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RESEARCH ARTICLE

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Inhibition of the inferior olive during conditioned responses in the decerebrate ferret

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Abstract Output from the interpositus nucleus can inhibit the inferior olive, probably via the GABA-ergic nucleo-olivary pathway. It has been suggested that the function of this inhibition might be to regulate synaptic plasticity resulting from parallel fibre/climbing fibre interaction in cerebellar Purkinje cells, by providing negative feedback information to the olive. Thus, when a learned response, generated by the interpositus nucleus, reaches a sufficient amplitude, the olive would be inhibited and further learning blocked. This suggestion was tested in a classical conditioning paradigm. Decerebrate ferrets were trained using electrical skin stimulation of the forelimb as the conditioned stimulus (CS) and periorbital stimulation as the unconditioned stimulus (US). Climbing fibre responses evoked in Purkinje cells by the US were recorded as surface field potentials in the part of the c3 zone controlling eyeblink. It was found that the CS did not inhibit the olive at the beginning of training, but when conditioned responses were large, the olive was inhibited by the CS in some animals. After a number of unpaired CS presentations, which caused extinction of the conditioned response, the inhibition disappeared. The size of individual conditioned responses correlated negatively with the size of the climbing fibre responses evoked by the US. Climbing fibre responses evoked by direct stimulation of the olive were also inhibited. It was concluded that cerebellar output during performance of a conditioned response inhibits the inferior olive. The results thus support the hypothesis of a cerebellar locus of conditioning and are consistent with the proposed role of cerebello-olivary inhibition.

Key words Cerebellum · Classical conditioning · Eyeblink · Inferior olive · Inhibition · Ferret

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Introduction

There is both anatomical and physiological evidence that the pathway from the interpositus nucleus to the inferior olive is inhibitory. The olive-projecting neurones in the interpositus have been shown to be GABA-ergic (Nelson and Mugnaini 1989). Direct stimulation of the brachium conjunctivum in cats produced a strong long-latency inhibition of the olive (Hesslow 1986). High-frequency climbing fibre activation, which is accompanied by increased firing in interpositus neurones, leads to a depression of the inferior olive which is dependent on the brachium conjunctivum and which can be abolished by injection of a GABA-blocking agent into the olive (Andersson and Hesslow 1987a, b; Andersson et al. 1988).

The physiological role of this inhibition is not known, but one possibility is suggested by the theory that the cerebellum participates in motor learning. It has been proposed by Marr (1969) and Albus (1971), and experimentally supported by Ito and coworkers (for review see Ito 1984), that climbing fibre impulses cause a modification of the parallel fibre – Purkinje cell synapses in the cerebellar cortex. A wiring diagram with the relevant structures for eyeblink conditioning is shown in Fig. 1. We have suggested that the function of the nucleo-olivary inhibition might be to regulate the size of learned responses (Andersson et al. 1988). When the interpositus neurones generate a response of a sufficient size, partly as a consequence of previous climbing fibre input to the cerebellar cortex, the olive would be inhibited and further strenghening of the response prevented. The nucleo-olivary inhibition would thus function as a negative feedback system regulating the amplitudes of learned responses.

A first step towards exploring this idea is to determine whether learned responses are indeed associated with an inhibition of the olive. The experimental work reported here was designed to do this in the context of classical eyeblink conditioning. There is now substantial evidence that the neural mechanisms supporting eyeblink conditioning are within the cerebellar cortex. Lesions of the an-

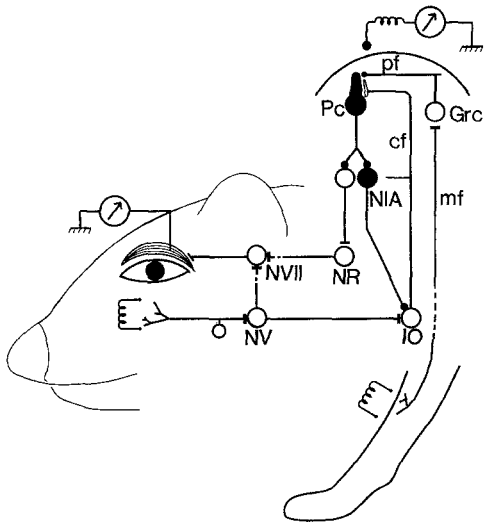


Fig. 1 Experimental set-up with wiring diagram of the circuit controlling eyeblink. Percutaneous stimulation electrodes were placed below the eye and on the forelimb. Recordings were made from the orbicularis oculi muscle and from the cerebellar cortex. Hypothetical pathway for the unconditioned stimulus is through the trigeminal nucleus (*NV*), the inferior olive (*IO*) and the climbing fibres (*cf*) to the Purkinje cells (*Pc*). Hypothetical mossy fibre input from the forelimb is via mossy fibres (*mf*), granule cells (*Grc*) and parallel fibres (*pf*). Output is from the cerebellar cortex to the anterior interpositus nucleus (*NIA*), red nucleus (*NR*) and facial nucleus (*NVII*). Notice also the inhibitory pathway from NIA to IO

terior interpositus nucleus (NIA) abolished conditioned eyeblink responses (CRs) in rabbits (McCormick and Thompson 1984; Yeo et al. 1985a). Lesions in the hemispheral part of lobule VI of the cerebellar cortex also abolished CRs, as did lesions of the inferior olive (Yeo et al. 1985b, 1986). Pharmacological inactivation of the NIA and overlying cortex with the GABA-agonist muscimol during training prevents learning, although such blockade of the red nucleus, an output relay form the cerebellum, does not have this effect (Krupa et al. 1993). It is puzzling that Welsh and Harvey (1991) found that animals could learn while the NIA was inactivated by lidocaine, but if the lidocaine injection was limited to the NIA, a cortical learning mechanism could have been unaffected. Further evidence for a cerebellar locus of conditioning is the observation that inhibition of interpositus neurones by local stimulation of small areas of the cat cerebellar cortex, identified as being involved in eyelid control, reversibly blocks CRs while having no effect on unconditioned responses (Hesslow 1994b). Purkinje cells in this area are inhibited by the conditioned stimulus (CS) in trained decerebrate ferrets, consistent with their role in generating the CR (Hesslow and Ivarsson 1994).

A possible mechanism behind conditioning in the cerebellum would be that the CS activates mossy and parallel fibres. The parallel fibre-Purkinje cell synapses would then be modified by climbing fibre input elicited by the unconditioned stimulus (US). This suggestion has been made by several authors and is based on extrapolation of current ideas about motor learning mentioned above. The relevant circuitry is illustrated in Fig. 1, in which the CS is a repetitive electrical stimulation of the forelimb and the US a periorbital electrical stimulus.

This cerebellar hypothesis of conditioning has been challenged by other investigators, who have observed recovery of CRs after cerebellar lesions (e.g. Welsh and Harvey 1989; Kelly et al. 1990). But some of these findings could not be replicated and others are open to serious criticism and alternative explanations (Hesslow et al. 1990; Nordholm et al. 1991; Yeo and Hardiman 1992; Ivarsson and Hesslow 1993; Hesslow 1994a, b, 1995). The cerebellar hypothesis of conditioning is therefore the most plausible of available alternatives.

An investigation of cerebello-olivary inhibition during conditioning could prove relevant to this problem. If CRs are generated by the interpositus nucleus, one would expect the olive to the inhibited during or just after the CR. An indication that this might be the case is the observation using multiunit recordings that there is reduced US-elicited spiking in the inferior olive of conditioned rabbits (Sears and Steinmetz 1991). It was not shown, however, that this effect resulted from an inhibition of the olive rather than a peripheral or pre-olivary mechanism.

In the present study we attempted to obtain clearer evidence of a conditioning-dependent depression of the relevant olivary cells and to show that this depression was due to inhibition of the olive rather than a pre-olivary sensory relay, such as the trigeminal nucleus.

The choice of ferrets rather than rabbits, the conventional species in studies of classical conditioning, was motivated by the fact that the zonal organization of the ferret cerebellum is very similar to that of the cat, in which this organization has been well characterized. It was crucial in the present study, as well as in other studies currently under way in our laboratory, to identify the area of the c3 zone which controls eyeblink – a relatively easy task in the ferret. In order to do this, however, it was necessary to make recordings from several cortical sites in each animal. This would be very difficult to do in intact ferrets. We have therefore tried to develop a decerebrate preparation which permits experimental intervention with a degree of precision unattainable in intact animals. It has previously been shown that the basic features of classical conditioning are present in decerebrate cats (Norman et al. 1974; Hesslow 1994b) and rabbits (Mauk and Thompson 1987).

Some of these results have been presented in preliminary form (Hesslow 1992).

Methods

Anaesthesia and surgery

The experiments were performed on 16 ferrets (0.6–2.5 kg). As explained below, six animals failed to give stable CRs and the results on olivary inhibition were based on the remaining ten subjects. 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 through which anesthetic gas was led, but when deep anaesthesia had been achieved, a tracheotomy was performed and the gas was led directly into a tracheal tube. The level of anaesthesia was monitored by testing withdrawal reflexes. The skull was opened on the left side and the caudal part of the left cerebral hemisphere was removed by aspiration up to the level of the thalamus. A medial caudal part of the right cerebral hemisphere was also removed so that the dorsal aspect of the brain stem, including the superior and inferior colliculi, was completely exposed at this level. The animals was then decerebrated by a section through the brain stem just rostral to the superior colliculus and the red nucleus. Bone and dura above the cerebellum was removed so that the pars intermedia of the cerebellum was exposed from the inferior colliculus to lobule VII. The cortex was covered with warm paraffin oil. After the 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 (4.5–5%, 120–160 mm Hg and 37.5–38.5° C respectively). The animal's head was fixed to a stereotaxic frame. To prevent drying of the cornea, artificial tears (hypromellose) were applied to the eyes at regular intervals.

Although the spine was clamped and fixed, some ferrets decerebrated at this level go through periods of increased excitability with brisk walking movements. Typically, these periods last for only 5–10 min, but the movements interfere with the recordings. To prevent this, the muscle relaxant alcurone (Norcuron, Organon Teknika) was sometimes given intravenously in low doses (0.2–0.4 mg) which were adjusted so that gross movements were eliminated while electromyogram (EMG) recordings from the eyelids could still be obtained. This was possible because low doses of curare affect eyeblink much less than gross movements. The animals were artificially respirated throughout the experiments. In experiments where the EMG activity in different recordings was 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. For instance, in this time the size of the unconditioned response, as judged by integration of the rectified EMG response, was not measurably different from that before curarization. Furthermore, all comparisons of EMG records were made using alternating test and control trials, so that records from periods with different levels of curarization would never be compared.

At the beginning of this study some animals developed oedema of the cerebellum and had to be excluded. This was probably caused by a combination of the trauma of decerebration and high blood pressure. In many animals the pressure reached very high levels, occasionally over 200 mm Hg during the first hours after decerebration. To counteract this, the antihypertensive drug dihydralazine (Nepresol, Ciba-Geigy) was given intravenously in small doses of 0.05 mg as soon as the blood pressure exceeded 150 mm Hg. This proved very effective in preventing oedema and no more animals were lost for this reason.

Recordings

Recordings of climbing fibre responses were made from the surface of the cerebellar cortex with monopolar silver ball surface electrodes. Areas in the c3 zone controlling eyeblink and forelimb were identified as described previously (Hesslow 1994a). The ground electrodes were inserted into the neck muscles. Usually, in recording field potentials from the cerebellar cortex, ground electrodes are used as references, but in non-curarized subjects such electrodes will pick up muscle activity. To prevent this, a second silver ball electrode was used as reference. It was placed on the cerebellar surface and care was taken to ensure that recordings were not dependent on the placement of the reference electrode. Thus, for each recording, three or four different placements on the cerebellar surface were tested and compared. Only events which were independent of these placements were counted as genuine. The eyeblink response was monitored by EMG recordings from the orbicularis oculi muscle through two stainless steel electrodes which were inserted into the eyelid 2–3 mm above the lateral margin of the left eye.

Electrophysiological recordings were converted to digital data with an AD converter (RC Electronics Inc.). Analysis of the data was performed with the Computerscope software package.

Training procedures

In all the animals, the CS was a 250-ms train stimulation (0.2-ms square pulses, 50 Hz) applied through two needle electrodes inserted through the skin on the medial side of the left upper forelimb. The tips were about 1 cm apart. The stimulation strength was adjusted so that the CS train was sufficient for evoking a small limb movement but single shock produced no visible reflex: this meant 0.5–1.0 mA.

In 8 pilot experiments, not included in the present data, a tone (1000 Hz) stimulus was used as the CS. The tone was presented through two plastic tubes placed with the openings about 5 mm from the ears. The intensity at the entrance of the external ear was 80–90 dB. As had previously been the experience with decerebrate cats, it turned out to be particularly difficult to condition the decerebrate ferrets to a tone CS (Hesslow 1994b). Only three of eight tested animals ever gave CRs to tone and none of these did so in more than about 25% of the trials. The results below are therefore based only on experiments in which forelimb stimulation was used as a CS.

The CS-US interval was 250 ms. In nine of the ten successfully conditioned animals the US consisted of electrical periorbital stimulation through two needle electrodes inserted into the skin of the left lower eyelid about 5 mm apart. Three square pulses of 0.5 ms duration and an interval of 20 ms were used.

In one animal the US was a similarly timed stimulation of the left forepaw and the EMG was recorded from the biceps brachii muscle to monitor conditioned flexion responses. In this animal, the CS was a train stimulation of the upper forelimb as described above, but the intensity of the stimulus was 100 μA. This was necessary because the usual CS produced EMG activity which would have been impossible to distinguish from CRs. With the weaker CS, there were clear short-latency alpha responses, but no EMG activity during the later two thirds of the CS-US interval before conditioning.

Training started 3–4 h after decerebration. The intertrial interval was 20–25 s throughout most of the training period, but was occasionally varied in a pseudorandom fashion for a few minutes at a time to ensure that the responses were not temporally conditioned. The animals were often paralysed during the first 2–3 h of training. The drug was allowed to wear off for brief periods to test for the presence of conditioned responses.

To control for sensitization and pseudoconditioning (Mackintosh 1974; Gormezano and Moore 1976), all the animals also received unpaired presentations of the CS and US. For technical reasons, the standard method of randomly presenting the stimuli was not employed. Instead, the CS-US interval was varied between 5 and 15 s pseudo-randomly during the test period. With an intertrial interval of 20–25 s, this amounted to pseudo-random unpaired CS-US presentations. Since extinction was almost complete within 100 trials in all animals except one, in which the CR frequency was reduced by about 50%, there can be little doubt that the responses were genuine CRs.

The background EMG activity was usually quite small and spontaneous eyeblinks were rare. There were often small conditioned responses during the initial phases of training which could not be unequivocally distinguished from spontaneous muscle activity, but when regular CRs appeared they were quite large (comparable to the unconditioned responses) and there was never any problem in distinguishing CRs from other types of activity.

Results

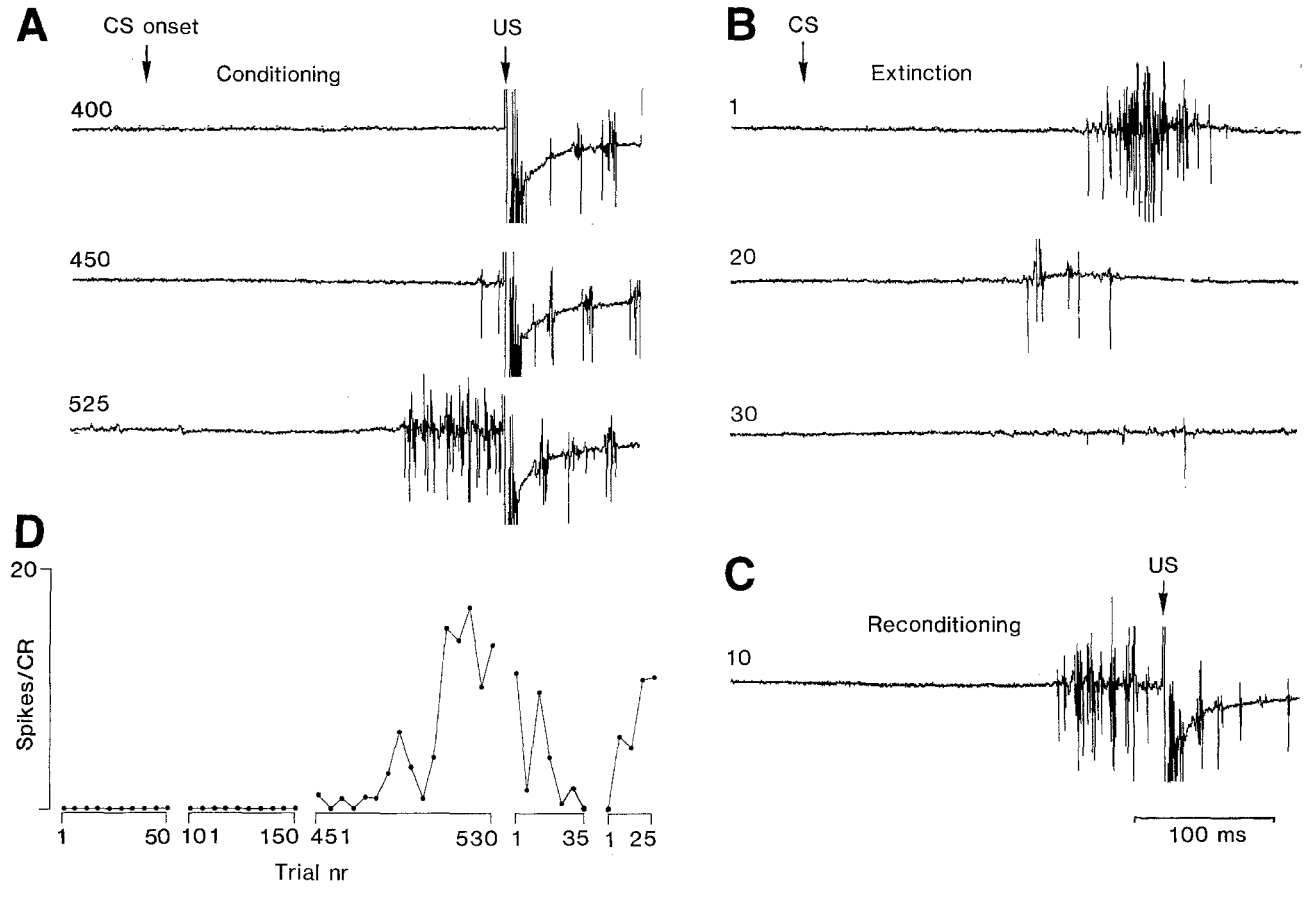
Characteristics of conditioning in decerebrate ferrets

Ferrets treated as described above could be maintained for up to 20 h (longer periods were never attempted). A problem which often occurred after termination of the anaesthesia was an increased blood pressure which caused oedema of the brain. This was successfully counteracted with dihydralazine. A second and more serious problem was spontaneous movements, which interfered with recordings and which could also damage the cerebellum by causing it to press against other structures such as bone edges and electrodes. Although the animals could be effectively immobilized with alcurone, it often happened that a certain test had to be aborted. Regrettably, for such reasons it was impossible to follow a predetermined experimental protocol in all animals.

CRs were observed in 13 of the 16 animals, and appeared to be normal CRs. They had latencies of 100–150 ms and the maximum EMG activity was usually about 25–50 ms before US onset. Typically, the animals would acquire CRs within 2–5 h (500–1000 trials) of training. In two animals, CR were observed after fewer than 100 trials, but even in these cases the responses gradually increased in size and did not rearch an asymptotic level until after more than an hour of additional training. In three animals, the conditioning never became robust. Although large and characteristically timed CRs were occasionally observed, they only occurred on a small number of trials. Thus, of the 16 subjects, three animals did not condition at all and three were conditioned very poorly. All these were excluded from analysis. The data on olivary inhibition below are thus based only the remaining ten animals. Of these, nine received forelimb CS and periorbital US while one animal received a (weak) forelimb CS and a forepaw US.

To exclude the possibilities of sensitization and pseudoconditioning, all animals were tested with unpaired CS and US stimulation as explained above. Although the rate of extinction was variable, nine of the animals showed almost complete extinction of CRs within 100 trials with not more than one or two CRs occurring dur-

Fig. 2A–D Conditioning in the decerebrate ferret. **A** Sample electromyogram (EMG) records from different parts of the training period. *Numbers* above records indicate the approximate trial number. In the *top record* no conditioned response is present. The *middle record* shows an early and the *bottom record* a later large conditioned response. **B** Records showing extinction of conditioned responses during trials in which the conditioned stimulus (*CS*), but no unconditioned stimulus (US) was applied. **C** Record of a conditioned response after ten trials of CS-US training. **D** Plot showing the average number of spikes (each average based on five trials) in the conditioned response (*CR*) during various phases of the training. Since the animal was curarized during some periods, it was not possible to include all trials. During the first three segments of the plot (trials 1–530) CS-US training was given. The fourth segment shows 35 extinction trials (CS only) and the fifth segment 25 conditioning trials (CS-US) which followed immediately after the extinction session



ing the last 20 trials. One animal did not show complete extinction and still gave CRs on about 50% of the trials after a 100-trial extinction period.

Because curarization was necessary during periods, so that EMG recordings could not be obtained continuously, it was not possible to construct complete acquisition curves, but we could usually get a good impression of both acquisition and extinction from the periods when the curarization had worn off. A good example from an animal in which most of the acquisition could be recorded is illustrated in Fig. 2. Figure 2A shows representative EMG records from the left orbicularis oculi muscle at various stages of training. The numbers above the records indicate the approximate trial from which the record was obtained. After the animal had been given about 600 trials in about 4 h, unpaired presentations of CS and US were given. This resulted in a gradual disappearance of the CRs. The responses also became erratically timed. When conditioning training was again applied, CSs came back rapidly (Fig. 2C).

The EMG in this case recorded several motor units. The responses were quantified by setting a window discriminator at a level which included the four to five units dominating the recording, and determining the average number of spikes in five consecutive trials. Plots based on five such periods are shown in Fig. 2D. The approximate trial is showed at the bottom. The first three plots are from the initial training period with paired CS and US presentations. The fourth plot is from the extinction session during which the animal received unpaired CS and US presentations. The fifth plot illustrates reacquisition of CRs.

There was no indication that the curarization had any effects on either acquisition or maintenance of CRs. Once stable conditioning had been achieved, a period of curarization did not change the size or probability of CRs. This is in line with classical findings showing that conditioning of skeletal responses is unimpaired by curarization (see e.g. Solomon and Turner 1962).

Inhibition of climbing fibre responses

The animals were trained until conditioning seemed robust. In paralysed animals the curare was occasionally allowed to wear off, to check for the occurrence of CRs. When clear CRs occurred on at least nine of ten consecutive trials, the effect on the inferior olive of preceding the periorbital stimulation by the CS was tested.

The area in the c3 zone which controls the orbicularis oculi muscle was identified as previously described for cats (Hesslow 1994a) and ferrets (Ivarsson and Hesslow 1993) by recording climbing fibre input from the periorbital area and by recording "delayed" EMG responses in the eyelid. The latter are responses which can be evoked by train stimulation of the same part of the c3 zone of the cerebellar cortex that receives climbing fibre input from the periorbital area. These responses have long latencies, typically 30–50 ms after termination of the stimulation, and they can be delayed by prolonging the stimulus train. Delayed responses probably result from activation of Purkinje cells, causing hyperpolarization followed by rebound excitation in the interpositus neurones. A site in the cerebellar cortex from which such responses can be evoked therefore probably projects to those neurones in the NIA which control eyeblink.

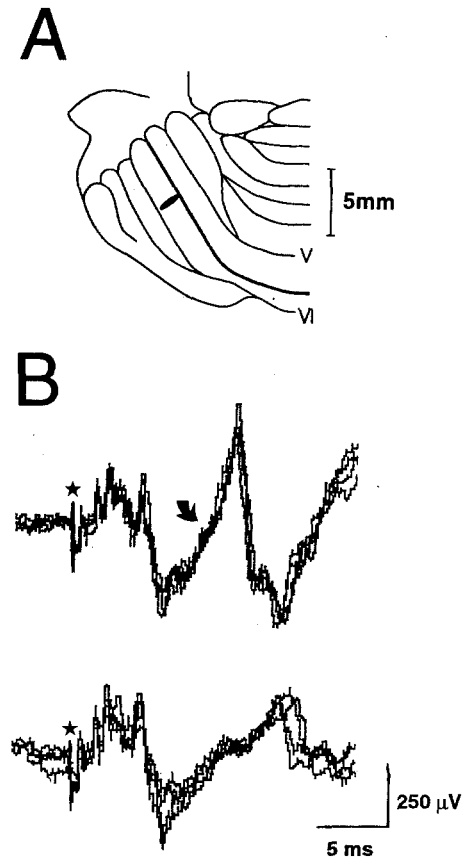


Fig. 3A, B Recording site and inhibition of climbing fibre responses. **A** Outline of the left anterior lobe of the cerebellum. Eyeblink-related area in the c3 zone in lobule VI is indicated in *black*. **B** *Upper panel*: Four superimposed climbing fibre responses (*arrow*) recorded from the area indicated in A on periorbital stimulation. Shock artefact is indicated by an *asterisk*. *Lower panel*: Records obtained from the same site when the periorbital stimulus (US) was preceded by the CS

The excitation of the olive by the US was tested by recording the field potentials elicited by the US in this area of the c3 zone (periorbital shock). Figure 3A shows the recording area in the c3 zone of lobule VI. The upper panel in Fig. 3B shows four superimposed records of typical mossy and climbing fibre (indicated by arrow) responses recorded in this area on periorbital stimulation. The climbing fibre response is preceded by a mossy fibre response. When the periorbital stimulus was preceded by a CS, the climbing fibre response was strongly inhibited, as shown in the lower panel of Fig. 3B. In contrast, the mossy fibre response was unaffected by the CS.

The results are summarized in Table 1, which shows the change in the amplitudes of climbing fibre responses to periorbital stimulation on trials on which the CS was

Table 1 Depression of the amplitude of climbing responses recorded from the cerebellar surface. Figures indicate the decrease in the amplitude of responses elicited by the US on paired CS-US trials compared with US alone trials. Animals are ordered by the magnitude of effect. Animal 6 received paw stimulation as US

Animal	% change
1	0
2	−1
3	−2
4	−2
5	−25
6	−52
7	−56
8	−57
9	−66
10	−74

presented. In four animals no significant effect was observed, but a clearcut and statistically significant CS-induced depression of the climbing fibre responses was observed in six of the ten animals (unpaired *t*-test, $P<0.0001$). The size of the depression varied between 25% and 74%. No inhibition was even observed of the mossy fibre responses.

Effects of conditioning and extinction

The fact that the climbing fibre responses to the US are depressed, when preceded by a CS, does not necessarily mean that the depression is causally related to the animal's emitting CRs. This could be an intrinsic property of the CS, for instance resulting from mutual inhibition between olivary cells. It has been shown that cells in the dorsal accessory olive which are activated by stimulation of the radial nerve are depressed by previous activation of cells responding to stimulation of the ulnar nerve (Andersson 1984). It is conceivable that the forelimb stimulus employed here as CS activates olivary cells which inhibit other olivary cells with a periorbital input. Another possibility is that the CS elicits responses in the same olivary cells as those activated by the US, which would lead to recurrent inhibition of these cells. No climbing fibre responses were recorded from the face area of the cerebellar surface during the CS, but it cannot be excluded that weak asynchronous activation occurred.

To exclude this possibility, and to determine whether the depression of the olive was dependent on the animal being conditioned, the effect of the CS was tested in three animals after both conditioning and extinction. The results were virtually identical in the three animals and only one case (the same one as in Fig. 2) is illustrated in Fig. 4. The animal received alternating sessions, each consisting of 20 trials, of control (US only) and test (CS-US) stimulation. The upper record in Fig. 4A shows the climbing fibre response (averaged over 10 trials) when only the US was given. The record below was from trials in which the US was preceded by the CS. This caused a clear reduction in amplitude of the climbing fibre response. The middle pair of records were obtained after the animal had received a period of extinction training. Altough the stimulation was the same, the inhibition of the climbing fibre response was markedly smaller. The bottom records show the reappearance of the inhibition after a new period of conditioning.

The plot in Fig. 4B is from the same experiment and shows the average climbing fibre response amplitude in alternating test and control sessions during periods of conditioning and extinction. The first test session was obtained after a period of extinction. This session is in-

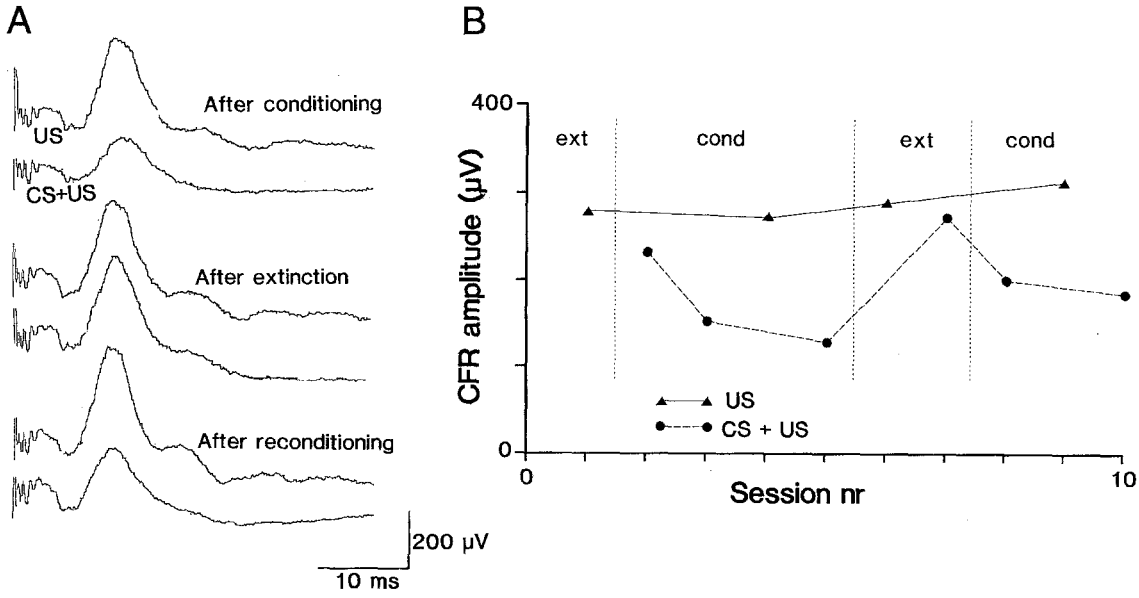


Fig. 4A, B Inhibition of climbing fibre responses after conditioning and extinction. **A** Averaged climbing fibre responses (averages of ten trials) recorded in the c3 zone of the cerebellar cortex. The *upper trace* in each pair shows the control response evoked by the US alone, and the *lower trace* the response to the US when preceded by CS. The *top pair* shows the depression of climbing fibre responses after about 600 trials of conditioning training, the *middle pair* after 40 extinction trials, and the *bottom pair* after 40 reconditioning trials. **B** Changes in the average amplitude of the climbing fibre responses over 20 consecutive trial sessions of conditioning (*cond*) and extinction (*ext*). Control sessions with only US presentation were alternated with test sessions with both CS and US

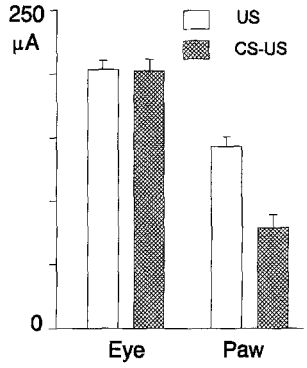


Fig. 5 CS inhibition of climbing fibre responses in face area of the cerebellar cortex to periorbital stimulation (*Eye*) and in forelimb area to stimulation of the paw (*Paw*) in an animal trained with paw stimulation as US

cluded in the conditioning period (cond) since the effect can only be tested by applying both CS and US. The inhibition was markedly increased during these conditioning sessions. Inhibition was then estimated after a new period of extinction. To prevent rapid reacquisition, each CS-US test trial was preceded by five extinction trials. This abolished the inhibition. Additional conditioning sessions caused the inhibition to reappear. Standard errors are not indicated in this plot, which was based on computer-averaged responses. In a few sessions, however, each individual response was measured and the standard errors were then found to be insignificant (less than 2% of the average amplitude).

Specifity of inhibitory effect

To determine whether the CS inhibition of the inferior olive was general or restricted to those parts of the olive activated by the training, climbing fibre responses were investigated in both the forelimb and the face areas of the cerebellar cortex. In the experiment illustrated in Fig. 5, the animal was trained with a forelimb CS and stimulation of the paw as US. This resulted in a robust conditioned EMG response in the biceps brachii muscle. Unpaired CS-US presentations caused extinction of this response. Climbing fibre responses to paw stimulation before training were not inhibited, but after training the amplitude of the climbing fibre response was markedly reduced by the CS. As can be seen in the left pair of bars in Fig. 5, there was no effect at all on climbing fibre responses evoked in the face area of the cerebellar cortex on periorbital stimulation.

Localization of inhibitory effect

The previous experiments do not demonstrate that the site of the inhibition is the inferior olive itself and they are consistent with a pre-olivary site of inhibition, for instance the trigeminal nucleus. The output from the NIA is relayed to the facial nucleus motoneurones via the red nucleus, which in turn projects to the trigeminal nuclei. It has been shown that stimulation of the red nucleus inhibits somatosensory transmission in the spinal trigeminal nucleus (Davis and Dostrovsky 1986). Thus, a CR-related depression of olivary firing does not necessarily involve the nucleo-olivary projection. To determine whether the olivary cells themselves are inhibited by the CS, climbing fibre responses were evoked by direct stimulation of the olive in two animals. Such stimulation can evoke both a direct response resulting from depolarization of the olivary cells or their axons and a synaptic response of longer latency resulting from activation of synaptic terminals or afferent fibres in the olive. An example of this is illustrated in the left-hand record (average of 10 trials) in Fig. 6A, which shows two climbing fibre responses in succession, the first one being a direct response with a latency of about 3 ms and the second one a synaptic response with a latency of 5.5 ms. When this stimulation was preceded by a CS in two animals, the synaptic but not the direct response was inhibited, as shown in the right-hand record in Fig. 6A. The effect is also shown in the bar charts of Fig. 6B.

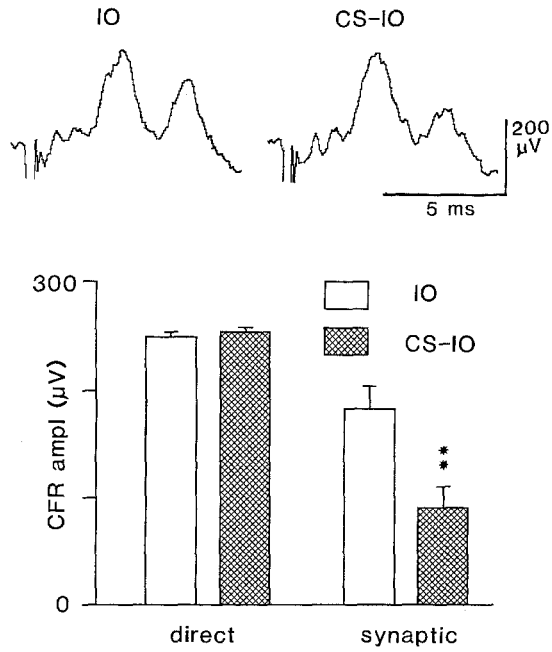


Fig. 6A, B Effect of CS stimulation on direct and synaptic climbing fibre responses evoked by stimulation of the inferior olive (*IO*). **A** Averaged responses recorded in the cerebellar cortex to olivary stimulation without (*left-hand record*) and with (*right-hand record*) a preceding CS. **B** Bar chart showing the amplitudes of climbing fibre responses evoked by direct (*left*) and synaptic (*right*) activation on IO only (*white*) and CS-IO (*hatched*) trials

Correlation between CR size and climbing fibre response amplitude

The experiment illustrated in Fig. 7 had two aims. One was to see whether there was a trial-by-trial correlation of the respective sizes of the conditioned response and the climbing fibre response. According to the hypothesis that both the CRs and the olivary inhibition are generated by cells in the NIA, a negative correlation would be expected. The second aim was to determine the approximate latency of the inhibition.

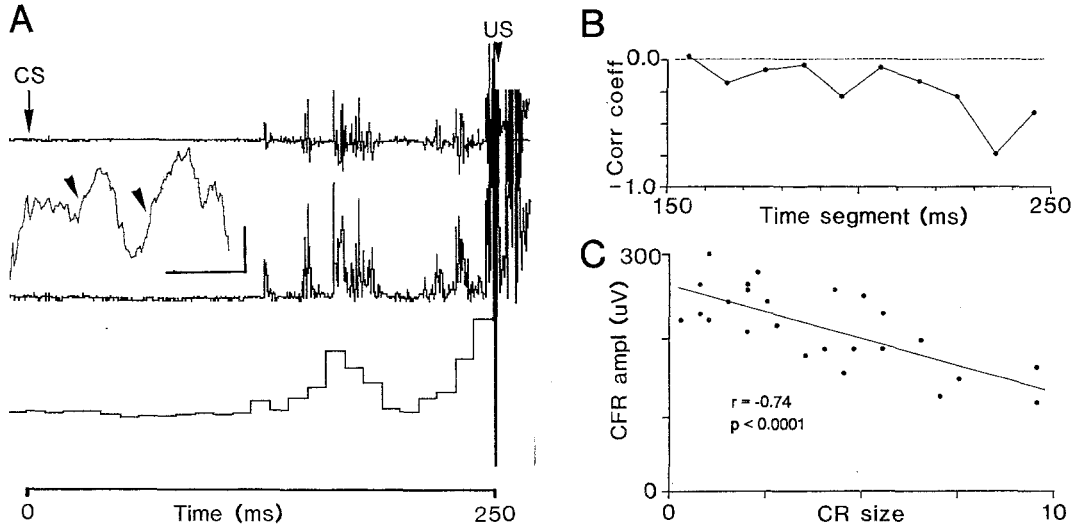


Fig. 7A–C Relationship between size of single CRs and of climbing fibre responses. **A** *Top record:* A sample CR. *Middle record*: The same response rectified. *Bottom*: The 10-ms integrals of the response. *Inset*: A mossy and climbing fibre response (*left* and *right arrowheads*) in the c3 zone. **B** CR-climbing fibre response correlation coefficient for all time segments between 150 and 250 ms of the CR. **C** Correlation between the size of the 230–240 ms part of CRs (*CR size*) and the amplutide of climbing fibre responses (*CRF ampl*)

Twenty-five consecutive CRs were quantified by rectification of the EMG response and integration over 10-ms intervals. Thus the size of the CR was determined for each 10-ms time segment of the CR. The steps in this procedure are shown in Fig. 7A. The upper trace shows a typical EMG record of a CR from this experiment. The next trace shows the same response after rectification of the EMG. Below is the result of integration of the rectified response. The correlation between CR size and climbing fibre response amplitude was then calculated for each time segment of the CR. The results of these calculations are shown in the plot in Fig. 7B, where the correlation between CR size and climbing fibre response amplitude is indicated for each time segment in the last 100 ms of the CS-US interval.

When the statistical significance of these correlations were tested (*t*-tests), only the last two of the ten segments correlated significantly with the climbing fibre responses. The individual measurements and the regression line are indicated for the second-last segment in Fig. 7C. The correlation coefficient for this segment was –0.74 (t=5.23, P<0.0001). Because different parts of the CR could correlate with each other, the correlation for the last tenth segment could be secondary to that of the ninth segment. For this reason, a multiple regression analysis was performed and partial correlations were calculated. This showed unequivocally that there was no true correlation between the size of the last CR segment and the climbing fibre response amplitude. For instance, when segment 10 was controlled, the correlation coefficient for segment 9 was –0.670, but when segment 9 was controlled, the correlation coefficient for segment 10 was only 0.007. This suggests that the inhibition must have a fairly long latency, far exceeding what is normal for monosynaptic effects (see Discussion for a calculation of the latency).

The amplitudes of the mossy fibre responses elicited by the US were also measured, but there was no correlation at all between these responses and CR size for any part of the CR.

Discussion

Conditioning in decerebrate ferrets

The general features of conditioning, such as rates of acquisition, extinction and reacquisition, in many of the decerebrate ferrets were comparable to what has previously been described in decerebrate and hemispherectomized cats (Norman et al. 1974; Hesslow 1994b) and also to what is known about conditioning in intact animals (Mackintosh 1974; Gormezano and Moore 1976). The rate of acquisition was rather slow in some animals, but it is likely that the time (and hence the number of trials) required to achieve stable conditioning included a recovery period. In some animals which had been trained before decerebration, CRs often did not appear until after a couple of hours (unpublished observations), suggesting that it may be the post-operative condition of the animal rather than insufficient training that explained a lack of response.

The temporal characteristics of the CRs were also normal. The CR latencies (about 100 ms) were typical for the interstimulus interval used here and the CRs reached their maximum just before the onset of the US. It was also demonstrated that they were not due to sensitization or pseudoconditioning. There can be little doubt that the responses were authentic CRs.

One difference between normal and decerebrate animals was that conditioning to tone was very poor in the latter. This was also the case in decerebrate cats (Hesslow 1994b). A possible reason for this is that transmission of sound information is depressed in the

acute decerebrates, either because of surgical trauma or because of the decerebration itself. Norman et al. (1974), observed normal conditioning to tone in hemispherectomized cats, but their animals had been allowed to recover for several days, whereas the experiments described above were acute and we only tested acquisition that occurred within a few hours. On the other hand, Mauk and Thompson (1987) found that rabbits which had acquired CRs while intact, emitted normal CRs immediately after decerebration. Hesslow et al. (1990) also observed normal acquisition to tone in acutely decerebrate rabbits. This suggests that there may be a species difference in the effects of acute decerebration.

On balance, there is good reason to assume that the mechanisms behind conditioning in the decerebrates are sufficiently similar to those in intact animals to justify the preparation employed here and also for basing general conclusions about classical conditioning on them.

CR-related inhibition of the inferior olive

If classically conditioned eyeblink responses are generated by the cerebellum, one would expect an increased activity in the NIA and the nucleo-olivary pathway and thus an inhibition of the inferior olive simultaneous to or just after the appearance of the CR. The evidence presented above clearly confirms this prediction. Climbing fibre responses in the c3 zone evoked by the US were strongly depressed by the CS in many of the animals in which conditioning was robust.

Since this inhibition disappeared when the animal was subjected to extinction by repeated presentations of the CS alone, and since the degree of inhibition correlated with the size of the CR, it is clear that the inhibition depended on the animal having learned the conditioned response.

The observation of a depression of climbing fibre responses synaptically elicited by direct stimulation of the inferior olive indicates that the target of the inhibition is the olivary cells. The fact that no effect was observed on mossy fibre responses is consistent with this conclusion. If, for instance, the inhibition resulted from a modulation of the trigeminal nucleus by the red nucleus (Davis and Dostrovsky 1986), one would have expected the mossy fibre responses elicited by the US to be depressed, but this was never observed.

The fact that no CR-related inhibition was observed in four of the ten animals is puzzling, but there are several possible explanations. Although some of the animals in which inhibition failed to occur gave large and regular CRs, it is possible that conditioning was nevertheless weaker in decerebrates than in intact animals. Another possibility is that inhibition occurs only in a small population of olivary cells which do not always terminate close to the cerebellar surface. Periorbital stimulation presumably elicits climbing fibre responses in areas not involved in control of eyeblink, for instance areas controlling neck muscles. There may also be a climbing fibre input to Purkinje cells which control different aspects of eyeblinks. Thus, inhibition could have occurred in some animals even though we failed to see the effect in surface recordings. It is also possible that the inhibition occurs in only a small proportion of trials. We have shown that the inhibition is related only to a narrow time segment of the CR. If the CRs were erratically timed, which they sometimes were, inhibition would not occur.

Latency of the inhibition

The analysis of the time course of the inhibition suggests that only that part of the CR occurring at least 10–20 ms before the US is related to the excitability of the inferior olive. If we assume that the inhibition involves the nucleo-olivary pathway, this would imply a latency of the inhibition from the brachium conjunctivum to the olive of about 20–30 ms or more. This is an unusually long latency, but it corresponds quite well with the observation that direct stimulation of the brachium conjunctivum in cats causes a depression of the olive with a latency of about 30 ms (Hesslow 1986).

These latencies were estimated by adding the component time lags under the assumption that the source of the inhibition is the nucleo-olivary pathway. To suppress the olive, the CR has to occur 10–20 ms before the US. Stimulation of the brachium conjunctivum causes an EMG response in the eyelid with a minimum latency of 4 ms. Since the stimulation of the brachium conjunctivum is likely to produce a rather unnaturally synchronous activation of the motor neurones, the true latency of an eyeblink may be longer. Thus, a CR occurring 10–20 ms before the US was produced in the NIA at least 5 ms earlier still, that is at least 15–25 ms before the US. The US in turn will elicit a response in the olive with a latency of about 5 ms. Adding this to the CR-US delay gives us an estimated latency of at least 20–30 ms. The minimum stimulation interval for the inhibition evoked by direct stimulation of the brachium conjunctivum in cats was usually about 40 ms, but this figure includes the delay from the peripheral stimulation to the olive of about 10 ms. Correcting for this, one would arrive at an estimated latency from the brachium conjunctivum to olive of 30 ms or more. This is a reasonably good correspondence.

Source of the inhibition

A straighforward interpretation of the results is that the CR is generated by the NIA which simultaneously sends inhibitory impulses to the inferior olive. It could of course be argued that there is facilitatory output from the cerebellum to some extracerebellar site of CR generation which in turn inhibits the olive. This is unlikely, however, because the nucleo-olivary pathway is currently the only known source of inhibitory input to the inferior olive. Furthermore, the unusually long latency of the CR inhibition of the olive is in good agreement with the latency of nucleo-olivary inhibition. Both these facts suggest that the inhibition is generated by the NIA neurones.

Possible functions of the inhibition

Given the reasonable interpretation that conditioned responses generated by the interpositus nucleus inhibit the inferior olive, what could its function be? Three possibilities should be considered.

1) Feedback regulation of the amplitude of learned responses. We have previously suggested that when the response reaches a certain amplitude, further learning would be blocked and the system would prevent acquisition of ever larger responses (Andersson et al. 1988).
2) Gating out of stimuli with no predictive value. This possibility is suggested by the blocking phenomenon (Kamin 1969; Mackintosh 1974). When an animal has acquired a response to one CS, for instance a tone, and is then trained with a compound CS, for instance tone plus light, it will not acquire a CR to the second CS. This makes physiological sense, because adding the light will not increase the animal's ability to predict the US. A cerebellar inhibition of the inferior olive would explain this phenomenon.
3) Regulation of the generalization of learning to other muscle groups. The training procedure used for conditioned eyeblink could conceivably lead to the learning of other responses as well, for instance a head withdrawal. Periorbital stimulation elicits climbing fibre responses not only in Purkinje cells controlling eyeblink, but also in cells which probably control other face and neck muscles. With continued training, one might expect not only an ever larger conditioned eyeblink to develop but also a more generalized defensive reaction including conditioned face twitching and head withdrawal. It is conceivable that such a spread of learning to synergistic responses needs to be regulated. The cerebello-olivary inhibition might accomplish this by inhibiting olivary cells projecting to neighbouring microzones in the cerebellar cortex.

These possibilities are not mutually exclusive, and the evidence presented above is consistent with all three suggestions. To test them, one would need to investigate the effects of inactivating the cerebello-olivary inhibition during prolonged conditioning. Such experiments are presently being carried out.

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