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Research report

The effects of hippocampal system lesions on a novel temporal discrimination task for rats

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Abstract

A novel, appetitive, Pavlovian conditioning task was used to assess interval timing. Experiment 1 showed that normal rats could discriminate between tones of 1.5 s and 0.5 s duration, or between tones of 12.0 s and 3.0 s duration. Learning was demonstrated by a greater duration of magazine responding in the period before the delivery of a food reward and after cessation of the CS+. Learning was, however, asymmetric as it was much quicker when the CS+ was the longer of the two durations (1.5 s and 12.0 s, respectively). Experiment 2 assessed the impact of fornix lesions on the acquisition of one version of this task (CS+ 1.5 s, CS− 0.5 s). No evidence was found of a change in discrimination learning following surgery. Experiment 3 examined whether rats with either fornix or hippocampal lesions affected discriminations between 12.0 s and 3.0 s stimuli. Again, there was no evidence of a lesion-induced deficit. T-maze alternation training confirmed the effectiveness of these lesions. The results not only reveal that neither the fornix nor the hippocampus is necessary for distinguishing temporal intervals within the ranges tested but also showed how under some circumstances these lesions can leave trace conditioning intact.

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1. Introduction

A fundamental aspect of time perception is the ability to measure intervals from seconds to minutes. This ability is regarded as a central element of cognition [5,39], and various models have been proposed to explain the nature of such interval timing. Influential models based around scalar expectancy theory, e.g. the pacemaker–accumulator model [5,16,17] assume that interval timing involves at least two distinct cognitive components. The first is an internal-clock process (pacemaker), while the second is a device used to compare a reference memory of previous outputs of the pacemaker against the current output to determine the interval between a start and stop signal. Monitoring the current output is often assumed to use working memory [4,5,18,53]. Support for the notion of at least two distinct processes includes evidence that the pacemaker (clock) relies on dopaminergic systems, while the reference memory component relies on cholinergic mechanisms [5,38–40].

A variety of brain structures have been implicated in timing. Prominent among these structures are the striatum and the prefrontal cortex [5,21,32,36,38,39]. It is also plausible that the hippocampus should play a key role [27], particularly in view of its connections, via the fornix, with both the striatum and the prefrontal cortex, and its critical involvement in some forms of memory. In spite of these links, the role of the hippocampus in interval timing remains uncertain. Neuropsychological studies of interval timing by amnesics with bilateral medial temporal damage (and hence hippocampal pathology) show that interval timing can remain intact for intervals of up to 20 s [50,54], although patients with right temporal lobe damage (including the hippocampus) show changes in decision-making processes for temporal duration [43]. While the famous amnesic HM appears to underestimate time intervals longer than 20 s [50], this deficit has been interpreted as a failure to encode or retrieve the task demands [12]. More reliable deficits have been reported for longer durations (1–8 min) in patients with unilateral medial temporal lobe lesions [44] that included the hippocampus.

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An alternative approach is to examine the effects of selective hippocampal damage in animals on various behavioural tests of interval timing [7]. One such test is the ‘peak procedure’. For this task the onset of a signal (e.g. a tone) marks the beginning of a period that culminates in food delivery if the animal makes a response after a preset period of time. Rats will show scalloped fixed-interval type responding after such training. On probe ‘peak trials’ (to study the full course of responding over time) the reward is not delivered but the signal continues, and trained rats show a peak in response rate around the time that the food would normally be delivered. Past studies using the peak procedure have reported that lesions of the fornix produce a shift to the left of the peak (i.e. as if the time of reinforcement occurred earlier than it really did) and an abnormal resetting of their internal clock when a gap is introduced [36,37,41,45]. Other tests that are said to invoke timing include differential reinforcement for low rates of responding (DRL) in which the animal is rewarded for not responding for a given interval. Lesions of the hippocampus and fornix often impair DRL performance [19,26,47,51,55], again suggesting a potential role of the hippocampal system in interval timing. Other results come from conditional discrimination tasks [22,41]. While rats with fornix lesions can learn duration discriminations such as temporal bisection [41], in which a rat might learn to press the right lever following a long tone for food but press the left lever after a short tone, the same lesions changed the point of subjective equality so indicating subtle deficits [41]. Likewise, hippocampal lesions only transiently impaired a conditional discrimination involving 2 s versus 8 s [22], but the rats were impaired on retaining this distinction after brief delays.

There are, however, a number of difficulties in interpreting these studies of hippocampal system function. First, some studies have failed to find peak procedure impairments in rats with hippocampal lesions [10,11]. Another issue concerns the task demands. Both the peak procedure task and the insertion of gap periods are potentially ambiguous to the animal [58]. The problem is that the animal needs not only to track time but also to discriminate what kind of trial is current, i.e. whether to keep on increasing responding, whether the current trial is a non-reinforced probe trial and thus to decrease responding, or whether the ‘gap’ is in fact an intertrial interval. Recent studies addressing this criticism [4] argue that a ‘time-sharing model’ provides a better account, although this model requires the control of attentional and memory resources that again could depend on the hippocampal system. As already noted, the peak procedure method involves probe trials in which a reward is withheld. Learning about these extinction trials will affect normal performance and, again, this might be altered by hippocampal damage [9,19]. A quite different approach is to use conditional discriminations such as the temporal bisection task. Again, this approach is potentially problematic as hippocampal system lesions can disrupt the learning of conditional rules [3,52,56]. Finally, a key aspect of the DRL task is the ability of the animal to withhold a rewarded response, something that hippocampal lesions may disrupt irrespective of the timing element [9,19].

In view of these potential problems, the present study introduced a novel test to assess whether either the fornix or the hippocampus is necessary for the discrimination of different temporal intervals. The goal was to employ a direct test with minimal task ambiguity and minimal demands on attentional resources, thereby removing peripheral features that could be affected by hippocampal damage. Rats were trained on an appetitive Pavlovian conditioning task in which one tone, for example the shorter tone, signalled the delivery of food (the CS+) after a 10 s trace interval and a longer tone was never followed by food after the trace period (the CS−). The expectation was that rats would spend more time with their head in the food trough during the trace period on CS+ rather than on CS− trials. In experiment 1 task parameters were explored with normal rats. In experiments 2 and 3 the effects of fornix and hippocampal lesions were tested on task acquisition.

2. Experiment 1: discriminating stimulus durations using an appetitive Pavlovian procedure

2.1. Method

2.1.1. Subjects

The subjects were 16 experimentally naive, male, Dark Agouti rats supplied by Harlan Olac, UK, that were approximately 10 weeks old at the beginning of testing. Throughout all three experiments, the rats were housed in pairs under diurnal conditions 14 h light/10 h dark. Prior to the start of each experiment, food was restricted until they reached 85% of their free-feeding weight which was then maintained throughout the study. All procedures were in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986 and associated guidelines.

2.1.2. Apparatus

Eight conditioning chambers (24.5 cm × 23.0 cm × 20.0 cm) were housed within separate light- and sound-attenuating chests. Fans in each chest provided background masking noise (72 dB, C-scale). The chambers consisted of three aluminium walls, and one clear, acrylic, side-wall. The ceilings were translucent white acrylic. Within each chamber there was a 5.0 cm × 6.0 cm recessed food magazine in the front wall into which 45 mg food pellets (traditional formula, P.J. Noyes, Lancaster, New Hampshire) could be delivered. The base of the food magazine was located 0.5 cm above the grid floor. Rats had to push open a clear acrylic flap to gain access to the magazine. Three pairs of photo-diode sensors were set into a 1 cm deep rectangular frame that surrounded the magazine entrance in such a manner that horizontal beams, 5 mm in front of the closed magazine flap were located 10, 20, and 30 mm above the grid floor. A RISC PC (Acorn Computers Ltd., Cambridge, England) programmed in Arachnid (Paul Fray Ltd., Cambridge, England) recorded the interruption of these beams and controlled the experimental events. A 5 Ω speaker in the centre of the ceiling delivered a 10 Hz tone at intensity of 80 dB.

2.1.3. Procedure

2.1.3.1. Pretraining.

Rats were initially given 3 days of magazine training in the conditioning chambers. The sessions lasted for 30 min during which one food pellet was dispensed into the food magazine at regular 1 min intervals. During the first session the acrylic flap of the magazine was taped open, but for the following two sessions it remained free. After 3 days, when the rats were retrieving all food pellets, they were divided into two groups for conditioning.

2.1.3.2. Discrimination training.

The basic procedure was the same for the two groups in the experiment, the key difference being in the duration of the tones to be discriminated. Group ‘Short’ received short stimuli (0.5 s vs. 1.5 s), while Group ‘Long’ heard longer stimuli (3.0 s vs. 12.0 s). A Pavlovian conditioning procedure was used so that rats received trials consisting of a tone followed by either food (when following the CS+) or no food (when following the CS−). The interval or “trace period” from the end of the tone to food delivery was 10 s for both groups. Dispensing of a food pellet was determined by the length
of the tone presented. Within each group (0.5 s vs. 1.5 s and 3.0 s vs. 12.0 s) the interval-food contingency was counterbalanced such that half of the rats received one contingency and the other half received the reverse contingency. Thus, for Group Short (n = 8), four rats were presented with 1.5 s tones that were always followed 10 s later with a food pellet and 0.5 s tones, which were never followed by a food pellet (Group Short, Subgroup A; Fig. 1, upper). The reverse contingency applied for the remaining four animals (Subgroup B). Similarly, for Group Long (n = 8), four rats were presented with 12.0 s tones that were followed 10 s later with food and 3.0 s tones that were not followed by food (Group Long, Subgroup A; Fig. 1). The remaining four rats received the reverse combination, i.e. 3.0 s then food, 12.0 s no-food (Subgroup B).

Other than the stimulus duration differences, the procedures were essentially identical for the two groups except for one additional feature in the protocol of Group Long. In Group Long one additional food pellet was dispensed at the termination of every tone (i.e. both reinforced and non-reinforced trials). This procedure was designed to counter the risk of possible mediating behaviours occurring during the presentation of these longer (3.0 s/12.0 s) tones. It was reasoned that the additional reinforcement ensured that the rat’s head would be in the magazine at the beginning of the trace period, irrespective of whether it was a CS+ or a CS− trial. It was anticipated that this could stop the development of different behavioural routines during the tone period that might help to distinguish the two delays. There was no additional reinforcement in the protocol for Group Short as it was deemed less likely that any mediating responses could occur given the much shorter (0.5 s/1.5 s) stimulus presentations. The reinforcement contingencies for Group Long Subgroup A are depicted in Fig. 1 (lower).

Each session contained 12 trials (six reinforced and six non-reinforced) that were presented in a pseudo-random order. The inter-trial-interval (ITI), as defined from the termination of one trial to the onset of the next trial, had a mean of 6 min (range 4–8 min).

2.2. Results

Discrimination learning was assessed by comparing the total duration of head entries in the food magazine at two time points within each trial; during the presentation of the tone, and during the trace period (10 s). The duration of head entries for each tone presentation were converted to a percentage of the total tone duration as the durations of the tone differed both within and between groups. In addition, the duration of head entries during the pre-CS period (5 s prior to the tone) were analysed to determine any changes in baseline activity.

For each time period, data were analysed using an analysis of variance (ANOVA) with Subgroup (A, B) as the between-subjects factor, Trial Type (CS+ vs. CS−) and sessions of the tone presented. Within each group (0.5 s vs. 1.5 s and 3.0 s vs. 12.0 s) the interval-food contingency was counterbalanced such that half of the rats received one contingency and the other half received the reverse contingency. Thus, for Group Short (n = 8), four rats were presented with 1.5 s tones that were always followed 10 s later with a food pellet and 0.5 s tones, which were never followed by a food pellet (Group Short, Subgroup A; Fig. 1, upper). The reverse contingency applied for the remaining four animals (Subgroup B). Similarly, for Group Long (n = 8), four rats were presented with 12.0 s tones that were followed 10 s later with food and 3.0 s tones that were not followed by food (Group Long, Subgroup A; Fig. 1). The remaining four rats received the reverse combination, i.e. 3.0 s then food, 12.0 s no-food (Subgroup B).

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Each session contained 12 trials (six reinforced and six non-reinforced) that were presented in a pseudo-random order. The inter-trial-interval (ITI), as defined from the termination of one trial to the onset of the next trial, had a mean of 6 min (range 4–8 min).

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For each time period, data were analysed using an analysis of variance (ANOVA) with Subgroup (A, B) as the between-subjects factor, Trial Type (CS+ vs. CS−) and sessions of
training (blocks 1–7) as within-subjects factors. Task performance was analysed in two different ways: (i) across all acquisition sessions (seven blocks of five sessions, Fig. 2A), (ii) across the last 10 sessions when performance appeared to reach an asymptote (Fig. 2B). Emphasis is given to responding during the ‘trace’ period as this provided the clearest indication of whether the duration discrimination had been acquired. While performance during the tone presentation is also considered, the brevity of the 1.5 s/0.5 s condition means that this analysis is likely to be insensitive for Group Short.

**Group Short (0.5 s/1.5 s tones).** Analysis of trace period responding across all session-blocks revealed a clear discrimination between the CS+ and the CS− (main effect of Trial Type, $F(1, 6) = 75.6, p < 0.001$), and also a Subgroup × Trial Type interaction, $F(1, 6) = 27.5, p < 0.005$. This interaction (Fig. 2A) reflects the superior discrimination performance when the CS+ was the 1.5 s tone (Subgroup A) rather than 0.5 s (Subgroup B). Consistent with this interaction, tests for simple main effects established an effect of Trial Type (CS+ vs. CS−) for Subgroup A, $F(1, 6) = 97.0, p < 0.001$ (1.5 s CS+, 0.5 s CS−), while for Subgroup B this difference just failed to reach significance, $F = (1, 6) = 5.97, p = 0.05$. By the final session blocks (Fig. 2B), the discrimination was significant for both subgroups [main effect of Trial Type, $F(1, 6) = 64.51, p < 0.001$; simple effects of Trial Type, $F_s(1, 6) > 11.39, p_s < 0.05$].

**Group Long (3.0 s/12.0 s tones).** Analysis of the trace period (Fig. 3A) across all session blocks indicated that rats in both subgroups discriminated the different tone durations. There was a main effect of CS+ versus CS−, i.e. of Trial Type, $F(1, 6) = 42.89, p < 0.005$. Tests for simple effects revealed that both subgroups learned their respective duration discriminations (Subgroup A, $F(1, 6) = 38.18, p < 0.001$; Subgroup B, $F(1, 6) = 9.51, p < 0.05$), although the discrimination appeared appreciably easier for Subgroup A (Fig. 3A). This greater difference between the CS+ and CS− trials in Subgroup A accounted for a Subgroup (A or B) × Trial Type interaction, $F(1, 6) = 12.32, p < 0.05$ over the last two blocks (Fig. 3B) which revealed that this CS+ CS− difference was only significant for Subgroup A rats (simple effects, $F(1, 6) = 52.45, p < 0.001$) although it was close to significance for Subgroup B rats ($F(1, 6) = 5.19, p = 0.063$).

### 2.3. Summary

While the rats could discriminate between the durations of the tones in the two discriminations (0.5 s vs. 1.5 s, and 3.0 s vs. 12.0 s) there was a clear asymmetry in learning between the different interval-food combinations: when the reinforced stimulus was longer than the non-reinforced stimulus, the interval-food associations were learnt more readily than in the reverse combination.

There was also evidence that the short stimulus (0.5 s/1.5 s) associations were learnt more readily than the longer (3.0 s/12.0 s) stimulus associations. While this difference might

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**Fig. 3.** (A) Duration of head entries in reinforced and non-reinforced trials during the pre-CS period and trace period for Group Long, Subgroup A (CS+ = 12.0 s, CS− = 3.0 s) and Subgroup B (CS+ = 3.0 s, CS− = 12.0 s). (B) The mean percentage of responding in the last two blocks (10 training sessions) when responding had reached asymptote for both subgroups during the pre-CS period, the trace period and presentation of the tone.
reflect the greater ease in distinguishing these particular durations, it might also reflect the one procedural difference between the two sets of discriminations. For the 3.0 s/12.0 s condition only, rats received an additional food pellet just as the tone finished. The purpose was to restrict possible mediating behaviours, but an inevitable consequence was that Group Long rats spent more time overall in the food hopper during the trace period than Group Short as they always started the period in this position.

Having established a protocol to test interval discriminations, the next stage was to examine the impact of lesions to the hippocampal system on task acquisition. Rats with fornix lesions (Experiment 2) were studied for a number of reasons. First, this tract conveys many cholinergic fibres, and cholinergic mechanisms are implicated in the reference component of interval timing. Second, this tract directly links the hippocampus with other regions (e.g. the striatum and prefrontal cortex) implicated in interval timing, and third, compared with surgery for hippocampal lesions, there is less likelihood of additional cortical damage.

3. Experiment 2: the effects of fornix lesions on a stimulus duration discrimination (1.5 s vs. 0.5 s)

For experiment 2, rats with fornix lesions and surgical controls were trained using the same procedure as the animals in Group Short from experiment 1. This particular duration discrimination (1.5 s vs. 3.0 s) was chosen as it was learnt more readily than the discrimination between the longer durations (12.0 s vs. 3.0 s). Only one stimulus-food combination was tested (CS+ 1.5 s, CS− 0.5 s) as the reverse discrimination proved much more difficult to acquire. After completion of discrimination training, subjects were tested in a T-maze with a spatial alternation task in order to confirm the effectiveness of the lesions [48].

3.1. Method

3.1.1. Subjects

The subjects were 14 experimentally naive, male, Dark Agouti rats that were maintained in the same manner as in experiment 1. The rats were approximately 10 weeks old and weighed between 226 and 300 g at the time of surgery. Eight rats received bilateral lesions of the fornix (FNX) by radiofrequency and six received sham lesions (SHAM).

3.1.2. Surgical procedures

Surgery was performed under aseptic surgical conditions. The rats were deeply anaesthetised with a mixture of isoflurane gas (Aerane liquid; Baxter Healthcare Ltd., UK) and oxygen and then placed in a stereotaxic head holder (Kopf Instruments, Tujunga, CA), with the incisor bar set at +5.0 mm to the horizontal plane. Diazepam (CP Pharmaceuticals Ltd., Wrexham, UK) was administered systemically (2.5 mg/kg) to prolong sleep after surgery. An incision was then made in the scalp and the skin retracted to expose the skull. A dorsal craniotomy was made directly above the target region and the dura cut to expose the cortex. Lesions were made by radiofrequency using an RFG4-A lesion maker (Radionics, Burlington). The electrode (0.3 mm tip length, 0.25 mm diameter) was lowered vertically, and at each site the temperature of the tip was raised to 75 °C for 60 s. The co-ordinates relative to ear bar zero were: (i) AP +5.3 mm, LAT ±0.7 mm, DV −3.7 mm from the top of the cortex; (ii) AP +5.5 mm, LAT ±1.8 mm, DV −3.8 mm from the top of the cortex. The control surgical procedures for this group used an identical procedure as described above except the electrode tip was lowered only 1.7 mm from the top of the cortex and the temperature of the tip of the electrode was not raised, but remained at body temperature.

After every surgery the skin was sutured together over the skull and antibiotic powder was applied to the wound (Acramide; Dales Pharmaceuticals, UK). All animals received 5 ml of glucose saline subcutaneously and the analgesic Meloxicam (1.0 mg/kg; Boehringer Ingelheim, Germany), and were then placed in a heated box until they showed signs of recovery. Behavioural testing began after a minimum of 10 days recovery from surgery.

3.1.3. Apparatus

Conditioning chambers. The same as those used in experiment 1.

T-maze. Spatial learning was assessed in a T-maze. The floor of the maze was made of wood and painted white. Each arm was 70 cm long and 10 cm wide. The sidewalls were made from clear Perspex and were 16.5 cm high. At the end of each arm was a sunken food well, 3.0 cm diameter, 0.75 cm deep. Four metal supports raised the floor of the maze 100 cm above the ground. The maze was located in a 3.0 m × 3.0 m room. From the maze, animals had full view of distal wall cues (1 picture/wall) and extra maze cues such as furniture.

3.1.4. Procedure

Stimulus duration conditioning. The procedure was the same as that in experiment 1. All rats underwent 3 days of magazine training and then went on to stimulus duration conditioning. In this procedure only short tones (1.5 s and 0.5 s) were presented and only one tone-food contingency was tested (CS+ 1.5 s tone, CS− 0.5 s tone). After 33 training sessions, the trace period was increased from 10 s to 20 s for a further nine sessions (sessions 34–42). Any details omitted were the same as for experiment 1.

T-maze alternation task. Pretraining began with nine sessions of habituation to the maze with the food wells in all three arms baited with sucrose reward pellets (45 mg, Noyes, Reward Pellets, Lancaster, NH). All habituation sessions lasted 5 min, and all rats learnt to explore the maze to gain food.

The rats were then trained with six trials per session (day) for 6 days. Trials consisted of a forced “sample” turn followed by a “choice” run. Forced turns were made by blocking one of the side arms of the T-maze with a metal barrier which fitted into the arms at the junction of the T. After turning down the “forced” arm the rat was allowed to eat two sucrose pellets that had previously been placed in the food well. Animals were then picked up from the end of the forced-choice arm and returned to the start arm, which was the same throughout the experiment. The animals were then given a free choice between the right and left turn arms, receiving a reward (further two pellets) if they turned in the direction opposite to that in the sample run (i.e. non-matching).

At the start of each session four rats were taken from the holding room to the experimental room in a sealed carry-box made of aluminium. The carry-box was placed on a table behind the T-maze during testing. All four rats were tested concurrently with each rat having one trial in turn, so that the inter-trial-interval (ITI) ranged from 3 to 4 min. All rats received five sessions and each session contained six trials, of which there were an equal number of forced right or left turns in a pseudorandom sequence. The interval between the end of the forced turn and the start of the choice run was around 10 s. The correct arm for the choice phase was baited before both components of the trial.

3.1.5. Histology

On completion of the experiment all animals were deeply anaesthetised with an intraperitoneal injection of pentobarbitone (Euthatal, Rhone Merieux) and perfused intracardially with saline followed by 10% formal saline. The brains were removed and post-fixed in 10% formal saline solution for a minimum of 12 h before being transferred to a 25% sucrose-saline solution for a minimum of 24 h. The brain was then cut on a freezing microtome into 40 μm coronal sections and the sections were mounted and then stained with cresyl violet (a Nissl stain).

3.2. Results

Histology. In four of the eight cases the lesions resulted in complete or very close to complete (90–100%) bilateral transection of the fimbria/fornix (Fig. 4). In three rats, the dam-
age was asymmetrical, so that in one hemisphere it involved approximately 80–100% of the tract and 60–70% in the other hemisphere. In one rat, the total bilateral damage was approximately 60% (Fig. 4). There was restricted damage to the most caudal portion of the septum in all rats. The largest and smallest of the FNX lesions are shown in Fig. 4.

3.2.1. Behaviour

Stimulus duration discrimination. The analyses mirrored those in experiment 1, but considered the pre-CS period as well as the tone period and the trace period. The primary focus was on duration of head entries during the trace period as this was the most sensitive measure in experiment 1, and the use of short tones meant that any behavioural differences during their presentation would be difficult to interpret. Because of the differences in the duration of the trace interval (10 s then 20 s), separate analyses were conducted upon the data collected during the first 33 training sessions (blocks 1–11) and the last nine sessions of training (blocks 12–14).

There was no evidence of differential responding during the pre-CS period for CS+ or CS− trials in either group (Fig. 5). There was, however, evidence of decreased responding over blocks 1–11 as revealed by a main effect of block, $F(10, 120) = 6.90, p < 0.001$. The lack of any group effect shows that the baseline levels of nose poking activity were not affected by the fornix lesions.

For blocks 1–11 (33 sessions), analysis of trace period responding (Fig. 5) showed that both SHAM and FNX-group rats discriminated the two intervals (main effect of Trial Type, $F(1,12) = 102.42, p < 0.001$) as rats responded significantly more following the CS+ (1.5 s tone). There was also a Trial Type × Block interaction, $F(10, 120) = 16.17, p < 0.001$, reflect-
ing improved discrimination with training. Similarly, during the 20 s trace period (blocks 12–14) both groups responded differentially to the CS+ and CS−, confirmed by a main effect of Trial Type, $F(1, 12) = 51.21, p < 0.001$. No lesion effects were detected in any of these analyses.

**T-maze alternation.** The mean percentage of correct responses over the six sessions for the FNX rats was significantly lower (71%) than that of the SHAM rats (94%). This difference was significant, $t(12) = 5.83, p < 0.001$.

### 3.3. Summary

Although there was clear evidence from the histology and from the very poor performance on T-maze task that the fornix lesions had been effective, there was no evidence that they had altered the rats’ ability to learn the interval discrimination. The pattern of discrimination appeared very similar to that seen in experiment 1, and discrimination performance did not seem to diminish when the trace interval was increased from 10 s to 20 s.

### 4. Experiment 3: the effects of hippocampal and fornix lesions on a stimulus duration discrimination (12.0 s vs. 3.0 s)

The lack of a lesion effect in experiment 2 raises the possibility that a direct hippocampal lesion might be more effective in disrupting interval discrimination if performance on this task relies on hippocampal fibres that follow routes other than through the fornix. For experiment 3 the hippocampal lesions were made by radiofrequency. Not only should this method help to limit damage to dorsal cortical regions (e.g. parietal) compared to the infusion of excitotoxins, but the additional damage to fibres of passage to and from the parahippocampal region also extends the impact of the surgery to regions closely related to the hippocampus. Furthermore, given the lack of a fornix lesion effect on distinguishing tones of 1.5 s from 0.5 s duration this final experiment compared tones of 12.0 s with 3.0 s. Unlike experiment 1 there was no additional food pellet presented at the end of the duration signal (the tone). This change in procedure was to reduce the potential for ceiling effects and, so, increase the sensitivity of the task. However, removal of this procedure meant there was a possibility that the rats might develop behavioural routines during the tone to help distinguish the two delays. Therefore, miniature cameras were inserted into the conditioning chambers to monitor behaviour throughout the trials. A further difference to the procedure was to train the rats throughout with a trace period of 20 s in order to make the task more demanding.

### 4.1. Method

#### 4.1.1. Subjects

The subjects were 26 experimentally naive male, Dark Agouti rats that were maintained in the same manner as in experiments 1 and 2. The rats were approximately 10 weeks old and weighed between 220 and 293 g at the time of surgery. One group of rats received bilateral lesions of the fornix (FNX; $n = 7$), a second group had bilateral hippocampal lesions (HPC; $n = 8$) and a third group served as a control group. This control group consisted of surgical sham animals (SHAM; $n = 11$) for both FNX and HPC procedures.

#### 4.1.2. Surgical procedures

The general procedure was the same as that in experiment 2, with the exception that for the hippocampal lesions the incisor bar of the Kopf frame was set at 0 mm to the horizontal plane and the temperature of tip of the radiofrequency probe was raised to 70 °C for 60 s at each lesion site. There were 17 sites bilaterally throughout the hippocampus (15 tract sites) for the HPC lesions. The co-ordinates were, relative to Bregma: $AP = 2.4, ML = 1.0, DV = 3.5$; $AP = 2.7, ML = 0.7, DV = 3.5$; $AP = 2.7, ML = 1.8, DV = 3.7$; $AP = 3.0, ML = 1.1, DV = 3.7$; $AP = 3.0, ML = 2.6, DV = 3.6$; $AP = 4.0, ML = 0.8, DV = 3.6$; $AP = 4.5, ML = 2.6, DV = 3.6$; $AP = 5.2, ML = 4.6, DV = 4.4$; $AP = 5.2, ML = 3.0, DV = 3.6$; $AP = 5.6, ML = 4.9, DV = 5.1$; $AP = 5.6, ML = 4.9, DV = 7.5$; $AP = 5.9, ML = 3.8, DV = 4.0$; $AP = 5.9, ML = 4.8, DV = 5.2$; $AP = 5.9, ML = 4.8, DV = 6.5$. The procedure for the fornix lesions was the same as that in experiment 2. The control surgical procedures were identical to those for actual FNX or HPC surgeries, with the exception that the electrode tip was lowered only 1.7 mm from the top of the cortex and the temperature of the tip of the electrode was not raised, but remained at body temperature. Five rats received sham FNX surgeries and six received sham HPC surgeries.

#### 4.1.3. Apparatus

**Conditioning chambers and T-maze.** These were unchanged from experiments 1 and 2, except for the addition of miniature CMOS black and white cameras (Maplin, UK) which were attached immediately outside the transparent sidewall of each conditioning chamber. These cameras were used to record the rats’ behaviour during the training sessions.

**Stimulus duration conditioning.** The procedure was essentially the same as that for experiments 1 and 2. All rats underwent 4 days of magazine training and then went on to stimulus duration conditioning. In this procedure only long tones (12.0 s and 3.0 s) were presented and only one interval-food combination was tested (CS+ 12.0 s, CS− 3.0 s). The trace period was now 20 s throughout conditioning and there was no additional food reward at the termination of the tones. The sessions were videotaped to examine whether there were behavioural differences during reinforced and non-reinforced trials and behaviour was scored by an observer blind to the group of each subject.

**T-maze alternation task.** The apparatus and procedure were same as experiment 2, however, there were only 3 days of habituation to the maze. Each of these habituation sessions lasted 20 min. Following habituation, the rats received six sessions (days) of training which consisted of six trials each. Only a subset of animals were tested on this task (SHAM, $n = 7$, randomly selected from 12).
4.1.4. Histology

The procedure was the same as in experiment 2.

4.2. Results

4.2.1. Histology

Fornix lesions. In five of the six FNX rats with full histology the lesions resulted in complete or near-complete (90–100%) bilateral transection of the fimbria/fornix. The remaining rat had lost approximately 90% of the fimbria/fornix in one hemisphere with approximately 70% of the tract lost on the other side. In two cases the corpus callosum was cut, with additional damage to restricted, adjacent parts of the ventral cingulate cortex as well as the medial aspect of the caudate–putamen (Fig. 6A). A seventh FNX rat died unexpectedly after completing all tests. This rat performed very poorly on T-maze alternation (mean 55% correct) despite appearing healthy throughout. This performance level is consistent with an extensive fornix lesion.

Hippocampal lesions. All hippocampal lesions resulted in substantial damage to the structure. Cell loss was most complete in the dorsal hippocampus, involving areas CA1, CA2, CA3, dentate gyrus and for most cases, the dorsal subiculum. There was consistent partial damage to the fimbria/fornix which, in some cases, was substantial. All rats had damage in the ventral hippocampus, but typically the lesions spared parts of the most posterior regions of the ventral hippocampus, which were, nevertheless, markedly shrunken. In half of the HPC cases restricted damage was present in the overlying cortex as a consequence of the lesion (Fig. 6B). The largest and smallest of the FNX and HPC lesions are shown in Fig. 6.

4.2.2. Behaviour

Stimulus duration discrimination. Unlike the previous two experiments, the duration of magazine entries was now measured at four time points during each trial, those being during the baseline period (5 s pre-tone), during the last 3 s of the tone presentation and during the first and second 10 s of the trace period. This more fine-grain analysis was in response to the null effect in experiment 2. For each time point, the data were analysed with a repeated measures analysis of variance (ANOVA) with Group FNX, HPC, SHAM as a between-subjects factor, Trial Type (CS+ vs. CS−) and training (blocks 1–8) as within
subjects factors. Once again there were additional analyses for just the final blocks, this time the final four blocks as opposed to the final three blocks, as the SHAM group showed evidence of learning from this stage of training. These additional data are presented as they provide the most sensitive test of whether either of the two lesion groups was impaired on the interval discrimination task.

(i) All eight blocks (24 sessions). Responding over the pre-CS period (Fig. 7) revealed no difference between upcoming CS+ or CS− trials. There was, however, a main effect of block, $F(7, 161) = 4.04, p < 0.001$, and a Group × Block interaction, $F(14, 161) = 2.93, p < 0.005$. This interaction arose because the pre-CS responding by the SHAM group decreased over training, $F(7, 17) = 3.641, p < 0.05$, but this was not found for the HPC or FNX groups.

Responding during the first 10 s of the trace period showed that the rats distinguished CS+ from CS− trials. There was also a significant Trial Type × Session interaction, $F(7, 161) = 12.89, p < 0.001$, reflecting the development of the discrimination. Tests for simple effects confirmed that SHAM- as well as FNX-group rats discriminated between CS+ and CS− trials, $F_s(1, 23) > 4.78, p < 0.05$, but again the performance of the HPC rats did not reach significance ($p = 0.066$). However, there was no Group × Trial Type interaction, $F < 1$, indicating that the development of the discrimination did not differ between the groups. The analysis also revealed a main effect of group, $F(2, 23) = 4.29, p < 0.05$, which reflected the higher overall levels of responding by the SHAM rats (irrespective of CS+ or CS− trials) and did not relate to the discrimination itself.

Responding during the second 10 s of the trace period, similarly, revealed an effect of Trial Type, $F(1, 23) = 18.87, p < 0.001$ (see Fig. 7). Again, there was a significant Trial Type × Session interaction, $F(7, 161) = 24.69, p < 0.001$, reflecting the development of the discrimination. Tests for simple effects confirmed that SHAM- as well as FNX-group rats distinguished between CS+ and CS− trials, $F_s(1, 23) > 4.78, p < 0.05$, but again the performance of the HPC rats did not reach significance ($p = 0.066$). However, there was no Group × Trial Type interaction, $F < 1$, nor Group × Trial Type × Session interaction, $F < 1$, indicating that the development of the discrimination did not differ between the groups. There was also a main effect of group, $F(2, 23) = 4.37, p < 0.05$, as overall responding by SHAM rats was greater than HPC and FNX rats.

Furthermore, there was evidence that rats could discriminate between the CS+ and CS− trials during the presentation of the tone (effect of Trial Type, $F(1, 23) = 22.55, p < 0.001$), due to greater responding during the last 3 s of the reinforced tone compared to responding during the 3 s non-reinforced tone. While this discrimination was found for SHAM- and
FNX-group rats (simple effects of Trial Type, \( s > 7.88, p_s < 0.05 \)) but not the HPC rats \((p = 0.15)\), there were no significant group effects.

(ii) Last four blocks (12 sessions): Analysis of data from the first 10 s of the trace period now showed that all three groups of rats distinguished the two different tone durations [effect of Trial Type, \( F(1, 23) = 30.0, p < 0.001 \)], simple effects analysis revealed that SHAM and HPC groups were discriminating the tone durations during this period, \( F(1, 23) = 27.9, p < 0.001; F(1, 23) = 7.01, p < 0.05, \) respectively] but that the difference in responding between the two tones for the fornix group did not quite reach significance \((p = 0.06)\). However, analysis of responding in the second 10 s of the trace period revealed an effect of Trial Type, \( F(1, 23) = 35.0, p < 0.001 \), and analysis of simple effects confirmed that all three groups were discriminating the tone durations, \( F_g(1, 23) > 6.65, p_g < 0.01 \), during this interval. There were no group differences regarding discrimination performance or Group \( \times \) Trial Type interactions (Fig. 8).

**T-maze alternation.** There was a highly significant group effect \( F(2, 18) = 48.7, \ p < 0.001 \), and post-hoc comparisons (Bonferroni) showed that the performance of SHAM–group rats (82% correct) was significantly better than that of the FNX \((p < 0.001)\) and HPC \((p < 0.001)\) groups (55% and 62% correct, respectively) across all sessions.

### 4.3. Summary

Although there was clear evidence from the histogram and from the very poor performance on the T-maze alternation task that the hippocampal system lesions had been effective, there was no evidence that the lesions had altered the ability to learn the interval duration discrimination (CS+ 12.0 s vs. CS− 3.0 s).

Thus, both the FNX- and HPC-lesioned rats exhibited significantly greater responding during reinforced trials compared to non-reinforced trials and their levels of discrimination did not differ from that of the SHAM controls. The pattern of discrimination appeared very similar to that seen in experiments 1 and 2, and discrimination performance in experiment 3 did not seem to suffer even though the trace interval \((20 \text{ s})\) was twice the length of that used in experiments 1 and 2. In fact, acquisition of the task in experiment 3 was considerably more rapid than that seen for the same condition in experiment 1. It is most likely that this advantage was a result of removing the reward provided at the end of every tone presentation. Finally, observation of the animals in the test boxes using video cameras failed to reveal any overt evidence of mediating behaviour to help solve the discrimination.

### 5. Discussion

Rats were trained using an appetitive Pavlovian conditioning procedure to test their ability to discriminate tones of different duration. In experiment 1, normal rats were able to discriminate tone lengths in two discriminations \((0.5 \text{ s vs. } 1.5 \text{ s}, \text{ and } 3.0 \text{ s vs. } 12.0 \text{ s})\), although there was a clear asymmetry as learning was much faster when the CS+ was longer than the CS− (i.e. when 1.5 s, and 12.0 s tones were the reinforced stimuli). Experiments 2 and 3 went on to examine the impact of lesions of the hippocampal system during task acquisition in view of the potential involvement of the hippocampus in assessing temporal duration [27,45]. While previous studies have largely focussed on the impact of fornix lesions [37,41,45], the present study also included hippocampal lesions.

Rats with fornix lesions (experiments 2 and 3) readily learnt to discriminate 0.5 s from 1.5 s tones, and 3.0 s from 12.0 s tones, and did not differ from SHAM controls. Likewise, rats with hippocampal lesions (experiment 3) learnt to discriminate tones of 3.0 s and 12.0 s duration and did not differ from the control rats. Even though the time between offset of the CS+ and food delivery (the trace interval) was increased from 10 s to 20 s, there was no evidence of a lesion effect. In contrast, both the fornix and the hippocampal lesions severely disrupted a spatial working memory task (T-maze), showing that the same lesions had been effective at disrupting other aspects of memory.

The first task is to consider why normal rats found it easier to show differential responding when the CS+ was the longer of the two stimuli (experiment 1). One reason concerns the inherent asymmetry of the situation for the animals [58]. The first 0.5 s of each Trial Type (CS+ and CS−) for Group Short, and the first 3.0 s of each Trial Type for Group Long is exactly the same. Consequently, the detection of the shorter duration can only occur at its offset (hence, during the trace period). In contrast, the detection of the longer duration can occur anytime after the short interval has ceased, and potentially before the longer interval has elapsed (not impinging on the trace period).

A closely related explanation for why discrimination learning was retarded when the longer stimulus was not reinforced \([1.5 \text{ s } \text{CS− (Group Short), } 12.0 \text{ s } \text{CS− (Group Long)}]\) is a phenomenon termed the ‘feature positive effect’. This effect refers
to the tendency in animals and humans to experience difficulty in processing non-occurrences [25]. When in experiment 1 the longer tones were non-reinforced (‘feature negative’), the CS— could, in fact, be regarded as consisting of consecutive blocks of the reinforced CS+. Using the example of Group Short, the 1.5 s CS— tone is built up of three consecutive blocks of 0.5 s CS+ tone. Thus, the positive feature is integral to the negative feature. However, in conditions in which the reverse combination of stimuli were presented (1.5 s CS+ vs. 0.5 s CS—), the positive feature (1.5 s) was not part of the negative feature (0.5 s) enabling it to be learnt more readily [2]. Although feature-positive and feature-negative discriminations can both be learned, it has previously been shown that feature-negative discriminations are inherently more difficult [20,24,25].

The main goal was to examine the effects of hippocampal system damage on interval timing. The lack of a deficit in the present study should be set in the context of repeated studies that have found impairments in interval timing following damage to the hippocampal system. One repeated finding is that fornix lesioned rats, and medial septum lesioned rats, respond as though time of reinforcement in the peak response task had occurred earlier than it did [37,41,42,45, but see 10]. Indeed, the hippocampus has been considered a prime candidate to provide the necessary memory component for comparisons within the ‘internal clock’ (pacemaker) component of interval timing [37,41,42], while the pacemaker is unaffected by hippocampal damage. The hippocampus also has direct connections with other regions, such as the striatum and prefrontal cortex, which are implicated in interval timing [5,21,32,36,38,42]. By these connections, reference memory for past intervals could be compared with the current interval (output from the pacemaker) for which the basal ganglia circuitry and dopaminergic neurotransmitters within striatal and/or cortical sites are believed to be involved [5,36,38]. In addition, hippocampal system damage also disrupts the ability of rats to remember a timed signal with a gap, as they appear to reset their clocks so only timing the signal after the gap and apparently forgetting that before the gap [41,42]. This resetting deficit is seen as a working memory deficit for the temporal problem [42].

In spite of much evidence concerning the hippocampus, there are reasons to question the importance of this structure for temporal processing. Evidence from peak response studies or gap intervals has received the criticism that such procedures contain ‘instructional ambiguity’ [57]. Thus, it has been argued that animals not only need to track time in this task but also discriminate what kind of trial is current. The task, therefore, becomes an conditional discrimination which is potentially problematic as hippocampal lesions can sometimes disrupt the learning of conditional rules [3,52,56]. While studies by Buhusi and Meck [4,5] suggest that these manipulations increase the loading on attentional and memory resources, rather than create ambiguity, it still remains the case that hippocampal system damage could affect performance indirectly.

This uncertainty prompted the present study which describes an alternative test of duration discrimination, then tests the sensitivity of the task to hippocampal system damage. With this goal in mind the durations were signalled by salient stimuli and the test conditions remained constant throughout (i.e. no extinction tests as occurs in probe trials). Furthermore, each session contained few trials but lengthy intertrial intervals (mean of 6 min). This procedure was designed to minimise ambiguity between intertrial intervals and the trace delay (of up to 20 s). The lack of a duration discrimination impairment after fornix lesions was consistent with an earlier study [41]. Likewise, the ability of the rats in the present study to perform the temporal discrimination task following hippocampal lesions complements a previous study using a conditional task which concluded that rats with hippocampal lesions can discriminate 8 s from 2 s [22]. In that conditional task, the rats with hippocampal lesions were, however, impaired in remembering the duration of exposure after a 10 s delay [22]. Other reasons to be uncertain about the importance of the hippocampus for duration judgements come from two studies that failed to find impairments using the peak response procedure after dorsal hippocampal lesions [10,11]. The conclusion is that hippocampal system lesions do not disrupt the measurement of duration, in accordance with the main findings of several other studies, rather they affect additional memory processes required to solve certain timing tests [22,40,41].

There are, however, some limitations with the present study. First, our procedure still has a potential working memory demand imposed by the fact that responding is often measured after the CS has terminated. Nevertheless, there was no evidence that this task was affected by the hippocampal lesions. Second, finer temporal discriminations might have been needed to reveal lesion-induced deficits, such as a shift forward in the point of subjective equality [41]. While this was beyond the goals of the present study, which sought to test the feasibility of this approach, it should be possible to use titration procedures in future studies. Third, while a duration discrimination task must tax timing, it may be solved by the relative duration of the signals rather than their absolute duration. A further issue concerned the used of trace conditioning.

An intrinsic element of the Pavlovian procedure adopted in this study was the use of trace conditioning, i.e. the imposition of a delay between the CS and the delivery or non-delivery of the food reward. The apparently normal ability of rats with hippocampal or fornix lesions to acquire this task, even with an extended trace period (20 s in experiment 3) seems to run counter to much evidence for the importance of the hippocampus for trace, but not delay, conditioning [1,8,33,34,35,57, but see 49]. This consensus raises the question of how our rats with effective hippocampal lesions were able to solve a task that depends on trace conditioning. One important point is that the very large majority of trace conditioning experiments use an aversive US+ (e.g. fear conditioning, air puff for eyeblink conditioning), sometimes combined with contextual cues as the CS. Very different conditioning arrangements were used in the present study, i.e. food as the US and discrete tones as the CS. With these differences in mind it is noteworthy that Jarrard and Davidson [23] also failed to find a trace conditioning impairment in rats with extensive hippocampal lesions trained to link auditory with visual stimuli for food reward.

While our data indicate that the hippocampus is not required for the discrimination of temporal duration [41], there is grow-
ing evidence of the involvement of the hippocampal system for tracking the temporal order of events [e.g., 13, 14, 28, 29, 31]. Although some studies have examined order information for spatial cues [30], and so might be prone to deficits from other causes, other studies have used non-spatial cues and have still reported order discrimination deficits after hippocampal system lesions [6, 15, 28]. This comparison between order information (impaired) and duration information (intact) is likely to prove informative in better understanding how tests of relative recency are solved.

References

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