Research Paper

Region- and receptor-specific effects of chronic social stress on the central serotonergic system in mice

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ABSTRACT

Serotonin (5-HT), via its receptors expressed in discrete brain regions, modulates aversion and reward processing and is implicated in various psychiatric disorders including depression. Stressful experiences affect central serotonergic activity and act as a risk factor for depression; this can be modelled preclinically. In adult male C57BL/6J mice, 15-day chronic social stress (CSS) leads to depression-relevant behavioural states, including increased aversion and reduced reward sensitivity. Based on this evidence, here we investigated CSS effects on 5-HT1A, 5-HT2A, and 5-HT2C receptor binding in discrete brain regions using in vitro quantitative autoradiography with selective radioligands. In addition, mRNA expression of *Htr1a, 2a, 2c* and *Slc6a4* (5-HT transporter) was measured by quantitative PCR. Relative to controls, the following effects were observed in CSS mice: 5-HT1A receptor binding was markedly increased in the dorsal raphe nucleus (136%); *Htr1a* mRNA expression was increased in raphe nuclei (19%), medial prefrontal cortex (35%), and hypothalamic para- and periventricular nuclei (21%) and ventral medial nucleus (38%). 5-HT2A receptor binding was decreased in the amygdala (48%) and ventral tegmental area (60%); *Htr2a* mRNA expression was increased in the baso-lateral amygdala (116%). 5-HT2C receptor binding was decreased in the dorsal raphe nucleus (42%). *Slc6a4* mRNA expression was increased in the raphe (59%). The present findings add to the translational evidence that chronic social stress impacts on the central serotonergic system in a region- and receptor-specific manner, and that this altered state of the serotonergic system contributes to stress-induced dysfunctions in emotional processing.

Introduction

The serotonergic system, including serotonin (5-HT, 5-hydroxytryptamine), its receptors and transporter (SERT), is implicated in stress responding (Ferrés-Coy et al., 2013; Sachs et al., 2015; Underwood et al., 2018; Van den Buuse and Hale, 2019) and the aetiology of depression (e. g. Stockmeier et al., 1998; Yohn et al., 2017). In animal studies, there is extensive evidence that serotonergic neurotransmission is sensitive to diverse stressors, and that stress-induced changes therein contribute to development of depression-relevant behaviours such as reduced interest in reward (Leventopoulos et al., 2009; Ferrés-Coy et al., 2013; Sachs et al., 2015; Yohn et al., 2017). Focussing on chronic exposure to stressors – an aetiological risk factor for depression and anxiety disorders (Keller et al., 2007) - experimental chronic stressors in rodents include repeated postnatal maternal separation, chronic unpredictable mild stress (CUMS) and various forms of chronic social stress (CSS). To-date, the majority of animal studies on the effects of chronic stress on the 5-HT system have been conducted in rats, with relatively few in mice (Pryce and Klaus, 2013).

Most studies of stress effects on the 5-HT system have focussed on the 5-HT1A receptor, for which there is also extensive human evidence for

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Abbreviations

Amy	amygdala
ARC	arcuate nucleus of the hypothalamus
BLA	basolateral amygdala
CSS	chronic social stress
DRN	dorsal raphe nucleus
Hipp	hippocampus
Hyp	hypothalamus
LC	locus coeruleus
mPFC	medial prefrontal cortex
NAcc	nucleus accumbens
RN	raphe nuclei
VMH	ventromedial nucleus of the hypothalamus
VTA	ventral tegmental area;
5-HT	5-hydroxytryptamine

changes in psychiatric disorders (Celada et al., 2013). The 5-HT1A receptor is both a presynaptic autoreceptor on 5-HT neurons in the raphe nuclei, and a postsynaptic receptor in prefrontal and frontal cortices, hippocampus, amygdala and septal nuclei. Activation or inhibition of 5-HT1A autoreceptors decreases or increases, respectively, the firing of 5-HT neurons and the concomitant 5-HT synaptic release and binding to post-synaptic receptors on neurons in target brain regions (Sharp and Hjorth, 1990; Polter and Li, 2010). Rat studies provide evidence for stress-induced decreased 5-HT1A binding. Thus, CUMS in adult rats led to a reduced 5-HT1A binding in hippocampus and not in raphe nuclei (Paré and Tejani-Butt, 1996), and adult chronic social-subordinacy stress also reduced hippocampal 5-HT1A binding (McKittrick et al., 1995). Chronic postnatal stress in rats led to reduced 5-HT1A binding in the dorsal raphe nucleus (DRN), anterior cingulate, motor cortex and ventral hippocampus in adulthood (Leventopoulos et al., 2009). With respect to *Htr1a* gene expression, postnatal stress led in adulthood to a decrease in DRN and an increase in amygdala (Bravo et al., 2014).

The 5-HT2A receptor is also affected by stress. In adult rats, chronic social subordinacy led to increased 5-HT2A binding in the parietal cortex (McKittrick et al., 1995). For *Htr2a* gene expression, in adult rats, stress induced by a learned-helplessness footshock paradigm resulted in down-regulation in the baso-lateral amygdala (BLA) (Jiang et al., 2009). In adult mice, CSS also resulted in reduced *Htr2a* expression in BLA (Azzinnari et al., 2014). To our knowledge there is little evidence to date for the effects of stress on 5-HT2C receptor status in rodents.

Turning to the 5-HT transporter, in ex vivo studies of SERT binding in adult rats, CUMS led to either an increase in the cortex or a decrease in the cortex and hippocampus, in a strain-dependent manner (Paré and Tejani-Butt, 1996). Chronic postnatal stress in rats led to reduced *Sert* mRNA expression in the DRN in adulthood (Bravo et al., 2014). Furthermore, there is evidence for inter-dependence of SERT and specific 5-HT receptors in the overall regulation of 5-HT signalling. For example, in rats, exogenous increase of *Slc6a4* and subsequently SERT binding in the DRN resulted in reduced 5-HT1A binding in DRN (Fabre et al., 2018). Accordingly, stress effects on one regulator of 5-HT signalling could well be the direct cause of changes in other 5-HT regulators.

As indicated above, whilst a large number of studies have investigated effects of chronic stress on brain and behaviour in mice, there is a paucity of data on stress effects on 5-HT receptor binding activity and gene expression, and on *Slc6a4* expression, in this species. The present study constitutes such an investigation, using a stressor of 15-day CSS in young adulthood, which is based on the resident-intruder paradigm (Pryce and Fuchs, 2017). Central effects of CSS include depression-relevant changes in resting-state brain functional connectivity (Grandjean et al., 2016) and increased levels of pro-inflammatory markers, in limbic and cortical brain regions, including the kynurenine metabolites of tryptophan, the 5-HT precursor (Fuertig et al., 2016; Bergamini et al., 2018). Furthermore, this manipulation leads to several depression-relevant behavioural states, as summarised in Table 1. According to the NIMH Research Domain Criteria framework (RDoC, https://www.nimh.nih.gov/research/research-funded-by-nimh/rdoc/), these behavioural changes include increased negative valence processing, as detected with Pavlovian aversion learning-memory and learned uncontrollability tests, and decreased positive valence processing, as detected with tests of Pavlovian (gustatory) reward learning, (gustatory) reward effortful motivation and social motivation. In addition, CSS mice exhibit deficits in operant behavioural tests with (gustatory) reward reinforcement but where decreased reward sensitivity and increased aversion sensitivity can both contribute to reduced performance, namely reversal learning tests (Table 1). These physiological and behavioural effects of CSS are obtained without dividing mice into susceptible versus resilient subgroups based on their subsequent passive avoidance of the aggressor mouse strain, the method used in some other studies (e.g. Golden et al., 2011). The inter-individual differences in the CSS mice are similar to those in the control group, and an "inclusive" experimental design is therefore used, as is the case with various other stressors e.g. chronic unpredictable mild stress (e.g. Tye et al., 2013; Willner, 2017).

There is substantial evidence that the 5-HT system is a major modulator of both aversion and reward processing (Cools et al., 2008). Serotonin is also integral to regulation of the behavioural processes impacted by CSS (Table 1), including Pavlovian aversion learning-memory (Sengupta and Holmes, 2019), learned uncontrollability (Amat et al., 2005; Pryce et al., 2012) and reversal learning (Ineichen et al., 2012; Bari et al., 2010). Furthermore, certain of these CSS-induced behavioural changes can be reversed by repeated antidepressant administration, including selective serotonin reuptake inhibitor (SSRI) and reduction of Pavlovian reward learning-memory and agomelatine (melatonin receptor MT1 and MT2 agonist and 5-HT2C antagonist) and increase in reversal learning (Fuertig et al., 2016; Bergamini et al., 2016). To enable the accurate, unconfounded study of the effects of CSS on the 5-HT system, it was essential to do so in mice that had not been tested behaviourally, given that stimuli such as electroshock will themselves impact on 5-HT function. Therefore, in the present study, in otherwise unmanipulated mice we investigated the effects of CSS on ligand binding activity of 5-HT1A, 5-HT2A and 5-HT2C receptors as well as the expression levels of their genes and that for SERT, in brain regions in which one or more of these receptors/genes are substantively expressed.

Table 1

Summary of behavioural effects of chronic social stress.

Domain	Behavioural process and test	CSS effect	Reference
Aversion	Pavlovian aversion learning-memory	Increase	(Azzinnari et al., 2014; Fuertig et al., 2016; Just et al., 2018; Cathomas et al., 2019)
Aversion	Learned uncontrollability	Increase	(Azzinnari et al., 2014)
Aversion	Physical fatigability	Increase	(Azzinnari et al., 2014; Van Buel et al., 2017)
Reward	Pavlovian reward learning	Decrease	(Kúkel'ová et al., 2018)
Reward	Reward effortful motivation	Decrease	(Kúkel'ová et al., 2018; Bergamini et al., 2016)
Reward	Social preference test	Decrease	(Cathomas et al., 2019)
Reward/ Aversion	Reversal learning	Decrease	(Bergamini et al., 2016)
Reward/ Aversion	Probabilistic reversal learning	Decrease	(Bergamini et al., 2016)

Experimental procedures

Animals and maintenance

Breeding of C57BL/6J mice and in vivo experiments were conducted at the Preclinical Laboratory for Translational Research into Affective Disorders, University of Zurich, under permits for animal experimentation (170/2012, 149/2015) issued by the Veterinary Office of canton Zurich. The details of animal maintenance are described in Azzinnari et al. (2014). Male offspring were weaned at 3 weeks of age and caged in groups of 2–3 littermates. At the age of 10–13 weeks and body weight 24.0–30.0 g they were included in the study. The male CD-1 mice (Janvier, Saint-Berthevin, France) that were the residents in the chronic social stress manipulation were aged 8 months, were ex-breeders, and caged singly at study onset. Cages were type 2 L (33 × 21 × 14 cm), were individually ventilated at 22–24 °C and 50–60% humidity and contained sawdust and paper tissue as nesting material. Mice were maintained on a reversed 12:12 h light-dark cycle with lights off 07:00–19:00 h. Complete-pellet diet (Provimi, Kliba Ltd., Kaiseraugst, Switzerland) and water were available ad libitum. All procedures were conducted during the dark phase of the cycle. The number of mice studied was the minimum necessary to achieve the study aims as based on previous neurobiological studies of CSS effects (e.g. Bergamini et al., 2018), and all unnecessary stress was avoided.

Chronic social stress

The CSS procedure is described in detail in Azzinnari et al. (2014). Briefly, the home cages of aggressive CD-1 mice were divided longitudinally into two compartments by a transparent, perforated Plexiglas divider. To prevent bite wounds, the lower incisors of CD-1 mice were trimmed every third day. On day 1 the CSS mouse was placed in the same compartment as the CD-1 mouse and behaviour was observed: the intruder and resident were maintained together for either 10 min maximum or 60 s cumulative attack time (chase, box, bite), whichever occurred sooner. Thereafter, the CD-1 mouse was transferred to the adjacent compartment and the two mice were maintained in sensory contact for 24 h. On each of days 2–15 the CSS – CD-1 mouse pairings were rotated, and the above procedure was repeated, so that each CSS mouse was placed together with a different CD-1 mouse each day. Each CSS mouse displayed submissive behaviour including vocalisation, but this did not prevent attacks by CD-1 mice; therefore, CSS mice experienced an uncontrollable social stressor. From day 15, each CSS mouse remained in the same divided cage next to the same CD-1 mouse without further attacks. Control (CON) mice were maintained in littermate pairs, which is the standard housing condition used in our laboratory; they were handled daily. Body weight was measured from 5 days prior to and throughout the CSS/CON procedure.

On day 17, i.e. 2 days after the CSS/CON procedure, mice were decapitated and the brain was rapidly dissected from the skull and rinsed with ice-cold saline solution. Brains for autoradiography were snap-frozen at − 40 °C in 2-methylbutane (Sigma-Aldrich) and stored at − 80 °C. Brains for RT-qPCR were embedded in optimal cutting temperature (OCT) compound and stored at − 80 °C. Brains were transferred from Zurich to University of Roehampton on dry ice.

Autoradiography

With the brains from one cohort of mice with ad libitum access to food prior to sample collection (CSS N = 8, CON N = 8), quantitative autoradiography of 5-HT1A, 5-HT2A and 5-HT2C receptors was performed. Frozen serial coronal 25 μm sections were cut that included medial prefrontal cortex (mPFC), nucleus accumbens (NAcc), ventral tegmental area (VTA), amygdala (Amy), hypothalamus (Hyp), dorsal raphe nucleus (DRN), hippocampus (Hipp) and locus coeruleus (LC). For each subject, region and receptor, 2–3 sections were used. Sections were mounted onto polysine-coated glass slides and stored at − 80 °C prior to autoradiography.

The following radioligands were used: [O-methyl-^{3}H]WAY 100635 as selective 5-HT1A receptor antagonist, [^{3}H]ketanserin as 5-HT2A receptor antagonist, and [^{125}I]DOI as 5-HT2C receptor agonist. Due to the relatively low specificity of the two latter radioligands, masking drugs were used to exclude interfering activities. [O-methyl-^{3}H]WAY 100635 (specific activity 80 Ci/mmol) and [^{125}I]DOI (specific activity 2200 Ci/mmol) were purchased from American Radiolabeled Chemicals Ltd, and [ethylene-^{3}H]ketanserin hydrochloride (specific activity 43 Ci/mmol), as well as methysergide, MDL 100907 and RS-102221, were purchased from Tocris/Biotechne Biosciences UK. Tritium microscales were purchased from Amersham UK. Tritium sensitive Hyperfilm BioMax MR (Kodak, UK) was used and all other chemicals were purchased from Sigma-Aldrich (Poole, UK).

Serotonin 5-HT1A receptor binding

The procedure was based on Leventopoulos et al. (2009). Brain sections were pre-incubated in 0.05 M Tris-HCl buffer pH 7.5 for 30 min at room temperature, and then incubated for 2 h at RT with 2 nM [O-methyl-^{3}H]WAY 100635 and with 10 μM pargyline, a monoamine oxidase inhibitor. Non-specific binding was determined using 10 μM unlabelled 5-HT. Slides were then washed in ice-cold Tris-HCl buffer for 2 min, dipped 3× in cold deionised water, and air dried for 24 h.

Serotonin 5-HT2A receptor binding

The procedure was based on Dawson et al. (2014). Brain sections were pre-incubated in 0.05 M Tris-HCl buffer with pH 7.5 for 5 min at RT and then incubated for 60 min at RT with 1 nM [^{3}H]ketanserin in 0.05 M Tris-HCl buffer pH 7.5, with 1 μM prazosin to block binding to adrenergic receptors and 100 nM tetrabenazine to block binding to the vesicular transporter. Non-specific binding was assessed in the presence of 200 μM methysergide (block solution). Slides were then washed 2 × 30 min in ice-cold Tris-HCl buffer, rinsed in cold deionised water for 10 s, and air dried for 24 h.

Serotonin 5-HT2C receptor binding

The procedure was based on Li et al. (2003). Brain sections were pre-incubated in assay buffer (0.05 M Tris-HCl buffer pH 7.5, 0.5 mM EDTA, 10 mM MgSO$_4$, 0.1% ascorbic acid, 0.1% bovine serum albumin, 10 uM pargyline) for 30 min at RT and then incubated for 1 h at RT in 0.2 nM [^{125}I]DOI with 100 nM MDL 100907. Non-specific binding was assessed using 10 μM RS-102221. Sections were washed in ice-cold assay buffer for 2 x 10 min, rinsed 3× with cold deionised water, and air dried for 24 h.

Air-dried slides were apposed to tritium-sensitive films and placed at 4 °C for the required period of exposure time: 8 weeks, 6 weeks and 2 days for [^{3}H]ketanserin, [O-methyl-^{3}H]WAY 100635 and [^{125}I]DOI, respectively. Autoradiograms were analysed using MCID™ (Version 7.0, Imaging Research Inc., Interfocus Ltd, U.K). Flat-field correction was applied. Tritium standards were used for calibration of tritium data density and such data were expressed as nCi/mg; the [^{125}I]DOI data were expressed as relative optical density (ROD). Blank sections were included and sampled to provide non-specific binding values. On each image, an anatomical region of interest (ROI) was drawn around the specific brain areas (mPFC, NAcc, VTA, Hipp, Hyp, Amy, LC and DRN) for quantification, based on a mouse brain atlas (Paxinos and Franklin, 2004) and the Allen Mouse Brain Atlas (Brain Explorer 2.3.2, Allen Institute from Brain Science). Specific binding of each ROI was calculated automatically by subtraction of non-specific binding from total binding.

Real-time quantitative PCR (RT-qPCR)

With the brains from another cohort of mice (CSS N = 12, CON N = 12), the expression levels of *Htr1a*, *Htr2a Htr2c*, and *Sert* (*Slc6a4*) were

investigated by means of RT-qPCR. These mice were fasted overnight prior to sample collection, for the purposes of studying gene expression of appetite hormones and their receptors (Carneiro-Nascimento et al., 2020). Given that fasting was restricted to the light/inactive phase when mice would anyway consume only a small proportion of their daily food intake, it would not be expected to impact substantively on genome expression. Brains embedded in OCT compound were mounted on the cryostat (Bright, model 5040 OTF/AS) and cut coronally into 300 μm sections at − 18 °C. Regions of interest were mPFC, NAcc, para-ventricular nucleus including periventricular nucleus of the hypothalamus (PVN), basolateral amygdala (BLA), arcuate nucleus of the hypothalamus (ARC), ventromedial nucleus of the hypothalamus (VMH), VTA and raphe nuclei (RN). In the cryostat with chamber temperature at − 24 °C, these regions were microdissected from the corresponding sections using tissue microdissection punches (Ø = 0.5 or 1 mm, model 57397, Stoelting Europe) and a mouse brain atlas (Paxinos and Franklin, 2004): mPFC (Ø = 1) at bregma 1.98–1.42; NAcc (Ø = 1) at bregma 1.42–0.74; PVN (Ø = 0.5) at bregma 0.02 to − 1.94; BLA (Ø = 1) at bregma − 0.82 to − 1.94; ARC (Ø = 0.5) at bregma − 1.34 to − 1.94; VMH (Ø = 0.5) at bregma − 1.34 to − 1.94; VTA (Ø = 0.5) at bregma − 2.92 to − 3.8 and RN (Ø = 1) at bregma − 4.36 to − 4.84.

From these tissue samples, total RNA was extracted using the RNeasy Micro Kit (Qiagen®). Tissue disruption was performed by adding QIAzol Lysis Buffer RLT (500 μL with 10 μL/ml (1%) β-mercaptoethanol) to each cryotube (Eppendorf, Protein LoBind) containing tissue and two stainless steel beads (Ø = 5 mm, QIAgen®). Tubes were placed in a TissueLyser LT (QIAgen®) for 3 min at 40 Hz. Subsequent extraction steps were performed according to the manufacturer's instructions, and each RNA sample was further digested with DNase I (QIAgen®).

Pre-designed KiCqStart® primer pairs for mouse *Htr1a*, *Htr2a* and *Htr2c* were purchased from Sigma-Aldrich. The *Slc6a4* primer pair sequence was obtained from Filipenko (2002) and synthesised by Invitrogen (ThermoScientific™). For the house-keeping gene β-actin (*Actb*), the primer pair was designed using NCBI Primer-BLAST, with the parameters set to create a product of 70–210 bp and primer melting temperature ranging from 57 °C to 63 °C. BLAT (BLAST-Like Alignment Tool) was used to check for primer sequence alignment. *Actb* was chosen as it is highly expressed throughout brain tissue (McKenzie et al. 2018) and is widely used for studies assessing gene expression in mice, including those performed on brain tissue from similar models (Martin et al., 2017). Working stocks were prepared containing both forward (FW) and reverse (RV) primers at a concentration of 10 μM.

Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Depending on the RNA yield for each brain region, different amounts of RNA were used: mPFC (85 ng), NAcc (110 ng), PVN (100 ng), BLA (100 ng), ARC (30 ng), VMH (50 ng), VTA (65 ng) and RN (100 ng). Specific quantification of cDNA targets was performed using the QuantiFast SYBR Green PCR Kit (QIAgen®) in the presence of ROX passive reference dye, according to the manufacturer's instructions. PCR cycle conditions were dependent on the primer efficiency of each target: *Htr1a,* 60 °C, 30 s, E = 106%; *Htr2a,* 60 °C, 30 s, E = 95%; *Htr2c,* 60 °C, 30 s, E = 99%; *Slc6a4,* 60 °C, 30 s, E = 100%; *Actb* 63 °C, 30 s, E = 103%. A melting curve was run for each PCR plate. Gene expression analysis was carried out using the $2^{-\Delta\Delta Ct}$ method, with *Actb* as the control gene for normalisation.

Statistical analysis

Statistical analysis of the CSS effects on serotonin receptor binding and mRNA expression in each region of interest was conducted using Statistical Package for the Social Sciences (version 22, SPSS Inc., Chicago IL, USA). For each receptor/transporter, an independent Student's *t*-test was conducted for each region. mRNA expression data are presented as fold change + standard error (SE).

Results

Serotonin receptor binding

In the first cohort of CSS and CON mice, for each of 5-HT1A, 5-HT2A and 5-HT2C receptor, radioligand binding to was present in each region of interest (ROI) (Fig. 1). For 5-HT1A receptor (Fig. 2A), [O-methyl-^{3}H] WAY 100635 binding was significantly higher in CSS mice compared with CON mice in the DRN (t(8.15) = −4.52, p < 0.01). This was the only region studied in which there was a significant difference between CSS and CON mice. For 5-HT2A receptor (Fig. 2B), the highest mean density of [3H]ketanserin binding was present in mPFC. There was significantly lower 5-HT2A binding in CSS compared with CON mice in the Amy (t(14) = 2.32, p < 0.05) and the VTA (t(13) = 6.91, p < 0.001), and no significant difference between the CSS and CON groups in the other ROIs. For 5-HT2C receptor (Fig. 2C), [125I]DOI binding mean density was highest in the DRN of CON mice, and was significantly lower in CSS compared with CON mice in this ROI (t(13) = 2.36, p < 0.05). There was no significant difference between the CSS and CON groups in any other region studied.

Serotonin receptor and transporter gene expression

In the second cohort of mice, expression of the genes *Htr1a*, *Htr2a*, and *Htr2c* was quantified by means of RT-qPCR in mPFC, NAcc, PVN, BLA, ARC, VMH, VTA and RN including DRN. For *Htr1a* (Fig. 3A), expression was significantly higher in CSS mice compared with CON mice in mPFC (t(19) = 2.92, p < 0.01), PVN (t(20) = 2.08, p < 0.05), VMH (t(20) = 3.15, p < 0.01) and RN including DRN (t(16) = 2.18, p < 0.05). There was no CSS effect in the other ROIs. *Htr2a* expression (Fig. 3B) was significantly higher in CSS mice compared with CON mice in the BLA (t(14.14) = 4.284, p < 0.001). There was no CSS effect in the other ROIs. Regarding *Htr2c* expression (Fig. 3C), there was no effect of CSS in any ROI. Expression of *Slc6a4* (Fig. 3D) was significantly higher in the RN including DRN of CSS mice compared with CON mice (t(18) = 3.11, p < 0.05).

Discussion

Serotonin neurotransmission has been the target of pharmaco-therapy for stress-related neuropsychiatric illnesses including depression and anxiety disorders for many years, and this interest continues. The focus on 5-HT as the target of antidepressant therapy has motivated preclinical and clinical studies of changes in the central 5-HT system in experimental animal models and human depression, including alterations in the binding activity of its receptors and transporter. Recent evidence indicates that 5-HT acts as a modulator of epigenetic processes as well as a neurotransmitter (Farrelly et al., 2019), further emphasising the importance of changes in its activity. Studies of human 5-HT receptors and SERT, by means of in vivo imaging with positron emission tomography (PET) or single photon computed tomography (SPECT) and post mortem binding assays, have demonstrated that depression is associated with changes in 5-HT status compared with healthy control subjects/control brain tissue (Drevets et al., 1999; Stockmeier, 2003; Newberg et al., 2015). The present mouse study aimed to assess the effects of chronic social stress on serotonin receptor binding and expression in discrete brain regions; these were selected on the basis that they are involved in the 5-HT signalling network, are important in emotional-stimulus processing, and/or impacted in the mouse model of CSS-induced depression-relevant behaviour. We observed region-specific CSS effects on the 5-HT receptors and transporter that contribute new evidence to the understanding of the inter-relationship between chronic social stress and the serotonergic system, as discussed below.
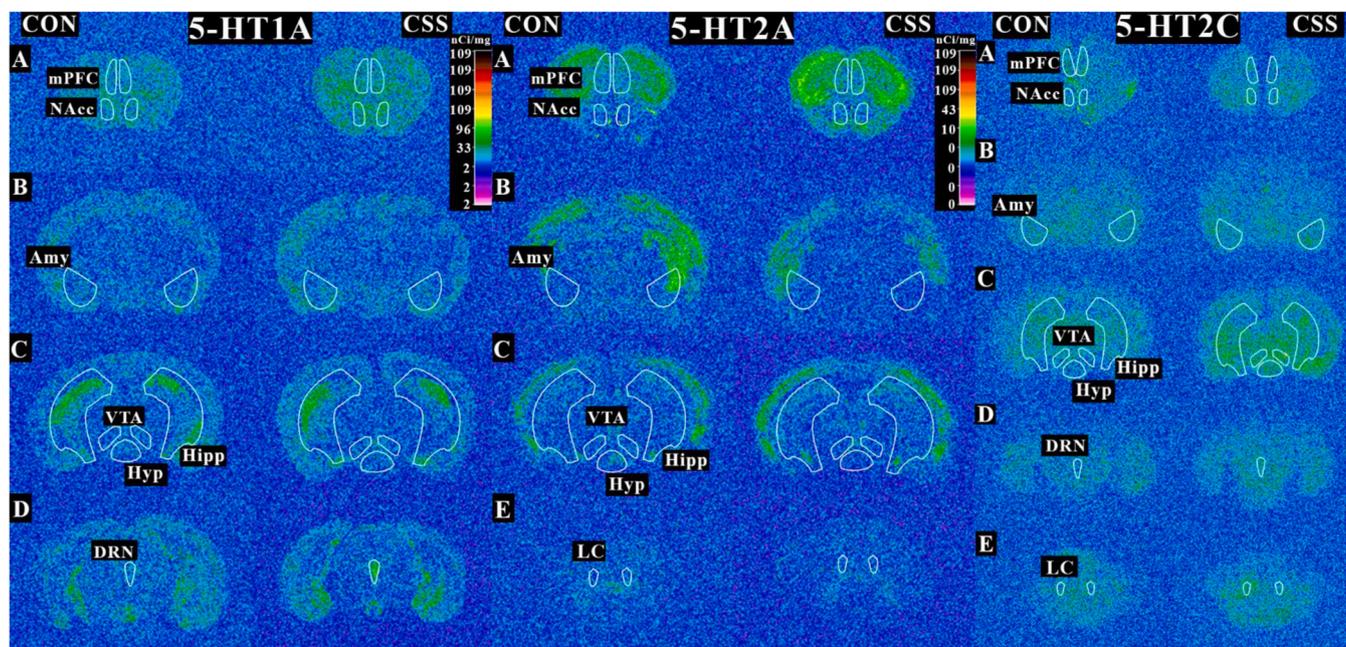
Fig. 1. Representative computer-enhanced autoradiograms of specific serotonin receptor binding in coronal brain sections from CON and CSS BL/6 mice: 5-HT1A [O-methyl-^{3}H]WAY 100635, 5-HT2A [^{3}H]ketanserin and 5-HT2C [^{125}I]DOI. ROIs are outlined in white: (A) Bregma 1.98: mPFC, NAcc; (B) Bregma − 1.06: Amy; (C) Bregma − 2.80: Hipp, VTA, Hyp; (D) Bregma − 4.16: DRN; (E) Bregma − 5.34: LC. Bregma levels are based on a mouse brain atlas (Paxinos and Franklin, 2004). Scale-bars are in nCi/mg. Abbreviations: mPFC, medial prefrontal cortex; NAcc, nucleus accumbens; Amy, amygdala; Hyp, hypothalamus; VTA, ventral tegmental area; Hipp, hippocampus; DRN, dorsal raphe nucleus; LC, locus coeruleus.

Serotonin 5-HT1A receptor and Htr1a

In the two study cohorts, CSS mice had increased 5-HT1A binding in DRN and increased *Htr1a* expression in RN, mPFC, PVN and VMH, respectively. Therefore, both gene expression and receptor binding were increased in the RN/DRN and gene expression specifically in the mPFC; whilst it could be a cohort effect, the latter finding might also be due to an increase in inhibitory miRNA function on *Htr1a* mRNA translation in the mPFC (see also discussion of *Htr2a* below). In the RN, 5-HT1A is a somatodendritic inhibitory autoreceptor located on 5-HT neurons; when activated by 5-HT it reduces neuronal serotonergic firing rate and 5-HT release in the projection regions in rodent (e.g. Albert and Vahid-Ansari, 2019) and human brains (Carhart-Harris and Nutt (2017) and references therein). Therefore, other things being equal, increased RN 5-HT1A binding would be expected to decrease 5-HT synaptic release in CSS mice. In preclinical studies, higher levels of 5-HT1A autoreceptors led to development of depression-relevant phenotypes in mice (Richardson-Jones et al., 2010). Chronic postnatal stress in rats led to reduced 5-HT1A binding in DRN and reduced reward motivation in adulthood, and both effects were reversed by chronic fluoxetine administration (Leventopoulos et al., 2009). Humans with major depressive disorder (MDD) exhibit increased 5-HT1A receptor binding in several brain regions including the RN (Hesselgrave and Parsey, 2013; Kaufman et al., 2016). Reduced 5-HT1A specific binding in the RN of depression patients has also been observed, however (Drevets et al., 1999; Sargent et al., 2000); in one study, the caudal DRN exhibited lower 5-HT1A binding whereas the rostral DRN exhibited higher binding in depression (Kaufman et al., 2016), suggests that 5-HT1A heterogeneity within the human raphe might account for these study differences.

Serotonin 5-HT2A receptor and Htr2a

We observed a decrease in the specific binding of 5-HT2A receptors in the amygdala and the VTA of CSS mice in comparison with CON mice. Using repeated uncontrollable electroshocks as a stressor (so-called learned helplessness) led to decreased 5-HT2A protein expression and

receptor density in the amygdala of rats (Wu et al., 1999; Jiang et al., 2009). In the amygdala, 5-HT signalling is an important modulator of aversion processing and changes therein are proposed to contribute to the pathophysiology of stress-related disorders (Holmes, 2008; Bocchio et al., 2016). In the VTA, the 5-HT2A receptor is important in modulating the mesocorticolimbic dopaminergic system, dysfunction of which contributes to reward processing pathologies (Doherty and Pickel, 2000). In clinical studies, 5-HT2A binding was increased in amygdala and prefrontal cortex in suicide/depression relative to control subjects (Hrdina et al., 1993), and decreased in the frontal cortex, occipital cortex, prefrontal cortex and anterior cingulate cortex in MDD patients (Nikolaus et al., 2016).

In the second mouse cohort, BLA *Htr2a* expression was increased in CSS versus CON mice. Taken together with the CSS-induced increase in amygdala 5-HT2A receptor binding identified in the first cohort, this would suggest that CSS induces impaired post-transcriptional processing of *Htr2a*. In addition, CSS-induced differences in in vivo protein half-life as well as membrane flip-flop translocation might contribute to the decreased 5-HT2A binding in CSS mice (Contreras et al., 2010; Gout et al., 2017). Whilst we do not consider it to be the explanation for the directional differences in CSS effects on receptor binding and gene expression, both for 5-HT2A and 5-HT2C (see below), it is worth noting that the mice studied in terms of receptor binding were not fasted overnight, whereas those studied in terms of gene expression were. However, as we state in the methods, the fast was during light phase when little food is consumed, rather than a 24 h fast including the dark phase which has been shown to affect expression of 5-HT receptor genes (Nonogaki et al.,2006). Furthermore, whilst it could be that the differences in receptor binding and mRNA findings are due to mouse cohort differences in CSS responsiveness, given the reproducibility of the CSS effect on other measures, including behavioural readouts (Table 1), this is unlikely.

Serotonin 5-HT2C receptor and Htr2c

Concerning the specific binding of 5-HT2C receptors, this was altered
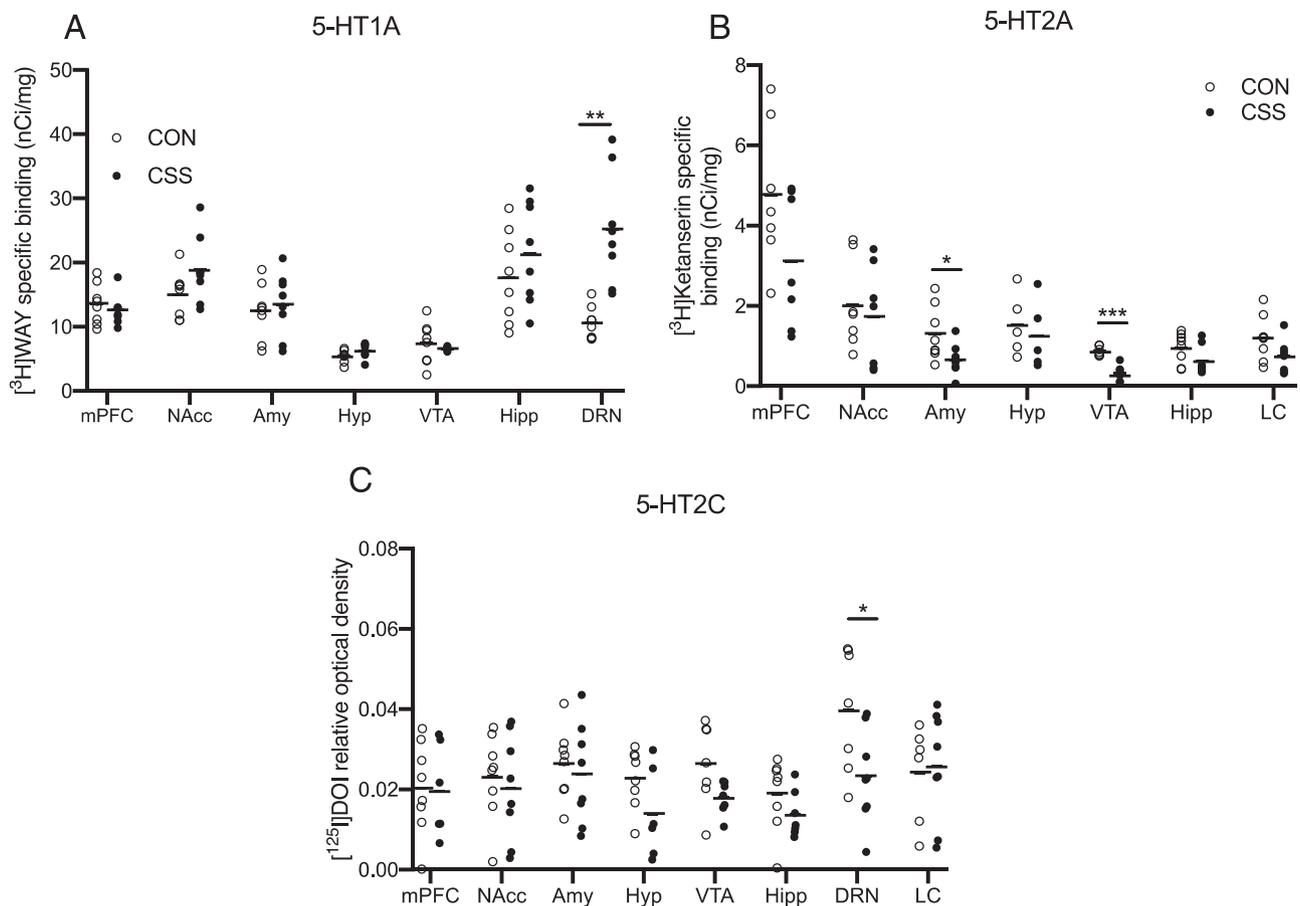
Fig. 2. Effects of 15-day chronic social stress (CSS, N = 8) versus control handling (CON, N = 8) on specific binding of (A) [O-methyl-^{3}H]WAY 100635, (B) [^{3}H] ketanserin and (C) [^{125}I]DOI at 5-HT1A, 5-HT2A and 5-HT2C receptor sites, respectively. Individual values and group means are shown. *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001 for CSS versus CON mice analysed by independent Student's t-tests. Mean $\pm$ SEM per receptor, region and group are detailed here. For (A) 5-HT1A: mPFC (CON: 13.5 $\pm$ 1.1, CSS: 12.6 $\pm$ 1.0), NAcc (CON: 15.1 $\pm$ 1.3, CSS: 18.8 $\pm$ 1.9), Amy (CON: 12.4 $\pm$ 1.5, CSS: 13.4 $\pm$ 1.8), Hyp (CON: 5.4 $\pm$ 0.4, CSS: 6.3 $\pm$ 0.5), VTA (CON: 7.4 $\pm$ 1.1, CSS: 6.6 $\pm$ 0.1), Hipp (CON: 17.7 $\pm$ 2.5, CSS: 21.4 $\pm$ 2.8), DRN (CON: 10.6 $\pm$ 0.9, CSS: 25.1 $\pm$ 3.1). For (B) 5-HT2A: mPFC (CON: 4.8 $\pm$ 0.7, CSS: 3.1 $\pm$ 0.6), NAcc (CON: 2.0 $\pm$ 0.4, CSS: 1.7 $\pm$ 0.5), Amy (CON: 1.3 $\pm$ 0.2, CSS: 0.7 $\pm$ 0.1), Hyp (CON: 1.5 $\pm$ 0.3, CSS: 1.3 $\pm$ 0.4), VTA (CON: 0.9 $\pm$ 0.04, CSS:0.3 $\pm$ 0.1), Hipp (CON: 1.0 $\pm$ 0.1, CSS: 0.6 $\pm$ 0.1), LC (CON: 1.2 $\pm$ 0.2, CSS: 0.7 $\pm$ 0.1). For (C) 5-HT2C: mPFC (CON: 0.02 $\pm$ 0.004, CSS: 0.02 $\pm$ 0.005), NAcc (CON: 0.02 $\pm$ 0.004, CSS: 0.02 $\pm$ 0.005), Amy (CON: 0.03 $\pm$ 0.003, CSS: 0.02 $\pm$ 0.004), Hyp (CON: 0.02 $\pm$ 0.003, CSS: 0.01 $\pm$ 0.005), VTA (CON: 0.03 $\pm$ 0.004, CSS: 0.02 $\pm$ 0.002), Hipp (CON: 0.02 $\pm$ 0.003, CSS: 0.01 $\pm$ 0.002), DRN (CON: 0.04 $\pm$ 0.006, CSS: 0.02 $\pm$ 0.004), LC (CON: 0.02 $\pm$ 0.005, CSS: 0.03 $\pm$ 0.005).

by CSS in the RN specifically, with CSS mice exhibiting an increase compared with CON mice. In rats, chronic early life stress also led to increased DRN 5-HT2C specific binding in adulthood (Leventopoulos et al., 2009). In the second mouse cohort, there was no CSS effect on *Htr2c* expression in any ROI including the RN. That 5-HT2C binding was increased by CSS in the absence of any change in gene expression might be explained by the alternative splicing of *Htr2c*. The splice variants include an mRNA for a truncated receptor isoform that is strictly located in internal membranes and, by means of hetero-dimerisation with a full-length receptor isoform, can reduce the overall constitutive activity of 5-HT2C receptors (Stamm et al., 2017). Thus, a possible shift in alternative RNA splicing towards increased formation of the truncated receptor isoform in CSS mice might contribute to decreased 5-HT2C membrane expression and therefore specific binding in the DRN.

Preclinical studies have shown that DRN 5-HT2C receptors are involved in the regulation of DRN 5-HT neuron firing (Boothman et al., 2003; 2006). Changes therein might contribute to animal depression-relevant behaviour and indeed human depression. The 5-HT2C agonist DOI reduces 5-HT neuron firing in rats (Boothman et al., 2003). Decreased DRN 5-HT2C binding as observed in CSS mice might lead to increased 5-HT neuron firing therefore, although this would also be expected to be counteracted by the increase in DRN 5-HT1A (autoreceptor) specific binding, as noted above.

Slc6a4

The expression of *Slc6a4* in the RN was increased in CSS mice compared with CON mice, similarly to a previous study that used repeated social stress (Filipenko et al., 2002). Assuming that the increase in RN *Slc6a4* mRNA predicts an increase in RN SERT protein, as observed by others (Zhang et al., 2012), 5-HT reuptake would be increased in target regions, leading to reduced synaptic levels of 5-HT (De Felice, 2016). Studies in MDD patients have yielded different findings for SERT binding: increased, decreased or unchanged levels of SERT binding have been reported, which reflects the complexity of the clinical condition and its comorbidities, the brain region studied and the methods used (Meyer, 2007). For example, increased SERT binding in MDD was observed by means of PET with [11C]DASB ligand, in the insula, thalamus and striatum (Cannon et al., 2007), whereas decreased SERT binding in MDD in the brainstem, midbrain and basal ganglia was also observed (Malison et al., 1998; Newberg et al., 2012, 2015).

Integration of serotonin findings with behavioural effects of CSS

To summarise the present findings, it is clear that CSS in mice induced changes in some major functional elements of the raphe region: it increased 5-HT1A and reduced 5-HT2C receptor binding in the DRN,
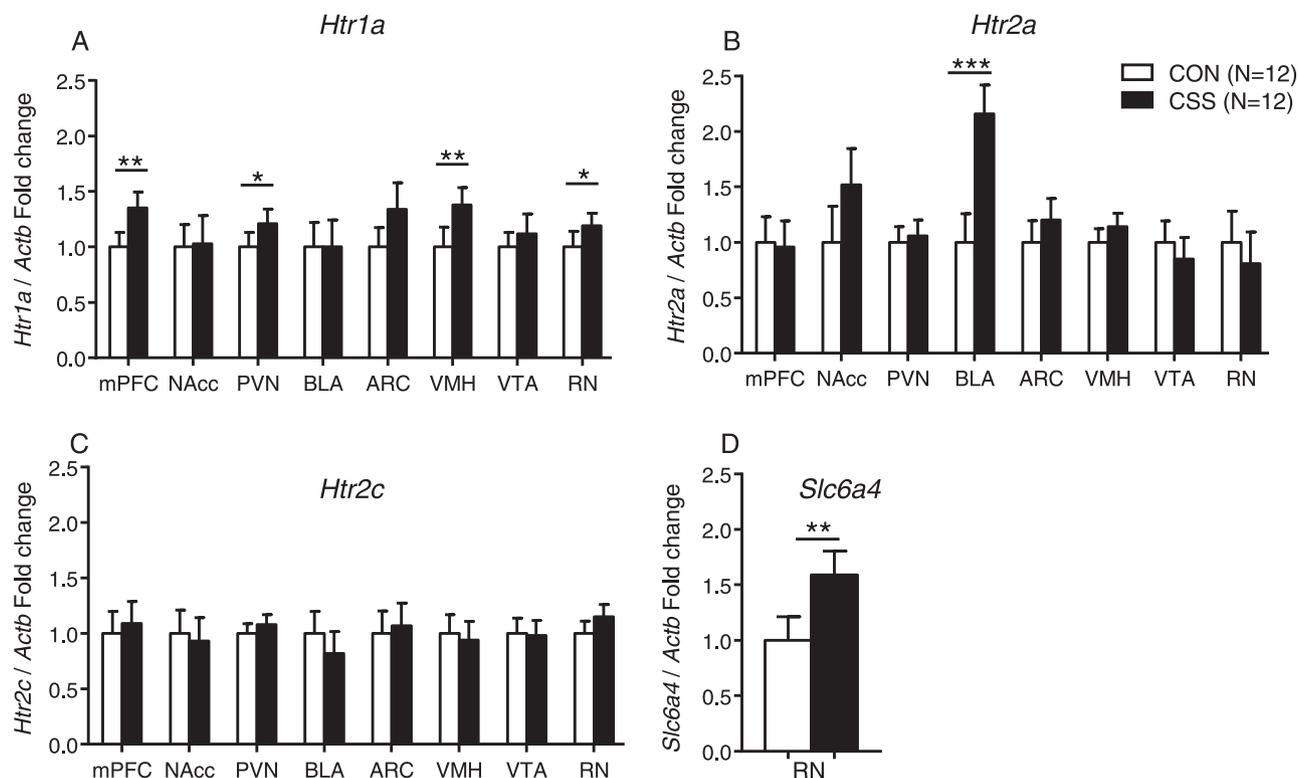
Fig. 3. Effects of 15-day chronic social stress (CSS, N = 12) versus control handling (CON, N = 12) on ROI-specific: (A) Htr1a mRNA expression; (B) Htr2a mRNA expression; (C) Htr2c mRNA expression; (D) Slc6a4 mRNA expression. Values were calculated as Log2 fold change relative to Actb. Values are presented as fold change + SE. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 for CSS versus CON mice analysed by independent Student's *t*-tests.

and increased the expression of *Slc6a4* in the RN, the latter effect suggesting increased SERT levels and serotonin re-uptake at the distal terminals of raphe axons. In addition, of the raphe 5-HT projection regions that were studied, the amygdala and ventral tegmental area had reduced 5-HT2A receptor binding in CSS mice. The combination of increased DRN 5-HT1A autoreceptor binding and increased RN *Slc6a4* expression suggests that tonic synaptic levels of 5-HT are decreased in CSS mice, and it is important to integrate these changes with the behavioural effects of CSS (see Table 1). Before doing so, it is important to emphasise that, as is the case for the physiological and behavioural effects demonstrated to date, for the 5-HT parameters investigated in this study, inter-individual differences in CSS mice were similar to those in CON mice, again demonstrating that CSS effects are not confined to a subgroup of mice (Pryce and Fuchs, 2017). With regards to Pavlovian aversion learning-memory, there is substantive evidence that 5-HT signalling in amygdala and hippocampus promotes these processes (Bauer, 2015). Based on this, the current findings suggest that altered 5-HT receptor and transporter expressions do not contribute to the increase in Pavlovian aversion learning-memory observed consistently in CSS mice (Table 1). However, it has also been proposed that low tonic synaptic 5-HT levels lead to a relative increase in the magnitude of the phasic response (i.e. high signal-to-noise ratio of 5-HT neurotransmission) to a high-valence aversive stimulus, including both unconditioned (e.g. electroshock) and conditioned (e.g. tone conditioned to electroshock) (Cools et al., 2008). In this latter context, it is highly relevant that the SSRI escitalopram, which would be expected to increase tonic 5-HT synaptic levels, reversed the CSS effect on Pavlovian aversion learning-memory (Fuertig et al., 2016). The same reasoning could also explain the increased learned uncontrollability observed in CSS mice in a two-way escape test (Table 1); reduced electroshock escape behaviour, indicative of learned uncontrollability, co-occurs with a (relatively) high phasic 5-HT release from DRN neurons in response to the aversive stimulus (Amat et al., 2005). With regards to positive valence/reward

processing, the CSS effects of decreased Pavlovian reward learning (Table 1) and decreased reward effortful motivation (Table 1) are likely more attributable to changes in dopamine, rather than 5-HT, neurotransmission (e.g. see Bergamini et al. (2018)). However, in operant reversal learning tests with reward reinforcement, where alternative choices are serially associated with both reward and non-reward (punishment), there is translational evidence for important involvement of 5-HT (Cools et al., 2008; Faulkner and Deakin, 2014). Chronic social stress mice display reduced reversal learning and probabilistic reversal learning, underlain by a reduced ability to discriminate the currently rewarded and non-rewarded stimuli (Table 1). In these same tests, the SSRI escitalopram increases performance in otherwise unmanipulated mice (Ineichen et al., 2012). In humans, both healthy probands in which 5-HT has been reduced pharmacologically and clinically depressed subjects, probabilistic reversal learning is also reduced, and it has been proposed that this effect is underlain by deficits in non-reward learning, mediated in particular by deficient 5-HT signalling in the amygdala (Faulkner and Deakin, 2014).

In addition to the study limitations alluded to above, some further points also merit mention. To increase the translational relevance of this study, it would have been interesting to include CSS and CON groups that had received SSRI administration; in particular to observe which, if any, of the CSS effects were reversed. Out of necessity, the resident-intruder stress model must be conducted with male mice which, given the higher prevalence of depression in women than men, is a limitation of the manipulation. Other chronic stressors, such as CUMS, can be conducted with males and females, but lack the aetiological validity of CSS (Pryce and Fuchs, 2017). Therefore, both stressors are relevant in the present context and it will be important to compare their respective effects on both translational readout tests of behaviour and on the 5-HT system in future studies.

Therefore, the present study demonstrates that chronic social stress in mice alters the central serotonergic system with respect to various and

region-specific changes in gene expression and binding activity of the 5-HT receptors and transporter. Taken together, these changes would be expected to decrease 5-HT signalling and to therefore contribute to some of the depression-relevant changes in emotional stimulus processing – namely increased aversion sensitivity and decreased non-reward learning – described for this mouse model.

Author contributions

S. Carneiro-Nascimento designed research, performed experiments, analysed data and wrote the manuscript. M. Buerge and H. Sigrist performed experiments. W. Powell and M. Uebel provided assistance with methodology and experiments. J. Opacka-Juffry, M. Patterson and C.R. Pryce designed research, acquired funds, supervised experimental work, and wrote the manuscript. C.R. Pryce revised the manuscript; all the co-authors approved the submitted manuscript.

Ethics statement

Breeding of C57BL/6J mice and in vivo experiments were conducted at the Preclinical Laboratory for Translational Research into Affective Disorders, University of Zurich, under permits for animal experimentation (170/2012, 149/2015) issued by the Veterinary Office of canton Zurich.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgements

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