

Learned Movements Elicited by Direct Stimulation of Cerebellar Mossy Fiber Afferents

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Summary

Definitive evidence is presented that the conditioned stimulus (CS) in classical conditioning reaches the cerebellum via the mossy fiber system. Decerebrate ferrets received paired forelimb and periocular stimulation until they responded with blinks to the forelimb stimulus. When direct mossy fiber stimulation was then given, the animals responded with conditioned blinks immediately, that is, without ever having been trained to the mossy fiber stimulation. Antidromic activation was prevented by blocking mossy fibers with lignocaine ventral to the stimulation site. It could be excluded that cerebellar output functioned as the CS. Analysis of latencies suggests that conditioned responses (CRs) are not generated by mossy fiber collaterals to the deep nuclei. Hence, the memory trace is probably located in the cerebellar cortex.

Introduction

In Pavlovian or "classical" conditioning, a neutral, "conditioned" stimulus (CS) is repeatedly followed by an "unconditioned" stimulus (US), which evokes a reflex response (Pavlov, 1927). In the standard eyeblink conditioning paradigm, the CS is often a tone, a light, or electrical skin stimulation. The US is usually an air puff to the cornea or a periocular electrical stimulus, which elicits a reflex blink (Gormezano et al., 1983). After a number of such stimulus pairings, the initially neutral CS will acquire the ability to elicit a blink, a "conditioned response" (CR). Several lines of evidence indicate that the cerebellum is critical for this type of learning (Thompson and Krupa, 1994; Yeo and Hesslow, 1998), although this conclusion is still opposed by many investigators (Bloedel and Bracha, 1995; De Schutter and Maex, 1996; Llinas et al., 1997).

Among those who are sympathetic to the idea that the memory trace is located in the cerebellum, it has still been controversial whether the critical site is in the deep cerebellar nuclei or in the cerebellar cortex. The second alternative is attractive on theoretical grounds; because of the massive convergence of sensory input in the mossy fiber/parallel fiber system (Ito, 1984), the cortex would seem ideally suited for associating widely divergent inputs with a certain output. Although some investigators have reported that lesions of the cerebellar

cortex abolish CRs (Yeo et al., 1985a), others have found no or only transient impairments of the CRs after cortical lesions and suggest that the memory trace is located in the anterior interpositus nucleus rather than in the cortex (McCormick and Thompson, 1984; Lavond et al., 1987).

It has been suggested, on anatomical grounds (Yeo et al., 1985b) and by analogy with other models of cerebellar learning, that the CS information is transmitted to the cerebellum by the mossy fiber/parallel fiber system and that the US activates the second major input to the cerebellum, the climbing fibers (Figure 1). According to the well-known model of cerebellar learning mainly associated with Marr (1969), Albus (1971), and Ito (1984) (for recent reviews, see Houk et al., 1996; Thach, 1996), the mossy fiber inputs to the Purkinje cells in the cerebellar cortex convey information about the context in which a movement is made, and the climbing fiber input from the inferior olive encodes errors in movement or position and elicits changes in the efficacy of context-encoding parallel fiber/Purkinje cell synapses. With each occurrence of the context, the movement pattern will gradually become more adaptive. As noted by Marr and Albus, this model fits very nicely with the classical conditioning paradigm.

One of the arguments for applying the Marr-Albus model to classical conditioning has been the demonstration, both in intact and decerebrate animals, that stimulation of the pontine nuclei or of the middle cerebellar peduncle (MCP) can be used as a CS (Steinmetz et al., 1986; Svensson et al., 1997) and that there is a transfer of learning such that an animal that has been trained with pontine nuclei stimulation as the CS will more rapidly (occasionally even immediately) learn to respond to a tone CS (Steinmetz, 1990). The MCP contains mossy fibers projecting to the cerebellum and mainly, although not exclusively, originating in the pontine nuclei, which receive convergent auditory, visual, and somatosensory inputs (Brodal and Bjaalie, 1992). The evidence from stimulation experiments has failed to convince many opponents of a cerebellar locus of conditioning. Even if mossy fiber activation can function as a CS, this does not show that the information from a peripherally applied CS must also be transmitted through this pathway. Indeed, it is well known that electrical stimulation of almost any site in the central nervous system, such as the cerebral cortex, can be used as an effective CS (Loucks, 1933; Doty et al., 1956; Doty, 1969). Yet, conditioning can proceed normally in decorticate and even decerebrate animals (Oakley and Russell, 1972; Norman et al., 1974; Mauk and Thompson, 1987; Hesslow, 1994). Presumably, stimulation of many different sites can, directly or indirectly, activate the true CS pathway. This would be expected with mossy fiber activation, particularly when pontine stimulation is used. Electrical stimulation in the pons is likely to activate descending or ascending fibers of passage and will certainly cause antidromic activation of afferent input from the cerebral cortex, the spinal cord, and the brainstem. Stimulation of mossy fibers in the MCP will cause antidromic activation, which, via

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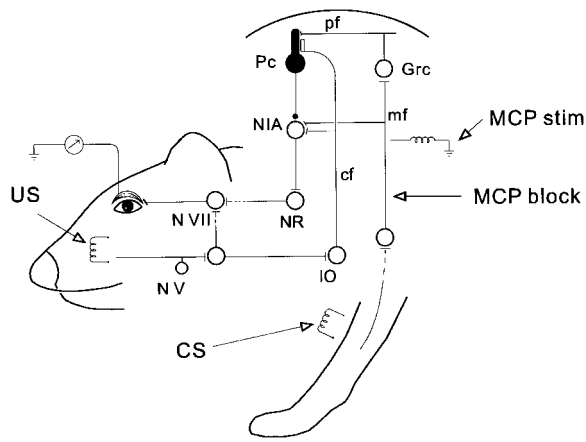


Figure 1. Experimental Setup and Simplified Wiring Diagram of the Neuronal Circuits Assumed by the Cerebellar Hypothesis of Conditioning

Percutaneous stimulation electrodes were placed in the lower left eyelid (US) and the left forelimb (CS). EMG recordings were made from the left orbicularis oculi muscle. The hypothetical pathway for the US signal is through the trigeminal nucleus (NV), the inferior olive (IO), and climbing fibers (cf) to the Purkinje cells (Pc). The hypothetical CS pathway for the forelimb is via mossy fibers (mf), granule cells (Grc), and parallel fibers (pf) to Purkinje cells. Both climbing and mossy fibers send collaterals to the anterior interpositus nucleus (NIA). Output from the cerebellar cortex goes via the NIA, red nucleus (NR), and facial nucleus (NVII). In all experiments, the middle cerebellar peduncle (MCP) was stimulated. In the experiment illustrated in Figure 4, transmission in the MCP was blocked ventral to the stimulation electrode by injection of 1 μ l of lignocaine.

collaterals, may affect many other structures in the central nervous system. Both pontine and MCP stimulation may cause cerebellar output, which could be associated with the US at a different site.

The experiments reported here were designed to overcome these problems by showing that (1) direct stimulation of the mossy fibers in the MCP in an animal previously trained to a peripheral CS can elicit a CR immediately, that is, without the animal ever having been trained to mossy fiber stimulation, (2) stimulation of the mossy fibers works as a CS even when antidromic activation has been excluded by blocking transmission in the MCP ventral to the stimulation site, and (3) activation of cerebellar output does not elicit CRs.

Results

The animals were trained with a 300 ms electrical train of stimuli to the forelimb (CS), followed by electrical periocular stimulation (US) for 3–5 hr, at which time they emitted CRs on 95%–100% of the trials. An electrode was then placed close to the center of the MCP, and a 50 Hz train of electrical stimuli to the MCP (25–90 μ A) was used as a CS. In two of the four animals tested, blink responses appeared on the very first trial (Figure 2A), that is, before MCP stimulation had ever been paired with the US. In the other animals, MCP stimulation did not elicit CRs on the first trial, but when the electrode was moved down 200–300 μ m, MCP stimulation elicited blinks on the third and fourth attempt, respectively. The latencies (100–200 ms) and topographies of these blink

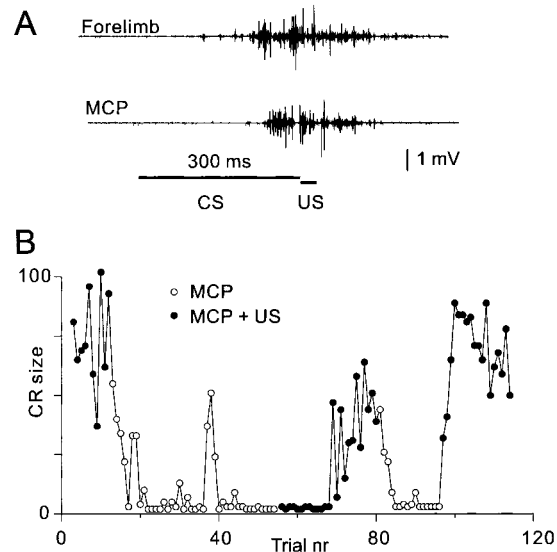


Figure 2. Conditioned Responses Evoked by Forelimb and MCP Stimulation

(A) The upper trace is a sample EMG record of a CR elicited in a trained animal by a forelimb percutaneous CS. The lower trace shows the first response to a train of stimuli to the MCP (50 Hz, 70 μ A).

(B) Sizes (in arbitrary units) of 120 consecutive EMG responses to MCP stimulation on paired MCP and US trials and on MCP alone trials. Responses were extinguished on MCP alone trials and reacquired on paired trials.

responses were similar to those elicited by the forelimb CS and were typical of CRs.

The MCP is situated immediately dorsal to the trigeminal nerve at this rostro-caudal level. Occasionally, especially if the animal moved, the MCP electrode penetrated deeper than intended, and the stimulation elicited electromyographic (EMG) activity with a latency that suggested that current had spread to the trigeminal nerve. An example is seen in Figure 4C (lower right). When the stimulation electrode was withdrawn about 100 μ m, these responses disappeared, while the long latency responses remained. Apart from this, there was no trace of any short latency EMG activity elicited by the MCP stimulation.

The observations that the MCP-elicited responses had normal latencies and topographies do not justify the conclusion that they were authentic CRs. They might merely reflect the hardwiring of the cerebellum. Three experiments were performed in order to determine if the responses to MCP stimulation were really learned and a result of the animal having been conditioned. First, MCP stimulation was given alone (one animal) or in a random temporal relation to the US (one animal). Unpaired MCP stimulation led to extinction of the responses, which were then reacquired quickly when MCP stimulation was again paired with the US. One of these experiments is illustrated in Figure 2B.

Second, if the MCP stimulation was exciting the CS pathway previously activated by the forelimb CS, one would predict that unpaired presentations of the forelimb CS, which lead to extinction of the forelimb-elicited CRs, would also lead to extinction of the MCP-elicited responses. This was indeed observed in two of two

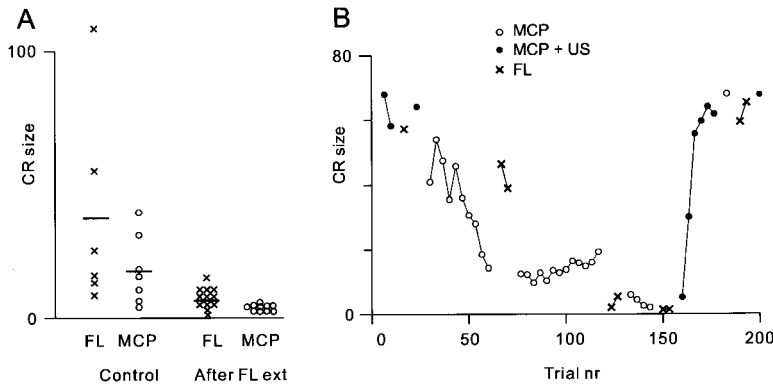


Figure 3. Interdependence of MCP- and Forelimb-Elicited CRs

(A) Dependence of MCP-elicited responses on previous conditioning to a forelimb CS. The animal had been trained to a forelimb (FL) CS until it emitted reliable CRs. Tests of unpaired MCP stimulation were interspersed between forelimb trials. Sizes (in arbitrary units) of single forelimb-elicited CRs (first column) and of MCP-elicited CRs (second column) are given. After 100 trials of unpaired forelimb stimulation, which caused extinction of forelimb-elicited CRs (third column), no responses were elicited by MCP stimulation (fourth column).

(B) Dependence of forelimb-elicited CRs on conditioning to MCP. The animal had been

trained with a forelimb CS and responded to MCP stimulation (each circle represents the average of five CRs). Presentation of unpaired MCP CSs caused extinction of MCP-elicited CRs. Unpaired forelimb stimulation was interspersed between MCP trials (each x represents a single CR). After extinction to the MCP CS, forelimb stimulation no longer elicited CRs. After paired MCP US stimulation had caused recovery of MCP-elicited CRs, forelimb stimulation could again elicit CRs.

animals tested. In the experiment illustrated in Figure 3A, MCP stimulation was tested in an animal that had already acquired CRs to a forelimb CS. When MCP stimulation was applied, it, too, reliably evoked blink responses. Since the experiment was intended to test the effect of conditioning with the forelimb CS, it was considered important to avoid any opportunity for the animal to associate MCP stimulation with the US. The US was therefore withheld on the MCP stimulation test trials. To prevent these test trials from causing extinction to MCP stimulation, only a small number of test trials were given. After testing for responses to MCP stimulation, the animal was subjected to 100 presentations of the forelimb CS alone, which caused extinction of forelimb-elicited CRs. When MCP stimulation was then applied, no CRs were present. Thus, the responses elicited by MCP stimulation were dependent on the animal's being conditioned to a peripheral CS.

Third, the reverse of the previous experiment was performed in one animal. If the MCP stimulation was exciting the pathway activated by the forelimb CS, extinction of the MCP-elicited CRs, induced by unpaired MCP stimulation, should lead to extinction of the forelimb-elicited responses as well. In the experiment illustrated in Figure 3B, the animal had been trained to a forelimb CS and also responded reliably to the MCP CS. Unpaired MCP stimulation was then given until it no longer elicited CRs. As predicted, when a forelimb CS was then presented, it, too, was unable to elicit CRs. To avoid any additional effects from conditioning with the forelimb CS, only a small number of test trials could be given. However, the experiment was repeated in the same animal with the same result.

As can be seen in Figure 3B, paired MCP and US stimulation induced a fast recovery of MCP-elicited CRs but also of forelimb-elicited responses. This was not a typical finding, however. In the two experiments previously described (Figure 3A), in which unpaired forelimb presentations had led to extinction of forelimb-elicited CRs (and indirectly, MCP-elicited CRs), we gave paired MCP and US presentations. Although the animals again responded to the MCP CS, we did not observe any responses to the forelimb CS.

To exclude the possibility that the MCP stimulus caused antidromic activation of mossy fibers, we blocked the mossy fiber transmission ventral to the stimulation electrode in two animals. If the CS information is transmitted via the mossy fibers, this should abolish responses to the forelimb CS but leave responses to MCP stimulation unaffected. A micropipette filled with 4% lignocaine solution was placed under visual guidance in the MCP about 2 mm rostral-ventral to the stimulation electrode, as illustrated schematically in Figure 1 and anatomically in Figure 4A. The animal emitted CRs both to forelimb and MCP stimulation. The lignocaine solution (1 μ l) was then injected through the micropipette. Within a few minutes, this completely abolished the CRs elicited by the forelimb CS but did not prevent CRs elicited by MCP stimulation (Figures 4B and 4C).

The experiments described above do not rule out the possibility that MCP stimulation caused cerebellar output, which activated a memory trace stored outside the cerebellum. However, it has been shown previously that an animal can acquire a CR normally when cerebellar output is blocked (Krupa and Thompson, 1995), suggesting that such output is not normally a part of the CS pathway. Furthermore, we have stimulated the superior cerebellar peduncle, the output pathway from the cerebellum, with a wide variety of parameters (1–300 μ A, 0.5–200 ms pulse trains, 50–500 Hz) in >50 trained and untrained animals in this and other studies (Ivarsson and Hesslow, 1993; Ivarsson et al., 1997). We have never observed in the eyelid long latency EMG responses that were not preceded by short latency responses. In every animal, we were able to elicit short latency responses (4–5 ms). Occasionally, longer latency responses were also observed, particularly with strong or repetitive stimulation, but they were then always preceded by large short latency responses. Examples from the present experiments are illustrated in Figure 4D.

Discussion

Before concluding that the mossy fiber afferents to the cerebellum constitute the CS pathway, the following

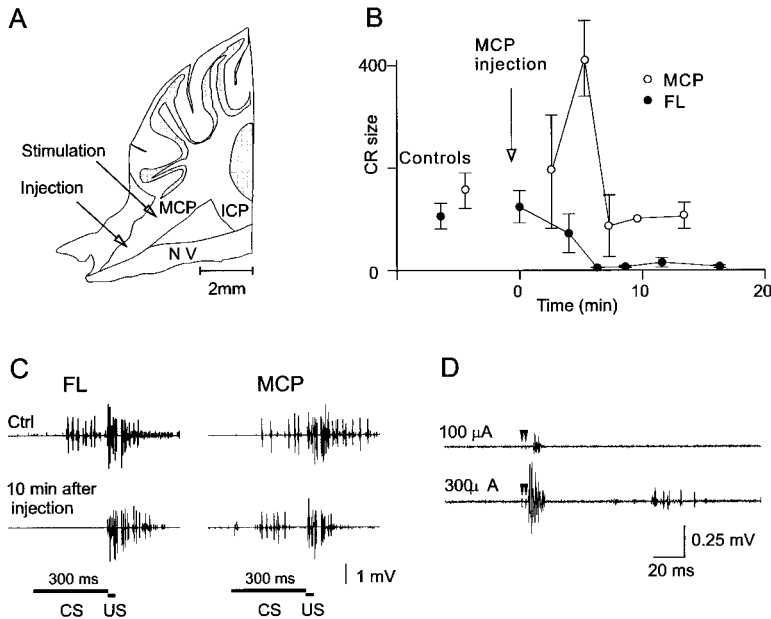


Figure 4. Effect of Blocking MCP Transmission on CRs Elicited by Forelimb and MCP Stimulation

After the animal had been trained to a forelimb CS, 1 μ l of lignocaine (4%) was injected into the MCP ventral and rostral to the stimulation site.

(A) Reconstruction from a histological section of stimulation and injection sites.

(B) Sizes (averages of five responses) of CRs elicited by forelimb and MCP stimulation before block (Control) and 15 min after injection of lignocaine. Forelimb-elicited CRs were abolished. After a transient increase, the sizes of MCP-elicited responses stabilized at about control level.

(C) Sample records of CRs from the same experiment.

(D) Eyelid EMG responses to stimulation of the superior cerebellar peduncle. Sample records of response to two pulses, 100 μ A (upper trace) and 300 μ A (lower trace). Arrowheads indicate stimulus artifacts.

questions must be answered: first, were the MCP-elicited responses really conditioned responses, second, were they elicited via the same pathways as the responses elicited by peripherally applied CSs, and third, did those pathways include the mossy fibers?

Are MCP-Elicited Responses True CRs?

It seems clear that the MCP-elicited responses were true CRs and that MCP stimulation did not merely activate a hardwired pathway to the orbicularis oculi motoneurons. The responses were adaptively timed, that is, their maximum amplitude occurred shortly before the US onset, and their topographies and latencies were quite similar to those of forelimb-elicited CRs. More importantly, they could be extinguished and reacquired by giving unpaired versus paired presentations of the MCP stimulus and the US.

Did MCP Stimulation Activate the Normal CS Pathway?

Not only were the MCP-elicited responses authentic CRs—they were also in an important respect the same CRs as those elicited by the forelimb CS. MCP stimulation did not elicit any responses after extinction of the forelimb-elicited CRs, and when the responses to the MCP CS had been extinguished, the forelimb CS could no longer elicit a CR. Thus, the MCP-elicited responses depended on conditioning to forelimb, and the forelimb-elicited responses depended on the ability of MCP stimulation to elicit CRs. These observations strongly suggest that MCP stimulation must have elicited the memory trace already established by pairing the forelimb CS with the US and that it thus activated the pathway actually utilized by the original CS. This conclusion is further supported by the observation that the MCP CS could elicit CRs on the first or the first few trials in which it was tested, that is, without ever having been paired with the US.

The conclusion that the MCP CS activated the CS pathway utilized by the forelimb CS is also supported

by previously published data showing that manipulation of the stimulation parameters of a MCP CS has effects that closely match those induced by similar manipulations of a forelimb CS. For instance, it was shown that increasing the train frequency of both forelimb and MCP stimulation shortened the CR latency (Svensson et al., 1997).

Does the CS Activate a Memory Trace in the Cerebellum?

In principle, MCP stimulation could inadvertently generate activity in several extracerebellar brainstem sites either by antidromic activation of mossy fibers and their collaterals or by causing output from the cerebellum. Such a signal might then activate a memory trace in the brainstem without involving the cerebellum in any essential way. Although it is rather implausible that such indirect activation of an alternative CS pathway would be able to mimic the activity generated by the forelimb stimulation so closely that it could also elicit CRs, it is not logically impossible.

However, the possibility that MCP stimulation worked via antidromic activation of extracerebellar sites is clearly excluded by the MCP blockade. The possibility that MCP stimulation generated cerebellar output, which in turn activated a memory store in the brainstem, may also be rejected because stimulating the cerebellar output pathway does not elicit any activity that resembles CRs. The only remaining possibility, then, is that the site of memory storage is in the cerebellum and that it is activated by a CS signal transmitted via mossy fibers in the MCP.

Why Did MCP Stimulation Not Elicit Other Learned Behaviors?

Although the finding that direct mossy fiber stimulation can elicit a previously stored memory trace is powerful evidence that the mossy fibers transmit the CS, it is also puzzling. If the MCP CS succeeded in mimicking the

activity generated in the mossy fibers by the forelimb CS so well that it elicited a similar response, why did it not also elicit other learned behavior? Presumably, the animals had learned many other things during their pre-experimental lives that might also be triggered by the activity elicited in the mossy fibers.

One possible answer is that decerebration changed the context in which previous learning had occurred. Classical conditioning is highly dependent on context (Rogers and Steinmetz, 1998). An animal learns to respond to the total stimulus situation, which consists of the experimentally manipulated CS in combination with other features of the experimental setup. CRs that have been acquired in a particular situation can disappear if the animal is transferred to a novel context. At the neuronal level, this presumably means that cerebellar learning is dependent on the background activity in mossy fibers and other cerebellar inputs. Since decerebration disrupts the forebrain input to the pontine nuclei and probably also has profound effects on the monoaminergic inputs, it represents quite a dramatic contextual change from everything that the animal has previously experienced and could have the effect that mossy fiber stimulation only elicits responses acquired after decerebration. In previous experiments (G. H. et al., unpublished data), in which we conditioned intact cats to a tone CS and then tested retention after decerebration, no CRs were ever observed. Mauk and Thompson (1987) did observe retention of CRs in rabbits after decerebration, but it is possible that rabbits are less dependent than cats on background input from the forebrain. These authors also used a somewhat less traumatic surgical technique.

An alternative explanation is that there was no occasion during the experiment for discrimination learning. When an animal first learns to respond to a particular CS, it also responds to many other stimuli. Such stimulus generalization is usually observed when the other stimuli belong to the same modality, such as different frequencies of a tone or a light, but there is also substantial transfer of learning between stimulus modalities (Thompson, 1959). It is possible that generalization reflects a convergence of information onto pontine neurons or granule cells. If other stimuli are not followed by the US, the animals learn to discriminate, that is, responses to nonreinforced stimuli are inhibited or extinguished. This could mean that many of those mossy fibers that could trigger CRs early in training would lose their ability to do so after the animal had undergone discrimination learning. As discrimination learning proceeds, a progressively smaller subset of mossy fibers would be able to elicit the response, and other mossy fibers may actually inhibit it. This is likely to have happened to most of the behaviors that the animals had acquired in their pre-experimental lives. Under natural conditions, responses that an animal has learned to perform in the presence of a certain CS are normally nonadaptive and nonreinforced under other stimulus configurations and would therefore extinguish in the presence of the latter. Responses that had been acquired by the subjects before the present experiments may therefore require a much more precise and patterned mossy fiber input to be elicited.

Similar considerations may explain the asymmetry in

the transfer of learning between MCP and forelimb stimulation. When an animal had learned to respond to a forelimb CS, it automatically responded to MCP stimulation, but when it had learned to respond to the MCP CS (after extinction of the forelimb-elicited CRs), it did not normally respond to the forelimb CS. When an animal is trained to a forelimb CS, the MCP stimulation will probably activate a substantial proportion of the fibers involved in this learning, including those transmitting input from the forelimb. But going from an MCP to a forelimb CS is probably different. When the animal is trained to the MCP CS, it will learn to respond to a much larger number of mossy fibers, and a forelimb stimulus can probably only activate a very small proportion of these. Thus, we should not expect the forelimb CS to elicit any CRs in this situation. That this did happen in one case may be due to the fact that the animal was reacquiring a previously learned response. Reacquisition is much faster than initial learning and could have happened well before any acquisition to other mossy fiber afferents had occurred. Steinmetz (1990) reported that animals that had learned to respond to stimulation of the pontine nuclei quickly (in a couple of cases, immediately) transferred to a tone CS. This result does not necessarily invalidate the argument above because Steinmetz stimulated the pontine nuclei rather than the MCP. This stimulation could have specifically activated a small population of pontine neurons with auditory input.

Cortical versus Nuclear Memory Trace

Although some investigators have reported that lesions of the cerebellar cortex abolish CRs (Yeo et al., 1985a), others have found no or only transient impairments of the CRs after cortical lesions and have suggested that the memory trace is located in the interpositus nucleus rather than in the cortex (McCormick and Thompson, 1984; Lavond et al., 1987).

Since the pontine mossy fibers (as well as climbing fibers) send collaterals to the interpositus nucleus (Brodal and Bjaalie, 1992; Shinoda et al., 1992), the present experimental results may seem consistent with both a cortical and a nuclear memory site, but in fact, there is one observation that favors the first alternative. Since we stimulated the mossy fibers in the MCP directly with a relatively high strength (up to 90 μ A), we must have produced a quite massive and synchronous mossy fiber input to the neurons of the anterior interpositus nucleus, and the resulting excitatory postsynaptic potentials (EPSPs) were probably much larger than those produced by a natural CS. If the mossy fiber collaterals are able to drive the interpositus neurons in spite of tonic Purkinje cell inhibition, as assumed by the nuclear learning hypothesis, one would therefore expect the MCP stimulus to excite these neurons and cause short latency EMG activity in the eyelid. Yet, in most cases (as seen in most of the sample records in the present paper), no trace of a short latency EMG activity was present. On some occasions, such activity did occur but could then be attributed to current spread to the trigeminal nerve.

It might be thought that excitation of interpositus neurons would be counteracted by Purkinje cell inhibition, but the route via granule cells and Purkinje cells would

not be sufficiently fast. When Shinoda et al. (1992) recorded from mossy fiber axons close to the dentate nucleus, the latencies of spikes evoked by pontine stimulation ranged from 0.3 to 1.3 ms. Antidromic spike latencies of pontine neurons activated from the dentate nucleus were 0.7–2.4 ms (Shinoda et al., 1987). The longer latency observed in the latter case is probably due to the fact that the mossy fiber collaterals entering the nucleus have smaller diameters than the main axons. A consequence of these observations is that the impulses reaching the deep nuclei cannot be delayed by more than 1 ms, compared with those reaching the granule cells of the cortex. Since the latter impulses must pass synapses on both granule cells and Purkinje cells as well as slowly conducting parallel fibers and Purkinje cell axons, it is difficult to imagine that Purkinje cell inhibition could block the early collateral input to the nuclear neurons.

This conclusion would be avoided if temporal summation of EPSP, resulting from mossy fiber impulses, caused a slow buildup of excitation in the interpositus neurons that reached its maximum after Purkinje cell inhibition had started. However, it has recently been shown that a single impulse in the mossy fibers is sufficient to evoke a CR (P. S. and M. I., 1999, submitted). It could also be argued that mossy fiber input causes an excitatory drive on the nuclear cells that can only be released during a delayed pause in Purkinje cell firing. The CR would thus be generated by the combined excitatory input throughout the CS–US interval and a delayed pause in Purkinje cell inhibition. But again, a single impulse in the mossy fibers suffices to elicit a CR, and it is not clear that this impulse can generate the requisite delayed and long-lasting excitation of the interpositus neurons. We grant that this cannot be excluded, but given the present state of knowledge about the cerebellar nuclei, it is difficult to see how the mossy fiber input to the interpositus nucleus could play a significant role in driving the CR.

In conclusion, the present results are strong evidence that the mossy fibers constitute the normal CS pathway, that the memory trace is located in the cerebellum, and that the cortex is a more likely site of memory storage than the cerebellar nuclei, all in good agreement with the Marr-Albus model of motor learning.

Experimental Procedures

Anesthesia and Surgery

The experiments were performed on five decerebrate ferrets (0.75–1.8 kg). The animals were deeply anesthetized with 1.5%–2% isoflurane (Abbot Laboratories, U. K.) in a mixture of O₂ and N₂O. They were initially placed in a box into which anesthetic gas was directed. When deep anesthesia had been achieved, a tracheotomy was performed, and the gas was then channeled directly into a tracheal tube. The level of anesthesia was regularly monitored by testing withdrawal reflexes.

After the head had been fixed to a stereotaxic frame, the skull was opened on the left side, and a substantial portion of the cerebral hemispheres and the thalamus was removed by aspiration. This exposed the left lateral part of the cerebellum and the superior and inferior colliculi of the brainstem. The surgery also exposed the MCP just as it enters the cerebellum and a small part of the underlying trigeminal nerve (Figure 4A). To gain access to the MCP, we sometimes had to remove the most lateral part of the inferior colliculus

by aspiration. When surgery was complete, the animals were decerebrated by a section with a blunt spatula through the brainstem ~1 mm rostral to the superior colliculus and the red nucleus. The anesthesia was then terminated. The completeness of the decerebration was always verified by postmortem examination. A pool of cotton-reinforced agar was constructed, and the exposed brain was covered with warm mineral oil. The animals were kept on artificial respiration throughout the experiment. The end-expiratory CO₂ concentration, arterial blood pressure, and rectal temperature were monitored continuously and were kept within physiological limits. During the whole experiment, infusion was given intravenously (glucose, 50 mg/ml; isotonic acetate Ringer solution; and Macrodex with NaCl, 60 mg/ml; proportion, 1:1:1; 1 ml/kg/hr).

Stimulation and Training

During initial training, the CS consisted of a 50 Hz train of 15 electrical stimuli, applied through two needle electrodes to the skin of the proximal left forelimb (1 mA, 0.2 ms, square pulses). The US consisted of periocular electrical stimulation (three square pulses, 0.5 ms, 3 mA, 50 Hz) delivered through two stainless steel electrodes inserted ~5 mm apart into the skin of the lower eyelid and starting 300 ms after the onset of the CS. The intertrial interval was 20 s.

The surgery had exposed the MCP, making it possible to insert electrodes and micropipettes into the MCP under direct visual guidance. A stimulation electrode made of tungsten wire (diameter, 50 μ m; deinsulated tip, 75 μ m) was inserted just rostral to the cerebellum into the MCP. The anode was placed either on the surface of the MCP or in the agar walls of the pool. The electrode placements were verified by histological examination after each experiment.

When MCP stimulation was used as the CS, it consisted of a train of 15 electrical stimuli (50 Hz, 0.1 ms negative square pulses, 25–90 μ A).

Recording

The CRs were monitored by recording EMG activity from the orbicularis oculi muscle through two stainless steel electrodes inserted about 2 mm apart in the lateral part of the left upper eyelid. The sampling period was 200 μ s. The EMG records were converted to digital data with an A/D converter from RC Electronics (Goleta, Ca). The size of the CRs was determined by rectifying the EMG signal and integrating the activity from 100 to 298 ms after CS onset with computer software developed in our lab. (For further details on these procedures, see Ivarsson et al., 1997.)

Lignocaine Injections

In two animals that responded with stable CRs both to a forelimb CS and a direct MCP CS, we blocked the transmission along the mossy fibers rostro-ventrally to the stimulating electrode, so that impulses from the forelimb, but not impulses generated by the MCP electrode, would be unable to reach the cerebellum. A micropipette (tip diameter, 50–100 μ m) filled with 4% lignocaine HCl (Xylocaine; Astra, Sodertalje, Sweden) was fastened to a Hamilton syringe, which was in turn attached to a micromanipulator. The pipette was inserted under visual inspection into the exposed MCP about 2 mm rostro-ventral to the MCP electrode and lowered about 1 mm into the MCP. The lignocaine solution (1 μ l) was then carefully injected into the tissue.

Histology

After each experiment, the animals were perfused with 4% formaldehyde. The cerebellum was removed and stored in formalin for at least 3 weeks. The tissue was placed in a sucrosephosphate buffer (0.2 M [pH 7.7]) solution and sectioned in 50 μ m slices. The slices were stained with cresyl violet and examined under a microscope.

Acknowledgments

This work was supported by grants from the Swedish Medical Research Council (09989) and from the Knut and Alice Wallenberg Foundation.

Received April 13, 1999; revised July 30, 1999.

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Note Added in Proof

The data referred to throughout as “P.S. and M.I., submitted” are now in press: Svensson, P., and Ivarsson, M. (1999). Short lasting conditioned stimulus applied to the middle cerebellar peduncle elicits conditioned eyeblink responses in the decerebrate ferret. *Eur. J. Neurosci.*, in press.