Conditioned Eyeblink Response Consists of Two Distinct Components

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Ivarsson, Magnus and Pär Svensson. Conditioned eyeblink response consists of two distinct components. J. Neurophysiol. 83: 796–807, 2000. The aim of these experiments was to obtain a detailed knowledge of how the orbicularis oculi muscle is activated during the execution of a conditioned eyeblink response (CR). This is the first critical step to understand the underlying neural mechanisms involved in the control of the CR. Decerebrate ferrets were trained in a classical conditioning paradigm. The conditioned stimulus (CS) was a train of electrical stimuli (15 pulses, 50 Hz, 1 mA) applied to the forelimb, and the unconditioned stimulus (US) was a train of electrical stimuli (3 pulses, 50 Hz, 3–4 mA) to the periorbital region. The CRs were studied by recording electromyograms (EMGs) from the orbicularis oculi muscle. The eyeblink CR in all animals showed a similar topography with at least two different components, CR1 and CR2, which were expressed at different rates. CR1 appeared first during acquisition, had a shorter onset latency, and was more phasic and more resistant to extinction than CR2. A marked pause in the muscle activity separated the two components. To control that the two-component CR were not species, paradigm or preparation specific, awake rabbits were trained with a tone CS (300 ms, 4 kHz, 64 dB) and a train of periorbital stimuli as US (3 pulses, 50 Hz, 3 mA). CR1 and CR2 were present in the rabbit eyeblink CR. The cerebellum is implicated in the control of CRs and to study whether separate neural pathways were responsible for CR1 and CR2, direct brachium pontis stimulation was used to replace the forelimb CS. CR1 and CR2 were present in the CR elicited by the brachium pontis CS. The presence of CR1 and CR2 after a unilateral lesion of the brachium conjunctivum shows that output from the contralateral cerebellar hemisphere was not the cause for any of the components. Other mechanisms that might be involved in the separation of the CR into two components are discussed. The results show that the eyeblink CR consists of at least two components, CR1 and CR2, which most likely originate either as a direct central command from the cerebellum or in the output pathway before the facial nucleus.

INTRODUCTION

Classical conditioning is a simple form of associative learning where a neutral stimulus (the conditioned stimulus, CS) is paired with a second stimulus (the unconditioned stimulus, US) that unconditionally elicits a specific reflex response (the unconditioned response, UR). After a number of paired presentations of the CS and the US, the previously neutral stimulus will acquire the ability to elicit a new response. This new response is termed the conditioned response (CR). The classical conditioning paradigm has been applied to a wide variety of different responses ranging from autonomic responses to precise motor responses like the eyeblink (Gormezano and Moore 1969). In the 1960s an animal preparation was developed by Gormezano and associates to provide the robust conditions needed to study the fundamental principles of classical conditioning (see Gormezano et al. 1983). In this setup the movement of the nictitating membrane (NM) of the rabbit is used as the behavioral measure. A tone (the CS) is paired with an airpuff (the US). The airpuff directed to the cornea of the rabbit elicits a reflex activation of the retractor bulbi muscle, which causes an eyeball retraction and thereby a passive extension of the NM. After repeated pairing of the tone and the airpuff, the tone acquires the ability to elicit a NM movement and eyeblink. Most studies on classical conditioning have used the movement of NM to measure the CR, but it has been shown that there is a close correspondence between the conditioned NM and the eyelid closure caused by the orbicularis oculi muscle (m.o.o.) (McCormick et al. 1982).

A detailed knowledge of how the m.o.o. is activated during the execution of an eyeblink CR is the first critical step to understand the underlying neural mechanisms involved in the control of a CR. This knowledge will make it possible to correlate neural activity with the actual behavior of the animal and also provide critical information for making computer models of the learning processes underlying classical conditioning.

In this report we aim to analyze the temporal characteristics of the eyeblink CR during acquisition, extinction, and in well-trained animals. It will be shown that the eyeblink CR recorded by electromyogram (EMG) from the m.o.o. is a complex response consisting of at least two distinct components, which are likely to originate from one cerebellar hemisphere or in the output pathway between the cerebellum and the facial nucleus.

Some of the results have been presented in a preliminary form (Ivarsson and Svensson 1997; Ivarsson et al. 1998a).

METHODS

Ferret experiments

ANESTHESIA AND SURGERY. The experiments were performed on 19 male ferrets (1.2–2.0 kg). The experimental procedures used were approved in advance by the local ethics committee. The animals were deeply anesthetized with isoflurane (Abbott Laboratories; 1.2–1.5% in a mixture of O₂ > 30% and N₂O < 70%). They were initially placed in a box into which the anesthetic gas was directed. Once deep anesthesia had been achieved, a tracheotomy was performed and the gas was led directly into a tracheal tube. The animals were then put on artificial ventilation. During surgery the depth of anesthesia was controlled by monitoring the absence of withdrawal reflexes. To prevent edema of the cerebellum and disseminated coagulation, 0.5 ml betamethasone (Betapred, i.v. 4 mg/ml; Glaxo Operations, Greenford, London) was given 30 min prior to surgery and every day thereafter. In order to prevent isoflurane induced cerebral edema, 1 ml betamethasone was administered prior to surgery and 1 ml betamethasone (Betapred, i.v. 4 mg/ml; Glaxo Operations, Greenford, London) was given intravenously immediately after induction.

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animals were killed by exsanguination through the femoral catheter. Tungsten wire electrode (50 μm diam and 75 μm deinsulated tip) was lowered 0.5–1 mm into the BP just where it enters the cerebellum. The location of the electrode was adjusted so that a train of stimuli 60–100 μA (50 Hz, 300 ms, 0.1 ms pulse duration) elicited a CR with similar onset latency as the forelimb CS. These parameters have been shown to elicit CRs in well-trained animals (Svensson et al. 1997). The stimulation site was verified histologically.

STIMULATION. The CS was a 300-ms, 50-Hz train of electrical stimuli (0.1 ms, square-wave pulse) applied through two needle electrodes inserted subcutaneously 15 mm apart on the proximal part of the left forelimb. The strength was 1 mA, which was usually sufficient to elicit weak reflex movements of the forelimb. In two animals trained to elicit stable CRs to a forelimb CS, the BP was stimulated to elicit weak reflex movements of the forelimb. In two animals trained to elicit stable CRs to a forelimb CS, the BP was stimulated directly. This has previously been used to activate the mossy fiber input to the cerebellum (Hesslow et al. 1996; Svensson et al. 1997). A (50 Hz, 300 ms, 0.1 ms pulse duration) elicited a CR with similar onset latency as the forelimb CS. These parameters have been shown to elicit CRs in well-trained animals (Svensson et al. 1997). The stimulation site was verified histologically.

The US was delivered through two needle electrodes, one inserted in the medial part of the upper eyelid and the other in the medial part of the lower eyelid (see Fig. 7). The US was a 50-Hz train of three electrical stimuli (0.5 ms duration, negative square-wave pulse) applied unilaterally or bilaterally starting 300 ms after CS onset. Three animals (F132, F147, and F151) were trained with a unilateral US, and the other 16 animals were trained with a bilateral US. The stimulus strength on both sides was 3–4 mA. The stimulus strength was chosen to reliably evoke a maximal eyelblink reflex.

Unpaired presentations of CS and US (ISI pseudorandomly changed between 1 and 15 s) caused a gradual decrease in magnitude of the CR until the response was extinguished. By applying paired trials the CR returned, which suggests that the responses were associative in nature (Fig. 1). This has been shown previously and suggests that neither sensitization nor pseudoconditioning cause the newly acquired responses in this preparation (Hesslow and Ivarsson 1996).

The intertrial interval (ITI) was kept constant at 20 s throughout most of the experiment. To exclude the possibility that the responses acquired during training were due to temporal conditioning, the ITI was occasionally increased in a pseudorandom manner to 25–60 s for at least 5 min. The acquired responses always remained time locked to the CS. This test was performed in every animal.

Surgery, mounting of the animal in a stereotaxic frame and placement of electrodes, etc., took 3–4 h. Then, either unilateral or bilat.

FIG. 1. Unpaired presentation of conditioned stimulus (CS) and unconditioned stimulus (US) in a well-trained animal. Each bar represents the average size of the conditioned eyelink response (CR; 60–298 ms from CS onset; ±SE) for a block of 10 consecutive trials. The CR size in each block is expressed in percentage of the average size during the 2 control blocks. Solid bars correspond to the left side and open bars to the right side.

Recoding

The eyelink responses on both sides were monitored by EMG recordings from the m.o.o. through two stainless steel electrodes ~5 mm apart. They were inserted into the upper or the lower eyelid as shown in Fig. 7.

A CR was defined as the muscle activity during the period 50–300 ms after CS onset that exceeded the spontaneous muscle activity 0–100 ms before CS onset. CRs were always easy to distinguish, and there were no problems in ruling out non-CRs. Due to the multiple spikes in the EMG recordings, the onset latencies of the components in Figs. 2 and 3 were judged by visual examination and set as the time when the rectified EMG activity was ~2.5 times the spontaneous activity.

To measure the size of muscle activity, the recorded EMG was first rectified and then integrated during specific time intervals of interest. The frequency of the CRs (Figs. 2 and 3) corresponds to the percentage of trials in which a CR is present during one session. One session consisted of 10 consecutive trials of either CS-US pairings or CS-alone trials.

To characterize the CR components objectively and to reduce the number of spikes in EMG recordings, the muscle activity was rectified and integrated in 5-msec bins in 20 consecutive trials with CRs. To be classified as a component, a minimum of two consecutive bins had to show muscle activity that was at least 10% of the maximum bin in each trial and a pause in muscle activity had to show activity that was <10% of the maximum value in at least two consecutive bins to be classified as a pause. These criteria were used to characterize the components and the pause separating them. The first muscle activity that was classified as a component during the period 50–300 ms was called CR1 and the second component CR2. With these criteria there were occasional more than two components in a trial (ranging from 1 to 6 components). In all the trials used for characterizing the CR, a first component was present in 100% of the trials, a second component in 86%, a third component in 37%, a fourth component in 13%, a fifth component in 3%, and a sixth component in 1% of the trials. Due to the low frequency and large variability of the latter components, we have chosen to include the third to the sixth components in the more stable second component and together call them CR2 in the rest of the paper.

For clarity in the presentation of the records, the raw EMG signal
(sampling frequency 5 kHz) was passed through a digital high-pass filter. In all figures, traces of the average and rectified EMG activity are shown, the signal has been smoothed (21 smoothing points). EMG records were converted to digital data with an A/D converter from RC Electronics (Goleta, CA), and the acquisition and analysis of the data were made by software from our own department.

MECHANICAL LESIONS AND HISTOLOGY. In two animals, a unilateral mechanical lesion of the main cerebellar outflow pathway, the brachium conjunctivum (BC), was made. Watchmaker's forceps were lowered at the border between the inferior colliculus and the cerebellum with a 2- to 3-mm distance between the tips and to a depth of 4–5 mm. The area between the tips was completely cut off by the forceps, and the completeness of the lesion was histologically verified postmortem.

The animals were perfused with 0.9% saline followed by 10% (wt/vol) formaldehyde in H₂O. After the experiments with BP stimulation and a mechanical lesion of the BC, the cerebellum was removed from the skull and stored in 10% formaldehyde in H₂O for at least 2 wk. Before sectioning the tissue was placed in a sucrose (30% wt/vol)-phosphate buffer (0.2 M, pH 7.7) solution. The brains were frozen and sectioned transversely in 50 or 60 μm slices. These were then mounted and stained with cresyl violet and examined under a microscope.

Rabbit experiments

Two male Swedish Loop rabbits (1.5–2 kg) were used to study the eyeblink CR in an awake, unanesthetized animal. The rabbits were housed individually and maintained with free access to food and water on a 12-h light/dark cycle. The experimental procedures used were approved in advance by the local ethics committee. During conditioning, the animals were placed in a sound-attenuated restraining box, and the eyeblink was measured with EMG electrodes placed in the upper eyelid, around 5 mm above the lateral margin of the eye. The CS was a 300-ms tone (4 kHz, 65 dB) delivered by a piezocrystal that was mounted centrally 15 cm from the rabbits auditory meatus. The background noise in the box was 40–42 dB. The US was a periorbital train of three electrical stimuli (50 Hz, 3 mA, 0.5 ms duration) delivered through two stainless steel electrodes placed in the frontal margin of the rabbit eyelid 10 mm apart. The stimulation elicited a forceful eyeblink in every trial. The conditioning training consisted of paired presentations of the tone CS and the periorbital US with an ISI of 300 ms. The intertrial interval was randomly changed between 20 and 25 s. The animals were placed in the restraining box for one session on three separate days before the conditioning training began to adapt to the environment. The animals were trained for one session consisting of 100 trials each day, and the first CRs appeared during the

FIG. 2. Acquisition of CR1 and CR2. A: acquisition sessions A1–A10, A16, and A18. Each session is illustrated by the rectified, smoothed, and averaged electromyogram (EMG) from the m.o.o. A sample record from each session is shown below the average. Pointers indicate the onsets of the CS (a) and the US (c). B: plot shows the sizes (±SE) of CR1 (■) and CR2 (○) during acquisition. The size is expressed as a percentage of the maximum size. Each value represents the average CR size from 10 consecutive trials. C: the average onset latencies of the CR1 (■) and the CR2 (○) from each acquisition session show how the latencies of the 2 components decrease gradually and then stabilize around a certain latency. D: frequency of CR1 (■) and CR2 (○) in each acquisition session.
second day of conditioning. The training was continued until CRs were elicited in at least 95% of the trials.

The EMG signal was sampled at a frequency of 5 kHz and converted to digital data with an A/D converter from National Instruments, and the acquisition of the data were made with Labview software developed in our lab.

RESULTS

Ferret experiments

ACQUISITION OF THE CR COMPONENTS. Approximately 1 h after the surgery was completed, the CS-US training began, and the acquisition of the CR was studied. The first CRs recorded in the m.o.o. were, in all animals, a single or a few spikes in the EMG with varying latency and frequency. The early activity increased in size and also became more stable in onset latency with additional CS-US pairings, but the duration of this early CR remained relatively short (always longer than 50 ms). With continued CS-US pairing a second, more complex later component of the CR evolved and increased in magnitude until it reached a stable level in both size and onset latency. A marked pause in the muscular activity between the “early” and the “late component” of the CR was present in all well-trained animals.

The first EMG activity that developed first in the CS-US interval during acquisition and was followed by a pause in the EMG activity was termed CR1, and the EMG activity that developed later during acquisition and was preceded by a pause was termed CR2. Figure 2 shows the acquisition of the two components in one animal trained with bilateral USs. This animal, together with one other animal, learned to elicit CRs unusually fast and was therefore not representative with respect to the “number of trials” necessary for acquisition in this preparation. However, the acquisition process of CR1 and CR2 was representative, and the separation of the components was very clear in this animal. Therefore for the sake of clarity this animal was chosen to represent the acquisition process of the CR. In Fig. 2A the acquisition process (A1–A10, A16, and A18) is shown as rectified, averaged, and smoothed EMG activity for each 10-trial session. Below every trace a representative record from each session is shown. The individual acquisition of the two components can here be followed session by session.

Figure 2B shows the increase in size of CR1 and CR2. The squares represent the average size of the muscular activity in 10 consecutive trials during the period 50–140 ms after CS onset, which includes as much as possible of CR1 and excludes as much as possible of CR2. The circles represent CR2, which corresponds to the EMG activity during the period 140–298 ms. The variation in onset latency of the CR made it difficult to make an exact measurement of the magnitude of the individual components and compare the size of the components
with each other. To study the process of acquisition for the CR1 and the CR2, the sizes of the components were expressed in relation to their own maximum response. It is clearly seen in this plot that the size of the components increased in size with training but at different rates. CR1 reached a stable size much earlier during acquisition than CR2. The onset latencies of CR1 and CR2 are shown in Fig. 2C, and the latencies of the two components were relatively stable before the response size had peaked. Figure 2D shows the corresponding frequency of the components during acquisition.

**Extinction of the CR Components.** The extinction process is not the reverse process of acquisition (Pavlov 1927; see Mackintosh 1974) and to study whether the components also are extinguished individually, seven animals were given CS alone trials. Data from this laboratory suggest that output from the two cerebellar hemispheres interact in the extinction process (Ivarsson et al. 1998b). This was suggested by the large difference in the number of trials necessary for extinguishing the CR with bilateral extinction (US withheld on both sides) compared with when unilateral extinction (US withheld on one side) was applied. For a possible bilateral interaction six (6/7) of the animals were trained with unilateral extinction training and four (4/7) with bilateral extinction. The unilateral extinction process is illustrated in Fig. 3A as a sequence of rectified, averaged and smoothed EMG traces (E1–E12), where each trace represents the average activity of one session. One EMG record from the m.o.o. in each session is shown below the trace, and together they show how the CR1 and CR2 were extinguished. Figure 3B shows the onset latency of the components of the CR during unilateral extinction in one animal. The contralateral reinforced side is not shown. Each point represents the average latency for each component during one session. The onset latency of the components gradually increased during extinction and became more irregular. The frequencies of the CR components are shown in Fig. 3C. The plots clearly show that CR2 was extinguished several sessions before CR1. The question can be raised whether it is CR1 that increases in latency during extinction or whether it is CR2 that is more resistant to extinction. The second possibility is unlikely because the CR2 amplitude decreases markedly well before the increase in CR1 onset latency (see Fig. 3A, especially E5–E7). In four animals bilateral extinction was performed, and this is shown in Fig. 4 for one of the sides in one animal. The extinction process is much faster during bilateral extinction, and Fig. 4 shows every EMG record from the m.o.o. during the extinction and reacquisition of CR1 and CR2. The records show that, as with unilateral extinction, the CR1 was more resistant to extinction even though bilateral extinction is much faster. The CR1 was more stable and was extinguished after CR2, and in this animal the reacquisition clearly showed that CR1 was reacquired before CR2 appeared. In most of the animals the reacquisition was very fast, making the separation of the CR into CR1 and CR2 difficult. Therefore no systematic study of the reacquisition was done even though Fig. 4 suggested that the reacquisition shows a similar pattern.

**Characteristics of CR1 and CR2.** Our data suggest that the CR consists of at least two components, which are acquired and extinguished at different rates. CR1 has shorter onset latency, reaches a stable magnitude earlier during acquisition, and appears to be more phasic than CR2. The CR was studied in detail in 16 animals, and the onset latency, the duration of CR1, and the pause between the two components were measured. Table 1 shows the description of the multicomponent CR in each animal. When the animal had reached a stable level of responding and gave CRs in >95% of the trials, 20 consecutive trials with CRs were used to characterize the CR in each animal. In the 9 animals that were trained bilaterally, the description of the CRs in both eyelids are shown. In two animals the EMG activity was recorded from both the upper and the lower part of the m.o.o. (F123 and F132). The average onset latency from CS onset to CR1 was 155 ± 2.0 ms (mean ± SE; n = 16). The average duration of CR1 was 44 ± 1.4 ms (n = 16). The average pause between the offset of the CR1 and the onset of CR2 was 21 ± 0.5 ms (n = 16). The characteristics of the CR complex on the right side were as follows: average onset latency for CR1 148 ± 2.9 (n = 9), average duration 43 ± 2.7 ms (n = 9), and average duration of the pause 28 ± 1.8 ms (n = 9). The duration of CR2 was not measured in these trials because the end of the activity could not be determined due to the presentation of the US. The duration of the CR2 was therefore measured on 6–10 CS-alone trials in each of 6 animals. The average duration was found to be 148 ± 20 ms. CR2 was a more complex response, which was more variable between animals.

Figure 5 shows the rectified, averaged and smoothed EMG activity for 10 trials in 16 animals. The onset latency of the CR varied slightly from trial to trial (see Table 1) and because an average of 10 consecutive trials would blur the separation of the different components in the CR, 10 trials with similar onset latency among the 20 trials used for the descriptive statistics
The examples from the two animals show that even if the dashed line shows the CR from the original analysis in Fig. 5, the onset latency. The averaged CR analyzed in this way for two of the periods corresponding to the CR trial with the longest US onset will occur at different times within the later parts of CR2 in varying degrees depending on the onset of the CR. This method of analysis has the disadvantage that the artifacts of the US and the CR as the trigger for the average trace. This was further tested in four animals by using the onset of the CR1 was seen in every animal, although in some of the average traces, the representative record below the trace clearly shows the pause and the two components. This was likely to be due to a combination of a short pause and varying activity in the m.o.o from the 10 trials are shown below each trace. To test whether the CR1 and CR2 were present in all parts of the m.o.o. and whether the components were truly separate muscular activity and not merely an artifact caused by muscular activity are a stable and prominent feature of the eyeblink CR. Together, Figs. 5 and 6 show that the two components separated by a pause in the muscular activity are a stable and prominent feature of the eyeblink CR. Correlation coefficients were calculated, and there was no significant correlation between the size of the CR1 and the duration of the pause in any of the animals (n = 4). Because the duration of the CR1 varies from trial to trial, the size was measured in every animal from the onset of the CR1 and for the period corresponding to the minimum duration of the CR1 among the trials used for correlation. In another two animals the size of the two components in CS alone trials were correlated and showed no significant correlation.

**ARE THE COMPONENTS “TRUE” COMPONENTS OF THE CR?** To test whether the CR1 and CR2 were present in all parts of the m.o.o. and whether the components were truly separate muscular activity and not merely an artifact caused by muscular activity with different latencies in the lower eyelid or neighboring facial muscles, we recorded from multiple sites in the

### TABLE 1. Characteristics of the multicomponent CR and UR

<table>
<thead>
<tr>
<th>Animal</th>
<th>CR1 latency</th>
<th>CR1 duration</th>
<th>CR1/CR2 pause</th>
<th>CR1 latency</th>
<th>CR1 duration</th>
<th>CR1/CR2 pause</th>
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<tr>
<td>F100</td>
<td>134 ± 1.8</td>
<td>36 ± 1.1</td>
<td>37 ± 1.3</td>
<td>176 ± 1.4</td>
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<td>162 ± 1.3</td>
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<td>194 ± 0.9</td>
<td>19 ± 0.3</td>
<td>21 ± 0.6</td>
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<td>34 ± 0.3</td>
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<td>108 ± 0.4</td>
<td>23 ± 0.2</td>
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<td>F107</td>
<td>163 ± 2.6</td>
<td>34 ± 0.6</td>
<td>19 ± 0.4</td>
<td>142 ± 2.1</td>
<td>85 ± 2.8</td>
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<td>F108</td>
<td>194 ± 1.1</td>
<td>26 ± 0.4</td>
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<td>152 ± 4.1</td>
<td>16 ± 0.5</td>
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<td>F110</td>
<td>148 ± 2.4</td>
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<td>F123</td>
<td>174 ± 0.6</td>
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<td>F127</td>
<td>160 ± 0.6</td>
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<td>F129</td>
<td>160 ± 0.5</td>
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<td>F130</td>
<td>97 ± 0.8</td>
<td>74 ± 2.7</td>
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<td>Mean</td>
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**Periorbital Stimulation**

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<th>UR1,2/UR3 pause</th>
<th>UR3 latency</th>
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<tr>
<td>F130</td>
<td>12 ± 0.1</td>
<td>36 ± 0.3</td>
<td>48 ± 0.2</td>
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<td>F147</td>
<td>10 ± 0.0</td>
<td>30 ± 0.2</td>
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<td>F165</td>
<td>15 ± 0.0</td>
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<td>Mean</td>
<td>12</td>
<td>34</td>
<td>46</td>
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**Rabbit experiments**

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<th>CR1 duration</th>
<th>CR1/CR2 pause</th>
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<td>R35</td>
<td>154 ± 0.9</td>
<td>88 ± 2.0</td>
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<td>R36</td>
<td>148 ± 1.1</td>
<td>70 ± 3.0</td>
<td>13 ± 0.4</td>
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<tr>
<td>Mean</td>
<td>151</td>
<td>79</td>
<td>14</td>
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</table>

Values are means ± SE expressed in ms, with 20 trials/animal. Characteristics of the multicomponent CR and UR. Top part (Left Eyelid and Right Eyelid) of the table shows the characteristics of the CR on the left and right side. The middle part of the table (Periorbital Stimulation) shows the characteristics of the UR elicited by a single periorbital stimulus. The bottom part of the table shows the characteristics of the CR in rabbits. CR, conditioned response; UR, unconditioned response.
eyelid. Figure 7 shows three consecutive trials recorded simultaneously from two different recording sites. The locations of the recording sites are shown in the schematic drawing of the ferret’s head. One electrode pair was placed in the upper eyelid ~5 mm from the lateral margin of the eye (I), and a second pair of electrodes was placed in the lower eyelid (II). The figure shows that the overall topography of the EMG activity in each trial from the two recording sites is almost identical. This suggests that the different components are present in all parts of the m.o.o. This conclusion is further supported by the cases where single motor units were firing in both CR1 and CR2 (e.g., the records from F101 and F135 in Fig. 5).

The CR is controlled by the cerebellum, and it has been shown convincingly that the output from the cerebellum is critical for eliciting a CR (see DISCUSSION) and also that there is a contralateral input which is important for the expression of the CR (Gruart and Yeo 1995; Ivarsson et al. 1997; Skelton 1988). A contralateral contribution to the cerebellar output might cause a separation of the CR into an early and a late component where the contralateral cerebellar hemisphere is generating one of the components. This was studied in two animals where the output pathway, the BC, was mechanically lesioned on one side. Figure 8A shows two sample records from one animal ~2 h after a lesion to the contralateral BC.

**FIG. 6.** CR1 and CR2 triggered on CR onset. Ten averaged, smoothed, and rectified records were triggered either on CR onset (—) or on CS onset (— —) in 2 animals (F142 and F151) to give a clearer representation of the CR topography, especially the pause between CR1 and CR2.
which clearly show that the separation into two components is still a prominent feature of the CR. This rules out that one of the components is generated by the contralateral cerebellar hemisphere, but does not imply anything about the contribution of the output from the contralateral cerebellar hemisphere in a nonlesioned animal. The reconstruction of the brain stem shows that the BC was completely lesioned unilaterally. This was also supported by the total lack of CRs on the lesioned side during the time studied (>2 h). The section of the brain stem of the second animal also showed that the BC was completely lesioned, no CRs were seen on the lesioned side, and the CR1 and CR2 was present on the nonlesioned side.

Figure 8B shows EMG records from two animals that were trained with a forelimb CS and showed a CR in >95% of the trials. The forelimb CS was then replaced by a direct stimulation of the brachium pontis (BP), which mediates the main input to the cerebellum (see METHODS) (Hesslow et al. 1996; Svensson et al. 1997). The CRs elicited by the BP-CS show muscular activity, with an early and a late component, that was similar to the CR1 and CR2 elicited by the forelimb CS. This was seen in both animals. This finding implies that the different components of the CR cannot be explained by some precer-ebellar temporal separation of the input to the cerebellum.

DIFERENT COMPONENTS OF THE UR. To compare the components in the CR with the known components of the reflexive blink response, three well-trained ferrets were presented with a single periorbital stimulus that was sufficiently intense (1–3 mA) to elicit a forceful eyeblink. The reflex blink has been described as consisting of several components. A supraorbital or a corneal stimulus has been shown to elicit a reflex response in the m.o.o. that consists of at least two different components in EMG recordings (Hiraoka and Shimamura 1977; Kugelberg 1952; Tamai et al. 1986). The early component, termed R1, has a short onset latency around 6–7 ms in the ferret. The late response or R2 is sometimes absent, but has a longer more variable onset latency, 10–15 ms in the ferret and a magnitude that is usually larger than R1 (Ivarsson and Hesslow 1993). The neural pathways for the R1 and R2 components have
Rabbit experiment

MULTICOMPONENT CR IN THE RABBIT. It is possible that the multicomponent CR is a specific characteristic of the decerebrate ferret’s eyelid blink CR, and to control for species, preparation and paradigm specificity, we trained two unanesthetized rabbits with a tone CS and periorbital stimulation as US. Figure 10 shows the rectified, smoothed, and averaged trace of 10 trials with similar CR onset latencies in two well-trained rabbits. The 10 trials were chosen among the 20 trials used for the descriptive statistics of the rabbit CR shown in Table 1. Underneath each trace a representative EMG record is shown. The CR1 and CR2 were clearly present in the rabbits conditioned eyelid blink too. Table 1 shows the characteristics of the CR in the two rabbits studied. The CRs in the two rabbits studied showed a more variable topography between animals than the CRs in the ferret experiments, but the separation of the eyelid blink CR into two components was clear.

DISCUSSION

The results clearly show that the eyelid blink CR in ferrets and rabbits consists of at least two distinct components: CR1 and CR2. In all animals studied the multicomponent CR was present in the EMG recorded from the m.o.o. The two components were acquired at different rates and have different topography, CR1 being more phasic and CR2 more tonic. CR1 has a short duration (43 ms on average) and a fast rise time in comparison with CR2, which shows a slower rise time and a considerably longer duration (148 ms on average in CS-alone trials). A more detailed analysis of CR2, as we have defined it in this paper, might reveal the presence of additional subcomponents. These might correspond to the successive sags in eyelid movement suggested by Delgado-Garcia and colleagues during an eyelid blink CR in cat (Gruart et al. 1995). The multicomponent CR is likely to be a general feature of the eyelid CR because it is present in both decerebrate ferrets and in awake rabbits. The components in rabbits were not as prominent and stable as in the decerebrate ferret and CR1 had different topographies in the two rabbits, but the components were clearly present in the eyelid CR of the rabbit. The results presented in this study show that the multicomponent eyelid CR was not species, paradigm, or preparation specific. Because we only describe the eyelid CR, it is impossible to say whether the division into two components is a general feature of motor CRs and whether it is present in for instance conditioned forelimb flexion.

Even if the two components appear to be separately acquired and extinguished, it is still possible that the input to the muscle consists of a continuous activity without any division into components. The appearance of two components could be a threshold effect so that only when the size of CR1 is saturated and reaches maximum that a later CR2 component is expressed. This seems unlikely because there is no significant correlation between the size of CR1 and the pause and there is neither any significant correlation between the size of CR1 and the size of CR2 in CS-alone trials in a well-trained animal. Records in Figs. 2 and 3 show examples of trials where relatively small CR1s are followed by relatively large CR2s (compare, for example, the records from A8 with A4 and E5 with E1). Could CR1 and CR2 merely be an effect of the way we...
recorded the eyeblink? The m.o.o. is described as being composed of two functional subdivisions: a palpebral portion, which is mainly involved in blinking, and an orbital portion, which is mainly involved in sustained closure of the eyelid (Gordon 1951; Patrinely and Anderson 1988). The palpebral division is further divided into a pretarsal and a preseptal portion. The pretarsal part is almost exclusively made up of phasic type 2 muscle fibers, whereas the majority of larger diameter type 1 muscle fibers are in the preseptal and the orbital part of the muscle (McLoon and Wirschafter 1991). The anatomic structure of the m.o.o. is therefore compatible with the idea that the multicomponent blink is caused by intrinsic properties. The different components in the CR might be the combined EMG activity from either the palpebral and the orbital parts of the m.o.o., or the upper and the lower eyelid or from other facial muscles. We usually only recorded from the upper eyelid, and activity from other muscles might be picked up by the recording electrode as a less phasic late component, i.e., CR2. This is not likely because the time delay for the CR2 is probably too long to be ascribed to activity from other muscles and the pause would most likely not be so pronounced. The average pause in the EMG activity between the CR1 and CR2 is 24 ms. The possible influence of activity from other muscles was also tested by recording simultaneously from one electrode in the lower and one in the upper eyelid. If activity from another part of the m.o.o. was causing CR2, the two recording sites would show different patterns of activation. Figure 7 shows that the EMG activity in the upper and lower eyelid are very similar. The records in Fig. 5 show that the different components can be seen in single motor units, and the presence of components in a single unit rules out the possibility of any activity from other muscles as the cause of multiple components in the CR. This is in agreement with previous studies on humans that the same motor unit can discharge in the R1 and the R2 of the unconditioned blink reflex as well as during voluntary contraction (Dengler et al. 1982).

Delgado-Garcia and associates have studied the kinematics of conditioned eyelid movements, and they suggest that the CR is formed by successive small downward sags of the eyelid, similar in amplitude to later components of the reflex blink, that increases in number until the eyelids are closed (Domingo et al. 1997; Gruart et al. 1994, 1995). Could the small successive sags of the eyelid correspond to the CR components discussed in this paper? It is possible but in the experiments by Delgado-Garcia and colleagues mainly a strong airpuff-CS that unconditionally elicits an eyelblink has been used. An airpuff-elicited eyelblink has been shown to consist of components (see discussion below) (Berthier 1992; Welsh 1992), and therefore any activity recorded during the CS period can either be a part of the airpuff-CS-elicited response or possibly the CR components described in this paper. These two possibilities cannot be distinguished in their training paradigm.

Mauk and colleagues have shown a short-latency CR in the eyelid movement after lesioning the anterior lobe of the cerebellum (Garcia and Mauk 1998; Perrett et al. 1993). It is, however, unlikely that the components we study are related to this early component for at least three reasons: 1) the cerebellum is intact in our preparation; 2) CR1 and CR2 are extinguished with unpaired and CS alone trials in contrast to Mauk’s short latency CR (Perrett and Mauk 1995); and 3) the onset latency and topography of Mauk’s short-latency CR are totally different from CR1 (Garcia and Mauk 1998).

Are the two components described in this paper present also in the conditioned nictitating membrane response (NMR)? The tight coupling of the activity in the retractor bulbi muscle and the m.o.o. during unconditioned and conditioned eyeblinks in the awake rabbit (Berthier 1992) and the “extremely close correspondence between the left eyelid [measured as multiple muscle unit activity] and NM response” (McCormick et al. 1982), would lead us to expect that several components are present in the NMR too. Welsh (1992) showed that the NM in rabbits during a CR accelerated in two peaks, which is in agreement with the EMG components we observed in our experiments. It was stated that the second peak of acceleration was weak during early parts of training but became more prominent over the conditioning sessions, and it was also more sensitive to electrolytic lesions of the cerebellum. However, it is difficult to make a conclusion about the underlying neural signal from the finding of two peaks of acceleration in the NM for several reasons. First, measuring the change in velocity of the passive NM movement would require the knowledge of both the resistance of the transducer and the viscoelastic properties of the NM to estimate the actual movement of the NM. Second, even if the transduction properties of the movement were known, the relation between the activation of the retractor bulbi muscle and the extension of the NM is uncertain (Berthier 1992). This is further suggested by comparing the findings of the two studies by Berthier (1992) and Welsh (1992). In the Berthier (1992) study an airpuff stimulus (0.6–1.1 kg/cm²) with a duration of 80 ms invariably elicited a long-latency EMG volley in the retractor bulbi muscle with an onset latency of ~70 ms. The two components, the R1/R2 complex and the long-latency volley, were clearly separable in the rectified and integrated EMG, but it could not be seen in the NM. On the other hand, in the Welsh (1992) study the kinematic analysis of the NMR after an airpuff (0.09–2.93 kg/cm²) of 100 ms failed to distinguish any noticeable components even though the responses were studied for the same length of time. Third, another complicating factor is that the movement of the NM is a passive consequence of the retraction of the eyelid, which is due to activation of both the extraocular muscles and the retractor bulbi (Berthier and Moore 1980; Evinger and Manning 1993).

We have established that CR1 and CR2 are prominent features of the eyeblink CR, but from where do the components originate? During the last 15 yr a considerable number of papers have demonstrated the importance of the cerebellum in classical conditioning. Lesion, inactivation, and microstimulation studies have outlined the neuronal circuit that is involved in the learning and performance of a CR and confirmed the importance of an intact cerebellar cortex and deep cerebellar nuclei for the generation of a CR (see Thompson and Krupa 1994; Yeo 1991; Yeo and Hesslow 1998 for reviews and further references). Inactivation studies have also shown that the extinction of a CR is dependent on intact cerebellar cortex and deep nuclei (Hardiman et al. 1996; Ramnani and Yeo 1996). Several recent studies have shown that the generation of a CR is dependent on input from the contralateral cerebellar hemisphere (Gruart and Yeo 1995; Ivarsson et al. 1997; Skelton 1988). There are several theoretical sites where the separation of the eyeblink CR into two components could take
place. The two components could be caused 1) on the input side to the cerebellum, 2) within one cerebellar hemisphere, 3) as an interaction between the two cerebellar hemispheres, 4) on the output side of the cerebellum before the facial nucleus, and 5) in the facial motoneurons or the m.o.o.

1) One possibility is that the input to the cerebellum carrying information about the CS follows several different pathways and this causes the separation into the CR1 and CR2 components. The direct stimulation of the BP elicits CRs with components, similar to the CRs that are elicited with the forelimb stimulation (Fig. 8B). This strongly suggests that the separation of the CR into components is not caused by any delays in the precerebellar input.

2) The components are unlikely to originate from a separation of the input to the cerebellum, but it is possible that the cerebellum itself is the source of the CR1 and CR2. There are eyeblink microzones in the C1 and C3 zones of the cerebellar cortex of cats and ferrets (Hesslow 1994a,b; Ivarsson et al. 1996). One possibility is that the microzones have different properties or different inputs and could therefore learn to generate different types of outputs with the characteristics of the CR1 and CR2 component. For example, the eyeblink area in the C1 zone could generate the CR1, and the C3 area could be responsible for the CR2 or vice versa. It could of course also be different cells with different properties within one microzone. These possibilities are under study at the moment. However, one finding by Lavond and associates that might suggest that a separation into two components is taking place in the cerebellar cortex is the single-unit recording from the interpositus nucleus with two distinct peaks in its firing during the CS period (see Fig. 3 in Clark et al. 1992).

3) The importance of the contralateral cerebellar hemisphere for the generation of a unilateral CR has been shown in several studies (Gruart and Yeo 1995; Ivarsson et al. 1997; Skelton 1988). Could it be that the contralateral hemisphere is generating one of the components in the eyeblink CR? For this reason we lesioned the outflow pathway from one cerebellar hemisphere (the BC) in two animals and studied the CR on the intact side (Fig. 8A). No apparent difference in the topography of the CR was seen after the lesion, and both the CR1 and CR2 were present when only the output from one cerebellar hemisphere was intact. It therefore seems highly unlikely that the contralateral cerebellar hemisphere is responsible for specific parts of the multicomponent CR.

4) A separation of, for example, a continuous burst of activity from the cerebellum can of course be separated into several components in any of the pathways from the anterior interpositus nucleus to the facial nucleus before the signal reaches the m.o.o. At this point it is impossible to say anything about this possibility.

5) Another possible cause for the separation of the CR into components might be that it is due to properties of the facial motoneurons. Some kind of recurrent inhibition could cause a pattern of oscillating muscular activity, but the pause of activity in the eyeblink CR or the unconditioned blink reflex is not likely to be due to this type of inhibition because there is no evidence for any recurrent inhibition in the motoneurons to the m.o.o. (Penders and Delwaide 1973; Sanes and Ison 1980). Even though the facial motoneurons are the final pathway for both the eyeblink CR and the unconditioned blink reflex and the overall pattern of m.o.o. activation is similar, the properties of the two eyeblinks are different. The components of the CR are acquired and extinguished individually, they are more variable in terms of size and onset latency from trial to trial and the pause between CR1 and CR2 is of short-duration (24 ms on average). This is in contrast to the blink reflex where the components are elicited reflexively and show the same pattern of activity with little difference in latency trial after trial and a pronounced pause (34 ms on average) in the muscular activity between the R1/R2 complex and the putative R3 component. It has also been shown by several investigators that parts of the UR and the CR pathways are functionally separate. Hence lesions or inactivations of the cerebellar circuitry involved in conditioning, abolish the CR without any attenuation of the size of the UR in any major way (Hesslow 1994b; Thompson and Krupa 1994; Yeo 1991). Some studies even report an increase in the size of the UR when the CR is abolished either by cortical lesions or BC inactivations (Baker et al. 1991; Ivarsson et al. 1997; Yeo and Hardiman 1992). It has been shown that there are two separate pools of motoneurons in the facial nucleus that project to the m.o.o (van Ham and Yeo 1996b), but it is impossible so far to draw any conclusion as to whether these separate motoneuron pools are involved in the separation of muscular activity into components. If the bursts of muscular activity in the CR and UR were caused by properties in the facial motoneurons, one would expect the CR bursts and pauses to be more similar to the muscular activity of the UR. We cannot rule out the possibility, but it seems unlikely that properties of the facial motoneurons are the cause of multiple components in the eyeblink CR. As discussed above and for the same reasons as for the facial motoneurons, it seems unlikely that the separation of the CR into components is caused by the muscle cells themselves.

In conclusion, we have shown that the eyeblink CR consists of at least two separate components in the m.o.o. The multicomponent CR studied in these experiments could be caused in many different ways, but the data in this study suggest that the presence of the CR1 and CR2 are neither caused by a separation in the CS input to the cerebellum, nor any properties of the facial motoneurons, nor any intrinsic properties of the m.o.o. To date, we cannot say whether the components are due to a direct central command from the cerebellum or if it is caused somewhere along the output pathway from one cerebellar hemisphere before the facial nucleus.

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