Markers of serotonergic function in the orbitofrontal cortex and dorsal raphé nucleus predict individual variation in spatial-discrimination serial reversal learning

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Abstract

Dysfunction of the orbitofrontal cortex (OFC) impairs the ability of individuals to flexibly adapt behavior to changing stimulus-reward (S-R) contingencies. Impaired flexibility also results from interventions that alter serotonin (5-HT) and dopamine (DA) transmission in the OFC and dorsomedial striatum (DMS). However, it is unclear whether similar mechanisms underpin naturally occurring variations in behavioral flexibility. In the present study we used a spatial-discrimination serial reversal procedure to investigate inter-individual variability in behavioral flexibility in rats. We show that flexibility on this task is improved following systemic administration of the 5-HT reuptake inhibitor citalopram and by low doses of the DA reuptake inhibitor GBR12909. Rats in the upper quintile of the distribution of perseverative responses during repeated S-R reversals showed significantly reduced levels of the 5-HT metabolite, 5-hydroxy-indoleacetic acid, in the OFC. Additionally, 5-HT_{2A} receptor binding in the OFC of mid- and high-quintile rats was significantly reduced compared with rats in the low-quintile group. These perturbations were accompanied by an increase in the expression of monoamine oxidase-A (MAO-A) and MAO-B in the lateral OFC and by a decrease in the expression of MAO-A, MAO-B and tryptophan hydroxylase in the dorsal raphé nucleus of highly perseverative rats. We found no evidence of significant differences in markers of DA and 5-HT function in the DMS or MAO expression in the ventral tegmental area of low- versus high-perseverative rats. These findings indicate that diminished serotonergic tone in the OFC may be an endophenotype that predisposes to behavioral inflexibility and other forms of compulsive behavior.

Key words: serotonin; dopamine; striatum; perseveration; monoamine oxidase; tryptophan hydroxylase

Introduction

Cognitive inflexibility is widely associated with depression (Dickstein *et al*, 2010), schizophrenia (Leeson *et al*, 2009), obsessive-compulsive disorder (OCD) (Chamberlain *et al*, 2006; Remijnse *et al*, 2006), and addiction (Ersche *et al*, 2008). The capacity to flexibility switch responding to changing stimulus-response (S-R) contingencies is widely assessed using reversal learning procedures; for example, in humans (Fellows and Farah, 2003; Murphy *et al*, 2002), non-human primates (Butter, 1969; Clarke *et al*, 2007; Dias *et al*, 1996; Groman *et al*, 2013) and rodents (Boulougouris *et al*, 2007; Chudasama and Robbins, 2003; McAlonan and Brown, 2003). Effective reversal learning requires a new S-R contingency to be learnt whilst ignoring competing interference from a previously learnt response. A failure to suppress previously learned responses is expressed behaviorally as increased response perseveration (Iversen and Mishkin, 1970).

Convergent evidence indicates that reversal learning is modulated by orbitofrontal-striatal mechanisms (Roberts, 2011). The OFC receives a dense serotonergic innervation from the dorsal raphé nucleus (DRN), which in turn provides regulatory input to the DRN (Azmitia and Segal, 1978; Peyron et al, 1998; Santana et al, 2004). In humans, the OFC is selectively activated during reversal learning (Hampshire and Owen, 2006) and damage to this region disrupts reversal learning in experimental animals (Bissonette *et al*, 2008; Boulougouris *et al*, 2007; Burke *et al*, 2009; Dias *et al*, 1996; Fellows *et al*, 2003). In contrast, a recent study by Rudebeck *et al* (2013) found that excitotoxic, fibre-sparing lesions of the macaque OFC had no effect on reversal learning performance. The basis for this discrepancy is unclear but may reflect cross-species differences in OFC anatomy and function together with variation in the methods used to assess reversal learning in different species. A role for 5-HT in reversal learning is substantiated by studies in humans involving dietary tryptophan depletion (Rogers *et al*, 1999) and in experimental animals depleted of 5-HT, both

globally (Mobini *et al*, 2000) and locally in the OFC (Clarke *et al*, 2004). In rats, 5-HT_{2A} and 5-HT_{2C} receptors bi-directionally modulate reversal learning (Boulougouris *et al*, 2008), putatively at the level of the OFC (Boulougouris and Robbins, 2010). Research also links the DMS and its DA, but not 5-HT, innervation to reversal learning (Castane *et al*, 2010; Clarke *et al*, 2011; O'Neill and Brown, 2007). Optimal DA levels in striatum are associated with improved reversal learning (Clatworthy *et al*, 2009; Cools *et al*, 2009) and in non-human primates flexible behavior depends in part on 5-HT and DA interactions in the OFC and striatum (Groman *et al*, 2013).

Recent evidence indicates that gene products associated with the metabolism and transport of 5-HT and DA may play a role in behavioral flexibility. Thus, variants of the 5-HT transporter (5-HTT) gene, *SLC6A4*, and of the dopamine transporter (DAT) gene, *SLC6A3*, predict reversal learning performance in humans (den Ouden *et al*, 2013) and *Slc6a4*-deletion mice more rapidly reverse visual discriminations than their unaffected littermates (Brigman *et al*, 2010). However, less is known about how the two isoforms of monoamine oxidase (MAO-A and MAO-B), tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH2) activity influences reversal learning despite their key role in the synthesis and degradation of biogenic amines (Shih and Thompson, 1999). Thus, by controlling 5-HT and DA homeostasis, MAO, TH and TPH2 may critically regulate flexible, goal-directed behavior.

Here we investigated the relationship between inter-individual variation in spatial reversal learning in rats and the natural heterogeneity that exists in 5-HT and DA functional markers in the OFC and DMS. We investigated the hypothesis that MAO, TH and TPH2 dysfunction in orbitofrontal-striatal circuitry may be linked to individual variation in spatial-discrimination serial reversal learning.

Materials and Methods

Subjects

Subjects were 192 male Lister-hooded rats (Charles River, Kent, UK), weighing 250-300g at the start of the experiment, and maintained at 85-95% of their free-feeding weight. Water was available *ad libitum*. Animals were group-housed, four per cage, and kept under a reversed light/dark cycle (white lights on/red light off from 19:00 to 07:00). Testing took place between 08:00 and 16:00. Four cohorts of rats were used for this study; each comprising 48 animals. These were destined for systemic drug administration (cohort 1), post-mortem monoamine analysis (cohort 2), *in-vitro* autoradiography (cohort 3), and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis (cohort 4). Cohorts 2-4 consisted of drug-naive animals only. All experiments were carried out in accordance with the UK (1986) Animal (Scientific Procedures) Act. Ten subjects were excluded from the study (four animals, each from cohorts 2 and 3, and one from both cohort 1 and cohort 4) because they failed to acquire a spatial discrimination during the acquisition of the task, as described below. In cohort 4, the posterior section of the brain was lost from 2 animals; these were excluded from the analysis of MAO expression in the DRN and VTA.

Behavioral apparatus

Testing was carried out in twelve 5-hole operant chambers (Med Associates, Georgia, VT), enclosed in a sound-attenuating box fitted with a fan for ventilation and masking of external noise. An array of five square nose-poke holes was set in the curved wall of each box. An infrared detector was positioned across each nose poke aperture. A yellow light emitting diode stimulus light was located at the rear of each aperture. On the adjacent wall a food magazine was located into which rodent food pellets (TestDiet®, Purina, UK) were delivered. The three inner apertures of the chamber were blocked using metal inserts so only

the two outermost holes remained unobstructed. The testing apparatus was controlled by Whisker Control software (Cardinal and Aitken, 2010).

Behavioral training

Subjects were initially habituated to the test apparatus over two days with each daily session lasting 20 min. During each session, the two stimulus lights, house-light and magazine light were illuminated, and the food magazine was filled with pellets. After the habituation phase, animals were trained to nose poke in the magazine to trigger the illumination of the stimulus lights and to respond in the holes for food delivery. This phase of training took place successively in each hole under a fixed ratio-1 schedule of reinforcement (FR1) to a criterion of 50 correct trials in 20 min, and thereafter, under FR2 and FR3 schedules to the same criterion. This schedule was used to eliminate the possibility of random, accidental nose poke responses. Responses in the unrewarded hole were not punished but omission errors resulted in a 5 sec time-out period, where all lights were extinguished. After the initial nose poke to trigger illumination of the stimulus lights, animals were required to make a response at the nose poke apertures within a 30 sec limited hold period. An inter-trial interval of 5 sec was introduced when responding had stabilized under a FR3 schedule.

Acquisition of spatial discrimination

After the initial training stage, subjects were trained on a two-hole discrimination task. A nose poke in the food magazine triggered the illumination of both stimulus lights. A sequence of 3 nose pokes in one of the holes resulted in reward (see **Fig. 1**). Three nose pokes in the "incorrect" hole resulted in a time-out and no reward. Rats were trained across sessions until they achieved a criterion of 9 correct trials across the previous 10 trials. "Correct" and "incorrect" holes were designated randomly and counterbalanced across subjects.

Within-session reversal learning

This session began with the illumination of both the house-light and magazine light. For individual rats, "correct" and "incorrect" holes were kept the same as those experienced in the acquisition of the spatial discrimination. After rats had reached criterion on this retention phase, the "correct" and "incorrect" holes were reversed such that the previously rewarded response now resulted in a time-out period, and the previously unrewarded response resulted in the delivery of a food pellet (see **Fig. 1**). Subjects completed three reversals, but no more, during the 1 hour session. We used this within-session serial reversal design because many animals display marked perseveration on the first reversal that they experience. Consequently, therefore, a single reversal does not effectively differentiate between good, middle and poor learners. Allowing animals to complete a second and third reversal in the same session provided a more sensitive method to categorize animals on the basis of perseverative responding.

Systemic drug administration

The selective 5-HT and DA reuptake inhibitors citalopram hydrobromide and GBR12909 dihydrochloride were purchased from Sigma (UK) and evaluated in the same subjects following a 1-week wash-out period between each compound. Drugs were administered intra-peritoneally (1 ml/kg, phosphate-buffered saline, PBS), starting with citalopram (PBS, 1, 3, 10 mg/kg) followed by GBR12909 (distilled deionised water, 1, 3, 10 mg/kg). Doses were selected according to previous research findings in Lister-hooded rats (Baarendse and Vanderschuren, 2012) and administered according to a fully randomized Latin square design. Drugs were administered 20 min prior to reversal learning, in a different room to the operant testing room. Each experiment started with a baseline retention session (day 1), followed by the test session where the drug was administered (day 2), and a third day where animals were

maintained in their home-cages. This cycle was repeated for each dose of drug administered. The criterion for retention and reversal sessions was the same as in initial testing.

Ex-vivo neurochemistry

Subjects were sacrificed by CO_2-induced asphyxiation and cervical dislocation. Brains were rapidly removed and placed on a steel dissection plate, cooled on dry ice, with the dorsal surface uppermost before being frozen at -80°C. Brains destined for qRT-PCR analysis were flash frozen in isopentane, at -30°C, to ensure minimal RNA degradation and stored at -80°C. Brains were sectioned in the coronal plane using a Jung CM300 cryostat (Leica, Wetzlar, Germany). For autoradiography, consecutive 20 μm slices throughout the OFC and striatum were mounted on Superfrost Plus microscope slides (Fisher Scientific, UK). Sections were stored at -80°C prior to being thawed at room temperature for processing. Samples destined for analysis by high-performance liquid chromatography (HPLC) and electrochemical detection (ECD), were sectioned into 150 μm consecutive slices and mounted on chilled microscope slides. Aliquots of tissue were removed using a micropunch of 1.2 mm diameter. Tissue from the medial and lateral OFC (mOFC, lOFC), and DMS (see **Fig. 2**) was extracted and frozen at -80°C. For qRT-PCR analysis, tissue was collected as described above for the HPLC-ECD study, and placed in RNA*later* stabilization reagent (QIAGEN, UK) for at least 1 hour at room temperature before being frozen at -20°C.

Neurochemical analysis

Samples were placed in 75 μl of 0.2M perchloric acid and kept on ice. Tissue samples were homogenized using an ultrasonic cell disrupter (QSonica LLC, Newton, CT, USA) and subsequently centrifuged at 6000 rpm for 10 min at 4°C. Twenty-five μl of the supernatant was collected for analysis. DA and 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HT and 5-

hydroxyindoleacetic acid (5-HIAA) were measured by HPLC-ECD, as described previously (Dalley *et al*, 2002). Quantification was achieved using a Coulochem II detector with an analytical cell (ESA model 5014B) and two electrodes in series (E1 -250 mV, E2 + 250 mV). The signal from E2 was integrated using computer software (Dionex Chromeleon, v6.8). The limit of detection varied between 5 and 10 fmoles for DA and DOPAC, and between 10 and 20 fmoles for 5-HT and 5-HIAA.

Ex-vivo receptor autoradiography

[3H]Citalopram (3127 GBq/mmol), [3H]ketanserin (1976 GBq/mmol), [3H]GBR12935 (1480 GBq/mmol) and [3H]raclopride (2812 GBq/mmol) were purchased from PerkinElmer (UK). Fluoxetine, mianserin, and mazindol were purchased from Sigma-Aldrich (UK); haloperidol was purchased from Tocris (UK). Duplicate, consecutive slides were pre-washed for 15 min at room temperature in 150 mM Tris-HCl (pH 7.4). Slides were incubated in a buffer containing the radioligand. For non-specific binding, additional cold ligand was added to the incubation buffer. Ligand concentrations and incubation times are given in **Table S1**. Following incubation, slides were washed twice in fresh 4°C buffer for 2 min and then rinsed in distilled-deionised water. Slides were air-dried for at least 2 hours before being fixed in paraformaldehyde vapour. These were subsequently apposed with tritium microscale standards (Amersham Biosciences, Freiburg, Germany) to a tritium-sensitive phosphor-imaging plate (FujiFilm, Tokyo, Japan). The plates were scanned using a FLA-5000 Bioimaging Analyser (Fujifilm) to digitize autoradiographs at 16-bit greyscale for image analysis. Region-of-interest analysis was conducted using IMAGE J (Abramoff *et al.*, 2004).

Gene expression

Messenger RNA was extracted from the frozen samples using the miRNeasy Micro Kit (QIAGEN, UK) with additional DNAse digestion. First-strand cDNA was synthesized from 5 ng total RNA with random hexamer primers using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, UK). SYBR green-based quantitative RT-PCR was performed on the CFX96 Touch thermal cycler (BioRad, UK). PCR was performed using 0.25 µM of each primer. The primer pairs, designed using Primer-BLAST software (NCBI) and purchased from Sigma Aldrich (UK), are given in **Table S2**. PCR conditions were as follows: 95°C for 5 minutes; 40 cycles at 95°C for 10 seconds; 60°C for 10 seconds, and 72°C for 1 minute.

PCR efficiencies for each gene were calculated using LinRegPCR (Freeware, HRFC, The Netherlands). Normalised relative quantities (NRQs) for all genes of interest were calculated using multiple reference genes (tubulin, actin, GAPDH and RLP19) and by adjusting for differences in PCR efficacy. The stability of each reference gene was assessed by calculating the gene stability value (M) and coefficient of variation (CV) in qBase+ (Biogazelle, Belgium). Reference genes that had mean CV and M values higher than 25% and 0.5, respectively, were excluded from further normalisation calculations.

Statistical analyses

Inferential statistics was carried out using SPSS for Windows (v.21). The main dependent variables analyzed were the total number of trials and errors to criterion. Errors made were refined by looking specifically at "perseverative" errors. Data were analyzed in moving windows of blocks of 10 trials. In the case where 7 or more commission errors (errors made due to an incorrect response being made, and not due to omissions) were made in a window of 10 trials, and where these were determined to be statistically significant (using Pearson's chi squared test, $p<0.05$), the errors were classed as "perseverative". Non-perseverative errors

were very small in number with many animals making no learning errors at all. Therefore, our analysis focused on perseverative errors as an index of behavioural flexibility. Dependent variables were measured across 3 reversals and the mean values used for statistical analysis. Subjects from each cohort were ranked for perseverative responses and divided into high-, mid- and low-perseveration groups based on the following criterion: High (upper quintile); Mid (middle quintile); Low (lower quintile). Behavioral data were analysed using repeated measures analysis of variance (ANOVA). When significant main effects or interactions were found, *post hoc* analysis using Fishers LSD test was performed. When the assumption of homogeneity of variance could not be met, a Games-Howell test was used. One-way ANOVA was used to compare *ex-vivo* monoamine and receptor levels and mRNA expression in high-, mid- and low-perseveration groups. Neurochemical variables were also regressed against perseverative responses for the low, mid, and high perseveration groups combined to determine the proportion of variance explained by the general linear model (R^2). Statistical significance was set at α=0.05.

Results

Behavioral screening

Rats were segregated into three groups according to their perseverative behavior on the first serial reversal learning session: (i) low-perseveration (first quintile); (ii) mid-perseveration (third quintile); (iii) high-perseveration (fifth quintile). The segregation of rats into quintiles not only allowed the inclusion of rats in the lower and upper regions of the distribution but also rats in the centre of the distribution. **Fig. 3A-D** shows the frequency distributions of perseverative responses, trials to criterion, and errors to criterion for the four cohorts of animals used in this study. Numerical data are shown in **Table S3.** The mean, median, and inter-quartile ranges of perseverative responses were: 33.4, 36.6 and 52.6 (cohort 1); 35.2,

35.9 and 30.2 (cohort 2); 28.4, 29.4 and 47.1 (cohort 3); 43.1, 41.6, and 23.9 (cohort 4). There was no significant difference in perseverative responses between the 4 cohorts ($F_{3,181}$=2.39; p=0.07). The overall distributions of perseverative responses (**Fig. 3B**), total trials (**Fig. 3C**), and total errors (**Fig. 3D**) were positively skewed (skewness: 0.073; 0.81; 0.91, respectively). Response latencies to initiate a new trial following a correct response were highly variable between high, mid, and low perseveration groups (cohort 1: means ± SEM: 4.33 ± 1.76 s, 3.74 ± 1.31 s and 3.10 ± 1.46 s, respectively). Although the data suggest that highly perseverative animals were slower to initiate a new trial this was not significant ($F_{2,24}$ = 0.158; p=0.855). Following an incorrect response, the latency values were 8.46 ± 2.54 s (highs), 7.57 ± 1.11 s (mids), and 6.33 ± 1.43 s (lows). Again there was no main effect of group ($F_{2,24}$ = 0.365; p=0.699) indicating that perseveration was not accompanied by apparent changes in motor behaviour.

5-HT reuptake inhibition facilitates reversal learning performance, similar to the effect of low-dose DA reuptake inhibition

To validate the sensitivity of the spatial-discrimination serial reversal task to altered levels of 5-HT and DA rats from the low-, mid- and high-quintile response-perseveration groups were tested after the administration of either citalopram or GBR12909. Citalopram produced a dose-related improvement in reversal learning as reflected by a decrease in the number of trials to reach criterion on the task (dose: $F_{3,63}$=3.38, p=0.023; **Fig. 4A**). *Post-hoc* tests revealed that the lowest (1 mg/kg; p=0.035) and highest (10 mg/kg; p=0.014) dose of citalopram significantly decreased total trials to criterion compared with the vehicle group. However, no significant dose x group interaction was observed ($F_{6,63}$=0.61, p=0.720). In contrast, GBR12909 produced a dose-dependent, biphasic effect on reversal learning (dose: $F_{3,60}$=4.544, p=0.006; dose x group interaction: $F_{6,60}$=0.89, p=0.511; **Fig. 4B**). *Post hoc*

analysis demonstrated that the highest dose of GBR12909 significantly increased total trials to criterion compared with the vehicle group (p=0.038) whereas the lowest dose (1 mg/kg) significantly decreased the total number of trials required to reach criterion (p=0.049).

Elevated perseverative behavior is associated with altered 5-HT metabolism in the OFC

Although no significant group differences were observed in 5-HT levels in the lOFC (group: $F_{(2,27)}$=2.05, p=0.150; **Fig. 5A**) a planned comparison between low- and high-perseverative rats in this region approached statistical significance (p=0.059). Levels of the 5-HT metabolite 5-HIAA were significantly decreased in both the mOFC (group: $F_{(2,27)}$=4.13, p=0.028) and lOFC (group: $F_{(2,27)}$=4.23, p=0.026) (**Fig. 5B**). A dimensional analysis of all three perseveration groups revealed that response perseveration was inversely correlated with 5-HIAA levels in the mOFC (R^2=0.17, p<0.01) and lOFC (R^2=0.12, p<0.05). *Post hoc* analysis using Fishers LSD test revealed a significant decrease in levels of 5-HIAA in the mOFC of high-perseverative rats with respect to mid-perseverative (p=0.028) and low-perseverative rats (p=0.014). In the lOFC, 5-HIAA levels were significantly decreased in high-perseverative rats compared with low-perseverative rats (p=0.008). However, indices of DA function in the OFC and DMS were not significantly different between low- and high-perseverative rats (see **Table S4**).

Elevated 5-HT_{2A} receptor binding in the OFC is associated with reduced perseveration

5-HT_{2A} receptor binding significantly varied in the mOFC (group: $F_{(2,33)}$=4.42, p=0.021) and lOFC (group: $F_{(2,33)}$=4.01, p=0.028) of low-, mid-, and high-perseverative rats (**Fig. 5C**). *Post hoc* Fisher's LSD tests showed that binding at 5-HT_{2A} receptors was significantly increased in the mOFC of low-perseverative animals compared with high- (p=0.029) and mid- (p=0.013) perseverative animals. Increased 5-HT_{2A} receptor binding was also present in the

lOFC of low-perseverative rats compared with mid- (p=0.023) and high- (p=0.028) perseverative rats. Decreased 5-HT_{2A} receptor binding in the OFC was not accompanied by significant changes in 5-HTT binding (**Fig. 5D**) nor was there a significant difference in binding of the DA transporter or D_2 receptors in either the OFC or DMS (see **Table S4**). The lack of relationship between perseverative behaviour and expression of 5-HTT and DAT was further supported by a lack of associated changes in *Slc6a4* and *Slc6a3* expression in the DRN and VTA (**Fig. 6A and Fig. 6B**).

Increased perseveration is associated with decreased TPH2 and MAO expression in the DRN and increased MAO expression in the OFC

TPH2 mRNA expression was significantly decreased in the DRN of highly perseverative rats ($F_{(2,23)}$=5.59, p=0.011, **Fig. 6A**). *Post hoc* Fishers LSD tests indicated a significant decrease in TPH2 expression in the DRN of highly perseverative rats compared with mid- (p=0.007) and low-perseverative (p=0.013) rats; however, correcting for a lack of homogeneity of variances, a Games-Howell *post hoc* analysis revealed that TPH2 expression in the DRN of highly perseverative rats differed significantly from mid-perseverative rats (p=0.048), but not from low-perseverative rats (p=0.081). Tyrosine hydroxylase (TH) mRNA expression in the VTA was not significantly different between the three groups (**Fig. 6B**). As shown in **Fig. 7A and Fig. 7B,** MAO-A and MAO-B mRNA expression was significantly decreased in the DRN of highly perseverative rats (MAO-A: $F_{(2,24)}$=5.03, p=0.016; MAO-B: $F_{(2,24)}$=4.15, p=0.030). A dimensional analysis of all animals (low, mid, high groups) revealed that response perseveration was inversely related to MAO-A mRNA expression in the DRN (R^2=0.23, p<0.05). *Post hoc* Fishers LSD tests showed a significant decrease in MAO-A expression in the DRN of highly perseverative rats compared with mid- (p=0.023) and low-perseverative (p=0.007) rats. MAO-B expression was significantly decreased in the DRN of

high-perseverative rats compared with mid- (p=0.020) and low-perseverative (p=0.024) rats. Conversely, MAO-A and MAO-B expression was increased in the lOFC of highly perseverative rats, as shown in **Fig. 7C and Fig. 7D** (MAO-A: $F_{(2,27)}$=5.49, p=0.011; MAO-B: $F_{(2,27)}$=11.1, p<0.001). In addition, response perseveration was positively correlated with MAO-B mRNA expression in the lOFC (R^2=0.13, p<0.05). *Post hoc* Fishers LSD tests showed a significant increase in MAO-A expression in the lOFC of highly perseverative rats compared with mid- (p=0.025) and low-perseverative (p=0.004) rats. Similarly, MAO-B expression was significantly increased in the lOFC of high-perseverative rats compared with mid- (p<0.001) and low-perseverative (p<0.001) rats. No significant differences were found for other 5-HT- (5-HT_{2A-2C} receptors) and DA-related ($D_{1/2}$ receptors) transcripts in either the OFC or DMS (see **Table S5**).

Discussion

The main findings indicate that naturally occurring perseverative behavior on a spatial-discrimination serial reversal learning task is associated with diminished 5-HT function and abnormal MAO-A and MAO–B expression in the OFC and DRN. Our findings implicate increased constitutive MAO-A and MAO-B mRNA expression, specifically in the lateral OFC, and decreased expression of these transcripts and TPH2 in the DRN as putative novel substrates underlying perseverative behavior. Although the reversal design was somewhat different from those used by other studies, in that there were up to 3 reversals within the session, rather than the typical single reversal, it is evident that performance was still dependent on dopaminergic and serotonergic modulation by selective reuptake inhibitors. We found that levels of the 5-HT metabolite, 5-HIAA, were significantly reduced in the OFC of highly perseverative rats compared with rats in the lower quintile of the perseveration-

response distribution. In addition, low levels of perseveration were also associated with increased 5-HT_{2A} receptor binding in the medial and lateral OFC. Whilst prior studies have demonstrated a role for 5-HTT (Holmes and Fam, 2013; Nonkes *et al*, 2012) and striatal DA-ergic mechanisms in reversal learning performance (Clarke *et al*, 2011; Collins *et al*, 2000; O'Neill *et al*, 2007) we found no evidence of abnormalities in binding at 5-HTT in high or low perseverative rats nor any alterations in several key indices of DA transmission in the DMS. These findings indicate that natural variation in serotonergic tone and MAO-A and MAO–B gene expression in the OFC and DRN, together with reduced TPH2 mRNA expression in the DRN, may underlie poor spatial-discrimination reversal learning in rats. Attenuated serotonergic function may thus be an endophenotype that biases behaviour toward perseveration when S-R contingences are reversed, a notion consistent with the effects of direct interventions that decrease central 5-HT function (Rogers *et al*, 1999) (Mobini *et al*, 2000) (Clarke *et al*, 2004).

The 5-HT metabolite, 5-HIAA, was significantly decreased in the medial and lateral OFC of rats selected for highly perseverative behaviour; this was accompanied by a trend significant reduction in 5-HT levels in the lateral OFC. These findings, together with the demonstration of improved behavioral flexibility after citalopram treatment, suggests a role of 5-HT in spatial reversal performance. Our results thus accord with the disruptive effects of dietary tryptophan depletion on reversal learning in humans (Rogers *et al*, 1999), which decreases central 5-HT transmission (Chase *et al*, 2011), as well as the effects of selective focal destruction of 5-HT terminals in the OFC of the marmoset monkey (Clarke *et al*, 2004). The observation that spatial reversal learning is facilitated by local administration of a 5-HT_{2C} receptor antagonist in the OFC (Boulougouris *et al*, 2010) lends further support to an involvement of orbitofrontal 5-HT mechanisms in spatial reversal performance. Interestingly, animals exhibiting highly flexible behavior in the present study showed the highest levels of

406 5-HT_{2A} receptor binding in the OFC. Activation of 5-HT_{2A} receptors on pyramidal projection
407 neurons in the PFC has previously been reported to increase the activity of serotonergic
408 neurons in the DRN (Puig *et al*, 2003). The resultant increase in 5-HT release in the PFC
409 (Puig *et al*, 2003) may be linked to enhanced fronto-striatal signalling and diminished
410 perseverative responding (Roberts, 2011). Thus, rats in the present study may have exhibited
411 improved behavioral flexibility as a result of increased 5-HT_{2A} receptor binding in the OFC, a
412 notion supported by evidence that 5-HT_{2A} receptor antagonists disrupt spatial reversal
413 learning in rats (Boulougouris et al., 2008).

414 In the present study, differential binding at 5-HT_{2A} receptors in the medial and lateral
415 OFC of low and highly perseverative rats was not accompanied by changes in 5-HT_{2A} mRNA
416 expression. This apparent anomaly suggests that the differences in binding associated with
417 perseverative behavior may reflect alterations in the binding affinity of 5-HT_{2A} receptors or a
418 change in the total pool of 5-HT_{2A} receptors available for binding in the OFC. At this point it
419 is difficult to discount the impact of factors such as receptor internalisation affecting receptor
420 density as distinct from (i) regulatory mechanisms involved in gene expression (Hitzemann *et*
421 *al*, 2007) and (ii) effects on transcript levels in projections to the OFC from non-serotonergic
422 fibres, notably those arising from the mediodorsal nucleus of the thalamus (Scruggs *et al*,
423 2000) and implicated in reversal learning performance (Chudasama *et al*, 2001).

424 MAO is the main enzyme responsible for the catalytic degradation of monoamines in
425 the brain, present in the synaptic cleft, axon terminals, and in some glial cells (Shih *et al*,
426 1999). The normal intraneuronal function of MAO is the catabolism of monoamine
427 transmitters not contained within synaptic vesicles. The novel finding of decreased MAO-A
428 and MAO-B gene expression in the DRN may be indicative of a general reduction in
429 serotonergic tone, a notion supported by the concurrent decrease in TPH2 expression in
430 highly perseverative animals, suggestive of reduced 5-HT synthesis in these animals. This

notion is supported by the accompanying reduction in 5-HIAA levels in the OFC and is consistent with the general view that reduced serotonergic transmission underlies poor reversal learning (Clarke *et al*, 2004; Kehagia *et al*, 2010). Although the mechanism underlying the hypothesized reduction in serotonergic tone in highly perseverative rats is unknown it is possible that decreased MAO activity in the DRN resulted in reduced 5-HT breakdown and consequently increased auto-inhibition of 5-HT neurons by somatodendritic 5-HT receptors (Liu *et al*, 2001). Intriguingly, highly perseverative rats exhibited increased MAO-A and MAO-B expression in the OFC. The mechanism underlying these strongly contrasting effects on MAO expression in the DRN and OFC is presently unknown. The hypothesized decrease in serotonergic tone in highly perseverative animals would, however, lead to long term compensatory effects on 5-HT transmission in the OFC, including alterations in 5-HT release and local metabolism by MAO present in the synapse and surrounding glial cells (Shih *et al*, 1999). Our results suggest the presence of at least two, functionally-distinct populations of MAO involved in 5-HT catabolism; one linked with DRN serotonergic neurons, the other putatively linked with extraneuronal processes in the OFC possibly linked to glial function.

We found that performance on the spatial-discrimination reversal task was dose-dependently affected by the DA re-uptake inhibitor, GBR12909, with low doses improving performance and higher doses impairing performance. This implies that DA may have a biphasic effect on reversal learning performance similar to dopaminergic modulation of other behaviours such as locomotor activity (Eilam and Szechtman, 1989). Such divergent effects may be mediated by inhibitory presynaptic D2 receptors responsible for controlling the rate of neuronal firing, synthesis and release of DA (Aghajanian and Bunney, 1977) with higher doses affecting postsynaptic DA receptors. However, despite these biphasic effects, we found no differences between high-perseverative and low-perseverative animals in levels of DA or

its metabolite DOPAC in the DMS. Striatal mechanisms have previously been linked to behavioral flexibility through selective lesion studies (Castane *et al*, 2010) and local DA depletion (Clarke *et al*, 2011; O'Neill *et al*, 2007), which have the common effect of impairing reversal learning. Striatal DA levels have also been shown to predict performance on outcome-specific reversal-learning tasks (Clatworthy *et al*, 2009; Cools *et al*, 2009). However, despite the DMS being a major output region of the OFC (Mailly *et al*, 2013; Schilman *et al*, 2008), our results suggest that absolute variations in *post mortem* DA content and DA transporter are not associated with natural variation in perseverative behavior following repeated spatial reversals. A similar conclusion was reached by a recent study in non-human primates (Groman *et al*, 2013), which found that interactions between 5-HT levels in the OFC and DA levels in the putamen predicted behavioral flexibility during reversal learning. Specifically, reversal of a novel visual discrimination in monkeys was impaired by relatively low levels of OFC 5-HT and putamen (but not caudate) DA and by relatively high levels of OFC 5-HT and putamen DA. The lack of similar interactions between 5-HT and DA in the present study may reflect differing task demands (i.e. spatial versus visual discrimination) possibly engaging associative-, as opposed to motor-related regions of the dorsal striatum (i.e., the putamen).

Research in primates and rats suggest that the OFC can be functionally segregated into medial and lateral subregions (Elliott *et al*, 2000; Iversen *et al*, 1970; Kringelbach and Rolls, 2004; Mar *et al*, 2011). The lateral OFC is implicated in cognitive control when previously rewarded responses require suppression (Elliott *et al*, 2000; Iversen *et al*, 1970), whereas the medial OFC has been hypothesized to play a role in assigning and adjusting subjective value to delayed and uncertain rewards (Kable and Glimcher, 2009). Our findings show that both subregions of the OFC are affected by abnormalities in their 5-HT innervation but only the lateral OFC shows constitutively increased MAO expression and a stronger trend

towards reduced 5-HT content. Collectively, therefore, abnormalities in the serotonergic modulation of the lOFC may account for impaired flexibility during reversal learning.

In conclusion our research adds to the extensive body of literature implicating a role of orbitofrontal 5-HT in flexible goal-directed behavior (Clarke *et al*, 2007; Hampshire *et al*, 2006; Schoenbaum *et al*, 2007). The main findings of this investigation support the novel hypothesis that subjects who naturally perseverate when S-R contingencies are reversed have reduced 5-HT tone in the OFC as a putative consequence of impaired afferent input from the DRN. In the present study the index of perseverative responding was used to stratify the subjects according to inflexible behavior. These errors may reflect compulsive responding, as expressed in brain disorders such as OCD, where acts are performed in a repetitive and habitual manner (Fineberg *et al*, 2009). Individuals diagnosed with OCD show impaired reversal learning and aberrant task-related OFC-striatal activity (Remijnse *et al*, 2006). Moreover, OFC hypoactivity and impaired reversal learning is reported in OCD patients and their first-degree relatives (Chamberlain *et al*, 2008). Since 5-HT_{2A} receptor availability, specifically in the OFC, predicts clinical outcomes in OCD (Perani *et al*, 2008), our findings suggest that naturally occurring response perseveration in rats may have utility as an endophenotype to investigate the neural basis of OCD and other compulsive brain disorders.

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Figure legends

Figure 1: Schematic illustration of the spatial discrimination reversal learning task. Rats were trained under a fixed-ratio (FR) schedule of reinforcement such that three consecutive nose pokes in the same aperture resulted in the delivery of a food pellet in the magazine. A failure to respond within 30 seconds (an 'omission') resulted in a 5 second timeout period. Following the acquisition of a spatial discrimination the contingency was reversed such that responses in the previously incorrect aperture were now correct (and vice versa). Animals completed three reversals ('x3') in a single 1 hour session.

Figure 2: Coronal (A) and sagittal (B) sections showing the regions of interest used for neurochemical assessment in post-mortem tissue of rats stratified according to low-, mid- and high-perseverative behavior on a spatial discrimination serial reversal task. Adapted from Paxinos and Watson (1998).

Figure 3: Cumulative frequencies of perseverative responses, total trials and errors to reach criterion, during initial testing, in all 4 cohorts. Cohort 1: systemic drug administration (n = 48), Cohort 2: HPLC-ECD analysis (n = 44), Cohort 3: autoradiography (n = 44), Cohort 4: qRT-PCR (n =47).

Figure 4: Effect of citalopram (n = 24) on (A) the number of trials to criterion; (C) incorrect trials to criterion and (E) percentage perseverative responses. Effect of GBR 12909 (n = 23) on (B) the number of trials to criterion; (D) incorrect trials to criterion and (F) percentage perseverative responses on the spatial reversal learning task. Data are means ± SEMs from a single reversal learning session. Asterisks denote a significant difference between the groups indicated: * for $p < 0.05$, ** for $p < 0.01$.

Figure 5: (A) Levels of 5-HT and (B) 5-HIAA (pmoles/mg) in the medial and lateral OFC of high (n=9), mid (n=10) and low (n=9) perseverative animals. (C) 5-HT_{2A} and (D) 5-HTT receptor binding in the medial and lateral OFC of high (n=9), mid (n=10) and low (n=15) perseverative groups. Data are means ± 1SEM. Asterisks denote a significant difference between the groups indicated: * for $p < 0.05$. + denotes p=0.059.

Figure 6: Expression of (A) *tph2* and *slc4a6* in the DRN and (B) *th* and *slc3a6* in the VTA of high (n=8), mid (n=8) and low (n=9) perseverative groups. Data are means ± 1SEM. Asterisks denote a significant difference between the groups indicated: * for $p < 0.05$.

Figure 7: Expression of (A) MAO-A and (B) –B in the DRN and VTA of high (n=8), mid (n=8) and low (n=9) perseverative groups. (C) MAO-A and (D) –B in the medial and lateral OFC of high (n=8), mid (n=10) and low (n=9) perseverative groups. Data are means ± 1SEM. Asterisks denote a significant difference between the groups indicated: * for $p < 0.05$, ** for $p < 0.01$.