Chapter 2.
General methods

SUBJECTS AND HOUSING CONDITIONS

Subjects were male Lister hooded rats (Harlan-Olac UK Ltd) housed in a temperature-controlled room (minimum 22°C) under a 12:12 h reversed light–dark cycle (before March 2000, lights off 08:30 to 20:30; after March 2000, lights off 07:30 to 19:30). Subjects were approximately 15 weeks old on arrival at the laboratory and were given a minimum of a week to acclimatize, with free access to food, before experiments began. Experiments took place between 09:00 and 23:00, with individual subjects being tested at a consistent time of day. Unless otherwise stated, subjects were experimentally naïve, housed in pairs, provided with free access to water, and maintained throughout the experiment at 85–90% of their free-feeding mass using a restricted feeding regimen. Feeding occurred in the home cages at the end of the experimental day. In behavioural tasks where a significant amount of food was provided in the experimental chambers, the control software reported the amount of food delivered and this was used to correct the amount of food given in the home cages. All experimental procedures were subject to UK Home Office approval (Project Licences PPL 80/00684 and PPL 80/1324).

SURGERY

General surgical technique

Animals were anaesthetized with Avertin (2% w/v 2,2,2-tribromoethanol, 1% w/v 2-methylbutan-2-ol, also known as tertiary amyl alcohol, and 8% v/v ethanol in phosphate-buffered saline, sterilized by filtration, 10 ml/kg intraperitoneally)[2] and placed in a Kopf or Stoelting stereotaxic frame (David Kopf Instruments, Tujunga, California, USA; Stoelting Co., Wood Dale, Illinois, USA) fitted with atraumatic ear bars (Figure 11). The skull was exposed and a dental drill was used to remove the bone directly above the injection and cannulation sites. The dura mater was broken with the tip of a hypodermic needle, avoiding damage to underlying venous sinuses such as the superior sagittal sinus. Lesions and cannulation were accomplished according to the atlas of Paxinos & Watson (1996) using bregma as the origin and with the incisor bar set at 3.3 mm below the interaural line.

At the end of the operation, animals were given 15 ml/kg of sterile 5% w/v glucose, 0.9% w/v sodium chloride intraperitoneally. They were then left for 7 days to recover, with free access to food, and were handled regularly. Any instances of post-operative constipation were treated with liquid paraffin orally and rectally. At the end of this period, food restriction was resumed.

Excitotoxic lesions

Fibre-sparing excitotoxic lesions were made with quinolinic acid (Sigma, UK) dissolved in 0.1 M phosphate buffer (PB; composition 0.07 M Na_2HPO_4, 0.028 M NaH_2PO_4 in double-distilled water, sterilized by filtration) to a concentration of 0.09 M and adjusted with NaOH to a final pH of 7.2–7.4, or with 0.06 M ibotenic acid (Sigma, UK) in the same vehicle. Toxin was infused using one of two systems:

a) through a 28-gauge stainless steel cannula (∅ 0.36 mm external, 0.18 mm internal; model C313, Plastics One, Roanoke, Illinois, USA, via Semat Technical Ltd, St Albans, UK) attached via polyethylene tubing to a

[2] Concentrations given as percentages are calculated as follows. A 1% solution, volume per unit volume (v/v), is a solution in which $^1/_{100}$ of the total volume is solute. A 1% solution, weight by unit weight (w/w), is one in which 1% of the total weight of the solution is solute; thus, a 1% solution implies 1 g of solute dissolved in 99 g of solvent. A 1% solution, weight by unit volume (w/v), is a solution of 1 g in a total volume of 100 ml (10 g l^{-1}); '100%' denotes 1 kg l^{-1}. Similarly, the notation '1:1000' denotes 1 g l^{-1} (1 mg ml^{-1}).

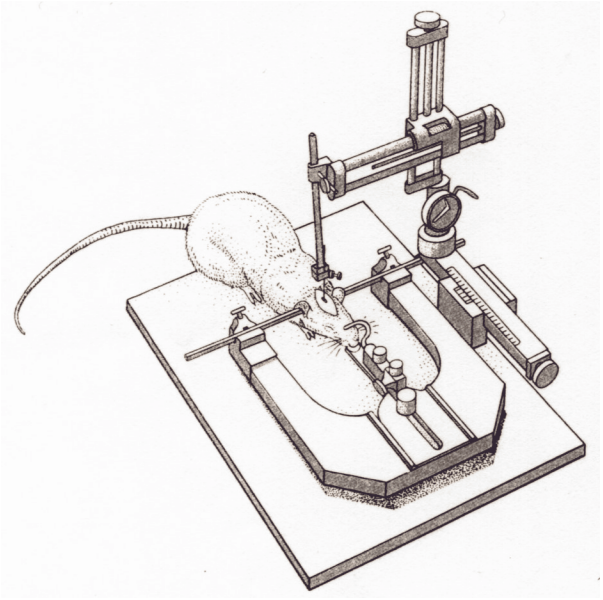


Figure 11. Stereotaxic frame, from Carlson (1991).

10-µl syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) mounted on a Harvard Apparatus (Edenbridge, UK) infusion pump;

b) through a micropipette, manufactured using a Stoelting (Illinois, USA) pipette puller (model 52500) from glass pipettes (∅ 1.2 mm external, 0.69 mm internal; Intracel Ltd, Royston, UK) to give a final tip diameter of 50–100 µm. The micropipette was glued to the tip of a 1-µl syringe (SGE Ltd, Milton Keynes, UK) using Araldite epoxy resin (Ciba, UK), and the syringe was hand-driven during infusions.

Infusion times are shown in Table 7. At each site the injector was left in place for a specified time following infusion (see table) to allow diffusion away from the injection site and so to minimize 'tracking' of the toxin along the path of the cannula. Sham lesions were made in the same manner except that vehicle was infused.

Table 7. Lesion coordinates. Dorsoventral coordinates are either from dura (D) or skull surface at bregma (SS). Along the anteroposterior (AP), mediolateral (ML), and dorsoventral (DV) axes, positive coordinates are in the anterior, left, and superior directions respectively.

Lesion	Toxin and delivery system	Sites per side	AP	ML	DV	Volume per site	Infusion time	Diffusion time
Anterior cingulate cortex (peri-genual lesion)	quinolinic acid, 0.09 M, via cannula	6	+1.2 +1.2 +0.5 +0.5 –0.2 –0.2	±0.5 ±0.5 ±0.5 ±0.5 ±0.5 ±0.5	–3.0 –2.2 –2.8 –2.0 –2.5 –2.0 (SS)	0.5 µl	1 min	1 min (lower sites); 2 min (upper sites)
Nucleus accumbens core	quinolinic acid, 0.09 M, via micropipette	1	+1.2	±1.8	–7.1 (SS)	0.5 µl	3 min	2 min
Nucleus accumbens shell	ibotenic acid, 0.06 M, via micropipette	3	+1.6 +1.6 +1.6	±1.1 ±1.1 ±1.1	–6.4 –6.9 –7.9 (SS)	0.1 µl 0.1 µl 0.2 µl	1 min 1 min 2 min	2 min 1 min 1 min
Medial prefrontal cortex	quinolinic acid, 0.09 M, via cannula	4	+3.8 +3.3 +3.3 +2.6	±0.5 ±0.5 ±0.5 ±0.5	–1.5 –3.0 –1.5 –1.5 (D)	0.5 µl	1 min	2 min

Implantation of bilateral intracranial cannulae

Holes were drilled in the skull as described above. Four stainless steel screws were placed on each side about the burr holes and a pair of 22-gauge, bevelled stainless steel guide cannulae (13.0 mm long, ∅ 0.7 mm external, ~0.4 mm internal; Coopers Needle Works, Birmingham, UK) were simultaneously lowered to the target position. Coordinates are given in Table 8, and a schematic is shown in Figure 12. With the cannulae held in place by inserters made of wire (∅ 0.36 mm) affixed to a steel frame, dental cement was applied around the cannulae and screws and allowed to dry. The inserters were then removed and the guide cannulae closed with stainless steel wire occluders (∅ 0.36 mm).

Table 8. Cannulae coordinates and intracerebral infusion parameters. First dorsoventral coordinate represents guide cannula tip location. Second coordinate represents injector tip location. Dorsoventral coordinates are from dura (D).

Cannulation	Drug	Sites per side	AP	ML	DV	Volume per site	Infusion time	Diffusion time
Nucleus accumbens	amphetamine sulphate, 0–20 µg	1	+1.6	±1.5	–5.0 –7.0 (D)	1 µl	1 min	2 min

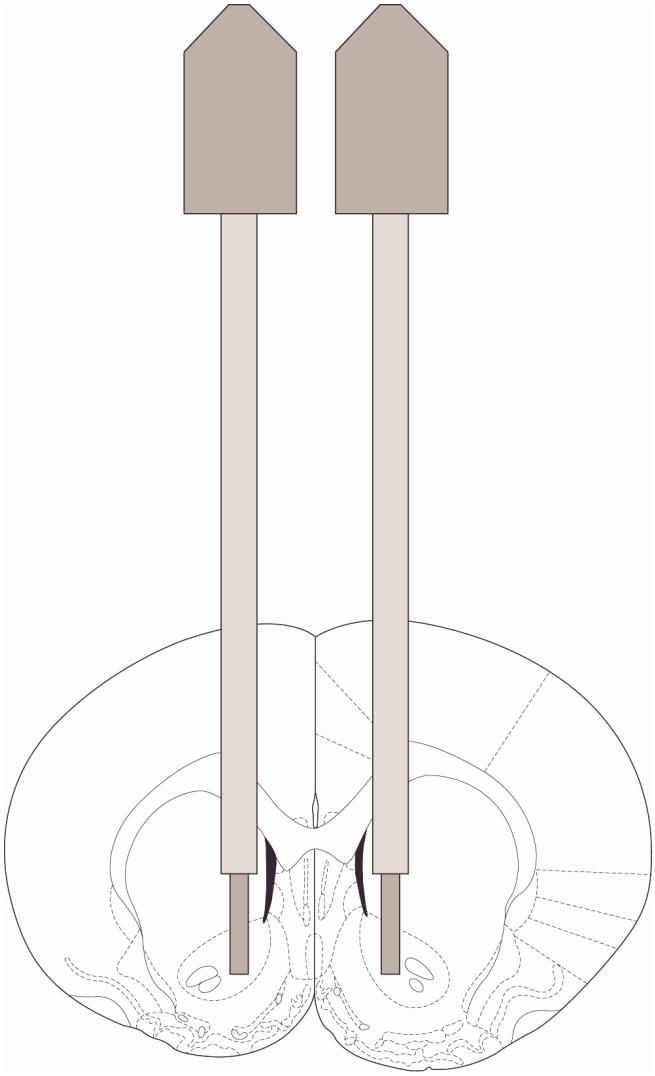


Figure 12. Intended location of guide cannulae (light grey) and injectors (dark grey), with the injector tip within the nucleus accumbens (1.6 mm anterior to bregma). The cannulae and injectors are drawn to scale, although the plastic injector mounting has been schematized. Brain section taken from Paxinos & Watson (1998).

HISTOLOGICAL ASSESSMENT

Perfusion and tissue fixation

At the end of experiments involving excitotoxic lesions or intracranial infusions, animals were deeply anaesthetized with Euthatal (pentobarbitone sodium, 200 mg/ml, minimum of 1.5 ml i.p.) and perfused transcardially with 0.01 M phosphate-buffered saline (PBS; composition 6.4 mM Na_2HPO_4, 1.47 mM NaH_2PO_4, 0.13 M NaCl, 2.68 mM KCl; the pK_a for this phosphate acid–base pair is 6.865, giving an approximate pH of 7.5) followed by 4% paraformaldehyde in PBS. Their brains were removed and postfixed in paraformaldehyde before being dehydrated in 20% sucrose for cryoprotection.

Nissl staining with cresyl violet

Cresyl violet stains for Nissl substance, the basophilic rough endoplasmic reticulum present in cytoplasm.

The brains were sectioned coronally at 60 μm thickness on a freezing microtome and every third section mounted on chrome alum (chromium potassium sulphate)/gelatin-coated glass microscope slides and allowed to dry. Sections were passed through a series of ethanol solutions of descending concentration (3 minutes in each of 100%, 95%, and 70% v/v ethanol in water) and stained for ~5 min with cresyl violet. The stain comprises 0.05% w/v aqueous cresyl violet (Raymond A. Lamb Ltd, Eastbourne, UK), 2 mM acetic acid, and 5 mM formic acid in water. Following staining, sections were rinsed in water and 70% ethanol before being differentiated in 95% ethanol. Finally, they were dehydrated and delipidated in 100% ethanol and Histoclear (National Diagnostics, UK) before being cover-slipped using DePeX mounting medium (BDH, UK) and allowed to dry.

The sections were used to verify cannula and lesion placement and assess the extent of lesion-induced neuronal loss. Lesions were detectable as the absence of visible neurons (cell bodies of the order of 100 μm in diameter with a characteristic shape), often associated with a degree of tissue collapse (sometimes with consequent ventricular expansion when the lesion was adjacent to a ventricle) and gliosis (visible as the presence of smaller, densely-staining cells).

Immunocytochemical staining for neuronal nuclei

Direct visualization of the location of neuronal nuclei was achieved using the NeuN antibody (Mullen *et al*., 1992).

The immunocytochemical procedure may be summarized as follows. A primary immunoglobulin-G (IgG) antibody is raised in a mammalian species against a specific target of interest. The tissue being investigated is incubated with the primary antibody, and then unbound primary antibody is washed off. Next, the sections are incubated in secondary antibody. The secondary antibody was raised in a second mammalian species against the constant (Fc) portion of the IgG molecule of the first species; it therefore binds to all IgG antibodies of the first species, including the primary antibody. The secondary antibody has biotin attached to it. To this is added a complex of avidin and biotinylated horseradish peroxidase (HRP). Avidin is a protein with multiple biotin binding sites; thus, it can 'bridge' the biotinylated secondary antibody to the biotinylated HRP, ultimately binding HRP to the primary antibody and to the target labelled by it. The location of HRP can be visualized because HRP is an enzyme that catalyses the oxidation by hydrogen peroxide of various substrates, including the chromagen diaminobenzidine (DAB); the DAB oxidation product is a visible, insoluble precipitate.

Following cutting at 40 μm, sections were washed in 0.01 M phosphate-buffered saline (PBS; pH 7.4) and placed into primary antibody (NeuN monoclonal mouse anti-neuronal nuclear protein; Chemicon International Ltd, Harrow, UK), and incubated overnight with gentle agitation. The primary antibody was made up at a concentration of 1:10,000 in 0.01 M PBS containing the non-ionic detergent 0.4% t-octylphenoxypolyethoxyethanol (Triton® X-100) to solubilize the protein. The next day, the sections were washed three times in 0.01 M PBS over 30 minutes and incubated in secondary antibody for 90 min on a rotary shaker; the secondary antibody was biotinylated rabbit anti-mouse IgG (Vectastain ABC Kit, Vector, Burlingame, CA) at 1:200 in 0.01 M PBS/0.4% Triton solution. The sections were washed again in 0.01 M PBS over 30 min, incubated with 1:200 avidin-biotin-horseradish peroxidase

complex (Vectastain ABC Kit, Vector, Burlingame, CA) in 0.01 M PBS for 1 h, and then washed again in 0.01 M PBS over 30 min. The immune conjugate was visualized by placing the sections in 0.05% 3,3´-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide in 0.01 M PBS. The visualization reaction was stopped by transferring the sections rapidly into cold PBS and washing thoroughly in 0.01 M PBS, after which the sections were mounted on gelatinized slides, dried, dehydrated through an ascending series of aqueous ethanol solutions (0%, 70%, 95%, 100% v / v), delipidated in Histoclear, and coverslipped with DePeX.

Neuronal loss was assessed as the absence of immunoreactive cells.

DEFINITION OF REINFORCEMENT SCHEDULES

The following reinforcement schedules are used or discussed in this thesis:

Continuous reinforcement (FR1). Every response is reinforced.

Extinction (ext). No response is reinforced.

Fixed ratio (FR). In an FR5 schedule, every fifth response is reinforced.

Variable ratio (VR). A ratio schedule in which the number of responses per reinforcer varies. If a single parameter is given (VR 5), the parameter is the mean number of responses required, with the probability distribution function (p.d.f.) unspecified.

Random ratio (RR). A random ratio schedule reinforces each response with a fixed probability. The parameter is the mean number of responses per reinforcement; thus an RR2 schedule is programmed as P(reinforcement | response) = 0.5.

Fixed time (FT). Reinforcement is delivered noncontingently at fixed, regular intervals.

Fixed interval (FI). After a successful response, reinforcement is not delivered until a certain time has elapsed. After this, the first response is reinforced.

Variable time (VT). A noncontingent schedule that delivers reinforcement after a certain time has elapsed since the previous reinforcement. If the interval is specified as a range (VT 10–30 s), a flat probability distribution of intervals is assumed (in this example, mean interval 20 s). If the interval is specified as a single number (VT 20 s) then this is the mean interval, with the p.d.f. unspecified.

Variable interval (VI). A contingent schedule; it is identical to a VT schedule, except that reinforcement is delivered for the first response following the interval.

Random time (RT). A form of VT schedule. Its objective is that the maximum interval between reinforcements is not specified (so that the probability of reinforcement does not increase to 1 at the end of the interval). A random time x schedule is typically programmed as P(reinforcement in each second) = $^1/_x$. The probability of reinforcement is therefore independent of the time since last reinforcement. The expected number of reinforcements follows a binomial distribution, i.e. $P(k \text{ reinforcements in } n \text{ seconds}) = C(n, k)p^k(1–p)^{n–k}$, where $C(n, k) = n! / \{k!(n – k)!\}$. The mean number of reinforcements in n seconds is np and therefore the mean inter-reinforcement time in seconds is $n / np = 1 / p$, the time for which the schedule is named. (The distribution of intervals follows the distribution function $P(\text{interval} = n) = (1 – p)^{n–1}p$; this distribution declines continuously so the mode is 1 s; for $p = ^1/_{30}$ the median is 21 s and the mean is 30 s.)

Random interval (RI). A random interval schedule sets up reinforcement on an RT schedule; the next response is then reinforced.

BEHAVIOURAL APPARATUS

Unless otherwise specified, behavioural testing was conducted in eight identical operant chambers (30 × 24 × 30 cm; Med Instruments Inc, Georgia, Vermont, USA; Modular Test Cage model ENV-007CT). The chamber layout is shown in Figure 13. Each chamber was fitted with a 2.8 W overhead house light and two retractable levers, 16 cm apart and 7 cm above the grid floor, with a 2.8 W stimulus light (∅ 2.5 cm) above each lever and one located centrally (all 15 cm above the floor). The levers measured 4.5 cm (W) × 1.5 cm (D) and required a force of approximately 0.3 N to operate. In between the two levers was an alcove fitted with a 2.8 W lightbulb (‘traylight’, replaced

in some experiments by a 60 mcd diffused green LED, RS Components Ltd, UK), an infrared photodiode, a dipper that delivered 0.04 ml when elevated through a hole in the magazine floor, and a tray into which could be delivered food pellets. The chambers were enclosed within sound-attenuating boxes fitted with fans to provide air circulation. The apparatus was controlled by software written by RNC in Arachnid (Paul Fray Ltd, Cambridge), a real-time extension to BBC BASIC V running on an Acorn Archimedes series computer.

DATA ANALYSIS

Data collected by the chamber control programs were imported into a relational database (Microsoft Access 97) for case selection and analysed with SPSS 8.01, using principles based on Howell (1997). Graphical output was provided by Microsoft Excel 97 and SigmaPlot 5.0. All graphs show group means and error bars are ±1 SEM unless otherwise stated.

Transformations. Skewed data, which violate the distribution requirement of analysis of variance, were subjected to appropriate transformations (Howell, 1997, §11.9). Count data (lever presses and locomotor activity counts), for which variance increases with the mean, were subjected to a square-root transformation. Homogeneity of variance was verified using Levene's test.

Analysis of variance. Behavioural data were subjected to analysis of variance (ANOVA) using a general linear model, using SPSS's Type III sum-of-squares method. Missing values were not estimated but excluded from analysis. All tests of significance were performed at $\alpha = .05$; full factorial models were used unless otherwise stated. ANOVA models are described using a form of Keppel's (1982) notation; that is, *dependent variable* $= A_2 \times (B_5 \times S)$ where A is a between-subjects factor with two levels and B is a within-subjects factor with five levels; S denotes subjects.

For repeated measures analyses, Mauchly's test of sphericity of the covariance matrix was applied and the degrees of freedom corrected to more conservative values using the Huynh-Feldt epsilon $\tilde{\varepsilon}$ (Huynh & Feldt, 1970) for any terms involving factors in which the sphericity assumption was violated. Thus, the same analysis with and without sphericity correction would be reported as follows:

$$\text{Uncorrected: } F_{10,160} = 2.047,\ p = .032$$
$$\text{Corrected: } F_{4.83,77.3} = 2.047,\ \tilde{\varepsilon} = 0.483,\ p = .084$$

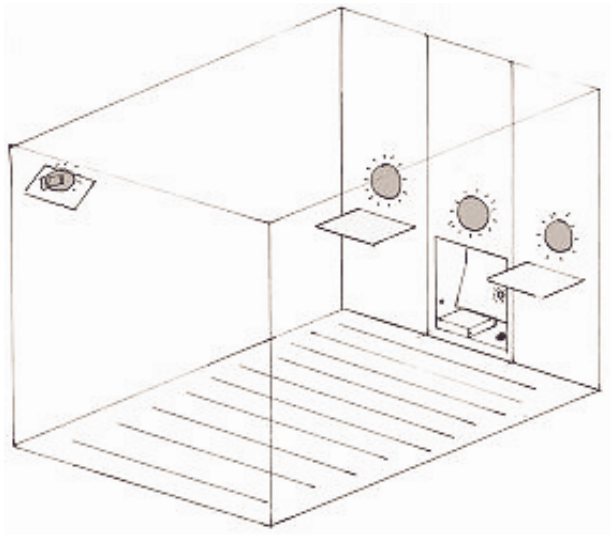
Post-hoc tests. Significant main effects of interest were investigated using pairwise comparisons with a Sidak correction. This is based on the observation that $\alpha_{\text{familywise}} = 1 - (1 - \alpha_{\text{each test}})^n$ when n tests are performed; the correction was made such that $\alpha_{\text{familywise}} = .05$.

Where main effects were found for between-subjects factors with three or more levels, post hoc comparisons were performed with the REGWQ range test (familywise $\alpha = 0.05$), or Dunnett's test in situations where several experimental treatments were compared with a single control group. These tests do not require the overall F for groups to be significant as they control the familywise error rate independently and test different hypotheses from the overall ANOVA, with different power (Howell, 1997, p. 351).

Where significant interactions were found following factorial analysis of variance, simple effects of a priori interest were calculated by one-way ANOVA and tested by hand against the pooled error term ($F = \text{MS}_{\text{factor}} / \text{MS}_{\text{pooled error}}$; critical values of F based on df_{factor} and $df_{\text{pooled error}}$). Multiple comparisons for simple effects were performed as described above but using the pooled error term.

Where significant interactions were found following repeated measures analysis, a pooled error term was used to test between-subjects simple effects of *a priori* interest, but separate error terms (i.e. plain one-way ANOVA) were used for within-subjects factors as sphericity corrections are inadequate if a pooled error term is used (Howell, 1997, p. 468).

A



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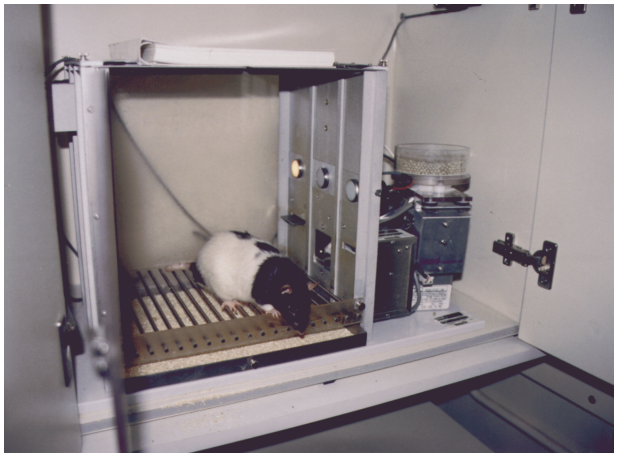

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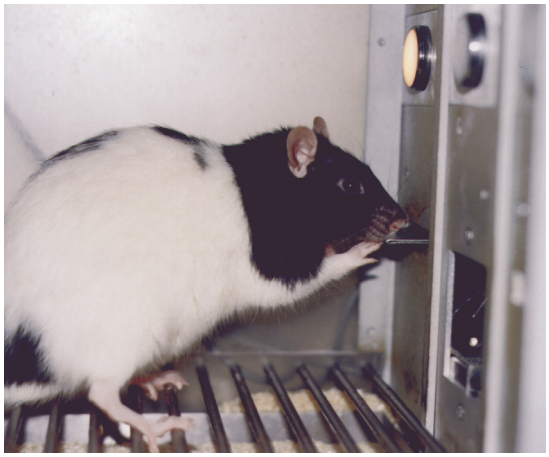

Figure 13. A: Sketch of the operant chambers used in several tasks. **B:** Photograph of such an operant chamber. **C:** Close-up of a rat in the chamber.