Animals. Fifty-six adult male Long-Evans rats (302-406 g prior to surgery; Monash Animal Services, Gippsland, Victoria, Australia; Experiment 1) were used. Rats were housed four per cage in ventilated Plexiglass cages in a temperature regulated (22 ± 1­°C) and light regulated (12h light/dark cycle, lights on at 7:00 AM) colony room. At least one week prior to behavioural testing, feeding was restricted ensuring that weight was approximately 95%, and never dropped below 85%, of ad libitum feeding weight. All animal research was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratories Animals (NIH publications No. 80-23, revised 1996) and approved by the University of New South Wales Animal Care and Ethics Committee.

Apparatus. Behavioural testing was conducted in eight identical operant chambers (30.5 x 32.5 x 29.5 cm; Med Associates) individually housed within a ventilated sound attenuating cabinets. Each chamber was fitted with a 3-W house light that was centrally located at the top of the left-hand wall. Food pellets (45 mg dustless precision grain-based pellets; Bio-serv, Frenchtown, NJ, USA) could be delivered into a recessed magazine located at the bottom centre of the right hand wall. The top of the magazine contained a white LED light that could be used as a visual stimulus. Access to the magazine was measured by infrared detectors at the mouth of the recess. Two retractable levers were located on either side of the magazine on the right-hand wall. A speaker located to the right of the house light could provide auditory stimuli to the chamber. In addition, a 5-Hz train of clicks produced by a heavy-duty relay placed outside the chamber at the back right corner of the cabinet was used as an auditory stimulus. The chambers were wiped down with 80% v/v ethanol between each session. A computer equipped with Med-PC software (Med Associates Inc., St. Albans, VT, USA) was used to control the experimental procedures and record data.

Surgery

Bilateral guide cannulae were surgically implanted targeting the lateral OFC. Rats were anesthetized with isoflurane, their heads shaved, and placed in a stereotaxic frame (World Precision Instruments, Inc., Sarasota, FL, USA). The scalp was incised, and the skull exposed and adjusted to flat skull position. Two small holes were drilled for the cannulae using a high-speed drill, and four holes were hand drilled on different bone plates to hold fixing screws. Bilateral stainless steel guide cannulae (26 gauge, length 5mm below pedestal; Plastics One, Roanoke, VA, USA) were lowered into the lateral OFC (AP: +3.5 mm; ML: ±2.2 mm; D-V: -4.0 mm from bregma). Cannulae were held in place by dental cement and anchored to the skull with 4 fixing screws. Removable dummy cannulae were inserted into the guide cannulae to prevent them from blocking. After one week of postoperative recovery, rats were returned to food restriction for 2 days prior to further testing.

Drug Infusions

During infusions, muscimol or saline was infused bilaterally into the lateral OFC by inserting a 33 gauge internal cannula into the guide cannula which extended 1mm ventral to the guide tip. The internal cannula was connected to a 25 *μ*l glass syringe (Hamilton Company, Reno, NV, USA) attached to a microinfusion pump (World Precision Instruments, Inc., Sarasota, FL, USA). A total volume of 0.5 *μ*l was delivered to each side at a rate of 0.25 *μ*l/min. The internal cannula remained in place for an additional 1 min after the infusion and then removed. During the infusion procedure animals were allowed to move freely in a bucket to minimize stress. Dummy cannulae were removed prior to, and replaced immediately after, infusions. For the two training sessions prior to infusions, all animals received dummy infusions which were identical to the infusion procedure, but no liquids were infused. These dummy infusions were performed to familiarize the rats with the microinfusion procedure and thereby minimize stress.

Drugs

The GABAA agonist muscimol (Sigma-Aldrich, Switzerland) was dissolved in 0.9% (w/v) non-pyrogenic saline to obtain a final concentration of 0.5 *μ*g/0.5 *μ*l. Non-pyrogenic saline 0.9% (w/v) was used as the saline control.

**Histology**

Magazine training

Prior to training, all rats were familiarised with retrieving rewards from the magazine in a session of magazine training that lasted approximately 32 mins. Rewards, consisting of two pellets delivered 0.25s apart, were delivered randomly throughout the session every 120s until 32 pellets were delivered. The house light was kept illuminated throughout the session.

**General training parameters**

All sessions consisted of a number of trials in which a 10s auditory and/or visual cue (CSs) were presented. Visual cues designated as X and Y were flashing panel lights (0.1 s illuminated, 0.1s off) or extinguishing the house light (identity counter balanced). Visual cue Z was always a flashing magazine light (0.1 s illuminated, 0.1s off) for all animals. Auditory cues A and B were a 5 Hz train of clicks or a 78 dB white noise (identity counter balanced). In experiment 2, auditory cue C was always an 84 dB, 2.6 kHz tone. On rewarded trials (denoted by the symbol ‘+’) a single reward pellet was delivered upon CS termination. On non-reinforced trials (denoted by the symbol ‘-’), no reward was delivered. The variable inter-trial-interval was 90s (± 45s). Only a single training session occurred per day. All animals were handled in the infusion bucket for 5 minutes prior to each session and handled similarly regardless of whether drug infusions were administered. This was done to equate handling cues and stress on all training days.

**Experiment 1**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Table 4. Pavlovian conditioned inhibition experimental design in Experiment 1c. | | | | |
| Stage 1 | Stage 2 | Stage 3 | Summation Test | Retardation Test |
| A+ | A+ |  |  |  |
|  | AX- |  | BX- | X+ |
| B+ |  | B+ | B- | Y+ |
|  |  |  | B+ |  |
|  |  |  | Y- |  |
| Z+ | Z+ | Z+ | Z+ | Z- |
|  |  |  |  |  |
| The design of experiment 1c intended to establish cue X as a conditioned inhibitor. The key aspects of the procedure are highlighted above the dashed line and additional cues are presented below the dashed line. A and B are auditory cues (white noise and click), X and Y are visual cues (house light off and panel lights on), Z was always a flashing magazine light and the symbols "+" and "-" denote reward and non-reward respectively. Infusion of saline or muscimol occurred during stage 2. | | | | |

Training

Stage 1 Acquisition (Days 1-4)

During each acquisition session a total of 36 trials were presented consisting of 12 A+, 12 B+ and 12 Z+ trials. Each session lasted 60 mins. On days 3 and 4, all animals received dummy infusions immediately prior to the session.

Stage 2 Feature negative training- Infusion (Days 5-10)

During each feature negative training session all animals received a total of 36 trials consisting of 10 A+, 20 AX- and 6 Z+ trials. The non-rewarded AX- trials consisted of the simultaneous presentation of the audio-visual cues A and X. The A+/AX- feature negative discrimination was used to establish cue X as a conditioned inhibitor. Prior to each feature negative training session all animals received an infusion of either muscimol or saline targeting the OFC.

Stage 3 Cue Training (Days 11-12)

During cue retraining sessions a total of 36 trials were presented, consisting of 18 B+ and 18 Z+ trials. This retraining was done to ensure that responding to cue B was high prior to the summation test. No infusions were administered prior to each session.

Stage 4 Summation probe test (Day 13)

The summation probe test consisted of 27 trials (45 mins session length) in the following order: first 3 Z+ and 3 B+ trials (order: Z+, B+, B+, Z+, B+, Z+). This rewarded start ensured high responding to the critical cue B. Then 2 B- and 2 BX- trials (Order randomised) were presented, followed by a Z+ trial. This cycle of 7 trials (B-/BX-/Z+) was repeated 2 more times. The B-/BX- cues were probe trials to test whether cue X had acquired inhibitory properties that transferred to cue B. The single rewarded Z+ trials were used to maintain responding throughout the probe trials. Finally, all animals received 6 presentations of Y- at the end of the session. This pre-exposure to cue Y was done to minimise any external inhibition that may occur during the retardation test that followed.

Stage 5 Retardation test (Days 14-16)

The retardation test sessions contained 36 trials consisting of 12 X+, 12 Y+ and 12 Z- trials. This test shows whether the prior inhibitory training with cue X impairs subsequent excitatory acquisition relative to the novel cue Y. The non-rewarded cue Z was designed to prevent animals from responding non-discriminatively to all cues during this acquisition session.

Consumption test (Days 17-18)

Following the retardation test, all animals were given a consumption test to assess whether muscimol infusions into the OFC impaired the motivation or timing of the consumption of pellets, which may have interfered with performance during the Stage 2 feature negative training under infusions. On day 17, all animals were given a dummy infusion immediately prior to entering the test chamber. Prior to the sessions, 40 pellets were placed in the magazine. All animals were given 30 minutes in the chamber. Magazine behaviour was recorded during this session for analysis, but there were no programmed events throughout the session. On day 18 all animals were infused with muscimol or saline before being entered for a consumption test identical to that on day 17.

**Experiment 2**

Training

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Table 5. Pavlovian conditioned inhibition experimental design in Experiment 1d. | | | | |
| Stage 1 | Stage 2 | Test | Summation Test | Retardation Test |
| A+ | AX- | A- |  |  |
|  |  |  | BX- | X+ |
| B+ |  |  | B- | Y+ |
| C+ | C- | C- | B+ |  |
|  |  |  | Y- |  |
| Z+ |  |  | Z+ | Z- |
|  |  |  |  |  |
| The design of experiment 1c intended to establish cue X was a conditioned inhibitor. The main design is depicted above the dashed line whereas additional control cues are depicted below the dashed line. A and B are auditory cues (white noise and click), C was always a tone, X and Y are visual cues (house light off and panel lights on), Z was always a flashing magazine light and the symbols "+" and "-" denote reward and non-reward respectively. Infusion of saline or muscimol occurred during stage 2. | | | | |

Stage 1 Acquisition (Pre-surgery Days 1-6)

During acquisition sessions there were a total of 36 trials, consisting of 9 A+, 9 B+, 9 C+ and 9 Z+ trials. Animals were entered for 2 sessions per day for stage 1 training for a total of 12 sessions across 6 days.

On the days following the final stage 1 acquisition session, animals were returned to free feeding and surgery was performed. Immediately following post-operative recovery all animals were returned to food restriction 2 days prior to re-acquisition.

Stage 1 Re-Acquisition (Post-surgery Days 7-9)

Sessions were identical to pre-surgical Stage 1 acquisition, except that only a single session was administered per day. On the final two days all animals received dummy infusions immediately prior each session.

Stage 2 Feature negative training - Infusion (Days 10-13)

During the feature negative training, each session consisted of 36 trials such that there were 18 AX- and 18 C- trials. Infusions of saline or Muscimol were administered immediately to separate groups (matched on performance to all cues) prior to each of these sessions.

Stage 3 Extinction test (Day 14)

During the extinction test there were a total of 24 trials consisting of 12 A- and 12 C- trials.

Stage 4 Summation test (Day 15)

The summation test was identical to that used **in experiment 1.**

Stage 5 Retardation test (Days 16-18)

The retardation test was identical to that used **in experiment 1.**

Histological Analysis

Following completion of behavioural testing, rats received a lethal dose of sodium pentobarbital. The brains were removed, frozen, and sectioned coronally at 40 *μ*m through the lateral OFC with a cryostat. Every third section was collected on a slide and stained with cresyl violet. The location of cannula tips was determined under a microscope by a trained observer using the boundaries defined by the atlas of George Paxinos and Watson (1998). Rats with cannulae placements outside the lateral or dorsolateral OFC were excluded from statistical analyses.

Data Analysis

CS responding was operationalized as the number of magazine entries during the 10s CS. PreCS responding was operationalized as the frequency of responding during the 10s immediately preceding the 10s CS and was used as a measure of baseline responding to the testing context.

All data were analysed with mixed ANOVAs, and significant interactions of interest were followed up with ANOVAs on the relevant subset of data. Following significant omnibus ANOVA tests, planned linear and quadratic orthogonal trend contrasts and their interactions between groups were analysed to assess differences in rates of responding.