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ARTICLE

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Serotonin at the level of the amygdala and orbitofrontal cortex modulates distinct aspects of positive emotion in primates

Mei-See Man[1,2]*, Yevheniia Mikheenko[1,2]*, Katrin Braesicke[1,2], Gemma Cockcroft[2,3] and Angela C. Roberts[1,2]

[1] *Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge, UK*
[2] *Behavioural and Clinical Neurosciences Institute, University of Cambridge, Downing Street, UK*
[3] *Department of Experimental Psychology, University of Cambridge, Downing Street, Cambridge, UK*

Abstract

Impaired top-down regulation of the amygdala, and its modulation by serotonin (5-HT), is strongly implicated in the dysregulation of negative emotion that characterizes a number of affective disorders. However, the contribution of these mechanisms to the regulation of positive emotion is not well understood. This study investigated the role of 5-HT within the amygdala and the orbitofrontal cortex (OFC), on the expression of appetitive Pavlovian conditioned emotional responses and their reversal in a primate, the common marmoset. Its effects were compared to those of the amygdala itself. Having developed conditioned autonomic and behavioural responses to an appetitive cue prior to surgery, marmosets with excitotoxic amygdala lesions failed to display such conditioned autonomic arousal at retention, but still displayed intact cue-directed conditioned behaviours. In contrast, 5,7-DHT infusions into the amygdala, reducing extracellular 5-HT levels, selectively enhanced the expression of appetitive conditioned behaviour at retention. Similar infusions into the OFC, producing marked reductions in post-mortem 5-HT tissue levels, had no overall effect on autonomic or behavioural responses, either at retention or during reversal learning, but caused an uncoupling of these responses, thereby fractionating emotional output. These data demonstrate the critical role of the amygdala in the expression of appetitive autonomic conditioning, and the region-selective contribution of 5-HT in the amygdala and OFC, respectively, to the expression of conditioned behaviour and the overall coordination of the emotional response. They provide insight into the neurochemical mechanisms underlying the regulation of positive emotional responses, advancing our understanding of the neural basis of pathologically dysregulated emotion.

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Introduction

Dysfunction in the orbitofrontal cortex (OFC) and amygdala has been implicated in a number of pathological conditions, including depression (Drevets, 2003, 2007), mania (Almeida *et al.* 2009), anxiety disorders (Etkin & Wager, 2007; Milad & Rauch, 2006), obsessive–compulsive disorder (Saxena & Rauch, 2000) and addiction (Childress *et al.* 1999; Grant *et al.* 1996). Research into the underlying causes of amygdala and OFC dysfunction has highlighted the importance of neuromodulatory systems including the ascending serotonin (5-HT) pathways. Pharmacological and genetic studies highlight the role of 5-HT in modulating emotional processing in the amygdala and prefrontal cortex of healthy people (Canli & Lesch, 2007; Cools *et al.* 2008) and patients with affective disorders (Harmer, 2008; Morilak & Frazer, 2004; Ressler & Nemeroff, 2000). The vast majority of these studies have focused on negative emotion. However, extensive research in animals has shown the vital role of the amygdala and OFC in positive affect (Balleine & Killcross, 2006; Everitt *et al.* 2003; Holland & Gallagher, 2004; Murray, 2007; Roberts *et al.* 2007) and recent findings have also highlighted the role of 5-HT in positive affect. Thus,

Address for correspondence: Professor A. C. Roberts, Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge, CB2 3DY, UK.
Tel.: 01223 339015 *Fax*: 01223 339014
Email: acr4@cam.ac.uk
* These authors contributed equally to this work.

peripheral administration of selective serotonin reuptake inhibitors (SSRIs), has implicated 5-HT in the modulation of both these structures during negative and positive emotion in humans (McCabe *et al.* 2010; Norbury *et al.* 2009). In addition, the tonic activity of dorsal raphe neurons, which include those that release 5-HT, has been shown to correlate strongly with future rewarding outcomes, linking 5-HT with reward expectation (Bromberg-Martin *et al.* 2010). Despite this, little is known of the specific actions of 5-HT at the level of the OFC and amygdala on positive emotion.

Positive emotion is frequently studied in the laboratory using a Pavlovian conditioning procedure, whereby a previously neutral stimulus acquires emotional significance and induces a positive emotional state, through its association with reward. Integral components of this emotional state are the accompanying physiological and behavioural responses. The amygdala has been identified as a key region for the acquisition of behavioural responses in this paradigm (Gallagher *et al.* 1990; Groshek *et al.* 2005; Parkinson *et al.* 2000) and studies in our laboratory have shown that the expression of conditioned autonomic arousal accompanying learned appetitive behaviour is also critically dependent on the amygdala (Braesicke *et al.* 2005). While the expression of conditioned responses is not dependent upon an intact OFC, we have shown that the regulation of such anticipatory positive arousal in the event of unexpected reward omission, and the coordinated adaptation of Pavlovian autonomic and behavioural responses when stimulus-reward contingencies change, is dependent upon the OFC (Reekie *et al.* 2008).

To directly assess the contribution of 5-HT at the level of the OFC and amygdala in positive emotion, the present study compares the effects of selective 5-HT depletions within either the amygdala or OFC, using the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT), on the expression and regulation of conditioned, appetitive autonomic and behavioural responses. For comparison purposes the effects of amygdala excitotoxic lesions were also investigated, as this particular version of the Pavlovian task in marmosets, with abstract auditory cues as the conditioned stimuli, had only been studied previously in the context of excitotoxic lesions of the OFC.

Materials and methods

Subjects

Sixteen common marmosets (*Callithrix jacchus*, five females, 11 males; average age 3.5 yr, range 2–6 yr) were pair-housed under temperature and humidity controlled conditions. [For details of diet see Supplementary Materials and methods (available online).] All procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986, under project licence 80/2225. Following completion of this study, 11 of the marmosets (four out of five controls, three out of four 5,7-DHT amygdala-lesioned animals and all four 5,7-DHT OFC-lesioned animals) were tested on an incongruency discrimination task and the excitotoxic amygdala-lesioned marmosets ($n=3$) were tested on the acquisition of a mild aversive Pavlovian procedure, the results of which have been reported, respectively, in Man *et al.* (2010) and Mikheenko *et al.* (2010). A further four marmosets received unilateral 5,7-DHT amygdala lesions to investigate lesion time-course (see Supplementary Materials and methods).

Test apparatus

Behavioural testing took place within a test apparatus as previously described by Reekie *et al.* (2008; Supplementary Materials and methods).

Cardiovascular measurements

Blood pressure (BP) and heart rate (HR) changes were measured remotely using a PhysioTel Telemetry system (Data Sciences, USA) as previously described in Braesicke *et al.* (2005; Supplementary Materials and methods).

Surgical procedures

Implantation of telemetric devices

Prior to the start of the study marmosets received an implant of a radiotelemetry probe (Chronic Use TA11PA-C40 Implant, Data Sciences, USA) into the descending aorta under general anaesthesia, according to procedures described previously (Schnell & Wood, 1993; Supplementary Materials and methods). During testing, the probe transmitted BP data, which was recorded and analysed offline.

Brain lesions

General neurosurgery and recovery procedures were performed as described in Supplementary Materials and methods. Excitotoxic lesions and lesions of the serotonergic innervation of the amygdala and OFC were performed as described in Table 1.

In-vivo *microdialysis*

Extracellular levels of 5-HT in the amygdala of two 5,7-DHT amygdala-lesioned and two sham-operated

Table 1. Parameters for the amygdala and OFC lesions including stereotaxic coordinates of each injection (based on the inter-aural plane) and the injection volume

	Coordinates (mm)			Volume injected (μl)	
Lesion area	AP	LM	V	Quinolinic acid	5,7-DHT[b]
Amygdala	9.3	±5.6	4.0	0.35	0.50
			5.0	0.35	0.50
OFC	16.75	±2.5	0.7[a]	–	0.40
		±4.8	0.7[a]	–	0.40
	17.75	±2.0	0.7[a]	–	0.40
		±4.8	0.7[a]	–	0.40
	18.5	±2.0	0.7[a]	–	0.60
		±4.0	0.7[a]	–	0.60

OFC, Orbitofrontal cortex; AP, anteroposterior; LM, lateromedial; V, ventral.
Excitotoxic lesions of the amygdala ($n=3$) were made by infusing 0.35 μl of 0.12 M quinolinic acid (Sigma-Aldrich, Germany), in 0.01 M phosphate buffer (pH 7.0), bilaterally. Injections were made at a rate of 0.05 μl/20 s through a glass cannula attached to a 2 μl Hamilton syringe (Precision Sampling Co., USA). Immediately after injection, the cannula remained in position for a further 4 min, before being withdrawn slowly. Lesions of the serotonergic innervation of the OFC ($n=4$) and amygdala ($n=4$) were made following a procedure adapted from Clarke *et al.* (2007) using 4 μg/μl 5,7-DHT (creatinine sulphate salt; Sigma-Aldrich, Germany) in 0.1% L-ascorbic acid (Sigma-Aldrich, USA) in 0.9% saline, infused bilaterally. The sham-operated control group ($n=5$) received vehicle infusion into either the OFC ($n=2$) or amygdala ($n=3$).
[a] 0.7 mm above base of brain.
[b] The noradrenaline (NA) uptake blocker, nisoxetine (50 mM; Sigma-Aldrich, Germany), and the dopamine (DA) uptake blocker, 1-(2-(bis-(4-fluorophenyl)methoxy)ethyl)-4-(3-phenylpropyl) piperazine dihydrochloride (GBR 12909, 2.0 mM; Sigma-Aldrich, Germany), were administered concomitantly to protect NA and DA innervation, respectively.

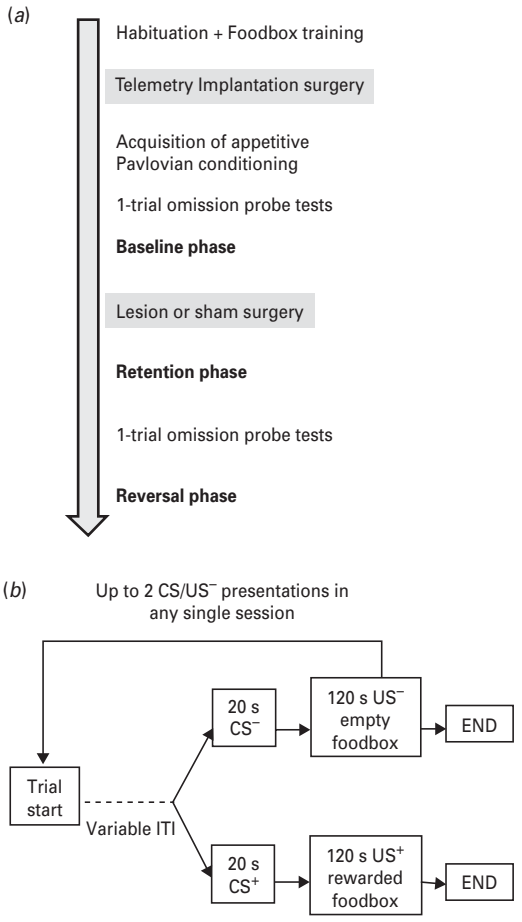

Fig. 1. The task design and set-up. (*a*) Flow diagram depicting the stages of training, surgery, probe tests (before and after surgery) and reversal. Prior to implantation of the telemetry probe, animals were habituated to the test chamber and received preliminary foodbox training, whereby, after a variable inter-trial interval (ITI), doors opened allowing access to a foodbox filled with marshmallow. (*b*) Schematic of a typical Pavlovian session where animals learnt to associate a 20-s tone or white noise (conditioned stimulus, CS) with immediate access to a highly palatable food reward (unconditioned stimulus, US$^+$) or an empty foodbox, (no reward, US$^-$).

control monkeys were measured by microdialysis, 8–10 months after lesion surgery. The marmosets were anaesthetized by isoflurane intubation (as detailed for brain lesions, Supplementary Materials and methods), and microdialysis probes were implanted acutely at the following stereotaxic coordinates based on the inter-aural plane: AP +9.3 mm, L −5.6 mm, DV +4.0 mm, centred on the basal nucleus. Details of probes, artificial cerebrospinal fluid composition and dialysis protocol are described in the Supplementary Materials and methods.

Study design

The design of the study (Fig. 1*a*) follows that described in Reekie *et al.* (2008). After telemetry implantation, animals underwent appetitive Pavlovian conditioning (Fig. 1*b*), in which one of two novel sounds (computer-generated white noise or 4 kHz tone) was presented for 20 s. One sound (CS$^+$) was associated with reward (US$^+$, marshmallow-filled foodbox) while the other sound (CS$^-$) was paired with no reward (US$^-$, empty foodbox). In any one session, 1–3 CS were presented,

which could include a single CS^- or multiple CS^-, but only one CS^+. A session started with the illumination of the house-light and CS were presented with a variable inter-trial interval (ITI) of 70–110 s. Immediately following CS^+, the house-lights were turned off, the foodbox lights turned on, and both clear and opaque doors of one foodbox opened, allowing 120 s access to the marshmallow reward inside. After CS^- presentation only the opaque door opened, allowing the animal to view an empty foodbox. The auditory CS continued to be played for the duration of the 120-s US period. Presentation of CS^+ terminated the session on completion of the reward period. Animals received no more than 5 CS^+ within a 2-wk period of 10 sessions. The pairing of sounds and outcomes was counterbalanced across subjects.

Training continued until subjects showed stable conditioning, as indicated by differential BP and behavioural responses to CS^+ and CS^- over approximately 1.5 wk ($p<0.05$). Subjects were then given two, one-trial probe sessions (probe tests 1 and 2) at least 1 wk apart, with additional re-conditioning trials in between.

Probe test 1: CS termination with reward omission

A single, 20-s CS^+ was presented. This was followed by termination of CS^+ and no presentation of reward (i.e. foodbox doors did not open).

Probe test 2: unexpected reward with CS omission

The US^+ was presented alone without any prior or accompanying CS^+.

Stable conditioned responding across 1 wk, post-probe tests was required before surgery could take place (pre-surgery baseline phase). Subsequently, animals received one of the following: excitotoxic lesions of the amygdala, selective 5-HT depletion of the amygdala or OFC, or sham control surgery. After post-operative recovery, animals were re-tested on the same Pavlovian conditioning procedure until stable differential BP and behavioural responses, comparable to those of pre-surgery, had been achieved (retention phase). Probe tests 1 and 2 were then presented (the same as in pre-surgery).

Reversal of reward contingencies

On completion of the two probe tests, animals were given an additional week of Pavlovian training to allow BP and behavioural responses to re-stabilize. Thereafter, CS outcome contingencies were reversed, such that the previously rewarded CS^+ became the CS^- and was no longer rewarded, while the previously unrewarded CS^- became the rewarded CS^+ (reversal phase). All subjects received a predetermined sequence of sessions. They were maintained on reversal until they reached a performance criterion of significantly ($p<0.05$) greater conditioned behavioural and autonomic responses to CS^+ than CS^- across consecutive sessions, that included 6 CS^+ presentations, or for a maximum of 100 sessions.

Behavioural and cardiovascular analyses

For the cardiovascular data, offline analyses involved the removal of abnormal spikes and outliers (typically BP values >400 mmHg or <0 mmHg), and then extraction of the minima and maxima of each heart-beat cycle to give systolic and diastolic events. Missing values of <0.4 s were filled with cubic spline interpolation, although longer disruptions were treated as missing values in the resulting dataset. For the 20-s CS period, the mean value for systolic and diastolic BP and HR was calculated and compared to mean baseline responding during the 20-s period immediately prior to the start of CS. Changes in systolic BP from baseline during the CS period was the primary measure for affective conditioned cardiovascular responses. For the US period, systolic and diastolic BP and HR was calculated over 60 s from when the animal first accessed the foodbox, compared to pre-CS baseline.

For behavioural data, all sessions were recorded onto DVD to be analysed post-testing. A researcher, blind to the experimental groups, scored all behaviours and latency measures.

CS-directed behaviour. The number of headjerks [HJs; CS-directed orienting responses consisting of a flick/snap of the head (Holland 1977; Reekie *et al.* 2008)] were counted during the 20-s CS period and subtracted from this was the number of HJs made during the immediately preceding 20-s baseline period.

US-directed behaviour. The number of looks (movements of the head) directed to the foodboxes was also scored during each CS and associated baseline period. In addition, at each US onset, the latencies to look, reach and eat (if US^+) were measured. The total amount of food consumed was also recorded.

Post-mortem neurochemical analysis

Post-mortem neurochemical analysis was used to determine the specificity and extent of the 5,7-DHT lesion in the OFC and amygdala (see Supplementary Materials and methods).

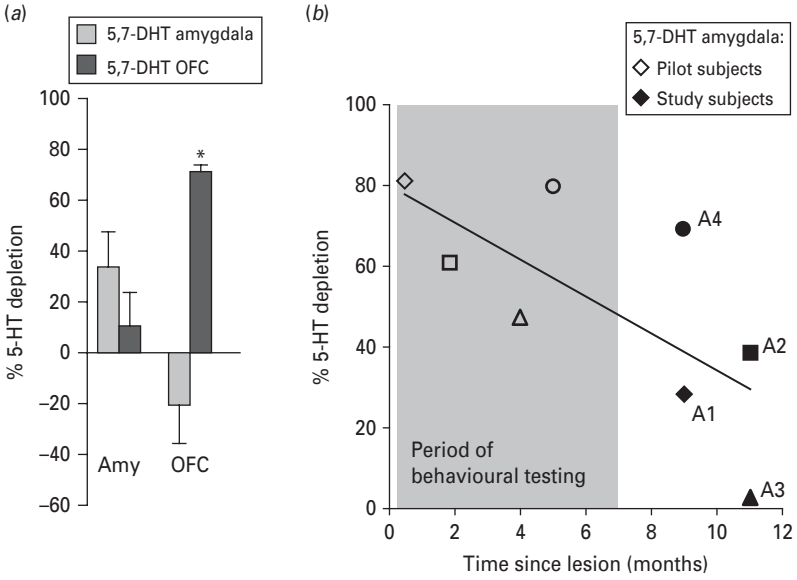

Fig. 2. Neurochemical verification of 5,7-DHT lesions. (*a*) % Depletions at 9–11 months post-surgery of 5-HT levels in the amygdala and orbitofrontal cortex (OFC) for 5,7-DHT amygdala-treated animals ($n=4$) and 5,7-DHT OFC-treated animals ($n=3$), calculated as a percentage change from control levels ($n=5$). Comparison of 5-HT levels across groups in 16 brain regions using ANOVA (with Sidak correction for multiple comparisons) revealed a significant main effect of group for 5-HT levels in OFC ($F_{2,11}=19.3$, $p=0.016$), but no significant effect in the amygdala ($F_{2,11}=0.26$, $p=1.0$, n.s.). Uncorrected *post-hoc t* tests demonstrated that the 5,7-DHT-lesioned OFC group had significantly lower 5-HT levels within the OFC compared to both controls ($t_6=8.9$, $p<0.001$) and 5,7-DHT-lesioned amygdala animals ($t_5=-4.9$, $p=0.005$). A similar reduction in 5-HT levels in the lateral PFC did not survive Sidak correction ($F_{2,11}=11.0$, $p=0.06$) although we have previously reported significant depletions in this area using this lesion method (Clarke *et al*. 2004, 2005, 2007; Walker *et al*. 2006, 2009). The 5,7-DHT-lesioned amygdala group did not have significant depletions of 5-HT in the amygdala when measured 9–11 months post-surgery [panel (*a*); individual subject values in panel (*b*)]. The most likely explanation is that recovery took place over time, since assessment did not take place until after completion of a second study described elsewhere (Man *et al.* 2010). Noradrenaline (NA) and dopamine (DA) were successfully protected in the targeted brain regions. Even at 2 wk, when 5-HT depletions within the amygdala are at their greatest, NA and DA remained relatively unaffected, i.e. +1.7% and −30.5%, respectively. In addition, there were no significant depletions of NA or DA in areas adjacent to the OFC (lateral, dorsal and medial PFC; all Fs <1, n.s.) in 5,7-DHT OFC-treated animals or in areas adjacent to the amygdala (hippocampus, peri-amygdala cortex, nucleus accumbens, putamen and hypothalamus) of 5,7 DHT amygdala-lesioned animals (all Fs <1, n.s.). (*b*) 5-HT depletion in amygdala at a range of time-points (2 wk, 2, 4, 5, 9, 11 months) following 5,7-DHT infusions into the amygdala. Grey bar indicates the period, post-lesion, during which behavioural testing in the current study occurred (2 wk–7 months). It should be noted that, at the time of behavioural testing, depletions should have been substantially larger than seen in the post-mortem analysis 9–11 months post-surgery.

Histological assessment of excitotoxic amygdala lesions

Animals with amygdala excitotoxic lesions were euthanased and their brains subsequently assessed for cell loss and gliosis (see Supplementary Materials and methods).

Statistical analysis

Data were analysed using SPSS for Windows (version 16.0, SPSS Inc., USA). Statistical significance was set at $p\leqslant 0.05$ and data were transformed as necessary if variances deviated significantly (as measured by Levene's test of homogeneity).

Results

Post-mortem tissue levels of 5-HT and the other monoamines

Injections of 5,7-DHT into the OFC caused a significant ($p<0.05$) reduction of post-mortem 5-HT tissue levels in the OFC and a substantial, but non-significant, reduction in the neighbouring lateral prefrontal cortex (Fig. 2*a*, Table 2), when measured 9–11 months post-surgery. In contrast, there was considerable recovery of post-mortem tissue levels of 5-HT in the amygdala across the same time period following 5,7-DHT injections into the amygdala (Fig. 2*a*, *b*; Table 2). However, extracellular levels of 5-HT in the amygdala in the

Table 2. Post-mortem 5-HT tissue levels

	Serotonin		
	Levels	% Depletion	
Brain region	Control	5,7-DHT OFC	5,7-DHT amygdala
OFC	0.8 ± 0.04 (0.77 to 0.99)	67.1 ± 4.5* (58.5 to 72.8)	−20.3 ± 15.0 (−48.3 to 21.7)
Amygdala	1.7 ± 0.3 (1.1 to 2.5)	12.5 ± 8.2 (−3.4 to 23.6)	33.9 ± 13.6 (2.0 to 68.0)
LPFC	0.9 ± 0.06	66.8 ± 4.8	1.7 ± 16.2
DLPFC	0.7 ± 0.1	31.0 ± 6.9	8.3 ± 16.5
MPFC	1.0 ± 0.06	41.1 ± 4.2	−5.9 ± 19.6
PM/M	0.7 ± 0.07	21.3 ± 2.3	0.6 ± 14.5
Peri-amygdala	1.6 ± 0.4	20.9 ± 13.1	33.2 ± 17.5
Hippocampus	0.9 ± 0.1	−0.2 ± 11.8	5.6 ± 34.8
C1	0.7 ± 0.07	9.0 ± 6.2	−21.2 ± 19.1
C2	0.8 ± 0.03	8.5 ± 5.3	−13.7 ± 18.0
Caudate 1	1.1 ± 0.3	−4.1 ± 9.4	−40.8 ± 13.4
Caudate 2	1.7 ± 0.1	18.4 ± 3.5	8.5 ± 20.1
Putamen 1	1.6 ± 0.2	12.0 ± 11.5	7.6 ± 3.4
Putamen 2	1.8 ± 0.2	14.8 ± 3.6	−3.8 ± 14.3
NAc	1.9 ± 0.3	−15.7 ± 1.7	−11.6 ± 14.5
Hypothalamus	2.8 ± 0.5	19.1 ± 2.6	−34.3 ± 22.0

OFC, Orbitofrontal cortex; LPFC, lateral prefrontal cortex; DLPFC, dorsal prefrontal cortex; MPFC, medial prefrontal cortex; PM/M, premotor/motor cortex; C1, anterior cingulate cortex; C2, mid-cingulate cortex; Caudate 1, anterior caudate; Caudate 2, mid-caudate; Putamen 1, anterior putamen; Putamen 2, mid-putamen; NAc, nucleus accumbens.
Mean levels of 5-HT (pmol/mg tissue weight ± S.E.M.) in the control group ($n = 5$), and depletions (% change from control levels of 5-HT ± S.E.M.) in marmosets with 5,7-DHT lesions of the OFC ($n = 3$) and amygdala ($n = 4$). Value ranges (in parentheses) are provided for OFC and amygdala regions of interest, but omitted from other brain regions for reasons of clarity.
ANOVA with Sidak correction for multiple comparisons revealed significant depletion of 5-HT in the OFC of 5,7-DHT treated OFC animals (* $p < 0.05$).

anaesthetized marmoset were considerably compromised at the time of behavioural testing (see below).

In-vivo *extracellular tissue levels of 5-HT and 5-HIAA*

Extracellular levels of 5-HT in both lesioned marmosets (Fig. 3*a*) were below the level of detection (2.0 fmol/20 min), in the baseline period and following K^+ challenge, even though subsequent post-mortem analysis of tissue levels of 5-HT had shown full recovery in one of these animals (A3; Fig. 2*b*). Amygdala concentrations of 5-HIAA (a 5-HT metabolite) in the lesioned animals were also approximately half that observed in controls (Fig. 3*b*).

Histological analysis

Histological analysis of excitotoxic amygdala lesions (Fig. 4) revealed that all three animals sustained damage to the central, basal, accessory basal and medial nuclei bilaterally, although the damage was partial in extent and the most anterior aspects of the basal and accessory basal nuclei were spared. There was also, primarily unilateral, lesioning of the lateral nucleus. The cortical nucleus was intact in all subjects. One animal showed damage to the very anterior tip of the hippocampus, but otherwise there was no damage outside of the amygdala.

Expression of conditioned and unconditioned cardiovascular and behavioural responses

Responses during the CS period

All animals showed differential cardiovascular (Fig. 5*a*; Supplementary Table S1) and behavioural responses (Fig. 5*b*) to CS^+ compared to CS^- prior to lesion surgery. Post-surgery, excitotoxic amygdala lesions significantly attenuated the expression of these conditioned BP responses, such that the responses to CS^+ became indistinguishable from responses to CS^- (Fig. 5*a*). In contrast, these same animals continued to show intact CS-directed conditioned behaviour to CS^+

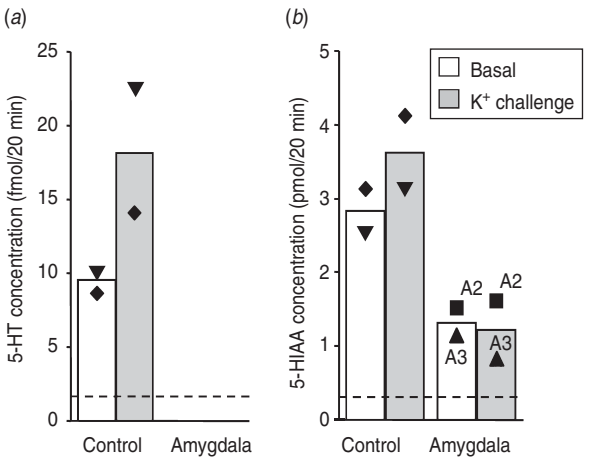

Fig. 3. *In-vivo* extracellular tissue levels of 5-HT and 5-HIAA. *In-vivo* extracellular concentrations of (*a*) 5-HT and (*b*) 5-HIAA in the amygdala of two sham-operated controls and two animals with 5,7-DHT lesions of the amygdala, measured after completion of reversal learning. The values reflect mean concentrations of three 20-min baseline (basal) dialysate fractions (taken after a 3-h equilibration period) and the subsequent 20 min dialysate fraction during which 75 mM potassium (K^+ challenge) was infused in the first 10 min. The dashed lines indicate the detection thresholds for 5-HT and 5-HIAA. Individual subject values for amygdala-lesioned animals are labelled (A2, A3) for ease of comparison with individual levels of post-mortem 5-HT depletion in Fig. 2*b*. Statistical analysis was not performed since $n=2$.

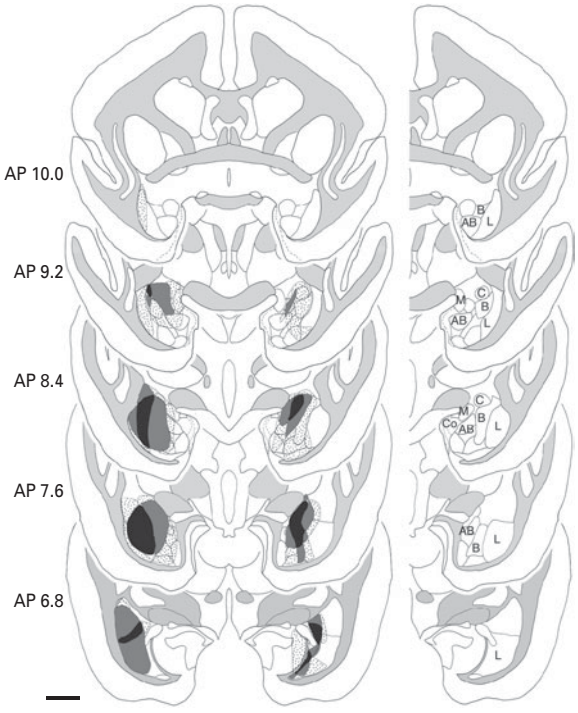

Fig. 4. Excitotoxic lesions of the amygdala. Schematic diagram of a series of coronal sections through the marmoset anterior temporal lobe illustrating the extent of cell damage following quinolinic acid infusion into the amygdala in three marmosets. Shadings indicate the area of cell loss in all (black shading), two (grey shading) or only one (stippled shading) marmoset. Amygdala nuclei are depicted on the adjacent half brain sections. AB, Accessory basal; B, basal; L, lateral; C, central; M, medial; Co, cortical. Scale bar, 1.5 mm.

(Fig. 5*b*). In contrast, 5,7-DHT amygdala lesions significantly enhanced the expression of CS-directed conditioned behaviour (Fig. 5*b*), which was accompanied by a somewhat (but non-significantly) augmented conditioned BP response (Fig. 5*a*). The conditioned BP (Fig. 5*a*) and behaviour (Fig. 5*b*) of 5,7-DHT OFC-lesioned animals did not differ from controls. Expression of conditioned responses was assessed within a month of surgery, when 5-HT depletions in the 5,7-DHT-lesioned animals would have been maximal (Fig. 2*b*).

A three-way ANOVA of systolic BP revealed a significant main effect of CS ($F_{1,12}=121.5$, $p<0.001$), a significant CS × group interaction ($F_{3,12}=5.8$, $p=0.011$) and an overall surgery × CS × group interaction ($F_{3,12}=12.07$, $p=0.005$). Further two-way ANOVAs (CS × group) demonstrated that pre-surgery, there was no difference between the groups (group: $F_{3,12}=2.1$; group × CS: $F_{3,12}=1.7$, n.s.), with all groups showing a clear conditioned rise in systolic BP to CS^+ and not CS^-. Post-surgery, however, a significant CS × group interaction ($F_{3,12}=22.3$, $p<0.0001$) was found. While there was a clear differentiation between CS^+ and CS^- responses in the control group ($t_8=4.4$, $p=0.002$) and both 5,7-DHT-lesioned groups (amygdala: $t_6=15.4$, $p<0.0001$; OFC: $t_6=3.6$, $p=0.01$), the excitotoxic amygdala-lesioned monkeys showed no difference in their responses to the two stimuli ($t_4=-0.12$, n.s.). A similar pattern was seen for diastolic BP (see Supplementary Results). Log-transformed HR data was more variable with no significant main or interaction effects (surgery: $F_{1,12}=2.5$; CS: $F_{1,12}<1$; group: $F_{3,12}=2.4$; surgery × CS × group interaction: $F_{3,12}=1.4$, all n.s.).

A three-way ANOVA of CS-directed behaviour (HJs) showed a significant main effect of CS ($F_{1,12}=91.4$, $p<0.001$), a significant CS × surgery interaction ($F_{1,12}=14.8$, $p=0.002$) and significant surgery × CS × group interaction ($F_{3,12}=3.7$, $p=0.04$). Examination of CS^+ only, revealed a significant group × surgery interaction ($F_{3,12}=5.6$, $p=0.012$), whereas no main or interaction effects for CS^- (all Fs $\leqslant 1$, n.s.) were found.

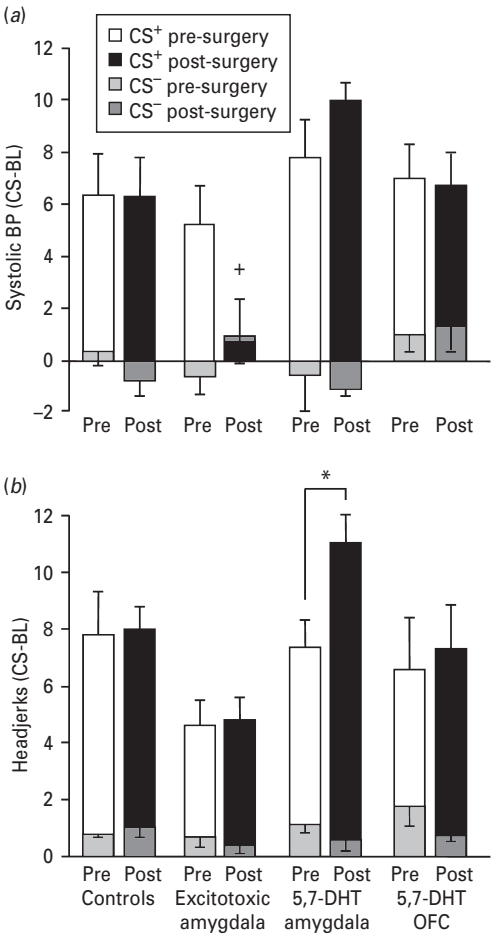

Fig. 5. Conditioned autonomic and behavioural responses pre- and post-surgery. Cardiovascular and behavioural changes to CS^+ and CS^- both pre- and post-surgery for the four experimental groups: controls ($n=5$), excitotoxic amygdala-lesioned ($n=3$), 5,7-DHT amygdala-lesioned ($n=4$), and 5,7-DHT OFC-lesioned ($n=4$) animals. (*a*) Conditioned systolic blood-pressure (BP) changes as calculated by total BP change during 20-s CS presentation, minus the preceding 20-s baseline period, mean across five sessions on reaching stable criterion. A differential rise to CS^+ compared to CS^- was seen in all groups pre-surgery. Post-surgery, excitotoxic amygdala lesions abolished the conditioned rise in BP to CS^+, such that there was no significant difference between the responses to CS^+ compared to CS^- (+), whereas all other groups still showed a significant effect of CS post-surgery. (*b*) Conditioned CS-directed behaviour expressed as number of headjerks (HJs), calculated by subtracting the number of HJs observed in baseline period from those observed during the CS period, mean across five sessions on reaching stable responding. A greater number of conditioned HJs was consistently observed in CS^+ compared CS^-, in all groups, pre- and post-surgery. 5,7-DHT amygdala-lesioned animals displayed a significant increase in HJs to CS^+ (* $p=0.03$) post-surgery.

Further *post-hoc* analysis for each group within CS^+ revealed a significant effect of surgery for the 5,7-DHT-treated amygdala group only ($F_{1,7}=7.8$, $p=0.03$, other groups all Fs <1, n.s.). Behavioural conditioning (i.e. greater number of HJs to CS^+ compared to CS^-) was observed in all groups before and maintained by all groups after surgery, except those animals treated with 5,7-DHT in the amygdala, which displayed significantly elevated HJs to CS^+, post-surgery (Fig. 5*b*).

Responses during the US period

Systolic and diastolic BP rose during the rewarded US period, over and above CS^+ period, partly in response to the sight of the reward in the open box as well as during consumption. However, there were no differences between these measures pre- and post-surgery (see Supplementary Results).

Additional measures

Analysis of additional behavioural measures revealed a significant main effect of CS on the number of looks towards the foodbox ($F_{1,12}=24.4$, $p<0.001$) such that animals looked significantly more during CS^+ compared to CS^- (Supplementary Table S2). There were, however, no effects of surgery, group or interaction effects (Fs <1, n.s.).

Reward omission and CS omission

There were no differences between the groups in their autonomic or behavioural responses during the two probe tests (Supplementary Results and Supplementary Tables S3 and S4).

Conditioned behavioural and autonomic responses following reversal of the contingencies

When the previously unrewarded stimulus became rewarded, and vice versa, all animals gradually reversed their conditioned responding, so that systolic BP and HJs became directed towards the new CS^+ (criterion of learning: CS^+ responses significantly greater than CS^- responses, $p<0.05$, over a block of sessions including 6 CS^+ presentations). There were no differences between the groups in the number of sessions taken to reach reversal criterion for either systolic BP (Table 3; $F_{2,12}<1$, n.s.) or behaviour (Table 3; $F_{2,12}<1$, n.s.). The final level of performance of the lesioned groups also did not differ from the control group (Table 3). Two-way ANOVAs of systolic BP and HJs at criterion demonstrated a significant main effect of CS for both measures (systolic BP: $F_{1,10}=46.2$, $p<0.0001$; behaviour: $F_{1,10}=55.9$, $p<0.0001$), but no effects of

Table 3. Reversal of conditioned systolic blood pressure (BP) and CS-directed behaviour

Group	Number of sessions to criterion		Systolic BP at criterion (mmHg)		Behaviour at criterion (no. HJs)	
	Systolic BP (mmHg)	Behaviour (no. HJs)	CS^+	CS^-	CS^+	CS^-
Control	39.20 ± 10.86	48.20 ± 8.49	4.22 ± 0.81 (3.97 ± 1.25)	0.46 ± 0.76 (0.85 ± 0.93)	4.17 ± 1.09	1.50 ± 0.53
5,7-DHT amygdala	52.50 ± 15.44	47.50 ± 14.53	5.40 ± 0.45 (3.45 ± 0.69)	0.02 ± 1.02 (−0.01 ± 1.33)	3.79 ± 0.50	1.00 ± 0.10
5,7-DHT OFC	64.00 ± 21.33	47.50 ± 16.22	5.34 ± 2.01 (3.53 ± 1.15)	1.24 ± 0.67 (0.88 ± 0.95)	5.71 ± 1.64	2.31 ± 0.75

Number of sessions to reach criterion for reversal of conditioned systolic BP and CS-directed behavioural responses (headjerks, HJs), and conditioned systolic BP and behavioural responses at criterion (mean over a block of sessions including 6 CS^+ presentations ± S.E.M.) for control ($n = 5$), 5,7-DHT amygdala ($n = 4$) and 5,7-DHT OFC-lesioned ($n = 4$) animals. As it was observed that post-reversal, many animals showed a delayed onset of the conditioned systolic BP responses during the 20 s CS period, their systolic BP responses at criterion were calculated as the mean of the last 10 s of the 20-s CS (control, $n = 3$; 5,7-DHT amygdala lesion, $n = 3$; 5,7-DHT OFC lesion, $n = 2$). Additionally, systolic BP responses calculated as the mean of the entire 20-s CS for all animals are given in parentheses.

group or CS × group interactions (all Fs < 1, n.s.), confirming that all groups showed higher conditioned systolic BP and behavioural responses to the new CS^+ by the end of the reversal.

Coupling of behavioural and autonomic responses

Previously, it has been shown that the coupling of autonomic and behavioural responses during reversal learning is dependent on an intact OFC (Reekie *et al.* 2008). Thus, in the present study we determined the extent to which conditioned autonomic and behavioural responses at the level of individual CS were correlated across reversal learning. ANOVA of r' (Fisher's transformation of the correlation coefficient, r) of the individual animals, in each of the three groups (Fig. 6*a*), revealed that the 5,7-DHT OFC-lesioned monkeys showed significantly lower r' scores, and thus a lower overall correlation of BP and HJs, than the other two groups ($F_{2,12} = 9.51$, $p = 0.005$). These effects were comparable to those seen in Reekie *et al.* (2008) following excitotoxic lesions of the OFC (mean r': control = 0.345, excitotoxic OFC lesion = 0.018; one-way ANOVA of square-root-transformed data: $F_{1,10} = 51.4$, $p < 0.0001$).

Further analysis to determine the extent of the uncoupling of these two emotional response outputs in the 5,7-DHT OFC-lesioned monkeys revealed that uncoupling was also present at retention, despite the monkeys at this stage showing similar overall conditioned performance to the other two groups. It can be seen in Fig. 6*b* that the 5,7-DHT OFC-lesioned group showed significantly lower correlations than controls and 5,7-DHT amygdala-lesioned monkeys (one-way ANOVA: $F_{2,12} = 5.01$, $p = 0.031$). It should be noted that they also showed a significantly lower correlation than they had displayed pre-surgery, with their mean score post-surgery being less than the 95% lower confidence limits of their pre-surgery score (Fig. 6*b*).

Discussion

Excitotoxic lesions of the amygdala abolished, selectively, the expression of conditioned autonomic arousal in anticipation of reward, in agreement with previous findings (Braesicke *et al.* 2005). They left intact, CS-directed and US-directed conditioned behaviour, in the form of HJs and 'looks' towards the foodbox, respectively, during the auditory CS. In contrast, 5,7-DHT infusions into the amygdala significantly enhanced the expression of CS-directed conditioned behaviour. 5,7-DHT infusions into the OFC were without effect on the expression of conditioned responses, consistent with previous findings with excitotoxic OFC lesions (Reekie *et al.* 2008). However, they did cause an uncoupling of the behavioural and autonomic responses both at retention as well as during reversal learning. In contrast, neither 5,7-DHT infusions into the amygdala or the OFC affected the number of sessions it took for the conditioned responses to reverse. Thus, taken together, these data affirm the role of the amygdala in the expression of appetitive conditioned cardiovascular arousal, and

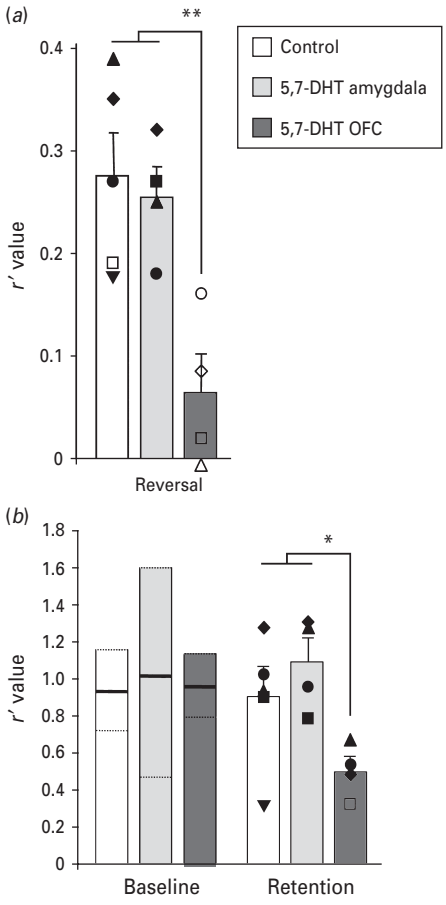

Fig. 6. Coupling of conditioned systolic blood pressure (BP) and CS-directed behavioural responses during reversal learning, retention and pre-surgery baseline. Mean correlation coefficients (r') for (*a*) systolic BP and CS-directed headjerks (HJs) at individual CS across reversal learning, and (*b*) at retention and pre-surgery baseline in control, 5,7-DHT amygdala and 5,7-DHT OFC groups. (*a*) Four out of five control subjects and all four 5,7-DHT amygdala-lesioned subjects showed a significant positive correlation between BP and HJs during reversal learning (significant correlation of individual monkeys indicated by filled symbols). In contrast, none of the four 5,7-DHT OFC-lesioned subjects displayed a significant correlation (indicated by unfilled symbols). Overall, the coupling of responses was significantly lower in the 5,7-DHT OFC-lesioned group compared to the other two groups (** $p=0.005$). (*b*) The coupling of responses in the 5,7-DHT OFC-lesioned group was also significantly lower during performance at retention (* $p=0.031$). Importantly, the groups did not differ from one another before surgery (baseline), as shown by their overlapping mean (solid lines) and 95% confidence intervals (dotted lines). Note that the mean of the correlations of the 5,7-DHT OFC-lesioned groups at retention, as well as the individual with the largest correlation (filled triangle), lay outside of the confidence intervals of their pre-surgery, baseline performance.

demonstrate the selective role of 5-HT in the regulation of conditioned response expression (at the level of the amygdala), and coordination (at the level of the OFC).

Amygdala, 5-HT and positive, emotionally salient stimuli

The conditioned HJs displayed by marmosets, triggered by a diffuse auditory CS, appear to be highly similar to those described in rats and are thought to reflect enhanced attentional processing of the stimulus that has acquired emotional significance through learning (Holland, 1977). The present finding of intact expression of HJs, following post-training amygdala lesions in marmosets is entirely consistent with studies in rats which have shown that such CS-directed responses are only dependent upon the amygdala, and specifically the central nucleus, for their acquisition, but not subsequent expression (Gallagher *et al.* 1990; Groshek *et al.* 2005). The possibility that intact HJs in the present study were due to spared functioning in the lateral nucleus, which was unilaterally lesioned, is unlikely, as basolateral lesions of the amygdala have no effect on the acquisition, or expression, of CS-directed behaviour (Hatfield *et al.* 1996).

In contrast to excitotoxic amygdala lesions, selective 5-HT depletion in the amygdala resulted in significantly enhanced expression of CS-directed behaviour, and a similar (but non-significant) augmentation of conditioned cardiovascular arousal responses. To our knowledge, these data are the first to demonstrate the role of amygdala 5-HT in appetitive Pavlovian conditioning, since to date most investigations into the neurochemistry of this process have focused on dopamine (e.g. El-Amamy & Holland, 2007; Phillips & Hitchcott, 2009; Rosenkranz & Grace, 2002). Increased responsiveness to CS following a reduction in 5-HT levels in 5,7-DHT amygdala-lesioned marmosets is consistent with results from electrophysiological studies demonstrating that amygdala 5-HT inhibits the excitatory glutamatergic input to projection neurons (such as the sensory input from auditory areas) by activating inhibitory GABAergic interneurons (Rainnie, 1999; Stutzmann & LeDoux, 1999; Stutzmann *et al.* 1998). Thus, a reduction in tonic 5-HT induced by the 5,7-DHT lesion in the present study may have caused reduced inhibitory tone in the amygdala and therefore greater responsiveness to emotionally salient stimuli, i.e. the CS^+.

The enhanced expression of CS-directed HJs in the 5,7-DHT amygdala-lesioned marmosets is somewhat similar to the enhanced approach responses of rats in

an appetitive Pavlovian conditioning paradigm following intracerebroventricular infusions of 5,7-DHT (Winstanley *et al.* 2004). The findings differ in that the overall increase in approach responses in rats was not specific to CS^+, unlike that in marmosets. Apart from the obvious difference in neuroanatomical selectivity between the two studies, the other major difference is that conditioning was trained pre- and post-surgery in marmosets and rats, respectively. While in marmosets the most salient stimulus post-surgery was the CS^+, in rats both CS^+ and CS^- were equally salient, at the beginning of learning. Thus, the overall effects of 5-HT depletions within the amygdala may be to enhance responding towards the most salient stimuli.

Consistent with the role of 5-HT in appetitive conditioning are the recent findings that putative 5-HT neurons in the dorsal raphe respond during reward expectation (Bromberg-Martin *et al.* 2010; Nakamura *et al.* 2008). Whilst it would seem that a deficit in such 5-HT signalling in the amygdala would impair CS-directed behaviour, this may be more likely to occur during acquisition, rather than during expression of well-learned behaviour, as in the present study. In addition, alterations in the balance of 5-HT receptor signaling, i.e. 1A, 2A and 2C, following 5-HT depletion (Cahir *et al.* 2007; Yatham *et al.* 2001) may contribute to the observed effects, although such changes may have less of an impact immediately post-surgery when the 5-HT levels are very low.

Relating our findings in the primate to human studies warrants caution, as both the behavioural measures and the ways of manipulating 5-HT function employed in human research are different from those used in animals. Although enhanced processing of positive stimuli following manipulations of the 5-HT system in humans has been reported, both behaviourally (Browning *et al.* 2007; Harmer *et al.* 2003, 2004; Hayward *et al.* 2005; Murphy *et al.* 2006, 2009), and neurally (Kemp *et al.* 2004; Kerestes *et al.* 2009; Norbury *et al.* 2009; but see Canli *et al.* 2005; Demaree *et al.* 2009; Murphy *et al.* 2009) whether this is the result of enhanced or reduced 5-HT signalling, or other compensatory changes, is unclear.

OFC, 5-HT and uncoupling of the emotional responses

Previous studies have demonstrated a role for 5-HT at the level of the OFC in the regulation of reward-related actions, and specifically in reversal learning in both monkeys and rats (Clarke *et al.* 2004, 2005, 2007; Lapiz-Bluhm *et al.* 2009; Man *et al.* 2010; Walker *et al.* 2006, 2009; Winstanley *et al.* 2006). Together these studies have led to the proposal that 5-HT facilitates the OFC's inhibitory, top-down control of responses orchestrated by subcortical regions, including the amygdala (Cools *et al.* 2008). We have previously shown that excitotoxic OFC lesions impair reversal learning and cause an uncoupling of behavioural and autonomic responses (Reekie *et al.* 2008). The present results extend these findings, implicating 5-HT within the OFC in the coordination of the emotional response. Thus, serotonergic dysfunction within the OFC caused an uncoupling of the autonomic and behavioural responses to appetitive stimuli, both during reversal learning and at retention. The finding that reversal learning, *per se*, was not affected by the 5,7-DHT OFC lesions is generally consistent with our previous studies investigating the effects of such lesions on an instrumental version of the discrimination reversal task. There, too, 5-HT depletions failed to affect performance on the first reversal of a series of reversals (Clarke *et al.* 2004, 2005, 2007). Very often, overall performance (errors to criterion) on subsequent reversals was also intact, the effect of the depletions being primarily an increase in perseverative responding to the previously rewarded stimulus (Clarke *et al.* 2005, 2007). Only when the intrinsic cell bodies of the OFC are destroyed is there an overall reversal impairment (Clarke *et al.* 2008; Reekie *et al.* 2008).

The finding of response uncoupling following OFC 5-HT depletions is of relevance to our understanding of emotion and, in particular, emotional dysregulation. Emotional states are the product of a variety of different response outputs, both physiological and behavioural, elicited by motivationally relevant external events. Somatic and autonomic interoceptive feedback arising from these response outputs has been proposed to contribute especially to subjective emotional experience or 'feelings' and to influence decision-making, effects dependent on the extent of the individual's interoceptive awareness (Craig, 2002; Critchley, 2009; Critchley *et al.* 2004; Damasio, 2003; Dunn *et al.* 2010; Gray *et al.* 2007; Nicotra *et al.* 2006). Moreover, it has been reported that ambiguous peripheral signalling may contribute to the emotional and behavioural biases in schizophrenia, depression, autism and anxiety (Hirstein *et al.* 2001; Paulus & Stein, 2006; Williams *et al.* 2007), many of which are treated by drugs that target the 5-HT system. Thus, we propose that uncoupling of autonomic and behavioural components of emotion and thus dysregulation of interoceptive feedback, as a consequence of 5-HT dysfunction within the OFC, may have long

term effects on overall emotionality and adaptive decision-making.

Additional considerations

The differential effects of selective amygdala depletion within the amygdala and OFC highlight the distinct roles played by 5-HT in the two structures. However, post-lesion levels of 5-HT in the amygdala did show some recovery over the course of the study, unlike 5-HT levels in the OFC. While there was a large 5-HT depletion (~80%) just 2 wk after the lesion, the depletion was approximately 50% by 4 months and further recovery was evident at 11 months. Partial recovery could explain the lack of an effect of 5-HT amygdala depletion on reversal learning, since the latter took place between 1 and 7 months post-surgery. In contrast, it cannot explain the lack of an effect on response coordination at retention, as seen following OFC 5-HT depletion, since retention took place 2 wk post-surgery, when amygdala depletion was maximal, and indeed had caused an enhancement of conditioned behaviour towards the appetitive stimulus. In addition, serotonergic neurotransmission in these lesioned animals was still significantly compromised during reversal learning as shown by subsequent *in-vivo* microdialysis. This demonstrated that the basal extracellular levels of amygdala 5-HT were below detection threshold (2.0 fmol) and did not exceed the threshold following potassium challenge, although it must be noted that the microdialysis sampled a discrete amygdala region (probably in the basal or lateral nuclei) whereas the post-mortem analysis included the entire amygdala. Finally, these same lesioned animals went on to display differential effects to controls on a subsequent instrumental reward learning test (Man *et al.* 2010). However, further research in animals allowing selective manipulation of 5-HT receptor signalling in the amygdala, will be vital to understanding of the underlying mechanism. In particular, it will be important to determine whether the behavioural effects following long-term changes in 5-HT levels induced by 5,7-DHT infusions could stem from adaptive changes in the function of specific 5-HT receptor subtypes within the amygdala, similar to the changes observed in other brain regions after prolonged alteration in 5-HT signalling (Cahir *et al.* 2007; Jennings *et al.* 2008).

In summary, we have demonstrated that 5-HT at the level of the primate amygdala and OFC modulates the expression and regulation, respectively, of conditioned Pavlovian responses in the appetitive setting. Thus, 5-HT modulates both positive and negative emotion, consistent with the effects of SSRIs on both positive and negative emotion in humans. It remains to be determined how 5-HT integrates emotional responding at these different levels of the control hierarchy.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/pnp).

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Statement of Interest

None.

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