Therefore, these mice were juveniles (PND 21) upon arrival and tested in the three-chamber social approach test at PND 28. Mice were group housed (4 mice allocated per cage such that all mice in the given cage were assigned to the same treatment group) in autoclaved individually ventilated cages (IVC; 39.1 cm length × 19.9 cm width × 16 cm height) with standard bedding on a 12:12 reverse light–dark cycle (lights on at 1900 h); food and water were available ad libitum. Refer to Figure 1 for an illustration of the experimental timelines. All procedures were carried out in accordance with the NHMRC Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013) and with approval from the La Trobe University Animal Ethics Committee. In addition, the research described here was conducted in compliance with the ARRIVE 2.0 Guidelines for Reporting Animal Research (Kilkenny et al., 2010; Percie du Sert et al., 2020). All efforts were made to limit the number of animals used and their suffering.

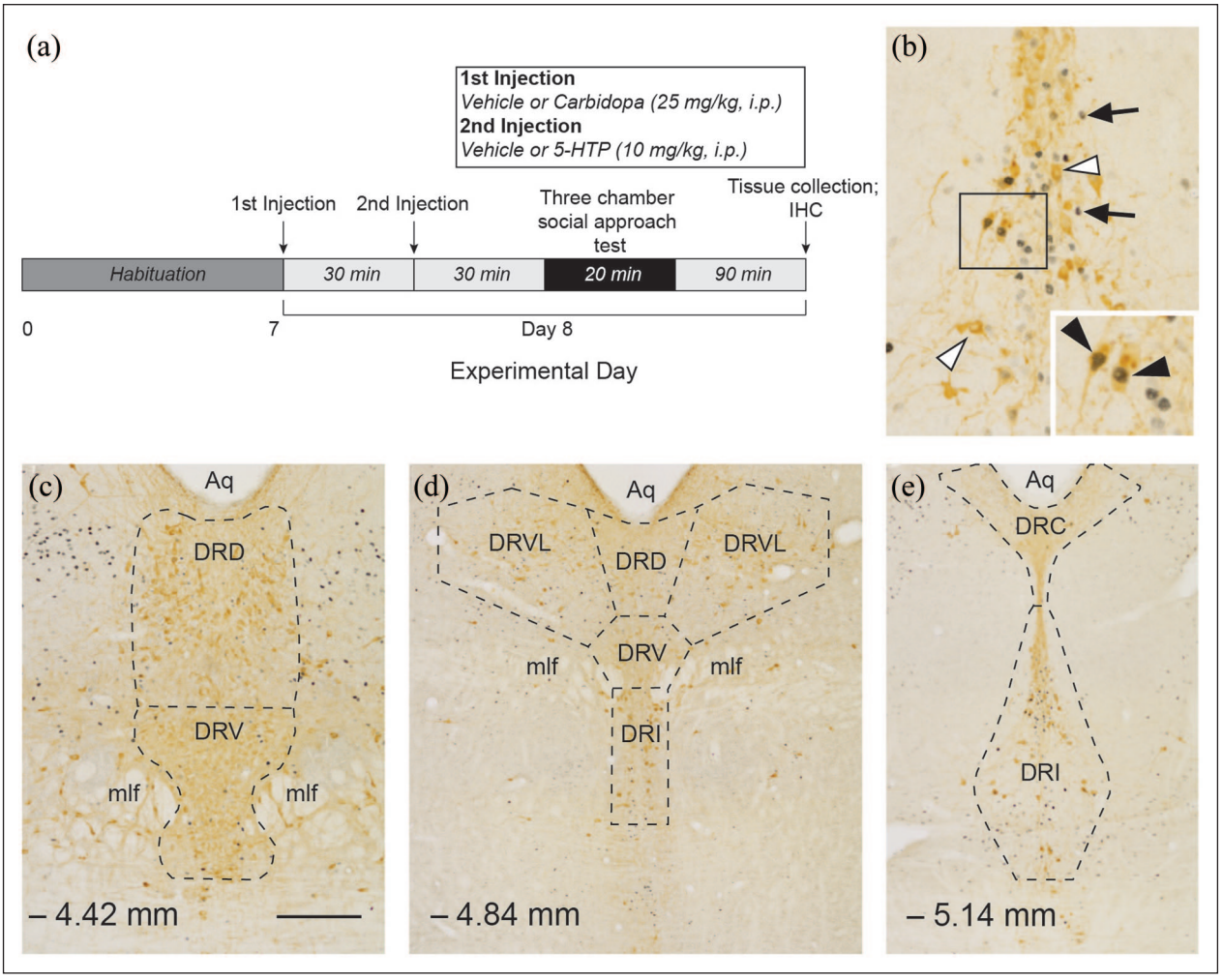

Figure 1. Schematic illustration of the experimental timeline (a), photomicrographs of c-Fos immunoreactivity in serotonergic neurons and non-serotonergic cells (b), and rostral to caudal levels (c, –4.42 mm; d, –4.84 mm; e, –5.14 mm bregma) of the dorsal raphe nucleus (DR) with both TPH2 and c-Fos staining selected for analysis. c-Fos-ir non-serotonergic cells are depicted by solid black arrows, TPH2-ir/c-Fos-immunonegative neurons are indicated by white arrowheads, and c-Fos-ir/TPH2-ir neurons are depicted by solid black arrowheads. Subregions of the DR are depicted by broken lines.

5-HTP, 5-hydroxytryptophan; Aq, aqueduct; DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part; DRVL, dorsal raphe nucleus, ventrolateral part; i.p., intraperitoneal; IHC, immunohistochemistry; mlf, medial longitudinal fasciculus. Scale bar = 200 μm.

Pharmacological treatment

BALB/c mice were assigned to one out of four drug groups prior to being exposed to the three-chamber social approach test, receiving two intraperitoneal (i.p.) injections, 30 min apart. Mice first received either an i.p. injection of 100 μL of vehicle (0.9% sterile saline) or an i.p. injection of 100 μL of 25 mg/kg carbidopa (Cat. No#H9772; Lot#BCBG8592V; Sigma-Aldrich, Castle Hill, NSW, Australia) dissolved in 0.9% sterile saline. Administration of carbidopa prior to 5-HTP has been previously shown to increase the availability of exogenous 5-HTP within the mouse brain, as carbidopa inhibits the metabolism of 5-HTP in both the periphery and in the blood-brain barrier (Magnussen, 1984). Mice then received either an i.p. injection of 100 μL of vehicle or 100 μL of 10 mg/kg 5-HTP (Cat. No#C1335; Lot#118M4107V; Sigma-Aldrich) dissolved in 0.9% sterile saline. Treatment groups were defined as follows: vehicle/vehicle (V/V), carbidopa/vehicle (C/V), vehicle/5-HTP (V/5-HTP) and carbidopa/5-HTP (C/5-HTP). The drugs and doses are based on previous research (Angoa-Pérez et al., 2012).

Three-chamber social approach test

Thirty minutes following the second injection of either vehicle or 5-HTP, social behaviour was measured using the three-chamber social approach test, a validated assay of social behaviour in mice (Nadler et al., 2004). Mice were tested one at a time under red light during the dark phase (~40 lux at the level of the mouse), between 3 and 6.5 h after lights-off, a time that corresponds with maximal expression of TPH2 in the DR (Malek et al., 2004). The testing order was alternated according to the mouse treatment

groups between each animal tested, and at no stage during behavioural testing were mice isolated in their home cage. The apparatus was an acrylic open top box (40 cm length × 60 cm width × 20 cm height) comprising three chambers separated by removable partitions, which when removed, reveal a small opening that allows free movement between chambers. Testing in the three-chamber social approach test comprised a 10 min habituation period and a 10 min experimental trial, with animals spending a total of 20 min in the apparatus across both periods. During habituation, mice were initially placed in the central chamber with partitions in place, for 10 min. Immediately following habituation, a stimulus mouse was placed inside the 'social' chamber inside a weighted wire enclosure (9 cm diameter × 10 cm height), and an empty wire cage placed inside the 'non-social' chamber. The partitions were then removed allowing the animal to freely explore all three chambers during the experimental trial for 10 min. The location of the stimulus mouse was alternated between testing of each experimental animal, and the apparatus was thoroughly cleaned using F10™ veterinary disinfectant to avoid scent carryover.

Behaviour analysis

A digital video camera was set in a fixed position overlooking the three-chamber social approach test, recording both the 10 min habituation period and the 10 min experimental trial. Behaviour was later analysed using EthoVision XT 10 behaviour-tracking software (Noldus Information Technology, Wageningen, The Netherlands). Behaviour from the experimental phase analysed included (1) the number of entries into each of the non-social and social chambers and cage zones (defined as a 2 cm radius surrounding the cage), and (2) the duration of time spent in the non-social and social chambers and cage zones. Time spent in the social chamber and social cage zone were treated as an indicator of social approach behaviour (Nadler et al., 2004; Yang et al., 2011).

Immunohistochemistry

Ninety minutes after the conclusion of testing in the three-chamber social approach test, mice were transcardially perfused. Tissue processing was conducted as previously described (Payet et al., 2018). Dual-label immunohistochemistry used two sets of midbrain sections, representing every third section throughout the DR. On day 1, tissue was rinsed twice in 0.05 M phosphate-buffered saline (PBS) for 15 min and then placed into 1% H_2O_2 in a 1:1 solution of 0.05 M PBS and 100% MeOH for 30 min. Brain sections were rinsed twice for 15 min in 0.05 M PBS and pre-incubated in 0.1% PBST for 30 min. Brain sections were transferred into the first primary antibody (rabbit anti-c-Fos; 1:2000; Cat#ABE457; Lot#28070411; Merck, Bayswater, VIC, Australia) in 0.1% PBST at room temperature overnight. On day two, tissue was rinsed twice in 0.05 M PBS, then incubated in the secondary antibody (biotinylated goat anti-rabbit; 1:500; Vector Elite; Cat# PK-6101; Abacus ALS, Meadowbrook, QLD, Australia) in 0.05 M PBS for 90 min. Brain sections were rinsed twice and then placed in an avidin–biotin–peroxidase complex (1:200; Vector Elite ABC kit; Vector; Cat# PK-6101, Abacus ALS) in 0.05 M PBS for 90 min. Tissue was rinsed twice in 0.05 M PBS for 15 min and placed into peroxidase chromogen substrate solution (Vector SG; Cat# SK-4700, Abacus ALS) for 20 min.

The procedure for TPH2 immunostaining was the same as for c-Fos, except for (1) the second primary antibody (1:2000; rabbit anti-TPH2; Cat# PA1-778, Lot# RK238110; Thermo Fisher, Scoresby VIC, Australia) and (2) the substrate, which consisted of 0.01 mg/mL of 3,3′-diaminobenzidine tetrahydrochloride (DAB; Cat# D9015; Sigma-Aldrich) and 0.0015% H_2O_2 in 0.05 M PBS for 32 min. Finally, sections were rinsed twice in 0.05 M PBS for 15 min.

Brain sections were rinsed (~5 s) in 0.15% gelatin (w/v) in distilled H_2O and then mounted onto microscope slides. Using a series of ethanol baths (70%, 95%, 100%), brain sections were dehydrated, then cleared in xylenes for 5 min. Coverslips were attached using Entellan mounting medium (Cat# IM0225; ProSciTech Pty. Ltd., Thuringowa, QLD, Australia).

Primary antibodies

The first primary antibody, rabbit anti-c-Fos (1:2000; Cat#ABE457; Lot#28070411; Merck, Bayswater, VIC, Australia), was used to identify expression of the immediate-early gene marker of cellular activation within the DR (Kovács, 1998). Molecular weight has been confirmed by the manufacturer (~60/56 kDa) and it is demonstrated to react with human and rat tissue and predicted to react with mice, by recognising the N-terminus of c-Fos. The rabbit anti-c-Fos antibody has been widely used across previous research (Lawther et al., 2018; Payet et al., 2018, 2021; Wilson et al., 2018).

The second primary antibody, rabbit anti-TPH2 (1:2000; Cat# PA1-778, Lot# RK238110; Thermo Fisher, Scoresby VIC, Australia), was used to identify serotonergic neurons within the DR by staining the soma of cells. Antibody specificity has been previously validated (Hale et al., 2011).

Cell counts

The DR contains anatomically and functionally distinct subpopulations of serotonergic neurons (for review, see Lowry et al., 2008). For example, several lines of evidence has shown that the dorsal (DRD), caudal (DRC) and ventrolateral (DRVL) parts of the DR can be activated by anxiogenic drugs (Abrams et al., 2005; Lawther et al., 2015; Staub et al., 2006). The ventral part of the DR (DRV) is associated with both social approach behaviour (Payet et al., 2021) and the anxiolytic effects of voluntary exercise (Greenwood et al., 2005), and finally the interfascicular part (DRI) is implicated in antidepressant-like behavioural effects (Lowry et al., 2007). Consequently, three rostrocaudal levels (−4.42 mm, −4.84 mm, and −5.14 mm bregma) of the DR of each mouse, including the DRD, DRV, DRVL, DRI and the DRC were selected for analysis. The specificity of the second primary antibody, rabbit anti-TPH2, has been previously validated (Hale et al., 2011), and it stains the soma of serotonergic neurons allowing for subregions to be defined as shown in Figure 1. We have previously described the morphological and anatomical characteristics used to delineate the borders of the DR (Hale et al., 2011). Briefly, the DRD is bordered dorsally by

the cerebral aqueduct, ventrally by the DRV, and laterally by clusters of large multipolar serotonergic cells that comprise the DRVL at the midrostrocaudal level (−4.84 mm bregma). The medial longitudinal fasciculi (mlf) laterally borders the DRV, which is particularly characterised by densely packed serotonergic neurons. Serotonergic neurons that comprise the DRC are positioned ventral to the cerebral aqueduct, and they descend ventrally into the DRI where a column of serotonergic neurons passes between the mlf (for review, see Hale and Lowry, 2011). Subregions selected for analysis were counted by an experimenter (AMR) blind to treatment condition. Round blue-black nuclei, darker than the background, were counted as c-Fos-immunoreactive (ir), while neurons with orange-brown staining, localised to the cytoplasm, were counted as TPH2-ir neurons. Orange-brown cells with blue-black nuclei were counted as c-Fos-ir/TPH2-ir double-immunostained neurons. All cells meeting these criteria identified within each subregion were counted regardless of size or volume. Photomicrographs were taken using a Nikon 90i upright microscope fitted with a Nikon DS-Fi1 digital camera (Coherent Scientific, Hilton, SA, Australia), using a 10× objective lens. Cells were manually counted using the cell counter function on ImageJ (version 1.52p).

Statistical analysis

All data were analysed using IBM SPSS version 27 for Windows (IBM Corporation, Armonk, NY, USA). For the behavioural data from the three-chamber social approach test, three statistical outliers were identified (1.25% of total data) using the Grubbs test (Grubbs, 1969) and excluded from the behavioural analyses. Locomotor activity (distance moved in cm) in the three-chamber social approach test, along with duration of time spent in, and entries into, the social and non-social chambers and cages zones were analysed using a one-way analysis of variance (ANOVA). Given a priori hypotheses, planned setwise comparisons were used to identify any significant differences between the C/5-HTP treated mice and the control treatments (V/V, C/V and V/5-HTP). Welch's test was used as a correction where group variances were found to be unequal, which is type 1 error robust, and uses non-integer degrees of freedom (Derrick et al., 2016). Preference for the social chamber versus the non-social chamber was analysed using a paired samples *t* test.

For the immunohistochemistry data, Grubbs test revealed nine statistical outliers (1% of total data) which were replaced using the Petersen method in order to run the repeated measures ANOVA (Petersen, 1985). Cell counts within the DR were analysed using a repeated-measures ANOVA for (1) c-Fos-ir/TPH2-ir neurons, (2) c-Fos-ir non-serotonergic cells and (3) total number of TPH2-ir neurons. Brain region was the within-subject factor (eight levels; DRD and DRV at –4.42 mm bregma; DRD, DRVL, DRV and DRI at –4.84 mm bregma; DRC and DRI at –5.14 mm bregma) and treatment condition (three levels; V/V, C/V, V/5-HTP and C/5-HTP) was between groups factor. The least significant difference (LSD) test was used for post hoc comparisons. Replacement values were removed for post hoc tests and in graphical representations of the data.

Correlations between social approach behaviour and c-Fos-ir/TPH2-ir neurons were conducted using a Pearson's Product Moment correlation coefficient, with two-tailed significance set at a priori $p=0.05$.

Results

Effects of 5-HTP supplementation on behaviour in the three-chamber social approach test

In the three-chamber social approach test, BALB/c mice treated with C/5-HTP both entered into, and spent significantly more time in the social cage zone than mice treated with V/V, C/V and V/5-HTP ($t_{(25)}=3.25$, $p=0.003$, Cohen's $d=-4.06$; $t_{(25)}=3.05$, $p=0.005$, $d=-3.81$). There was no difference between the number of entries into, or the amount of time spent in the non-social cage zone ($t_{(26)}=0.83$, $p=0.413$, $d=-1.03$; $t_{(25)}=0.73$, $p=0.474$, $d=-0.91$; Figure 2). BALB/c mice treated with C/5-HTP entered the social chamber more than mice treated with V/V, C/V and V/5-HTP ($t_{(26)}=2.16$, $p=0.040$, $d=-2.68$). However, there was no difference in the duration of time spent in the social chamber ($t_{(21.47)}=1.80$, $p=0.086$, $d=-1.61$). There was no difference in the number of entries into, or the duration of time spent in the non-social chamber ($t_{(26)}=0.95$, $p=0.349$, $d=-1.18$; $t_{(26)}=0.93$, $p=0.363$, $d=-1.15$; Figure 3).

Irrespective of their treatment condition, BALB/c mice did not show a preference for the social or the non-social chambers. Paired samples *t* tests were used to compare duration of time spent in the social to the non-social chamber within each treatment group, finding no significant differences (V/V, $t_{(7)}=0.61$, $p=0.563$, $d=0.22$; C/V, $t_{(6)}=-1.33$, $p=0.233$, $d=-0.50$; V/5-HTP, $t_{(6)}=1.27$, $p=0.251$, $d=0.48$; C/5-HTP, $t_{(7)}=0.01$, $p=0.991$, $d<0.01$). BALB/c mice treated with C/5-HTP demonstrated higher levels of locomotor behaviour in the three-chamber social approach test than mice treated with V/V, C/V and V/5-HTP ($t_{(26)}=-3.75$, $p=0.001$, $d=-4.65$; Figure 4).

Effects of 5-HTP supplementation on c-Fos expression in serotonergic neurons and non-serotonergic neurons

c-Fos-ir/TPH2-ir neurons in the DR. Treatment with C/5-HTP did not have a significant effect on c-Fos expression of serotonergic neurons within subregions of the DR (region × treatment interaction: $F_{(21,\ 182)}=0.80$, $p=0.718$, $\eta_p^2=0.08$; treatment main effect: $F_{(3,\ 26)}=0.04$, $p=0.991$, $\eta_p^2<0.01$; region main effect: $F_{(7,\ 182)}=16.65$, $p<0.001$, $\eta_p^2=0.39$). Regardless of the results reported for the omnibus test, evidence indicates proceeding to post hoc analysis to avoid possible false negative results is important (Chen et al., 2018). Consequently, post hoc least significant difference (LSD) tests revealed c-Fos expression in serotonergic neurons within the DRC (–5.14 mm bregma) were significantly reduced in C/V-, V/5-HTP- and C/5-HTP-treated mice compared to V/V-treated mice (Figure 5).

C-Fos-ir non-serotonergic cells in the DR. A repeated measures ANOVA analysing expression of c-Fos-ir non-serotonergic cells revealed a significant interaction between subregions of the DR and treatment conditions (region × treatment interaction: $F_{(5.08,\ 44.02)}=2.71$, $p=0.032$, $\eta_p^2=0.24$; treatment main effect: $F_{(3,\ 26)}=4.18$, $p=0.015$, $\eta_p^2=0.33$; region main effect: $F_{(1.69,\ 44.02)}=446.81$, $p<0.001$, $\eta_p^2=0.95$; Table 1). Expression of non-serotonergic neurons across multiple subregions of the DR was higher in BALB/c mice treated with C/V. At –4.84 mm bregma,

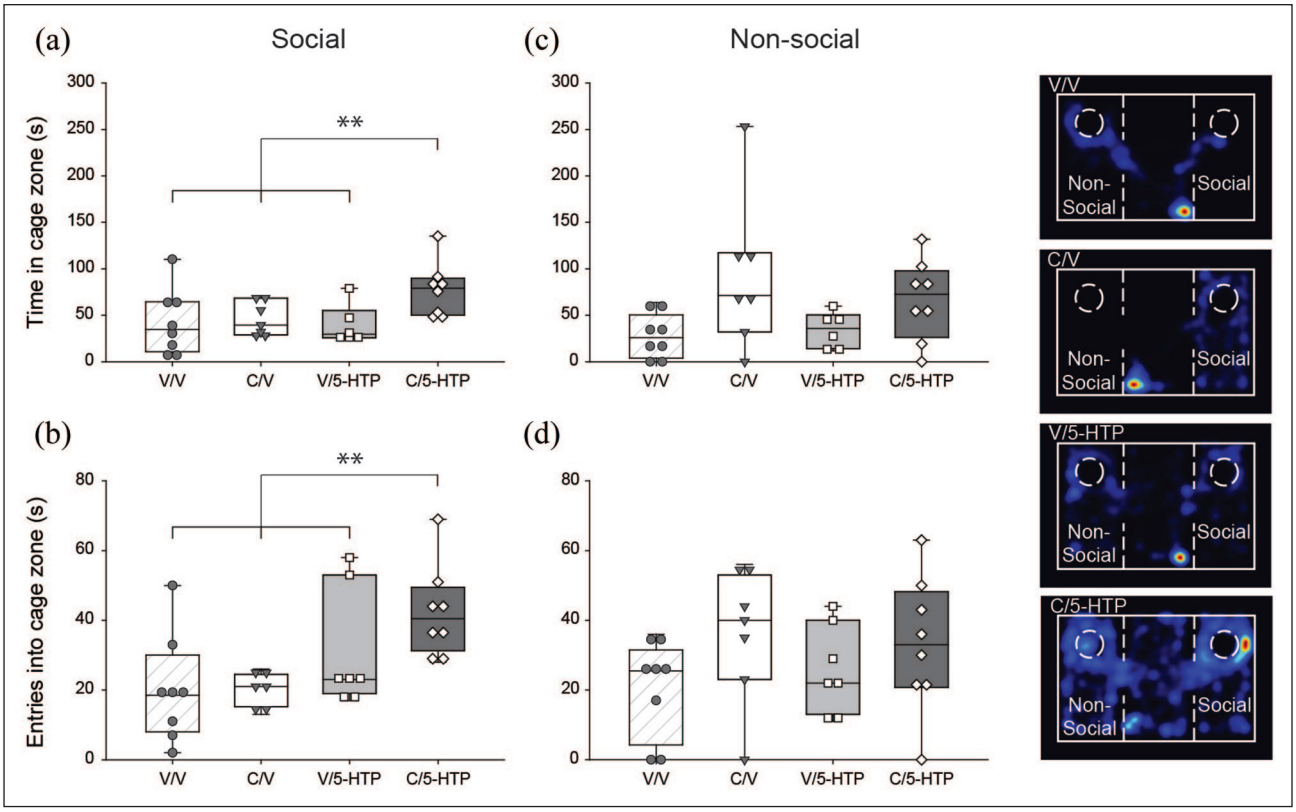

Figure 2. BALB/c mice treated with carbidopa followed by 5-HTP (C/5-HTP) demonstrated increased social interaction compared to other treatment conditions. (a) Box plot illustrating the duration of time spent in the social cage zone (V/V, $n=8$; C/V, $n=7$; V/5-HTP, $n=6$; C/5-HTP, $n=8$). (b) Box plot illustrating the frequency of entries into the social cage zone (V/V, $n=8$; C/V, $n=6$; V/5-HTP, $n=7$; C/5-HTP, $n=8$). (c) Box plot illustrating the duration of time spent in the non-social cage zone (V/V, $n=8$; C/V, $n=7$; V/5-HTP, $n=6$; C/5-HTP, $n=8$). (d) Box plot illustrating the frequency of entries into the non-social cage zone (V/V, $n=8$; C/V, $n=7$; V/5-HTP, $n=7$; C/5-HTP, $n=8$). (e) Heatmaps generated using EthoVision XT illustrating an example of mouse location in the three-chamber social approach test for a mouse treated with vehicle only (V/V), carbidopa followed by vehicle (C/V), vehicle followed by 5-HTP (V/5-HTP) and carbidopa followed by 5-HTP (C/5-HTP). The dotted circles represent the location of the cage placed in each chamber. ** $p<0.01$ versus V/V, C/V and V/5-HTP.

differences were found within the DRD (compared to both V/V and V/5-HTP groups), the DRV (compared to V/V), the DRI (compared to V/V) and the DRVL (compared to both V/V and V/5-HTP groups), while at –5.14 mm bregma, differences were found in the DRI (compared to V/V, V/5-HTP and C/5-HTP). Refer to Table 1 for further differences found.

Total TPH2-ir neurons in the DR. The analysis of counts of total TPH2-ir neurons revealed no interaction between region and treatment condition. However, there was a significant main effect for brain region (region × treatment interaction: $F_{(9.33, 80.85)}=0.92$, $p=0.515$, $\eta_p^2=0.10$; treatment main effect: $F_{(3, 26)}=0.62$, $p=0.611$, $\eta_p^2=0.07$; region main effect: $F_{(3.11, 80.85)}=87.58$, $p<0.001$, $\eta_p^2=0.77$).

Correlation analysis. Activation of serotonergic neurons within multiple subregions of the DR did not significantly correlate with entries into, or the duration of time spent in the social cage zone (data not shown). In animals treated with C/5-HTP, time spent in both the social chamber ($r=0.82$, $p=0.014$) and cage zone ($r=0.89$, $p=0.003$), as well as entries into the social cage zone ($r=0.91$, $p=0.002$), positively correlated with activation of serotonergic neurons in the rostral DRD (–4.42 mm bregma). Within the midrostrocaudal DR (–4.84 mm bregma), activation of serotonergic neurons in the DRD positively correlated with time spent in the social cage zone ($r=0.74$, $p=0.037$), and in the DRVL there was a positive correlation with the entries into the social chamber ($r=0.71$, $p=0.049$).

Discussion

Acute 5-HTP supplementation combined with carbidopa increased social approach behaviour in juvenile male BALB/c mice. An increase in locomotor behaviour was also observed, suggesting 5-HTP treatment likely led to a reduction in state anxiety-like defensive behaviour, which manifested in an increase in exploratory drive. Administration of 5-HTP with carbidopa did not have a marked effect on activation of serotonergic neurons across subregions of the DR. However, social approach behaviour in mice receiving both carbidopa and 5-HTP was associated with activation of serotonergic neurons in the rostral and midrostrocaudal DRD. Furthermore, following

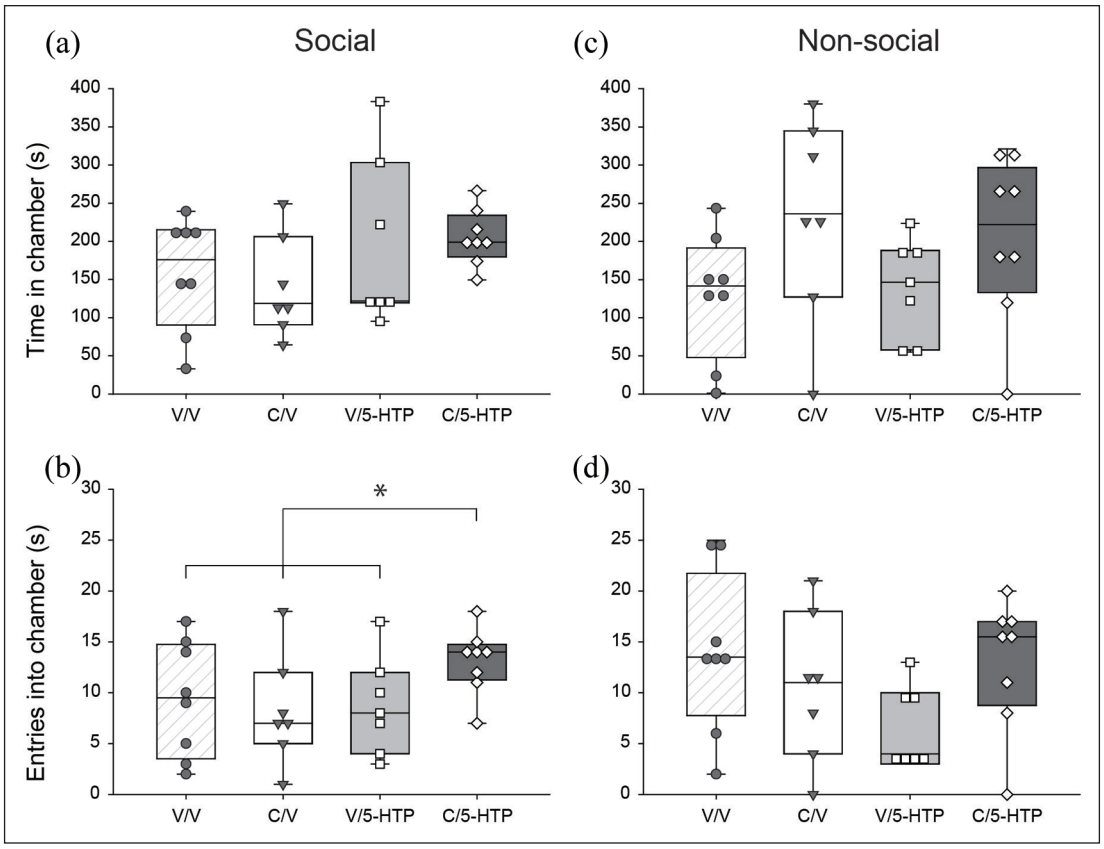

Figure 3. BALB/c mice treated with carbidopa followed by 5-HTP entered the social chamber more frequently compared to other treatment conditions. (a) Box plot illustrating the duration of time spent in the social chamber (V/V, $n=8$; C/V, $n=7$; V/5-HTP, $n=7$; C/5-HTP, $n=8$). (b) Box plot illustrating the frequency of entries into the social chamber (V/V, $n=8$; C/V, $n=7$; V/5-HTP, $n=7$; C/5-HTP, $n=8$). (c) Box plot illustrating the duration of time spent in the non-social chamber (V/V, $n=8$; C/V, $n=7$; V/5-HTP, $n=7$; C/5-HTP, $n=8$). (d) Box plot illustrating the frequency of entries into the non-social chamber (V/V, $n=8$; C/V, $n=7$; V/5-HTP, $n=7$; C/5-HTP, $n=8$). * $p<0.05$ versus V/V, C/V and V/5-HTP.

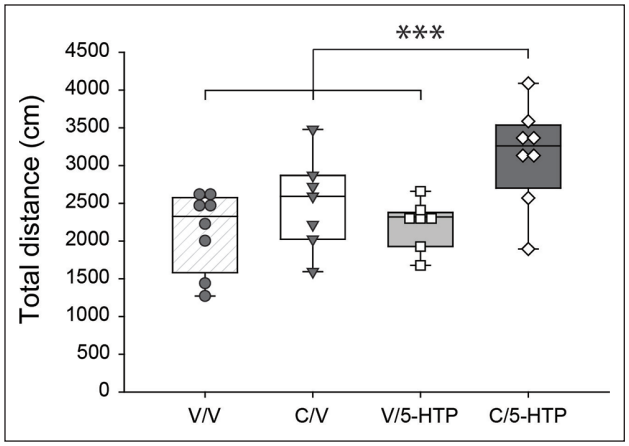

Figure 4. Box plot illustrating the differences in total distance moved in the three-chamber social approach test (V/V, $n=8$; C/V, $n=7$; V/5-HTP, $n=7$; C/5-HTP, $n=8$). ***$p<0.001$ versus V/V, C/V and V/5-HTP.

treatment with carbidopa and vehicle there was an increase in c-Fos protein expression in non-serotonergic neurons within the midrostrocaudal DRD, DRVL, DRV and DRI, along with the caudal DRI. Taken together, these findings are consistent with the hypothesis that increasing central serotonin synthesis via acute supplementation with 5-HTP can increase social approach behaviour via a reduction in state anxiety-like defensive behaviour. However, given that there were no differences in serotonergic activation within the DR, this suggests that mechanisms other than activation of the serotonergic neurons themselves are important for the 5-HTP effects on social behaviour.

Acute supplementation with 5-HTP following administration of carbidopa increased social approach behaviour in juvenile male BALB/c mice. Importantly, this increase was also found in comparison with mice injected with both vehicle and 5-HTP, suggesting that 5-HTP needs to cross the blood-brain barrier for a social behavioural effect to be observed. This finding is consistent with the research showing that the acute dietary tryptophan enhancement can increase sociability and serotonin turnover in BTBR mice (Zhang et al., 2015). In BALB/c mice, however, tryptophan depletion, but not supplementation, has an anxiolytic effect (Biskup et al., 2012; Browne et al., 2012). Previous observations suggest that following the tryptophan supplementation, tryptophan levels within the brainstem and hippocampus (Browne et al., 2012) and 5-HTP content within the prefrontal cortex, frontal cortex and the hippocampus (Biskup et al., 2012) are not increased, suggesting that the supplementation in these studies

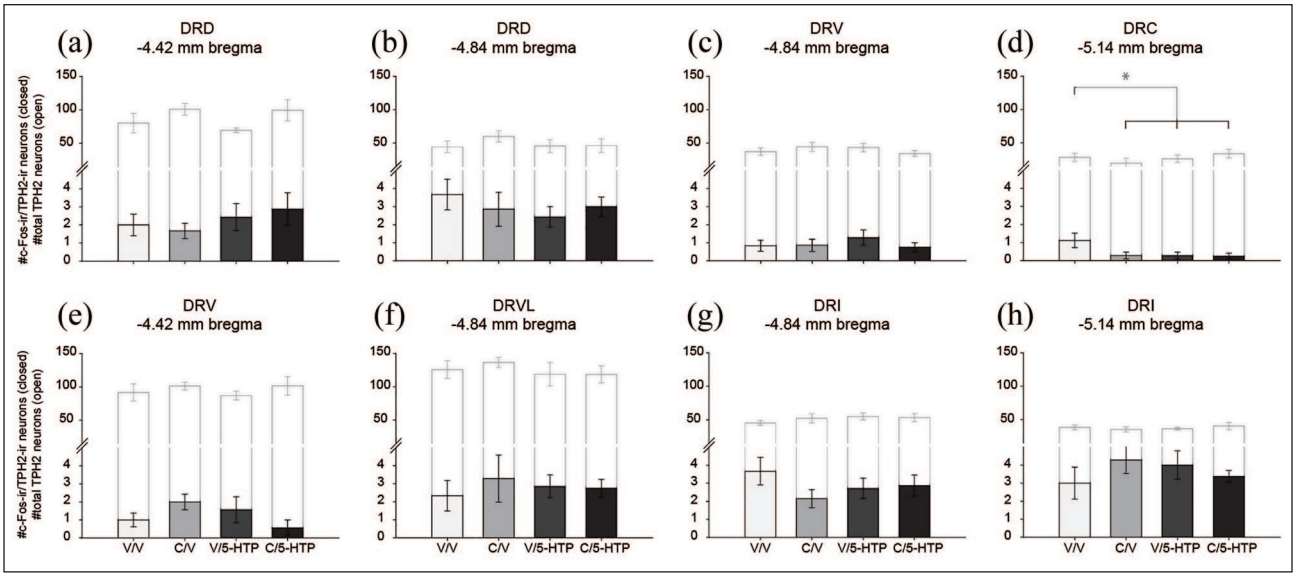

Figure 5. Bar graphs illustrating the effects of treatment with vehicle only, carbidopa/vehicle, vehicle/5-HTP or carbidopa/5-HTP on c-Fos-immunoreactive (ir)/TPH2-ir neurons and total number of TPH2-ir neurons in the dorsal raphe nucleus. c-Fos/TPH2-ir neurons are depicted in the closed bars, and total TPH2 neurons are depicted in the open bars.

Data are presented as mean ± SEM. *$p < 0.05$.

DRC: dorsal raphe nucleus, caudal part; DRD: dorsal raphe nucleus, dorsal part; DRI: dorsal raphe nucleus, interfascicular part; DRV: dorsal raphe nucleus, ventral part; DRVL: dorsal raphe nucleus, ventrolateral part.

Table 1. Effects of treatment condition on numbers of c-Fos-ir non-serotonergic cells within the dorsal raphe nucleus.

Brain region	Treatment groups			
	V/V	C/V	V/5-HTP	C/5-HTP
–4.42 mm bregma				
DRD	59.0 (7.9)	64.7 (9.4)	64.0 (7.6)	77.3 (9.5)
DRV	25.0 (4.3)	19.5 (1.6)	27.6 (3.4)	30.5 (3.6)[a]
–4.84 mm bregma				
DRD	31.6 (1.7)	58.1 (7.8)[d]	36.8 (2.9)	48.8 (4.6)[b]
DRVL	224.2 (19.9)	319.1 (32.3)[d]	242.9 (16.7)	268.6 (13.7)
DRV	24.2 (2.7)	41.0 (4.5)[c]	34.6 (4.6)	32.0 (3.5)
DRI	20.6 (1.2)	31.3 (3.8)[c]	26.9 (1.9)	28.8 (2.2)[b]
–5.14 mm bregma				
DRC	17.5 ((3.9)	18.4 (1.8)	15.3 (3.2)	17.8 (2.0)
DRI	58.0 (5.8)	88.0 (3.0)[e]	61.1 (5.6)	69.8 (6.2)

DRC: dorsal raphe nucleus, caudal part; DRD: dorsal raphe nucleus, dorsal part; DRI: dorsal raphe nucleus, interfascicular part; DRV: dorsal raphe nucleus, ventral part; DRVL: dorsal raphe nucleus, ventrolateral part; i.p., intraperitoneal.

Values represent raw cell counts of one section per subregion per animal presented as mean (SEM). Denotes statistically significant difference: [a] C/5-HTP compared to C/V, [b] C/5-HTP compared to V/V, [c] C/V compared to V/V, [d] C/V compared to V/V and V/5-HTP, [e] C/V compared to V/V, V/5-HTP and C/5-HTP.

did not substantially increase the rate of brain serotonin synthesis. However, in a *Tph2* knockout model, 5-HTP supplementation with carbidopa restored brain serotonin synthesis to wild-type levels, and a subsequent reduction in marble burying and impulsive aggressive behaviour was reported (Angoa-Pérez et al., 2012). Consistent with the hypothesis that normalised serotonin synthesis capacity is important for the expression of emotional behaviour, in BALB/c mice the antidepressant effects of the selective serotonin reuptake inhibitor, citalopram, are dependent on increasing serotonin synthesis capacity using dietary tryptophan supplementation (Cervo et al., 2005). We have previously shown that BALB/c mice exhibit reduced levels of sociability and 5-HTP accumulation in the rostral and midrostrocaudal DR (Russo et al., 2019). Therefore, these findings taken together suggest that increasing brain serotonin synthesis via 5-HTP supplementation can increase social approach behaviour.

Alternatively, the increased social approach behaviour displayed by mice treated with 5-HTP and carbidopa may represent an increase in exploratory drive, rather than sociability per se. Mice treated with carbidopa and 5-HTP did not show a preference for the social chamber over the non-social chamber, entered the social chamber more frequently and covered more overall distance during the experimental phase of the test. These results suggest that 5-HTP treatment may have had an anxiolytic effect, resulting in increased exploration. Interestingly, a reduction in anxiety-like defensive behaviour which manifested as increased exploratory behaviour has previously been observed in adult rats that were chronically treated with 5-HTP between PND 1 and PND 21 (Blazevic et al., 2012). Furthermore, it is important to acknowledge that while BALB/c mice demonstrate reduced sociability, this has been consistently shown in the three-chamber social approach test (Brodkin et al., 2004; Fairless et al., 2012; Russo et al., 2019; Sankoorikal et al., 2006), a test which prevents direct contact between the experimental and stranger animal. However, recent evidence suggests that BALB/c mice have a qualitatively different approach to social behaviour compared to other strains like the C57BL/6J mouse, showing a preference for facial but avoiding anogenital investigation (Arakawa, 2018). Most importantly, it was found that the BALB/c mice did not show a reduction in sociability with a stranger mouse when direct contact is allowed (Arakawa, 2018). Therefore, as BALB/c mice have a phenotype characterised by increased anxiety-like defensive behavioural responses (Bouwknecht and Paylor, 2002; Lepicard et al., 2000; Priebe et al., 2005; Russo et al., 2019) and displayed no social preference, these findings suggest that treatment with 5-HTP could have an anxiolytic effect, which in this instance has manifested in increased social approach behaviour. Furthermore, these findings are important in the context of interpreting the results from the three-chamber social approach test when using BALB/c mice, suggesting that anxiety-like defensive behaviour is a key factor driving behaviour in this apparatus.

Supplementing serotonin synthesis via 5-HTP and carbidopa did not have a marked effect on the activation of serotonergic neurons in subregions of the DR. This is consistent with evidence that suggests increase in serotonin synthesis can activate inhibitory somatodendritic 5-HT_{1A} receptors on serotonergic neurons, in turn inhibiting serotonin neuronal firing rates (Evans et al., 2008). Previously, in vitro evidence has demonstrated that following exposure to tryptophan, 5-HT_{1A} receptor mediated inhibition of serotonergic firing rates occurs within the raphe nuclei (Evans et al., 2008; Liu et al., 2005). Furthermore, this can be reversed using a central AADC inhibitor like NSD10-15 (Evans et al., 2008). Inhibition of serotonergic neurons within subregions of the DR has also been found following both acute and chronic treatment with fluoxetine in BALB/c mice exposed to the three-chamber social approach test (Payet et al., 2018). This is particularly interesting given fluoxetine has been shown to reduce the rate of serotonin synthesis within the DR following both acute and chronic administration (Mück-Šeler et al., 2002). However, we found that the social approach behaviour in mice treated with carbidopa and 5-HTP was associated with activation of serotonergic neurons in both the rostral and midrostrocaudal DRD. This is consistent with evidence demonstrating that serotonergic activation in the rostral DRD is associated with social approach behaviour following fluoxetine treatment (Payet et al., 2018), while deep brain stimulation of the DRD has an anxiolytic effect in rats (Wscieklica et al., 2017). There is considerable evidence suggesting that the DRD subnucleus plays an important role in modulating anxiety-related behaviour (Matthiesen et al., 2020; Spiacci et al., 2012; Spiacci et al., 2016). Therefore, this suggests that the anxiety-like defensive behaviour may have been an important factor driving the increased social approach behaviour observed in BALB/c mice treated with carbidopa and 5-HTP. Taken together, to better understand how the 5-HTP supplementation alters behaviour, investigating serotonergic activation in alternative regions is important; however, the evidence suggests that the DRD plays a role in social approach behaviour.

Activation of cell populations within the forebrain may explain why there was not a marked effect of serotonergic activation within the DR. Importantly, while the majority of serotonin is synthesised in the soma, research has shown that it is also synthesised within the axon terminals projecting to the forebrain (Mück-Šeler and Diksic, 1996). Further, administering a 100 mg/kg dose of 5-HTP has been shown to increase forebrain serotonin by 353% 20 min post injection (Trulson and Jacobs, 1976). The role of forebrain regions in social behaviour and anxiety-like defensive behavioural responses is well-documented. For example, increased c-Fos expression is seen within multiple forebrain regions including the medial prefrontal cortex, orbitofrontal cortex and the lateral amygdala following social play behaviour in male Wistar rats (van Kerkhof et al., 2014). In addition, increased activation within the basolateral amygdala occurs in response to social compared to non-social behaviour within the three-chamber social approach test (Ferri et al., 2016). With respect to stress and anxiety-like defensive behaviour, evidence has shown that following controllable stress, serotonin is reduced within the prefrontal cortex and the hypothalamus, along with an increase in serotonin turnover in the hypothalamus of BALB/c mice (Kasabov et al., 2019). Furthermore, serotonin turnover is increased in the striatum in response to both acute and chronic stress, while it is increased in the hippocampus following chronic stress (Browne et al., 2011). In male Wistar rats, deep brain stimulation of the DRD has an anxiolytic effect in the elevated T-maze along with increased activation within the medial amygdala, lateral septum and cingulate cortex (Wscieklica et al., 2017). Taken together, these findings suggest that a forebrain anxiety network likely plays an important role in both social and anxiety-like defensive behaviour. Therefore, involvement of such a network could potentially explain the increased exploratory drive observed in BALB/c mice following 5-HTP and carbidopa supplementation.

Although speculative, an alternate hypothesis is that 5-HTP supplementation could lead to ectopic serotonergic activation within dopaminergic neurons. Evidence has previously shown that when administered via intraperitoneal injection, L-DOPA, the immediate dopamine precursor, can be decarboxylated to dopamine by AADC within serotonergic neurons in the DR (Arai et al., 1994). Moreover, serotonin can be synthesised within dopaminergic neurons of the substantia nigra pars compacta, with evidence demonstrating that the decarboxylation of 5-HTP by AADC to serotonin occurs when 5-HTP is administered via intraperitoneal injection (Arai et al., 1995) and via oral gavage

(Lynn-Bullock et al., 2004). Consequently, despite no marked effect on the activation of serotonergic neurons within the DR following administration of 5-HTP in our study, it remains possible that there were ectopic changes within populations of dopaminergic neurons, such as those in the substantia nigra pars compacta. Interestingly, optogenetic stimulation of serotonergic terminals in the substantia nigra and ventral tegmental area both lead to an increase in serotonin levels, reduced immobility time in the forced swim test and increased distance travelled in the elevated plus-maze (Ohmura et al., 2020). While this hypothesis was not directly tested, ectopic serotonergic activation within dopamine neurons remains possible. Therefore, this should be considered in subsequent research involving 5-HTP supplementation, so as to determine whether ectopic serotonergic activation within dopamine neurons in the substantia nigra pars compacta plays a role in anxiety-like defensive behaviour.

Treatment with carbidopa alone increased activation of non-serotonergic neurons within multiple subregions of the DR. Specifically, increased activation was found within the midrostrocaudal DRD, DRVL, DRV and DRI, as well as in the caudal DRI. Peripheral decarboxylase inhibitors increase central availability of exogenous 5-HTP (Magnussen, 1984) and central levels of serotonin (Itskovitz et al., 1989). However, this finding was unexpected given that the carbidopa is not thought to pass the blood brain-barrier (Porter et al., 1962; Yee et al., 2001), and when administered alone, it has no effect on endogenous central 5-HT, noradrenaline or dopamine in *Tph2* knockout mice, nor does it affect 5-HTP or L-DOPA within the cortex or striatum of wild-type mice (Carli et al., 2015). In addition, humans treated with carbidopa, endogenous 5-HTP is not synthesised in sufficient quantity in the periphery to affect brain serotonin functioning (Young et al., 1982). Given this evidence, an explanation for the observed increased activation of non-serotonergic neurons within BALB/c mice treated with carbidopa remains unclear.

As previously discussed, anxiety-like defensive behaviour could be a key factor driving behaviour in the three-chamber social approach test, particularly when BALB/c mice are tested. Given BALB/c mice show no social deficit when direct contact is allowed (Arakawa, 2018), using an assay of social behaviour which does not restrict social contact such as the reciprocal social interaction test (Silverman et al., 2010) should be considered to provide a more comprehensive profile of social behaviour in BALB/c mice following 5-HTP supplementation. In addition, more direct measures of anxiety-like defensive behaviour may help further elucidate the behavioural effects of 5-HTP treatment. For example, we have previously shown that BALB/c mice display elevated levels of anxiety-like defensive behaviour relative to C57BL/6J mice using the elevated plus-maze (EPM; Russo et al., 2019). Importantly, past research has demonstrated that the EPM is a mild stressor (Rodgers et al., 1999), and when using multiple behavioural assays, animals should be tested in the least invasive test first to ensure valid results (Sukoff Rizzo and Silverman, 2016). Exposing animals to a mildly stressful stimulus prior to the three-chamber social approach test may confound the results. Therefore, including an additional assay of anxiety-like defensive behaviour such as the EPM in future research is warranted, but requires careful consideration of the research design. Here, we report results from male BALB/c mice only, limiting the ability to generalise across sexes. However, recent work indicates that acute treatment with fluoxetine increased social avoidance in female BALB/c mice in the three-chamber social approach test (Payet et al., 2021) which is consistent with behavioural outcomes in male BALB/c mice (Payet et al., 2018). As such, further research is warranted to determine if 5-HTP supplementation alters behaviour consistently between male and female BALB/c mice. Finally, the use of home-cage controls would provide further context helping to understand the relationship between exposure to the three-chamber social approach test, DR activation and 5-HTP treatment.

The present experiment has provided evidence that supplementation of serotonin synthesis, via the administration of 5-HTP with carbidopa, can increase social approach behaviour in BALB/c mice. The increase in social approach behaviour likely occurred via the attenuation of anxiety-like defensive behaviour in these mice, and therefore, the current evidence contributes to the body of research suggesting serotonergic dysfunction is implicated in social and anxiety-like defensive behaviour. While we did not find evidence that 5-HTP treatment had an effect on serotonergic neuronal activation within the DR, we did observe an association between social approach behaviour and serotonergic activation in the DRD of mice treated with carbidopa and 5-HTP. To better understand the mechanisms that drive alterations to behaviour following 5-HTP and carbidopa treatment, further research should explore the effects of 5-HTP supplementation on both activation of serotonergic neurons and serotonin turnover in the forebrain. In conclusion, 5-HTP supplementation increases social approach behaviour, likely via the attenuation of anxiety-like defensive behavioural responses; as such, continued investigation of the mechanisms through which 5-HTP supplementation modulates behaviour is required.

Acknowledgements

AMR and MWH designed the research; AMR, JMP and MWH conducted the research; AMR and MWH analysed the data; AMR prepared the figures and drafted the manuscript; AMR, JMP, SK, JAL, CAL and MWH revised the manuscript. MWH supervised the research.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the La Trobe University Research Focus Area: Understanding Disease. AMR and JMP were supported by an Australian Postgraduate Award.

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