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Short-lasting conditioned stimulus applied to the middle cerebellar peduncle elicits delayed conditioned eye blink responses in the decerebrate ferret

P. Svensson and M. Ivarsson
Section for Neurophysiology, Department of Physiological Sciences, Sölvegatan 19, S-223 62 Lund, Sweden

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Abstract

In delay eye blink conditioning, the conditioned stimulus (CS) ends at the time of the unconditioned stimulus (US). If the CS duration is decreased, there will be a 'trace' period with no ongoing CS before the onset of the US. During this period some neural activity has to continue after the CS offset to: (i) permit association between the CS and the US; and (ii) elicit a conditioned response appearing after the CS offset. In this study we test the role of the cerebellum in maintaining CS activity required for eliciting a conditioned response after the CS offset. Decerebrate ferrets were trained in a delay conditioning paradigm with an electrical stimulation of the forelimb as CS and of the periorbital area as US. The conditioned responses in the upper eyelid were monitored with electromyographical techniques. In well-trained animals, test CSs of short duration down to 0.2 ms were applied to the forelimb or the middle cerebellar peduncle, while the interstimulus interval between CS onset and US onset was kept constant at 300 ms. Test CSs of short duration applied to the forelimb elicited conditioned responses. More importantly, also a short-lasting CS to the middle cerebellar peduncle could elicit conditioned responses. The results indicate that precerebellar CS pathways are not required for maintaining the neural activity that elicits conditioned responses after the CS offset. It is suggested that neurons maintaining such activity are located in the cerebellum, either the cortex alone or the cortex and the deep nuclei.

Introduction

Several studies have indicated that the mechanism of association in the classical eye blink conditioning paradigm is located in the cerebellum, probably parts of lobule HV and HVI projecting to the nucleus interpositus anterior (Thompson & Krupa, 1994; Yeo & Hesslow, 1998; Ivarsson, 1998).

Based on the characteristics of the cerebellar cortical architecture it was hypothesized that cerebellar mossy fibre input represents the context that triggers a conditioned response (CR) in a behavioural context (Marr, 1969), and that mossy fibre activity can be the conditioned stimulus (CS), in a classical learning sense, that elicits CRs in a neuronal circuit (Albus, 1971). The mossy fibres as CS pathway in the eye blink conditioning paradigm has received experimental support by the anatomical finding of projections from several mossy fibre sources to lobule HVI (Yeo *et al*., 1985). Furthermore, lesions of the pontine nuclei or the middle cerebellar peduncle abolished eye blink responses and direct stimulation of the pontine nuclei, the lateral reticular nuclei or the middle cerebellar peduncle could function as CS during CR acquisition (Steinmetz *et al*., 1985, 1986; Solomon *et al*., 1986; Lewis *et al*., 1987; Steinmetz *et al*., 1987; Lavond *et al*., 1987; Steinmetz, 1990; Tracy *et al*., 1998). Interestingly, it has also been shown that middle cerebellar peduncle stimulation can, without any further training, elicit 'true' eye blink CRs previously acquired to a peripheral CS (Hesslow *et al*., 1999).

Correspondence: Dr P. Svensson, as above.
E-mail: Par.Svensson@mphy.lu.se

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It is thus relatively clear that the mossy fibres transmit the CS to the cerebellum, but less clear to what extent the CS is processed before reaching the cerebellum. Some processing is required in the trace conditioning paradigm where neural activity has to bridge the trace interval between the CS offset and the US onset to: (i) permit association between the CS and the US; and (ii) to elicit a CR.

A suggested location for the neurons maintaining the CS activity after CS offset is the hippocampus as hippocampectomy abolished trace conditioned eye blink CRs (Moyer *et al*., 1990; Weiss *et al*., 1999). Also, structures in the CS pathway afferent to the cerebellum may be involved. The pontine nuclei, e.g. contain interneurons that may sustain the CS activity in the mossy fibres (Mihailoff *et al*., 1988; Brodal & Bjaalie, 1992). The cerebellum itself may contribute to the sustained CS activity. For example, sustained activity elicited by short stimuli has been identified in granule cells *in vitro* (Larson-Prior *et al*., 1995; Larson-Prior & Church, 1997).

The aim of this paper was to evaluate the role of the cerebellum in maintaining the neural activity that elicits CRs after the CS offset. First, it was tested if such a mechanism is operative in the decerebrate ferret. The animals were delay conditioned with a forelimb CS and subsequently tested with short-lasting CSs applied to the forelimb. Second, the role of the cerebellum in maintaining the CS-evoked neural activity was tested by applying short-lasting CSs directly to the mossy fibres in the middle cerebellar peduncle.

Preliminary results have previously been published (Svensson *et al*., 1997a).

Materials and methods

Anaesthesia and surgery

Twenty-one male ferrets, weighing 0.75–2.7 kg, were used for this study. Each animal was anaesthetized with isofluorane (Abbot Laboratories, England; 1.5–2% in a mixture of O_2 and N_2O). When deep anaesthesia had been achieved, a tracheotomy was performed and the gas was then channelled directly into a tracheal tube. Testing withdrawal reflexes monitored the depth of anaesthesia. If withdrawal reflexes occurred, the level of anaesthesia was increased and the surgery was stopped until the reflexes had disappeared. The end-expiratory CO_2 concentration, arterial blood pressure and rectal temperature were monitored continuously and kept within physiological limits. Throughout the experiment the animals were artificially ventilated and a continuous infusion was given into the femoral vein [1 mL/kg/h of a mixture of equal volumes: glucose in H_2O (50 mg/mL; Kabi Pharmacia AB, Sweden), macrodex in 0.9% NaCl solution (60 mg/mL; Medisan Pharmaceuticals, Sweden) and isotonic acetate Ringer (Pharmacia & Upjohn AB, Sweden)]. The study has been reviewed and approved by the local Swedish ethical committee.

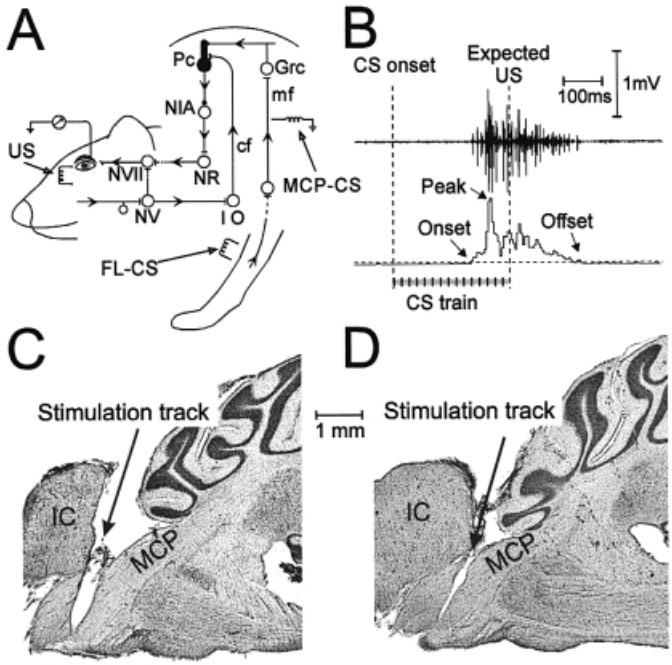

FIG. 1. Experimental and technical information. (A) Experimental set-up and simplified, hypothetical wiring diagram. The unconditioned stimulus (US) is hypothesized to be transmitted via the trigeminal nucleus (NV), the inferior olive (IO) and climbing fibres (cf) to the Purkinje cells (Pc) in the cerebellar cortex. The hypothetical pathway to the Purkinje cells for the conditioned stimulus (CS) is via mossy fibres (mf), granule cells (Grc) and parallel fibres (pf). Output from the PC in the cerebellar cortex is via the anterior interpositus nucleus (NIA), red nucleus (NR) and facial nucleus (NVII). Test CSs of different duration were applied to the forelimb (FL-CS) or the middle cerebellar peduncle (MCP-CS). The CR was recorded as EMG from the upper eyelid. (B) Sample record. The EMG activity, recorded from the orbicular oculi muscle on a CS alone trial, is high-pass filtered (top), and high-pass filtered, rectified and integrated in 5-ms bins (bottom). The horizontal bar in the histogram indicates the threshold of 200% of the spontaneous activity. (C and D) Anatomical localization of stimulation track. Sagittal sections of cerebellum and brainstem from two animals showing the tracks from which CRs were elicited. A stimulus intensity of 75 μA (C) and 100 μA (D), respectively, was required for eliciting CRs. MCP, middle cerebellar peduncle; IC, inferior colliculus.

The animal's head was fixed in a stereotaxic frame. The skull was opened on the left side, and the caudal half of the right and left cerebral hemispheres, and a substantial part of the thalamus on the left side were removed by aspiration. The aspiration exposed the cerebellum and the superior and inferior colliculi. The animal was then decerebrated by sectioning the brain stem with a blunt spatula 1–2 mm rostral to the superior colliculus, so that the red nucleus was spared. The completeness of the decerebration was verified in all cases by post-mortem examination. In experiments involving stimulation of the middle cerebellar peduncle, the peduncle was exposed on the left side by removing the most lateral part of the tentorium. Bleeding was controlled with gelfoam (Johnson & Johnson Medical, England). After decerebration the anaesthesia was terminated, and the mixture of O_2 and N_2O was replaced with a mixture of O_2 and air. A cotton-reinforced agar pool was built around the opening in the skull and was filled with warm mineral oil.

Stimulation and recording

The CS was either a peripheral stimulation of the left forelimb or a direct stimulation of the mossy fibres in the middle cerebellar peduncle. The peripheral stimulation was a 50 Hz square pulse train of 1 mA (pulse width 0.2 ms) applied subcutaneously through a pair of needle electrodes (insulated syringe tips) inserted ~5 mm apart through the skin. This intensity sometimes gave a small flexion reflex of the forelimb but never any eye blink. During the conditioning phase the CS consisted of 15 pulses, but later in the experiments the CS duration was decreased as described in the Results section.

The unconditioned stimulus (US) was a periorbital electrical stimulation through two stainless steel electrodes (insulated insect needles, de-insulated tip of ~100 μm), the tips of which were inserted ~5 mm apart into the skin of the medial part of the periorbital area. Three square pulses of 0.5 ms duration (50 Hz) and a stimulus intensity of 3 mA were used. This stimulus intensity elicited a reliable blink reflex.

The blink responses were monitored as EMG activity recorded from the orbicularis oculi muscles through two stainless steel electrodes (insulated insect needles, de-insulated tip 100 μm) inserted through the skin 2–3 mm dorsocaudal to the lateral margin of the left eye. The experimental set-up is schematically shown in Fig. 1. Electrophysiological recordings and analysis were performed with computer software developed in our laboratory. The sampling rate was 5 kHz.

In eight well-trained animals, the forelimb CS was replaced with direct stimulation of the middle cerebellar peduncle. The middle cerebellar peduncle had been exposed during the surgery so that a tungsten needle electrode (de-insulated tip diameter ~20 μm and length ~75 μm) could be positioned under visual guidance at the surface of the middle cerebellar peduncle just as it enters the cerebellum, ~5 mm lateral to the medial bone crest of the parietal bone. The electrode was lowered vertically 0.5 mm to 1.5 mm and the depth of the electrode tip was adjusted so that CRs could be reliably elicited with a 50 Hz train of 15 square pulses (pulse width 0.2 ms). Other stimulus parameters are described in the 'Test procedure' section and in the 'Results' section. The reference electrode was a silver ball (diameter ~300 μm) placed on the ventral surface of the inferior colliculus. If the electrode was lowered too deep (~1–3 mm below the surface) the trigeminal nerve was activated, which elicited short-latency EMG components as previously described (Svensson *et al*., 1997b). The stimulation site was always verified by histological examination. In Fig. 1C and D, sections from two animals show the electrode tracks from which CRs were elicited.

Training procedure

The training consisted of paired presentations of the peripheral 15-pulse CS and the US. The interstimulus interval (ISI) between the CS onset and the US onset was always 300 ms. In previous work in our laboratory neither sensitization nor pseudoconditioning has been observed with these parameters (Hesslow & Ivarsson, 1996; Ivarsson *et al*., 1997).

The intertrial interval (ITI) was kept constant at 20 s throughout most of the experiment. In order to exclude the possibility that the responses acquired during training were due to temporal conditioning, the ITI was occasionally increased in each animal to 30–60 s in a pseudorandom manner over at least 45 trials. The acquired responses always remained time locked to the CS. This test was performed in every animal.

Test procedure

When robust CRs were elicited by the forelimb CS consisting of 15 pulses, test CSs were applied to the forelimb in all animals. The forelimb test CSs were later replaced by test CSs applied directly to the middle cerebellar peduncle in eight animals. On the test trials the CS consisted of 15 pulses or was decreased to 11, 6, 2 or 1 pulses, while the ISI and the pulse frequency was kept at 300 ms and 50 Hz, respectively, and the current intensity was kept constant. To avoid new associations between the short-lasting CS and the US, the test CSs, except the 15-pulse CS, were never applied together with the US. Each CS duration was applied either as single trials or in groups of 10 trials. Possible order effects of CS duration were avoided by presenting CSs of different duration in a random order. Occasionally, some CS durations were tested more than once and in other animals some durations were not tested. Before a new CS duration was tested, each test trial or group of 10 test trials was followed by at least five consecutive reinforced trials with a CS consisting of 15 pulses applied to the forelimb or the middle cerebellar peduncle, respectively.

Analysis

In order to reduce the baseline shifts caused by the movement of the eyelid during the eye blink, the recordings were run through a digital high-pass filter before analysis started. The electromyographic (EMG) signal was rectified and the CR probability, size, onset latency, peak latency and duration were analysed. The CR size was defined as the integrated EMG activity from 60 to 800 ms after the CS onset.

To standardize the measurements of the CR onset, the CR peak and the CR offset, the EMG activity was integrated over 5-ms bins. Only EMG responses with an onset between 60 and 800 ms after the CS onset and with a peak amplitude of at least 200% of the spontaneous activity were classified as CRs. In most cases, however, there was little doubt concerning the occurrence of a CR. The CR onset latency was the time interval from the CS onset to the first 5-ms bin exceeding the average spontaneous EMG activity prior to the CS onset by 200%. The CR peak latency was the latency from the CS onset to the start of the bin with the highest amplitude. The duration of the CR was the difference between the offset and the onset of the CR, and the offset was the end of the last 5-ms bin that exceeded the spontaneous activity prior to the CS onset by 200%. A sample record showing a CR is illustrated in Fig. 1B. The same response is also presented as a diagram of the integrated activity per bin.

Histology

After the experiment the animals were perfused with sodium chloride followed by 10% (w/v) formaldehyde (Merck, Germany) in phosphate buffer (0.2 M, pH 7.7) solution. The cerebella were removed from the skull, stored in 10% formaldehyde for at least 3 weeks, frozen and sectioned either sagittally or transversally in 50-μm slices in a cryostat. The slices were mounted and stained with cresyl violet and then examined under a microscope.

Statistics

All test trials were included when analysing the CR probability, but trials without CRs were excluded when analysing the CR latency, CR duration and CR size. The effect of shortened CS duration was analysed by calculating the correlation between the number of pulses in the CS train and specific characteristics of the CR, e.g. CR onset latency, CR duration or CR size. The correlation was statistically evaluated with an *F*-test. Differences from zero were considered significant when $P < 0.05$.

Results

Short-lasting CSs applied to the forelimb or the middle cerebellar peduncle can elicit CRs

All animals were initially trained to a forelimb CS consisting of 15 pulses (50 Hz), and they emitted robust CRs (CRs in >95% of the

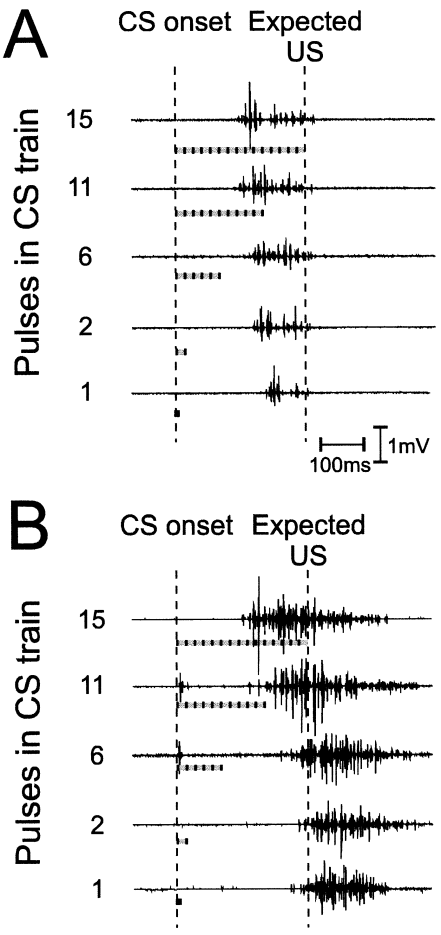

FIG. 2. Sample EMG records from the orbicularis oculi muscle. The high-pass filtered EMG records show CRs elicited on single CS alone test trials in one animal by CS trains of different duration (15, 11, 6, 2, or 1 pulses) applied to the forelimb (A) or the middle cerebellar peduncle (B; stimulus intensity 28 μA). Short-lasting middle cerebellar peduncle CS-elicited CRs indicated that neural activity was maintained for almost 300 ms. The time for the expected US is indicated.

trials for at least 1 h) within usually 4–7 h (~ 350–600 trials) of conditioning. In order to test if decerebrate ferrets could maintain the CS activity from the CS offset to the CR, shorter CS trains of 11, 6, 2 or 1 pulses were then applied to the forelimb. To avoid possible learning effects of the short-lasting CSs, each test CS was applied on single non-reinforced trials and was followed by at least five reinforced trials with the 15-pulse forelimb CS. Figure 2A shows sample records of CRs elicited by forelimb CSs of different duration. It was possible to elicit CRs with all tested CS durations, even with a CS consisting of a single pulse (0.2 ms).

The maintained neural activity in the decerebrate ferret, necessary to bridge the interval from CS offset to the CR, can in principle be set up anywhere along the CS pathway from the periphery to the cerebellum. In order to test the involvement of precerebellar sites in maintaining the CS-induced neural activity after CS offset, short-lasting CSs were applied to the middle cerebellar peduncle in animals previously trained to forelimb CS. The electrode tip had been placed at a site in the middle cerebellar peduncle from which it was possible to elicit CRs with a 15-pulse CS. A site was found in four animals within 10–20 CS alone trials. In the other four animals 10–20 trials were not sufficient, and to avoid possible extinction effects during the following search the middle cerebellar peduncle CS was reinforced. The required intensity for eliciting CRs varied between animals from 14 to 128 μA (median 67 μA). Recently, CS applied directly to the middle cerebellar peduncle in animals previously trained to a forelimb CS has been shown to elicit cerebellar-dependent and learning-dependent 'true' CRs without further training (Hesslow *et al*., 1999; see Discussion). As also found by Hesslow and co-workers (Hesslow *et al*., 1999), no systematic differences in CR onset or CR size were observed between CRs elicited by the middle cerebellar peduncle CS and the forelimb CS in the present study.

When CRs were reliably elicited by the middle cerebellar peduncle CS consisting of 15 pulses, single test trials of different duration were applied to the middle cerebellar peduncle. Also here, each test CS was applied on non-reinforced trials and was followed by at least five trials with reinforced middle cerebellar peduncle CS consisting of 15 pulses. Figure 2B shows sample records of CRs elicited by middle cerebellar peduncle CS consisting of 15, 11, 6, 2 and 1 pulses. All tested durations of the middle cerebellar peduncle CS could elicit CRs, interestingly also the CS consisting of 1 pulse (0.2 ms).

Occasionally, the middle cerebellar peduncle CS not only elicited a CR but also a short-latency and short-lasting EMG component (Fig. 2B, records 2 and 3). This early component was present in less than 0.5% of all trials with CR in six animals. In one animal the early component was present in ~ 60% of the CR trials and in another animal the CRs could not be separated from the CR. The presence of the early component was not related to the amount of training or the duration of the CS, but rather the position of the electrode tip. The short-latency EMG activity in the orbicular oculi muscle was probably caused by an activation of the trigeminal nerve as lowering the electrode too deep into the trigeminal nerve evoked the short-latency component (see Discussion; Svensson *et al*., 1997b).

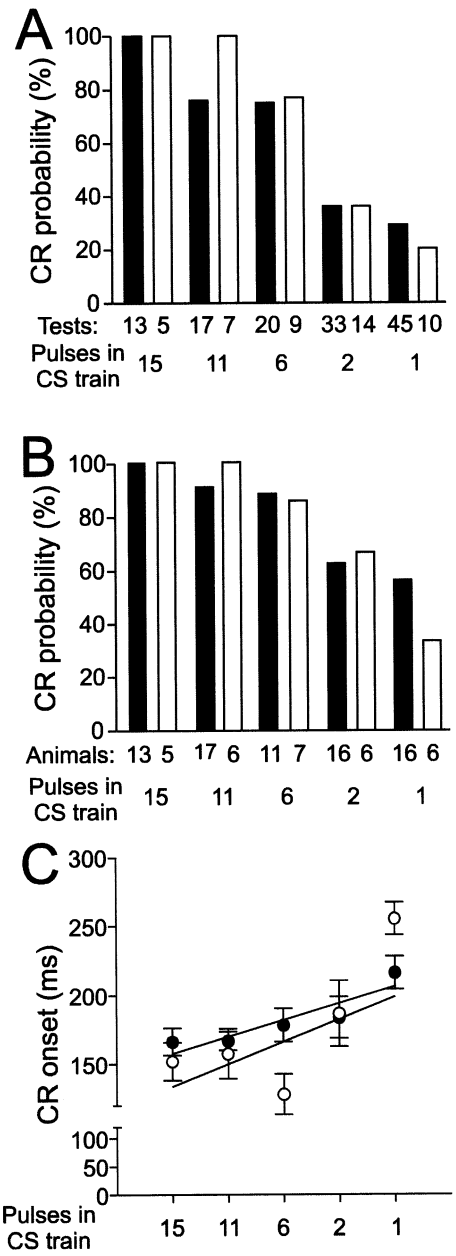

FIG. 3. Effect on CR probability and CR onset latency tested on single CS alone trials. (A and B) CR probability in each test (A; average of tests) and in each animal (B; average of tested animals) to CSs of different durations applied to the forelimb (solid bars) and the middle cerebellar peduncle (open bars). (C) Latency to the onset of CRs elicited by CSs of different durations applied to the forelimb (solid circles; mean ± SEM) and the middle cerebellar peduncle (open circles; mean ± SEM). The lines represent the linear regression (upper line, forelimb CS; lower line, middle cerebellar peduncle CS).

Characteristics of the CRs elicited by short-lasting CSs

Even though the short-lasting CSs could elicit CRs, some CR properties were changed. The CR probability decreased with shorter CSs as illustrated in Fig. 3A. The solid bars in Fig. 3A show that the forelimb CSs consisting of 15 pulses elicited CRs in all tested trials, and that the CR was present in 76, 75, 36 and 29% of the trials when the CS train was decreased to 11, 6, 2 and 1 pulses, respectively. The open bars in Fig. 3A show that the middle cerebellar peduncle CS consisting of 15 and 11 pulses elicited CRs in all tested trials, and that the CR was present in 77, 36 and 20% of the trials when the middle cerebellar peduncle CS was decreased to 6, 2 and 1 pulses, respectively.

Some animals were occasionally tested more than once with a certain CS duration and other animals not at all in a pseudorandom way. This makes it possible that a few animals contribute more to the CR probability than other animals when expressed as percentage of tests. Therefore, we also analysed the probability of an animal to elicit a CR. In Fig. 3B the solid bars show that the forelimb CS elicited CRs in all tested animals when the CS consisted of 15 pulses,

and that the CR was present in 91, 88, 62 and 56% of the animals when the CS train was decreased to 11, 6, 2 and 1 pulses, respectively. The open bars show that the middle cerebellar peduncle CS consisting of 15 and 11 pulses elicited CRs in all tested animals, and that the CR was present in 86, 67 and 33% of the animals when the middle cerebellar peduncle CS was decreased to 6, 2 and 1 pulses, respectively.

As illustrated in the sample records in Fig. 2A and B, and in the diagram in Fig. 3C, the CR latency increased with shorter CS duration. The negative correlation between the CS duration and the CR latency was weak but statistically significant in tests with the forelimb CS (solid circles, $r^2 = 0.16$, $P = 0.002$), as well as with the middle cerebellar peduncle CS (open circles, $r^2 = 0.16$, $P = 0.04$).

In the sample records in Fig. 2A and B, CRs elicited by shorter CSs had shorter duration. When all data were taken into account, however, this relationship was found to be weak and statistically non-significant for the forelimb CS ($r^2 = 0.05$, $P = 0.13$) and the middle cerebellar peduncle CS ($r^2 = 0.12$, $P = 0.14$). The CR size and CR peak latency were also analysed, but no systematic changes were found.

Effects of preceding US on the CR

A plausible explanation for the relationship between the CS duration and the CR probability is that a short-lasting CS will produce less temporal summation. The short-lasting CS will therefore decrease the likelihood that the CS signal reaches the firing threshold in synapses along the CS–CR pathway. This is, however, not the only possibility. An alternative explanation is that the CS, in addition to activating the CS–CR pathway, induces a non-specific increase in excitability or arousal. Thus, the decrease in CR probability associated with the short CS duration could reflect a decrease in general excitability. This was difficult to test but, instead of manipulating CS properties, we attempted to evaluate the excitability effects of a preceding US. It is known that the US has effects on general excitability as indicated by the finding that the CR probability decreased in well-trained subjects when the US intensity was decreased (Trapold & Spence, 1960).

In the previously described experiments, only single tests with short-lasting CSs were inserted into series of reinforced 15-pulse CS. In order to assess the effect of a preceding US, some animals were presented with a group of 10 non-reinforced test trials, one particular CS duration for each group. CS trains of 15, 11, 6, 2 and 1 pulses were tested. The forelimb CS was tested in 15 animals and the middle cerebellar peduncle CS in six of these. Not all animals were tested and all animals that were tested did not receive all five CS durations mainly because the quality of the subject had deteriorated.

In the 10-trial test, as in the single-trial test, short lasting CSs decreased the CR probability (Fig. 4A and B). The CR was present in 96% of trials tested with a forelimb CS of 15 pulses and decreased to 62%, 53%, 33% with a CS of 11, 6 and 2 pulses, respectively, and there was no difference in the CR probability to one and two pulses (34% vs 33%). The CR was present in 96% of trials tested with a middle cerebellar peduncle CS consisting of 15 pulses, and decreased to 92%, 40%, 26% with a CS consisting of 11, 6 and 2 pulses, respectively (Fig. 4B). The one pulse CS, tested in three animals, did not elicit any CRs. The probability is analysed as percentage of tested 10-trial blocks. Note that not only the number of tested 10-trial blocks is indicated but also the number of tested animals.

The 10 trial test allowed statistical testing of the effect of the shortened CS duration within the same subject. Some CS durations were occasionally tested in the 10 trial test more than once in the same animal, but the statistical testing was always performed on data from consecutive 10 trial tests, one test for each CS duration. Because not all CS durations were tested and because not all tests elicited CRs in the same animal, the within subject analysis was restricted to

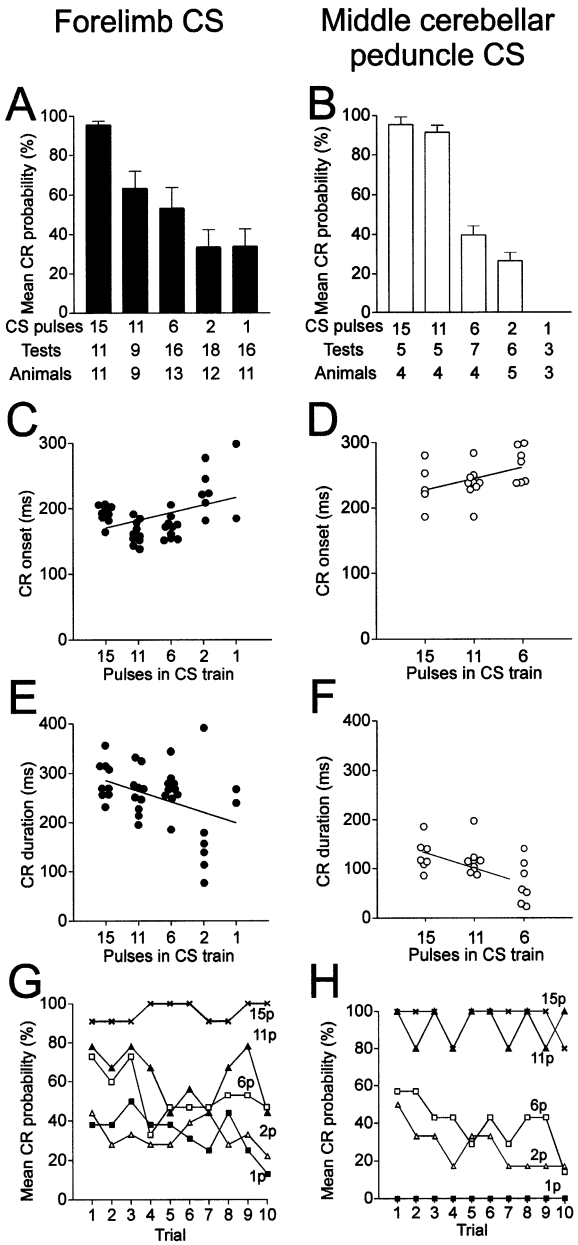

FIG. 4. Effect of decreased CS duration tested in groups of 10 consecutive CS alone test trials. (A and B) CR probability with CSs of 15, 11, 6, 2 and 1 pulses, respectively, applied to the forelimb (A; mean ± SEM) and middle cerebellar peduncle (B; mean ± SEM). The middle cerebellar peduncle CS of 1 pulse did not elicit any CRs in three tested animals. (C and D) CR onset latency in one representative animal. The CR onset latency increased with shorter CS duration of the forelimb CS (C) and the middle cerebellar peduncle CS (D). Each circle represents the onset latency of one CR and the line represents the linear regression. (E and F) CR duration in one representative animal. The CR duration decreased with shorter duration of the forelimb CS (E) and middle cerebellar peduncle CS (F). Each circle represents the duration of one CR and the line represents the linear regression. Data in C and D were calculated from the same CRs as data in E and F, respectively. (G and H) CR probability in each trial. The crosses represent mean values for CR probability to a CS of 15 pulses, solid triangles 11 pulses, empty squares 6 pulses, empty triangles 2 pulses and solid squares 1 pulse. Diagrams in the left column represent data from CRs elicited by the forelimb CS and in the right column from CRs elicited by the middle cerebellar peduncle. G and H are based on the same raw data as A and B, respectively.

animals that elicited CRs to at least three of the tested durations. The CR onset latency increased (statistically significant) in 9 of 13 animals to shortened forelimb CS, and in 3 of 4 animals to shortened middle cerebellar peduncle CS. Figure 4C and D illustrates data from one animal with longer CR latencies (statistically significant) to shorter CS duration when applied to the forelimb (Fig. 4C) and to the middle cerebellar peduncle (Fig. 4D).

In contrast to the single-trial test, the 10 trial test revealed an effect on the CR duration. Shorter CSs elicited CRs with shorter (statistically significant) duration in 11 out of 13 animals when applied to the forelimb and in all four animals when applied to the middle cerebellar peduncle. The CR durations illustrated in Fig. 4E and F are obtained from the same trials as in Fig. 4C and D, respectively. The CR size and the CR peak latency was also analysed but no systematic changes were found.

In Fig. 4C–F, the CRs elicited by the middle cerebellar peduncle have longer onset latencies and shorter durations compared with CRs elicited by forelimb CSs. However, other animals showed the opposite relation and no systematic differences were found.

There was no major difference in CR probability between the single-trial test and the 10-trial test. However, it is possible that the effect of withholding the US only appeared after a number of non-reinforced trials. Figure 4G illustrates the change in CR probability over trials. The mean CR probability decreased gradually over trials when the CS consisted of a single pulse ($r^2 = 0.31$, $P = 0.03$), but not to any of the other CS durations. Note that when the CS was six pulses (illustrated with empty squares), the CR probability dropped between the third and fourth trial, but the correlation was not statistically significant ($P = 0.14$). No statistically significant changes in CR latency were observed over trials.

The middle cerebellar peduncle CS seemed to induce a somewhat more pronounced effect compared with the forelimb CS (Fig. 4H). The mean CR probability decreased gradually over trials when the middle cerebellar peduncle CS consisted of six or two pulses ($r^2 = 0.52$, $P = 0.02$ and $r^2 = 0.62$, $P < 0.01$, respectively), but not when the CS consisted of 11 or 15 pulses. No statistically significant changes in CR latency were observed over trials.

Discussion

This study shows that very short forelimb CSs can emit CRs in animals trained to a 300 ms delay paradigm. More importantly, in these animals also very short stimuli applied directly to the middle cerebellar peduncle could emit CRs. This indicates that structures in the precerebellar CS pathways, e.g. the pontine nuclei, are not required for maintaining the elicited neural activity after CS offset necessary for emitting a CR. We also show that the CR probability is lower and the CR onset latency is longer to shorter CS durations.

Responses elicited by short-lasting middle cerebellar peduncle CS have, throughout this paper, been considered as 'true' CRs. We have recently shown that direct stimulation of the middle cerebellar peduncle in animals trained to a forelimb CS can elicit blinks without further training (Hesslow *et al*., 1999). It was demonstrated that these responses were 'true' learning-dependent CRs. For instance, they were extinguished by unpaired middle cerebellar peduncle stimulation, and if the CRs to the forelimb CS were extinguished, middle cerebellar peduncle stimulation no longer elicited any CRs.

Structures involved in maintaining the CS activity

The involvement of structures in the precerebellar CS pathway, e.g. mossy fibre sources, in maintaining the evoked neural activity after CS offset has not previously been possible to elucidate. Although direct stimulation of the pontine nuclei as CS could function as CS in a trace paradigm (Steinmetz, 1990; Tracy & Steinmetz, 1999), such stimulation is likely to activate pontine interneurons and bypassing axons, making it difficult to exclude CS processing in the pontine nuclei or possible efferent structures. Furthermore, direct application of short electrical stimulation (0.1 ms, 70–200 μA) to the cerebellar cortex or white matter could elicit CRs in delay-conditioned rabbits (Perrett, 1998), but the role of the mossy fibres was not elucidated here as it was difficult to exclude activation of other cerebellar components. The middle cerebellar peduncle CS used in the present study, however, has recently been shown to trigger CRs independently of precerebellar structures (Hesslow *et al*., 1999). A blockade of the middle cerebellar peduncle rostroventral to the stimulation site did not affect the middle cerebellar peduncle-elicited CRs, indicating that CS-evoked neural activity must have propagated to the cerebellum.

Postcerebellar structures in the CS–CR pathway could be involved in maintaining the CS-evoked neural activity. The CR may be elicited via, or delayed in, such postcerebellar structures. However, this seems improbable. Firstly, stimulation of the superior cerebellar peduncle elicited short-latency EMG responses and no CRs in trained as well as untrained animals (Ivarsson & Hesslow, 1993; Hesslow *et al*., 1999). Secondly, blockade of the superior cerebellar peduncle permits acquisition but not expression of CRs (Krupa & Thompson, 1995). Thirdly, cerebellar neural responses reveal patterns similar to the CR (McCormick & Thompson, 1984; Berthier & Moore, 1986; 1990; Hesslow & Ivarsson, 1994).

The distinction between pre- and postcerebellar structures becomes less clear when considering the fact that several loops that project from postcerebellar to precerebellar structures may be involved in cerebellar function (Tsukahara *et al*., 1983; Houk *et al*., 1996). It could be, for instance, that the interposito-pontine projection induces some pontine activity that maintains the CS activity. In intact classically conditioned rabbits, however, no such sustained activity was recorded in the pontine nuclei. Instead, only tone-related short-latency responses and CR-related long-latency responses were recorded (Clark *et al*., 1997). Also, the lack of effect on middle cerebellar peduncle-elicited CRs by rostroventral blockade, as described above, excludes suggested support by such activity.

Although animals decerebrated just rostral to the red nucleus can acquire conditioned eye blinks in the delay paradigm (Mauk & Thompson, 1987; Svensson *et al*., 1997b), it may well be that precollicular structures, e.g. the hippocampus, are involved in other aspects of CR generation. The hippocampus has been suggested to maintain the CS activity during the 'trace' period in trace conditioning (Port *et al*., 1986; Moyer *et al*., 1990). In the present study, decerebrate animals could bridge the interval from the CS offset to the CR, indicating that precollicular structures are not necessary for the maintenance of CS activity to trigger the CR. However, the interval tested here was at most 300 ms, and we can not exclude that bridging of longer intervals requires precollicular structures. It is also important to point out that the animals in the present study were initially conditioned to a delay paradigm and then tested with short-lasting CSs, making it impossible to conclude that precollicular structures are not involved in CR acquisition in the trace paradigm.

The cerebellar cortex as well as the deep nuclei can be involved in maintaining the CS activity. However, it seems unlikely that the deep nuclei alone maintain the CS activity. This should require either slow-propagating mossy fibres or some receptor property maintaining the activity. None seems to be present as stimulation of the pericruciate cortex, the pontine nuclei and the middle cerebellar

peduncle elicits short-latency EPSPs in the interpositus and dentate nuclei (Tsukahara *et al*., 1968; Shinoda *et al*., 1987; 1992; Gould *et al*., 1993). On the other hand, the cerebellar cortex seems to contain plausible substrates. For instance, Larson-Prior observed that granule cells *in vitro* could respond with sustained firing which outlasted a mossy fibre stimulus by a couple of hundred milliseconds (Larson-Prior *et al*., 1995; Larson-Prior & Church, 1997). The cortex may also act in cooperation with the deep nuclei so that Purkinje cell-induced rebound-excitation of the nuclear cells delays the eye blink CR (Ito & Yoshida, 1966; Jahnsen, 1986; Andersson & Hesslow, 1987; Llinás & Mühlethaler, 1988; Ivarsson & Hesslow, 1993).

Methodological considerations

Stimulation applied directly to the central nervous system may not only activate the intended structures but can also spread and activate unintended structures. Cerebellar sections confirmed that the CS was applied to the middle cerebellar peduncle. However, three animals required relatively high CS intensities ($\geqslant 100\,\mu A$) to elicit CRs, opening the possibility that spread of current to other nearby structures elicited the CRs. Previous studies indicate that this is unlikely. Direct stimulation of the trigeminal nerve, situated just ventro-lateral to the stimulation site in the middle cerebellar peduncle, always elicited short-latency EMG responses in the orbicularis oculi muscle and never any responses that resembled CRs (Svensson *et al*., 1997b). Also, direct stimulation of the superior cerebellar peduncle, just medial to the stimulation site, evoked a short-latency EMG response in the orbicularis oculi muscle (Ivarsson & Hesslow, 1993). In contrast, CRs elicited from the middle cerebellar peduncle were rarely accompanied by short-latency EMG responses.

On the change in CR probability and latency

The ability of short-lasting forelimb CSs to elicit CRs extends previous results where a tone CS of 12 ms and 50 ms duration elicited CRs in delay-conditioned rabbits, and confirms that shorter CSs elicit fewer CRs with longer latency (Kehoe & Napier, 1991a,b). One possible explanation for the decrease in CR probability is that a short-lasting CS is equivalent to a low-intensity CS. In this case the CS would not elicit the neural activity in the CS pathway required to reach the intensity threshold for eliciting CRs.

Another possibility is that the decreased CR probability is a result of an insufficient level in general excitability, normally required for CR expression. If so, not only the CS but also the US may change the general excitability and thereby the CR probability. An effect of the US on the general excitability would be reflected in the CR probability when comparing the single-trial test with the 10-trial test. However, although the CR duration was affected, neither the overall CR probability nor the CR latency was different from the single-trial test, indicating a minor effect of the US on general excitability.

The decrease in CR probability to shorter CSs may arise because a short-lasting CS is perceived as a new CS, as the animal never experienced the short-lasting CS during the training phase. Such stimulus generalization, indicated by a decrease in CR probability, has previously been observed. When the frequency or amplitude of a tone CS was either decreased or increased, the CR probability was decreased (Moore, 1972). Also an increase of the CS duration in the trace paradigm decreased the CR probability (Kehoe & Napier, 1991a,b).

In the 10-trial test, a decrease in CR probability over trials was observed to the shortest CS trains. This may not only reflect a decrease in excitability or a learning generalization, but also the presence of an extinction process. However, a pure extinction process is probably not the only explanation as a decrease in CR probability over trials then also would have appeared with longer test CS durations. Furthermore, extinction within 10 trials seems abnormally fast as extinction in the decerebrate ferret normally requires 30–80 trials (Ivarsson *et al*., 1998). Also, the decrease in CR latency, normally present during extinction, was not observed.

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Abbreviations

CR, conditioned response; CS, conditioned stimulus; ISI, interstimulus interval; ITI, intertrial interval; US, unconditioned stimulus.

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