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Serotonergic depletion increases conditioned suppression to background stimuli in the rat

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Dark Agouti rats were lesioned by intra-ventricular injection of 5,7-dihydroxytryptamine (DHT) and, 2 weeks later, learning was tested in a conditioned suppression of drinking procedure. Lesioned and vehicle-injected control rats were conditioned with a discrete stimulus (tone or light conditioned stimulus, CS) twice paired with footshock (unconditioned stimulus), with or without a 30-s trace interval between these events to produce strong and weak learning conditions (a trace conditioning effect). During this conditioning session, the alternate stimulus (light or tone) was presented continuously in the background. Since the 5,7-DHT lesion also reduced the baseline licking response in the experimental chambers, we used drinking during the first minute, when this non-specific effect was minimal, as the dependent variable. We tested conditioning to target CS and to the alternative experimental background stimulus in exactly the same way in the same rats. We found that a level of serotonergic depletion without any intrinsic action on the trace conditioning effect nevertheless increased conditioning to the alternative background stimulus, irrespective of trace interval or stimulus modality. Thus, for both light and tone stimuli, the effect of serotonergic depletion depended only on the discrete target versus diffuse background role of the stimulus in use. These findings have implications for the modification of human cognition by serotonergic drugs.

Key words: classical conditioning; rat; serotonin depletion

Introduction

There is mounting evidence that serotonin (5-HT) is important in a number of cognitive brain disorders and their medication (e.g. depression, dementia and schizophrenia). In addition, recreational drugs that affect the serotonergic system, such as 'Ecstasy' (MDMA, 3,4-methylenedioxymethamphetamine), produce cognitive disturbances (Curran and Travill, 1997). However, whilst these clinical associations heighten interest in the relationship between normal thought processes and the serotonergic system, controlled experiments are needed to specify more exactly how the serotonergic system is involved in aspects of cognitive (dys)function. In the present experiment, we measured the extent to which rats learned about an experimental background, manipulating the level of learning it supported through the use of trace conditioning in which there is a delay between target conditioned stimulus (CS) and unconditioned stimulus (UCS) (Odling-Smee, 1975; Marlin, 1981; Tanner *et al.*, 1987).

Whilst previous work suggests some role for the serotonergic system in conditioning to contextual stimuli, 'context' tends to confound differences in the physical and functional nature of stimuli. For example, context has a containing function in that it provides the background against which other stimuli are presented. This means that contextual stimuli are typically extended in time or diffuse. Conventional contexts are also typically multimodal in that they are produced by a configuration of environmental stimuli. Whilst conditioning to conventional context is typically tested by place preference (Odling-Smee, 1975), for more direct comparison, it is necessary to test conditioning to discrete and diffuse cues in the same way.

For this reason, our behavioural procedures differ from those used previously (based on Odling-Smee, 1975). In the present study, lesioned and vehicle-injected control rats were conditioned with a discrete stimulus (tone or light CS) twice paired with footshock UCS, with or without 30-s trace interval between these events to produce strong and weak learning conditions (a trace conditioning effect). During this conditioning session, the alternate stimulus (light or tone) was presented continuously in the background. Then, in the conditioned suppression test, we measured the disruption in responding produced by the target and background stimuli (separately presented, cf. Tsaltsas *et al.*, 1983; Tanner *et al.*, 1987). With this kind of procedure, our experimental 'context' is provided by a unimodal diffuse background cue. Previous studies showing that the serotonergic system is necessary for conditioning to background used a polymodal context (Wilkinson *et al.*, 1995). However, testing conditioning to target and background stimuli in

the same way goes some way towards unconfounding the functional role of the background stimulus from the differences that arise from its physical differences. Since (like Wilkinson *et al.*, 1995) we used intra-ventricular (i.c.v.) 5,7-dihydroxytryptamine (DHT) with likely non-specific effects, we monitored drinking in the experimental chambers over the 10 days before the experimental stages were run.

Materials and methods

All procedures were conducted under the UK Animals Scientific Procedures Act 1986 (Project Licence number PPL 40/1423).

Animals

Fifty-six Dark Agouti male rats (Harlan, Bicester, UK) were given 20 days to acclimatize to their keeping conditions. Rats were operated on (at 200–245 g) over 8 days and given at least 2 weeks to recover before the start of water deprivation, when 48 rats (26 lesioned and 22 vehicle-injected controls) went into the behavioural phase of the experiment (at 200–240 g). Water was removed on the day before shaping and on the 17 days of the experiment, water access was restricted to 1.5–2 h per day between 13.00 h and 15.00 h. The time and duration of water access varied slightly to keep the maximum duration of deprivation below 23 h for every rat. On all but the conditioning day, the rats also had access to water for 15 min in the conditioning chambers.

Throughout rats were maintained on a 12 : 12 h light : dark cycle (lights on 08.00 h to 20.00 h) and tested during the light phase. They were usually housed in pairs except during post-operative recovery when they were housed singly. Food (B&K Universal, Hull, UK) was always available.

Surgery

Rats were pre-treated with desipramine (10 mg/kg i.p., Sigma, Poole, UK) to protect noradrenergic neurones (Bjorklund *et al.*, 1975; Azmitia and Segal, 1978). After 30 min, they were anaesthetized with halothane (4% in oxygen, Zeneca, Macclesfield, UK) and placed in a stereotaxic frame with atraumatic ear bars. Anaesthesia was maintained using 1–2% halothane during surgery. In the lesioned (DHT) group, 150 mg 5,7-DHT creatine sulphate (Sigma) in 10 ml 0.1% ascorbate artificial cerebrospinal fluid (aCSF: 60 mM NaCl, 10 mM $NaHCO_3$, 1.2 mM KCl, 250 mM NaH_2PO_4, 1.3 mM Na_2HPO_4, 250 mM Na_2SO_4, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$) was infused bilaterally (5 µl per ventricle, 5 µl per min) by micro-syringe (SGE, Milton Keynes, UK) at the following co-ordinates (in mm from bregma): – AP, – 0.8; ML ± 1.5 and V – 3.8 mm from dura. The needle was left *in situ* for a further 4 min after the infusion was completed. All measurements were taken with the incisor bar set at 0 mm below the interaural line. The vehicle-injected (VEH) control group was given the same treatments, except that there was no 5,7-DHT in the ascorbate aCSF. Immediately after surgery, all rats received 2 ml glucose/saline s.c. to prevent post-surgical dehydration and aid recovery. Thereafter, they were given additional bedding and palatable foods until food and water intake normalized and their weights stabilized. Also during recovery, they were handled (using protective gloves) until reactivity subsided.

Of the 56 rats operated on, we lost six under anaesthesia as a result of respiratory depression, one was killed immediately after surgery and one died later.

Apparatus

All behavioural procedures took place in fully automated chambers, housed within sound-attenuating casings that contained ventilating fans (Cambridge Cognition, Cambridge, UK). The inner conditioning chambers were plain steel-plate boxes (25 × 25 × 22 cm high), with a Plexiglas door 19 × 27 cm at the front. The roof was also steel plate with a loudspeaker inset through which the auditory stimulus was presented. The floor was a shock grid with steel bars 1 cm apart and 1 cm above the upper lip of a sawdust tray that was a further 7 cm deep. The water spout was mounted in the same wall as the levers, 5 cm above the grid floor and connected to a lickometer supplied by a pump (Cambridge Cognition). A photobeam detected each broken contact with the water spout as a lick and this triggered water delivery from the pump, set to deliver 0.05 ml per lick. The levers were retracted and inoperative throughout the session.

We used two stimuli as target CS and background in a fully counterbalanced design. One was a click set at 2 Hz, sound level 70 dB including background, so that pulses of noise were separated by 0.5-s intervals. The alternate target/background was provided by flashing three stimulus lights (positioned in the wall above the retracted levers) and the houselight together, both on and off at 0.5-s intervals. There was no other illumination in the chambers, except when water was available the water spout was also illuminated. Footshock was delivered through the grid floor by a constant current shock generator (MISAC Systems, Newbury, UK).

Stimulus control was by a RISC PC programmed in Basic, with additional interfacing using an Arachnid extension (Cambridge Cognition).

Behavioural procedures

The experiment was run in a single replication over a 3-week period: 3 days of shaping in the latter half of the first week, followed by 10 days of pretraining; then the conditioning sequence on the following 4 consecutive days. All training and testing was during the light phase of the cycle between 08.00 h and 14.00 h.

Pre-experimental

The rats were shaped (first in pairs) over 3 days until they contacted the water spout and drank freely from it. Individual rats were then assigned to the same box (counterbalancing for lesion and behavioural condition) on all days of pretraining and for the duration of the experiment. On each of 10 days, they had access to water, timed for 15 min after the rat first contacted the water spout. The purpose of this phase was to ensure that rats drank freely and to measure any baseline differences between rats assigned to different experimental conditions. On each of the 10 days of pretraining, we recorded the total number of licks completed in the session. On the final 2 days, we also recorded (1) the time to first lick and (2) the number of licks in each of 15 1-min ‘bins’ of time.

These sessions also involved habituation to the context provided by the experimental chambers. No experimental stimuli were presented in this phase, except for the stimulus light that illuminated the water spout.

Conditioning

There was 1 day of conditioning in which each squad of rats received two conditioning trials (with or without a trace interval) within a single session. In the background, the first stimulus (e.g. flashing stimulus lights) was on for the whole the session (total duration just over 15 min). After 5 min, the second stimulus (5-s duration CS, e.g. click) preceded a footshock (UCS, 0.5 mA, 1-s duration). The interval between CS and UCS was either 0 or 30 s (Marlin, 1982; Honey and Hall, 1992). In the 0-s condition, shock was contiguous with CS offset. In the 30-s condition, the first stimulus continued in the background during the 30-s interval. After a further 5 min, there was an identical second conditioning trial. The rats were then left in the conditioning chambers for 5 more min (in which time the background stimulus continued).

Throughout conditioning, there was no access to water. The water spout stimulus light was not illuminated in this phase. Given the absence of licking activity, there was nothing to record.

Reshaping

The reshaping day followed the same procedure used on the pre-experimental days. Reshaping had two purposes: (1) to re-establish drinking after conditioning to allow the subsequent conditioned suppression test; (2) to provide a measure of conditioning to the context provided by the experimental chambers. We also recorded (1) the time to first lick and (2) the number of licks in each of 15 1-min 'bins' of time as a measure of contextual conditioning.

Test

Rats were given access to water again and we tested conditioning to each of the (click and light) stimuli presented in turn over 2 consecutive days. Conditioning was measured as the disruption in responding: the greater the disruption (conditioned suppression), the stronger the association with shock. To allow the conditioning test to proceed, rats must first drink to provide a baseline. Only after each rat made 50 licks was the target or background stimulus (flashing light or click) presented. Either test stimulus then continued for a total of 15 min.

We tested conditioning to the designated CS and the background stimulus for each rat in exactly the same way and in a counterbalanced order. Thus, on the first test day, half the rats received the stimulus that had been presented as CS on the conditioning day and half were presented with the stimulus that had been in the background. Whichever stimulus was presented on test day 1, the alternative stimulus was presented on test day 2.

The number of licks in each of 15 1-min 'bins' of time provided a measure of the strength of conditioning based on the pattern of drinking during the entire duration of stimulus presentation. This measure is not affected by differences in latency to drink and allows good differentiation between levels of conditioning at various levels of suppression. To anticipate, the suppression levels observed in the present study were quite low so drinking in the first minute (licks-1) provided the most sensitive measure of learning.

Tissue extraction and high-performance liquid chromatography (HPLC)

At the end of the experiment, animals were killed after concussion of the brain by striking the cranium, followed by cervical dislocation before consciousness could be regained. They were then decapitated and the brains removed. The hippocampus, hypothalamus, frontal cortex and ventral striatum were rapidly dissected over ice, placed individually in eppendorf tubes, frozen on dry ice and stored at – 80 °C until extraction.

Tissue samples were weighed frozen, then thawed and 1 ml of extraction solution (0.02% $Na_2S_2O_5$ and 0.1 M perchloric acid) added, then probe sonicated (Soniprep 150 ultrasonic disintegrator, Fisher Scientific, Loughborough, UK), spun at 3500 r.p.m. for 10 min and the supernatant stored at –80 °C. All samples for a particular region were extracted at the same time.

5-HT was separated and measured in the brain region extracts using HPLC with electrochemical detection (ECD) using a Hypersil C_{18} column (3 mm ODS; 10 cm × 4.6 mm internal diameter) with a mobile phase containing 0.15 M $Na_2H_2PO_4$, 1 mM EDTA, 0.5 mM 1-octanesulphonic acid and 14% methanol adjusted to pH 3.4 with phosphoric acid (1 M) and a flow rate of 0.3 ml/min. Tissue samples were extracted in antioxidant (1 ml 0.1 M perchloric acid containing 1.6 mM sodium metabisulphite), followed by sonication (30 s) on ice and centrifugation. Resultant supernatants were filtered (0.45 mm syringe filters, Gelman Sciences, Northampton, UK) and 5-HT quantified by ECD (Antec detector, Presearch Ltd, Hitchin, UK) using a potential of 0.65 V. Minimum level of detection was 20 fmol/20 ml injected onto the column.

There was an HPLC exclusion criterion for the 5,7-DHT lesioned rats. Lesions were considered ineffective if 5-HT levels were 2 SD above the mean for the lesioned group in two of the three brain regions for which we had complete data.

Statistical analysis

All statistical tests use an alpha of 0.05. The HPLC output was analysed separately for each of the brain regions. The dependent variable was 5-HT. The analysis of the data from hypothalamus is based on a smaller sample since over one-half the data were lost because of freezer failure.

Behavioural analyses were by analysis of variance (ANOVA). We first checked for lesion effects on drinking over pretraining in a mixed design with 10 levels of Day. In further analyses within 15-min sessions, the repeated measures factor, Time, had 15 levels. Here, we also checked that there were no pre-existing differences between the rats depending on the experimental conditions-to-be. Each of the factors of Lesion, Trace and Stimulus had two levels. At reshaping, we also measured the latency to first lick after the footshocks delivered on the preceding day. The conditioning results were analysed in a $2 \times 2 \times 2$ design with the same factors of Lesion × Trace × Stimulus. Here, the dependent variable was drinking in the first minute (a sensitive measure of learning and, as it turned out, little affected by the non-specific effects of the lesion). To further correct for the baseline differences in water consumption, the conditioning analyses were also run with the reshape latency as covariate.

Day was not included as a factor as it was confounded with the target/background stimulus identity. To anticipate, the effects of interest did not depend on which stimulus was designated target and which background. Nevertheless, we present the data separately for the tone and light as target versus background since the equivalent responses to these stimuli, irrespective of their functional role (as target versus background), shows the generality of the observed effects.

There were 11 rats allocated to each of the trace conditions in the vehicle-injected group and 13 rats allocated to each of the trace conditions in the lesioned group, counterbalancing for the identity

Table 1 Levels of serotonin as nmol/mg by brain region for samples extracted 6–7 weeks postoperative

	Mean (± SEM)[a]		
Region	VEH[c]	DHT[d]	% Loss[b]
Frontal cortex	400.3 (± 61.8)	92.6 (± 13.5)	77
Hippocampus	205.1 (± 42.2)	89.2 (± 41.8)	56.5
Ventral striatum	460.1 (± 69.2)	107.9 (± 18.0)	76.5
Hypothalamus	139.8 (± 26.1)	94.5 (± 17.4)	32

Sample sizes: VEH $n = 22$, DHT $n = 24$, except in hypothalamus VEH $n = 7$, DHT $n = 12$. [a]SEM, standard error of the mean; [b]% Loss, percentage depletion (by); [c]VEH, vehicle-injected control group; [d]DHT, 5,7-dihydroxytryptamine-injected lesion group.

of the target stimulus as far as possible (n was 5 or 6 per cell for the vehicle group and 6 or 7 per cell for the lesioned group).

Results

Assay by HPLC

Univariate F-tests confirmed that the serotonergic depletion was significant in frontal cortex [$F(1,46) = 13.02$, $p < 0.001$], hippocampus [$F(1,46) = 4.40$, $p < 0.05$] and ventral striatum [$F(1,46) = 15.73$, $p < 0.001$]. As might be expected, the degree of the depletions varied somewhat across brain regions and it was not significant in hypothalamus [$F(1,17) = 2.25$] (Table 1).

Two rats in the DHT group met the exclusion criterion, with 5-HT levels much more like those seen in the vehicle (VEH) group (and clearly within two standard deviations of the VEH mean). It turned out that both of these rats had been allocated to the same behavioural group (light as target CS with 30-s delay before shock UCS, for which the original $n = 7$). Since there were no significant effects of Stimulus we can collapse across that condition, so the two exclusions left us with 11 rats in the trace conditioned DHT lesion group.

On exclusion of these rats, the serotonergic depletion remained significant in frontal cortex [$F(1,44) = 25.65$] and ventral striatum [$F(1,44) = 26.17$], but was now marginal in hippocampus [$F(1,44) = 3.79$, $p = 0.06$].

Behaviour

Effects of the lesion on drinking

Over the 10 days of pretraining, the lesioned rats drank less overall in the experimental chambers compared with the VEH controls. This effect of the lesion was confirmed statistically by the main effect of Lesion in analysis of lick totals [$F(1,46) = 15.74$, $p < 0.001$]. As expected, rats drank more as they became accustomed to the experimental chambers and this was confirmed statistically by a main effect of Days, [$F(9,414) = 15.71$, $p < 0.01$], but the Lesion × Day interaction was not significant [$F(9,414) = 1.75$].

Since this was a highly significant effect of the 5,7-DHT lesion, drinking provided us with a useful behavioural assay for the effectiveness of the serotonergic depletion. Figure 1 shows that that the two 'DHT' rats that were outliers on assay by HPLC were also behavioural outliers here in that their drinking scores clearly resembled the VEH rather than the DHT group.

This confirms the conclusion that these rats belong in the vehicle population. As would be expected, their exclusion makes little difference to the results of the analysis of drinking over the 10 days [Lesion, $F(1,44) = 15.79$; Days $F(9,396) = 13.82$; Lesion × Days $F(9,396) = 1.35$]. All subsequent analyses of drinking exclude these outliers.

On the final day of pretraining (day 10) we also looked at the pattern of drinking in the 15-min session. The rats had been randomly allocated to the delay and stimulus conditions-to-be and ANOVA confirmed that the experimental groups were well matched for baseline drink rates since there was no overall effect of Trace or Stimulus [$F(1,38) < 2$].

However, there was an overall effect of Lesion [$F(1,38) = 14.98$, $p < 0.01$]. There was a main effect of Time [$F(14,532) = 166.87$, $p < 0.01$] because rats drank less in successive minutes of the session (Table 2). The Lesion × Time interaction was not significant [$F(14,532) = 1.55$] but the effects of lesion were small in the first and final minutes of the session (Table 2). On analysis of the first minute of drinking (licks-1), there was no effect of Lesion or Trace (the latter a condition-to-be) [$F(1,38) = 1.55$ and 0.01, respectively] but there was an effect of Lesion on latency to drink [$F(1,38) = 13.92$, $p < 0.01$] (Fig. 2).

We saw the same pattern of drinking on the immediately preceding days but the 'bins' breakdown for day 9 was lost because of a technical failure so we show day 8 for comparison (Table 2).

On the reshaping day (just after conditioning and immediately preceding the test days), again there was a significant overall effect of Lesion on drinking [$F(1,38) = 21.04$, $p < 0.001$] and this time also on the pattern of drinking over time in the bins analysis [$F(14,532) = 5.56$, $p < 0.01$]. In the bins analysis, there was also a main effect of Time [$F(14,532) = 130.48$, $p < 0.01$]. No other effects or interactions were significant [all $F < 1.5$]. Again there

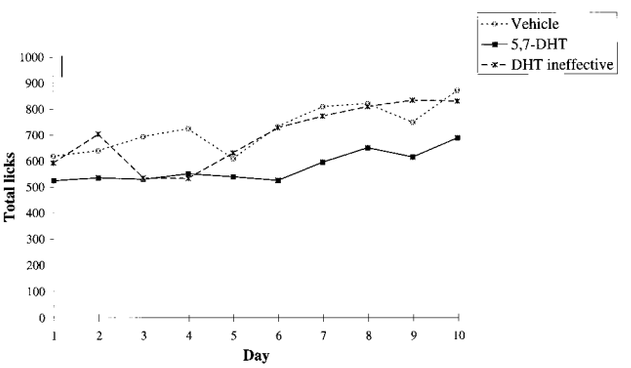

Figure 1 Total number of licks made on each of 10 pretraining days. Vehicle = injected with artificial cerebrospinal fluid; 5,7-DHT = injected with 5,7-dihydroxytryptamine; DHT ineffective = 5,7-DHT lesion shown ineffective at assay. Bar at top left shows 2 standard error of the difference of the mean, taken from the appropriate stratum of the analysis of variance, to allow approximate between-groups comparisons

Table 2 The pattern of drinking over successive minutes of two pretraining sessions (days 8 and 10)

Min	Lesion	Day 8 mean (± SEM)[a]	Day 10 mean (± SEM)
1	VEH[b]	196.86 (± 7.83)	195.77 (± 6.96)
	DHT[c]	186.42 (± 8.82)	182.88 (± 9.45)
2	VEH	150.77 (± 9.10)	167.64 (± 5.80)
	DHT	143.58 (± 8.85)	137.71 (± 11.57)
3	VEH	117.68 (± 10.36)	130.95 (± 10.66)
	DHT	107.13 (± 11.70)	114.50 (± 9.65)
4	VEH	98.86 (± 9.25)	102.82 (± 10.63)
	DHT	63.25 (± 8.04)	74.71 (± 10.20)
5	VEH	62.14 (± 6.77)	65.86 (± 6.70)
	DHT	44.63 (± 7.49)	55.21 (± 8.91)
6	VEH	47.18 (± 7.44)	56.41 (± 10.21)
	DHT	34.13 (± 8.21)	33.04 (± 7.81)
7	VEH	38.55 (± 8.74)	43.59 (± 8.21)
	DHT	22.79 (± 5.76)	31.13 (± 6.61)
8	VEH	27.09 (± 6.60)	26.32 (± 6.80)
	DHT	10.42 (± 2.84)	9.58 (± 3.95)
9	VEH	22.64 (± 5.37)	24.68 (± 5.66)
	DHT	16.63 (± 3.88)	18.08 (± 6.04)
10	VEH	16.59 (± 4.57)	27.05 (± 4.99)
	DHT	6.17 (± 2.32)	7.21 (± 2.67)
11	VEH	12.27 (± 3.87)	5.77 (± 2.19)
	DHT	7.38 (± 4.40)	14.29 (± 4.35)
12	VEH	4.45 (± 1.74)	7.14 (± 2.42)
	DHT	6.54 (± 2.70)	9.08 (± 3.39)
13	VEH	9.50 (± 3.89)	6.23 (± 2.84)
	DHT	0.17 (± 0.13)	2.92 (± 1.65)
14	VEH	7.36 (± 2.98)	6.95 (± 3.67)
	DHT	1.00 (± 0.72)	0.25 (± 0.21)
15	VEH	9.18 (± 3.05)	4.82 (± 2.67)
	DHT	0.75 (± 0.60)	1.00 (± 0.78)

[a]SEM, standard error of the mean; [b]VEH, vehicle-injected control group; [c]DHT, 5,7-dihydroxytryptamine-injected lesion group.

was an effect of Lesion on latency to drink [$F(1,38) = 5.23$, $p < 0.05$] (Fig. 2) in the absence of an effect of Lesion on the licks-1 measure [$F(1,38) = 0.75$].

Thus, whilst there were effects of the lesion on drinking, licks-1 was consistently insensitive to this effect, so we used this early drinking score to measure the level of conditioning to the experimental stimuli (see below).

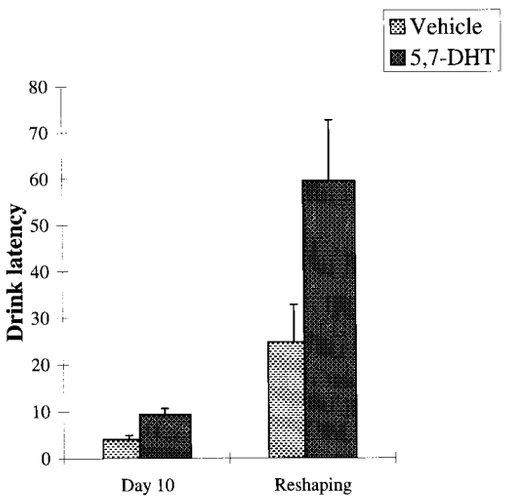

Figure 2 Time to start drinking (in seconds), shown separately for day 10 of pretraining and the reshaping day. Vehicle = injected with artificial cerebrospinal fluid; 5,7-DHT = injected with 5,7-dihydroxytryptamine. Bars show the within-subject standard error bars to allow all between-groups comparisons

Effects of the lesion on conditioning to discrete stimuli

The lesion tended to increase conditioning to target [$F(1,38) = 3.99$, $p < 0.06$] (Fig. 3). This non-significant tendency of the 5,7-DHT lesion was the same for both click and light stimuli because there were no significant Lesion × Stimulus interactions [both $F(1,38) < 1$].

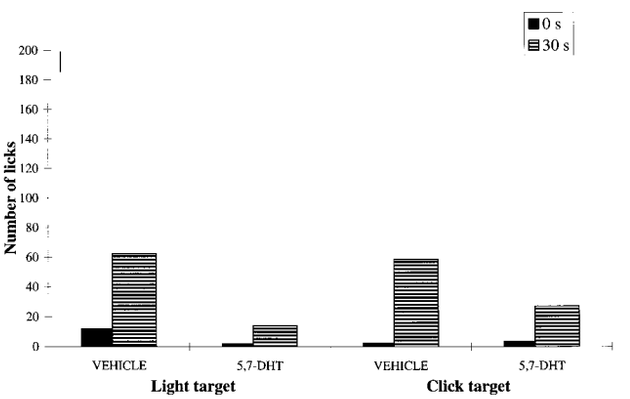

Figure 3 Conditioned suppression of drinking to the target stimulus measured as the number of licks in the first minute of stimulus presentation, shown separately for the light and click stimuli and for each of the lesion and delay conditions. VEHICLE = injected with artificial cerebrospinal fluid; 5,7-DHT = injected with 5,7-dihydroxytryptamine; 0 s = conditioned at 0-s delay; 30 s = conditioned at 30-s delay. Bar at top left shows two standard error of the difference of the mean, taken from the appropriate stratum of the analysis of variance, to allow approximate between-groups comparisons

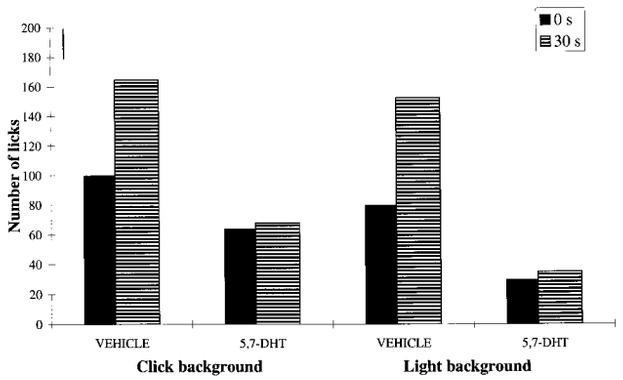

Figure 4 Conditioned suppression of drinking to the background stimulus measured as the number of licks in the first minute of stimulus presentation, shown separately for the click and light stimuli and for each of the lesion and delay conditions. VEHICLE = injected with artificial cerebrospinal fluid; 5,7-DHT = injected with 5,7-dihydroxytryptamine; 0 s = conditioned at 0-s delay; 30 s = conditioned at 30-s delay. Bar at top left shows 2 standard error of the difference of the mean, taken from the appropriate stratum of the analysis of variance, to allow approximate between-groups comparisons

Effects of the lesion on trace conditioning

The extension of the interval from 0 to 30 s weakened conditioning. Statistically this was confirmed by a significant main effect of Trace on conditioning to the target [$F(1,38) = 10.28$, $p < 0.005$] (Fig. 3). The level of suppression to each of the two stimuli (presented in a counterbalanced design) was comparable (Fig. 3). There was no significant Trace × Stimulus interaction for conditioning to target or background [both $F(1,38) < 1$].

However, the lesion neither impaired nor enhanced the observed trace conditioning effect because there was no significant Lesion × Trace interaction for conditioning to target [$F(1,38) = 2.56$]. The Trace × Lesion × Stimulus interaction was also insignificant [$F(1,38) < 1$]. Thus, despite the effects of the lesion on drinking and its probable effect on conditioning, it had no effect on the difference between conditioning at 0-s and 30-s delay. This was true for both click and light stimuli as CS.

Effects of the lesion on conditioning to background stimuli

There was a significant effect of Trace during presentation of the background stimulus [$F(1, 38) = 5.95$, $p < 0.05$]. This means that the amount learned about the background stimulus depended on whether there was a good alternative predictor of shock or not. The pattern of effects observed was that, overall, the rats learned relatively less about the background in the 30-s delay condition (Fig. 4).

In both delay conditions, the lesion increased conditioning to background (Fig. 4). Statistically, there was a main effect of Lesion [$F(1,38) = 24.62$, $p < 0.001$] and a significant Trace × Lesion interaction [$F(1,38) = 4.48$, $p < 0.05$]. Figure 4 shows that this arose because the lesion abolished the difference in suppression to the alternate stimulus otherwise seen between the 0-s and 30-s delay groups.

The experimental background may have been ineffective as a contextual stimulus. We therefore also examined conditioning to the contextual stimuli provided by the experimental chambers on the reshaping day by Trace and Lesion. However, there was no effect of Trace on total licks [$F(1,42) < 1$] or on the pattern of drinking over the 15-min session, F (14,588) = 0.72. Analysis of drink latency and licks-1 also showed no main effect of Trace [$F(1,42) = 0.005$ and 2.71, respectively] and there were no Trace × Lesion interactions [$F(1,42) < 1$].

That said, visual inspection of the overall drink latencies on day 10 and the reshaping day suggests that the time to start drinking was further increased by contextual conditioning to cues provided by the box, irrespective of delay condition (Fig. 2). This effect looks greater in the DHT-lesioned group. If the lesion affected drinking in the absence of any effect on contextual conditioning, we would expect to see a difference between the VEH and DHT groups of the same order of magnitude to that seen on day 10 (around 5 s) against a different baseline due to the expected suppression to the context in which the shock had been given. Figure 2 shows that the observed difference was in fact much greater (some 30 s). However, ANOVA with Day as a factor showed that the lesion effect was not different at reshaping because whilst there was an overall effect of Lesion [$F(1,42) = 6.29$, $p < 0.05$], the Trace × Day and Lesion × Trace × Day interactions were not significant [maximum $F(1,42) = 0.25$].

Figure 2 also shows that post hoc comparisons using the within-subject standard error bars confirm that whilst there was a substantial change in the general level of suppression to the contextual cues provided by the experimental chambers, the DHT-lesioned rats were slower to drink both before and after conditioning.

Allowing for differences in baseline drinking

When we ran the analyses with reshape latency as covariate, to further cater for lesion effects due to an effect on drinking (or contextual conditioning, but see above), the differential effect of Lesion on conditioning to target [$F(1,37) = 2.18$] and background, [$F(1,37) = 16.65$, $p < 0.001$] became even clearer. There was still a significant effect of Trace for both conditioning to target [$F(1,37) = 10.21$, $p < 0.003$] and background [$F(1,37) = 7.29$, $p < 0.01$] and a Trace × Lesion interaction [$F(1,37) = 6.99$, $p < 0.05$] for conditioning to background only. No other interactions were significant [maximum $F(1,37) = 2.84$].

Discussion

These results show that serotonergic depletion can increase conditioning to a ‘contextual’ background stimulus, irrespective of the level of conditioning that background stimulus would normally support (which was manipulated by the use of a trace interval between CS and UCS). What mattered was not whether the stimulus under test was light or tone, but rather whether it was presented as target or background. Thus, the effect of the serotonergic depletion depended on the discrete versus diffuse nature of the stimuli in use, irrespective of the modality of the stimulus. This dissociation became even clearer when the rats’ hesitancy to drink in the experimental chambers was partialled out (using the reshaping drink latency as covariate).

The trace conditioning effect was unaffected by the lesion in that, in both lesioned and non-lesioned groups, conditioning to target CS was weaker when that stimulus was separated from the UCS by a 30-s interval, compared with the behavioural control conditions in which it was presented contiguously. The inclusion of

trace-conditioned groups enabled us to assess the effect of the lesion relative to different control (non-lesioned) levels of learning. Comparison of the different behavioural groups then allows us to distinguish lesion effects that depend on the nature of the stimulus in use from those that might instead arise from a general increase in conditioning with an aversive UCS, through some form of 'rate-dependent' effect (Kelleher and Morse, 1968; Rawlins *et al.*, 1980). On such an account, lesion-induced increases in conditioned suppression, measured as decreased drinking, would be greater the higher the baseline lick rate indicated in the vehicle-injected group. This would explain the bigger statistically reliable effect of the lesion on conditioning to the background stimuli and the lack of difference in conditioning between the 0-s and 30-s groups: if we simply assume that the more the rats drink to begin with, the bigger the lesion effect. We discount this possible explanation of our test results because there is no such rate-dependent effect in the pattern of drinking shown on the preconditioning days. There we saw a modest depression in licking (of up to only around 20 licks) in the lesioned rats relative to the vehicle-injected controls across a baseline that varied from 200 to 20 licks per minute (Table 2).

In the vehicle-injected group, as would be expected, conditioning to the background was overall weaker and, superimposed on this effect, the trace interval had a secondary effect on conditioning to background: the weaker the conditioning to target, the weaker the conditioning to background. This finding suggests that the background stimulus became associated with the target through second order conditioning and this would explain why its associative strength should follow the same pattern of effects (depending on how good a predictor of shock the target was). There is evidence for the formation of this kind of higher order conditioning (Marlin, 1981; Pearce *et al.*, 1981; Rescorla, 1984). Such within-compound associations are favoured by relatively few CS–UCS pairings (Marlin, 1982), as here, and temporal similarity of the stimuli in use (Testa, 1975). Alternatively, the temporal similarity between the flashing light and click stimuli may have promoted generalization from the target to background cue (Thomas and Basbaum, 1972). A third possibility is that the CS–UCS associative unit might have been associated with the background as an occasion-setter, though in this case we would not necessarily expect the background stimulus to acquire excitatory properties (Bouton and Swartzentruber, 1986).

Furthermore, rather than an effect on contextual conditioning that happened (in this case) to involve second order conditioning, the pattern of results observed here may straightforwardly reflect heightened second order conditioning. If so, it would follow that serotonergic depletion should heighten second order conditioning irrespective of the discrete versus diffuse nature of the cues in use and this possibility could be tested using two discrete cues in the same conditioned suppression procedure, to measure the effects of serotonergic depletion on standard second order conditioning. However, we would need to explain the dissociation between the effects of the serotonergic depletion on first and second order conditioning (and without reference to the discrete versus diffuse nature of the cues in use).

A treatment that was without significant effect on first order conditioning might nevertheless affect second order conditioning because of the increased information load involved in forming an associative chain. Second order conditioning might, for example, require relational processing (Eichenbaum *et al.*, 1994). However, whilst the possibility that second order conditioning is what is being affected by the serotonergic depletion (because in vehicle-injected animals, the level of conditioning seen to the background stimulus follows that seen to the target), the lesion increased conditioning to background equally for both contiguously and trace conditioned groups. Thus, the fact that the serotonergic depletion increased conditioning irrespective of the informativeness of the second order conditioned stimulus would still need explanation (and we have already discounted a rate-dependent explanation of the observed effects). A simple increase in second order conditioning should have left the 0-s conditioned group more suppressed to the background than the 30-s trace group at a higher level of overall suppression.

This increase in conditioning to the diffuse background cue used here appears to differ from the effect on contextual conditioning reported earlier (Wilkinson *et al.*, 1995). Explanations of this apparent discrepancy are considered below. We first consider the level of serotonergic depletion produced by our lesion and its non-specific effects.

The level of serotonergic depletion

At assay, this lesion was less than that produced in other studies using the same surgical procedure (Wilkinson *et al.*, 1995; Lister *et al.*, 1996). The main part of the experiment was over 5 weeks after surgery because the rats needed a minimum of 2 weeks recovery before they could be put on water deprivation and a further 13 days to complete shaping and pretraining days. At the time of the assay, the depletion was substantial but incomplete (by 32–77%). The delay on measuring the extent of the lesion (imposed by the behavioural testing schedule) meant that sprouting could have (further) reduced the size of the lesion (see, for example, Clewans and Azmitia, 1984). Some of the variation in the size of the lesion by region is likely to be because 5,7-DHT given i.c.v. would have less effect on median than dorsal raphe (Azmitia and Segal, 1978; Park *et al.*, 1993).

Whilst experimental depletions of around 90% are more typical, various levels of serotonergic depletion have been found to have significant behavioural effects (Hole *et al.*, 1976; Lorens *et al.*, 1976; Vanderwolf *et al.*, 1989; Williams *et al.*, 1990; Cassaday *et al.*, 1993; Ison *et al.*, 1996). It is possible that a more complete lesion would have different effects from the relatively modest levels of depletion achieved here. However, it seems likely that effects should only be qualitatively different with a bigger dose of neurotoxin (administered in the same way) if this had the consequence that further brain regions were significantly depleted. A more wide-ranging serotonergic depletion could both have direct effects on the psychological processes of interest and introduce (for our purposes) non-specific effects (see below). Given that non-specific effects would inevitably be greater with a larger lesion, follow-up work should target likely terminal regions.

Of the terminal regions that we sampled, serotonergic depletion was clearly significant in frontal cortex and ventral striatum. Of these two regions, a role for ventral striatum seems particularly likely in that excitotoxic lesions here have been found to increase conditioning to contextual cues (Parkinson *et al.*, 1999). In this study, contextual conditioning was measured using a place preference conditioning procedure like that used by Wilkinson *et al.* (1995). However, the finding of Parkinson *et al.* (1999) held for the core but not the shell subregion of the nucleus accumbens and our assay does not enable us to distinguish where within ventral striatum serotonergic depletion may have been effective.

The rat equivalent of frontal cortex is not necessary for other selective learning processes (Joel *et al.*, 1997), but that does not allow us to exclude its role in the present study. Neither can we discount a role for other brain regions not specifically sampled for our assays by HPLC, particularly where there is other evidence that they play a role in contextual fear conditioning (Vazdarjanova and McGaugh, 1999; Bucci *et al.*, 2000).

Conventional lesions to the hippocampus have been reported to increase contextual fear conditioning (Winocur *et al.*, 1987). However, in a trace conditioning paradigm like the one used here, conventional hippocampal lesions did not affect conditioning to a temporally extended background stimulus (Rawlins and Tanner, 1998). Anyway, our serotonergic depletion was marginal in hippocampus.

Non-specific effects of the lesion

General serotonergic depletion affecting activity at a range of receptor subtypes would be expected to have very widespread effects (Leonard, 1994). Our procedures control for non-specific effects such as response disinhibition or increased shock sensitivity through comparison of conditioning groups. In addition, since the lesion reduced drinking over a 15-min session, we used early drinking (licks-1), when this effect was insignificant, as a measure of conditioning. Whilst licks-1 at test reflects a slightly different experimental time epoch at test in that it excludes the first 50 licks made prior to stimulus presentation, it is still the most appropriate measure of early drinking given that rats initially drink at around 200 licks per minute on the baseline days. Since these first 200 licks are little affected by the lesion prior to conditioning, non-specific effects provide no obvious account of lesion effects on conditioning observed in this response window. Anyway, the covariance analysis provided further confirmation that baseline lick rates were an inadequate account of the observed effects.

Lesion effects on drinking might secondarily influence conditioning since water deprivation increases fear conditioning to contextual but not discrete stimuli in rats (Maren *et al.*, 1994). However, there is no reason to suppose that lesion effects on thirst might mediate the increase in conditioning to background that we saw because the lesion increased the time taken to begin drinking and decreased the overall volume of water taken, consistent with decreased rather than increased thirst. The observed increase in conditioning to background reflected in response inhibition similarly finds no account in terms of the response disinhibition typically produced by such lesions (Tye *et al.*, 1977).

Conditioning to context

The increase in conditioning to background is an apparently opposite effect to the impaired contextual conditioning found using a conventional polymodal context (Wilkinson *et al.*, 1995). Before we discuss this apparent discrepancy, we first consider the possibility that, rather than treating our experimental background as context, the rats treated the various stimuli provided by the experimental chambers as the 'real' context. The experimental envelope was an extended modality-specific stimulus that might well differ functionally from the polymodal configuration of stimuli that normally go to make up context (Tsaltas *et al.*, 1989; Rawlins and Tanner, 1998). However, analysis of drinking on the reshaping day did not support this interpretation. There was no evidence from the lick scores that, in the 30-s delay conditions, the rats instead conditioned to the alternative contextual stimuli provided by the experimental chambers. We also used the time to start drinking to determine conditioning to these more conventional contextual stimuli. Whilst the lesion seemed to increase conditioning to experimental context measured by drink latency on the reshaping day, statistically, this effect was no more than would be expected on the basis of its known effects on drinking (e.g. relative to day 10).

If the background was an effective context, the observed result, of a direct rather than an inverse relationship between conditioning to target and background, is inconsistent with some theory (Rescorla-Wagner, 1972) and data (Odling-Smee, 1975). However, others have proposed that conditioning to discrete and contextual cues can proceed independently (Gibbon and Balsam, 1981). This contrasting position is supported by data showing that conditioning to context is either unrelated to conditioning to target (Balsam and Gibbon, 1988; Williams *et al.*, 1992) or this relationship is weaker than would be predicted by the Rescorla-Wagner (1972) model (Tanner *et al.*, 1987).

Place preference procedures with distinctive alternate contexts (such as those used by Wilkinson *et al.*, 1995) are clearly different measures of the level of conditioning to contextual cues that may not reproduce where the context is provided by a temporally extended background stimulus (Winocur *et al.*, 1987; Rawlins and Tanner, 1998). In contrast to earlier findings using spatial multimodal stimuli (Wilkinson *et al.*, 1995), the present study demonstrates an increase in conditioning to the 'context' provided by a single diffuse stimulus that was functionally but not qualitatively different from an alternative discrete CS.

Conclusions and implications

We found that a level of serotonergic depletion without intrinsic effect on trace conditioning, nevertheless increased learning to a background stimulus, irrespective of trace interval and stimulus modality. The discrepancy with the impaired contextual conditioning previously found after the same lesion (Wilkinson *et al.*, 1995) could be due to the different forms of 'context' available to the rat (and therefore the way the stimulus was encoded), or the apparently smaller depletion in our study. However, our lesion had other behavioural effects, for example, it decreased drinking in the present study.

The increase in conditioning to background in the absence of any significant increase in conditioning to the target CS (and irrespective of the different levels of learning supported through variation of the trace interval) could still be due to heightened (breadth of) attention rather than an effect on contextual conditioning as such. This is a testable explanation of the apparent discrepancy with the Wilkinson *et al.* (1995) study: On this account, serotonergic depletion should also decrease the salience overshadowing normally observed when two discrete stimuli compete for associative strength. That is the increased ability to condition to alternative stimuli should be observable irrespective of the diffuseness of the competing stimuli in use. Such an effect could be mediated by lesion effects on arousal (Easterbrook, 1959).

The size of depletion we have is relevant to considering the cognitive effects of MDMA that arise from similar (modest but widespread) depletion of brain 5-HT. Whilst it is known that acute treatment with MDMA can result in cognitive deficits (Curran and Travill, 1997; Frederick and Paule, 1997), there is little, if any, direct behavioural data on its longer-term effects in rats. Since the spread and size of the serotonergic depletion produced by

treatment with MDMA in Dark Agouti rats is highly comparable to that seen here (Colado *et al.*, 1993; O'Shea *et al.*, 1998) our results suggest that (in consequence of its longer term neurotoxic effects) MDMA will similarly result in increased conditioning to diffuse background stimuli.

This effect of serotonergic depletion also has implications for the likely effects of other serotonergic drugs such as the atypical antipsychotics or the selective serotonergic reuptake inhibitors (SSRIs). These are difficult to predict precisely, but, in other conditioning tasks, serotonergic depletion and 5-HT antagonists can have similar effects (Cassaday *et al.*, 1993; Shadach *et al.*, 2000). Similarly, chronic treatment with 5-HT agonists such as the SSRIs would be expected to have cognitive effects like those of a serotonergic depletion. For example, in a recent study of human volunteers, buspirone and fluvoxamine had effects on aversive conditioning that were attributed to the suppression of neural activity in the dorsal raphe (Hellewell *et al.*, 1999).

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