CLASSICAL CONDITIONING OF EYEBLINK IN DECEREBRATE CATS AND FERRETS

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SUMMARY

Several lines of evidence suggest that the cerebellum is involved in classical conditioning of the eyeblink response. For instance, lesions to the interpositus nucleus abolish both learning and retention of conditioned responses. There is strong disagreement about the precise nature of the cerebellar involvement, however. Although many now believe that it is the site of learning, is has also been argued that the cerebellum is merely necessary for the normal performance of conditioned responses. In order to clarify the role of the cerebellum in conditioning, electrophysiological techniques were applied to decerebrate cats and ferrets, which can acquire normal conditioned responses. Four small discrete areas of the cerebellar cortex have been identified which seem to control the orbicularis oculi muscle. Electrical stimulation of these areas, which inhibits neurones in the interpositus nucleus, completely suppresses a conditioned response but has only a weak effect on the unconditioned response. Recordings from Purkinje cells in one of these areas show firing patterns which are consistent with their being involved in the learning of the conditioned response. In combination with results from other groups, these findings provide strong support for the cerebellar learning hypothesis.

INTRODUCTION

If a stimulus which elicits a reflex eyeblink response, such as an air-puff to the cornea, is repeatedly preceded by a neutral stimulus, such as a tone, the neutral stimulus will gradually acquire the ability to elicit an eyeblink. This is an example of classical or Pavlovian conditioning, a form of associative learning first described by Pavlov. He originally trained dogs to respond with salivation to sound, but the same basic technique has later been applied to a wide variety of autonomic as well a skeletal muscle responses (Gormezano and Moore, 1976). In all cases, an unconditioned stimulus (US) initially causes an unconditioned response (UR). A neutral conditioned stimulus (CS) initially has no effect, but after training elicits a conditioned response (CR).

Although Pavlov suggested that the neural plasticity mediating this learning was located in the cerebral cortex, it has been known for some time that conditioning, at least of somatic responses, can occur in decorticate, hemispherectomized or hippocampectomized rabbits and cats, leaving the brain stem and cerebellum as the only possible sites of learning (Oakley and Russell, 1972; Norman, Villablanca, Brown, Schwafel and Buchwald, 1974; Schmaltz and Theios, 1972). The cerebellum, with its massive convergence of sensory information via the mossy and parallel fibres to the Purkinje cells, satisfies the anatomical

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requirements of a structure suitable for associative learning and has also been implicated in other forms of motor learning (Ito 1984). It is therefore a plausible site of classical conditioning.

There is now strong evidence for this hypothesis. Eyeblink conditioning is absent or at least highly abnormal in animals with lesions of the anterior interpositus nucleus (NIA), the hemispheral part of lobule VI of the cerebellar cortex or to the inferior olive (McCormick and Thompson 1984; Yeo, Hardiman and Glickstein 1985a,b, 1986). Pharmacological blockade of the NIA and overlying cortex during conditioning prevents learning, although such blockade of the red nucleus, an output relay from the cerebellum, does not have this effect (Krupa, Thompson and Thompson, 1993).

Several writers have proposed that the CS would reach the Purkinje cells in the cerebellar cortex via the mossy and parallel fibres. When this input is paired with climbing fibre input elicited by the US, changes would occur in the parallel fibre/Purkinje cell synapses.

Both the results and the interpretation of these studies have been challenged by other investigators. For instance, Welsh and Harvey (1989) reported that a substantial proportion of animals with NIA lesions were able to relearn after prolonged training. CRs in these animals occurred with lower frequencies, were smaller and had longer latencies but they were not abolished. But, since there is a bilateral output from the cerebellum, at least in the ferret, it cannot be excluded that these responses were due to the contralateral cerebellar hemisphere (Ivarsson and Hesslow, 1993). Kelly, Zuo and Bloedel (1990) observed that learned responses in animals which had been trained after decerebration survived complete removal of the ipsilateral cerebellum. But, this finding could not be replicated by Hesslow, Hardiman and Yeo, (1990). Furthermore, other evidence suggests that the responses observed by Kelly *et al.*, may not have been true CRs (Nordholm, Lavond, Thompson, 1991).

The precise role the cerebellum in conditioning is still controversial, however, and can only be clarified by physiological investigation of the relevant neuronal elements. A few studies employing physiological techniques have been published previously, for instance recordings from Purkinje cells (Berthier and Moore, 1986), but it was not known if these units were actually related to the control of eyeblink.

METHODS

Applying physiological techniques to intact animals is very difficult, for technical as well as for ethical reasons, and we have therefore used decerebrate cats and ferrets. These preparations permit a higher degree of experimental control and freedom. It has been shown by several investigators that forebrain structures are not necessary for conditioning and that normal conditioning can be obtained in decerebrate cats and rabbits (e.g. Norman *et al.* 1974).

The animals were anaesthetized with halothane and then decerebrated by sectioning the brain stem just rostral to the superior colliculus and the red nucleus. After decerebration, the anaesthesia was terminated. Surface recordings were made with monopolar silver ball electrodes. For single unit recordings, glass-coated tungsten wires were used.

The CS was a 300 ms, 50 Hz train of stimuli applied via needle electrodes inserted through the skin of the medial side of the proximal left forelimb. The US was a 50 Hz, 60 ms train of stimuli (0.2 ms, negative square pulses), to the lower eyelid delivered through a pair of stainless steel needles inserted about 1 mm into the skin. The CS-US interval was 300 ms. It was ensured, by observing extinction after unpaired presentations of the CS and US, that the responses were 'true' CRs and not due to sensitization or pseudoconditioning.

Identification of Eyeblink Areas in the Cerebellar Cortex

Studies of the climbing fibre projection to the cerebellar cortex have revealed the presence of sagitally 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 zones can be further subdivided into microzones on the basis of the topography of the climbing fibre input. Here, we will only consider the c1 and c3 zones of the intermediate part of the cerebellar cortex. Lesion studies have implicated the NIA and the dorsal accessory olive in normal conditioning (Yeo *et al.*, 1985a,b, 1986). The NIA is

innervated by Purkinje cells in the c1 and c3 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.

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 and Schouenborg, 1991). Thus, one would expect the zones projecting to the NIA to contain microzones which have climbing fibre input from the cornea and the periorbital area and which control the orbicularis oculi muscle. This has now been demonstrated in the cat.

The recording and stimulation arrangement is illustrated in Fig. 1 A. Four areas in the cerebellar cortex of the cat, which control the orbicularis oculi muscle are shown in B. These areas were identified by recording climbing fibre input from the cornea and skin around the eye and by recording 'delayed' EMG responses in the eyelid. The latter can be evoked by train stimulation at certain sites in the cerebellar cortex. They 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 cell axons which causes hyperpolarization followed by rebound excitation of interpositus neurones. A site from which such responses can be evoked therefore probably projects to those neurones in the NIA which control eyeblink (Hesslow, 1994a).

Inhibition of CRs by Cortical Stimulation

If the cerebellum has a critical role in the performance of CRs, cortical stimulation during the execution of a CR would be expected to inhibit it. Furthermore, cortical stimulation should inhibit CRs more effectively than URs. Conversely, if the cerebellum has no particular relationship with the CR and solely provides background excitation and facilitation of the motoneurones in the facial nucleus, CRs should be inhibited to the same degree as the URs.

Fig. 2 shows rectified and averaged EMG recordings of CRs and URs in a cat. When a single shock was applied to the cerebellar cortex, the CR was completely suppressed. This effect on the CR was topographically specific. When a stimulus was applied to a site in the periphery or outside an eyeblink area, there was little or no inhibition of the CR (Hesslow, 1994b).

When the UR was preceded by a cortical stimulus, some depression of this response was observed, but it was slight compared to the effect on the CR. It could be argued that the difference in effect on CR and UR reflects a difference in excitatory drive. If the motoneurones are excited far above threshold during the UR and only slightly above threshold during the CR, a certain amount of inhibition could have a much stronger effect on the latter. This suggestion may be rejected, however, since the inhibition of the UR was insignificant even when a weak US was used, and the EMG activity in the UR was considerably smaller than in the CR. This suggests that the effect on the CR is not just due to a loss of background facilitation of the motoneurones in the facial nucleus. The CR is more critically dependent upon the cerebellum than is the UR.

It cannot be excluded, of course, that the cerebellum exerts a tonic facilitation of some other brain stem structure, which is involved in generating the CR but not the UR. If such a structure used the red nucleus as an output pathway, cerebellar cortical stimulation would cause a loss of background excitation which could block the CR. In the absence of a plausible candidate structure, apart from the cerebral cortex, which we know is not necessary for conditioning, this suggestion must be regarded as *ad hoc*. The most reasonable explanation for the inhibition of CRs is, therefore, that they are generated in cells in the NIA, which are inhibited by stimulation of the cerebellar cortex.

Single Unit Recordings

Microelectrode recordings have been made from Purkinje cells, Golgi cells and from NIA neurones in decerebrate ferrets. The number of cells in the latter two categories is still too small to permit any conclusions, but there are observations, consistent among the more 150 Purkinje cells now studied in an eyeblink-related area of the c3 zone (Hesslow and Ivarsson 1994). These may, with some simplification, be summarized as follows:

a) Purkinje cells in naive animals respond weakly or not at all to the CS. An example of a cell recorded in a naive animal is shown in Fig. 2 A. Slight increases or decreases in firing were observed in many neurones, but the changes in firing frequency were less than 10%. It is very difficult to judge the importance of such responses and it cannot be excluded that even weak changes in firing are important at the population level.

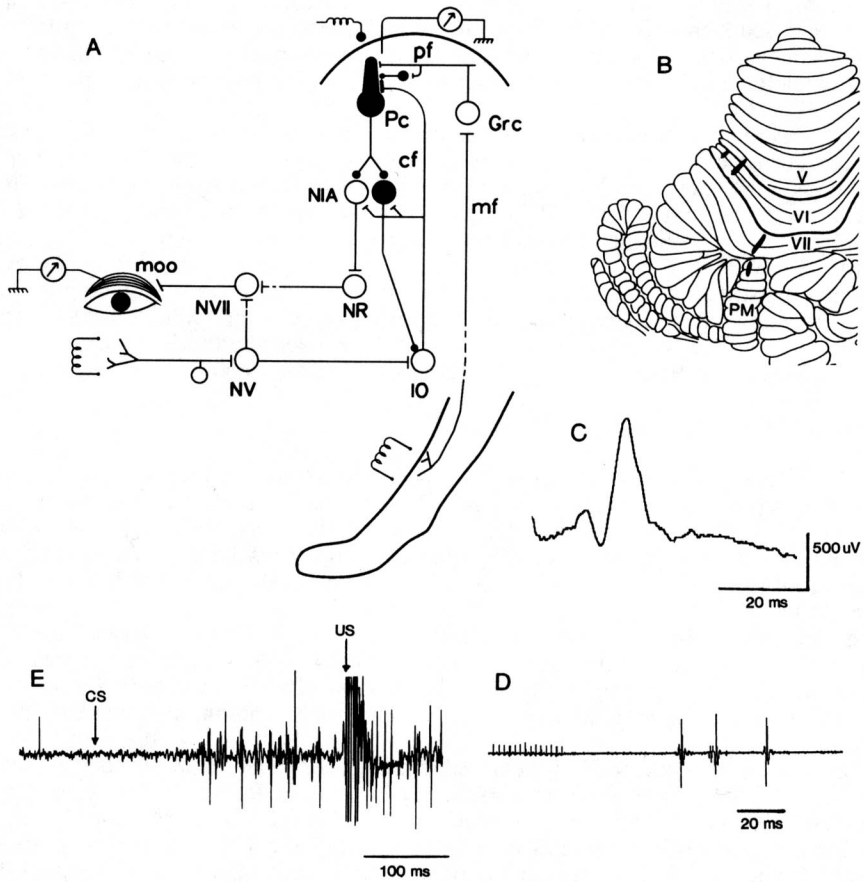

Figure 1. A: Experimental setup and diagram of the circuit controlling eyeblink. Recording electrodes in the orbicularis oculi muscle (moo) and in the cerebellar cortex. Stimulation electrodes are in the cerebellar cortex, the lower eyelid (US) and the forelimb (CS). The US pathway is through the trigeminal nucleus (NV), the inferior olive (IO) and the climbing fibres (cf.) to the Purkinje cells (Pc). A hypothetical CS pathway from the forelimb is via mossy fibres (mf), granule cells (Grc) and parallel fibres (pf). Output from the cerebellar cortex is to the anterior interpositus nucleus (NIA), red nucleus (NR) and facial nucleus (NVII). **B**: Outline of the cerebellar surface of the cat with eyeblink areas indicated in black. **C**: Climbing fibre response recorded from the cerebellar surface in this area. **D**: Delayed EMG response in the eyelid evoked by stimulation of the same area. **E**: Conditioned response.

b) In the trained animals, a certain proportion of Purkinje cells responded with a marked (sometimes complete) suppression of simple spike firing during the later parts of the CS-US interval. An example is shown in Fig. 2 B. The suppression was sometimes preceded by a period of excitation. About 15-25% of the cells (depending on the criteria used) showed this inhibitory response. Because of the variability between cells in the degree, latency and duration of the simple spike suppression, this figure unavoidably involves some simplification.

c) The remaining Purkinje cells in conditioned animals show the same kind of behaviour as cells in naive animals, that is weak or no responses to the CS.

These observations are consistent with the cerebellar hypothesis of conditioning. Suppression of simple spikes would be expected to cause a disinhibition of the target

neurones in the NIA which would lead to excitation, via the red nucleus, of motoneurones in the facial nucleus. It cannot be excluded, that the Purkinje cell responses observed were effects of changes in other brain stem structures such as feedback from the motoneurones, but since the latency of the inhibition was often considerably shorter than the CR latency this seems unlikely.

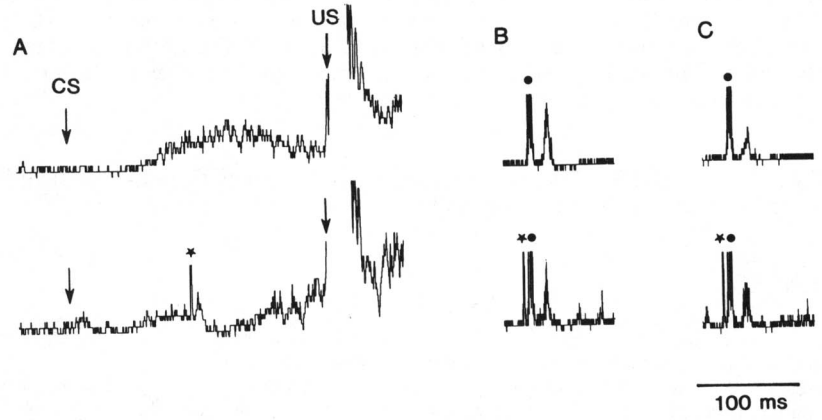

Figure 2. Comparison of the effect of cerebellar cortical stimulation on conditioned and unconditioned responses. **A**: The upper trace is an average of rectified EMG recordings from 20 conditioning trials. The lower trace shows effect of cortical stimulation. Arrows indicate onset of CS, US and cortical stimulus (Ctx). **B**: The upper trace shows the rectified average of 40 EMG responses to a very weak US and the lower trace is the response to the same stimulus preceded by a cortical stimulus.

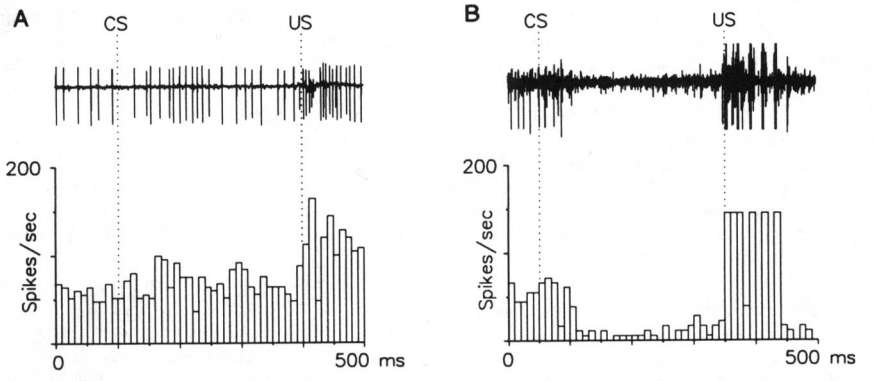

Figure 3. Sample records and time histograms from non-responsive (**A**) and responsive (**B**) Purkinje cells in conditioned ferrets.

These findings may be simply and powerfully interpreted in terms of the long term depression (LTD) of parallel fibre/Purkinje cell synapses, which is induced by simultaneous parallel fibre and climbing fibre input to the Purkinje cells, a mechanism which probably plays a role in the adaptation of the vestibulo-ocular reflex (Ito 1984). As suggested by several authors, and confirmed by data described above, the CS activates the mossy fibre/parallel fibre input while the US activates climbing fibres to the Purkinje cells controlling the orbicularis oculi muscle. Parallel fibres activated by the CS will influence the Purkinje cells directly via the excitatory synapses as well as indirectly via inhibitory stellate and basket cells. It is conceivable that the CS before training activates both these mechanisms so that excitation and inhibition are balanced and the net effect on the Purkinje cell therefore is close to zero. During conditioning, the paired parallel fibre and climbing fibre input would then cause a progressively more pronounced depression of the excitatory parallel fibre - Purkinje cell synapses whereafter the dominating effect of the CS would be interneuronal inhibition of the Purkinje cells.

CONCLUSIONS

The hypothesis, that the neural plasticity underlying classical conditioning is located in the cerebellum, has met fierce opposition, and the critics have often pointed out that the empirical evidence is not conclusive. However, since the cerebellum is the only structure remaining in the decerebrate, which is known to have the requisite anatomical connections as well as a mechanism of synaptic plasticity (LTD), it is an *a priori* plausible site of motor learning. Furthermore, although evidence in empirical science is never conclusive in any strict sense, there are now a number of diverse findings in the literature which can be effectively and parsimoniously explained by the cerebellar hypothesis, and no plausible alternative has so far been suggested.

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