# Inhibition of classically conditioned eyeblink responses by stimulation of the cerebellar cortex in the decerebrate cat

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1. The purpose of the present study was to test the hypothesis that neurones in the anterior interpositus nucleus, under the control of Purkinje cells in the c1 and c3 zones of the cerebellar cortex, exert some control over classically conditioned responses. In particular, the experiments were designed to determine whether the cerebellar control of conditioned and unconditioned responses is different.

2. The experiments were performed on cats decerebrated rostral to the red nucleus under halothane anaesthesia. The cats were conditioned using either a 1000 Hz tone or trains of stimuli through the skin of the proximal forelimb as the conditioned stimulus, and periorbital electrical stimulation as the unconditioned stimulus.

3. A large proportion of the animals acquired conditioned responses at normal rates. It could be shown that these were true conditioned responses and did not result from sensitization or pseudoconditioning. For instance, unpaired presentations of conditioned and unconditioned stimuli caused rapid extinction.

4. Cerebellar areas controlling eyeblink were identified by recording climbing fibre responses in the cerebellar cortex and recording EMG activity in the eyelid evoked by stimulation of the cerebellar cortex. When single shocks of 40–70 $\mu$A were applied to these areas during the emission of conditioned eyeblink responses, the latter were strongly inhibited. The inhibition had a latency of about 10 ms and a duration of 25–75 ms.

5. It was shown that this inhibition of the conditioned responses was topographically specific and could only be evoked from cortical sites identified as controlling eyeblink. Stimulation of the periphery of an eyeblink area caused little or no inhibition.

6. The effect of cortical stimulation on unconditioned reflex responses in the orbicularis oculi muscle was also tested. Some inhibition of unconditioned responses was observed, but quantitative analysis showed that this inhibition was considerably weaker than the corresponding inhibition of conditioned responses. The magnitude of the inhibition was determined for unconditioned responses of different sizes including responses which were weaker than the conditioned responses.

7. It is concluded that conditioned eyeblink responses are under strong cerebellar control from areas in the c1 and c3 zones receiving climbing fibre input from the periorbital area. This effect is not likely to be due to a reduction in the background facilitation of facial motoneurones. In contrast, the weak inhibition of the unconditioned responses was probably due to this mechanism. The results, therefore, suggest that the conditioned responses are dependent on the cerebellum in a way that is not true of unconditioned responses.

Evidence from lesion experiments has suggested that the cerebellum is essential for the normal expression and possibly also for the learning and retention of the classically conditioned eyeblink response in rabbits. According to initial reports, lesions of the nucleus interpositus anterior (NIA) ipsilateral to the trained side permanently abolished conditioned responses (CRs) but had no effects on the unconditioned responses (URs) (McCormick & Thompson, 1984; Yeo, Hardiman & Glickstein, 1985). Both the results and the interpretation of these early studies have later been challenged by other investigators (Bloedel, 1987; Welsh & Harvey, 1989; Kelly,

Zuo & Bloedel, 1990; Welsh, 1992). For instance, in one study a substantial proportion of animals with NIA lesions were able to relearn after prolonged training (Welsh & Harvey, 1989). CRs in these animals occurred with lower frequencies, were smaller and had longer latencies, but they were not abolished. It was also found that cerebellar nuclear lesions did have an effect, albeit much smaller, on the UR. Amplitudes were decreased and the latency to peak amplitude was increased by the lesions.

If lesion effects are not specific to the CRs, there is no reason to interpret them as evidence for a special role of the cerebellum in conditioning. A possible explanation for the effects of lesions is that the cerebellum facilitates the CR through tonic background excitation. Disrupting the outflow from the cerebellum would thus increase the threshold for activating the eyeblink motoneurones. The fact that the UR is not abolished by cerebellar lesions could merely be a result of a stronger excitatory drive in the reflex pathway or of different dynamics of the response (Welsh, 1992). It has long been known that the cerebellum exerts a tonic control over lower motor centres and that cerebellar damage can cause tonic changes in the excitability of motoneurones (Dow & Moruzzi, 1958). It would not be surprising, therefore, if CRs were also influenced by cerebellar lesions.

In the preceding paper, four discrete areas were identified in the c1 and c3 zones of the cerebellar cortex of the cat, which are probably important loci of eyeblink control in the cerebellum (Hesslow, 1994). Trains of stimuli to these areas of the cerebellar cortex evoked 'delayed' EMG responses in the orbicularis oculi muscle with a latency of about 50 ms after termination of the stimulation. Evidence was presented which suggests that these responses were caused by hyperpolarization of the NIA neurones followed by rebound depolarization and excitation. The delayed responses can be completely abolished by further cortical stimulation timed about 10 ms before the expected onset of the response. Cortical stimulation could also inhibit spontaneous background EMG activity in the orbicularis oculi muscle. These results thus suggest that stimulation of the cerebellar cortex is a very precise technique for rapid and reversible manipulation of the excitability of NIA neurones which may be helpful in resolving the problem stated above.

If the cerebellum has a role in the execution of conditioned responses, stimulation of the cerebellar cortex during the execution of a CR would be expected to inhibit the response. This effect should be topographically specific, that is, inhibition should only be obtained from those areas of the cerebellar cortex which have previously been shown to control the orbicularis oculi muscle. Furthermore, cortical stimulation should inhibit conditioned responses more effectively than unconditioned responses. Conversely, if the cerebellum merely provides background excitation and facilitation of the motoneurones in the facial nucleus, CRs should be inhibited to the same degree as the URs.

A schematic outline of the experimental set-up is shown in Fig. 1.

The experiments were performed on decerebrate cats, a preparation which offers a number of advantages. It would be very difficult, both for ethical and technical reasons, to test the suggestions made above in intact animals. Use of decerebrate animals permits a much higher degree of experimental control and precision. Although this preparation is abnormal in many respects, it has been shown by a number of investigators that normal conditioning can be achieved in decorticate or hemi-spherectomized rabbits and cats (Oakley & Russell, 1972; Norman, Villablanca, Brown, Schwafel & Buchwald, 1974).

A preliminary communication of some of these results has been given (Hesslow, 1990).

## METHODS

Many of the techniques employed in the present work, especially the methods of anaesthesia and surgery, were similar to those described in detail in the preceding paper (Hesslow, 1994). The description given below therefore focuses on techniques specific to the experiments described in this paper. Where the methods overlap, only a brief summary will be given.

### Subjects

The results presented here were based on experiments on nine cats (2·5–4·0 kg) some of which were also used in the experiments described in Hesslow (1994). This number represents only those animals which acquired stable CRs. An additional twenty animals were used in pilot experiments in order to find stimulation parameters which would be effective for establishing reliable conditioning.

### Anaesthesia and surgery

Before decerebration, the animals were deeply anaesthetized with halothane (1·2–1·5 % in a mixture of $O_2$ and $N_2O$). They were artificially ventilated throughout the experiment. An opening was made in the skull on the left side, and a substantial proportion of the forebrain was removed on both sides by aspiration. The aspiration completely exposed the brainstem at the level of the thalamus and superior colliculus. The animal was then decerebrated by section with a blunt spatula through the brainstem just rostral to the superior colliculus and the red nucleus. The completeness of the decerebration was always verified by postmortem examination. The remaining parts of the forebrain showed no sign of continuing activity. The anaesthesia was terminated a few minutes after the decerebration. The end-expiratory $CO_2$ concentration, arterial blood pressure and rectal temperature were continuously monitored and kept within physiological limits. The animal's head was fixed to a stereotaxic frame. A pool was built around the cerebellum with cotton-reinforced agar and the cortex was covered with warm paraffin oil. Further details of the surgery are described in Hesslow (1994).

In order to prevent walking movements which would interfere with recordings, the animals were sometimes curarized for periods of variable lengths. Alcurone (Norcuron; Organon Teknika, Boxtel, The Netherlands) was then given intravenously in low doses which were adjusted so that gross movements were eliminated. Before electromyographic (EMG)

recordings, the curarization was allowed to wear off for at least 1 h. It had been determined in pilot experiments that this was sufficient to eliminate any substantial effect on the EMG. As an extra precaution, all test–control comparisons were made with alternating trials and within short periods of time, so that EMG records from periods with different levels of curarization would never be compared.

### Stimulation and recording

Probable areas of eyeblink control were identified in all experiments by recording climbing fibre responses and by eliciting delayed responses by stimulation of the cerebellar cortex (Hesslow, 1994). Recordings of climbing fibre responses were made from the cerebellar surface with monopolar silver ball electrodes (diameter 100–150 $\mu$m). Since the animals were not always curarized, reference electrodes placed in or close to musculature would pick up or, since the same electrodes were used for stimulation, elicit irrelevant muscle activity. In order to prevent this, a second silver ball electrode was used as a reference electrode and placed on the cerebellar cortex or on

the wall of the agar pool. Care was taken to ensure that recordings were not dependent on the precise placement of the reference electrodes. The stimulation and recording arrangement is shown in Fig. 1.

Stimulation of the cerebellar cortex was done with a constant-current generator through the same monopolar silver ball electrodes used for surface recordings. Cathodal square wave pulses of 200 $\mu$s duration were used. Cortical stimulation with the purpose of eliciting delayed EMG responses in the orbicularis oculi muscle employed 200 Hz pulse trains with durations of 30–40 ms and stimulation strengths of 250–400 $\mu$A. Stimulation in order to inhibit conditioned and unconditioned eyeblinks was always single cathodal pulses of 40–70 $\mu$A. In order to ensure that observed effects were not due to the anode, extreme care was always taken to test several different placements of both electrodes.

The eyeblink responses were monitored by EMG recordings from the orbicularis oculi muscle as described in the preceding paper. Examples are shown in Fig. 2A–C.
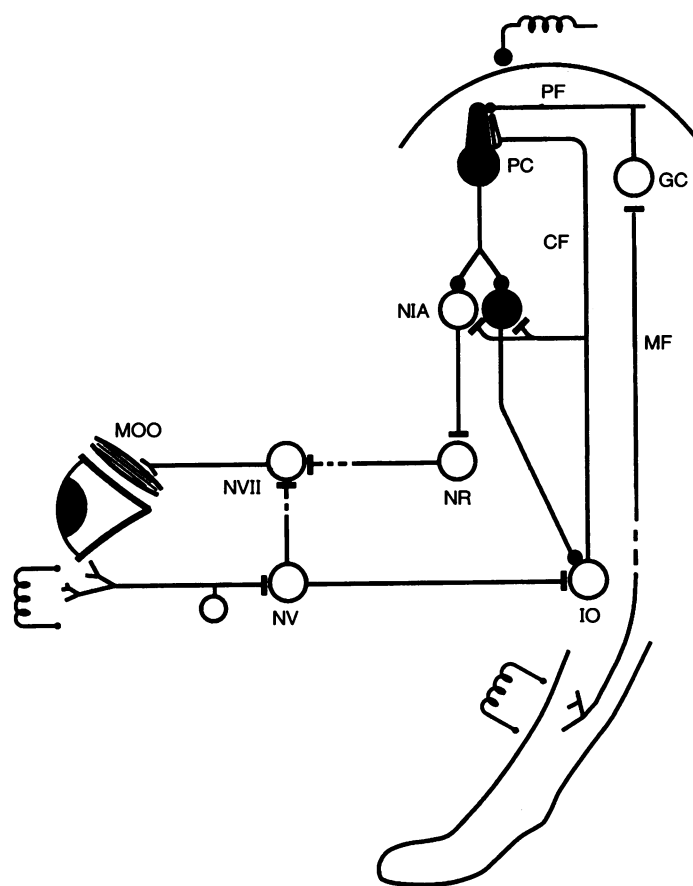


**Figure 1. Experimental set-up with hypothetical wiring diagram of the circuit controlling eyeblink**

EMG recordings were made from the orbicularis oculi muscle (MOO). Recordings of surface potentials were made from the cerebellar cortex via a silver ball electrode. The same electrode was used for stimulation of the cortex. Percutaneous stimulation electrodes were placed in the lower eyelid. The pathway for the periorbital stimulus, the US, is through the trigeminal nucleus (NV), the inferior olive (IO) and the climbing fibres (CF) to the Purkinje cells (PC). A hypothetical CS pathway from the forelimb is via mossy fibres (MF), granule cells (GC) and parallel fibres (PF). Mossy fibre input from the periorbital area is not shown. Output from the cerebellar cortex to the nucleus interpositus anterior (NIA), red nucleus (NR) and facial nucleus (NVII). Note also inhibitory pathway from NIA to IO.

Stimulation of the periorbital area was achieved with two needle electrodes inserted through the skin of the lower eyelid, about 5 mm apart.

EMG data was passed through an analog-to-digital converter from RC Electronics Inc. (Goleta, CA, USA) and analysed with Computerscope software from the same company. In many cases, shock artifacts generated by the periorbital stimulus were so large that they prevented visualization of the EMG activity. In some of these cases, the data were passed through a software high-pass filter. EMG responses were sometimes quantified by rectifying and averaging the EMG signals. In other cases, single motor unit activity was analysed with the help of a window discriminator. All these operations were performed with computer software.

### Conditioning procedure

Surgery, mounting of the animal in a stereotaxic frame and placement of electrodes etc. took 5–6 h. Conditioning was then started and continued until the animal began to show signs of conditioning. During the conditioning period, recordings were made from the cerebellar cortex in order to identify areas of eyeblink control. Conditioning had to be discontinued for periods of varying duration in order to test if delayed responses could be evoked.

Two types of conditioned stimulus (CS) were used. In initial experiments the CS was a 1000 Hz tone stimulus. The tone was presented through plastic tubes with a diameter of 6 mm placed 5 mm from the ear. The intensity at the entrance of the external ear was 80–90 dB. This form of conditioning turned out to be rather unsuccessful – only two out of fifteen animals learned – and was discontinued.

In the remaining experiments the CS was a 250 ms, 100 Hz train of stimuli (0·1 ms, square wave pulses) applied via two needle electrodes inserted through the skin of the medial side of the proximal left forelimb. The strength was adjusted so that it was above the threshold for evoking climbing fibre responses in the forelimb area of the c3 zone (200–500 $\mu$A). Sometimes this stimulation also elicited weak reflexive forelimb movements. In pilot experiments lower stimulation strengths were used which were just above threshold for evoking mossy fibre responses in the cerebellar cortex, but this did not produce reliable conditioning.

The unconditioned stimulus (US) was a brief train of electrical shocks, each one being a 0·5 ms, negative square wave pulse, to the lower eyelid delivered through a pair of stainless-steel needles inserted about 1 mm into the skin. The strength was about twice the strength for evoking maximal climbing fibre responses in the c3 zone (usually about 2–3 mA). The frequency and duration of the pulse train was sometimes varied during the experiment. This was necessary, because recordings were often made from the cerebellar cortex during conditioning. Since shock artifacts would otherwise make the recordings impossible to interpret, responses evoked by the US could only be studied when single shocks were used. When no cerebellar recordings were made, three to four shocks were used with an interval of 5–10 ms between each shock.

The CS–US interval was 250 ms. The inter-trial interval was kept constant at about 20 s throughout the experiment. It was varied pseudorandomly for occasional periods of a few minutes at a time.

Conditioning was continued until CRs occurred in at least 80 % of the trials. In order to ensure that the responses were 'true' CRs and not due to sensitization and pseudoconditioning

(Mackintosh, 1974; Gormezano & Moore, 1976), the animals then received unpaired presentations of the CS and US, where the CS–US interval was varied between 5 and 15 s. This was done for 15–30 min after which time the responses had been extinguished in all animals. Conditioning was then resumed and continued until stable CRs had returned. Periods of testing the effects of cortical stimulation were then alternated with periods of conditioning until the experiments were terminated 18–22 h after the start of surgery.

## RESULTS

### Acquisition and extinction of conditioned responses

Conditioned responses developed in nine animals. In two of these the CS was a tone and in the others it was percutaneous train stimulation of the forelimb. Conditioning failed or was too unstable in the other twenty animals, but this number understates the success rate which gradually increased during the course of the project. For instance, of the last five animals, four acquired CRs. However, this was only true when forelimb stimulation was used as CS. Tone stimulation only succeeded in two out of fifteen animals. The improved results during the project were probably due both to improvements in surgical techniques and to changes in stimulation parameters. In a number of pilot experiments, the CS strength was adjusted so that it evoked mossy fibre but not climbing fibre field potentials in the forelimb part of the c3 zone of the cerebellar cortex. This was usually unsuccessful, but when the CS intensity was increased so that it evoked climbing fibre field potentials, most animals acquired conditioned responses.

The animals that learned the CR seemed to do so quite normally. It usually took 3–5 h before the first CRs appeared. With an inter-trial interval of 20 s and occasional periods of pseudorandom variations of the inter-trial interval, this corresponds to about 500–900 trials. It is possible that some of the initial conditioning was ineffective and that the first hours after decerebration were actually needed for recovery from the surgical trauma. Some observations are consistent with this suggestion. For instance, in two cases, where conditioning was not started until 3–4 h after decerebration, the acquisition was more rapid. No attempts were made to study this systematically.

Conditioned responses were initially small and irregular and it sometimes took an additional 1–2 h of conditioning to obtain a reliable CR. The responses always occurred towards the end of the CS–US interval with a mean latency close to 100 ms. Typical CRs from three different animals are shown in Fig. 2A. A fourth animal, shown in Fig. 2B had bilateral responses.

Once the animals had acquired CRs, these were remarkably stable. Various kinds of interference with the animal, such as injecting drugs or moving electrodes over the cerebellar surface, would temporarily abolish the responses, but when the experimental conditions were

stable, there was usually little variation in the responses. This is illustrated in Fig. 2C, which shows five consecutive responses from the same animal.

Unpaired presentations of the CS and US always caused extinction. In most animals this was quite rapid. Responses usually disappeared almost completely within 30–50 trials (10–15 min), but in one animal about 100 trials had to be given before extinction was complete. When conditioning with paired CS–US presentation was resumed, CRs usually reappeared within ten to twenty trials.

Because the animals were curarized during parts of the experiments, it was not always possible to construct learning curves for the whole acquisition period. Often the animals were curarized for the first 2–3 h of conditioning and the curare was then allowed to wear off about every half-hour, so that the presence of CRs could be checked. The exact trial in which responses first occurred was therefore not always known. However, the example shown in Fig. 2D was representative of most experiments. CRs were quantified by counting the mean number of spikes
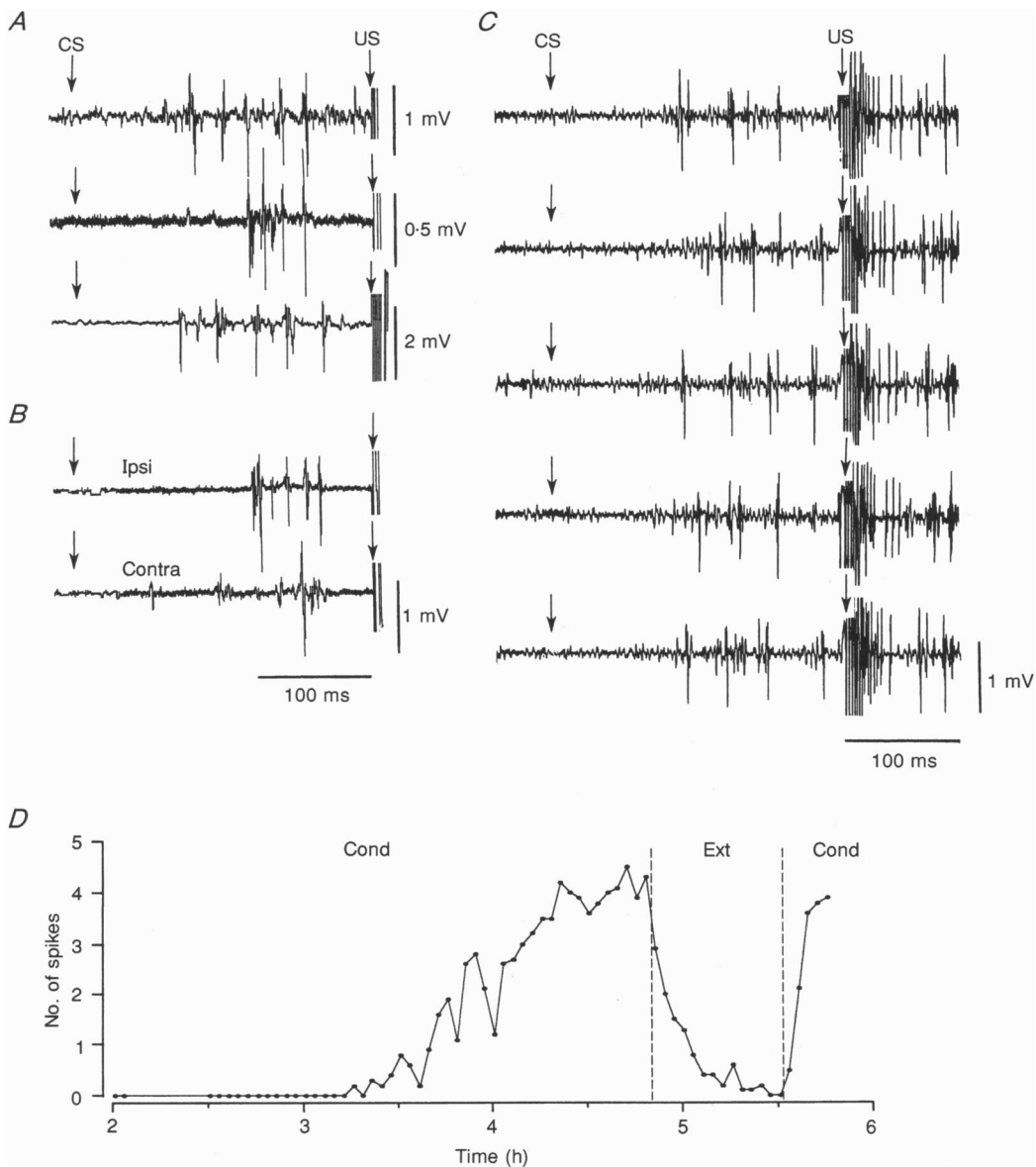


Figure 2. Conditioning
A, shows typical EMG recordings of conditioned responses (CRs) from the left orbicularis oculi muscle in three different animals. B, recordings from a fourth animal from the ipsilateral (Ipsi) and contralateral (Contra) sides. C, five consecutive CRs, illustrating the consistency of responses in the same animal. CS and US indicate the onsets of conditioned and unconditioned stimuli. D, learning curve from one animal. The plot shows acquisition during conditioning (Cond), extinction during unpaired CS and US presentations (Ext) and reacquisition when conditioning was resumed (Cond). Changes in stimulation parameters indicated by dashed lines. Each observation point represents mean number of spikes in 10 trials.

during ten trials. In the case illustrated, this could only be done after 2 h, and there was a brief period of curarization in which no measurements are indicated in the plot.

The part of the plot to the left of the first dashed line (Cond) shows acquisition during paired CS–US presentation. When conditioning was firmly established, unpaired CS and US presentations were tested. As seen in the plot between the two dashed lines (Ext), this led to extinction within about fifty trials. There were occasional odd spikes for a longer period. The last part of the plot shows reacquisition when CS–US presentations were again paired (Cond).

## Inhibition of conditioned responses by cortical stimulation

When conditioning had been obtained in an animal, an eyeblink related area was identified in the c3 zone of the cerebellar cortex by recording climbing fibre responses on periorbital stimulation and by recording EMG responses evoked in the orbicularis oculi muscle on cerebellar cortical stimulation as described in Hesslow (1994). The effect of applying a single electrical shock to the centre of this area on a CR was then tested. The upper record in Fig. 3*A* (4 superimposed traces) shows a typical conditioned EMG response to ipsilateral forelimb stimulation. When a cortical stimulus (Ctx) of 70 μA was applied at two different times during the response (middle and lower records), there was substantial inhibition of the EMG activity beginning about 10 ms after the shock and lasting for 25–50 ms.

Similar results were obtained in all animals. The duration of the inhibition was typically 25–50 ms as in the example above, although it could sometimes outlast the

whole CR. An example is shown in Fig. 3*B*. When the electrode was positioned in the centre of the eyeblink area, the stimulation strength required for inhibiting the conditioned response was always 40–70 μA, which is about the same as that required to inhibit delayed EMG responses evoked by cortical stimulation (Hesslow, 1994). Such low stimulation strengths were sufficient only with an optimal placement of the electrode (cf. below). The amount of inhibition of the CR was similar in all cases, i.e. motor units were always completely silenced for at least 20 ms, and multiunit EMG activity was reduced to background levels or below.

In those cases where a single shock produced a brief inhibition, the CR could be suppressed for longer periods by applying a train of pulses. However, such stimulation also produced delayed EMG responses like those described in Hesslow (1994), this made the results difficult to interpret. The stimulation also seemed to interfere with the conditioning. After application of trains of stimuli in ten to twenty trials, the CRs became erratic and variable in latency and amplitude. Therefore, only single-shock stimulation was tested systematically.

Stimulation of the cerebellar cortex was also tested in one experiment in eyeblink areas in the c1 zone in lobules VI–VII and in the very lateral part of lobule VI. The effects were the same as those described above.

## Topographical specificity of the inhibition

In order to determine if the inhibition of CRs by cerebellar cortical stimulation was specific to areas tentatively identified as controlling eyeblink, the effects of stimulating different cortical sites were investigated in all experiments. One case is illustrated in Fig. 4. A dorsal view of the left
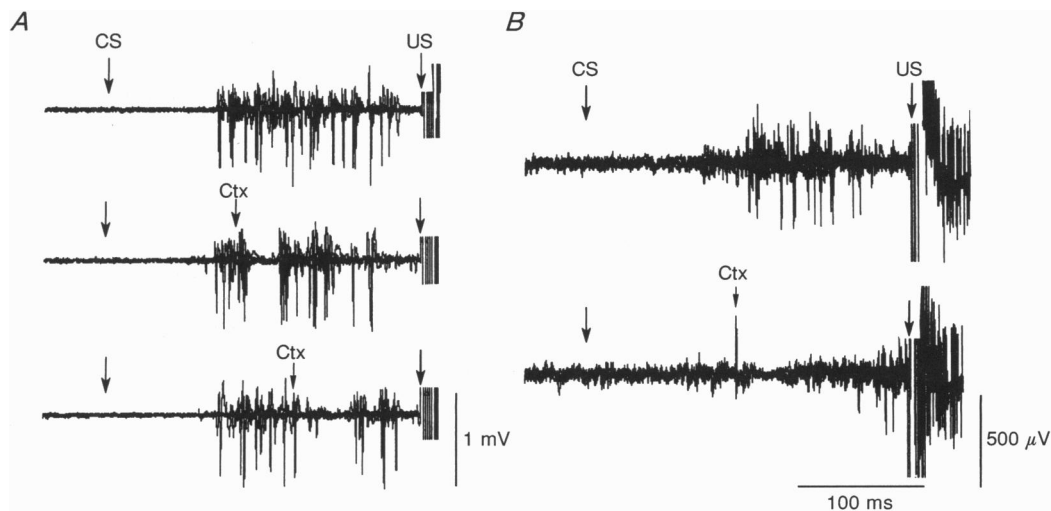


**Figure 3. Inhibition of conditioned responses by stimulation of the cerebellar cortex**
Upper record in *A* shows control CR (4 superimposed records). The middle and lower records show inhibition of the CR when a single shock (70 μA) was applied to the 'eyeblink area' in the c3 zone of the cerebellar cortex at two different time points after the onset of the conditioned stimulus. CS, US and Ctx indicate onset of conditioned, unconditioned and cortical stimuli, respectively. *B*, records from a different experiment in which inhibition of the CR was of longer duration.

*Cerebellum and classical conditioning*

anterior lobe of the cerebellar cortex of is shown in Fig. 4A. The eyeblink area in the c3 zone in lobule VI was identified as described previously and is indicated by hatching. Since the identification is based on the gradual reduction of climbing fibre field potential amplitudes and gradually increasing stimulation thresholds for evoking delayed eyeblink responses, the exact borders of the area are somewhat arbitrary. Three test sites, one in the centre and two in the periphery of the eyeblink area, are indicated by numbers. The distance between the sites was about 500 μm. The recordings shown in Fig. 4B correspond to the sites indicated with numbers in Fig. 4A. The left and right arrows in the trace corresponding to site 1 indicate the onsets of mossy and climbing fibre field potentials respectively. Note how the amplitude of the climbing fibre

potential decreases outside the hatched area. Figure 4C shows the effect of a single cathodal shock (70 μA) applied to site 2 in Fig. 4A. The upper trace shows three superimposed EMG records with CRs appearing with a latency of about 100 ms after the onset of the conditioned stimulus. The lower trace shows similar records when a single shock was applied to the cerebellar cortex.

A comparison of the effects of stimulation at the different cortical sites indicated in Fig. 4A is shown in the post-stimulus time histograms in Fig. 4D. The uppermost histogram is a control based on twenty conditioning trials. After collecting data for the control, a single electrical stimulus was applied in each trial to one of the cortical sites indicated in Fig. 4A during the CR. The three histograms below the control, each one based on twenty trials, were
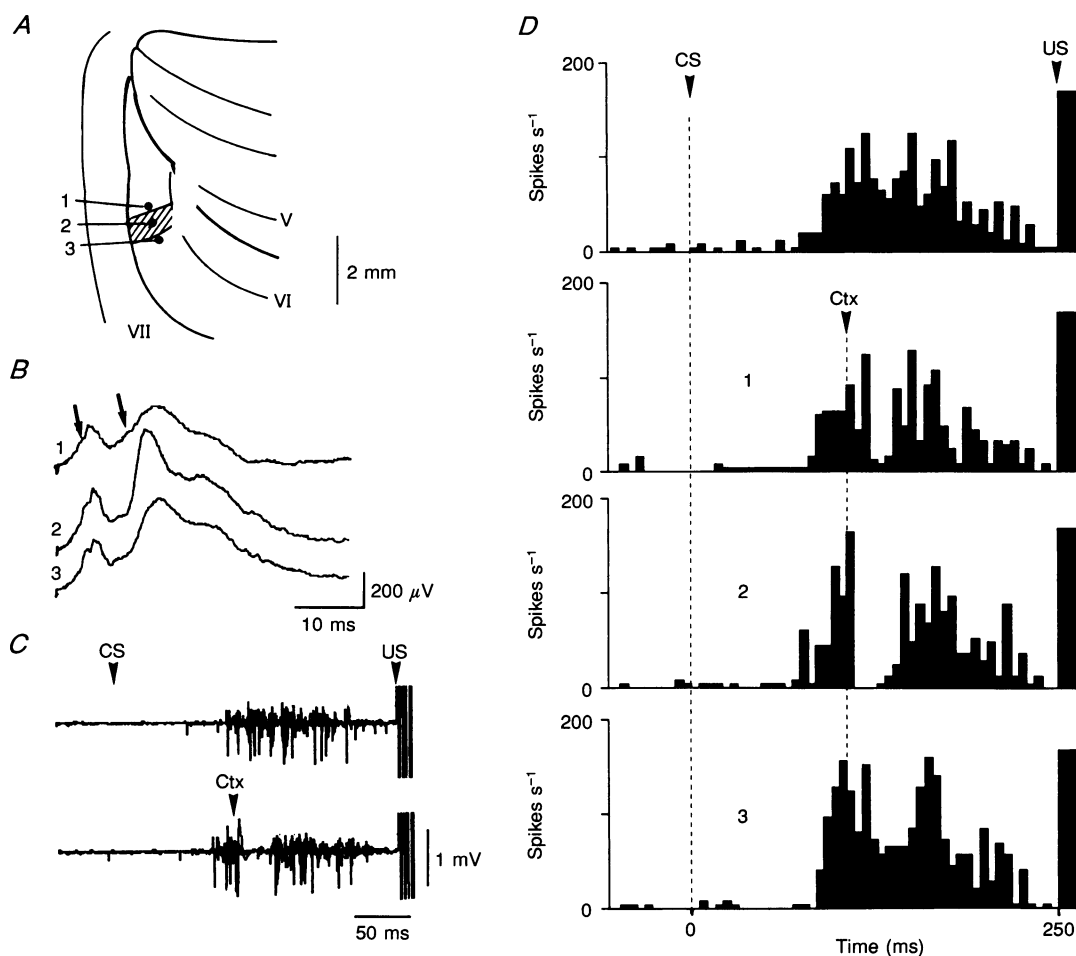


Figure 4. Cerebellar inhibition of conditioned responses: comparison of different sites

A, outline of the cerebellar cortex with lobules V, VI and VII indicated. The hatching indicates the area receiving climbing fibre input on periorbital stimulation and from which delayed EMG responses could be evoked by trains of stimuli to the cortex. Three recording and stimulation sites are indicated by numbers. B, mossy fibre (left arrow) and climbing fibre (right arrow) responses recorded from the cerebellar surface in the sites indicated in A. C, EMG recordings (3 superimposed traces) of CRs. The upper record shows the control CR and the lower record the CR when a single 70 μA shock was applied to the cortex at site 2. CS, US and Ctx indicate onset of the conditioned, unconditioned and cortical stimuli, respectively. D, peristimulus time histograms of the CRs recorded without (upper histogram) and with application of cortical stimulation to sites 1–3. Each histogram based on 20 trials. The bin width is 5 ms.

obtained with such cortical stimulation. When cortical stimuli were applied in a large number of trials, there were sometimes changes in the CRs which became more irregular and erratically timed. In order to prevent this from occurring, about twenty conditioning trials without cortical stimulation were given between the test sessions. These conditioning trials also served as extra controls to ensure that the conditioned response was similar throughout the experiment.

When the stimulation was applied to sites 1 and 3 in the periphery of the eyeblink area, the latency of the inhibition was longer, its duration shorter and its size smaller than when the stimulus was applied to the centre of the eyeblink area, i.e. site 2.

The topographical specificity of the inhibition described above was seen in all animals, although the inhibition could be quantified only in two cases. Since the interval between conditioning trials should not be less than 20 s, many trials were needed for each site and extra control sessions were necessary between test sessions, these experiments took several hours to complete and the preparations were not always in a stable state for sufficiently long periods of time. It was clear, however, even from the less systematic observations in the other experiments, that CRs could never be inhibited by stimulation of sites which were outside the eyeblink areas unless the stimulation strength was increased to several hundred microamps.

## Effect of cortical stimulation on conditioned and unconditioned responses

The inhibition of CRs by cerebellar cortical stimulation could be attributed to a temporary interruption of the background excitation generated by interpositus cells in the motoneurones producing the eyeblink. Therefore, a number of experiments were performed with the specific purpose of comparing the effects of such stimulation on conditioned and unconditioned responses.

There are several problems involved in making such comparisons. The conditioned and unconditioned responses have quite different topographies. The CR is a relatively weak and long-lasting response. The unconditioned eyeblink response consists of two or more components, a short latency response (sometimes called R1) with large amplitude and short duration (typically 2–5 ms) and one or more slower responses (sometimes called R2 and R3) which together may last for 100 ms or more. The early UR is a disynaptic reflex whereas the later response components depend on largely unknown pathways which may include the cerebellum (Hiraoka & Shimamura, 1977). It is only the early disynaptic response that is an appropriate object of comparison here. These components are not always easy to separate in the very strong muscle activity generated by the unconditioned stimulus in a typical conditioning experiment (cf. Figs 1C and 5A), but when a weaker single-shock periorbital stimulus is applied, only the early response appears (cf. Fig. 5B).

Since the UR is usually much stronger than the CR, it presumably involves a much stronger excitatory drive on the motoneurones, possibly far above the threshold for eliciting a maximal response. A removal of one source of excitatory drive could thus have a powerful effect on the CR and yet only a weak effect on the UR. It is therefore necessary to adjust the strength of the unconditioned stimulus so that it elicits a response which is comparable to the CR.

An example of such an experiment is illustrated in Fig. 5. A sample EMG record of both conditioned and unconditioned responses is shown in the upper trace in Fig. 5A. Low frequency components ($< 100$ Hz) were filtered out in order to reduce the shock artifact generated by the unconditioned stimulus. An eyeblink area was identified in the c3 zone in lobule VI of the cerebellar cortex as described previously. The effect of applying a single shock (indicated by star in Fig. 5) to this area during the CR is shown in the lower EMG record in Fig. 5A. The response was completely suppressed for a period of approximately 50 ms beginning about 10 ms after the cortical stimulus.

The UR in Fig. 5A is considerably larger that the CR, and in order to obtain responses which could be meaningfully compared with the CR, weaker single-shock periorbital stimuli were presented. The upper trace in Fig. 5B shows an example of such a response. When the periorbital stimulus was preceded by a shock to the cerebellar cortex, timed about 10 ms before the periorbital stimulus, so that the maximum inhibition should coincide with the unconditioned eyelid response, there was no observable effect on the latter, as illustrated by the lower trace in Fig. 5B.

In order to compare these effects more precisely, unfiltered EMG recordings of conditioned and unconditioned responses were rectified and averaged. The upper trace in Fig. 5C shows a control CR, whereas the lower trace shows the effect of cerebellar cortical stimulation. Periorbital stimuli of different strengths, ranging from 25–500 $\mu$A, were tested in order to elicit eyeblink responses which were comparable in amplitude to the CR. Two examples are shown in Fig. 5D and E. Both amplification and time scale are the same as in Fig. 5C in order to facilitate comparison. The control response (upper trace) in Fig. 5D has a slightly larger amplitude than the CR. When preceded by a cerebellar stimulus it was depressed, but much less so than the CR. When the UR is quantified as the area under the curve, the depression was about 15 % in Fig. 5D. The effect of cerebellar stimulation on smaller URs was also tested, but no stronger depression was observed. An example is shown in Fig. 5E where the amplitude of the control UR was smaller than the CR. In this case the test response was actually increased by about 10 %.

Although the URs shown in Fig. 5D and E were means of forty responses, the stimulation strength was only slightly above the threshold for evoking a response and

there were some fluctuations in UR size due to unsystematic fluctuations in excitability. This was not a problem in the cases illustrated (although the 10 % increase in Fig. 5E was probably due to such fluctuations), but it was not possible to study responses reliably, that were significantly smaller than those shown in Fig. 5E.

Systematic comparisons of the inhibition of the CR and UR, such as those illustrated in Fig. 5, were made in three cases. The results were very similar in all of these. When the response size was quantified as the area under the averaged rectified EMG response, the strongest inhibition

of the UR that was observed was a 25 % reduction. Changing the timing of the cortical stimulus did not measurably affect the strength of the inhibition as long as the UR was within the 10–50 ms time window.

Since the technique of rectification and averaging may be questioned on methodological grounds (Poliakoff & Miles, 1992), a different kind of analysis was also attempted. As can be seen in the record in Fig. 5A, it was possible to separate a single motor unit in the EMG and thus obtain an estimate of the mean spike frequency during both CRs and URs. The inhibition of these responses by a cortical stimulus could then
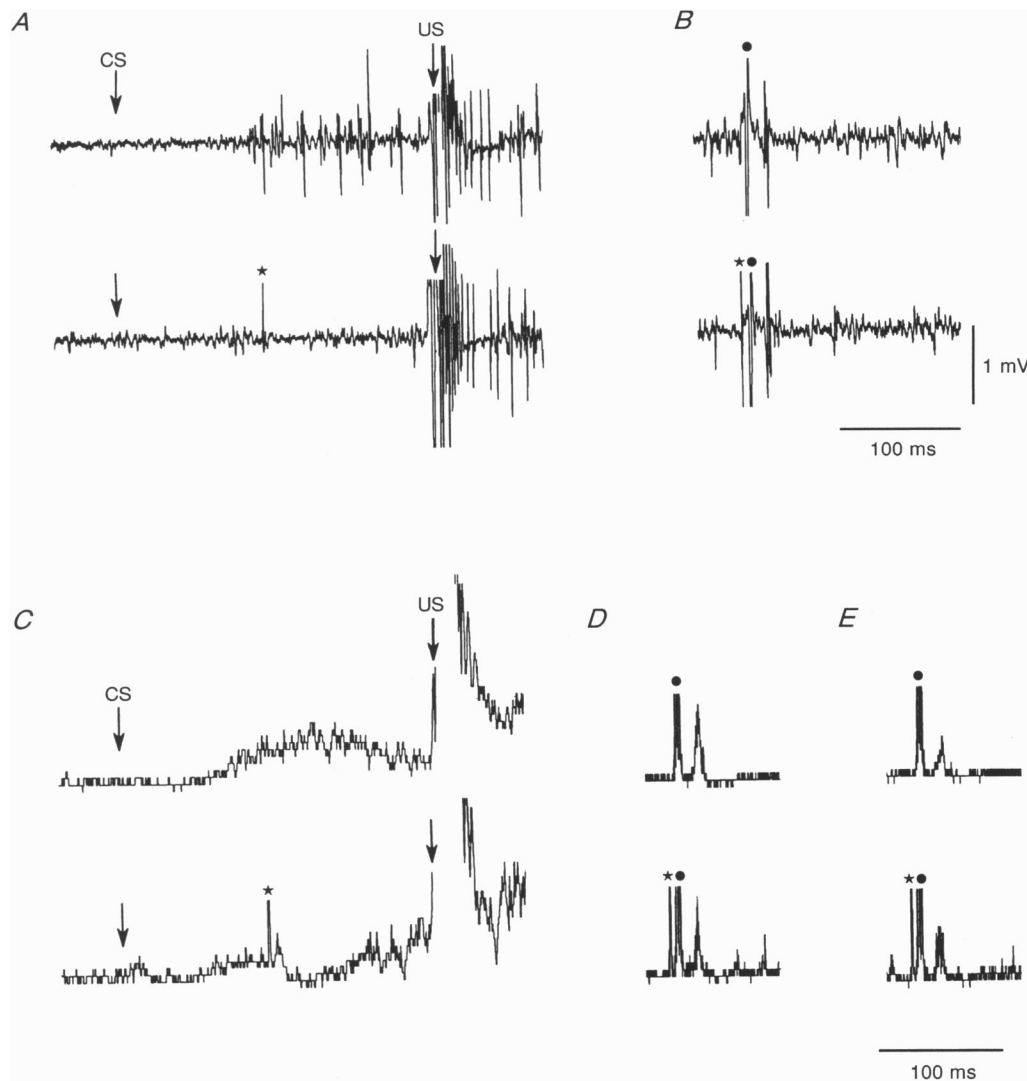


Figure 5. Comparison of the effect of cerebellar stimulation on conditioned and unconditioned responses in one experiment

A, upper trace is a sample EMG record of CR and UR. The lower trace shows a similar record when a single shock was applied to the c3 zone of the cerebellar cortex. Arrows indicate onset of CS, US and cortical stimulus (★). B, the upper trace shows the orbicularis oculi EMG response to a weak periorbital stimulus (●) and the lower trace shows the response to the same stimulus preceded by cortical stimulus (★). Low frequency components were filtered out to reduce shock artifact in A. C, the upper trace is the mean of rectified (unfiltered) EMG recordings from 20 conditioning trials. The lower trace shows effect of cortical stimulation. D, means of 40 rectified EMG responses to a weak periorbital stimulus (●). The upper trace is a control while the response in the lower trace was preceded by a cortical stimulus (★). E, similar to D, but responses were evoked by an even weaker periorbital stimulus.

be quantified as changes in spike frequency. However, this kind of analysis gave results which were virtually identical to those shown in Fig. 5. The spike frequency during the CR was reduced by 100 % whereas the suppression of the frequency during the UR ranged from 5 to 30 %, even when the UR was considerably weaker than the CR.

Less systematic observations were made in all of the other experiments. Although it was not always possible to compare large numbers of responses, these observations were consistent with the conclusions made above. The inhibition of the UR was always small, whereas the CR was always completely inhibited.

## DISCUSSION

### Inhibitory effects of cerebellar stimulation

The main result of the present work is that single-pulse stimulation of eyeblink areas in the c1 and c3 zones of the cerebellar cortex during performance of conditioned eyeblink responses inhibited the CRs for a period of at least 25–50 ms. This effect was quite strong – the orbicularis oculi motor units were completely silenced in all animals. Such stimulation also inhibited unconditioned responses, but this effect on the URs was never as strong, even when very weak URs were studied.

The mechanism behind these effects might be a hyperpolarization of neurones in the nucleus interpositus anterior due to activation of inhibitory Purkinje cell axons. Several investigators have shown that cathodal stimulation of the cerebellar cortex activates climbing fibres and that this indirectly elicits responses in Purkinje cells which in turn hyperpolarize the deep cerebellar nuclear cells (Ito & Yoshida, 1966; Armstrong, Harvey & Schild, 1973; Andersson & Oscarsson, 1978).

This suggestion is consistent with the previously described observations that stimulation of the cerebellar cortex could inhibit eyeblinks evoked by trains of stimuli to the cortex, probably via inhibition of NIA neurones (Hesslow, 1994). This cerebellar cortical stimulation may, therefore, be used as a technique for reversibly inactivating neurones in the cerebellar nuclei. It differs from other techniques in being much more rapid in onset and offset and also in being topographically precise.

When the stimulus electrode was at an optimal location on the cerebellar cortex, the stimulus strength required to block the CR was only 40–70 $\mu$A. This is much lower than the strengths usually needed to activate units in the cerebellum with ball electrodes. Andersson & Oscarsson (1978) for instance, used 500–800 $\mu$A stimuli in the b zone in order to elicit IPSPs in the lateral vestibular nucleus. Armstrong et al. (1973), using 100 $\mu$s cathodal pulses, found thresholds for activating climbing fibres above 150 $\mu$A. It is likely, however, that the stimulation electrodes were not as optimally placed as in the present experiments. Furthermore, these investigators studied graded responses which may have required the activation of a large number of units in order to be detectable. The stimulus strength

required to evoke delayed responses was also higher, 250–400 $\mu$A (Hesslow, 1994). It is possible that in order to elicit a delayed response, which requires a sufficiently deep IPSP in the NIA to generate a rebound, a large number of Purkinje cells must be activated whereas, in order to block the CR, only a few of the cells projecting to eyeblink neurones in the interpositus need to be activated.

The effect of cortical stimulation on CRs would be explained if the NIA was the main source of excitatory drive to the motoneurones during the CR. By inhibiting the NIA neurones, cortical stimulation could remove this drive and thus abolish the CR. Inhibition of NIA neurones could also inhibit the CR if the motoneurones were highly dependent on background excitation from the cerebellum (see below).

The topographical specificity of the inhibition is also consistent with this interpretation. As expected, the strongest inhibition was obtained from the centre of the eyeblink area whereas weaker inhibition was observed when the periphery of the eyeblink area was stimulated.

Inhibition of NIA neurones could also explain the effect of cerebellar cortical stimulation on unconditioned eyeblinks. It was shown in Hesslow (1994) that cerebellar cortical stimulation can suppress the spontaneous background EMG activity in the orbicularis oculi muscle, suggesting that there is a tonic excitatory cerebellar outflow to the motoneurones. This tonic activity probably contributes to the UR, and it is therefore to be expected that cerebellar stimulation would also inhibit this response. The magnitude of the inhibition would depend on the relative contribution of the NIA and of the unconditioned reflex pathway to the excitation of the motoneurones. The latter will normally dominate, but when the periorbital stimulus is sufficiently weak, the contribution from the NIA would be substantial and so would cause inhibition. Although alternative interpretations are certainly possible, the data presented in Fig. 5 are consistent with the suggestion that the inhibition of the UR derives mainly from the loss of background excitation.

The fact that decerebrate rather than intact animals were used in this study probably does not limit the generality of the findings. Evidence from several investigators indicates that classical conditioning in decerebrate animals is comparable to normal intact animals. Acquisition and extinction of conditioned responses in hemispherectomized or decorticate dogs, cats and rabbits is quite similar to what has been observed in intact animals (Bromiley, 1948; Oakley & Russell, 1972; Norman et al. 1974; Kelly et al. 1990; Hesslow, Hardiman & Yeo, 1990). Intact rabbits which were decerebrated after training and acquisition of CRs retained the learning (Mauk & Thompson, 1987). The results reported here are quite consistent with this literature. Rates of acquisition, extinction and reacquisition as well as timing and topography of conditioned responses were similar to those previously found in intact animals (Mackintosh, 1974; Gormezano & Moore, 1976). The fact

that conditioning sometimes required a larger number of trials than is typical in the literature, may be explained by the fact that the decerebrate animals required a certain recovery period, so that the first trials did not actually contribute to the conditioning. It should also be noted that the rate of acquisition, in terms of the number of trials required, is considerably slower, even in intact animals, when all training takes place on the same occasion. There is good reason, therefore, to believe that the mechanisms behind the acquisition and generation of conditioned responses are essentially the same in decerebrate and intact animals and results obtained from the former also hold for the latter.

## Implications for classical conditioning

It is highly likely that the inhibitory effects of cerebellar stimulation on both conditioned and unconditioned responses result from inhibition of interpositus neurones. It is less clear how the inhibition of NIA affects the responses. The strong effect of cerebellar stimulation on conditioned responses could be taken as evidence that these are generated by the cerebellum, but there are also other possibilities. It has been suggested by several authors that the mechanism whereby cerebellar lesions abolish conditioned eyelid responses is nothing more than the disfacilitation caused by interrupting background excitation from the interpositus nucleus, and hence the red nucleus, on the motoneurones of the facial nerve (Bloedel, 1987; Welsh & Harvey, 1989; Kelly *et al.* 1990; Welsh, 1992). Indeed, it is well known that cerebellar lesions produce tonic changes in the excitability of other reflex systems and it would be surprising if the eyeblink was different (Dow & Moruzzi, 1958).

It might be thought that such a depression of the excitability of the motoneurones would not differentially affect movements evoked in different ways and conditioned and unconditioned responses should be affected to a similar extent, but this is not necessarily true. If one were to compare the depression of responses to the conditioned and unconditioned stimuli, as these are normally applied in standard conditioning experiments, it is quite likely that the CR would be more powerfully depressed for the simple reason that it involves a much weaker drive to the motoneurones, possibly just above threshold. The UR on the other hand could result from a drive stronger than that required to excite the motor neurones maximally. In that case, a small loss of background excitation might have no effect on the response. A similar line of reasoning could be applied to the effect of cerebellar cortical stimulation on CRs and URs.

This argument probably does not apply to the results presented above. Firstly, when very weak periorbital stimuli were used in eliciting URs, so that the EMG activity in the orbicularis oculi motoneurones was lower than during CRs, stimulation of the cerebellar cortex still always produced a weaker inhibition of the URs. Secondly,

there was no indication that small responses were inhibited more strongly than large ones. If anything, the opposite was true. Thus, although effects of this kind are very difficult to evaluate, it does not seem likely that the complete suppression of the CR by cortical stimulation results merely from a loss of tonic background excitation of the motoneurones from the cerebellum.

This does not exclude the possibility that the cerebellum exerts a tonic facilitation of some other brainstem structure, which is involved in generating the CR but not the UR. It is conceivable, for instance, that the CR is generated in an extracerebellar structure which uses the red nucleus as an output pathway. The present results do not exclude this possibility. However, there is at present insufficient knowledge of the anatomical pathways which would be required of such an hypothesis and it is therefore difficult to evaluate.

Another possibility is that the cerebellum is involved in certain important aspects of the CR, such as its timing and amplitude. The precise timing and control of response amplitude are characteristic features of a conditioned response. It is evident that these features are learned, since they depend on the timing of the CS and US presentation, upon CS and US intensities and other background factors (Gormezano & Moore, 1976). The cerebellum may contribute to the development of the latency and amplitude characteristics of the CR, either by learning within the cerebellar circuitry, or alternatively, by supplying inputs to other sites of plasticity. Both suggestions would explain why lesions and cerebellar cortical stimulation have stronger effects on CRs than on URs and the second suggestion is consistent with the observation that CRs can occur after removal of the cerebellum (Kelly *et al.* 1990). Both are consistent with the role in motor control traditionally ascribed to the cerebellum.

In conclusion, the evidence suggests that CRs depend on the cerebellum in a way that is not true of URs. The results are consistent with the hypothesis, that the cerebellar cortex is importantly involved in the control of some aspects of conditioned responses, although it is at present difficult to say anything about the precise nature of this control. Furthermore, the results confirm the suggestion that plausible control loci are the sites in the c1 and c3 zones previously identified as 'eyeblink areas' (Hesslow, 1994).

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