Reversible inactivations of the cerebellum prevent the extinction of conditioned nictitating membrane responses in rabbits

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1. Studies show that reversible inactivation of the anterior interpositus nucleus (AIP) of the cerebellum with muscimol (a $GABA_A$ agonist) prevents acquisition of the classically conditioned nictitating membrane response (NMR) in the rabbit. Here, we have used reversible inactivations of the AIP with muscimol to investigate the role of the cerebellum in the extinction of this response.

2. Experimental subjects were implanted with cannulae targeted to the AIP, through which muscimol could be infused via an injector cannula. This experiment was divided into three phases lasting 4 days, separated by 3 day intervals. Experimental and unoperated control subjects received acquisition training in phase 1; in phases 2 and 3 they received extinction training.

3. Presentation of the conditioned stimulus (CS) alone in phase 2 produced normal extinction in control subjects. Muscimol inactivation of the AIP in experimental subjects during phase 2 prevented extinction of conditioned responses (CRs), shown by initial high CR frequency in the first post-drug session of phase 3, which then extinguished in a manner indistinguishable from controls in phase 2.

4. Our findings support the suggestion that similar cerebellar circuitry is engaged in acquisition and extinction of NMR conditioning.

The neuronal architecture and connectivity of the cerebellar cortex have inspired several models that assign an important motor learning function to the cerebellum (Marr, 1969; Albus, 1971; Ito, 1972; Gilbert, 1974). These models are controversial, but recent evidence continues to indicate motor learning functions in the cerebellum (see Thach, 1996, for review).

The classically conditioned nictitating membrane response (NMR) of the rabbit has been used extensively to study the neural mechanisms of associative learning of a simple motor response (see Thompson & Krupa, 1994). A tactile or electrical unconditional stimulus (US) applied to the periorbital region always elicits an unconditioned reflex (UR) blink of the nictitating membrane. The NMR can be associatively conditioned by presenting an auditory conditioned stimulus (CS) that initially does not elicit a response, followed by the US. After repeated, paired presentations of these stimuli, the CS comes to evoke a classically conditioned NMR.

Lesions of parts of the olivocerebellar system (including cerebellar cortical lobule HVI) profoundly impair conditioned responses (CRs) during acquisition training and retention testing (Yeo, Hardiman & Glickstein, 1985*b*; see Yeo, 1991; Thompson & Krupa 1994). Thus, the neural circuitry essential for this simple form of associative learning includes these parts of the cerebellum. Lesions of the cerebellum may abolish CRs because a site of essential plasticity has been destroyed. Alternatively, sites which are crucial for CRs and which are efferent from the cerebellum may require cerebellar input to learn and/or express CRs (Welsh & Harvey, 1989). Studies employing permanent lesions cannot differentiate between the above possibilities and therefore have not localized essential neural plasticity for NMR conditioning to the cerebellum.

Reversible lesions can overcome many of the drawbacks associated with permanent lesions in studies of learning and memory. If acquisition occurs while the cerebellum is inactivated, CRs will be evident when the blockade is lifted; CRs will not be evident if the cerebellum is essential for acquisition. Such a result does not prove that there is essential plasticity in the cerebellum but, assuming such plasticity is not extensively distributed, further inactivations in efferent and afferent pathways can be used to identify a set of candidate structures within which essential plasticity must be present.

Reversible lesion studies have been used to test for a cerebellar role in NMR conditioning. Some have found CRs after training during cerebellar inactivation (Welsh & Harvey,

1991), but others found no evidence for acquisition in previously naive subjects during cerebellar inactivations (Krupa, Thompson & Thompson, 1993; Nordholm, Thompson, Dersarkissian & Thompson, 1993); in addition, reversible inactivation of the brachium conjunctivum does not prevent NMR conditioning (Krupa & Thompson, 1995). These authors have concluded that the essential memory trace for NMR conditioning is within the cerebellum (see Discussion).

One problem with these reversible lesion experiments is that an essential contribution to learning of a particular brain region is judged by the absence of learned responses after the inactivation. Other factors unrelated to learning may produce this result. If there are extended drug effects into the post-inactivation phase and the target area is essential for response expression (as is the anterior interpositus nucleus, AIP), then initial failure to produce learned responses would relate to such extended drug effects and learning may have occurred during the inactivation. This problem may be overcome by analysing extinction learning. In NMR conditioning, extinction is observed as the gradual waning of CRs with repeated presentation of the CS alone in the absence of the reinforcing US after previous acquisition. Several features of extinction learning, including the phenomenon of spontaneous recovery (see Mackintosh, 1974) indicate that, like acquisition, extinction is an active learning process. It is highly probable that the neural circuitry supporting extinction learning is closely related to circuitry essential for acquisition learning.

In extinction learning, it is the absence of CRs which demonstrates learning, in contrast to acquisition, in which the presence of CRs indicates learning. If NMR extinction learning can be prevented using reversible inactivations in the AIP then this will be revealed as the presence of CRs in the post-inactivation phase. Such a result would rule out extended drug effects and demonstrate that the cerebellum is essential for extinction of NMR conditioning.

The purpose of the present study is to examine the role of the cerebellum in extinction of NMR conditioning. After acquisition training, experimental subjects were given a period of extinction training during inactivation of the AIP with muscimol, and then a period of extinction training without AIP inactivation. Control subjects received the same behavioural training but without the muscimol blockade. Results are consistent with the suggestion that muscimol inactivation of the AIP prevents extinction of classically conditioned nictitating membrane (NM) responses.

METHODS

Subjects

The subjects were fifteen male Dutch belted rabbits weighing 1·8–2·5 kg. They were housed individually, allowed food and water *ad libitum* and maintained on a 12 h light–dark cycle.

Surgery

Nine rabbits underwent surgery for the implantation of a guide cannula directed towards the right anterior interpositus nucleus (AIP). Each rabbit was anaesthetized using a fentanyl–fluanisone mixture (Hypnorm, Janssen, Oxford, UK; 0·1–5·0 mg kg^{-1}, I.M.) with a supplement of benzodiazepam (Valium, Roche, Welwyn Garden City, UK; 0·5 mg kg^{-1}, I.V.) and was then intubated with an endotracheal tube and the head placed in a stereotaxic instrument (Kopf, Tujunga, CA, USA), aligned with the stereotaxic co-ordinate system of Matricali (1961). Naloxone hydrochloride (Narcan, Du Pont; 5–7 mg kg^{-1}, I.V.) was used to antagonize the fentanyl and anaesthesia was maintained throughout the operation using halothane (1·5–2·5%) in a nitrous oxide–oxygen mixture (1 : 3). The scalp was reflected and bone and dura removed to expose the right cerebellar cortex. The approximate position of the cerebellar nuclei was defined stereotaxically (λ − 5 mm AP, λ + 4 mm ML, λ − 10 mm DV), and their exact position was then determined by electrophysiological recording using tungsten microelectrodes. We looked for rapid cellular activity below the deepest cortical layer ventral to the rostromedial part of the paramedian lobe. We then searched for an eyeblink-related area in the deep nuclei. Microelectrode tracks were made until cellular activity that correlated with eyeblinks evoked by gentle tactile stimulation of the cornea periocular area was recorded. In order to evoke blinks whilst recording, we maintained a level of anaesthesia that completely suppressed the toe-pinch response but which did not entirely suppress the corneal blink reflex. A 24 gauge stainless-steel cannula guide was then implanted 1 mm caudal and 1 mm dorsal to the position of the activated cells. The placement of the guide tip avoided damage to the connections of these cells and the orientation of the guide avoided damage to parts of the overlying cortex that are important for NMR conditioning (Yeo *et al.* 1985*b*). Exposed brain was covered with sterile absorbable gelatin foam and the cannula guide was fixed to the skull with dental cement. The scalp was sutured around the cannula guide and the animal removed from the stereotaxic instrument. Sodium chloride–glucose (0·18%–4%, 10–20 ml S.C.) was given to supplement fluid and electrolyte levels. Postoperative analgesia was provided by buprenorphine (Temgesic, Reckitt & Colman, Hull, UK; 50 μg kg^{-1}) each day for 3 days and antibiotic cover was by chloramphenicol (chloromycetin succinate, Formitalia Carlo Erba Ltd, Milton Keynes, UK; 7·5 mg kg^{-1}) twice per day for 3 days. All surgical subjects were maintained in a quiet recovery room separate from the main colony for these 3 days and their behaviour was closely monitored. All cannulated rabbits resumed normal feeding, drinking and grooming within 12 h and they exhibited no obvious motor deficits. All subjects were allowed 2 weeks for recovery.

Preparations for conditioning

All cannula-implanted and non-operated control rabbits were prepared for measurement of nictitating membrane movements. Each subject was placed in a Perspex restraining stock and a rubber foam-padded end-stop was positioned to hold each subject firmly. The ears were gently held between rubber foam pads to restrain head movements. Two to three drops of ophthalmic anaesthetic (proxymetacaine hydrochloride; 0·5% w/v; Ophthaine, Squibb, Dublin, Ireland) were administered to the right eye so as to wet the surface of the nictitating membrane and the sclera. After 5 min, a sterile, fine monofilament suture (Ethicon, Prolene, 5/0) was passed through the superficial soft tissue on the lateral edge of the right nictitating membrane and tied to form a very small loop (diameter,

1·5 mm). This loop afforded a simple, atraumatic coupling for the nictitating membrane movement transducer (see below) and so it was left in place throughout the experiment. Topical chloramphenicol eye ointment (1% w/w; Daniels Pharmaceuticals, Derby, UK) was applied to the right eye of each subject on each day throughout the experiment and no subject developed infection or irritation of the eye or periocular areas. The subjects were then returned to their home cages where they resumed normal feeding and grooming behaviours. We saw no evidence of discomfort caused by the suture loop.

At the beginning of the adaptation session (see below), two standard 12 mm stainless-steel Michel clips served to deliver stimulation to the facial skin surrounding the eyelids. One was placed approximately 5 mm behind the lateral canthus of the eye and the other was placed approximately 5 mm below the centre of the lower eyelid. The 12 mm clips were gently attached to the superficial skin layer using a standard applicator without the need to remove underlying fur. Again, when the subjects were returned to their home cages at the end of the adaptation session, the rabbits made no attempts to remove the clips and we saw no other evidence of discomfort.

Conditioning apparatus and stimuli

The rabbits were trained using techniques similar to those first developed by Gormezano, Schneiderman, Deux & Fuentes (1962) and described in an earlier paper (Yeo & Hardiman, 1992). At the beginning of each daily behavioural testing session, each subject was placed in the Perspex restraining stock (see above for details). Subjects adapted well to this restraint and remained passive during the behavioural testing sessions of up to 1 h duration. A low-torque potentiometer mounted upon a simple alloy cradle was positioned above the dorsal aspect of the head. Posteriorly, the cradle included a smooth, curved alloy ring which passed around the base of the ears and anteriorly, smooth alloy extensions stabilized the cradle on each side of the muzzle. This arrangement allowed the body of the potentiometer to be atraumatically fixed relative to the head. The shaft of the potentiometer was then coupled to the nictitating membrane, via a lever and miniature universal joint, with a small stainless-steel hook that was passed through the suture loop (Gormezano & Gibbs 1988). This coupling of the potentiometer and suture allowed isotonic transduction of NM movement and eliminated the need for a restoring force on the transducer. This isotonic transduction is directly comparable with that used in recent studies by Welsh & Harvey (1989, 1991) and with those from our own laboratory (Gruart & Yeo, 1995).

Each subject was then placed in a ventilated, sound-attenuating chamber facing a loudspeaker mounted centrally. The conditioned stimulus (CS) was a 1 kHz sine wave tone of 310 ms duration and an intensity of 63 dB (A scale) (reference 20 μN m^{-2}). Background noise produced by ventilation fans was 54 dB (reference 20 μN m^{-2}). The unconditioned stimulus (US) was periorbital electrical stimulation. Each US was a 60 ms train of three biphasic pulses of intensity 2·5 mA applied to the periorbital region of the face through the stainless-steel clips described above. On paired trials the interstimulus interval between the CS and US onset was 250 ms. The intertrial interval was randomly selected between 25 and 35 s. This US level was sufficient to evoke a clear blink of the external eyelids and a closure of the nictitating membrane that did not habituate with repeated presentations. On initial stimulation, small movements of the neck and limb muscles could sometimes be seen but these habituated rapidly over a few trials.

Behavioural training

Session types. There were three different types of sessions.

(i) Adaptation. Subjects were placed in the restraining stock, fitted with the nictitating membrane transducer cradle and the Michel clips (see above) and placed in the conditioning chamber for 1 h – equivalent to the duration of one conditioning session. There were no presentations of either the CS or the US during this period.

(ii) Acquisition training. Subjects were placed in the restraining stock, fitted with the nictitating membrane transducer cradle and placed in the conditioning chamber. Each session consisted of 100 trials. In ninety trials the CS and US were paired and in ten trials the CS was presented alone. A CS alone was presented in every tenth trial.

(iii) Extinction training. Subjects were placed in the restraining stock, fitted with the nictitating membrane transducer cradle and placed in the conditioning chamber. Each session consisted of 100 trials. The CS was presented alone in every trial.

Experimental design. Two weeks after surgery, the monofilament loop was sutured in the right nictitating membrane and 3 days later all subjects underwent a session of adaptation. On the following day, they entered the experiment in phase 1. There were three phases, and 3 days between each phase.

(i) Phase 1. All subjects received four daily sessions of acquisition.

(ii) Phase 2. All subjects received four daily sessions of extinction training. Experimental subjects received muscimol infusion prior to each session; 1·54 nmol muscimol in 1 μl solution (muscimol hydrobromide; RBI; in 50 mM phosphate-buffered saline, pH 7·4) was injected over 1 min into the right cerebellum of each cannulated subject. The injection was via a 36 gauge cannula inserted through the 24 gauge implanted guide cannula and protruding 0·5 mm below the tip of the guide. The injection was, therefore, 0·5 mm above cells with activity related to eyeblinks. The cannulated subjects were then left for 1 h in their home cage before training.

(iii) Phase 3. All subjects received four daily sessions of extinction training. If experimental subjects had extinguished in phase 2, they would show the same frequency of CRs as control subjects in phase 3. However, if muscimol infusions prevented extinction, then the subjects would extinguish at the same rate as controls extinguishing for the first time (control subjects in phase 2).

Subject groups. Nine subjects underwent surgery in which a guide cannula was implanted above the right cerebellar nuclei. Five subjects in the cannulated group had very low frequencies of CRs during the first extinction session with muscimol blockade in phase 2 (see Results) and so were assigned to the 'effective' group. The remaining four animals had much higher CR frequencies during this session (see Results) and were assigned to the 'ineffective' group ($n = 4$). The control group consisted of six subjects.

Histology

At the end of the experiment rabbits with implanted cannulae were injected with heparin sodium (500 units kg^{-1}, I.V.) and an overdose of pentobarbitone sodium (90 mg kg^{-1}, I.V.). Each rabbit was perfused through the aorta with 0·9% saline followed by 4% formaldehyde and the brain was removed from the skull. The brain was then embedded in 10% gelatin and placed in a solution of 20% sucrose formalin for 3 days. Frozen sections, 60 μm thick, were cut in the transverse plane. Alternate sections were mounted onto gelatinized slides and stained for Nissl substance with Cresyl Violet.

Data analysis

Conditioned responses. A conditioned response (CR) was defined as a nictitating membrane response with amplitude greater than or equal to 0·5 mm and with onset latency greater than 35 ms from CS onset (Yeo, Hardiman & Glickstein, 1985*a*). All responses occurring from CS onset to 1000 ms after CS onset were recorded. Responses on paired and unpaired trials occurring between 35 and 250 ms from CS onset were defined as CRs. The frequency of responses whose latency to onset occurred between 250 and 1000 ms in the extinction phases was calculated for each group, in order to determine whether response onset latency changed during extinction training. These were expressed as a function of the number of trials presented. In order to show within-session effects, data were plotted graphically in ten trial blocks (9 paired trials + 1 CS-alone trial for phase 1, and 10 unpaired trials for phases 2 and 3). Where necessary, within-session frequency graphs were superimposed on block frequency graphs. Data from the paired and unpaired trials were treated similarly, i.e. occurrence of CRs could only be determined within the CS–US interval. We applied the same analysis to extinction learning trials which, although consisting entirely of CS-alone presentations, were analysed for CR frequency during the same time window. Statistical analysis was performed on session and block CR frequency.

Statistical analysis. *A priori* comparisons of CR frequencies of groups across blocks and sessions was achieved using three-way analysis of variance (ANOVA). In addition, a two-way ANOVA and the Student–Newman–Keuls test were applied for *post hoc* analysis where required.

RESULTS

The principal findings of this study were that muscimol infusions into the anterior interpositus nucleus (AIP) completely prevented the extinction of conditioned responses.

Histological verification and group assignment

There were nine cannulated subjects. Five cannulated subjects (E1–E5) were included in the effective group. They showed very few CRs during the first extinction session of phase 2 (range, 0–2·5%). Cannula placements of these subjects were located between approximately 0·5 and 1·5 mm from anterior parts of the interpositus nucleus (see Figs 1 and 2). In four of these subjects (E1–E4), the guide cannulae traversed white matter, slightly dorsal to or just below the surface of posterior parts of the interpositus nucleus. We have previously shown that damage in this area does not impair conditioned responses (Yeo *et al.* 1985*a*). In one other case (E5), the cannula tip was located in the caudal aspect of the interpositus nucleus.

The remaining four subjects showed high CR frequencies in session 1 of phase 2 (range, 42–100%). Cannula placements of these subjects were located between 2 and 5 mm from the AIP. Placements in two of these subjects were located close to the surface of the cerebellum, in the white matter between vermis and paramedian lobe, dorsal to the AIP (I6 and I7). In another subject, a cannula tip was located in the cerebellar cortex of the paramedian lobe (I8), and another at the base of lobule VI of the cerebellar cortex (I9). These subjects constituted the ineffective group.

Behavioural results. All probability values result from analysis of variance tests unless stated otherwise.

Phase 1. There were significant effects of session ($F_{3,33} = 40{\cdot}5$; $P < 0{\cdot}05$) and block ($F_{9,18} = 6{\cdot}15$; $P < 0{\cdot}05$), consistent with increases in CR frequency during learning. Groups (Fig. 3*A*; $F_{2,11} = 0{\cdot}26$, $P < 0{\cdot}05$) and all interactions were not significant factors, indicating that the three groups learned at similar rates.

Phase 2. Before each session of phase 2 all cannulated subjects received injections of muscimol. Figure 3*B* shows that subjects in the effective group produced very few CRs during this stage (0·52%).

Subjects in the control group showed a pattern of extinction typical for this type of conditioning. There was no significant group-by-session interaction between the CR frequencies of the ineffective group and the control group ($F_{3,24} = 0{\cdot}22$; $P > 0{\cdot}05$), but there was a significant group-by-block interaction ($F_{9,72} = 7{\cdot}10$; $P < 0{\cdot}05$). Therefore, subjects in the ineffective group extinguished normally over sessions (see session CR frequencies in Fig. 3*B*), but had different patterns of CR frequencies within the sessions. Inspection of Fig. 3*B* reveals that the ineffective group had elevated levels of spontaneous recovery at the beginning of each session (see block CR frequencies in Fig. 3*B*).

Phase 3. All subjects were given continued extinction training for four more sessions (Fig. 3*C*). The cannulated subjects did not receive muscimol in this phase.

At the beginning of session 1, the ineffective group and control group showed low CR frequencies, which extinguished to baseline levels by session 4. However, subjects in the effective group showed very high CR frequencies, which then extinguished with continued training (see comparison with control group, below). There were significant main effects for groups ($F_{2,11} = 8{\cdot}14$; $P < 0{\cdot}05$), confirming that CR frequencies were different between groups.

Consistent with the very low session CR frequencies in both the ineffective and control groups in this phase, the main effect for sessions was not significant ($F_{3,33} = 2{\cdot}58$; $P > 0{\cdot}05$), and there was no significant interaction between groups and sessions ($F_{6,33} = 1{\cdot}44$; $P > 0{\cdot}05$). The significant main effect for blocks ($F_{9,99} = 10{\cdot}55$; $P < 0{\cdot}05$) and significant interaction between groups and blocks ($F_{18,99} = 3{\cdot}22$; $P < 0{\cdot}05$) are clearly due to large within-session differences across groups – the cannulated group extinguished from high CR frequency to a low CR frequency, in contrast to the CR frequencies of the ineffective and control groups, which showed low levels of extinction within each session.

By inspection of Fig. 3*C*, it is clear that the main effect of groups was due to greater CR frequencies produced by the effective group. Over sessions, a Student–Newman–Keuls test revealed that there was no significant difference between the ineffective and the control group (two-way ANOVA, $F_{2,58} = 0{\cdot}025$, $P < 0{\cdot}05$; Student–Newman–Keuls, $p = 2$,

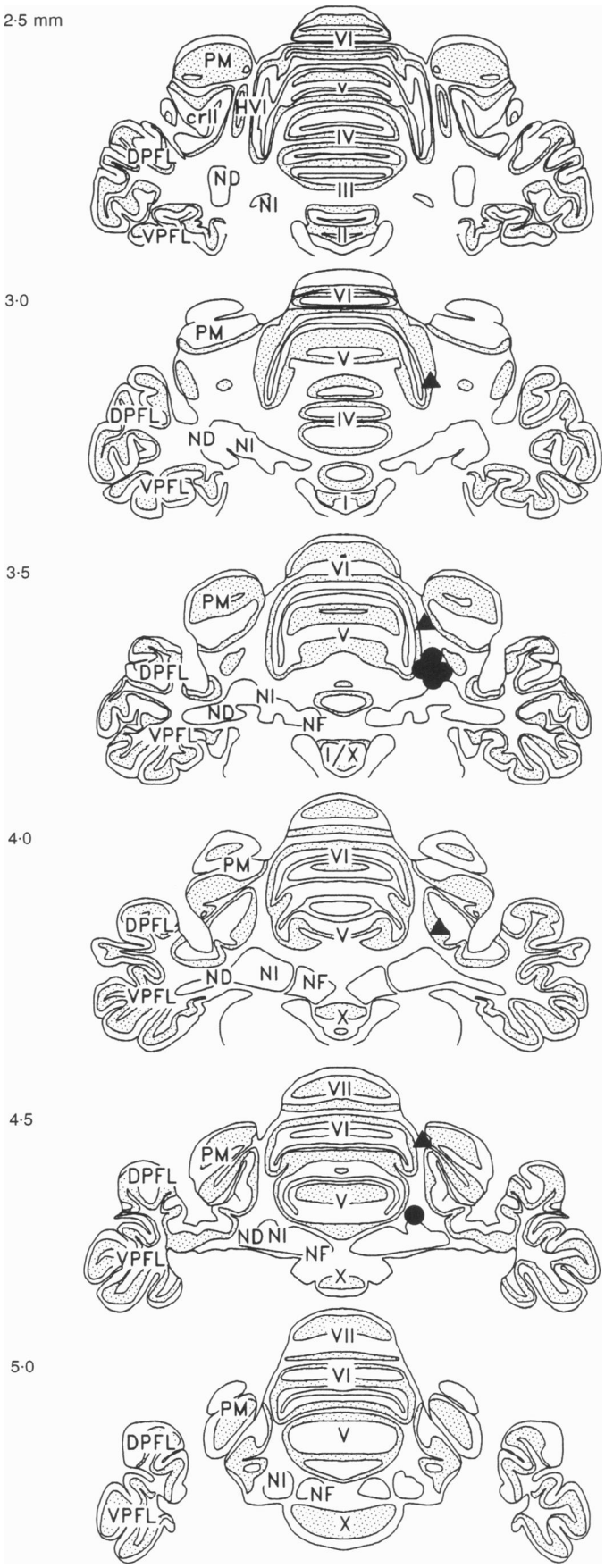

Figure 1. Reconstructions of cannulated subjects

Transverse sections through the cerebellum and brainstem at 0·5 mm intervals. Section identifier is distance from λ. Each symbol represents the site of injection in a cannulated subject. ●, effective placements; ▲, ineffective placements. DPFL, dorsal paraflocculus; crII, crus II; HVI, lobule HVI; ND, dentate nucleus; NF, fastigial nucleus; NI, interpositus nucleus; PM, paramedian lobe; VPFL, ventral paraflocculus.

Table 1. Frequency of responses occurring between 250 and 1000 ms as percentages ± S.D. of all responses

	Control group	Ineffective group	Effective group
Phase 2	1·04 ± 2·11	0·95 ± 1·65	2·1 ± 3·08
Phase 3	0·4 ± 0·64	1·27 ± 1·25	0·5 ± 1·52

$q = 0{\cdot}04$, $P > 0{\cdot}05$), but there was a significant difference between the effective and control groups (Student–Newman–Keuls, $p = 3$, $q = 4{\cdot}07$, $P < 0{\cdot}05$), and between the effective and ineffective groups (Student–Newman–Keuls, $p = 2$, $q = 3{\cdot}60$, $P < 0{\cdot}05$).

Comparison of effective group (phase 3) and control group (phase 2). A comparison between the effective group in phase 3 and the control group in phase 2 (Fig. 3*D*) revealed no significant main group effect ($F_{1,9} = 0{\cdot}2$, $P > 0{\cdot}05$). There were also no group-by-session ($F_{3,27} = 1{\cdot}29$, $P > 0{\cdot}05$) or group-by-block interactions ($F_{9,81} = 0{\cdot}7$, $P > 0{\cdot}05$). There were significant block effects ($F_{9,81} = 16{\cdot}7$; $P < 0{\cdot}05$) and session effects ($F_{3,27} = 4{\cdot}76$; $P < 0{\cdot}05$). Therefore, subjects in the effective group extinguished at a rate very similar to that of control group subjects extinguishing for the first time.

Analysis of responses occurring between 250 and 1000 ms. The number of responses occurring after stimulus offset was very low (Table 1). The tendency for CR onset latency to change in the extinction phases was not significant.

In summary, these results show that the muscimol blockade of the cerebellum appears to prevent the extinction of conditioned NMRs.

DISCUSSION

It is now well established that destructive lesions of the cerebellum and its associated circuitry can abolish or impair conditioned reflex responses of the nictitating membrane (for reviews, see Thompson, 1986 and Yeo, 1991). Whether cerebellar lesions selectively impair learning or whether they produce more general deficits in the performance of conditioned responses is controversial. It is clear that destructive lesions of the cerebellar nuclei can lead to changes in the reflex NM response to periocular stimulation. These changes include an extension of the response rise time and decreased response frequencies at low stimulus intensities close to threshold (Welsh & Harvey, 1989). The presence of these deficits may relate to depressed excitabilities of the brainstem reflex pathways for motor expression of both conditioned and unconditioned NM responses. So the interpretation that permanent lesions of the cerebellar nuclei produce only learning deficits is confounded.

Studies using cerebellar cortical lesions have produced evidence that supports the cerebellar learning hypothesis more clearly because the lesion effects upon CRs and URs are strongly dissociated. Lesions of lobules HVI, and parts of HVII and HVIII (medial Crus I and rostral paramedian

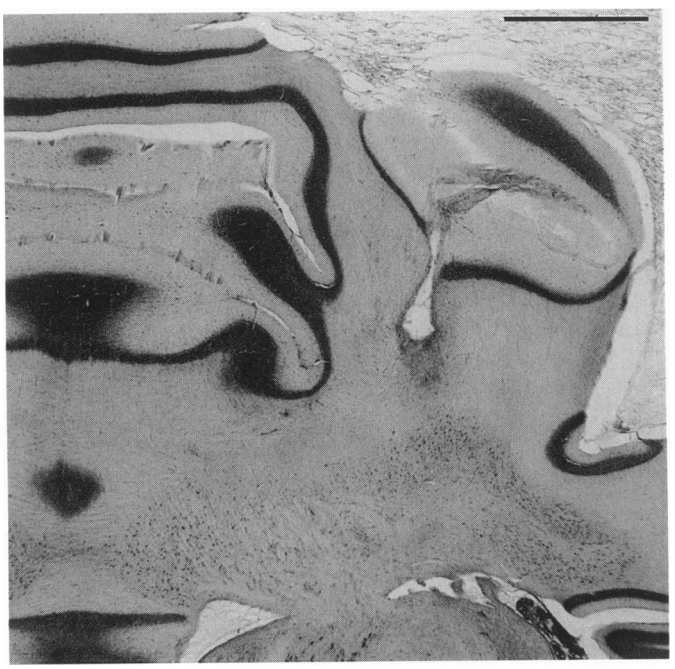

Figure 2. Photomicrograph of transverse, Nissl-stained section through the cerebellum and brainstem of subject E1 (approximately 3·5 mm caudal to λ)

The cannula track penetrated through the paramedian lobe, terminating just above lateral parts of posterior interpositus nucleus. Scale bar, 2 mm.

lobe) abolish or greatly impair the amplitude of NM CRs, whilst UR amplitudes are enhanced (Yeo & Hardiman, 1992; Gruart & Yeo, 1995). These findings are consistent with a disinhibition of brainstem NM reflex pathways following the loss of Purkinje cell inhibition upon the cerebellar nuclei. That CRs are no longer expressed, even though brainstem reflex pathways have enhanced excitabilities, is highly consistent with the suggestion that essential plasticity for CRs is lost following the cortical lesion. Destructive lesions have proved extremely valuable in identifying candidate areas for the location of neural plasticity supporting NMR conditioning. However, destructive lesions are less useful for further analysis of the role of the cerebellum in NMR conditioning in particular, and in motor learning in general, because of the excitability changes that attend them.

Reversible lesions have been used to analyse cerebellar contributions to NMR conditioning free from performance-related effects, but the findings are inconsistent. In the first investigation there was initial conditioning to a light CS followed by a further single session of training to an auditory CS during lidocaine inactivation (1 μl bolus followed by 1 μl min^{-1} 4% w/v solution) of the cerebellar nuclei (Welsh & Harvey, 1991). After recovery from the lidocaine, CRs were present to the auditory CS, consistent with the suggestion that NMR conditioning is not dependent upon cerebellar function. Others have tested for acquisition during repeated daily sessions of inactivation of the cerebellar nuclei using high doses (14 nmol) of muscimol (Krupa *et al.* 1993) or very high doses (up to 16 μl of 32% w/v solution) of lidocaine (Nordholm *et al.* 1993) and found no evidence for CRs after the effects of the drug are presumed to have disappeared, and have claimed that the cerebellum is directly implicated in *de novo* NMR conditioning.

In both *de novo* acquisition experiments, the inactivating drugs were repeatedly infused at high doses so the initial

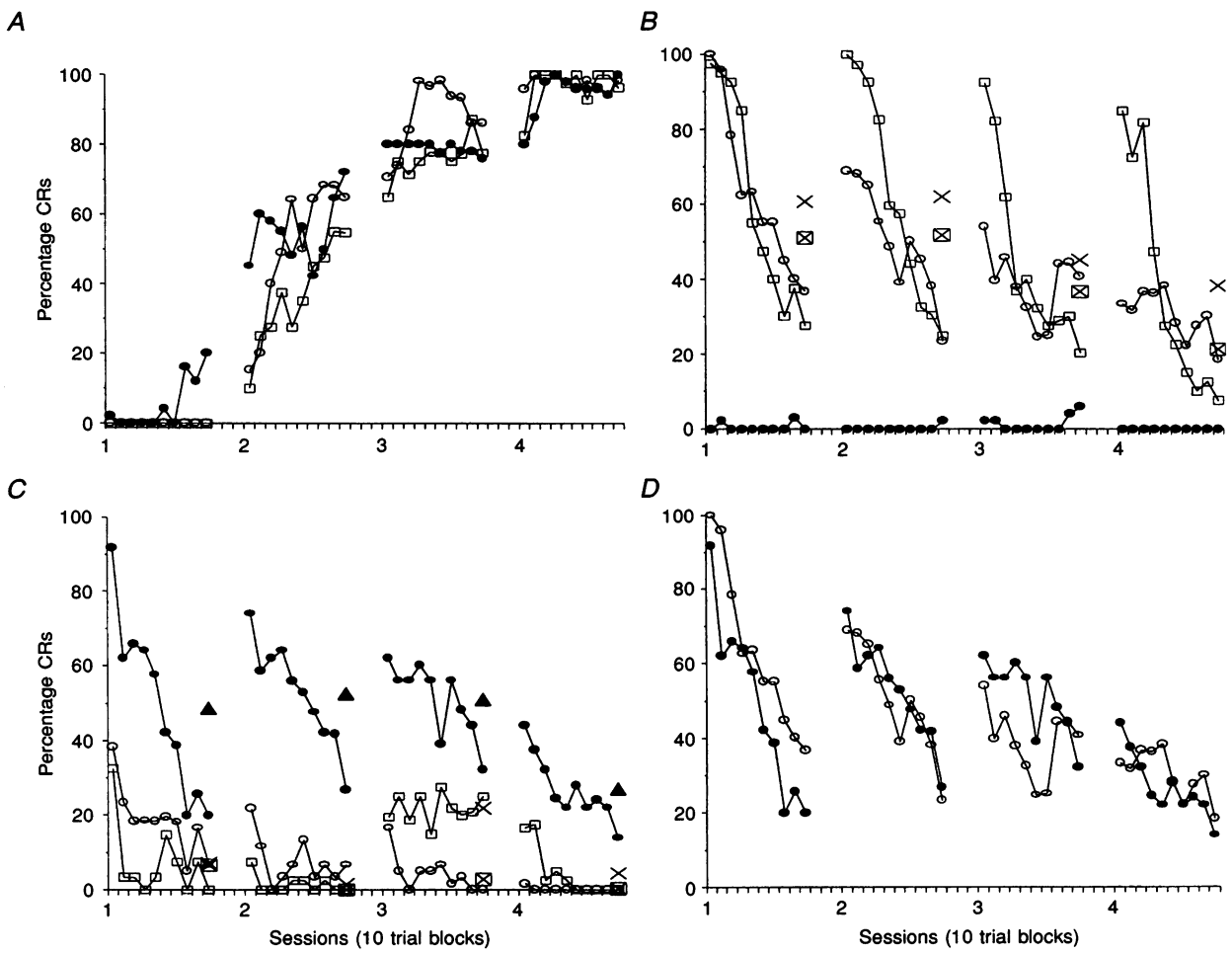

Figure 3. CR frequency across blocks of 10 trials per session

A, acquisition in phase 1; *B*, extinction under muscimol in phase 2; *C*, continued extinction without muscimol in phase 3. *D*, comparison of effective group in phase 3 with control group in phase 2. For markers joined by lines, each marker represents the CR frequency across a block of 10 trials: ●, effective group; ○, control group; □, ineffective group. Large markers at the end of sessions indicate mean CR frequencies across the session: crossed squares, control group; ▲, effective group; ×, ineffective group. CRs in the effective group are virtually absent in phase 2, and so the data points are close to the x-axis.

absence of CRs after training during cerebellar inactivations might be due to a carry-over of residual drug effects into the following acquisition sessions, rather than an absence of learning under the blockade. In the present experiment we have tested this possibility by examining extinction learning. In extinction it is the absence of CRs which demonstrates learning, in contrast to acquisition, in which the presence of CRs represents learning. We found that subjects given four sessions of extinction training under muscimol blockade of the cerebellum displayed high frequencies of NM CRs after the effects of the drug had dissipated and that four more sessions of continued extinction training subsequent to the blockade resulted in behaviour which was similar to that of controls, extinguishing over four sessions for the first time. The presence of CRs after extensive extinction training indicates firstly, that following repeated infusions at low doses, there are no extended residual effects of muscimol on the performance of CRs and secondly, that no learning takes place during the blockade.

The most direct interpretation of our findings is that extinction learning of the classically conditioned NMR is critically dependent upon the cerebellum and so is prevented by cerebellar inactivation. But there are other possibilities. Drugs may have three kinds of effect on classical conditioning. Firstly, they may impair motor expression of CRs through effects on motor systems. Secondly, they may alter the sensory properties of the CS and US, which would in turn affect learning and performance of CRs. Thirdly, they may impair learning-related plasticity (Schindler & Harvey, 1990). We consider our results in the context of these possibilities.

Does muscimol infusion in the cerebellar nuclei affect the motor expression of CRs?

All subjects in the effective group, with cannula locations close to the AIP, were trained to asymptotic levels of CR frequency in phase 1 before administration of the muscimol. In all these cases, muscimol infusions in phase 2 completely abolished performance of CRs. It is most likely that during phase 2, motor expression of CRs was prevented. However, the high frequency of CRs present immediately upon testing in phase 3, after the effects of muscimol had dissipated, indicates that there were no deficits of motor expression extending into phase 3 and validates the reversible lesion technique as free from performance deficits during the post-inactivation phase. It is not possible to extend this particular validation to an earlier study of acquisition using higher doses of muscimol (Krupa *et al.* 1993) but a recent study from our laboratory shows the production of new CRs is prevented with intracerebellar infusions of muscimol at the same low dose as used here (Hardiman, Ramnani & Yeo, 1995).

The ineffective group extinguished at the same rate as the control group, but showed enhanced spontaneous recovery at the beginning of each session in phase 2, though not in phase 3. The cannula placements in this group were not as close to the AIP as those in the effective group and were mainly dorsal to it. Muscimol infusions would have invaded cortical areas dorsal to the AIP more quickly in the ineffective group and agonized $GABA_A$-mediated transmission by cortical interneurons. We suggest that muscimol spread was not optimally localized to block cortical function in all NM response-related areas but was sufficient to produce some disinhibition of the NM-related areas in the cerebellar nuclei, resulting in small enhancements of reflex expression and thus the enhanced spontaneous recovery. The increased spontaneous recovery is apparent and similar to 'performance' changes that have been noted in other studies.

Does muscimol infusion in the cerebellar nuclei affect sensory properties of the CS or US?

We have considered and rejected the possibility of residual muscimol effects upon motor expression in the critical post-inactivation sessions. The remaining possibility, other than that the drug impaired learning-related plasticity, is that the drug affected the sensory properties of the CS and US. In the present extinction learning study, there is no presentation of the US during the muscimol phase, so any possible effects that muscimol may have upon the sensory properties of the US would not be relevant to extinction learning.

If muscimol affected the sensory properties of the CS, then state-dependent learning (SDL) may have occurred. Here, subjects learn in the drug condition but cannot retrieve this information unless they are returned to the drug condition. If a drug alters the properties of the CS, subjects will first associate the CS and US. But then, after the drug effects have dissipated, the CS will be perceived as being novel, and subjects will need to re-learn the association in the drug-free state. Two independent sets of associations may therefore be formed – one in the drug condition, and the other in the no-drug condition.

To observe SDL, behaviour in the drug condition is often compared with behaviour afterwards in the no-drug condition. However in our study, CR performance was abolished by the drug in the effective group so overt behaviour during muscimol inactivation could not be observed. If SDL had taken place, then subjects in the effective group may have extinguished covertly under muscimol in a state-dependent manner in phase 2, and may not have shown retention for the extinction of CRs because there was no muscimol in phase 3. Thus, CRs may have been unextinguished at the beginning of phase 3. Alternatively, the subjects may not have extinguished in phase 2 because muscimol inactivation of the cerebellum impaired learning-related plasticity. In choosing between these options, we consider the following points.

SDL could not have occurred due to muscimol effects in areas surrounding the target area or through the circulation into more widespread brain areas because the extinction behaviour of the ineffective group during and after muscimol infusion was similar to that of the control group (see above). In phase 2, CRs of the ineffective group were

not abolished by muscimol and extinguished like those of control subjects in phases 2 and 3. If muscimol had produced SDL effects in the ineffective group, then subjects would have extinguished in phase 2, and would show no signs of having extinguished in phase 3 (i.e. high CR frequencies which would have declined with further training). However, there was a continual decline in CR frequency in phases 2 and 3, so extinction in phase 2 was continuous with, and not dissociated from, extinction in phase 3.

A final alternative possibility is that SDL effects may be purely local and restricted to the target area of the infusion. In the present experiment, extinction learning may have occurred during muscimol inactivation of the AIP within the olivocorticonuclear compartment disrupted by drug infusion but in a state-dependent manner. Thus, with inactivation studies, we cannot distinguish between the possibility that there is SDL within cerebellar circuitry or the possibility that learning within cerebellar circuitry is disrupted. But for the purposes of identifying the locations of plasticity essential for NMR conditioning, the distinction between these possibilities is of little importance since both indicate plasticity within the cerebellum.

Anatomical and temporal specificity of inactivation

Since providing anatomical evidence for the convergence of NMR conditioning-related information in lobule HVI of the cerebellar cortex (Yeo *et al.* 1985*b*) we have tested for cerebellar cortical mechanisms for NMR conditioning (Hardiman & Yeo, 1992; Yeo & Hardiman, 1992; Gruart & Yeo, 1995). Our findings are consistent with other models of motor learning in the cerebellar cortex (Marr, 1969; Albus, 1971; Ito, 1972; Gilbert, 1974) and with recent physiological studies of cerebellar cortex during eyeblink conditioning in decerebrate cats (Hesslow, 1994*a*, *b*). Our use of muscimol inactivations of the cerebellar nuclei to test cerebellar involvement in the NMR extinction learning may, therefore, appear surprising. However, we suggest that inactivation of the cerebellar nuclei provides an effective disruption of all related cerebellar cortical and olivary regions through losses of nucleo-olivary inhibition and disruption of nucleocortical inputs.

We have assessed the approximate spread of muscimol and also the time taken for behavioural effects to disappear. We have considered evidence from our own laboratory and other sources. Muscimol doses varied, but in all cases, the injection volumes were 1 μl. Mink & Thach (1991) injected 8·8 nmol of muscimol into the dentate nucleus of monkeys (approximately 6 times the dose used in our study). Behavioural effects began about 3 min after the injection and disappeared between 7 and 12 h later. Robinson, Straube & Fuchs (1993) also injected 8·8 nmol muscimol into the caudal fastigial nucleus of monkeys and found behavioural changes starting 10 min after injection and lasting for 2–3 h, with no residual effects 24 h later. Their estimated radius of spread was up to 2·5 mm. In their analysis of NMR conditioning in rabbits, Krupa *et al.* (1993) injected tritiated muscimol into the AIP and autoradiography revealed that their dose of 14 nmol did not spread outside the cerebellum within 2 h.

Since the dose used in our study (1·54 nmol) is only 11% of the dose used by Krupa *et al.* (1993), our dose too would not have spread outside the cerebellum in significant concentrations during a conditioning session. When muscimol was injected up to 2 mm from the AIP, CRs were abolished consistent with permanent lesion studies (Yeo *et al.* 1985*a*). However, if muscimol was injected further away from the AIP, CRs were not abolished. Assuming that muscimol abolishes CRs by acting at the AIP, it is highly unlikely that the injection bolus spread beyond 2 mm or in significant concentrations beyond the cerebellum. Our preliminary data indicate that an injection of 1·54 nmol takes effect within 5 min. Behavioural effects (depressed CR frequency or absent CRs) persist for approximately 7 h in overtrained animals, after which CRs recover completely, and there are no effects on CRs after 24 h (N. Ramnani, unpublished observation).

Therefore, the behavioural time course of muscimol in our study was considerably less than 24 h, and the approximate spread of muscimol was approximately 2 mm. The effects of muscimol in one session of training could not have intruded into the next.

Muscimol infusion in the cerebellar nuclei prevents plasticity for extinction learning of the classically conditioned NMR

Krupa *et al.* (1993) prevented the development of CRs with doses of muscimol infusions in the AIP much higher than those used in the present experiment. If such doses do not produce SDL or significant post-inactivation performance deficits, then the cerebellum is clearly implicated in the acquisition of NMR conditioning. Here we have demonstrated that, at low doses, muscimol inactivations of the AIP do not produce general state-dependent learning effects or significant post-inactivation performance deficits and so we conclude that our inactivations have prevented the plasticity associated with the extinction of NMR conditioning. In a related acquisition study (Hardiman, Ramnani & Yeo, 1995) we have also shown that the same low dose of muscimol prevents the development of CRs in naive animals.

On this evidence alone, we cannot conclude that essential plasticities for the acquisition and extinction of NMR conditioning are confined to the cerebellum. The cerebellum may supply inputs essential for acquisition and extinction to efferent targets but recent work has shown that this possibility is unlikely. Reversible inactivation with tetrodotoxin of the brachium conjunctivum, the major efferent pathway from the cerebellum, does not prevent acquisition (Krupa & Thompson, 1995). These findings are consistent with the suggestion that acquisition learning, and now extinction learning too, are critically dependent upon cerebellar mechanisms.

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