Benzodiazepine impairment of perirhinal cortical plasticity and recognition memory

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Abstract
Benzodiazepines, including lorazepam, are widely used in human medicine as anxiolytics or sedatives, and at higher doses can produce amnesia. Here we demonstrate that in rats lorazepam impairs both recognition memory and synaptic plastic processes (long-term depression and long-term potentiation). Both impairments are produced by actions in perirhinal cortex. The findings thus establish a mechanism by which benzodiazepines impair recognition memory. The findings also strengthen the hypotheses that the familiarity discrimination component of recognition memory is dependent on reductions in perirhinal neuronal responses when stimuli are repeated and that these response reductions are due to a plastic mechanism also used in long-term depression.

Introduction
Benzodiazepines are very widely prescribed human medicaments because of their anxiolytic and sedative actions; however, at higher doses they can also cause amnesia (Curran, 1991; Lister, 1995; Buffett-Jerrott & Stewart, 2002). Notably the benzodiazepine lorazepam produces a temporary anterograde amnesia in humans that has a number of similarities to the permanent anterograde amnesia that follows medial temporal lobe lesions. These similarities include a loss of recognition memory (Brown et al., 1982; Brown & Brown, 1990; Buffett-Jerrott & Stewart, 2002; Huron et al., 2002). While rodent studies have shown that lorazepam impairs the performance of a variety of memory tasks, e.g. delayed matching to position, nonspatial reference memory in the cued radial arm maze, habituation to a new environment, and passive avoidance (Jensen et al., 1979; Venault et al., 1986; Cole & Hillmann, 1994; Steckler et al., 1998; Olaman & McNaughton, 2001), there are no full reports of the effects of lorazepam or other benzodiazepines on recognition memory in rats. Benzodiazepines have well established actions upon GABA_A receptors (Izquierdo & Medina, 1991; Greenblatt, 1992; Sigel & Buhr, 1997; Rudolph et al., 1999) and, correspondingly, a tendency to hyperpolarize cell membranes. Hence it is to be expected that they would affect the generation of synaptic plasticity where this is driven by depolarization-dependent calcium entry. Accordingly, by so influencing plastic changes, benzodiazepines might be expected to affect the ability to form new memories. However, though there are reports of benzodiazepine interference with long-term potentiation (LTP) (Del Cerro, Jung & Lynch, 1992; Evans & ViolaMcCabe, 1996; Mori et al., 2001), there is no single study closely linking the actions of benzodiazepines to both plasticity and memory mechanisms. We here seek parallel benzodiazepine effects on recognition memory and perirhinal cortical plasticity.

Mounting evidence indicates that the familiarity discrimination component of recognition memory depends on the reductions in the responses of neurons of the perirhinal cortex of the temporal lobe that occur when a visual stimulus is re-encountered (Brown et al., 1987; Desimone, 1996; Ringo, 1996; Brown & Xiang, 1998; Murray & Bussey, 1999; Aggleton, 2001; Brown & Bashir, 2002). In particular, ablation of perirhinal cortex in rats and monkeys produces severe impairments in visual recognition memory tasks that depend on familiarity discrimination for their solution (Gaffan & Murray, 1992; Mumby & Pinel, 1994; Aggleton et al., 1997; Murray & Bussey, 1999; Buffalo et al., 2000; Brown & Aggleton, 2001). Furthermore, differential neuronal responses on stimulus repetition are the only possible neuronal substrate identified within perirhinal cortex that could subserve general, long-term familiarity discrimination (Brown & Xiang, 1998; Xiang & Brown, 1998; Brown & Aggleton, 2001; Brown & Bashir, 2002). Additionally, there is evidence that these response changes are first generated in perirhinal and/or adjacent visual association cortex (Brown & Xiang, 1998; Xiang & Brown, 1998; Brown & Aggleton, 2001; Brown & Bashir, 2002). The change in neuronal activation for familiar compared to novel stimuli is so large that it can be imaged in rats using immunohistochemistry for the protein products (Fos) of the immediate early gene c-fos (Zhu et al., 1995, 1996; Wan et al., 1999). Further, theoretical modelling (Bogacz & Brown, 2003) indicates that familiarity discrimination could be performed by networks relying on synapse-specific plasticity involving mechanisms such as those underlying LTP or long-term depression (LTD).

Here we test the hypothesis that benzodiazepine (lorazepam) administration impairs recognition memory as well as perirhinal plasticity. Additionally, we test the hypotheses that differential perirhinal neuronal responses to novel and familiar stimuli underlie familiarity discrimination and that these differential responses rely upon a synaptic plastic mechanism used in LTP and/or LTD. Thus we seek the effects of lorazepam upon familiarity discrimination behaviour, perirhinal neuronal activity changes produced by novel compared to familiar stimuli (as measured by Fos expression), and plasticity (LTP and LTD) induced in perirhinal slices.

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Materials and methods
Male, pigmented rats (DA strain, Bantin and Kingman, Hull, UK, weighing 150–250 g at the start of the experiment) were used in all experiments. The animals were housed under a 12-h light : 12-h dark cycle (light phase 18:00–6:00 h). Behavioural training and testing were conducted during the dark phase of the cycle. All animal procedures were performed in accordance with United Kingdom Animals Scientific Procedures Act (1986) and associated guidelines and approved by the University of Bristol Animal Affairs Ethical Committee. All efforts were made to minimize the number of animals used.

All statistical tests were two-tailed and used a significance level of 0.05.

Experiment 1: object recognition
Systemic injection
Subjects and apparatus
Two groups of 15 and of 12 rats were used. Exploration occurred in an open-topped arena (H × W × D = 150 × 90 × 100 cm) made of wood. The walls of the arena were covered with a black cloth so that no external stimuli could be seen during the experiment, and the floor was covered with sawdust. An overhead camera and a video recorder were used to monitor and record the animal’s behaviour for subsequent analysis. The stimuli presented were triplicate copies of objects made of glass or plastic that varied in shape, colour and size (9 × 8.5 cm to 25 × 10 × 5 cm), and were too heavy for the animal to displace.

Training
After being handled for a week, the animals were habituated to the arena without stimuli for 10–15 min daily for 2 days prior to the commencement of the spontaneous recognition procedure. The procedure itself comprised two phases separated by a delay: an acquisition or sample phase followed by a test phase. In the sample phase, duplicate copies (A1 and A2) of an object were placed near the two corners at both ends of one side of the arena (10 cm from each adjacent wall); see Fig. 1a. The animal was placed into the arena facing the centre of the opposite wall and then allowed a total of either 25 s of exploration of A1 and A2, or 4 min in the arena. Exploratory behaviour was defined as the animal directing its nose towards the object at a distance of <2 cm. Any other behaviour such as looking around while sitting on or resting against the object was not considered as exploration.

Two studies were run. In the first, using a group of 15 rats, the delay between the test phase and the sample phase was 20 min. In the second, using a group of 12 rats, the sample phase was repeated at 4, 24 and 28 h, allowing a maximum of 100 s exploration of the object. Between the sample phases, the animal was moved back to its home cage. The test phase then followed 20 h after the last sample phase (i.e. 48 h after the first sample period). In the test phase, the animal was replaced in the arena, presented with two objects in the same positions: one object (A3) was the third copy of the triplicate set of the objects used in the sample phase and the other was a novel object (B3); see Fig. 1a. The positions of the objects in the test phase and the objects used as novel or familiar were counterbalanced between the animals in a group and between the drug-treated or control groups. Lorazepam (0.25 mg/kg; Wyeth laboratories, UK) or equivalent volume of vehicle (normal saline and propylene glycol) was administered by i.p. injection, with the experimenter being blind to whether the injection was of drug or of vehicle.

Two designs were used in the second study with a 20 h delay. (i) To examine if lorazepam is involved in the acquisition stage of memory, the injection was given 30 min prior to the commencement of the each sample phase. After another 5 days, an injection of the other substance (vehicle or lorazepam in a cross-over design) was given and the animal was tested again. (ii) To study if lorazepam is involved in retrieval or consolidation, but not in acquisition, the injection was given after acquisition, 30 min before the test phase. Again, testing of retrieval following injection of the other substance was 5 days later. The same 12 rats were used in both designs with a period of >2 weeks between the two. As no impairment was found for acquisition at 20 min delay in the first study, an effect on consolidation and retrieval was not sought.

Localized infusion into perirhinal cortex
Cannulation surgery
Each of 8 rats was anaesthetized with isoflurane (induction 4%, maintenance 2–3%). The rat was secured in a stereotaxic frame with the incisor bar set at 3.3 mm below the interaural line. Two stainless steel guide cannulae (26 gauge, Plastics One, Semat, UK) were implanted through burr holes in the skull at an angle of 20° to the vertical, using the coordinates: AP −5.6 mm, L ± 4.47 mm (relative to bregma), V −6.7 mm (relative to surface of the skull). The guide cannulae were anchored to the skull by two stainless steel skull screws (Plastics One, Semat, UK) and dental cement. Between infusions cannulae were closed by dummy cannula. Following surgery, the animal was given fluid replacement therapy (5 mL saline, s.c.) and analgesia (0.05 mL Temgesic, i.m.), and was housed individually. It was allowed to recover for at least 10 days before habituation to the testing arena began.

Histology
At the end of the experiment, each rat was anaesthetized with Dothetal and perfused transcardially with phosphate buffered saline followed by 4% paraformaldehyde. Its brain was postfixed in paraformaldehyde for a minimum of 2 h before being transferred to 30% sucrose in 0.2 M phosphate buffer and left overnight. Coronal sections were cut at 50 µm on a cryostat and stained with cresyl violet. Cannulae locations were checked against standardized sections of the rat brain (Swanson, 1998).

Drug delivery
Lorazepam dissolved in propylene glycol was diluted with an equal volume of sterile 0.9% saline solution. Control animals received 0.9% saline. Propylene glycol was not added to the saline for this study as it had had no effect on memory when administration was systemic nor on plasticity when added to the bathing medium in the in vitro experiments. Injections were made into the perirhinal cortex through a 33 gauge infusion cannula (Plastics One, Semat, UK) attached to a 5 µL Hamilton syringe via a length of polyethylene tubing. A volume of 1.0 µL (equivalent to 2 µg) was injected into each hemisphere over a 2-min period, using an infusion pump (Harvard). The infusion cannula was then left in place for a further 5 min. Object recognition testing began 15 min after the start of drug infusion and followed the procedures detailed above. The delay between the sample and test periods was 20 min. As for the systemic injections, half the animals were given saline infusions followed a week later by lorazepam, while the other half received lorazepam followed 1 week later by saline.
Fig. 1. Measuring the effects of lorazepam on recognition memory. (a) Preferential exploration task. In the sample phase of this task the animal is allowed to explore two copies (A1 and A2) of the same object. After a delay, in the test phase, the time the animal spends exploring a novel object (B1) is compared to the time spent exploring a third copy (A3) of the previously explored object. (b) Effect of systemic lorazepam on preferential exploration. The histogram bars indicate the relative time (DR) spent exploring the novel compared to the previously seen object when lorazepam or vehicle (control) was administered before the sample phase, or after the sampling phase but before the test phase. The sample-test delay was 20 h. Lorazepam was without significant effect when administered after sampling but before the test period. However, when the drug was given before sampling, it significantly (*P < 0.025) reduced discrimination between the novel and previously seen object compared to controls. Thus the effect of lorazepam was on acquisition rather than consolidation or retrieval. (c) Histological localization of implanted cannula. The position of the implanted cannula allowing localized drug infusion into perirhinal cortex is shown opposite an atlas (Swanson, 1998) diagram of the corresponding brain section. (d) Effect of localized infusion of lorazepam on preferential exploration. The DIs indicate significant (*P < 0.05) impairment in the discrimination of a novel from a familiar object in the lorazepam compared to the saline group. The saline (**P < 0.01) but not the lorazepam group discriminated significantly. The sample to test delay was 20 min.
Statistical analysis

All measures of exploration were made with the experimenter blind as to the treatment. The discrimination index (DR) was calculated as the difference in time spent by each animal exploring the novel compared to the familiar object divided by the total time spent exploring both objects. For the systemic injections only the first 2 min of each 3-min test session were analysed as this period has been shown to be the most sensitive measure of discrimination (Dix & Aggleton, 1999). Comparisons between the vehicle and drug-treated groups used a within-subject analysis of variance (ANOVA) or paired t-tests.

Experiment 2: immunohistochemical staining for Fos

Subjects, apparatus and stimuli

Six rats were used in the lorazepam group and seven in the drug-free control group. Each animal was trained in an open-top Perspex viewing chamber (45 × 30 × 35 cm) which was placed in a larger, dimly lit (1 lux) matt black metal box (80 × 90 × 100 cm). Additional infrared illumination (15 W bulb, with Kodak Beehive Safelamp filter, Wratten series 2, Kodak Ltd, London, UK) allowed continuous observation of the animal’s behaviour by means of a TV camera (VISTA, NCD 360, Japan). All the walls inside the chamber were black except the front which was of translucent perspex. An observing hole (6 cm above the base and 3 cm in diameter) in the front wall permitted the animal just to reach and lick a metal tube that delivered drops of diluted blackcurrant juice. The tube was located outside the chamber, 7 cm from the hole. Two one-way mirrors (20 × 18 cm, forming the front of the object boxes) were symmetrically placed 25 cm from the hole on each side. Two objects, one behind each mirror, were presented simultaneously so that one object was within the monocular visual field of the right eye while the other within that of the left eye (Fig. 2a). Each object was 30 cm from and 10° above the animal’s eye when the animal’s head was positioned in the hole. A black partition ensured that the animal’s left eye could not see the right object and its right eye could not see the left object (Zhu et al., 1996). The sides and the back of the object boxes were covered with black cloth and objects within them could be illuminated by small light bulbs concealed from the animal’s view. When illuminated, the objects appeared against a black background. The illumination was 88 lux where the objects were displayed and 22 lux where the animal was. Objects became visible to the animal when, and only when, the lights were turned on in the compartments behind the mirrors.

Fig. 2. Measuring the effect of lorazepam on neuronal activation. (a) Paired viewing procedure. In this procedure, while a rat licks for juice with its head in an observing hole, a novel and a previously seen object are simultaneously displayed such that each of the rat’s eyes can see only one of the objects. The objects are placed behind one-way mirrors and become visible to the rat only when lights are turned on in the compartments behind the mirrors. (b) Brain areas sampled for Fos. The areas sampled are shown on frontal sections at the indicated distances behind Bregma (Swanson, 1998). EC, entorhinal cortex; HPC, hippocampus; OCC, occipital visual cortex; PRH, perirhinal cortex; SC, superior colliculus; TE, temporal visual association cortex area Te2. (c) and (d) Fos counts for control (c) and lorazepam-treated (d) groups. The normalized counts of Fos stained nuclei are shown for the indicated areas. For the control animals significantly greater activation was produced by novel than familiar objects in areas TE and PRH (*P < 0.05), but no area showed a significant difference for the lorazepam group.

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were turned on. The visual stimuli were 240 (in 8 sets of 30) different 3-D objects (toys, junk items, etc.) varying in size (from 3 × 3 × 3 cm to 6 × 6 × 6 cm), and in shape, colour and material. Two sets (F1 and F2) were chosen as familiar objects while the other 6 sets (N1-N6) were of novel objects.

**Behavioural procedure**

The behavioural procedure comprised pretraining, training and testing stages. During these stages the animals were allowed *ad libitum* access to water for 2 h at the end of each training day. Each animal was pretrained for 2 d inside the viewing chamber, without presentation of objects, to poke its head through the observing hole. When the animal’s head was positioned inside the hole, an infrared beam was interrupted, signalling the computer (Viglen Pentium PC, 486DX2, UK) to start a trial by switching on the light that illuminated the inside of the object box for 2 s. When the lights went out, a drop of diluted blackcurrant juice was delivered. The detection of the presence of the head, the onset and offset of lights and juice delivery were all controlled by the computer. The animal’s behaviour was video-monitored and recorded for off-line analysis. During the subsequent training, which lasted 6 days, there were two morning sessions and one afternoon session each day. The second morning session followed the first session without a break; the afternoon session started 3 h after the end of the morning sessions. In each session, two sets of 30 objects were presented inside the object boxes, one set in each box, one object from each set being presented simultaneously on each of the 30 trials. In the morning sessions the two sets that were to be presented repeatedly, R1 and R2, were presented twice, R1 to one side and R2 to the other side. In each afternoon session, one eye was presented with one of the 6 sets of novel objects while the other eye was shown set R1. A different novel set was used each afternoon to familiarize the animal with seeing novel and previously seen stimuli simultaneously. The different sets of novel and repeatedly seen stimuli were presented so that by the end of the experiment, each eye had seen the same number of novel and repeated stimuli, and both eyes had seen set R1 the same number of times. The last novel set (N6) was shown with R1 on the last afternoon, 1.5 h before the animal was anaesthetized deeply with pentobarbitone and perfused with 0.1 M phosphate buffer followed by 4% paraformaldehyde (pH 7.4). The set of objects used as N6 for one animal became set R1 for the next, so that stimulus materials were counterbalanced across animals. Further, the eye (left or right) that viewed the novel stimuli in the final test was also counterbalanced across animals.

**Drug treatment**

Each animal treated with lorazepam (0.25 mg/kg i.m., Wyeth laboratories, UK) was injected twice per day on the first five training days, 20 min before the training sessions in the morning and afternoon. On the final day, the injection was given only in the morning, so that no injection was given before the final test session. The time span from the drug injection in the morning to the final test in the test in the afternoon was 3.5 h, by which time the brain concentration of lorazepam would be extremely low (Lister et al., 1983). Lorazepam administration produced no observable gross behavioural changes.

**Tissue processing procedure**

After perfusion the brain was removed and placed for 12 h in 4% paraformaldehyde followed by 24 h in 30% sucrose. The immunohistochemical and counting procedures followed those of Zhu et al. (1995). Briefly, coronal sections (25 μm) were cut on a cryostat, and floating sections processed using a primary antibody and the avidin-biotin-complex (ABC, Vector Laboratory, UK) method. The primary antibody, kindly provided by Dr D. Hancock (Biochemistry of the Cell Nucleus Laboratory, Imperial Cancer Research Institute, London, UK), was a rabbit polyclonal directed against the N-terminal region of rat c-fos peptide and is c-fos specific (Zhu et al., 1995). The secondary antibody was biotinylated goat antirabbit (Vector Laboratory Inc., California, US). DAB was employed for visualization of Fos immunoreactivity and the automated counting of stained nuclei was carried out using an image analysis system (SeeScan Ltd, Cambridge, UK). Sections were visualized using a Zeiss Axioskop light microscope and images (512 × 512 pixels) were displayed on the computer screen at an effective magnification of 200 times and captured at a 256 grey level resolution. Sections were rejected if staining was not uniform on both sides and artefact-free. A nucleus was counted if one or more pixels was > 30 grey levels darker than the darkest pixel surrounding the image of the nucleus. This threshold was chosen because it gave automated counts that corresponded closely to those obtained in pilot manual counts and was that used in previous studies (Zhu et al., 1995, 1996; Wan et al., 1999). Counts above threshold were obtained from the right and left hemispheres for rectangular areas (0.94 × 0.67 mm) from two sections for each brain region. The rectangular area was such that all layers of the cerebral cortex were counted. All tissue processing and counting was performed with the experimenter blind as to which eye had seen the novel set.

**Statistical analysis**

Each count of Fos stained neurons was first normalized by dividing it by the corresponding mean for the particular area averaged across both hemispheres for a particular animal. This removed the difference in mean absolute counts between the areas and between the lorazepam-treated and control rats (Table 1). The normalized counts were then subjected to an analysis of variance with factors: rat, area, drug-treatment and novelty/familiarity of stimuli.

**Experiment 3: synaptic plasticity in perirhinal slices**

**Subjects and tissue**

Results reported in this study were obtained from slices of perirhinal and adjacent cortices (entorhinal cortex and association visual cortex, area Te2) prepared from 21 rats, as described previously (Ziakopoulos et al., 1999); see also Fig. 3. Animals were anaesthetized with halothane and the brain was removed rapidly and placed in ice-cold artificial cerebrospinal fluid (aCSF; bubbled with 95% O₂ with 5% CO₂) which comprised (mM): NaCl, 124; KCl, 3; NaHCO₃, 26;
NaH₂PO₄, 1.25; CaCl₂, 2; MgSO₄, 1; d-glucose, 10. A mid-sagittal cut was made through the brain, the rostral and caudal parts were removed by cutting at ~45° to the dorsoventral axis (see Fig. 3) and the remaining tissue mounted onto a vibroslice (Campden instruments, Sileby, UK). Slices (400 μm) were submerged in aCSF at room temperature (20–25 °C). A single slice was placed in a submerged recording chamber (28–30 °C, flow rate ~2 mL/min).

**Electrophysiological techniques**

For LTP experiments, standard extracellular recording techniques (with 4 m NaCl-filled glass electrodes) were used to obtain field potentials from the intermediate part (~ layer II/III) of the perirhinal cortex (directly beneath the rhinal fissure). Field potentials were recorded using an Axopatch 2D amplifier (Axon Instruments, Foster City, CA). Pipette (4–7 MΩ) solutions (280 mosm, pH 7.2) comprised in (mM): CsMeSO₄, 130; NaCl, 8; Mg-ATP, 4; Na-GTP, 0.3; EGTA, 0.5; HEPES 10; QX-314, 6. Neurons recorded in layer II/III were voltage clamped at ~70 mV. To induce homosynaptic LTD, low frequency stimulation (LFS: 200 stimuli, 1 Hz) was delivered to one input only. Lorazepam (1 μM; when used) and picrotoxin (5 μM) were perfused for the duration of the experiment. The amplitude of the evoked excitatory postsynaptic currents (EPSCs) was measured and expressed relative to the normalized preconditioning baseline. Effects of LFS were measured at appropriate time points (averaged over a 5-min period) after delivering LFS. LTD was defined as a decrease in the EPSCs 20–25 min after LFS.

**Statistical analysis**

For any particular experimental protocol, data was collected from only one slice or one neuron per animal. No consistent differences were established between responses evoked by stimulating to the entorhinal or temporal side of the rhinal sulcus and therefore their data have been pooled. Data were analysed using the LTP program (Anderson & Collingridge, 2001). Data are expressed as the mean ± SEM. Significance for within slice measures was tested using paired t-tests and for between slice measures using independent t-tests, or analysis of variance with repeated measures, as appropriate.

**Results**

**Experiment 1: familiarity discrimination behaviour**

Recognition memory (familiarity discrimination) was measured by comparing the time during a test phase that a rat spent exploring a novel object compared to an object encountered previously during a sampling phase.

**Systemic administration of lorazepam**

**Preferential exploration**

Lorazepam administered 30 min prior to the sample (acquisition) phase produced a significant impairment in familiarity discrimination when the delay from sampling to test was 20 h. Under these conditions, the relative time spent exploring a novel compared to a previously encountered object during the test phase was significantly impaired when the animals were given lorazepam rather than vehicle (discrimination index, DR; n = 12 rats; ANOVA, F₁,₁₁ = 7.75, P < 0.025); see Fig. 1a and b. The mean times spent with the novel and previously seen objects were 22.3 ± 2.0 and 10.6 ± 1.1 s, respectively, without lorazepam, and 17.6 ± 1.6 and 13.3 ± 0.9 s with lorazepam; the rats discriminated (P < 0.05) between the objects under both conditions, but with lorazepam the group’s performance was significantly worse than in the control condition. In contrast, lorazepam administered after sampling but 30 min prior to the test phase was without consistent effect (F₁,₁₁ < 1; P > 0.05); see Fig. 1b. In this case, the mean time spent on the novel and previously seen object, respectively, was 28.4 ± 2.4 and 18.0 ± 1.0 s without...
lorazepam, and 24.0 ± 3.2 and 15.8 ± 1.9 s with lorazepam; the group was equivalent in its discrimination ($P < 0.05$) between the objects in both conditions. These results using a 20 h delay demonstrate that lorazepam interfered with acquisition or consolidation, but not retrieval.

Lorazepam did not cause significant impairment when the delay was 20 min ($n = 15$ rats; $F_{1,14} < 1$, $P > 0.05$; DR = 0.40 ± 0.07 for control and 0.47 ± 0.08 for lorazepam group), in contrast to the results obtained with local infusion of lorazepam (see below).

**Exploration during the sample period**

In the 20-h delay experiment, the mean time spent exploring the two copies of the object during the sample (acquisition) phase did not differ significantly between the control and the lorazepam-treated groups (24.5 ± 0.3 and 23.1 ± 0.7 s, respectively). However, on average, the rats took a shorter time to complete this exploration when given vehicle than lorazepam (133 ± 10 compared to 201 ± 12 s, respectively; $t_{11} = 4.35$, $P < 0.001$). Nevertheless, as in both conditions the animals spent an equivalent time exploring the objects, the lorazepam memory impairment cannot readily be explained as due to differential exposure to what had to be remembered.

**Localized infusion of lorazepam in perirhinal cortex**

To exclude the memory impairment being due to possible generalized actions of lorazepam on other brain regions or on global variables such as alertness, the drug was delivered locally to perirhinal cortex by implanted cannulae.

**Preferential exploration**

Lorazepam (2 µg per side) impaired familiarity discrimination when infused bilaterally into perirhinal cortex; see Fig. 1c and d. The infusions were given before the sample (acquisition) phase. The delay between the sample and test phases was 20 min. When saline was infused before sampling, the rats spent significantly more time exploring the novel than the previously encountered object (21.6 ± 4.7 and 12.3 ± 6.4 s, respectively; $n = 8$ rats; DR difference, $t_{5} = 4.56$; $P = 0.003$). In contrast, there was no significant preference when lorazepam was infused before sampling (novel, 18.6 ± 3.3 s, previously seen, 15.5 ± 5.1 s; DR difference, $t_{5} = 1.51$; $P > 0.1$); the interaction between drug and preference was significant (repeated measures ANOVA of DR values, $F_{1,7} = 6.33$, $P = 0.04$).

**Exploration during the sample period**

The memory impairment was not explicable as being due to any significant difference between the groups in the mean time to complete exploration during the sample period (control, 210 ± 11 s; lorazepam, 218 ± 10 s; $t_{5} < 1$, $P > 0.1$), nor in the mean total time spent exploring the two objects (control, 33 ± 9 s; lorazepam, 33 ± 8 s; $t_{5} < 1$, $P > 0.1$). Thus the impairment in discriminating the previously encountered from the novel object indicates that lorazepam can disrupt recognition memory even when administration is local to perirhinal cortex and the delay period is only 20 min.

**Experiment 2: differential neuronal activation measured by Fos**

Neuronal activity in response to presentation of novel and familiar stimuli was assessed using counts of neuronal nuclei immunohistochemically stained for Fos protein. Counts in the brain hemisphere that was opposite to the eye that had viewed novel objects were compared with counts in the other hemisphere that was opposite to the eye that had viewed objects previously shown repeatedly. Thus comparisons were within rat. The novel and previously seen objects were shown at the same time using the paired viewing procedure (Fig. 2a). The delay from the last time the previously seen objects were shown to the final test showing was 3 h. Lorazepam was administered before each presentation of the repeatedly shown objects except for the final, test comparison. Thus at test both the novel and the previously shown stimuli were viewed by both the lorazepam-treated and control rats without lorazepam having been recently administered. By the time the animals were perfused for immunohistochemical processing ~5 h had elapsed since lorazepam had been administered.

**Differential neuronal activation for novel and repeatedly seen stimuli**

Fos stained nuclei were counted in six brain areas; see Fig. 2b and Table 1. For analysis, the counts were normalized by dividing each count by the mean for the rat and the area. In control rats the number of Fos stained nuclei was significantly higher in the hemisphere opposite the novel stimuli than in that opposite the repeatedly shown stimuli (ANOVA, $F_{1,66} = 19.58$, $P < 0.001$); see Fig. 2c. In particular, the counts were significantly (design comparisons, $P < 0.05$) higher for novel than familiar stimuli in perirhinal cortex (PRH) and area Te2 (TE), areas that have previously been implicated in familiarity discrimination (Zhu et al., 1995; Zhu et al., 1996; Wan et al., 1999; Brown & Aggleton, 2001). For no other area was such a comparison significant. In contrast, in the rats that had been given lorazepam so as to block acquisition on all the occasions when the repeatedly shown stimuli were presented prior to the final test, there was no significant difference between the hemispheres overall (ANOVA of Fos counts, $F_{1,53} = 0.75$, $P > 0.1$); neither was there a significant difference for any individual area, including PRH and TE; see Fig. 2d. Moreover, when the results for the areas (PRH and TE) that have been implicated in familiarity discrimination were considered, a significant interaction was found between the Fos expression produced by novel and repeatedly shown stimuli and whether the rats had received lorazepam or not (ANOVA, $F_{1,33} = 5.85$, $P = 0.021$). Thus lorazepam treatment prevented the normally greater neuronal activation produced in PRH and TE by novel compared to previously seen stimuli.

**Behaviour**

The lorazepam-treated animals’ behaviour during the final test session was compared with that of the drug-free animals (see Table 2). There were no significant differences between the lorazepam-treated and control animals in the following measures: total time taken for the test session; mean time the animals spent grooming; locomotion during the session; and the (small) number of trials for which there was a failure to maintain head position.

**Fig. 4.** Measuring the effects of lorazepam upon plasticity. (a) and (b) Effect of lorazepam on LTP in perirhinal slices. Mean normalized evoked potentials are shown before and after high frequency stimulation (HFS) for slices perfused with propylene glycol vehicle (a) or lorazepam (b). Individual traces from the time points indicated are shown above the mean traces. In each slice, HFS was delivered to either the temporal or the entorhinal side input while the other input was used as a control for nonspecific or heterosynaptic effects of the HFS. No significant differences were found between which of the two inputs received HFS and their data have therefore been pooled. LTP was significantly ($P < 0.01$) impaired in the presence of lorazepam; the potentials returned to baseline after ~1 h. (c) Effect of lorazepam on LTD in perirhinal slices. Mean normalized excitatory postsynaptic currents (EPSCs) are shown before and after low frequency stimulation (LFS) for control and lorazepam-perfused slices. LTD was significantly ($P < 0.01$) impaired in the presence of lorazepam; the potentials returned to baseline levels in < 20 min. As for (a) and (b), open triangles, input receiving LFS; filled dots, control input.

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a) propylene glycol (PPG)  
PPG + HFS  
1 & 2 superimposed  

Propylene glycol  

Normalised response  

180  
160  
140  
120  
100  
80  
60  

0  40  80  120  160  
Time (min)  

0.5 mV  
10 ms  

1 HFS  

b) lorazepam  
lorazepam + HFS  
1 & 2 superimposed  

Lorazepam  

Normalised response  

180  
160  
140  
120  
100  
80  
60  

0  40  80  120  160  
Time (min)  

0.5 mV  
10 ms  

1 HFS  

c) Normalised peak EPSC (% of baseline)  

0  50  100  150  

0  10  20  30  40  50  
Time (min)  

Control  
1μM Lorazepam  

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TABLE 2. Behaviour of control and lorazepam-treated rats during testing in the paired viewing procedure.

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total duration of test (min)</td>
<td>Lorazepam-treated</td>
</tr>
<tr>
<td>9.58 (5.23–7.18)</td>
<td>5.85 (4.47–7.37)</td>
</tr>
<tr>
<td>Head turns (number)</td>
<td>4.33 (1–8)</td>
</tr>
<tr>
<td>Wandering off (number)</td>
<td>3.16 (0.8)</td>
</tr>
<tr>
<td>Grooming (min)</td>
<td>0.05 (0–0.16)</td>
</tr>
</tbody>
</table>

Mean data and ranges are shown.

**Experiment 3: synaptic plasticity in perirhinal slices**

**Single evoked potentials**

Extracellular field potentials were recorded directly beneath the rhinal sulcus in layer II/III of perirhinal cortex (Fig. 3). No consistent differences were established between single or potentiated/depressed responses evoked by stimulating to the entorhinal or temporal side of the rhinal sulcus and therefore their data have been pooled. Propylene glycol vehicle (1 μM) did not cause any changes in the evoked field potentials; however, lorazepam (1 μM) caused a decrease (18 ± 6%) in these evoked field potentials. To allow for this decrease, the initial stimulation voltage used was higher in the lorazepam slices so that after stabilization baseline responses with lorazepam were the same amplitude as those in control slices. Hence the mean response in the presence of propylene glycol (0.44 ± 0.03 mV) was not significantly different from that when lorazepam was present (0.44 ± 0.03 mV).

**Long-term potentiation**

In the control slices in the presence of 1 μM propylene glycol, stable LTP (122 ± 6%; n = 4, t₃ = 3.55, P < 0.05) was obtained, lasting for at least 120 min; see Fig. 4a. However, statistical analysis established a significant interaction between the time after HFS and the presence or absence of lorazepam (ANOVA with repeated measures F₁,₅₉ = 1.53; P < 0.01). Although there was no significant difference from LTP in control slices at 30 min, by 90 min lorazepam had blocked LTP (92 ± 3%; n = 7; t₄ = 2.57, P < 0.05); see Fig. 4b.

**Long-term depression**

Low frequency stimulation (LFS) induced stable LTD of the evoked EPSCs (65 ± 5% of baseline; n = 5 neurons from 5 slices; t₄ = 6.9, P < 0.01) lasting > 30 min in the control slices. In contrast, in the presence of 1 μM lorazepam there was no change from baseline (97 ± 4%; n = 5 neurons from 5 slices; t₄ < 1; P > 0.1), and the impairment compared to controls was significant (t₈ = 3.55, P < 0.01); see Fig. 4c.

Thus lorazepam blocked both LTP and LTD in perirhinal slices, however, the impairment of LTD was evident at < 20 min whereas for LTP there was no impairment until > 30 min.

**Discussion**

The findings establish for the first time that administration of a benzodiazepine, lorazepam, has consistent effects on putative memory mechanisms at the behavioural, systems and cellular levels of analysis. This consistency of action provides experimental support to the hypotheses that: (i) perirhinal neuronal response reductions underlie recognition memory (familiarity discrimination) (ii) these response reductions rely upon a mechanism used in LTD and/or LTP, and (iii) lorazepam interferes with both of these processes and with recognition memory. Thus, in particular, the Fos immunohistochemical results establish that lorazepam can disrupt the characteristic differential activation of perirhinal neurons by novel and familiar stimuli. Furthermore, although a variety of effects of benzodiazepines on plasticity have been reported in other areas (Brown, M.W. et al., 1983; Del Cerro et al., 1992; Evans & ViolacMcCabe, 1996; Mori et al., 2001; Akhondzadeh et al., 2002), the results also provide the first evidence that activity-dependent LTD can be impaired by a benzodiazepine, and that LTD and LTP in the perirhinal cortex can be blocked by such a drug.

The action of lorazepam on synaptic plasticity is likely to be due to the well documented benzodiazepine enhancement of GABA-ergic transmission (Rudolph et al., 1999). There are at least two potential mechanisms that may explain the inhibition by lorazepam of both LTD and LTP. First, enhanced GABA-ergic transmission increases the local hyperpolarization of cell membranes. Increased hyperpolarization results in a decrease in the likelihood of depolarization-dependent calcium entry, for example through reduced NMDA receptor activation. The involvement of NMDA receptor activation in perirhinal plasticity is well established (Ziakopoulos et al., 1999). Second, the enhancement of GABA-ergic mechanisms is likely to disrupt the pattern of transmission in local circuitry and thereby also disrupt the network conditions essential for the generation of plasticity. These effects of lorazepam provide possible mechanisms through which benzodiazepines may impair recognition memory and also cause amnesia. The reason for the differential temporal effects of lorazepam on LTP and LTD is not clear, but may reflect the differing degrees of depolarization and consequent postsynaptic calcium concentration changes required for the induction and maintenance of these forms of plasticity (Cho et al., 2001). In particular, the calcium concentration changes at induction may have failed to activate intracellular signalling pathways necessary to the long-term maintenance of LTP.

The results of the behavioural experiments using systemic delivery demonstrate that the effect of lorazepam was on acquisition or consolidation and not retrieval, as impairment was only found when the drug was present during acquisition and not when the drug was present during test (retrieval). This finding accords with impairments found previously in human studies (Brown, J. et al., 1982, 1983; Curran, 1991; Lister, 1995; Steckler et al., 1998; Buffett-Jerrott & Stewart, 2002). It was because of lorazepam’s action on acquisition or early consolidation that in the immunohistological imaging experiment the drug was given each day prior to presentation of the repeatedly shown stimulus set but not during the critical final test comparison. In the present experiments, the action could be on either acquisition or early consolidation as effects were sought and established at long intervals, i.e. up to 20 min for LTD, > 1 h for the blockade of LTP, 20 min for the behavioural amnesia following local infusion, 20 h for systemic delivery, and > 3 h for the Fos impairment. Lorazepam infused locally into perirhinal cortex impaired recognition memory when the retention interval was 20 min while LTD was not impaired until after such an interval. Accordingly, the memory impairment is more likely to be due to interference with plastic mechanisms used in LTD than in LTP. This conclusion is consistent with the suggested greater role for synaptic weakening than synaptic strengthening in familiarity discrimination (Brown & Bashir, 2002) – though it should be noted that all models have a requirement to maintain average synaptic strength and so must also use synaptic changes that counterbalance the primary change (Bogacz & Brown, 2004).
The failure to find an impairment at a delay of 20 min using systemically administered lorazepam may have been due to the dose level being insufficiently high. (Thus only one dose was tested as higher doses could not be tested because they resulted in gross behavioural impairments.) Nevertheless, systemic delivery did produce an impairment at a delay of 20 h; this might have been due to the disruption of LTP-like in addition to LTD-like mechanisms and/or to the increased difficulty of the task at a longer delay.

There was evidence against the effects of lorazepam being secondary to global effects on alertness or attention. Thus, impairment in recognition memory was found with localized administration of lorazepam into perirhinal cortex by cannula: such administration did not produce generalized behavioural effects. In the paired viewing apparatus there was no difference in behaviour between the control and the previously drug-treated animals during the final test. Additionally, for the final test comparison in the immunohistochemical experiment in the paired viewing apparatus, both the novel and repeatedly seen pictures were presented simultaneously and without lorazepam having been recently administered so as to minimize any potential attentive effects of the drug (although this procedure does not exclude any drug effect on attention during previous presentations). However, the present results do not exclude an action of lorazepam on visual perceptual processes as well as recognition memory: there is evidence that perirhinal cortex is involved in perceptual as well as mnemonic processes (Buckley & Gaftan, 1998; Murray & Bussey, 1999). In this context, it should be noted that, in vitro, in addition to an action on plasticity, lorazepam produced a reduction in the size of the potential evoked by baseline stimulation, no doubt due to the potentiation of GABA-ergic transmission (Greenblatt, 1992; Sigel & Buhr, 1997; Rudolph et al., 1999; Ziaikopoulos et al., 2000). Nevertheless, the results demonstrate that lorazepam impairs plastic and mnemonic processes whatever its other effects might be.

As administration was systemic in the Fos experiments, the effects of lorazepam might not be due to an action on perirhinal cortex alone. Indeed, in the lorazepam-treated animals, novel stimuli failed to produce greater neuronal activation than the previously seen stimuli in area Te2 as well as in perirhinal cortex. However, there was a memory deficit when lorazepam was administered locally to perirhinal cortex. Thus an action in perirhinal cortex alone is sufficient to impair recognition memory. Moreover, although lorazepam may have actions in other areas, the observed disruption of mechanisms necessary for LTD and LTP in perirhinal cortex could itself be sufficient to explain the drug’s impairments of memory and differential neuronal activation.

Thus the results establish that benzodiazepine (lorazepam) administration can produce parallel impairments of mnemonic and plastic processes. They therefore provide additional support to the idea that mechanisms involved in LTD and LTP may also be crucial to memory. Moreover, they provide further evidence for the role of perirhinal cortex in recognition memory and of the value of the perirhinal cortex as a site for investigating plastic processes involved in memory.

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Abbreviations

DI, discrimination index; EC, entorhinal cortex; HFS, high frequency stimulation; HPC, hippocampus; LFS, low frequency stimulation; LTD, long-term depression; LTP, long-term potentiation; OCC, occipital cortex; PoRH, postrhinal cortex; PRH, perirhinal cortex; RS, rhinal sulcus; SC, superior colliculus; TE, temporal visual association cortex area Te2.

References


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