Correspondence between climbing fibre input and motor output in eyeblink-related areas in cat cerebellar cortex

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1. The purpose of the present work was to identify sites in the cerebellar cortex which are likely to control eyeblink. This work was motivated by findings suggesting that the cerebellum is involved in the learning and/or performance of the classically conditioned eyeblink response. The identification was based on climbing fibre input to the cortex and on the effects of electrical stimulation of the cerebellar cortex in cats decerebrated rostral to the red nucleus.

2. The cerebellar surface was searched for areas receiving short latency climbing fibre input on periorbital electrical stimulation. Four such areas were found in the c1 and c3 zones of lobules VI and VII in the anterior lobe of the cerebellum and in the c3 zone in the paramedian lobule.

3. Electrical stimulation of the cerebellar cortex with trains (150–400 Hz) of at least 10 ms duration evoked two types of EMG response in the orbicularis oculi muscle. An early response, time-locked to the onset of the stimulation, was unrelated to climbing fibre input and a delayed response, time-locked to the termination of the stimulation, could only be evoked from areas which received short latency climbing fibre input from the eye, that is, the c1 and c3 zones. The delayed responses had long latencies (up to 50 ms) after the termination of the stimulus train and could be delayed further by prolonging the stimulation.

4. Both types of response were abolished by injections of small amounts of lignocaine into the brachium conjunctivum.

5. A number of characteristics of the delayed responses are described. They could be inhibited by a further shock to the same area of the cerebellar cortex. Their latency could be increased by increasing the stimulation frequency. The period between stimulation and appearance of the response often showed a decrease in spontaneous EMG activity.

6. There was a close topographical correspondence between input and output. Delayed responses could be evoked from all four of the areas in the c1 and c3 zones which have climbing fibre input from the periorbital area. They could not be evoked from other areas. In contrast, early responses were only evoked from areas without such climbing fibre input.

7. It is proposed that the delayed responses were generated by activation of Purkinje cell axons leading to hyperpolarization and a subsequent rebound depolarization and activation of cells in the interpositus nucleus. The cortical areas are therefore probably involved in the control of the orbicularis oculi muscle.

8. The implications for the functional organization of the cerebellum as well as for its role in classical conditioning are discussed. In particular, it is noted that the results are consistent with the hypothesis that there are cerebellar microzones controlling single muscles or muscle groups and receiving climbing fibre input which signal errors in the behaviour of the corresponding muscle.

Recent evidence suggests that the cerebellum may be necessary for normal acquisition of conditioned eyeblink responses. Classical, or Pavlovian, conditioning of eyeblink responses has been widely used as an experimental protocol in learning studies. If a neutral stimulus, such as a tone, is repeatedly paired with a stimulus which reliably elicits an eyeblink, such as an air puff to the cornea, an animal will learn to blink in response to the tone alone. It is now established that this kind of learning does not require either the neocortex or the hippocampus (Oakley & Russell, 1972; Schmaltz & Theios, 1972). In contrast, lesions of the nucleus interpositus anterior (NIA) of the cerebellum or of the superior cerebellar peduncle have been reported to abolish conditioned eyeblink responses ipsilateral to the lesions and to prevent their reacquisition (McCormick & Thompson, 1984; Yeo, Hardiman & Glickstein, 1985*a*).

In experiments in rabbits, lesions of other cerebellar or cerebellar-related structures have resulted in conflicting findings. According to Yeo, Hardiman & Glickstein (1985*b*) lesions of the lobulus simplex (HVI) of the cerebellar cortex permanently abolished conditioned responses, while McCormick & Thompson (1984) found no loss of conditioned responses after cortical lesions. More recently, Lavond, Steinmetz, Yokaitis & Thompson (1987) reported that, although cortical lesions initially abolished conditioned responses, the rabbits soon relearned the response.

Some writers reject altogether the idea that the cerebellum is the site of the synaptic plasticity underlying classical conditioning (Bloedel, 1987; Welsh & Harvey, 1989). Indeed, Kelly, Zuo & Bloedel (1990) reported that when eyeblink conditioning had been established in rabbits which had previously been decerebrated, cerebellectomy did not abolish the responses.

It is not possible to resolve these difficulties at present, and the hypothesis of cerebellar learning must still be regarded as unproven. However, in view of the profound effects of cerebellar lesions on conditioned responses found by almost all investigators, it seems clear that the cerebellum is involved in the control of at least some aspects of these responses. One possible reason for the conflicting results is the fact that it has not been known from where exactly in the cerebellum eyeblink is being controlled. If areas controlling eyeblink are not confined to HVI, variations in the extent of lesions could have very different effects.

In order to investigate more precisely what the role of the cerebellum is in conditioning, it is essential to determine which parts of the cerebellar cortex are related to eyeblink responses. Such a localization is not only necessary for a proper interpretation of lesion effects, but it is also a crucial prerequisite for meaningful physiological studies, such as recording neuronal activity. In the work presented here, this problem was addressed by investigating the climbing fibre input from the periorbital area to the cerebellar cortex and by mapping areas from which eyeblinks could be evoked by electrical stimulation.

Both anatomical and physiological studies of the climbing fibre projection to the cerebellar cortex have revealed the presence of sagittally oriented zones of Purkinje cells, each receiving input from a distinct part of the olive and each projecting to a specific portion of the cerebellar nuclei (Oscarsson, 1980; Ito, 1984). The eight zones which have been identified (in cats) are the a, x and b zones in the vermis, the intermediate c1, c2 and c3 zones and the hemispheric d and y zones. These zones can be further subdivided into microzones on the basis of the topography of the climbing fibre input. Lesion studies have implicated the anterior interpositus nucleus and the dorsal accessory olive in normal conditioning. The anterior interpositus nucleus is innervated by Purkinje cells in the c1, c3 and y zones, which are precisely the zones which receive their climbing fibre input from the dorsal accessory olive. It is these zones, therefore, that are likely to participate in the control of eyeblink. The present study, therefore focused on climbing fibre input to these zones.

It has been suggested that the climbing fibre input to a certain area of the cerebellar cortex is related to its functions such that, for instance, the areas of c3 receiving forelimb input would control forelimb muscles and, further, that each microzone in the forelimb area would control a single muscle or muscle group. (Oscarsson, 1980; Ito, 1984; Ekerot, Garwicz & Schouenborg, 1991). If this conception is true, one would expect the zones projecting to the anterior interpositus nucleus to contain microzones which have climbing fibre input from the cornea and the periorbital area and which control the orbicularis oculi muscle.

In order to determine if areas receiving climbing fibre input from the periorbital area were related to output in a way consistent with the suggestions made above, the cerebellar cortex was stimulated electrically while recording EMG activity in the orbicularis oculi muscle. It was found that such stimulation could elicit eyeblinks and that there are at least four different areas in the c1 and c3 zones which receive climbing fibre input from the periorbital area and from which EMG activity can be evoked by electrical stimulation. It is probable that these are areas which control eyeblink activity. Preliminary communications of some of the results have been made (Hesslow, 1990).

METHODS

Anaesthesia and surgery

The experiments were performed on eighteen cats (2·5–4·3 kg), some of which were also used in the experiments described in the accompanying paper. The animals were deeply anaesthetized with halothane (1·2–1·5 % in a mixture of O_2 and N_2O). They were initially placed in a box into which anaesthetic gas was directed, when deep anaesthesia had been achieved, a tracheotomy was performed and the gas was then channelled directly into a tracheal tube. The level of anaesthesia was monitored by testing withdrawal reflexes. In

order to prevent oedema of the cerebellum and disseminated coagulation, ampicillin (Ampivet; Novo, Bagsværd, Denmark; 10 mg, I.V.) and betamethasone (Betapred; Glaxo, Greenford, UK; 1 mg, I.V.) were given at the start of each experiment.

The skull was opened on the left side, and a substantial proportion of the forebrain on both sides was removed by aspiration. The remaining parts showed no sign of continuing activity. 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. Bleeding was controlled with Spongostan (Ferrosan, Søborg, Denmark). The bony tentorium and the dura above the cerebellum were removed. The cerebellar cortex was usually exposed caudally to the rostral border of the paramedian lobe. In some experiments, blood vessels connecting the dura and the cerebellum in the medial parts of lobules VI and VII (corresponding to the c1 zone) limited the area which could be studied. In some experiments the skull was removed even further caudally, so that the whole of the left paramedian lobe was exposed.

After decerebration the anaesthesia was terminated. 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 then built around the cerebellum with cotton-reinforced agar and the cortex was covered with warm paraffin oil.

The vertebral column was clamped and fixed, but cats decerebrated at this level can produce walking movements which would interfere with recordings. In order to prevent this, the muscle relaxant alcurone (Norcuron; Organon Teknika, Boxtel, The Netherlands) was given intravenously in low doses (about 0·5 mg) which were adjusted so that gross movements were eliminated while EMG recordings could still be obtained. This necessitated artificial ventilation. In experiments where different EMG recordings were to be compared, 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. In order to prevent drying of the cornea, artificial tears containing hypromellose (Isopto-plain; Alcon, Forth Worth, TX, USA) were applied to the eyes at regular intervals.

Stimulation and recording

Recordings of climbing fibre responses were made from the cerebellar cortex with monopolar silver ball electrodes (diameter 100–200 μm). The reference electrodes were usually in the neck muscles. Since the animals were not completely curarized, the electrodes sometimes picked up muscle activity. In such cases a second silver ball electrode was used as a reference electrode and placed on the cerebellar cortex. Care was taken to ensure that recordings were not dependent on the precise placement of the reference electrodes. The eyeblink response was monitored by EMG recordings from the orbicularis oculi muscle through two stainless steel electrodes about 5 mm apart, which were inserted into the upper eyelid about 5 mm above the margin. In some cases, the electrodes were placed so that a single motor unit could be isolated. This then permitted quantification of the responses by peristimulus time histograms.

Stimulation of the cerebellar cortex was done with the same monopolar silver ball electrodes used for surface recordings. Cathodal square wave pulses of 200 μs duration were used. The stimulus strength was between 50 and 800 μA. Pulse trains were used with frequencies of 150–400 Hz and durations of 10–150 ms, although usually the train duration was 30–40 ms. The anode was placed on the cerebellar surface. As with the recordings, different electrode placements were tested in order to ensure that the responses were not generated through the anode.

Stimulation of the periorbital area was done through two needle electrodes inserted through the skin of the lower eyelid, about 5 mm apart. The stimulus intensity was adjusted so that it was above threshold for evoking maximal climbing fibre responses in the c3 zone of the cerebellar cortex. This usually meant 2–3 mA.

Electrophysiological recordings were converted to digital data with an analog-to-digital converter from RC Electronics Inc. (Goleta, CA, USA). Analysis of the data was performed with their Computerscope software package.

Lignocaine injections

In three experiments, 0·5–1·0 μl of 4% lignocaine HCl (Xylocaine; Astra, Södertälje, Sweden) was injected into the brachium conjunctivum in order to block cerebellar output. The brachium was first localized by tracking with a stimulation electrode according to stereotaxic co-ordinates (3–3·5 mm lateral to the mid-line and 1–2 mm ventral to the horizontal zero plane as defined in Berman, 1968) at the border between the cerebellum and the inferior colliculus until a site was found from which EMG responses in the eyelid could be evoked with weak stimulation (< 20 μA). The same co-ordinates were then used to place a micropipette (tip diameter about 50–100 μm) attached to a Hamilton syringe. The injection site was later verified histologically.

RESULTS

Climbing fibre input from the periorbital area

The climbing fibre input to lobule V of the anterior lobe has been well characterized by a number of investigators (Oscarsson, 1980; Armstrong, 1990) and it is easy to identify the different zones by recording the characteristic climbing fibre responses from the cerebellar surface. Figure 1 shows the results from a typical experiment in which such recordings were made on both ipsi- and contralateral stimulation of the forelimbs and of the periorbital area. Figure 1*A* shows a dorsal view of the left cerebellar cortex, including lobules IV–VI and part of lobule VII. The c1, c2 and c3 zones are outlined schematically.

The c2 zone (shaded area in Fig. 1*A*), which is characterized by wide receptive fields, bilateral input and long latencies on forelimb stimulation (usually about 16–25 ms for ipsilateral and 20–30 ms for contralateral stimulation) received input from the periorbital area with slightly shorter latencies (15 and 18 ms respectively for ipsi- and contralateral stimulation). Examples of such responses are shown in Fig. 1*B*. The upper traces in the two pairs of

records were obtained on ipsilateral and the lower traces on contralateral periorbital stimulation. The climbing fibre input to the c1 and c3 zones from the forelimbs is only ipsilateral and has short latencies, usually about 13–15 ms. This input (hatched area in Fig. 1*A*) extended into lobule VI. Within the forelimb area of the c3 zone, a wedge-shaped area with short latency climbing fibre input from the face was found. This area extended from the caudalmost part of lobule Vc into lobule VI. This input was ipsilateral and had short latencies (9–12 ms). Records of these responses are shown in Fig. 1*B*. The recording sites are indicated in Fig. 1*A*. Clearly, this face area was in the c3 zone.

In some experiments, a face area could also be identified in the c1 zone. This was more caudally located, however, usually in lobule VII. As noted above, large blood vessels often made this area inaccessible and it was not studied systematically.

Eyelid responses evoked from the cerebellar cortex

Electrical stimulation of the cerebellar cortex with single shocks usually evoked no EMG responses in the eyelid (orbicularis oculi muscle) even with stimulus strengths as high as 1 mA. In most experiments, however, EMG activity could readily be evoked by trains of stimuli with frequencies above 100–150 Hz and durations over 10 ms. This EMG activity was of two distinct types, here called 'early' and 'delayed' responses, respectively. Examples are given in Fig. 2*A* and *B*, which show superimposed EMG responses in the orbicularis oculi muscle on stimulation of the cerebellar cortex (200 Hz, 400 μA, 30 and 60 ms, respectively). The records in Fig. 2*A* are early responses evoked from site 1 in Fig. 2*C*. This type of response was time-locked to the beginning of the stimulus train and usually had a latency of about 15–30 ms. The responses shown in Fig. 2*B*, evoked from site 2 in Fig. 2*C*, were time-locked to the termination of the stimulus train and had much longer latencies. Thus, when the duration of the stimulus train was increased from 30 to 60 ms, as shown in the lower records in Fig. 2*A* and *B*, the early responses evoked from site 1 had the same latency, whereas the late responses evoked from site 2 were delayed when the stimulus train was prolonged, and the latency measured from the end of the stimulus train was approximately the same, about 50 ms.

The latency of delayed responses was typically 40–60 ms, although latencies down to 25 ms were occasionally observed. The duration of the delayed responses was highly variable. In some cases the stimulation evoked only a single spike, whereas in others the response continued for 50–70 ms. To some extent this variability reflected general fluctuations in excitability, characteristic of the decerebrate preparation. Some animals went through cycles of about 30–60 min with periods of vigorous walking movements and lively reflexes alternating with periods during which the animal was still and the reflexes weaker.

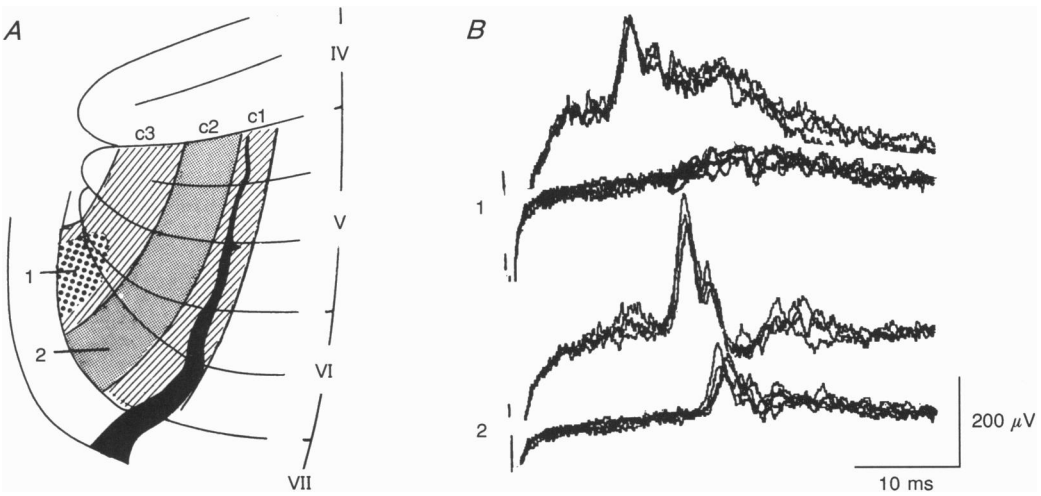

Figure 1. Location of the c1, c2 and c3 zones in lobules V and VI
A, outline of the left anterior lobe of the cerebellar cortex from one experiment. The hatched areas in c1 and c3 receive climbing fibre input from the ipsilateral forelimb. Shaded area is the c2 zone. The paravermal vein is shown in black. Dotted area receives climbing fibre input from the periorbital area with a latency of 10 ms. *B*, surface recordings from the cerebellar cortex. The upper two records are from site 1 in the c3 zone on periorbital stimulation on the left and right sides respectively. Ipsilateral stimulation evoked characteristic climbing fibre field potentials. The lower two records are from site 2 in the c2 zone and shows typical bilateral field potentials with longer latencies.

The stimulus strengths required to evoke delayed responses were highly variable between experiments. There was also a marked variability within experiments which reflected the excitatory state of the animal. The thresholds were rarely less than 150 μA and sometimes as high as 800 μA, even in the absence of curarization. In order to obtain responses which were sufficiently regular to allow systematic study, it was generally necessary to use

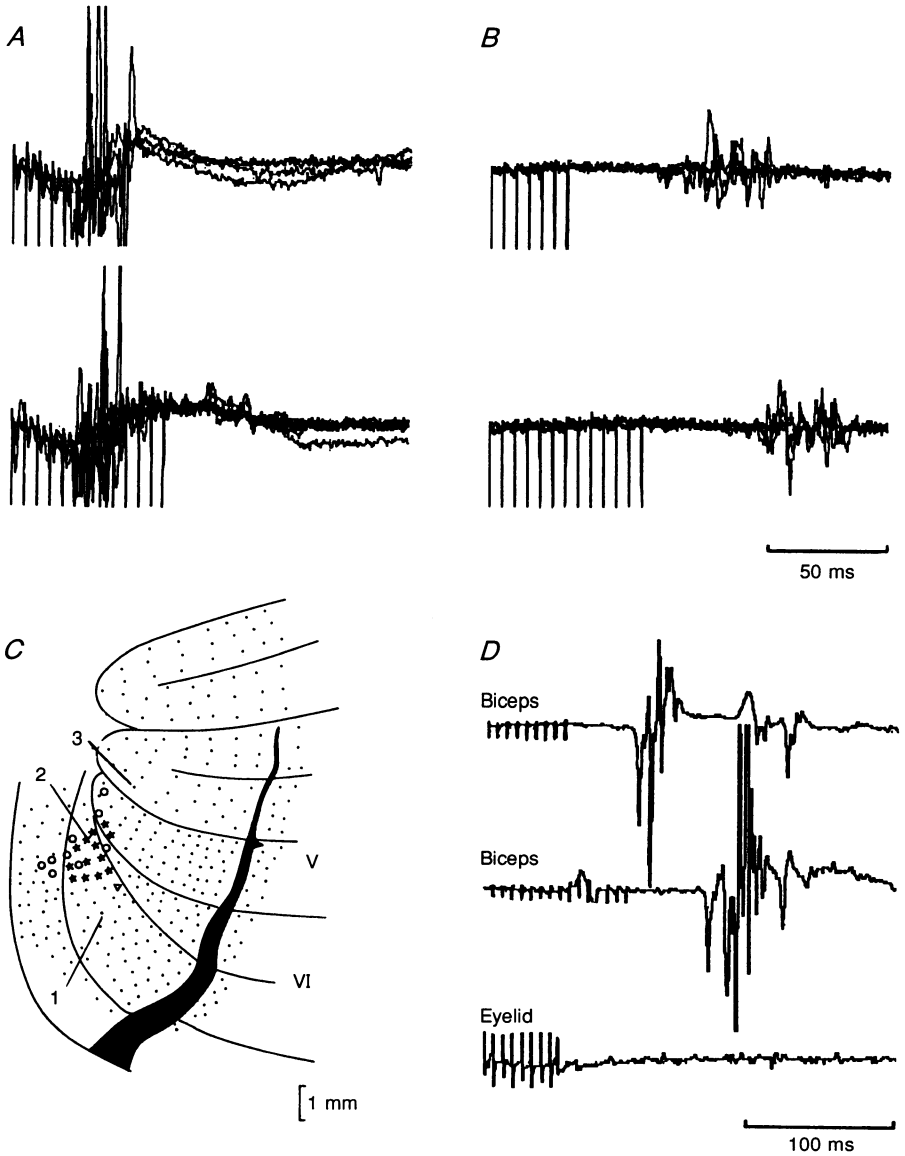

Figure 2. EMG responses in orbicularis oculi and biceps brachii muscles evoked by trains of stimuli to cerebellar cortex in the same experiment as Fig. 1

A, responses evoked in the orbicularis oculi muscle from site 1 in the c2 zone in lobule VI (shown in *C*) with trains of 30 (upper records) and 60 ms (lower records) duration respectively. Stimulation frequency was 200 Hz and the strength 400 μA. *B*, orbicularis oculi responses on 30 and 60 ms train stimulation of site 2 in the c3 zone. Stimulation parameters same as in *A*. *C*, correspondence between climbing fibre input from the periorbital area and delayed EMG responses evoked by cortical stimulation. Dorsal view of lobules V and VI of the left pars intermedia of the cerebellar cortex. Each dot represents a site investigated in one experiment. Triangles indicate sites of short latency (< 12 ms) climbing fibre field potentials on periorbital stimulation. Circles indicate sites from which delayed EMG responses could be evoked (30 ms trains, 200 Hz, 400 μA). Stars represent sites satisfying both criteria. Also indicated are the sites from which the early and delayed responses shown in *A* and *B* were evoked (1 and 2 respectively) and the area from which the forelimb responses shown in *D* were evoked (3). *D*, delayed EMG responses recorded from the biceps brachii muscle evoked by trains of stimuli to the forelimb area of the c3 zone (site 3 in *D*). Upper and middle traces demonstrate the delayed nature of the response. This stimulation evoked no response in the eyelid (bottom trace).

stimulus strengths at least twice that of the threshold, usually 300–500 μA. Both anodal and cathodal stimulation was tried. Early responses could be evoked by both types of stimulation, but only cathodal stimulation evoked delayed responses.

Early responses could be evoked from many parts of the cerebellar cortex and no consistent topographical pattern could be observed. In contrast, delayed responses were evoked only from areas receiving climbing fibre input from the face. This topographic specificity is illustrated in Fig. 2*C*, which shows a view of the anterior lobe of the cerebellar cortex. A large number of cortical sites were investigated by first recording surface potentials on periorbital electrical stimulation and then by recording EMG activity evoked in the orbicularis oculi by trains of stimuli to the cerebellar cortex (30 ms, 200 Hz). Sites from which delayed EMG responses could be evoked and sites at which short latency climbing fibre responses could be recorded are indicated with symbols.

The correspondence between climbing fibre input and motor output only holds for short latency climbing fibre responses and delayed EMG responses. The delayed responses shown in Fig. 2*B* were evoked from the site indicated with 2 in Fig. 2*C*. Climbing fibre responses with longer latency were normally recorded medial to this area, in the c2 zone, but delayed responses were never evoked from c2. Early responses were evoked from many areas, including the c2 zone. The early responses in Fig. 2*A*, for instance, were evoked from site 1, which was in c2. The only consistent observation regarding the topography of early responses was that they were never evoked from the centre of the areas receiving short latency climbing fibre input, although they could often be evoked from the periphery of such areas.

The same pattern of a close correspondence between climbing fibre input and of delayed responses to cortical stimulation was also observed for the forelimb, although it was not examined in detail. Thus, the site indicated by 3 in Fig. 2*C* was in the forelimb area of c3 and received short latency (< 14 ms) climbing fibre input from the superficial radial nerve. Delayed EMG responses were evoked in the biceps brachii muscle from this site (shown in Fig. 2*D*, upper and middle traces) but not in the eyelid (lower trace).

In a few initial experiments, EMG recordings were also made from other muscles of the face and neck in the hope that it would be possible to find a correspondence between sites in the cerebellar cortex and single face muscles. Although occasionally cortical stimulation evoked responses only or mainly in the eyelid, the stimulation usually resulted in responses in other face muscles as well. These attempts were therefore discontinued.

Evidence that the delayed, but not the early, responses arise via activation of Purkinje cell axons to the NIA neurones which control eyeblink, is presented below. Since the purpose of the present investigation was to identify cortical sites of eyeblink control, mainly delayed responses were investigated in these experiments. Unless otherwise specified, 'EMG response' or 'eyeblink response' thus refers to this type of response.

Nature of delayed responses

The observations that delayed responses were time-locked to the termination of the stimulation and that the latency after termination was so long, suggest that they result from hyperpolarization of the neurones in the NIA anterior followed by a rebound excitation. The experiments described in this section were designed to examine this possibility.

If the responses are mediated by a period of inhibition, factors which increase the duration of the IPSP in the NIA should increase the latency of the responses, measured from the termination of the stimulation. Thus, increasing the duration, frequency or strength of the cortical stimulus should increase the amplitude and duration of the IPSP in the NIA neurones and delay the rebound excitation that generates the EMG response.

In one set of experiments, the duration of the stimulus train was varied while other parameters were held constant. The result of one such experiment is illustrated in Fig. 3*A* and shows examples of responses in a single motor unit evoked by stimulus trains of three different durations, while Fig. 3*B* shows the quantitative relationship between stimulus duration and latency, measured from the termination of the stimulus train. Inspection of the EMG records in Fig. 3*A* indicates that the latency is relatively constant, but the regression line reveals a small but systematic increase in latency ($r = 0{\cdot}79$, $P = 0{\cdot}0005$).

Increasing the frequency in the stimulus train, at a constant duration, also prolongs the latency of the response. This is illustrated for three different frequencies in Fig. 3*C* and *D*. Again, the effects were quite consistent and statistically significant ($r = 0{\cdot}91$, $P < 0{\cdot}0005$). It was not possible to study the effects of varying stimulus strength. When the stimulus strength was close to threshold, the responses were too irregular to permit systematic study. When the strength was sufficiently above threshold for evoking regular responses, the latency variations seemed small.

Cerebellar damage has long been known to produce tonic changes in neuronal excitability (Dow & Moruzzi, 1958). It has recently been shown that cerebellar lesions produce a small decrease in the amplitude of unconditioned nictitating membrane responses (Welsh & Harvey, 1989), suggesting that the cerebellum exerts a tonic facilitatory influence on the eyeblink motoneurones. If stimulation of the cerebellar cortex inhibits the interpositus neurones controlling eyeblink, one would expect a reduction in the spontaneous background EMG activity during the stimulation and during the period between the stimulation and the appearance of the response. This was confirmed in many experiments. In some animals, no background

activity could be recorded, but in all eight cases where background activity was present, it was reduced by the cortical stimulation. Two cases are illustrated in Fig. 4*A* in the form of superimposed raw EMG records. In the upper trace, it can be seen that during and after the stimulation there was a period of reduced EMG activity preceding the onset of the response. The second case, from a different animal, shows a reduction in the background activity even when the stimulation strength was below threshold for eliciting delayed responses. The stimulus strength in this case was 150 μA and the threshold was about 300 μA.

The suggested mechanism underlying delayed responses also implies that it should be possible to inhibit them by stimulating the cerebellar cortex at the time when the NIA neurones are supposed to be active. This was tried in five animals and the expected inhibition was always observed. The upper record in Fig. 4*B* shows a typical delayed response evoked by a train of stimuli (400 μA) to the c3 zone. EMG activity in fifty trials was rectified and then averaged. When a single 50 μA shock was applied to the same site just before the expected onset of the response, the response was almost completely abolished. The latency of this inhibition was usually about 10 ms. It was a regular and noteworthy finding that although the stimulus strength required to evoke reliable delayed responses was usually very high, in the order of 300–500 μA, a much weaker shock (30–50 μA) was usually sufficient to inhibit the response.

Stimulating the cerebellar cortex will activate not only Purkinje cell axons projecting to the NIA. The stimulation will also produce antidromic activation of both mossy and climbing fibres. Thus, the responses evoked by cortical stimulation could be mediated by brainstem systems which receive collaterals from climbing or mossy fibres. In order to test this possibility, the effect of injecting lignocaine into the brachium conjunctivum, the output pathway from the NIA,

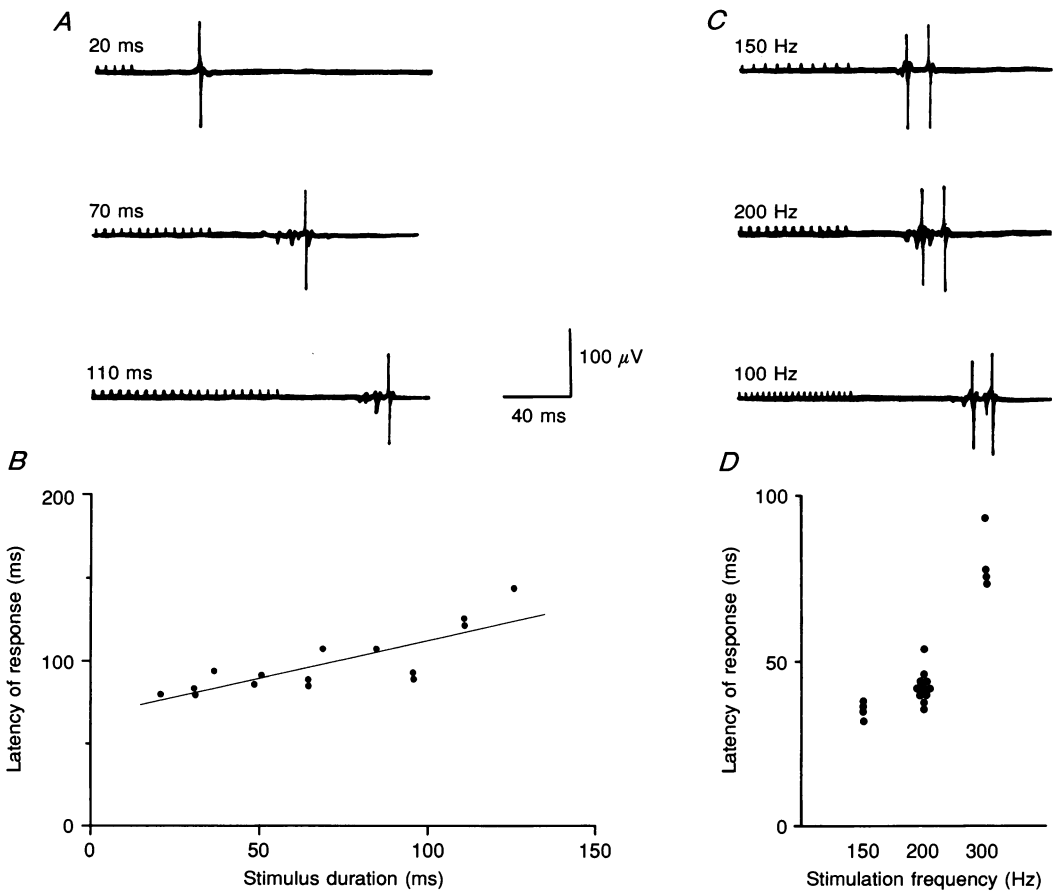

Figure 3. Effects of stimulus duration and stimulation frequency on latency of delayed responses

A, records of EMG responses in the orbicularis oculi muscle evoked with three different stimulus durations at 200 Hz. *B*, scatter plot and regression lines of stimulus duration *versus* latency of response. Latency measured from termination of the stimulation train. $r = 0{\cdot}79$, $P = 0{\cdot}0005$. *C*, records of EMG responses evoked with three different stimulation frequencies, 150, 200 and 300 Hz respectively, and constant duration. *D*, scatter plot of stimulation frequency *versus* latency from end of stimulation.

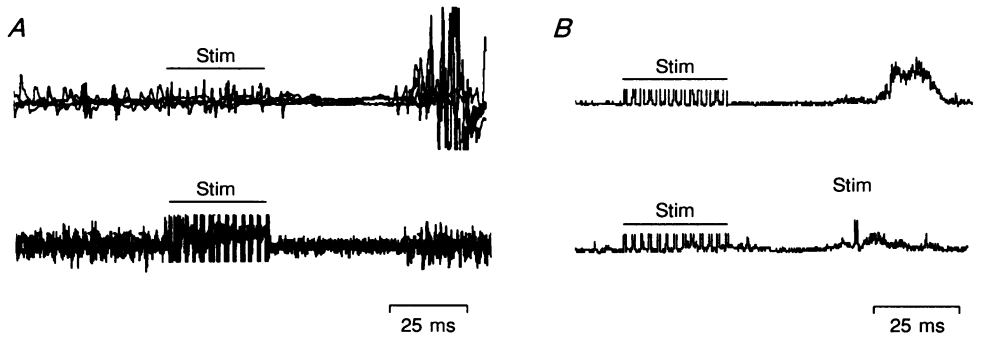

Figure 4. Inhibition of ongoing EMG activity by stimulation of the cerebellar cortex
A, superimposed EMG records from two different experiments showing a decrease in spontaneous EMG activity during and after stimulation. Stimulus strength was above (400 μA, upper record) and below (150 μA, lower record) the threshold for eliciting delayed responses. Note that shock artifacts in the upper traces have amplitudes similar to the EMG. *B*, upper trace is a rectified and averaged EMG record (based on 50 trials) of a typical delayed response evoked by cortical stimulation (500 μA, 300 Hz, 33 ms). The lower record shows the effect of applying a single stimulus (50 μA) to the same cortical site 36 ms after the end of the train.

was examined in three animals. These injections abolished both early and delayed responses. The results of one such experiment are shown in Fig. 5. Figure 5*A* and *B* show early and delayed responses, respectively. The upper traces show control responses before injection of 1·0 μl of lignocaine. Within 1 min of the injection, both types of response were dramatically reduced (middle traces), and both had reappeared 15 min later (bottom traces).

Correspondence between climbing fibre input and motor output

The results illustrated in Fig. 3 suggest a close correspondence between short latency climbing fibre input and motor output. This correspondence was investigated in more detail in a further set of experiments. An example is shown in Fig. 6. A number of sites were investigated in

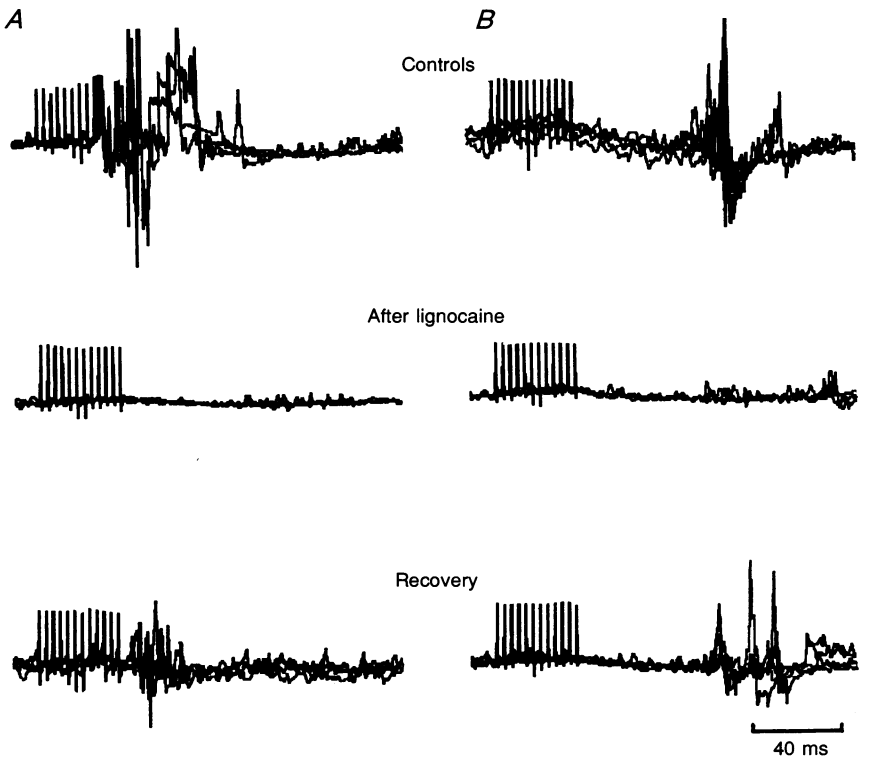

Figure 5. Effect of injecting 1 μl lignocaine into the brachium conjunctivum on early (left column) and delayed (right column) responses
Upper records (4 superimposed) are controls. Middle traces were obtained 5 min after the lignocaine injection. Lower traces were obtained after a recovery period of 15 min.

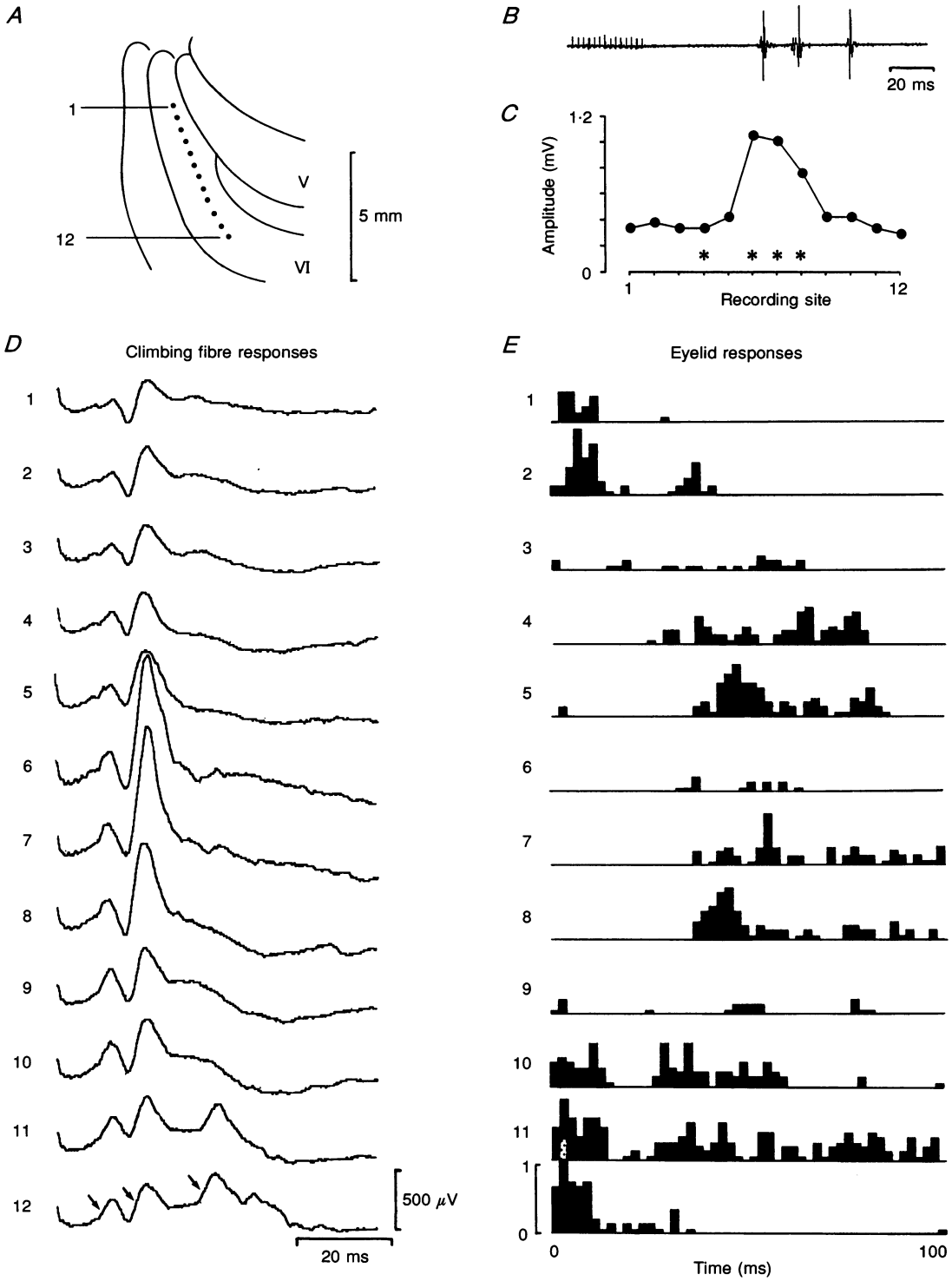

Figure 6. Delayed responses evoked from different sites across a cortical zone
A, sites of recording and stimulation in lobule VI of the left anterior lobe. *B*, sample record of delayed response evoked by stimulation of site 7. *C*, correspondence between climbing fibre field potential amplitude and sites from which delayed responses were evoked. Plot shows amplitudes (baseline to peak) of the field potentials (shown in *D*) in sites 1–12. Asterisks indicate sites from which only delayed responses (shown in *E*) with long latencies (> 30 ms) were evoked. *D*, field recordings from cerebellar surface on ipsilateral periorbital stimulation. Each record is an average of 20 trials. The three arrows in trace 12 indicate (from left to right) a mossy fibre response, a short latency climbing fibre response characteristic of the c3 zone and a long latency climbing fibre response in the c2 zone. Numbers 1–12 correspond to the sites indicated in *A*. *E*, post-stimulus time histograms of unit EMG activity after stimulation of the same sites of the cerebellar cortex (400 Hz, 35 ms, 500 μA). Histograms start at the termination of stimulation.

lobule VI, along a folium approximately perpendicular to the orientation of the zones. Figure 6*A* shows a dorsal view of the cerebellar cortex including the left lobules V and VI with twelve recording and stimulation sites indicated. Averaged field potentials from the cerebellar surface from each site are shown in Fig. 6*D*. The largest responses are in sites 6–8. The latency and the location of these responses indicates that they are in the c3 zone. Each site was also stimulated with a 40 ms pulse train (200 Hz, 500 μA). A sample response evoked by this stimulation is shown in

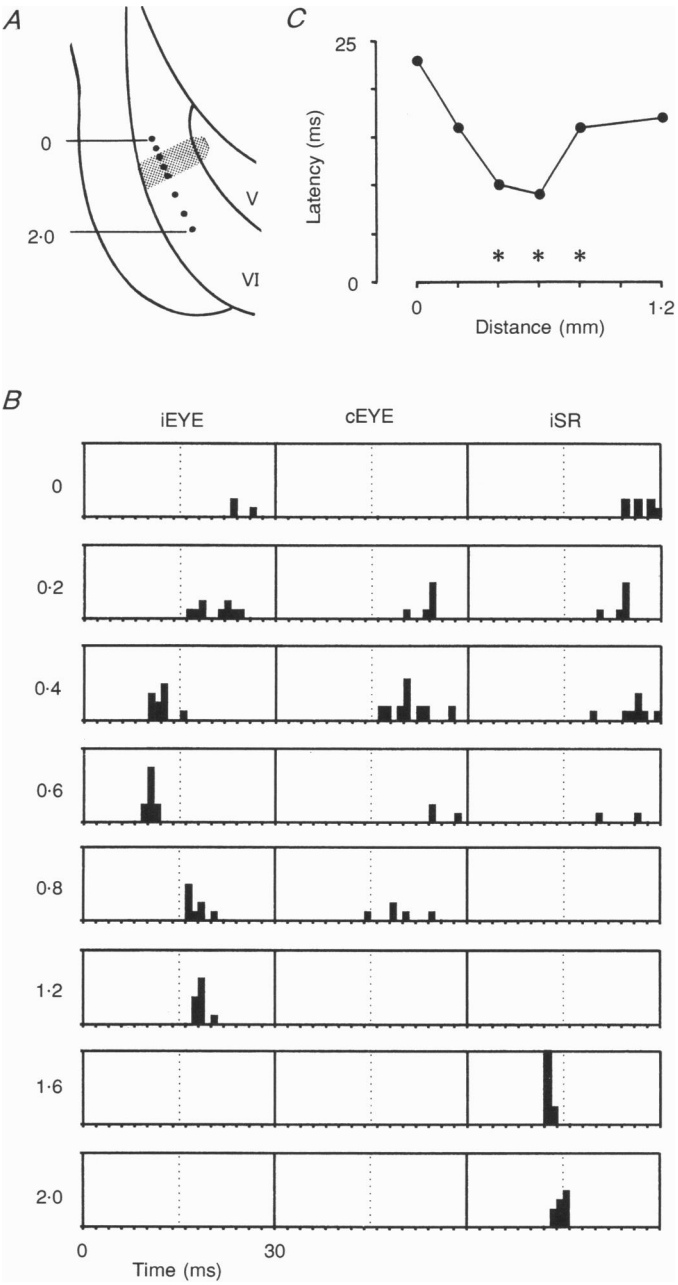

Figure 7. Latencies of unitary climbing fibre responses in c3 zone in lobule VI
A, location of recording tracks in the cerebellar cortex. Shaded area indicates zone from which delayed EMG responses (latency > 30 ms) could be evoked (400 Hz, 35 ms, 150 μA). *B*, post-stimulus time histograms showing distribution of climbing fibre responses on ipsilateral (left column) and contralateral (middle column) periorbital stimulation and stimulation of the ipsilateral superficial radial nerve (right column). Numbers to the left correspond to sites in *A* and indicate distances (in mm) from site 0. Each histogram was based on 20 trials with the first cell encountered in the track or, if this unit was lost, the second one up to a depth of 100 μm. The time span of each histogram is 30 ms. *C*, plot of latency of climbing fibre responses against distance from reference site. Asterisks indicate sites from which delayed responses were evoked.

Fig. 6*B*. Post-stimulus time histograms showing the activity of this motor unit during the 100 ms after the cortical stimulation are shown in Fig. 6*E*. From sites 1–2 and 10–12, typical early responses were evoked. Delayed responses (here defined as those having post-termination latencies of at least 30 ms) were evoked from sites 4–8. The activity evoked from sites 3–5 and 9 (possibly also 10 and 11) seemed to be a mixture of the two types of response. In histograms 3 and 5, for instance, there was probably both a small early response and a delayed response. Notice how the latency of the delayed response increases from histogram 3 to 4 and from 4 to 5. Due to possible fluctuations in excitability, the size of the responses do not permit any firm conclusions. The correspondence between long latency delayed responses and amplitude of climbing fibre responses, however, is clear. This is also shown in Fig. 6*C*, where climbing fibre amplitude is plotted against site and the sites from which only delayed responses were evoked are indicated by asterisks.

Similar mapping experiments, with similar results, were made in six other animals, although in four of these they were not as complete. In some cases, recordings had to be discontinued because of gross movements by the animal, so that only a smaller number of sites could be investigated. Even if there was less precision in the mapping in these cases, the basic pattern was always observed. The centre of the area with short latency climbing fibre input corresponded closely with the centre of the area from which delayed responses were evoked.

In the experiment illustrated in Fig. 7, microelectrode recordings were made from individual Purkinje cells in a number of tracks along a 2 mm segment across the c3 zone in the left cerebellar cortex (Fig. 7*A*). Purkinje cells were identified by the appearance of climbing fibre responses, which are easily recognizable in the form of characteristic complex spikes or by large dendritic EPSPs in the superficial layers of the cortex (Eccles, Ito & Szenta'gothai, 1967). The post-stimulus time histograms in Fig. 7*B* show the latency of climbing fibre responses in each track on stimulation of the left lower eyelid, the right lower eyelid and the left superficial radial nerve. Climbing fibre input from the ipsilateral periorbital area was recorded in six of the eight tracks, covering a width of 1·2 mm. Most of these had quite long latencies, for example, more than 25 ms in track 0. The shortest latency encountered was 9–10 ms in tracks 0·4–0·6. Delayed EMG responses could only be evoked from the cerebellar surface above three of the tracks (0·4–0·8). These are indicated by asterisks in the plot of latency (of climbing fibre responses) against distance. Thus, the correspondence between short latency climbing fibre input and motor output is quite striking.

Such a good correspondence was not obtained in all experiments. Usually, the area from which delayed responses could be evoked was wider than in this case. In many experiments, the width of this zone was about 1–2 mm as for instance in the experiment shown in Fig. 6. To some extent this was related to stimulus strength. In order to construct the time histograms of Fig. 6*D*, it was necessary to employ a stimulus strength which evoked delayed responses with a high degree of regularity, in this case 500 μA. When weaker stimuli could be used, the area from which responses could be evoked was considerably narrower, down to about 0·5 mm. Because of the labile nature of the responses, however, it was difficult to obtain a reliable estimate of the area. In the experiment illustrated in Fig. 7, the stimulus strength used was 150 μA and delayed responses occurred in about 50 % of trials.

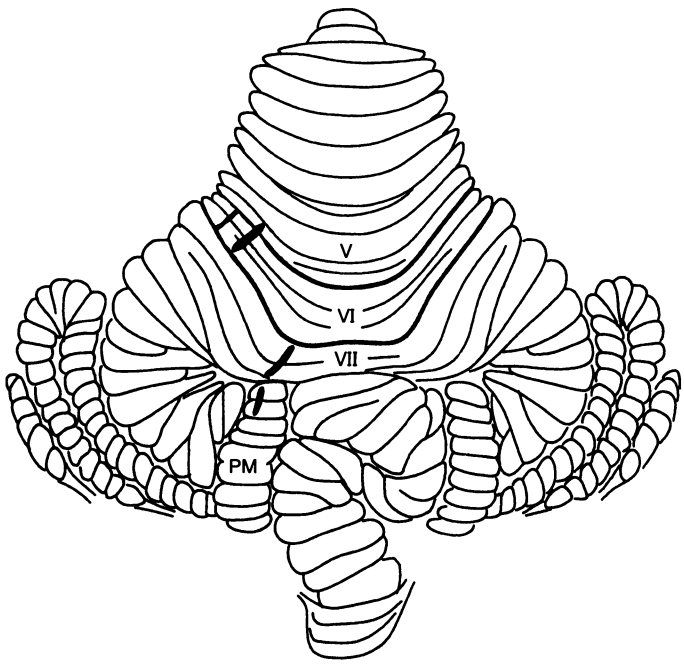

Figure 8. Schematic drawing of the cat cerebellum (adapted from Larsell, 1970)
Areas in lobules V, VI, VII and paramedian lobule (PM) receiving short latency climbing fibre input from the periorbital area and from which delayed EMG responses could be evoked in the orbicularis oculi muscle are indicated in black.

It is worth noticing that the units with the shortest latency input from the ipsilateral eye also received a long latency input from the contralateral eye as well as some input from the forelimb.

Other loci of eyeblink control

Similar mapping experiments in other animals confirmed the above results and also revealed the existence of at least three additional areas that might be involved in eyeblink control. One of these was in a more lateral part of lobule VI. It could be either a more lateral part of the c3 zone or the y zone. This was seen in all of the six animals in which this part of the cortex was investigated. A third area was observed in four out of four animals in a medial part of in lobule VII, extending into VI, and was probably in the c1 zone. Finally, a fourth area was located in the most rostral part of the c3 zone of the paramedian lobule and was seen in both animals studied. In all of these cases both short latency climbing fibre responses and delayed eyelid responses were present. These findings are summarized in Fig. 8. Although all these areas could be identified in all animals in which they were accessible, there was some variability in their extent and exact location. For instance the c3 area in the anterior lobe frequently extended into the most caudal folium of lobule V. The c1 area in lobule VII was highly variable both in its exact location but also in the consistency of the climbing fibre input and the regularity with which eyelid responses could be evoked from it. Because of the limited accessibility of this area, it was not studied systematically.

DISCUSSION

The nature of cortically evoked responses

Electrical stimulation of the cerebellar cortex in order to elicit movements has been employed by a number of previous investigators, e.g. Clark (1939), McDonald (1952) and others. Since the stimulus parameters employed in these studies were very different from the ones used here, comparisons are difficult.

In the present study, trains of stimuli to the cerebellar cortex produced two distinct types of EMG activity in the orbicularis oculi muscle, early and delayed responses. The early responses are characterized by a fixed latency (usually 15–30 ms) and by the fact that the cortical sites from which they can be evoked have no clear relationship to climbing fibre input, although they were often evoked from areas adjacent to those receiving climbing fibre input. The delayed responses, on the contrary, have long latencies which are time-locked to the termination of the stimulus train and can only be evoked from sites which receive short latency climbing fibre input.

Since both types of responses were abolished by lignocaine injections into the brachium conjunctivum, it is reasonable to assume that they are mediated by the interpositus nucleus. The evidence strongly suggests that the delayed EMG response is generated by a rebound excitation after inhibition of neurones in the NIA. This mechanism is illustrated in Fig. 9. Cortical stimulation could excite the interpositus neurones in two ways, either (a) by direct excitation through antidromic activation of climbing or mossy fibre collaterals or nucleo-cortical fibres or (b) by rebound excitation after inhibition through activation of Purkinje cells. IPSPs have been observed, for example, in the lateral vestibular nucleus on cathodal stimulation of the cortex with stimulus strengths similar to those employed here (Ito & Yoshida, 1966; Andersson & Oscarsson, 1978*a*). Rebound excitation in interpositus neurones after inhibition has been described both *in vivo* (Ito & Yoshida, 1966; Andersson & Hesslow, 1987) and *in vitro* (Jahnsen, 1986).

Several observations suggest that this is the mechanism behind the delayed EMG response. The response was time-locked to the termination of the stimulus train and could be delayed by prolongation of the train. The response had a very long latency after the stimulus termination, often more than 50 ms. The latency could be increased by increasing stimulation frequency and thus, presumably, the amplitude and duration of the IPSP in the NIA neurones. Stimulation in the centre of the zone produces responses with longer latencies than stimulation in the periphery, where presumably a smaller number of blink-related Purkinje cells would be activated. In the interval between the stimulus train and the response, the level of background EMG activity was reduced, indicating a decrease in excitatory drive on the motoneurones. Finally, the delayed response could be inhibited by appropriately timed stimulation of the cerebellar cortex.

It is difficult to know exactly how the cortical stimulation activated Purkinje cells, but it seems likely that both climbing fibre and parallel fibre terminals were excited by the stimulation. Of these mechanisms, climbing fibre activation was probably more important, because it would be more efficient in triggering the Purkinje cells. Furthermore, antidromic activation of the climbing fibres would, via collaterals and via electrotonic coupling in the olive, activate a large number of Purkinje cells in the same microzone. The fact that very high stimulus strengths were necessary to produce the delayed responses, however, suggests that a large group of Purkinje cells had to be activated in order to generate a sufficient IPSP. It is noteworthy that, although several shocks with a stimulus strength of 300–500 μA were necessary to evoke a delayed response, a single shock of 50 μA was sufficient to block the response. It is possible that weaker stimuli were sufficient to produce an IPSP in the NIA neurones, but that a rebound excitation which would cause a delayed response required a larger IPSP produced by activation of a larger number of Purkinje cells.

The early EMG responses may have resulted from antidromic activation of mossy fibres, which via collaterals

would excite the NIA neurones or activation of nucleocortical fibres. Cortical stimulation probably also activated climbing fibres antidromically, and impulses in climbing fibre collaterals to the NIA could have contributed to the response. However, if this were the major mechanism contributing to the early responses, one would have expected a stronger topographical relation to the climbing fibre input from the periorbital area. The early responses had rather long latencies, usually more than 15 ms. Together with the fact that these responses required trains of stimuli, this suggests that they require temporal summation.

It is puzzling that early responses were not evoked from sites which were effective in producing delayed responses. It seems unlikely that these areas would lack the relevant mossy fibre input. A possible explanation is that stimulation of eyeblink areas of the cerebellar cortex activated the neuronal elements responsible for the early responses, i.e. probably mossy fibres, but that Purkinje cell inhibition prevented early responses from occurring. Stimulation outside the eyeblink area could activate the same mossy fibres but would not cause inhibition of the NIA neurones which mediate eyeblink. This would explain the observation that early responses were often evoked from areas adjacent to those from which delayed responses were evoked. This interpretation is consistent with the observations by Andersson & Oscarsson (1978*a*) that early EPSPs in the lateral vestibular nucleus, probably due to activation of mossy fibre collaterals, were only evoked from sites not containing Purkinje cells projecting to this nucleus (i.e. sites outside the b zone).

Identification of loci of eyeblink control

The findings presented above are essentially consistent with earlier studies showing that the main mossy and climbing fibre input from the face is to lobule VI. Snider & Stowell (1944), Miles & Wiesendanger (1975*a*, *b*) and Cody & Richardson (1979) all found powerful input from trigeminal afferents to the hemispheral part of lobule VI and to adjacent areas in lobules V and VII. Eyeblink has also been observed after intracerebellar stimulation of the border between lobules V and VI in monkeys (Ron & Robinson, 1973). These studies were made before the zonal organization of the cerebellar cortex was clarified (Oscarsson, 1980) and it is impossible to know which zones were studied.

As summarized in Fig. 9, four different areas were found which are likely to control eyeblink. Two of these were located in HVI, one medial and one lateral. The medial area was clearly in the c3 zone. It was continuous with the forelimb area of the c3 zone in lobule V and had short latency climbing fibre input from the cornea and periorbital area. The input was mainly, although not exclusively, ipsilateral. The more lateral area could either be in the y zone or in the lateral part of c3 (Ekerot & Larson, 1979). A third area of climbing fibre input was located in the c1 zone of lobule VII and a fourth in the c3 zone of the paramedian lobe. It can by no means be excluded that there are other areas in the cerebellar cortex which are directly or indirectly involved in eyeblink control. Indeed, it seems likely that there is a c1 representation in the paramedian lobe. Since only the

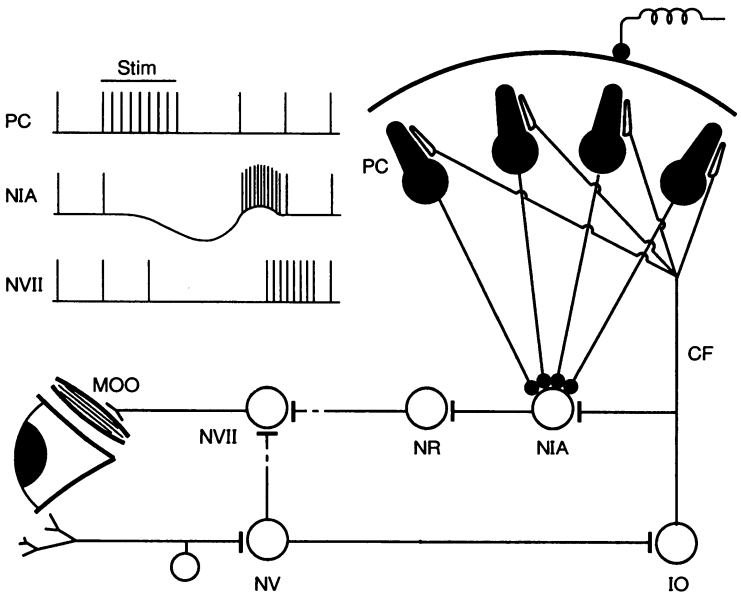

Figure 9. Wiring diagram of cerebellar circuit controlling eyeblink
Purkinje cells (PC) in a c3 microzone receive climbing fibre input (CF) from the eye via the trigeminal nucleus (NV) and the inferior olive (IO). The output from the cortex is via the nucleus interpositus anterior (NIA), the red nucleus (NR) and the facial nucleus (NVII) to musculus orbicularis oculi (MOO). The inset shows hypothesized activity in Purkinje cells, interpositus neurones and motoneurones.

surface of the cerebellum was investigated, areas which are buried in the fissures would be missed. Furthermore, Nagao, Ito & Karachot (1984) have reported that eyeblinks could be evoked from the rabbit flocculus. This finding is puzzling, because the flocculus presumably does not project to the NIA. However, this area in the flocculus may be concerned with a different aspect of eyelid control. Eyelid movements may accompany reflex eye movements which are known to be controlled by the flocculus.

Since all the areas identified here project to the NIA and since delayed responses could be evoked from all of them, it seems likely that they all contribute to the control of the orbicularis oculi muscle. However, there are two methodological problems which complicate this interpretation. The first concerns the resolution of the stimulation technique. Since quite high stimulus strengths were usually necessary to evoke reliable EMG responses, it is likely that a large part of the zone had to be stimulated. But this means that the width of the area from which responses could be evoked was probably considerably wider than the area containing the relevant Purkinje cells. Thus, although the centre of the area defined by input corresponded well with the centre of the area defined by motor output, it was impossible to compare the extent of these two entities. It is a reasonable working assumption, however, that Purkinje cells controlling eyeblink are confined to the area with the shortest latency climbing fibre input.

A second problem derives from the fact that the short latency climbing fibre input is paired, so that, for instance, some olivary cells project to both c1 and c3. Antidromic stimulation of climbing fibres in one zone will, by axon reflexes as well as through electrotonic coupling in the olive, evoke climbing fibre responses in the other zone (Armstrong & Harvey, 1966). Thus, even if only one zone in a pair controlled eyeblink, it would be possible to evoke delayed responses from both of them.

Studies of the climbing fibre input to the cerebellar cortex suggest that the functional unit of the cerebellum is a microzone or microcomplex, and it has been proposed that each microzone controls a certain motor mechanism, for instance a single muscle or muscle group (Andersson & Oscarsson, 1978*b*; Oscarsson, 1980). The climbing fibre input to the microzone signals a problem that could be corrected by changing the activity in the muscles controlled by that zone and does this by depressing the efficacy of the parallel fibre synapses which are active at the time (Marr, 1969; Albus, 1971; Gilbert, 1974; see Ito, 1984 for further references).

With respect to eyeblink, this implies the existence of one or more microzones controlling the orbicularis oculi muscle and receiving climbing fibre input whenever something that threatens the eye occurs. The correspondence between climbing fibre input and motor output demonstrated here is thus in good agreement with this view of cerebellar function.

Implications for classical conditioning

Although the experiments reported here do not address the issue of cerebellar learning *per se*, they do have some relevance for the interpretation of lesion studies. Yeo *et al.* (1985*b*) reported that lesions including most of the ipsilateral cortex of lobule HVI, but sparing NIA, abolished conditioned responses and prevented relearning. Others have found that cortical lesions have only small or transitory effects on conditioned responses (Lavond *et al.* 1987). Yeo & Hardiman (1992) also found some recovery after HVI lesions. Some authors have suggested that these results could be reconciled if there were additional areas of eyeblink control outside HVI. The present findings of areas in lobules V and VII as well as in the paramedian lobe support this interpretation. These areas might, perhaps after a period of adaptation, take over the control function lost by the lesion.

It should be stressed here, that the lesion studies cited above have all used rabbits and it is possible that the exact location of eyeblink areas differ between species. However, the basic findings that there are several such areas located in the c1 and c3 zones probably holds for rabbits as well.

Another limitation of the generalizability of the present results stem from the fact that many studies focus on a different aspect of the eyeblink. In rabbits, the eyeblink not only involves closure of the eyelids but also horizontal movement of the nictitating membrane. The latter is caused by the retractor bulbi muscle which is innervated by neurones in the accessory abducens nucleus (Gray, McMaster, Harvey & Gormezano, 1981). Thus, the eyelid and nictitating membrane responses are controlled by different cranial nerves. Although it seems likely, it is not necessarily the case that the same cerebellar sites control both responses.

It is now clear that conditioning leads to bilateral learning. For instance, Disterhoft, Kwan & Lo (1977), using tone conditioning in rabbits, observed small conditioned responses on the side contralateral to training. Weak contralateral responses were also observed in decerebrate cats when ipsilateral forelimb stimulation was used as the conditioned stimulus (Hesslow, 1994). It has been observed (e.g. Yeo *et al.* 1985*a*) that when ipsilateral conditioned responses have been abolished by unilateral lesions, subsequent training on the other side leads to much more rapid acquisition. Since the ipsilateral responses were abolished, the contralateral responses were presumably mediated by the contralateral side. If the unconditioned stimulus reaches the cerebellum via the climbing fibres, then the finding of a weak contralateral climbing fibre input to the c3 zone might explain these observations.

It was suggested by Yeo, Hardiman & Glickstein (1985*c*) that the pathway for the conditioned stimulus is through mossy fibres originating in the pontine nuclei or the nucleus reticularis tegmenti pontis and projecting to the cerebellar cortex while the unconditioned response would

be relayed through the climbing fibres. A problem with this hypothesis is that there may be no or only sparse mossy fibre collaterals to the NIA from the pontine nuclei (McCrea, Bishop & Kitai, 1977; Dietrichs, Bjaalie & Brodal, 1983). The afferents transmitting the CS input would thus not be able to drive the interpositus neurones and only non-specific mossy fibre inputs from other sources could provide such drive. Since the Purkinje cells are inhibitory, it would then be difficult to understand how the Purkinje cells could generate the conditioned response. The strong rebound activity after inhibition of the nuclear cells observed in the present work suggests a possible mechanism for generating the conditioned responses without a concomitant specific excitation of the NIA. This mechanism would also account for the otherwise puzzling fact that the minimum latency of conditioned eyeblink responses is usually more than 100 ms. Normally, the latency of the conditioned response is such that the maximum closure of the eye coincides in time with the appearance of the unconditioned stimulus. When the interstimulus intervals are as short as 50–100 ms, however, this no longer holds. The latency of the conditioned response never seems to go below 70–80 ms (Mackintosh, 1974).

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