HIGHLIGHTED TOPIC | Neural Control of Movement

Relative contributions of eyelid and eye-retraction motor systems to reflex and classically conditioned blink responses in the rabbit

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The eye blink reflex has been extensively used in studies of associative learning in different species, with emphasis on the rabbit (14, 16, 19, 30, 45–47). Blinks result from the action of the extraocular recti muscle produced very small eyelid movements and/or muscle lesions were studied in behaving rabbits. Reflex and conditioned eyelid responses were recorded in 1) controls and following 2) facial nerve section, 3) retractor bulbi muscle removal, and 4) facial nerve section and retractor bulbi muscle removal. Animals were classically conditioned with a delay paradigm by using a tone (350 ms, 600 Hz, 90 dB) as conditioned stimulus, followed 250 ms later by an air puff (100 ms, 3 kg/cm²) as unconditioned stimulus. Conditioned eyelid responses generated in the absence of the facial motor system (i.e., by the almost sole action of the retractor bulbi motor system) presented a wavy profile, due to the successation of eye-retraction movements. Learned eyelid responses generated in the absence of the eye-retraction motor system (i.e., by the almost exclusive action of the facial motor system) were similar to those of controls, but were reduced in amplitude and peak velocity. Finally, the isolated action of the extraocular recti muscle produced very small eyelid movements during both reflex and learned eyelid responses. Although each of these motor systems could act independently of the others, the motor result of their joint action did not coincide with the simple addition of their separate actions. Both facial and eye-retraction motor systems appear to be necessary for normal eyelid closure during blinking in rabbits. Central reorganization to compensate for loss of either of these systems may explain why the response of each system in isolation cannot be added linearly to obtain normal blink response magnitudes and profiles.

facial motor system; motor compensation; motor learning; orbicularis oculi muscle; retractor bulbi muscle

THE EYE BLINK REFLEX HAS BEEN EXTENSIVELY USED in studies of associative learning in different species, with emphasis on the rabbit (14, 16, 19, 30, 45–47). Blink responses result from the action of separate groups of muscles (11, 29). First, the orbicularis oculi muscle closes the eyelids, where the tonically active levator palpebrae muscle is inhibited. Additionally, the retractor bulbi muscle retracts the eyeball into the orbit and facilitates the downward movement of the upper eyelid. The nictitating membrane passively follows eyeball retraction, with a nasalteto-temporal displacement. Besides corneal protection, eyelid movements have other functions (16) that imply further coordination with other motor systems, for example, when the eyelids accompany eye movements during vertical saccades (11), or during both emotionally and voluntarily triggered facial expressions (34).

Most studies on classically conditioned eye blinks were carried out by recording nictitating membrane responses (14, 36, 41, 45, 47). The results obtained have contributed extremely important data to the understanding of the basic mechanisms underlying motor learning. Nevertheless, the passive character of nictitating membrane displacements makes the quantitative analysis of the involved muscle forces difficult, particularly with respect to their frequency domain properties. In recent years, the search coil in a magnetic field technique (13), or a variation of the Hall effect (25), has been successfully applied to record eyelid movements in mice, guinea pigs, rabbits, cats, and humans (10, 11, 16, 19, 21, 25, 43). An interesting finding using this technique was the presence of a neural oscillator (27) underlying the generation of reflex and learned eyelid responses (9, 19). Although not explicitly indicated, the presence of oscillatory movements has also been observed in learned eyelid responses in humans (30), in nictitating membrane movements (44), and in the electromyographic (EMG) activity of the orbicularis oculi muscle during classical conditioning of the blink responses in rabbits (4). The fact that the same oscillatory frequency (~20 Hz for cats) has been recorded for eyelid responses (16), resting membrane potential of facial motoneurons (28, 42), and even the motor cortex (1) suggests that oscillations present in reflex and learned blinks are the result of a neural background activity necessary for their appropriate execution (27).

The facial motor nucleus (and its corresponding cranial nerve) is frequently used as a model system to study neurochemical, cellular, and functional responses to somatic or axonal damage. The advantages of this experimental model are the large size of the facial motor nucleus and of its motoneurons, the accessibility of the facial nerve, and the easy recognition of motor deficits produced by damage to the VIIth cranial nerve and of its subsequent regeneration (26, 37). Moreover, this motor system has been widely studied after hypoglossal-facial anastomosis in human patients or as a convenient animal model of neuronal plasticity (18, 20, 33, 37). The detailed analysis of the EMG activity of the orbicularis oculi and other facial muscles can help to explain the putative

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compensatory mechanisms reported in this motor system following facial nerve reconstruction in humans and other experimental manipulations (37, 39, 40).

The present study was aimed at detecting early compensatory mechanisms (7) contributing to the recovery of missing eyelid responses when one of the motor systems involved in blink responses is damaged. For this purpose, one group of rabbits received a complete transverse section of the facial nerve; a second group had the retractor bulbi muscle resected; and a third group of animals both received a facial nerve section and had the retractor bulbi muscle resected. A fourth group of animals acted as controls. All animals were implanted with upper eyelid coils and bipolar EMG electrodes in the orbicularis oculi muscle and, when it was not removed, in the retractor bulbi muscle. The compensatory contribution of surviving motor systems was recorded from 2 wk after surgery. Eyelid movements and the EMG activity of the remaining muscles were recorded and analyzed. Blinks were evoked by air puffs to the cornea, and the animals were also trained with a delay classic conditioning paradigm. All animals of each experimental group were able to evoke reflex and learned eyelid responses. However, a kinetic and frequency domain analysis of both kinds of response revealed some important differences between them, depending on the motor systems involved in the reflexively evoked or learned eyelid responses.

**MATERIALS AND METHODS**

**Subjects.** Experiments were carried out on 24 adult male rabbits (New Zealand White albino) weighing 2.4–2.9 kg on arrival at the animal house. Animals were obtained from an authorized supplier (Iffa-Credo, Lyon, France). All experimental procedures were carried out following the guidelines of the European Union Council (86/609/ EU) and Spanish regulations (BOE 67/8509-12, 1988) for the use of laboratory animals in chronic experiments. Four experimental groups were used: 1) controls; 2) facial nerve section; 3) retractor bulbi muscle removal; and 4) facial nerve section and retractor bulbi muscle removal. All animals were implanted with search coils for the chronic recording of left upper eyelid movements, and with electrodes in the left orbicularis oculi muscle to record the EMG activity of this muscle. For animals in groups 1 and 2, two extra electrodes were implanted in the retractor bulbi muscle for EMG recordings.

**Surgical procedures.** Animals were anesthetized with an intramuscular mixture of ketamine (35–50 mg/kg), acepromazine (0.3 mg/kg), and xylazine (5 mg/kg), following a protective injection of atropine sulfate (0.4 mg/kg im) to prevent unwanted vagal reflexes. Animals were then distributed randomly in four experimental groups, depending on the kind of peripheral manipulation (Fig. 1D): 1) controls (n = 6); 2) transverse section of the left facial nerve (n = 6); 3) complete removal of the retractor bulbi muscle (n = 6); and 4) transverse section of the left facial nerve and removal of the retractor bulbi muscle (n = 6). Animals of the control group did not suffer any change in their peripheral nerves or in their muscles. In the six animals with a facial nerve section, the left main trunk of this nerve was

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**Fig. 1.** Diagrams illustrating the different experimental groups and the surgical manipulations carried out in the present study. A: one of the surgical manipulations consisted of sectioning the facial nerve trunk at the stylomastoid foramen level to disconnect the orbicularis oculi muscle from its parent motoneurons. B: a second surgical manipulation was the complete removal of the retractor bulbi muscle, responsible for eye retraction. C: recording electrodes. A small stainless steel coil was implanted in the left upper eyelid for recording eyelid movements. Bipolar hook electrodes were implanted in the orbicularis oculi and in the retractor bulbi muscles of the same side to record their electromyographic (EMG) activity. D: the 24 rabbits used in this study were divided into 4 experimental groups: controls (n = 6); animals with a facial nerve (n.) section (n = 6); animals with the retractor bulbi muscle (m.) removed (n = 6); and animals with a facial nerve (n.) section and the retractor bulbi muscle (m.) removed (n = 6).
exposed peripherally up to the stylomastoid foramen and then transected at 5 mm from the foramen with a microknife (Fig. 1A). The proximal stump of the facial nerve was displaced caudally and sutured to the nearby ear muscle with two 11-0 atrumatic sutures (Ethilon EH 7438G, Ethicon, Germany) to prevent orbicularis oculi reinnervation. For the group with the retractor bulbii muscle resected, the superior rectus muscle was de-inserted, and a long incision was made in the conjunctival membrane to allow free access behind the eyeball, where the retractor bulbii muscle is located, around the optic nerve. The four limbs of the retractor bulbii muscle were carefully resected with the help of a hook needle (Fig. 1B). Finally, the superior rectus muscle was reattached to its insertion in the eye. Six of the animals suffered both peripheral manipulations, namely, section of the left facial trunk for the group with the retractor bulbii muscle resected, the superior rectus muscle was de-inserted, and a long incision was made in the conjunctival membrane to allow free access behind the eyeball, where the retractor bulbii muscle is located, around the optic nerve. The four limbs of the retractor bulbii muscle were carefully resected with the help of a hook needle (Fig. 1B). Finally, the superior rectus muscle was reattached to its insertion in the eye. Six of the animals suffered both peripheral manipulations, namely, section of the left facial trunk

In the same surgical session, animals were implanted with a five-turn coil (3-mm diameter) in the center of the left upper eyelid, close to the lid margin (Fig. 1C). Coils were made of Teflon-coated stainless steel wire (A-M Systems, Everett, WA) with an external diameter of 50 μm. The low weight (10–15 mg) of the coils did not impair eyelid movement or cause any lid drooping compared with the contralateral (right) upper eyelid. All of the animals were also implanted with bipolar hook electrodes in the left orbicularis oculi muscle. One electrode was placed 1–2 mm posterior to the external canthus, close to the zygomatic subdivision of the facial nerve, and the other was implanted 1 mm lateral to the eyelid coil. The EMG recording electrodes were made of the same wire as the eyelid coils.

For those animals of the control and the facial nerve section groups, a second bipolar hook electrode (made of the same wire) was implanted in the retractor bulbii muscle. With the use of the same approach as in retractor bulbii removal, one of the electrodes was implanted in the dorsal limb of the muscle and the other in the medial one. These electrodes were sutured to the orbit to keep their position during eye retraction. A silver electrode (1-mm diameter) was attached to the animal’s skull as a ground. Terminals of the eyelid coil and of the EMG electrodes were bared to a nine-pin socket. The connector cemented to the cranial bone.

When the recording sessions were finished, animals were deeply reanesthetized with pentobarbital sodium (50 mg/kg ip) and perfused transcardially with a water solution of 9% saline and 4% paraformaldehyde. The correct location of EMG and coil electrodes was checked afterwards. It was also checked that the retractor bulbii muscle did not regenerate following ablation.

**General conditions for recording sessions.** Recording sessions began 2 wk after surgery and lasted ~80 min per day. Each animal was placed in a Perspex box, designed to mildly restrain its movements (14). The box was placed on a recording table and surrounded with an opaque plastic cloth. During the experiments, the recording room remained softly illuminated, with a 60-dB background white noise. Animals stayed sitting for 1 h, during which period 10 air puffs of 100-ms duration and 3 kg/cm² pressure were presented randomly. Data illustrated in Fig. 2 are from the second recording session, when
a set of 50 air puff stimuli, of the same duration and pressure as in the first, was presented at intervals of 60 ± 10 s. Data shown in Figs. 3–7 were obtained from six consecutive sessions of classic conditioning of eyelid responses using a delay conditioning paradigm.

Recording and stimulating techniques. Eyelid movements were recorded with the search coil in a magnetic field technique (16). Each coil was calibrated with a transparent protractor placed sagittally to the animal’s head and with its center located at the external canthus of the lid. During the calibration process, animals were seated in the restraining box with the head immobilized, while soft taps evoked eyelid closures. Under these conditions, the maximum range of the upper eyelid displacement was 30–48° for all of the animals. The gain of the recording system was adjusted to yield 1 V per 10°. The EMG activity of the orbicularis oculi and retractor bulbi muscles was recorded by using a differential amplifier (AM 502 Tektronix, Beaverton, OR) at a bandwidth of 1 Hz to 10 kHz.

Before conditioning sessions, reflex eyelid responses were obtained by submitting all of the animals to a set of 50 air puffs at a pressure of 3 kg/cm² and lasting 100 ms. To avoid blink fatigue and to allow the upper eyelid to return to the opening position, air puffs were presented at a random time interval of 50–70 s. As described in detail elsewhere (19), air puffs were applied through an open plastic pipette attached to an articulated metal holder fixed to the animal’s socket. The tip of the pipette was oriented toward the upper part of the cornea and placed 1 cm away from it.

Conditioned eyelid responses were obtained with the use of a delay paradigm. A tone of 350-ms duration, 600-Hz frequency, and 90-dB intensity was used as conditioned stimulus (CS). The tone was followed 250 ms from its onset by an air puff of 100-ms duration and 3 kg/cm² pressure directed at the left cornea as unconditioned stimulus (US). The two stimuli terminated at the same time. Each animal was trained for 6 consecutive days. A conditioning session lasted for ~70 min and consisted of 66 trials separated by random intervals of 50–70 s. Six of these trials were considered as test trials, in which the CS was presented alone. An animal was considered conditioned when it produced a conditioned response during the CS–US interval on 80% of the trials.

Data collection and analysis. The precise timing of air puff stimuli reaching corneal and skin mechanoreceptors was determined at the beginning of the recording sessions with a microphone located at the same site as the eye. The recorded signal was rectified, integrated, and led into the computer as 1-V rectangular pulses for latency measurements. The vertical and horizontal position of the left upper eyelid, the unresected EMG of orbicularis oculi and retractor bulbi muscles, and rectangular pulses corresponding to air puffs, or to conditioned and unconditioned stimuli, were stored digitally on an eight-channel videotape recording system (Neurodata, New York, NY). Data were transferred offline through an analog-to-digital converter (CED 1401 plus, Cambridge, UK) at 5 kHz to a computer for quantitative analysis. The amplitude resolution of the acquisition system was 12 bits. Various commercial computer programs (Spike2 and SIGAVG from CED; MATLAB, The Mathworks; and Corel Draw, Corel, Ontario, Canada) were used to represent single, overlapped, and raster profiles of eyelid position, velocity, and acceleration, and of EMG muscle activity. These programs also allowed the measurement of latency, duration, amplitude, area, and velocity of evoked eyelid movements and/or the EMG activity of recorded muscles. EMG analyses were carried out after rectification. Eyelid velocity and acceleration were computed digitally as the first and second derivative of eyelid position traces, after low-pass filtering of the data at −3-dB cutoff at 50 Hz and a zero gain at ~100 Hz (9). This low-pass filtering removed the noise without altering the data, at the frequencies of analysis (<40 Hz). Color rasters were represented with the help of a program written by R. Fernández-Mas (Instituto Mexicano de Psiquiatría, Mexico).

Statistical analyses were carried out by using the SPSS 11.0 for Windows package (SPSS, Chicago, IL), with a statistical significance level of P < 0.05. Mean values for the latency, duration, amplitude, velocity, and acceleration of eyelid responses, as well as for the latency and peak amplitude and area of EMG recordings, were calculated from 120 measurements collected from a minimum of three animals. Values are presented as means ± SD, except for Fig. 5, in which, for the sake of clarity, the SE is indicated. The parametric differences between measurements obtained from the four groups of animals were compared throughout conditioning by using a two-way (group × time) analysis of variance [multivariate ANOVA (MANOVA)] with repeated measurements in the second factor. The polynomial contrast test of MANOVA was used to verify any statistically significant trend in learned eyelid responses along the six sessions. The power of the spectral density function of interstimulus interval from eyelid acceleration recordings was calculated by using a Morlet wavelet transform to define the relative strength of the different frequencies present in eyelid acceleration responses (6). This fast wavelet transformation algorithm permitted the use of a 32-point moving window relative to 0.25-Hz discrete analyses for spectral density function analysis (35). Significance of power spectrum peaks was tested with the χ² distributed test for spectral density functions (9).

RESULTS

General considerations. Animals recovered from surgical manipulations carried out on their eyelid and eye-retraction motor systems 1 wk after surgery, and they appeared similar to controls after 2 wk, when recording sessions were started. From the first day of recording, animals with a facial nerve section presented a noticeable eyelid reflex response to corneal air puffs. These reflex responses were maintained across successive recording sessions. In these animals, the orbicularis oculi muscle was not responsible for this eyelid displacement, because no EMG activity was recorded from it. Animals with the retractor bulbi muscle resected showed identifiable reflex eyelid responses (although smaller than those of controls) to corneal air puffs, from the first recording session. These animals showed completely normal (comparable with that of the control group) EMG activity in the orbicularis oculi muscle, meaning that no damage was produced in this muscle during the surgical procedures. Air puffs presented to the ipsilateral cornea in animals with a facial nerve section and the retractor bulbi muscle resected evoked an almost unnoticeable blink reflex during the first day of conditioning. These small reflex eyelid responses did not increase across the successive recording sessions.

Kinetic characteristics of reflexively evoked blinks in the four experimental groups. Figure 2 illustrates five superimposed examples of blinks evoked in experimental and control animals by an air puff presented to the left (operated side) cornea. Recordings were made 2 wk after surgery. In all cases, a closure of the left upper eyelid could be recorded, even in those animals with a facial nerve section and the retractor bulbi muscle resected. In general, noticeable differences were found in eyelid displacement profiles and kinematics after peripheral surgery for the different experimental groups. Animals with a facial nerve section showed a more wavy profile in eyelid displacements, and, as commented on before, these downward sags presented larger peak velocities than those recorded in controls. Animals with the retractor bulbi muscle resected showed eyelid responses similar in shape to controls, although of longer onset latency, smaller maximum amplitude, and lower
peak velocity. Animals with a facial nerve section and the retractor bulbi muscle resected showed very small reflex eyelid responses, probably evoked by the cocontraction of the extraocular recti muscles (8).

The onset latency of reflex eyelid responses was measured from air puff initiation (vertical dashed line in Fig. 2, A and B) to the initial downward inflexion evoked in eyelid position recordings (a in Fig. 2B). Animals from the control group showed reflex eyelid responses with a latency of 15.1 ± 1.6 ms (Fig. 2C). Animals with a facial nerve section presented latency values similar to those of controls (14.9 ± 2.2 ms). In contrast, animals with the retractor bulbi muscle resected and those with a facial nerve section and the retractor bulbi muscle resected showed larger reflex eyelid response latencies than did controls (21.1 ± 3.9 and 21.8 ± 6.2 ms, respectively). In both cases, latency differences with the control group were statistically significant (P < 0.001; n = 120), following the contrast test of the analysis of variance.

The time to peak amplitude of a reflex eyelid response was measured as the time from the initiation of eyelid displacement to its maximum amplitude (b in Fig. 2B). The time to peak of reflex eyelid responses in controls was 87.8 ± 14.4 ms (Fig. 2E). Similar values for reflexively evoked blinks were recorded in animals with a facial nerve section (83.1 ± 13.4 ms). Animals with the retractor bulbi muscle resected showed a slightly, but statistically significant, shorter duration in the time to peak amplitude during reflex blinks (72.8 ± 21.0 ms; P < 0.05; n = 120). Animals with a facial nerve section and the retractor bulbi muscle resected presented an eyelid response of 107.3 ± 19.5 ms duration, a value significantly longer (P < 0.001; n = 120) than that in controls.

The maximum amplitude of the blink responses was measured from the opened (rest) position of the upper eyelid to the lowest position during the eyelid-closing displacement (c in Fig. 2B). Animals from the control group presented the largest eyelid displacement, with a mean amplitude of 14.9 ± 4.2° (Fig. 2D). Animals with a facial nerve section presented eyelid responses with a smaller amplitude than controls (9.3 ± 5.3°). For those animals with the retractor bulbi muscle resected, the mean value of their maximum eyelid response amplitude was even smaller (5.8 ± 2.2°). Animals with a facial nerve section and the retractor bulbi muscle resected showed reflex eyelid responses of very small amplitude (1.74 ± 1.18°). Each of the three groups of animals with manipulations of the facial nerve and/or the retractor bulbi muscle showed eyelid displacements smaller than in controls, and each of these differences was statistically significant after contrast analysis in the MANOVA test (P < 0.001; n = 120).

Peak eyelid velocity was calculated for the down phase of the reflexively evoked blink (d in Fig. 2B). Mean maximum velocity of reflex eyelid responses in controls was 547.5 ± 208.0°/s (Fig. 2F). Surprisingly, animals with a facial nerve section presented reflex eyelid responses faster than in controls, with a mean peak velocity of 809.4 ± 497.4°/s, which was statically significant (P < 0.001; n = 120). In contrast, animals with the retractor bulbi muscle resected, and those with a facial nerve section and the retractor bulbi muscle resected, presented a lower peak velocity, with means of 260.9 ± 137.7 and 94.9 ± 35.3°/s, respectively. In both cases, the differences were also statistically significant (P < 0.001; n = 120).

**Acquisition of learned eyelid responses in the four experimental groups.** The acquisition curves for eyelid-conditioned responses collected from the four experimental groups through the six consecutive sessions are presented in Fig. 3. A downward eyelid displacement was considered a conditioned response if it appeared at the CS-US interval in the absence of any other eyelid movement during the preceding 500 ms. During the first conditioning session, only animals from the control group showed any conditioned eyelid responses. An animal was considered conditioned when it was able to produce 80% of conditioned responses. This criterion was reached only in the fifth or sixth session for the controls, animals with retractor bulbi muscle resected, and animals with facial nerve section and retractor bulbi muscle resected. In contrast, the group of animals with a facial nerve section reached criterion from the fourth session. Animals with eyelid or eye-retractor motor system manipulations showed acquisition curves similar to those of controls. Surprisingly, those animals with a facial nerve section or a facial nerve section and the retractor bulbi muscle resected showed slightly more conditioned responses than the control group in all conditioning sessions after the first one. The polynomial contrast test of MANOVA indicated a statistically significant linear trend in learned eyelid responses along the six sessions for the four experimental groups (P < 0.001).

**Profiles and kinetic characteristics of eyelid-conditioned responses.** Further insight into the production of learned eyelid responses can be obtained from the representation of their profiles, evoked by the sole CS presentation (Fig. 4). This allowed the observation of complete conditioned eyelid responses, without masking by the unconditioned ones, evoked by US presentations. These recordings were obtained from the sixth conditioning session, when all of the animals of the different experimental groups had reached the maximum level of learning.

A typical conditioned eyelid response in a control animal consisted of a fast downward displacement followed by a
slower upward movement to return the eyelid to the initial (resting) position (Fig. 4A, left). The eyelid displacement was not, in fact, smooth, but was instead made up of two or more downward components, which could be easily followed in eyelid velocity profiles (Fig. 4A, middle). The EMG activity of the orbicularis oculi and retractor bulbi muscles was also recorded and represented (Fig. 4A, right). The EMG activity of the orbicularis oculi muscle started ~4 ms before the initiation of eyelid displacement and consisted of a burst of activity of similar duration to that of downward eyelid movement. The EMG activity of the retractor bulbi muscle was initiated almost simultaneously. Spike amplitudes recorded from the retractor bulbi muscle were usually larger than for the orbicularis oculi muscle and lasted until the eyelid reached its previous (opened) position.

Fig. 4. Profiles of eyelid position and velocity and of the EMG activity of orbicularis oculi and retractor bulbi muscles during conditioned eyelid responses. In all cases, the CS was a tone of 600 Hz in frequency and 90 dB in intensity, lasting for 350 ms. The US was an air puff to the left cornea, of 100 ms duration and 3 kg/cm² pressure, which started 250 ms after tone onset. These examples were obtained from test trials, i.e., when the US was omitted (represented with dotted lines), during the 6th conditioning session. A–D: experimental groups. Left: 5 superimposed examples of conditioned eyelid responses obtained for each experimental group. Middle: single trace of eyelid position and velocity of conditioned eyelid responses from each experimental group. Right: single examples of eyelid position and orbicularis oculi and retractor bulbi muscle EMG activity, obtained from representative animals during their conditioning, are shown for each experimental group. Thick horizontal dashed lines (C, D) indicate the absence of EMG recordings from the retractor bulbi muscle in groups in which this muscle was removed. Vertical dashed lines indicate CS onset. Calibrations in A are also for B–D.
position. Another particularity of the EMG activity of the muscle responsible for eye retraction was the presence of a remaining tonic activity that continued for a few seconds after the end of the conditioned eyelid response.

Conditioned eyelid responses recorded in animals with a facial nerve section showed an evident wavy profile compared with those recorded in controls (Fig. 4B, left). Different downward components, or sags, were detected during both downward and upward components of the conditioned eyelid response. This was further confirmed in eyelid velocity traces, where each component of the eyelid position was accompanied by its corresponding wave in the eyelid velocity profile (Fig. 4B, middle). No EMG activity was recorded from the orbicularis oculi muscle, as a consequence of facial nerve section (Fig. 4B, right). This was a further test of the success of the surgical procedure. The profile of EMG activity of the retractor bulbi muscle in animals with a facial nerve section was identical in both amplitude and duration to the one described for controls and during the remaining tonic activity.

Animals with the retractor bulbi muscle resected presented smaller and slower conditioned eyelid responses than did controls (Fig. 4C, left and middle). Nevertheless, the EMG activity recorded in the orbicularis oculi muscle was similar in duration and profile to the one recorded in the same muscle in controls (Fig. 4C, right). Animals with a facial nerve section and the retractor bulbi muscle resected had very small conditioned eyelid responses (Fig. 4D, left) that were accompanied by almost unnoticeable eyelid velocity profiles (Fig. 4D, middle). Obviously, no EMG activity was available in this latter experimental group, because the retractor bulbi muscle was removed and the orbicularis oculi was denervated.

For quantitative purposes, the onset latency, latency to the peak response, maximum amplitude, and peak velocity of conditioned eyelid responses were recorded and analyzed for the six conditioning sessions (Fig. 5). A comparative analysis was carried out for the EMG activity of the orbicularis oculi muscle between the control group and animals with retractor bulbi muscle resected (not illustrated). In this latter case, all of the parameters measured (onset latency, duration, integrated activity, peak amplitude, and latency to the peak amplitude) presented no statistically significant differences from controls. This result was interpreted as an absence of orbicularis oculi muscle short-term compensation after retractor bulbi muscle removal.

The latency of conditioned responses to CS onset (a in Fig. 5A) decreased slightly during conditioning (Fig. 5B). The mean value for the onset of conditioned eyelid responses in controls was $184.5 \pm 31.59$ ms for the first conditioning session and...
131.2 ± 23.5 ms for the sixth. Onset latencies measured in the group with facial nerve section (186.2 ± 31.9 ms for the second session and 130.9 ± 29.8 ms for the sixth), with the retractor bulbi muscle resected (180.1 ± 25.1 ms for the second session and 133.8 ± 17.5 ms for the sixth), and with the facial nerve section and retractor bulbi muscle resected (160.8 ± 25.7 ms for the second session and 144.0 ± 30.2 ms for the sixth) were not significantly different from those presented by controls.

The successive conditioning sessions produced an increase in the time to peak (b in Fig. 5A) of conditioned eyelid responses (Fig. 5C) in the four experimental groups. Similar values were recorded for controls (from 76.8 ± 18.2 ms in the second session to 93.5 ± 38.8 ms in the sixth session) and for the groups with facial nerve section (73.2 ± 21.2 ms for the second session to 118.0 ± 36.6 ms in the sixth session) and facial nerve section and retractor bulbi muscle resected (from 78.7 ± 27.1 ms in the second session to 121.1 ± 37.9 ms in the sixth session). Animals with the retractor bulbi muscle resected showed latencies to peak amplitude (from 78.7 ± 27.1 ms in the second session to 132.57 ± 23.15 ms in the sixth session) longer than, although not statistically different from, controls.

The maximum amplitude (c in Fig. 5A) of conditioned eyelid responses increased through the six conditioning sessions (Fig. 5D). In animals of the control group, the maximum amplitude of their conditioned eyelid responses increased from 5.6 ± 0.4° in the second session to 14.6 ± 0.7° in the sixth session. Animals with a facial nerve section also showed an increase in the maximum amplitude of their eyelid responses across conditioning (from 7.4 ± 4.9° in the second session to 12.6 ± 7.1° in the sixth session; see Fig. 5D). Animals with the retractor bulbi muscle resected showed conditioned eyelid responses smaller than controls. In all of the sessions, these differences with controls were statistically significant (P < 0.001); the maximum amplitude values for conditioned eyelid responses in this group ranged from 1.9 ± 1.1° in the second session to 11.6 ± 8.2° in the sixth session. As already described for eyelid reflex responses, animals with a facial nerve section and the retractor bulbi muscle resected showed conditioned eyelid responses of very small amplitude in all of the conditioning sessions. Maximum amplitude values of conditioned eyelid responses for this group ranged from 0.7 ± 0.4° in the second session to 2.8 ± 1.8° in the sixth session, and they were significantly smaller (P < 0.001) than those evoked in controls.

Animals of the control group showed an increase in the peak velocity (d in Fig. 5A) of conditioned eyelid responses across conditioning sessions, presenting a statistically significant linear trend (P < 0.001) when the polynomial analysis was applied (Fig. 5E). Peak velocities ranged from 40.5 ± 9.5°/s in the first session to 318.5 ± 17.2°/s in the sixth session. Interestingly, animals with a facial nerve section showed statistically significantly (P < 0.001) larger peak velocities for conditioned eyelid responses (254.5 ± 20.8°/s in the second session to 400.5 ± 17.7°/s in the sixth session), when compared session by session with values obtained in controls. Animals with the retractor bulbi muscle resected and those with a facial nerve section and the retractor bulbi muscle resected presented significantly (at least P < 0.001 for all sessions) smaller peak velocity values in their conditioned eyelid responses than the control ones, from the second to the sixth conditioning sessions. Peak velocity values of conditioned eyelid responses ranged from 42.5 ± 3.6°/s in the second session to 146.0 ± 3.6°/s in the sixth session in animals with the retractor bulbi muscle resected, and from 11.3 ± 1.2°/s in the second session to 84.5 ± 6.8°/s in the sixth session in animals with a facial nerve section and the retractor bulbi muscle resected.

Evolution of eyelid-conditioned responses. The raster displays show in Fig. 6 illustrate the evolution of the conditioned and unconditioned eyelid responses of one representative animal from each experimental group through the six conditioning sessions. Each raster was prepared by using 11 eyelid position recordings selected from each conditioning session. The control group showed a smooth increase in the amplitude of the conditioned eyelid responses and a gradual decrease in their onset latencies (Figs. 5A and 6A). It can be seen how the conditioned eyelid response built up after repeated presentation of the paired CS-US stimuli. As indicated before, animals with a facial nerve section readily showed conditioned eyelid responses (Fig. 5B), increasing in number across conditioning, as did control animals (Fig. 3). It is important to note that, in animals with a facial nerve section, conditioned eyelid responses built up through the successive conditioning sessions with a larger variability in latency and maximum velocity, giving a more disorganized view of conditioned response formation (Fig. 6B). Another characteristic of learned eyelid responses of this experimental group was the pronounced wavy profile. Typically, after a facial nerve section, the conditioned eyelid responses built up from the addition of successive pullings of the eye back into the orbit. In contrast, the removal of the retractor bulbi muscle produced conditioned eyelid responses similar to those of controls, although with smaller amplitude and lower velocity (Figs. 5C and 6C). In this latter case, conditioned eyelid responses presented a smooth build-up across the six conditioning sessions, with a profile similar to that obtained for control animals. Finally, those animals with a facial nerve section and the retractor bulbi muscle resected presented very small conditioned eyelid responses across conditioning sessions (Figs. 5D and 6D). Clearly, the extraocular muscles could not achieve the same level of performance as the eyelid-closure or eye-retractor systems.

In the group with a facial nerve section and the retractor bulbi removed, both conditioned and unconditioned eyelid responses were completely integrated (see Fig. 6 and Fig. 7, parts I and 2). For the other groups, the unconditioned eyelid response to US presentation maintained a definite separation from the learned response across the conditioning sessions.

Frequency-domain analyses of conditioned eyelid responses. Figure 7 shows how the conditioned eyelid responses were produced across the six conditioning sessions. To illustrate how a learned eyelid response was built up, we have represented (Fig. 7A) all of the trials recorded from a representative control animal during the six conditioning sessions using a color code. At the beginning of the conditioning, it was easy to identify the green and red bands corresponding to the positive and negative peaks of eyelid acceleration profiles during reflex eyelid responses (Fig. 7A2). Along the successive conditioning sessions, it was possible to identify new bands preceding the former, corresponding to the newly formed conditioned eyelid responses. It is interesting that these learned eyelid responses surpass in amplitude the reflex one (Fig. 7A2). This can also be seen in the single record illustrated in Fig.
7A1. It is important to point out the fixed timing in eyelid displacement signaled by the temporal appearance of the different bands, even during the conditioned eyelid responses, where the stimulus that acts as a trigger was weaker than the unconditioned one. A complementary frequency-domain analysis was done using eyelid acceleration recordings collected across conditioning (Fig. 7A3). The evolution of the power spectral density across the conditioning sessions revealed a statistically significant \((P < 0.01)\) dominant peak frequency in a range between 8 and 18 Hz for all control animals.

The representation of eyelid acceleration recordings across conditioning for those animals with a facial nerve section showed similar green and red bands as those in controls, indicating a similar process for the formation of the conditioned eyelid response (Fig. 7B2). It was interesting that, in this case, the easily distinguishable color pattern corresponding to unconditioned eyelid responses did not change when the conditioned eyelid response appeared. Thus, in this group of animals, conditioned eyelid responses were added to, but not completely fused with, the unconditioned ones. A single example of this case can be seen in Fig. 7B1. Moreover, although it was still possible to see the positive and negative peaks of eyelid acceleration during the conditioned responses, they appeared with a more disorganized pattern. The frequency analysis of eyelid acceleration recordings in this group presented a broad band of statistically significant \((P < 0.01)\) frequencies, ranging between 5 and 25 Hz (Fig. 7B3). Thus, and in contrast to results obtained in controls, the range of significant frequencies got broader throughout conditioning sessions, and the power spectral density decreased considerably. The presence of harmonics of the dominant frequencies was another clear difference with the spectral analysis carried out for the control group.

When the retractor bulbi muscle was removed, the evolution of eyelid acceleration recordings across conditioning sessions showed clear differences from controls (Fig. 7C2). Similar to results collected from animals with a facial nerve section, a clear fixed pattern of green and red bands corresponding to unconditioned eyelid responses could be seen. In all of the animals tested, the conditioned eyelid responses were smaller in amplitude than the control ones, and the corresponding eyelid acceleration values were far from the ones reached by the reflex responses. Single examples of this relation between position and acceleration profiles corresponding to conditioned responses.
and unconditioned eyelid responses are shown in Fig. 7C1. The spectral analysis of eyelid acceleration recordings of a representative animal in this group showed a similar range of statistically significant ($P < 0.05$) frequencies (8–18 Hz) to that in the control group (Fig. 7C3), but with a smaller power spectral density. Only during the sixth conditioning session was the power spectrum that was measured from eyelid acceleration profiles of animals with the retractor bulbi muscle resected similar to the one recorded for the animals in the control group.

**A Control**

1. Conditioned responses

2. Evolution of eyelid acceleration across conditioning sessions

3. Evolution of power spectral density across conditioning sessions

**B Facial n. section**

**C Retractor bulbi m. resection**

**D Facial n. section & retractor bulbi m. resection**
Finally, in those animals with a facial nerve section and the retractor bulbi muscle resected, no eyelid acceleration could be noted in the color code across the different conditioning sessions, when the calibration used for the other groups was maintained (Fig. 7D2). The same null recording was obtained for the power spectral density analysis (Fig. 7D3). Thus the small conditioned responses and the eyelid acceleration recorded in this group (Fig. 7D1) were imperceptible for the frequency-domain analysis carried out in this study.

**DISCUSSION**

**General overview.** Two different approaches are usually followed for measuring classically conditioned eyelid responses: 1) nictitating membrane displacement produced mainly by the retractor bulbi motor system (14, 36, 41, 45) or 2) upper eyelid course during blinking produced by the facial motor system (16, 19, 25). Surprisingly, no direct attempts had yet been made to explain the exact contribution of each motor system in any of the recorded responses. The profiles and metric properties of reflex and conditioned eyelid responses in conscious animals when eyelid and/or eye-retraction systems are removed have been shown in this study. Special care was taken to start conditioning sessions early after animals recovered from surgery, to avoid late compensatory processes produced by high-level neural centers, as similar processes have been described in the vibrissal motor system in rats (12), and adaptive processes have been proposed for muscle control during facial expressions in humans (34).

The present results indicate that the activation of the facial motor system innervating the orbicularis oculi muscle, in the absence of the retractor bulbi system, produces a movement similar to that observed in controls, although much reduced in amplitude and peak velocity. When the conditioned response was produced by the (almost) exclusive action of the retractor bulbi motor system, the evoked eyelid response presented a wavy profile, probably due to the successive eye-retraction movements characteristic of this motor system. Acting independently, both motor systems seem strong enough to protect the eye from external, potentially noxious stimuli. Finally, the isolated action of the extraocular recti muscles produces only very small eyelid responses during both reflex and learned movements. These miniscule eyelid displacements are not sufficient for protection of the corneal surface. Although each of the three motor systems considered here can act independently of the others, the motor result of the three working together does not coincide with a simple linear addition of each system acting separately. Compensatory effects generated following the lack of one of the involved systems during blinking could explain the magnitude and profiles of eyelid displacements during reflex and learned responses produced by the others.

**Contribution of the facial motor system to classically conditioned eyelid responses.** The zygomatic branch of the facial nerve innervates the orbicularis oculi muscle, which is the main one responsible for active eyelid closure in any kind of eyelid response. The retractor bulbi muscle removal approach permits a detailed analysis of that eyelid motor system, mixed with only the very small contribution of the extraocular recti muscles.

Many clinical and experimental studies have addressed the basis of the successful regeneration of the facial nerve after injury (38). A widely made conclusion is that, besides a more or less complete regeneration of all myelinated neurons, the precise and selective innervation of an adequate percentage of motoneurons in their specific target sites is necessary (47). Otherwise, the regenerated axons with inappropriate reinnervation will actually hinder the recovery of the proper movements (20). In this study, the facial nerve was not allowed to reinnervate any facial muscle, including the orbicularis oculi, to prevent any dysfunctional eyelid movement caused by inappropriate innervation.

Eyelid response profiles after retractor bulbi removal paralleled those of controls but with the amplitude and (especially) the peak velocity diminished. It seems that the orbicularis oculi muscle by itself is not sufficient for the vigorous eyelid displacement obtained during reflex and learned eyelid responses. In this regard, electrophysiological recordings carried out in the facial and the accessory abducens nuclei (where many retractor bulbi motoneurons are located) during eyelid responses in alert cats showed that the latter nucleus is usually involved in large eyelid responses but not in smaller ones (42). A spectral analysis of reflex and conditioned eyelid acceleration profiles showed a significant 8- to 10-Hz oscillation within a broad band of frequencies, ranging between 4 and 15 Hz in normal, intact rabbits (19). In the present study, rabbits maintained the same dominant peak frequency for eyelid responses, but with a diminished spectral power as a consequence of the smaller amplitude of eyelid responses, following removal of...
the retractor bulbi muscle. This means that the oscillatory component recorded from eyelid responses comes mainly from the facial motor system and that the principal mechanisms for an appropriate execution, in time and space, of eyelid movements (9, 27) probably act through this motor system.

**Contribution of the eye-retraction motor system to classically conditioned eyelid responses.** The specific contribution of the eye-retraction motor system to reflex and learned blinks can be studied by the denervation of the orbicularis oculi muscle. Reflex eyelid responses evoked by the (almost) sole contribution of the eye-retraction system showed latencies similar to those of controls, but shorter than the values recorded for air puff-evoked nictitating membrane displacements (31, 41).

An interesting finding was that the EMG activity of the retractor bulbi muscle in rabbits is maintained during both reflex and conditioned eyelid responses. This could be different in cats, because electrophysiological recordings from the accessory abducens nucleus showed that only vigorous eye blinks, such as reflexively induced ones, are accompanied by activation of the motoneurons in that nucleus (42). Thus, in cats, as opposed to rabbits, the eye-retraction motor system makes no apparent contribution to the generation of conditioned eyelid responses (18, 42).

Although eyelid displacements evoked by the retractor bulbi muscles displayed sags in their velocity profiles, these did not have the same dominant frequency as the ones produced by the facial motor system (8–10 Hz; see Ref. 19). The wavelet analysis carried out here showed a wider range (5–25 Hz) of dominant frequencies for the eye-retraction motor system. It would be interesting to perform a wavelet analysis of nictitating membrane responses to determine the spectral components present in the wavy profiles seen by eye in nictitating membrane recordings obtained by other groups (3, 4, 45).

Compared with controls, conditioned eyelid responses recorded in rabbits with a facial nerve section presented more disorganized profiles, giving an image of eyelid position and acceleration profiles far from the smooth outlines obtained in control animals across the learning process. This corroborates the proposal that the eyelid motor system, rather than the eye-retraction motor system, is the main one responsible for smooth and precise eyelid responses. Moreover, the retractor bulbi muscle in isolation evokes wavy eyelid displacements with smaller total amplitude and larger peak velocity than in controls. These findings confirm that the eye-retraction motor system produces eyelid movements that are the result of repetitive (at 5–25 Hz) pulling of the eyeball back into the orbit, instead of the smooth contraction built up by the orbicularis oculi muscle. Moreover, the retractor bulbi muscle is not exclusively necessary for nictitating membrane displacements, because the transection of the sixth cranial nerve, which innervates the greater part of it (2), produces only some 50% reduction in its displacement (5).

It has been reported that rapid (in <4 h) functional changes take place in the motor cortex of the rat following a facial nerve lesion (26). Thus it is possible that cortical effects on eyelid responses are present when one of the involved motor systems is removed. This could explain the rather strong eyelid responses recorded after the denervation of the orbicularis oculi muscle. Because the latencies of reflex and conditioned eyelid responses were similar to those obtained in controls, the possible contributions of adaptive cortical circuits have to be made in the later components of such responses.

**Contribution of the extraocular recti muscles to classically conditioned eyelid responses.** The small maximum amplitude of eyelid responses evoked with the sole action of the extraocular recti muscles (−0.5−3° for reflex and ≈0.5−4° for conditioned) is similar in size to the eyelid downward sags that have been proposed as the minimum constitutive (or quantum) element for an eyelid response (19). The limited range of those eyelid responses evoked by the sole contribution of the extraocular recti muscles makes any eye-protective role of this system improbable. The role of the extraocular recti muscles for reflex and learned eyelid responses is also irrelevant in cats, in which <10% of abducens nucleus motoneurons present some related firing during active eye retraction (8).

Transection of the six extraocular muscles has no effect on nictitating membrane responses in otherwise intact animals (5). The authors conclude that these muscles may play a role that is redundant in intact animals, because their denervation abolishes the nictitating membrane response only when the abducens nerve, also innervating the greater part of the retractor bulbi muscle (2), is also transected. Finally, it is known that the sole inhibition of levator palpebrae muscle can produce downward eyelid saccades (10, 11). The EMG activity of the levator palpebrae was not recorded in the present study, but it has to be considered for a more complete picture of muscles involved in blinking.

**Early compensatory effects between eyelid and eye-retraction motor systems.** Acute section of the facial nerve in cats produces an absence of orbicularis oculi EMG activity, as in rabbits, but it is accompanied by a total lack of eyelid responses (18, 20). Only weeks after surgery can cats close their eyelids in the absence of orbicularis oculi EMG activity, a response that has to be evoked almost exclusively by the action of the eye-retraction motor system. This is not so in the case of the rabbit, in which the retractor bulbi muscle has a more direct cooperative role with the orbicularis oculi muscle for the execution of reflex and learned eyelid responses. The different functional organization of neural circuits controlling nictitating membrane and eyelid motor learned responses in cats and rabbits is not exclusive to these two motor systems, because similar differences between species have been described for the oculomotor system (15).

The precise displacements during the different types of eyelid response seem to need the synergistic contribution of the three motor systems studied here, because, as illustrated in Fig. 7, the evolution of eyelid acceleration across the conditioning sessions showed that only control animals were capable of appropriately removing their unconditioned responses following the appearance of the conditioned one. Rabbits with a facial nerve section, or the retractor bulbi muscle resected, could produce noticeable conditioned eyelid responses, but they were unable to diminish the unconditioned eyelid responses (evoked by US presentation) following the appearance of the conditioned one.

The possible cooperative role between orbicularis oculi and retractor bulbi motoneurons could be facilitated if they shared some premotor nuclei. Some authors have found various brain stem nuclei that send information to both motor systems (22, 23, 32). The principal trigeminal nucleus and the adjacent rostral pars oralis of the spinal trigeminal nucleus project to the...
respective motoneuron pools innervating the orbicularis oculi and the retractor bulbi muscles (44). These nuclei represent putative common coordination centers supporting eye blink conditioning after receiving the corresponding motor commands from the circuits responsible for learned and nonlearned eyelid responses. An example of the integrative role of some brain stem common centers is the M group, a mesencephalic premotor nucleus with neurons projecting to motoneurons innervating levator palpebrae, superior rectus, and frontalis muscles (24). This projection suggests a role in the coordination of the upper eyelid and eye during vertical saccades. A gradient of compensatory adjustments and/or functional substitutions carried out by eyelid and eye-retraction motor systems seems to occur when rabbits, cats, and humans are compared. For rabbits, no real compensation is necessary because both (facial and eye-retraction) motor systems have a prominent role in normal eye blinks. In cats, the gain of the eye-retraction motor system can clearly increase when the eyelid motor system is removed, but this process has a slow time constant (weeks). Humans are devoid of both a nictitating membrane and an eye-retraction motor system, so there is no possible motor compensation following a complete disconnection of the facial motor system (20).

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