

Kinetic and Frequency-Domain Properties of Reflex and Conditioned Eyelid Responses in the Rabbit

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Gruart, Agnès, Bernard G. Schreurs, Eduardo Domínguez Del Toro, and José María Delgado-García. Kinetic and frequency-domain properties of reflex and conditioned eyelid responses in the rabbit. *J. Neurophysiol.* 83: 836–852, 2000. Eyelid position and the electromyographic activity of the orbicularis oculi muscle were recorded unilaterally in rabbits during reflex and conditioned blinks. Air-puff-evoked blinks consisted of a fast downward phase followed sometimes by successive downward sags. The reopening phase had a much longer duration and slower peak velocity. Onset latency, maximum amplitude, peak velocity, and rise time of reflex blinks depended on the intensity and duration of the air puff-evoking stimulus. A flashlight focused on the eye also evoked reflex blinks, but not flashes of light, or tones. Both delayed and trace classical conditioning paradigms were used. For delayed conditioning, animals were presented with a 350-ms, 90-dB, 600-Hz tone, as conditioned stimulus (CS). For trace conditioning, animals were presented with a 10-ms, 1-k/cm² air puff, as CS. The unconditioned stimulus (US) consisted of a 100-ms, 3-k/cm² air puff. The stimulus interval between CS and US onsets was 250 ms. Conditioned responses (CRs) to tones were composed of downward sags that increased in number through the successive conditioning sessions. The onset latency of the CR decreased across conditioning at the same time as its maximum amplitude and its peak velocity increased, but the time-to-peak of the CR remained unaltered. The topography of CRs evoked by short, weak air puffs as the CS showed three different components: the alpha response to the CS, the CR, and the reflex response to the US. Through conditioning, CRs showed a decrease in onset latency, and an increase in maximum amplitude and peak velocity. The time-to-peak of the CR remained unchanged. A power spectrum analysis of reflex and conditioned blink acceleration profiles showed a significant ≈ 8 -Hz oscillation within a broadband of frequencies between 4 and 15 Hz. Nose and mandible movements presented power spectrum profiles different from those characterizing reflex and conditioned blinks. It is concluded that eyelid reflex responses in the rabbit present significant differences from CRs in their profiles and metric properties, suggesting different neural origins, but that a common ≈ 8 -Hz neural oscillator underlies lid motor performance. According to available data, the frequency of this putative oscillator seems to be related to the species size.

INTRODUCTION

The nictitating membrane/eyelid response has been used for more than 60 yr as a primary experimental model for studying the neuronal organization of reflex responses and, more importantly, for understanding the mechanisms underlying motor

learning. Although early studies were carried out mostly in humans (Berstein 1934; Marquis and Porter 1939), the rabbit has been the animal of choice during the past four decades (Gormezano et al. 1962). Most of these studies were carried out by recording the nictitating membrane response (that passively follows eyeball retraction) as a measure of sensorimotor changes taking place in blink-related circuits during learning of different conditioning paradigms (Gormezano et al. 1983; Schreurs and Alkon 1990; Thompson and Krupa 1994; Welsh 1992; Woody 1986).

Although available data in rabbits have been extremely important for understanding the basic processes related to the acquisition of new motor skills, the passive character of nictitating membrane displacements makes certainly difficult its use in quantitative studies of their kinetic and, mostly, frequency-domain properties. The search coil in a magnetic field technique has been successfully used in humans, cats, rabbits, and guinea pigs (Evinger et al. 1984, 1991; Gruart et al. 1995) for the precise recording of upper eyelid movements. Because the kinetics of conditioned eyelid responses have been recently described in cats (Gruart et al. 1995), the enormous amount of available information on nictitating membrane conditioning in rabbits suggests that a similar study of lid kinetics should be carried out in the latter, for comparative and integrative purposes.

Moreover, a recent description of the frequency-domain properties of reflex and conditioned eyelid reactions in cats has suggested the existence of a ≈ 20 -Hz oscillator underlying lid movements (Domingo et al. 1997). Although not explicitly reported, human eyelid learned responses present evident discontinuities in downward lid displacements (Marquis and Porter 1939). A similar oscillation can be observed in nictitating membrane movements (Welsh 1992) or in the electromyographic (EMG) activity of the orbicularis oculi muscle during classical conditioning of blinking responses in rabbits (Berthier 1992). The reported oscillatory behavior (at ≈ 20 Hz) of pericruciate cortex neurons in the cat during the conditioning of eyelid movements (Aou et al. 1992) suggests that these oscillations are not the result of the inertial and viscoelastic properties of moving lids, but a basic, neural background for the appropriate execution of motor responses (Llinás 1991). Also, cat cerebellar interpositus neurons seem to fire in an oscillatory way during reflex blinks, precisely following the successive eyelid excursions (Gruart and Delgado-García 1994). A similar oscillation has been reported underlying firing properties of facial motoneurons innervating the orbicularis oculi muscle in

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alert cats (Trigo et al. 1999a) and in vitro studies in rats (Magariños-Ascone 1999). It seems interesting to study the frequency-domain properties of rabbit eyelid responses as a way to determine whether such oscillatory mechanisms are present in lid motor performance, and to compare them with available data in other species (Domingo et al. 1997). Moreover, a complete characterization of eyelid reflex and conditioned responses will help to the identification of functional properties of underlying neural circuitry.

The present experiments were carried out in rabbits provided with an upper lid search coil and with EMG recording electrodes implanted in the ipsilateral orbicularis oculi muscle. Reflex blinks were induced by puffs of air and by electrical stimulation of the ipsilateral supraorbital branch (SO) of the trigeminal nerve. Effects of other blink-evoking stimuli (light flashes, tones) were also examined. The profile and metric properties of evoked lid responses were analyzed as a function of the intensity and duration of air puff stimulation. Quantitative relationships between maximum lid velocity and duration of the closing (downward) phase of the blink and lid displacement were determined and discussed in relation with available data for cats (Gruart et al. 1995) and humans (Evinger et al. 1991). Reflexively evoked blinks were also compared with the activity induced in the EMG of the orbicularis oculi muscle. Animals were trained with trace and delayed conditioning paradigms. In both cases, the unconditioned stimulus (US) was a long, strong air puff. For trace conditioning, a short, weak air puff (which finished 250 ms before US onset) was used as a conditioned stimulus (CS), whereas delayed conditioning was achieved with a long-lasting tone (which finished simultaneously with the US). The topography and kinetics of eyelid conditioned responses (CRs) were analyzed with the same mathematical procedures used for reflex blinks. Finally, frequency-domain properties of reflex and conditioned eyelid responses were studied as well, and the results compared with available data for the same motor system of other species (Domingo et al. 1997). A preliminary report of this work has been presented in abstract form (Gruart et al. 1997).

METHODS

Subjects

Experiments were carried out on 10 adult rabbits (New Zealand white albino) weighing 2.3–2.7 kg on arrival from an authorized supplier (Iffa-Credo, France). Animals were prepared for the chronic recording of left upper eyelid displacements and of EMG activity of the ipsilateral orbicularis oculi muscle. In two of the rabbits, the SO nerve was stimulated. All experimental procedures were carried out in accordance with the guidelines of the European Union Council (86/609/EU) and following the Spanish regulations (BOE 67/8509–8512) for the use of laboratory animals in chronic experiments.

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. Mepivacaine hydrochloride (2%) was injected routinely into wound margins. A five-turn coil (3 mm diam) was implanted into the center of the left upper eyelid as close as possible to the lid margin. Coils were made of Teflon-coated stainless steel wire (A-M Systems, Everett, WA) with an external

diameter of 50 μm . Coils weighed 10–15 mg and did not impair movement or cause any lid drooping compared with the contralateral (right) upper eyelid. Animals were also implanted with bipolar hook electrodes in the left orbicularis oculi muscle. These electrodes were made of the same wire as the coils and bared ≈ 1 mm at the tip. One of the orbicularis oculi EMG electrodes was fixed 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. In two of the rabbits, a second bipolar hook electrode (made of the same wire) was implanted. One of the electrode tips was fixed at the SO nerve, and the other was anchored 1 mm laterally. A silver electrode (1 mm diam) was attached to the skull as a ground. Finally, terminals of lid coil, and of EMG, SO, and ground electrodes were soldered to a nine-pin socket. All of these connectors were covered with acrylic resin, and the whole system was attached to the skull with the aid of four small screws fastened and cemented to the bone.

At the end of the recording sessions, animals were deeply anesthetized with pentobarbital sodium (50 mg/kg ip) and perfused transcardially with saline and 10% Formalin. The proper location of recording lid coil and EMG electrodes was then checked.

Recording sessions

Recording sessions began 2 wk after surgery. Sessions of ~ 80 min per day, to a total of 10–12, were carried out with each animal. Each rabbit was placed in a Perspex restrainer specially designed for limiting the animal's movements (Gormezano et al. 1983). The box was placed on the recording table and was surrounded by a black cloth. The recording room was kept softly illuminated and a 60-dB background white noise was switched on during the experiments. For all the subjects, the first session consisted of the adaptation of the rabbit to the restrainer and to the experimental conditions. Animals remained sitting for 1 h, and they were presented, at random, with 6 air puffs of 50 ms of duration and 3 k/cm^2 of pressure. Data illustrated in Figs. 1 and 2 were obtained during the second recording session, when a set of 48 air puff stimuli of different pressures and duration were presented randomly at time intervals of 20–40 s. Data shown in Figs. 4–10 were obtained from classical conditioning of eyelid responses using two different conditioning paradigms as described below. Four animals were assigned at random to each of these paradigms. The remaining two animals were submitted to seven recording sessions to collect reflexively evoked blinks for parametric analyses. Data from these sessions are presented in Figs. 3, 9, and 10.

Recording of eyelid movements and EMG activity of the orbicularis oculi muscle

Eyelid movements were recorded with the magnetic field search coil technique. As described in detail elsewhere (Gruart et al. 1995), eyelid coils were calibrated with a transparent protractor placed sagittally to the animal's head and with its center located at the external canthus of the lids. During eyelid calibration, rabbits were seated in the Perspex restrainer with the head immobilized, and eyelid closures were evoked with light taps. Upper eyelid maximum opening ranged from 32 to 48° for the 10 animals. For the sake of homogeneity, the gain of the recording system was adjusted to yield 1 V per 10°. The EMG activity of the orbicularis oculi muscle was recorded with the help of differential amplifiers (AM 502 Tektronix) at a bandwidth of 0.1 Hz to 10 kHz.

Stimuli evoking movements

Before classical conditioning sessions were started, the eight animals received a set of air puff stimuli of different pressures (1, 2, 3, and 4 k/cm^2) and duration (10, 50, and 100 ms). To allow a complete return of the upper lid to its resting position during air puff presentations, stimuli were applied with a random time interval of 20–40 s.

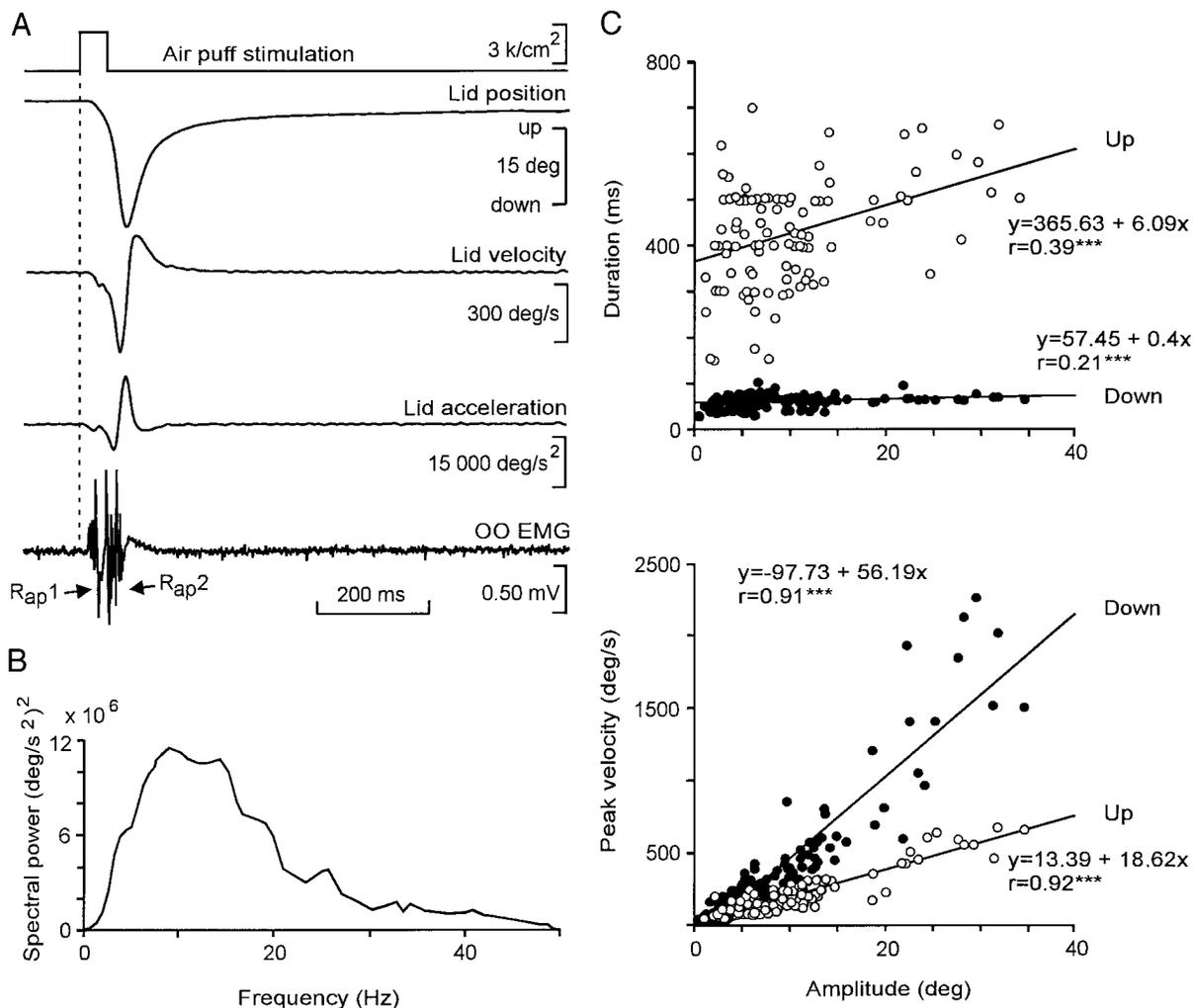


FIG. 1. Kinetic and frequency-component characteristics of reflex blinks. *A*: eyelid response to a 50-ms, 3-k/cm² air puff presented to the ipsilateral cornea. From top to bottom are illustrated eyelid position, velocity, and acceleration, and the electromyographic (EMG) activity of the orbicularis oculi muscle (OO EMG). Note that the reflex response has 2 downward components, designated R_{ap}1 and R_{ap}2 in the EMG trace. *B*: mean power spectra averaged from 10 acceleration records of lid reflex responses to 50-ms, 3-k/cm² air puffs, including the one illustrated in *A*. *C*: duration (above) and peak velocity (below) of air-puff-evoked blinks as a function of their maximum amplitude in the downward (●) and upward (○) eyelid displacements. Each regression line was obtained from 100 eyelid responses from 4 different rabbits. Asterisks (***) for regression lines in *C* indicate $P < 0.0001$.

With respect to air puff duration and intensity, the order of presentation was also determined at random. Air puffs were applied through the opening of a plastic pipette (3 mm diam) attached to a metal holder fixed to the animal's nine-pin socket. This allowed the pipette to follow the spontaneous movements of the animal's head. The tip of the pipette was placed 1 cm away from the upper part of the cornea. The latency of the air puff in reaching corneal and lid mechanoreceptors was calculated 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 fed into the computer as 1-V square pulses for latency measurements.

Two of the rabbits were not subjected to the classical conditioning procedure but were presented with different blink-evoking stimuli. Auditory stimulation consisted of two different tones (600 and 6,000 Hz) applied for 10–100 ms at 90 dB. The loudspeaker was placed 1 m in front of the animal's head. Bright full-field flashes lasting for ≈1 ms were used as visual stimulation. These two animals also received a set of air puff stimuli of different pressures and duration, as indicated above. In addition, sets of air puffs were applied to the whiskers, and superior and inferior eyelids. A set of single SO stimuli (cathodal,

square, 50 μs, <1-mA pulses) was also presented to these two animals. To avoid habituation or response fatigue, stimuli of the same modality, but of different values, were presented in blocks of 10–50 stimuli at varying intervals (20–40 s). A minimum of 4–6 min was allowed between successive presentations of stimuli of different modalities.

Occasionally, an external 10-turn coil was located on the nose or under the mandible for recording movements during the smelling or eating of food. This coil was made of 1-mm diam, enamel-coated copper wire.

Classical conditioning paradigms

Classical conditioning of eyelid movements was achieved by the use of either delayed or trace conditioning paradigms. For delayed tone-air puff (T-AP) conditioning, a 350-ms, 600-Hz, 90-dB tone was presented to the animal as CS. The tone was followed 250 ms from its onset by a 100-ms, 3-k/cm² air puff directed to the left cornea as US. The tone and the air puff co-terminated. For trace weak air puff–strong air puff (ap-AP) conditioning paradigm, animals were pre-

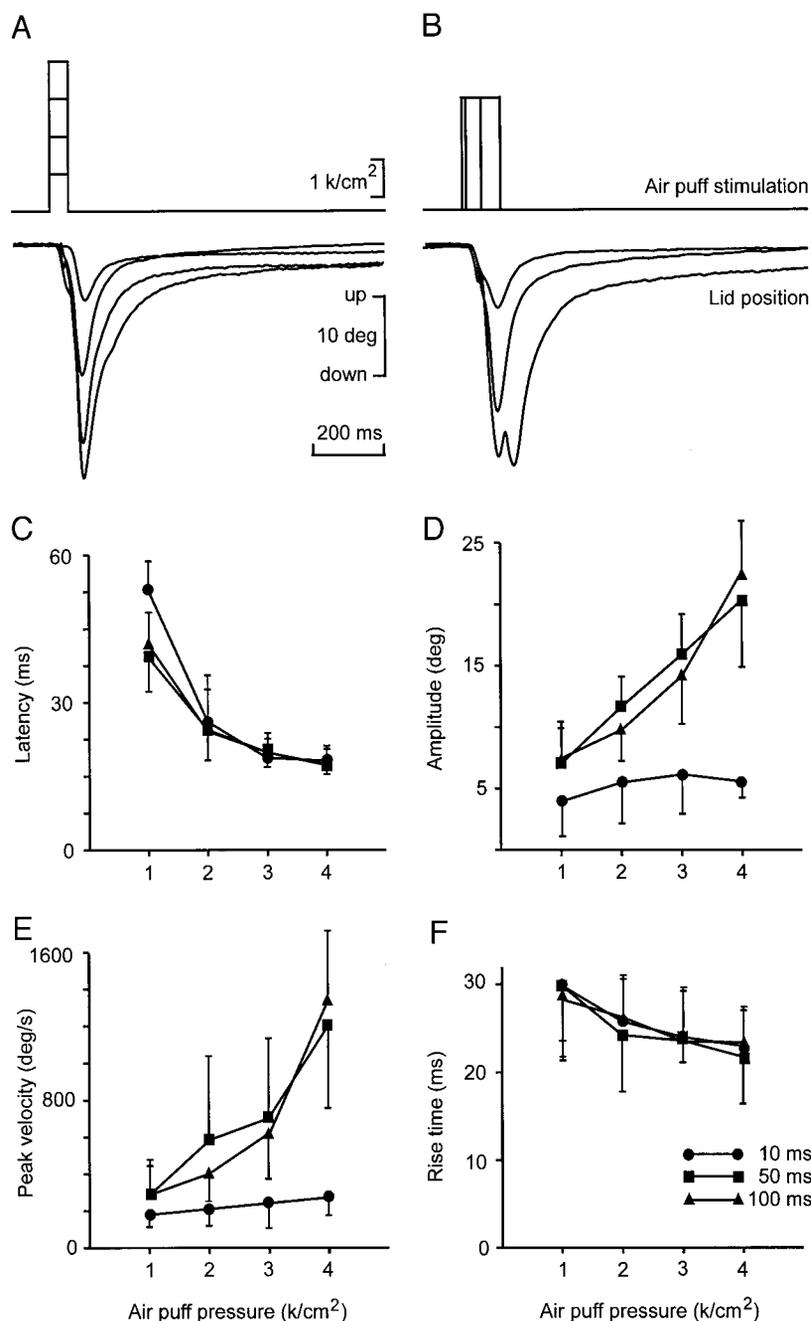


FIG. 2. Parameter measurements of reflexively induced blinks as a function of the duration and pressure of air puffs applied to the ipsilateral cornea. *A* and *B*: overlapped records of lid position from blinks evoked by air puffs of increasing pressure (*A*) or duration (*B*). *C–F*: latency (*C*), amplitude (*D*), peak velocity (*E*), and rise time (*F*) of lid position in the downward direction during air-puff-induced blinks as a function of stimulus pressure. The duration of applied air puffs was 10 ms (●), 50 ms (■), and 100 ms (▲). Each symbol corresponds to the mean value of 16 responses from 4 different animals. Calibrations in *A* are also for *B*.

sented with a short (10 ms), weak (1 k/cm²) air puff as CS, followed 250 ms later by the same US as that presented during the delayed conditioning paradigm. Both CS and US were presented to the left side. Each of the classical conditioning paradigms was used to train four animals.

For both conditioning paradigms, the conditioning session consisted of 66 trials separated at random by 50- to 70-s intervals. Six of the 66 trials were test trials, and the CS was presented alone. The daily conditioning session lasted for ≈80 min, and each animal was trained for seven successive days. An animal was considered to be conditioned when it was able to produce 95% of CRs per session to the CS-US paired presentation.

Ancillary observations

To complete data illustrated in Fig. 11, additional experiments were carried out in four Wistar rats (230–260 g) and three pigmented

guinea pigs (420–580 g) obtained from local official suppliers. Those animals were prepared for the chronic recording of eyelid position as described here for rabbits. During recordings sessions ($n = 2$ per animal) they were only presented with air puffs as blink-evoking stimulus. No attempt was made to obtain classically conditioned blinks from them. The holding, stimulation, and recording systems were adapted to animals' size.

Data collection and analysis

The horizontal and vertical position of the upper eyelid, the unrectified EMG activity of the orbicularis oculi muscle, and 1-V rectangular pulses corresponding to blink-evoking stimuli, or to CSs and USs presented during conditioning sessions, were stored digitally on an eight-channel videotape recording system. Data were transferred later through an analogue digital converter (CED 1401-plus, CED,

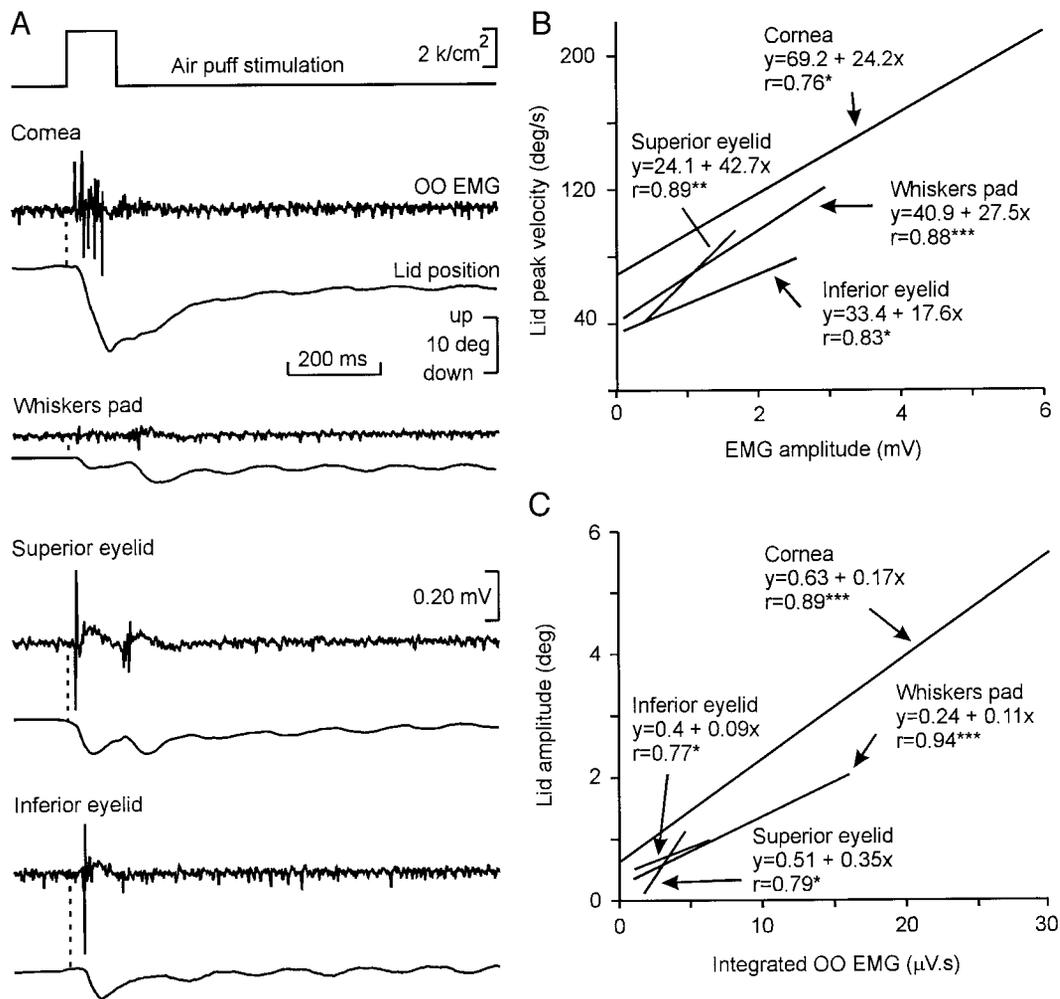


FIG. 3. Characteristics of blinks evoked by air puffs applied to different parts of the animal's face. *A*: records of orbicularis oculi muscle activity (OO EMG) and eyelid movement in response to 100-ms, 3-k/cm² air puffs directed to the ipsilateral cornea, whiskers pad, and superior and inferior eyelids. *B*: lid peak velocity as a function of peak OO EMG amplitude for 40 eyelid responses. *C*: lid maximum amplitude as a function of integrated OO EMG for 40 eyelid responses. Asterisks for regression lines in *B* and *C*: * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$. Calibrations and legends in *A* are for all examples.

Cambridge, UK) to a computer for quantitative off-line analysis. Data were sampled at 1,000 Hz, with an amplitude resolution of 12 bits.

Commercial computer programs (Spike2 and SIGAVG from CED; MATLAB, The Mathworks; and Corel Draw, Corel Corporation, Ontario, Canada) were used to display single, overlapping, averaged, and raster representations of eyelid position, velocity, and acceleration, and of EMG activity of the orbicularis oculi muscle. These programs also allowed the quantification, with the aid of cursors, of the onset latency, latency to the peak, amplitude and peak velocity of the eyelid displacement, and the onset latency, peak amplitude and area of the rectified EMG activity of the orbicularis oculi muscle.

Some analyses required the development of new programs. Velocity and acceleration traces were computed digitally as the first and second derivative of lid position records, following low-pass filtering of the data (-3 dB cutoff at 50 Hz and a zero gain at ≈ 100 Hz). As explained in detail elsewhere (Domingo et al. 1997), the power of the spectral density function (i.e., the power spectrum) of selected data were calculated using a fast Fourier transform to determine the relative strength of the different frequencies present in lid displacements and EMG recordings. The power spectra of lid movements were calculated exclusively from the corresponding acceleration (Domingo et al. 1997; Wessberg and Vallbo 1995). Concisely, acceleration recordings were divided into 1.024-s segments, starting 100 ms in advance to any blink-evoking stimulus. Segments containing CRs

were selected exclusively from those obtained during the presentation of the CS alone. This design allowed the complete evoked response (from eyelid, nose, and mandible) to be contained in the segment, with a spectral resolution of 0.97 Hz. The spectral power of EMG records was calculated in the same way. Autocorrelation of acceleration recordings as illustrated in Fig. 8*E*, and cross-spectral values between acceleration and EMG recordings as illustrated in Fig. 8*D*, were calculated according to available statistical tools (Bendat and Piersol 1986; Domingo et al. 1997). The coherence spectrum was normalized to a 0 to 1 scale.

Statistical analyses were carried out using the SPSS package (SPSS, ILL), for a statistical significance level of $P = 0.05$. Mean values are followed when necessary by their standard deviations (SD). Unless otherwise indicated, mean values were calculated from ≥ 16 measurements collected from a minimum of 2 animals. Latency measurements were carried out with the help of a special graphic program determining the intersection point between resting and deflected eyelid positions. The rise time of downward phase of reflex responses (Fig. 2*F*) was calculated with the help of the same program. Statistical differences of mean values were determined by ANOVA. Regression analyses were carried out using ≥ 100 measurements collected from at least two animals. Peaks of power spectra were tested with the χ^2 -distributed test for spectral density functions.

RESULTS

General characteristics of experimentally induced eyelid movements

Before presentation of any kind of blink-evoking stimulus, animals rarely made spontaneous blinks (1–2 per hour). Once air puff stimulation sessions were started, 60% of rabbits made spontaneous eye blinks of large amplitude, mostly within a few tenths of a second after evoked reflex responses.

Air-puff-induced blinks consisted of a fast downward phase followed by a much slower upward movement until the lid's initial position was reached (Fig. 1A). For air puffs of ≥ 50 ms duration, a double slope was noticed during the initial fast downward phase. This double slope was more easily detected in the lid velocity and acceleration profiles (Fig. 1A). Mean latency of blinks elicited by 50-ms, 3-k/cm² air puffs was 19.8 ± 4.4 ms (mean \pm SD; $n = 16$). For this stimulus, the downward phase of the elicited blink presented a mean amplitude of $14.2 \pm 7.9^\circ$ ($n = 16$), which was not enough to produce the complete closure of the eye, even with the addition of the upward movement of the lower lid (not measured). Mean peak velocity reached by the lid during its downward trajectory was $626.3 \pm 386.5^\circ/\text{s}$ ($n = 16$). The time elapsed from stimulus presentation until maximum lid displacement was 94.3 ± 19.3 ms ($n = 16$). When an air puff lasting for 100 ms was applied, the initial, fast downward lid displacement was followed by successive small sags also in the direction of closure (Fig. 2B).

Although in some sessions the number of blink-evoking stimuli was considerable, animals always reopened the eye after stimulation. The duration of complete reopening of the lid to the initial position could last >1 s. For this reason, eyelid upward movements were measured until 90% of the total eyelid displacement was reached. The mean duration of upward phases of eye blinks induced by 50-ms, 3-k/cm² air puffs was 463 ± 92.1 ms ($n = 16$), and the mean peak velocity reached during upward displacements was $243.3 \pm 19.3^\circ/\text{s}$ ($n = 16$). The time constant, calculated as the time needed by the lid to return two-thirds of the distance to its initial position showed a mean value of 73.2 ± 21.2 ms ($n = 16$).

Air puffs presented to the cornea evoked a double EMG activation of the orbicularis oculi muscle at 12.5 ± 1.8 and 28.7 ± 3.5 ms ($n = 16$), as computed for stimuli of ≥ 3 k/cm² of pressure. Thus muscle activity preceded the initial downward phase of the blink by 7 ± 1.2 ms ($n = 16$). For air puffs of 50 ms and 3 k/cm², the peak amplitude of the rectified EMG showed a mean value of 1.3 ± 1.2 mV ($n = 16$), and its total area had a mean value of 21.82 ± 18.9 $\mu\text{V} \cdot \text{s}$ ($n = 16$). The area of the rectified EMG was calculated for the 300 ms from stimulus presentation (see Gruart et al. 1995).

Power spectrum analyses were carried out to determine the frequency components of the successive downward waves of the air-puff-evoked blinks. The mean power spectra of 10 acceleration records from air-puff-evoked blinks showed a dominant ($P = 0.01$) peak at ≈ 8 Hz over a broadband of frequencies between 4 and 15 Hz (Fig. 1B).

No eyelid movement could be reflexively induced by flash or tone stimulation in any of the animals. As already described (Manning and Evinger 1986), only when a flashlight was located very close to the cornea did the animal close its eyelids. The mean power spectra of flashlight-evoked blinks showed a dominant peak ($P < 0.001$) at a lower (6 Hz) frequency.

Relationships of amplitude with duration and peak velocity for air-puff-evoked blinks

As illustrated in Fig. 1, mean values presented by the down- and upward phases of reflex blinks showed a significant variation when air puff parameters were modified. All the animals tested ($n = 6$) showed a statistically significant ($P < 0.0001$) linear relationship between the peak velocity achieved during the downward phase of the blink and the maximum amplitude of the evoked movement (Fig. 1C, *bottom*). The same significant ($P < 0.0001$) linear relationship was found for the upward phase of reflex blinks, but with a slope of one-third the value for the downward phase. These results indicate that for a 10° movement of the upper eyelid, if the movement is in the downward direction its maximum velocity will be threefold that when the movement corresponds to the upward phase of air-puff-induced blinks.

Although the relationship between the maximum amplitude of eyelid reflex responses and their duration was also statistically significant ($P < 0.0001$), it showed very low coefficients of correlation ($r < 0.4$) for both upward and downward phases of the blink (Fig. 1C, *top*). The low slope recorded for this relationship (0.4 ms/deg for the downward phase and 6.1 ms/deg for the upward phase) is in agreement with the constancy in rise time of the downward phase during the presentation of air puffs of different intensity and/or duration (see Fig. 2F). According to these amplitude-peak velocity and amplitude-duration relationships, the duration of a 10° lid opening movement induced by a puff of air is ≈ 15 times longer than the duration of lid displacement in the closing direction during the same blink.

Dependence of air-puff-evoked blinks on stimulus parameters

Results of the quantitative study of lid movements during the presentation of air puff stimuli of different pressures and duration are shown in Fig. 2. The latency to blink onset decreased with the increase in air puff pressure, and it was unrelated to stimulus duration (Fig. 2C), except for 10-ms, 1-k/cm² air puffs, which rarely evoked lid responses, and when they did, the responses showed a large onset latency (52.6 ± 7.4 ms). Latency values decreased in a statistically significant ($P < 0.01$), exponential-like manner, from 52.6–41.6 ms for responses evoked by air puffs of 1 k/cm² to 18.3–17.7 ms those ones evoked by air puffs of 4 k/cm². Maximum amplitude of reflexively evoked downward lid movements increased linearly ($P < 0.01$) with increased air puff pressure for stimuli lasting 50 and 100 ms (Fig. 2D). Mean maximum amplitude values increased from 6.8 – 7.3° for air puffs of 1 k/cm² to 20.4 – 22.7° for air puffs of 4 k/cm². No significant trend was observed in the mean maximum amplitude for reflex responses evoked by stimuli of 10 ms during increasing air puff pressures. In this latter case, blink amplitude remained between 3.8 and 6.1° for the different air puff pressures.

Peak velocity of the downward phase of air-puff-evoked blinks increased with a statistically significant ($P < 0.01$) linear trend with air puff pressure for stimuli of 50 and 100 ms (Fig. 2E). Mean peak velocity values increased from 278.7 – $284.1^\circ/\text{s}$ for 1 k/cm² air puffs to $1,210$ – $1,352^\circ/\text{s}$ for 4 k/cm² air puffs. Although mean peak velocity for 10-ms air puffs in-

creased slightly with the increase in air pressure, no significant trend was observed. The mean peak velocity values for 10-ms air puffs ranged from 175.5 to 274.2°/s.

The rise time of the downward phase of air-puff-evoked blinks was calculated as the time spent by the lid in producing 80% of its displacement, as measured from the initial 10% to the final 90% of lid displacement. Mean values for the rise time were similar (26.4–21.7 ms) for pressures of 2, 3, and 4 k/cm² regardless of the duration of the stimulus (Fig. 2F). However, rise time mean values (28.6–30.1 ms) of reflex blinks evoked by 1-k/cm² air puffs were significantly ($P < 0.01$) longer than those obtained for air puff presentations of 2–4 k/cm². In fact, responses to air puffs of 1 k/cm² (and especially of 10 ms duration) were both small and variable, as observed in the four parameters measured.

Figure 3 illustrates the EMG activity of the orbicularis oculi muscle and lid movements of different responses evoked by the same 100-ms, 3-k/cm² air puff when applied ipsilaterally to different parts of the animal's face: cornea, whiskers pad, and superior and inferior eyelids. Mean onset latencies of all these responses had similar values around 16.5 ms, except for lid responses during inferior eyelid stimulation, which was ≈ 32.5 ms. The amplitude of the air-puff-evoked eyelid responses as well as the profile were modified depending on the stimulus application site. The maximum eyelid displacement and the largest EMG-rectified area were recorded for stimuli applied to the cornea. Stimuli applied to whiskers and superior and inferior eyelids evoked lid movements of about one-third those evoked by corneal stimulation. Frequently, an oscillatory activity of ~ 8 Hz was noticed in lid position traces in correspondence with vigorous movements of the animal's nose after receiving an air puff to the whiskers. The oscillation of the lid in the absence of any noticeable EMG activity helped to determine the foreign origin of the oscillation recorded with the eyelid coil (see *Comparison of power spectra from eyelid, nose, and mandible movements*).

As shown in Fig. 3, B and C, lid movements evoked by corneal, whiskers pad, and eyelid (superior and inferior) stimulation shared similar, statistically significant ($P < 0.05$) relationships for EMG amplitude versus lid peak velocity, as well as for integrated EMG area versus lid amplitude plots. All the coefficients of correlation for the plotted data were $r > 0.7$. Regression lines for data obtained after superior eyelid stimulation with air puffs showed higher slope values than those obtained by stimulating the cornea, the whiskers or the inferior eyelid for the same set of air puffs.

Selected animals ($n = 2$) were presented with electrical stimulation of the ipsilateral SO nerve. The relatively weak SO nerve stimulation (< 1 mA) used here was able to evoke early and stable EMG responses at a mean latency of 9.1 ± 1.8 ms, and late and more variable responses at > 20 ms. Both early and late EMG evoked responses were named R1 and R2 following Kugelberg (1952). The EMG activity of the orbicularis oculi muscle preceded lid movement by 4–5 ms (not illustrated).

Kinetic characteristics of eyelid conditioned responses

Raster displays shown in Fig. 4, A and B, illustrate the evolution through seven successive classical conditioning sessions of the blink reflex of one of the animals trained with the

T-AP paradigm. The CS in this paradigm was a 350-ms, 90-dB, and 600-Hz tone followed at 250 ms from tone onset by a US consisting of a 100-ms, 3-k/cm² air puff. In three rabbits trained with the T-AP paradigm, clear conditioned responses appeared during the third conditioning session, and in the fourth animal appeared during the second conditioning session. The CRs seemed to move forward from the UR as small, downward waves or sags toward CS onset. Once animals were consistently conditioned, they showed an eyelid response in which the CR was integrated with the UR. Rabbits presented only a few responses in a ramplike manner even after seven sessions of training. The evolution of the CR could be seen more clearly during test trials when the US (Fig. 4B) did not follow the CS. Two of the animals sometimes showed late downward lid displacements just after the end of unconditioned responses during paired trials, or following CRs during CS-alone trials. As clearly illustrated in Fig. 6C, onset latency of CRs decreased through the successive conditioning sessions from a mean value of 215.7 ± 23.2 ms for the first conditioning session to 121.4 ± 20.5 ms for the seventh. Mean and standard deviation values were calculated for the four animals trained with the T-AP paradigm.

In the ap-AP paradigm, the CS consisted of a 20-ms, 1-k/cm² air puff applied to the ipsilateral cornea followed 250 ms later by a 100-ms, 3-k/cm² air puff as US. The raster displays shown in Fig. 5, A and B, illustrate the evolution through seven consecutive sessions of blink conditioning in one of the animals trained with the ap-AP paradigm. During the first two conditioning sessions, the reflex response to the CS increased in amplitude and peak velocity in a clear process of sensitization. This alpha response started to decrease during the fourth session and almost completely disappeared during the seventh session (see Figs. 5B and 6, C–F). For all four animals, the CR evoked with the ap-AP paradigm appeared during the second conditioning session. After the second session, the CR was a succession of downward, small sags that produced an almost complete lid closure (Fig. 5B). Because the reflex response to the short air puff (CS) appeared in almost all the traces throughout conditioning, the complete response topography consisted of an alpha response to the CS, the CR, and the reflex response to the US. Only late in conditioning (i.e., 5th–7th session, see Fig. 5B) were ramplike CRs obtained with onset in coincidence with CS reflex response onset, and maximum amplitude at the time of the unconditioned response to US. In those cases, the CS alpha response seemed integrated with the CR.

As illustrated in Fig. 6C, mean latency for CR onset across the conditioning sessions decreased from 228 ± 18.6 ms to 191.4 ± 9.6 ms ($n = 4$ animals) for the ap-AP paradigm. Mean latency values for CRs for the ap-AP paradigm were computed without the inclusion of the alpha response evoked by the short, weak air puff used as CS (see Fig. 6B). This decrease in onset latency values through the seven conditioning sessions was even more evident with the T-AP paradigm (from 215.7 ± 23.2 ms for session 1 to 121.4 ± 20.5 ms for session 7). From session 2 to session 7, all the CRs obtained with the ap-AP paradigm showed significantly ($P < 0.05$) longer onset latency values than that shown by CRs obtained with the T-AP paradigm.

The time-to-peak amplitude of the CR measured from CS onset (Fig. 6D) did not show any significant variation across the seven conditioning sessions for either paradigm, as computed for data collected from CS-alone trials. Obviously, be-

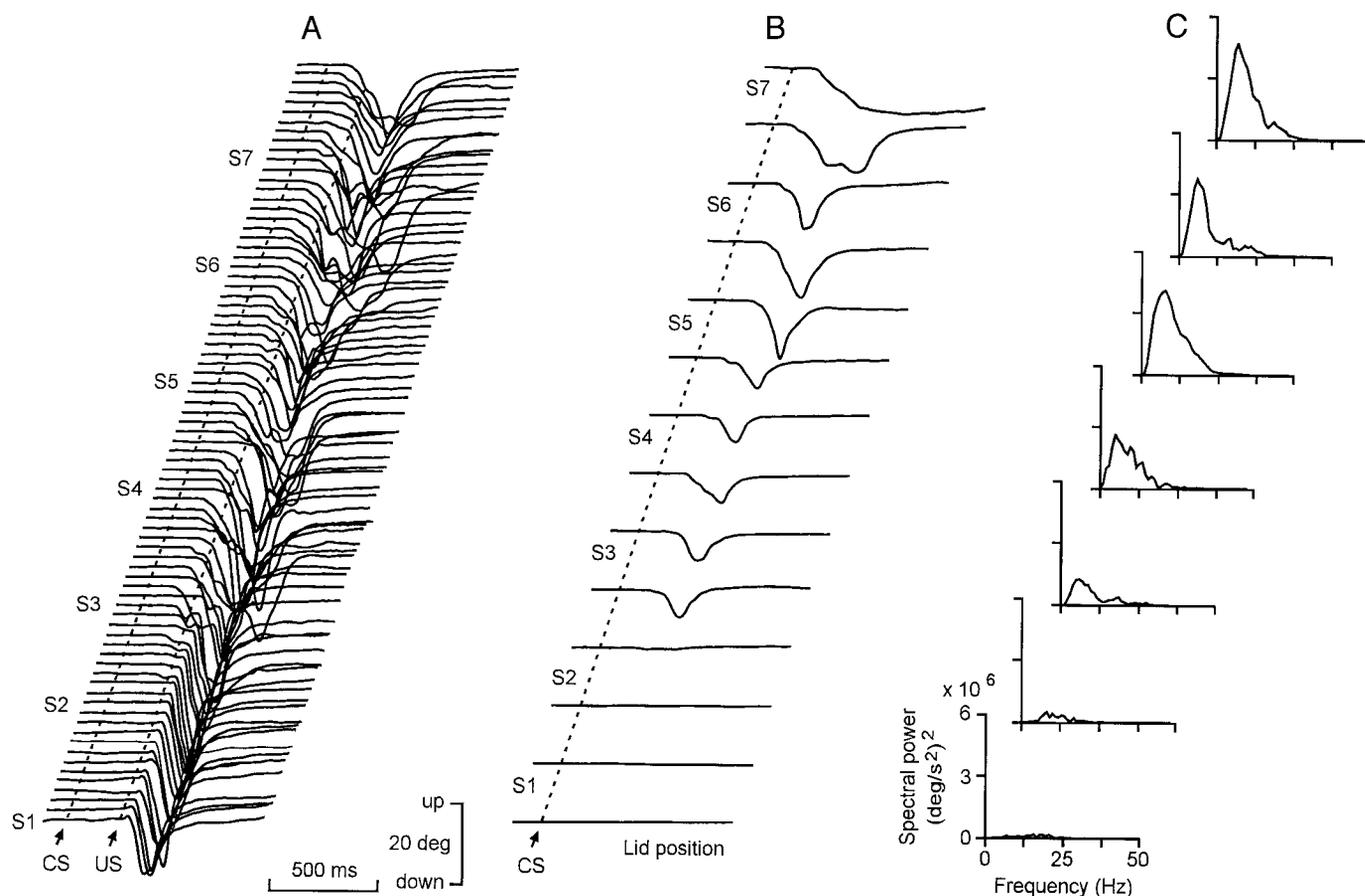


FIG. 4. Raster display of session evolution of conditioned responses during paired and unpaired presentation of a tone as conditioned stimulus. Conditioned stimulus (CS) consisted of a 350-ms, 90-dB, 600-Hz tone followed 250-ms later by an unconditioned stimulus (US) consisting of a 100-ms, 3-k/cm² air puff directed to the ipsilateral (left) cornea. *A*: selected records from the 7 consecutive conditioning sessions. Eleven of the 60 paired conditioned responses (CRs) are presented for each session. Note the progressive appearance of the CR. Arrows and dotted lines indicate CS and US presentations. *B*: records corresponding to the 1st and last CRs to CS-alone presentations for each of the 7 consecutive conditioning sessions. Arrow and dotted line indicate the conditioned stimulus onset. For *A* and *B*, the session number is indicated. *C*: mean power spectra of 6 acceleration records from CRs during CS-alone presentations for each conditioning session. Calibrations in *A* are also for *B*.

cause the latency for CS onset decreased through the conditioning sessions (Fig. 7C), the duration of CRs increased with training. Thus mean time-to-peak CR values measured from CR onset increased from 60.2 ± 17.8 ms (session 1) to 134.6 ± 6.8 ms (session 7) for those CRs evoked by the T-AP paradigm, and from 47.2 ± 15.5 ms (session 1) to 91.3 ± 3.9 ms (session 7) for those obtained with the ap-AP paradigm. In both cases, data were collected from test trials (not illustrated).

Mean values of the maximum amplitude of CRs showed a statistically significant ($P < 0.001$) linear trend through different conditioning sessions, as illustrated in Fig. 6E, during T-AP and ap-AP conditioning paradigms. From session 3 to session 7, differences observed between CRs obtained with both paradigms were statistically significant ($P < 0.05$). During the seventh session, the maximum amplitude of the CR was large enough to produce a complete eyelid closure in one of the animals (trained with the T-AP paradigm). The other animals had an upper eyelid displacement around two-thirds of the complete closure, for animals submitted to T-AP conditioning, and around one-half for those submitted to ap-AP conditioning. Individual differences could depend on the animal's strategies for motor learning and, perhaps, on the precise location of the coil in the upper eyelid.

With regard to maximum CR amplitude, peak velocity had a statistically significant linear trend ($P < 0.01$) across the successive training sessions (Fig. 6F) for both conditioning paradigms. Although mean peak velocity values from session 2 to session 7 were smaller for the ap-AP than for the T-AP paradigm, no significant differences were recorded, probably due to the wide variability of these values, as noticed in the high standard deviation values.

The kinetic characteristics of the CRs in CS alone trials were also studied. As illustrated in Fig. 7, D and F, the peak velocity of CRs obtained during T-AP or ap-AP conditioning paradigms increased linearly as a function of their maximum amplitude, with coefficients of correlation, r , between 0.75 and 0.96 ($P < 0.01$), for both downward (Fig. 7D) and upward (Fig. 7F) CR phases. Moreover, slopes of regression lines computed from data of CR downward phases were twofold those for the return movement. These results indicate that for a 10° CR, the peak velocity during the downward phase was twice the peak velocity during the upward phase. Maximum CR amplitude values during ap-AP conditioning paradigms were significantly ($P < 0.01$) smaller than those recorded for reflex blinks, whereas maximum CR amplitude values during T-AP conditioning paradigms were significantly ($P < 0.01$) larger than

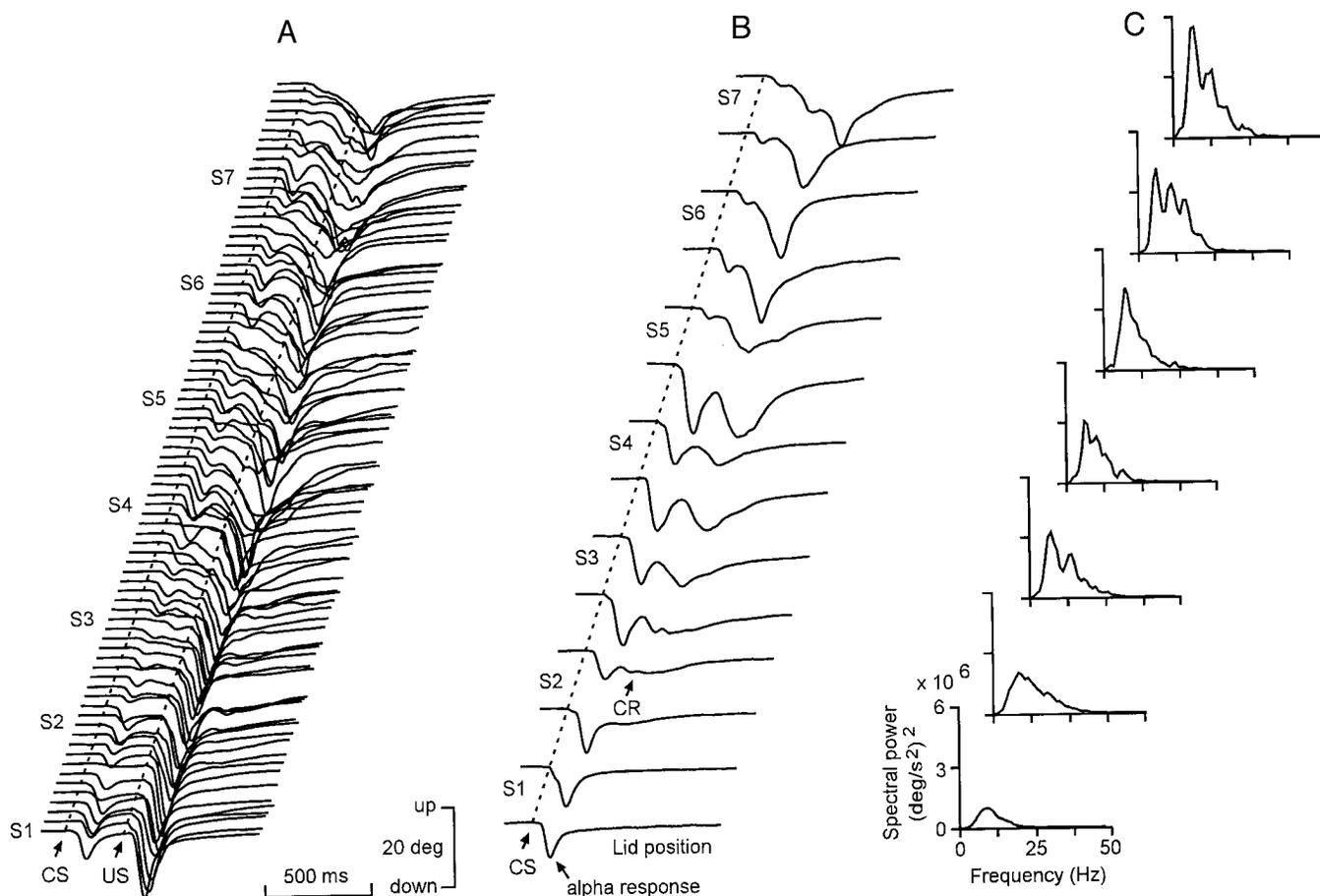


FIG. 5. Raster display of session evolution of conditioned responses during paired and unpaired presentation of a weak air puff as conditioned stimulus. Conditioned stimulus (CS) consisted of a 10-ms and 1-k/cm² air puff followed 250 ms later by an unconditioned stimulus (US) consisting of a 100-ms, 3-k/cm² air puff directed to the ipsilateral (left) cornea. *A*: selected records from the 7 consecutive conditioning sessions. Eleven of the 60 paired conditioned responses (CRs) are presented for each session. Note the progressive appearance of the CR preceded by an early alpha response. Arrows and dotted lines indicate CS and US presentations. *B*: records corresponding to the 1st and the last CRs to the CS-alone presentation for each of the 7 consecutive conditioning sessions. Arrow and dotted line indicate the conditioned stimulus onset. For *A* and *B*, the session number is indicated. *C*: mean power spectra of 6 acceleration records corresponding to CS-alone presentations from each conditioning session. Calibration in *A* are also for *B*.

those recorded for reflex responses to 50-ms, 3-k/cm² air puffs (Figs. 1 and 7). However, CR peak velocity values during both paradigms reached one-sixth the peak amplitude values for reflex blinks, for downward (Fig. 7D) and upward (Fig. 7F) lid displacements. As in the case of air-puff-evoked lid movements, very low coefficients of correlation were obtained between duration and maximum amplitude of CRs for downward (Fig. 7C) and upward (Fig. 7E) eyelid movements for both conditioning paradigms.

Frequency- and time-domain analyses of conditioned eyelid and orbicularis oculi EMG responses

Figure 8 illustrates the mean power spectra of selected segments of lid acceleration records during the performance of CRs evoked by the tone CS. These power spectra showed a significant ($P < 0.001$) peak at ≈ 8 Hz accompanied by other peaks at lower and, particularly, higher frequencies (Fig. 8B). The mean power spectra of selected EMG segments during the same evoked CRs are shown in Fig. 8C. In this case, EMG records were not rectified but were filtered to avoid their

high-frequency components' masking the signal. The EMG mean power spectra showed a broad, dominant peak at 6–10 Hz, although other significant peaks were recorded, especially at higher frequencies. The coherence between acceleration (Fig. 9B) and EMG (Fig. 9C) mean power spectra from reflex responses is shown in Fig. 9D and was significant (99% confidence limits) for frequencies < 18 Hz. The coherence between acceleration and EMG power spectra from CRs evoked during ap-AP and T-AP conditioning paradigms ($n = 2$ animals each) was always significant (99% confidence limit) for frequencies between 2 and 20 Hz.

The autocorrelation function of acceleration records was also calculated to check the rhythmicity of acceleration profiles for CRs obtained during ap-AP and T-AP conditioning paradigms (Fig. 8E). This autocorrelation function showed repeated peaks at ≈ 125 -ms intervals. Nevertheless, the autocorrelation time of the signal was rather short, fading away in two to four waves, possibly due to the shortness (< 0.5 s) of the evoked response (see Domingo et al. 1997).

As illustrated in Figs. 4 and 5, eyelid displacement during CRs did not occur in all-or-nothing fashion, but seemed to

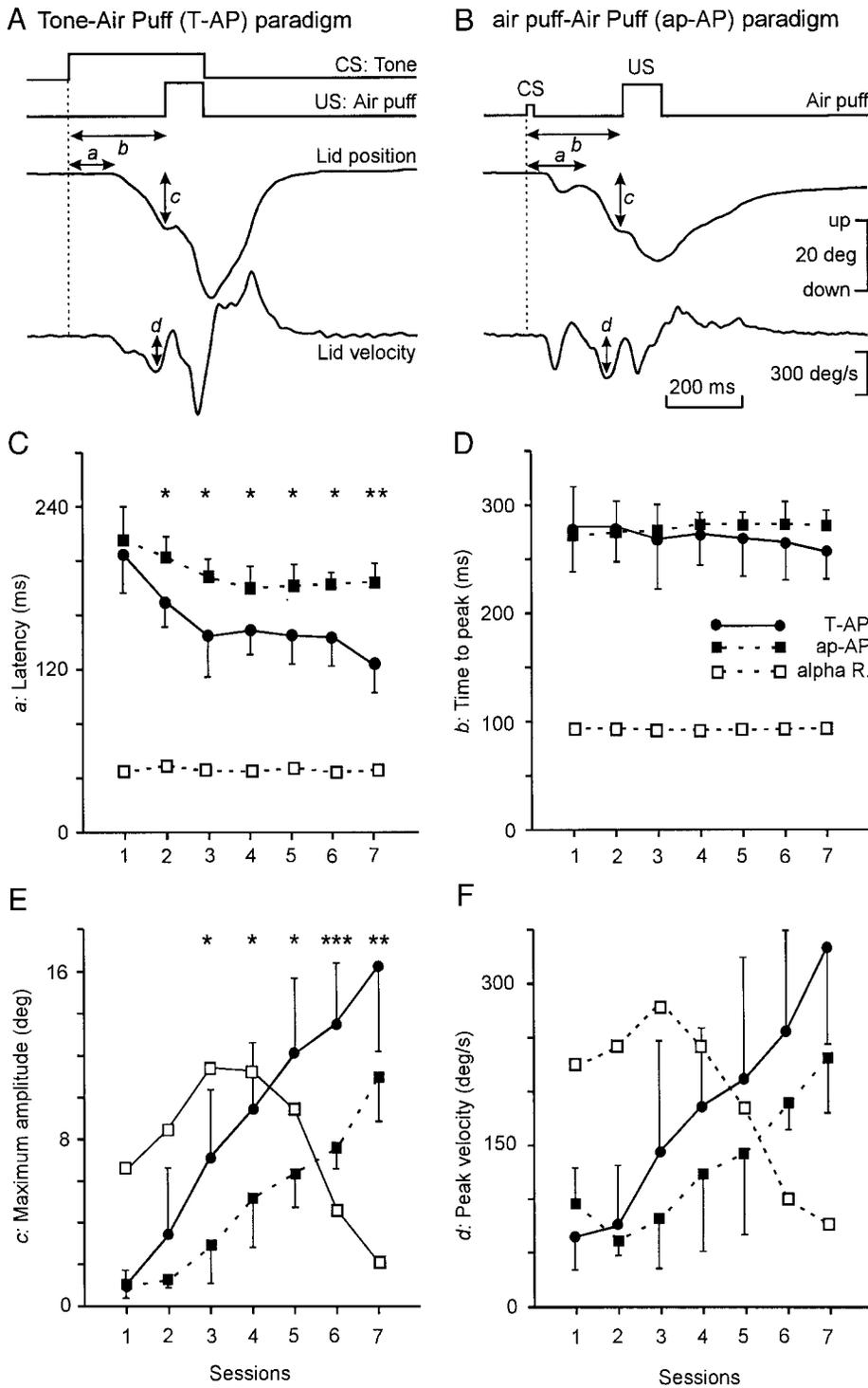


FIG. 6. Quantitative analysis of conditioned response evolution through conditioning sessions. **A**: examples of eyelid position and velocity during the paired presentation of a conditioned stimulus (CS, tone of 350 ms, 90 dB, and 600 Hz) followed 250 ms later by an unconditioned stimulus (US, air puff of 100 ms and 3 k/cm²). **B**: records of eyelid position and velocity during the paired presentation of a CS (air puff of 10 ms and 1 k/cm²) followed 250 ms later by a US (air puff of 100 ms and 3 k/cm²). In **A** and **B** are indicated the measured parameters of the conditioned responses (CRs): *a*, onset latency; *b*, time-to-peak amplitude; *c*, maximum amplitude; *d*, peak velocity. **C–F**: time histograms of CR evolution through the 7 conditioning sessions. Each symbol represents the mean and standard deviation values from the 4 animals trained with the T-AP paradigm (●), and the 4 trained with the ap-AP paradigm (■). Mean values for alpha responses present during ap-AP conditioning are also illustrated (□). Parameters represented in **C–F** correspond to those indicated in **A** and **B**. Asterisks for significant differences between plotted data in **C–F**: * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$. Calibrations in **B** are also for **A**.

increase by the successive inclusion of waves, or quanta of lid displacement, which added to the downward movement produced by the preceding wave. The increase in the number of waves present in CRs during 7 consecutive conditioning sessions with the T-AP paradigm (Fig. 4B) produced a progressive increase in the height of a significantly ($P < 0.01$) dominant frequency of ≈ 8 Hz, measured in acceleration profiles of CRs. In the same way, CRs recorded during the ap-AP conditioning paradigm also seemed to be elaborated by the addition of successive downward waves or sags. The increase in the num-

ber of waves present in CRs during consecutive conditioning sessions (Fig. 5B) produced a parallel increase in the height of the dominant ≈ 8 Hz component ($P < 0.001$), also computed from the acceleration records of CRs (Fig. 5C).

Comparison of power spectra from eyelid, nose, and mandible movements

It could be argued that frequency-domain properties ascribed here to eyelid reflex and learned responses could be motor

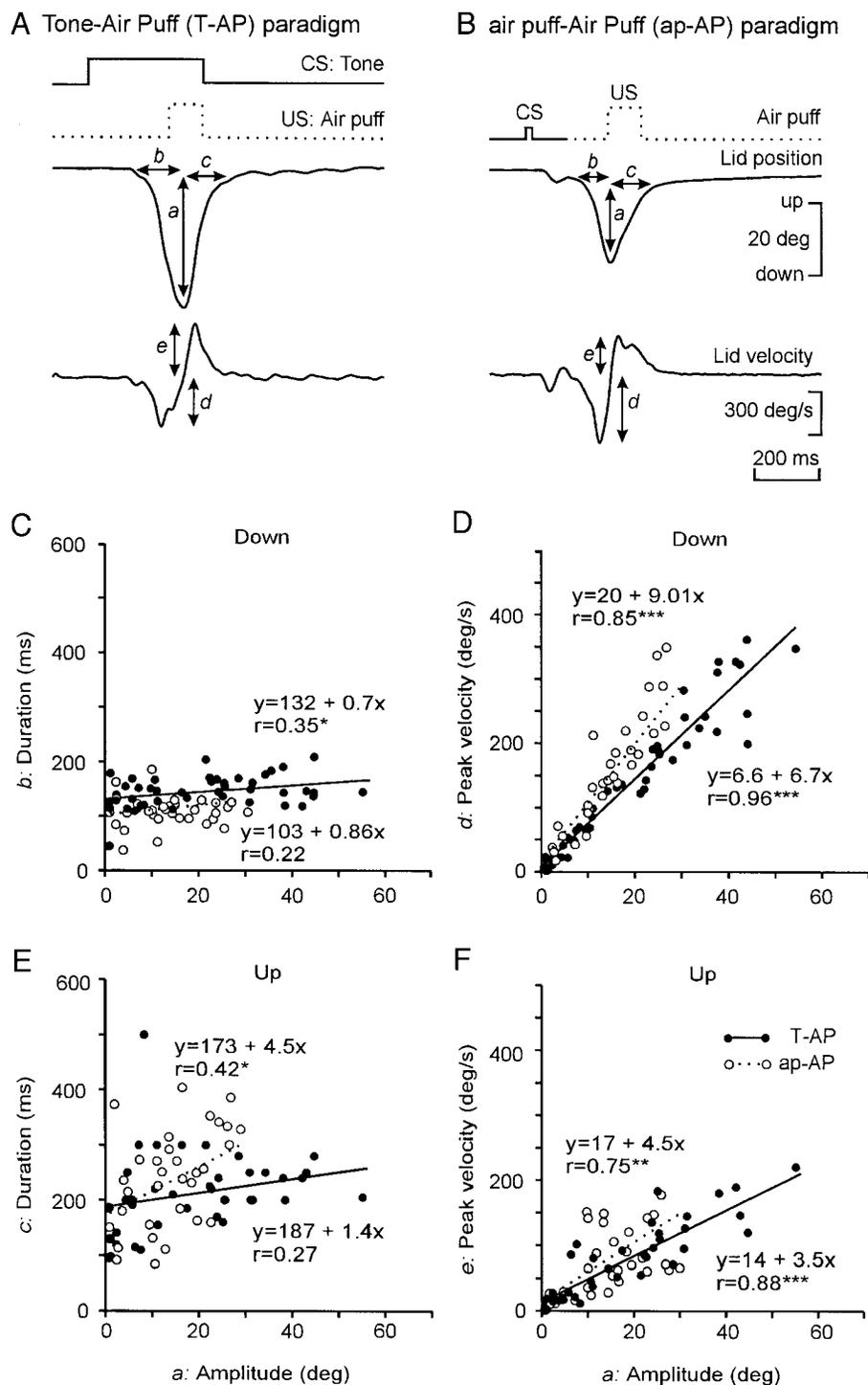


FIG. 7. Kinetics of conditioned responses. *A* and *B*: examples of eyelid position and velocity for tones (350 ms, 90 dB, 600 Hz) presented alone as conditioned stimulus (CS) during T-AP classical conditioning (*A*), and for air puffs (10 ms, 1 k/cm²) also presented alone as CS during ap-AP conditioning (*B*). In both examples, the measured CR parameters are indicated: *a*, maximum amplitude; *b*, duration of the downward phase; *c*, duration of the upward phase; *d*, peak velocity of the downward phase; and *e*, peak velocity of the upward phase. Data for regression lines in *C–F* were collected exclusively from CRs in response to CS-alone presentations. *C* and *D*: duration (*C*) and peak velocity (*D*) of the downward phase of CRs as a function of their maximum amplitude. *E* and *F*: duration (*E*) and peak velocity (*F*) of the upward phase of CRs as a function of 90% of their maximum amplitude. Each regression line was calculated from 100 measurements from 4 rabbits. Continuous regression lines and filled circles are for CRs obtained with the T-AP paradigm, and dotted regression lines and open circles are from CRs obtained with the ap-AP paradigm. Asterisks for regression lines in *C–F*: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Calibrations and legends in *B* are also for *A*.

artifacts propagated from other facial muscles, mostly those controlling nose or mandible movements. To test this possibility, nose movements were recorded while the animal was smelling a piece of apple placed 1 cm from its nose, and mandible movements were recorded when the animal was eating pieces of carrot. For the sake of comparison, nose and mandible position during these movements and eyelid position during reflex and learned lid responses are shown in Fig. 9, *A–D*. As already illustrated (Figs. 1, 8, and 9*E*) both reflex and classically conditioned eyelid responses present a dominant peak at ≈ 8 Hz in their corresponding power spectra within a

broadband of lower and higher frequencies (Fig. 9*E*, arrows A and B). The power spectra of acceleration profiles corresponding to nose movements showed a significant ($P < 0.001$) dominant peak of ≈ 8 Hz (Fig. 9*E*, arrow C). In this case, it was a very well-defined frequency domain with a narrow range of other accompanying peaks, from 7 to 10 Hz. As illustrated in Fig. 9*C*, nose movement proved to be a small-amplitude, but stereotyped movement. The amplitude of nose movement was only $\frac{1}{5}$ to $\frac{1}{10}$ the amplitude of reflex and conditioned eyelid responses, a fact that may explain the smaller peak in the spectral power of nose movement when compared with corre-

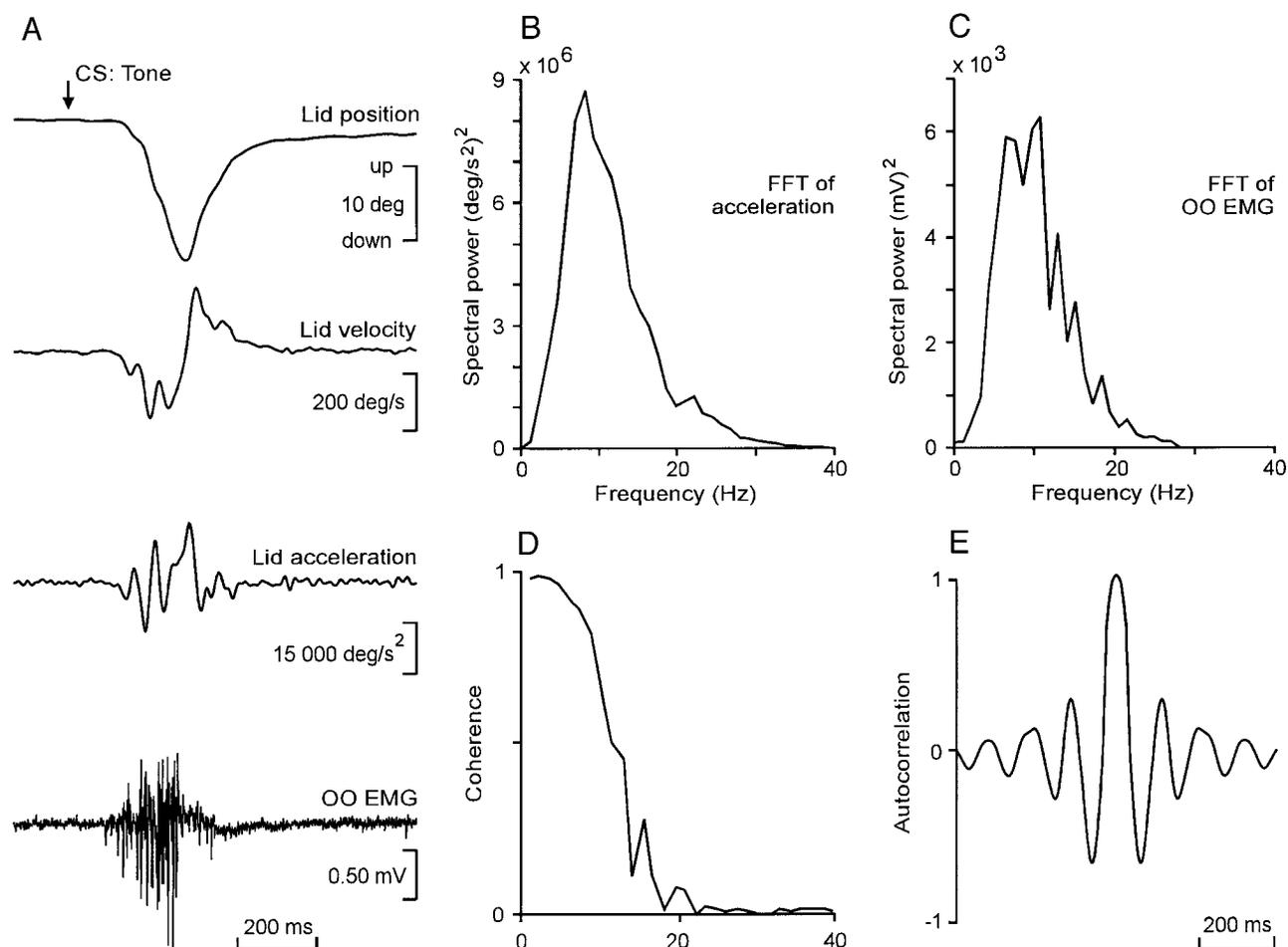


FIG. 8. Frequency- and time-domain analyses of eyelid conditioned responses (CRs). *A*: from top to bottom are illustrated the position, velocity and acceleration of the upper eyelid, and the electromyographic activity of the orbicularis oculi muscle (OO EMG) of a CR obtained from a T-AP conditioning paradigm. The CR was obtained during a CS-alone presentation. *B*: mean power spectra from 6 acceleration profiles obtained from CS-alone presentations during the 7th conditioning session for the same animal and paradigm illustrated in *A*. *C*: mean power spectra obtained from the 6 EMG records of the CRs used in *B*. *D*: coherence spectra between acceleration (data illustrated in *B*) and EMG (data in *C*) records, normalized to 0 and 1 values. *E*: autocorrelation function of the acceleration record in *A*. FFT, fast Fourier transform.

sponding power spectra for lid movements. The power spectra of acceleration records corresponding to mandible movements showed a significant ($P < 0.001$) peak at ≈ 4 Hz (Fig. 9E, arrow D), accompanied by two other peaks of lower spectral power values at 6 and 9 Hz.

Spectral power of acceleration profiles corresponding to reflex blinks evoked by air puffs of increasing pressure also increased in their relative strength around the dominant frequency of ≈ 8 Hz (Fig. 10A). The substantial increase of blink response when the air puff was increased from 3 k/cm^2 to 4 k/cm^2 was accompanied by a corresponding increase in the power spectra. Similarly, the increase in the duration of the reflex-evoking air puffs also increased the height of the corresponding dominant frequency (≈ 8 Hz) in the power spectrum. As shown in Fig. 4C for T-AP, and in Fig. 5C for ap-AP conditioning paradigms, the spectral power of acceleration profiles of CRs from successive conditioning sessions showed an increase in the height of the corresponding dominant frequency (≈ 8 Hz) in parallel with the appearance of new waves in eyelid position traces during the progressive build-up of the CR (Fig. 10C).

DISCUSSION

General overview

The present experiments show the profiles and metric properties of reflex and conditioned eyelid responses in conscious rabbits using the search coil in a magnetic field technique as a precise way of recording lid movements. Previous reports of this technique in different species (Evinger et al. 1984, 1991; Gruart et al. 1995) prompted us to apply it to the study of eyelid blinks in rabbits, because this is the species of choice for studying classical conditioning of the nictitating membrane/eyelid response. Because eyelid coils have negligible mass, they do not interfere with lid displacements, allowing a power spectrum analysis to be applied to selected lid responses (Bendat and Piersol 1986; Domingo et al. 1997; Wessberg and Vallbo 1995). We show here that the kinetics of lid CRs is different from that of reflexively evoked blinks. Moreover, we also show that an ≈ 8 -Hz oscillator characterizes both reflex and conditioned eyelid responses, and probably its generator circuit underlies the genesis and control of those lid responses. According to available data, the dominant frequency of this

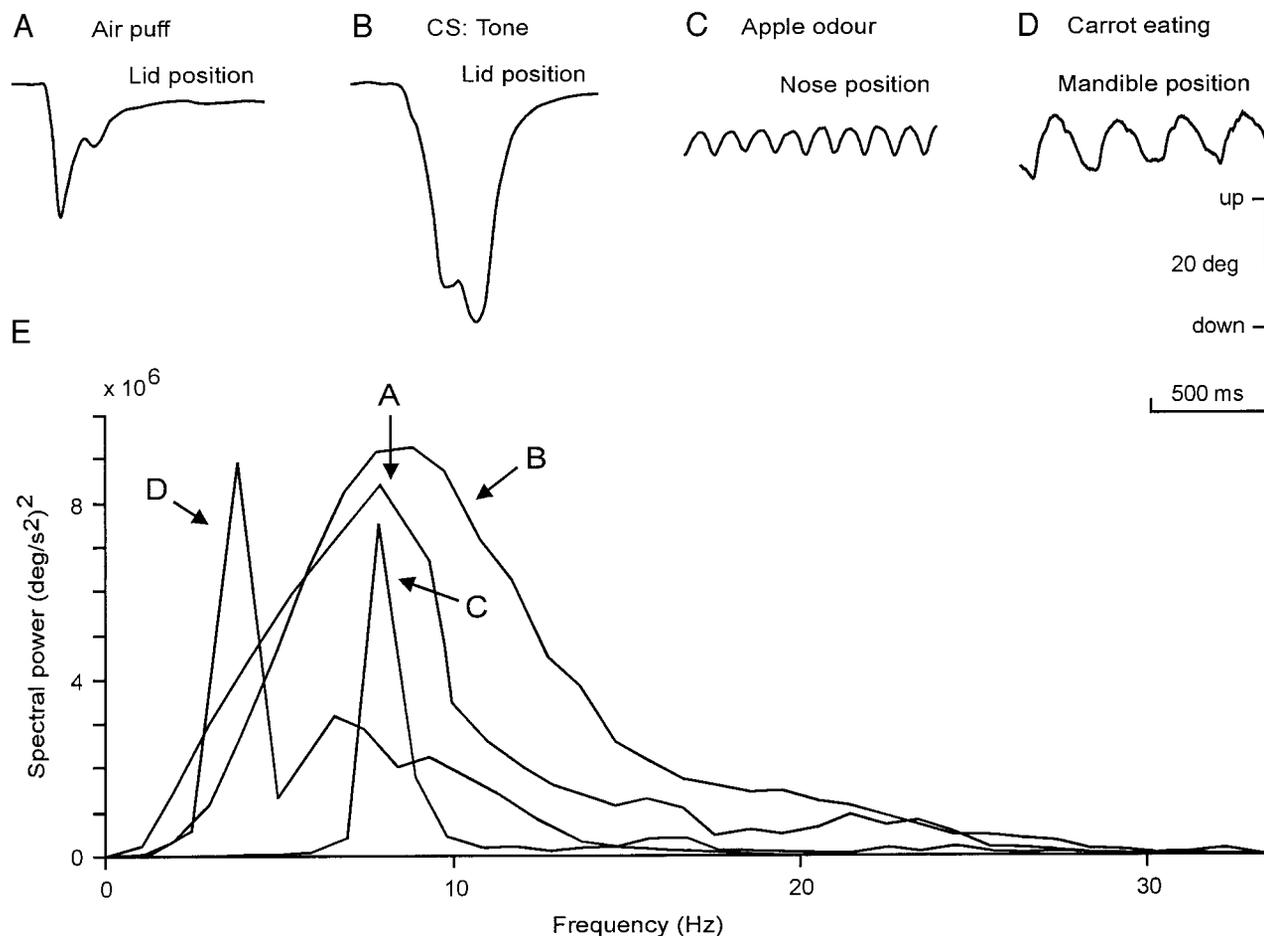


FIG. 9. Frequency domain analyses of eyelid, nose, and mandible movements. A–D: position records of reflex (A) and classically conditioned (B) eyelid responses, nose motion during apple smelling (C), and mandible movement during carrot eating (D). Calibrations in D are also for A–C. E: power spectra of 6 acceleration records (1.024 s each) during the movements illustrated in A–D. Note differences in the dominant peak of the power spectrum for each type of movement.

neural oscillator is species-specific, being adapted to the inertial and viscoelastic needs of each species (Fig. 11).

Metric properties of reflex blinks

According to the present study, reflex eye blinks in rabbits have a shorter latency than reported values for nictitating membrane responses following air puff presentations or the electrical stimulation of the SO nerve. The mean latency of reflex eyelid blinks to air puff stimuli was ≈ 20 ms, which is 25–70% shorter than the corresponding values for air puff-evoked nictitating membrane displacements (Marshall-Goodell et al. 1992; Thompson and Krupa 1994). The latency of reflex eyelid blinks in response to SO nerve stimulation was also 35–45% shorter than nictitating membrane displacements evoked by similar stimuli (Marshall-Goodell et al. 1992). In general, reflex eyelid blinks presented a more wavy appearance and a more consistent profile than reflexively evoked nictitating membrane responses. When compared with the same motor response in cats (i.e., an animal of similar body weight), reflex blinks in rabbits presented a longer latency and rise time and a lower peak velocity (Gruart et al. 1995). Moreover, the time constant of lid upward phases for rabbits (≈ 73 ms) presented almost a value double that reported for cats (≈ 37 ms) (Gruart et al. 1995). Apart from quantitative differences in the behav-

ioral characterization of reflex blinks between different species, the present data show that blink topography (i.e., latency, amplitude, duration, and peak velocity) in the rabbit is highly dependent on the intensity and duration of the applied stimulus (Manning and Evinger 1986; Schreurs and Alkon 1990). Moreover, rabbits did not seem to respond to light flashes or tones of similar intensity to those able to evoke reflex blinks in cats.

The electrical activity of the orbicularis oculi muscle preceded the onset of lid movement by 7 ms, a value larger than the one reported for cats (≈ 4 ms) (Gruart et al. 1995), but shorter than in humans (10–12 ms) (Evinger et al. 1991). As already reported for humans (Kugelberg 1952) and cats (Gruart et al. 1995), the initial downward phase of reflex blinks in rabbits was the result of a double activation of the orbicularis oculi muscle, evoked either by puffs of air (R_{ap1} and R_{ap2}) or by electrical stimulation of the SO nerve (R1 and R2). As already described (Lindquist and Mårtensson 1970), the weak SO stimulus used here (<1 mA) evoked a stable R1 response and a late, more variable R2 response in the EMG activity of the orbicularis oculi muscle.

In agreement with previous descriptions for humans (Evinger et al. 1991) and cats (Gruart et al. 1995), the peak velocity of the initial downward phase of reflex blinks in rabbits was a linear function of total lid displacement; that is,

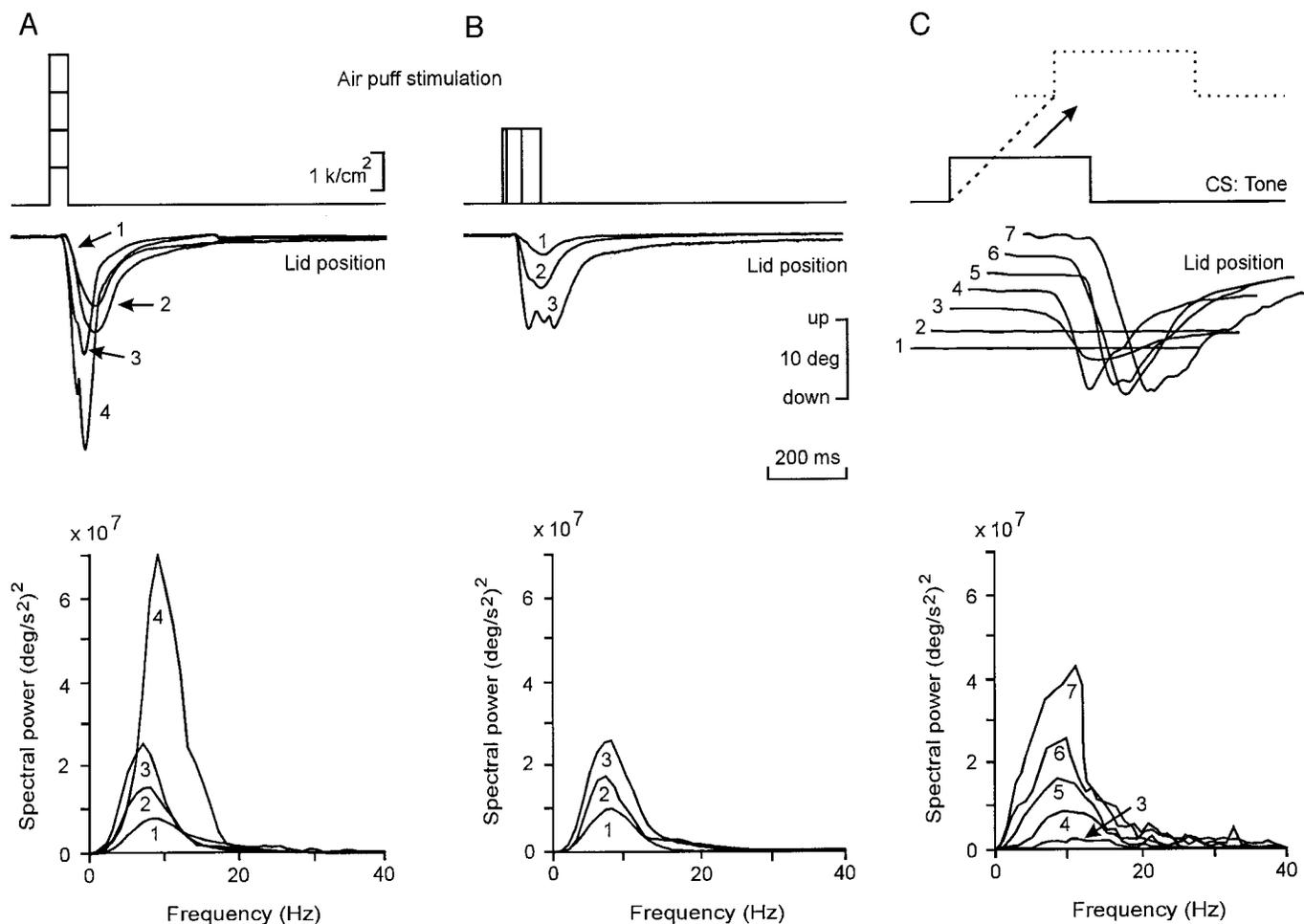


FIG. 10. Frequency domain analyses of reflex and conditioned responses as a function of pressure, stimulus duration, and session evolution. *A* and *B*: eyelid position traces during increasing pressure (*A*) or duration (*B*) of air puffs presented to the ipsilateral cornea. *C*: eyelid position for conditioned responses (CRs) obtained by the sole presentation of the conditioned stimulus (CS: a 50-ms, 90-dB, 600-Hz tone) during a T-AP conditioning paradigm. Histogram in *A* shows the mean power spectra of 24 acceleration profiles recorded during air puff presentations for 50 ms and with 1 (1), 2 (2), 3 (3), and 4 (4) k/cm^2 of air pressure. Histogram in *B* shows the mean power spectra of 18 acceleration profiles recorded during 2- k/cm^2 air puffs of 10 (1), 50 (2), and 100 (3) ms. Histogram in *C* shows the mean power spectra of 42 acceleration profiles recorded from CRs in response to the CS-alone presentation through successive conditioning sessions (1–7). Calibrations in *B* are also for *A* and *C*.

larger lid responses were achieved by increasing the velocity of the movement. Accordingly, the rise time of the eyelid closing response remained unchanged for the whole range of lid responses, and, obviously, lid movements were not linearly related to the duration of the movement. Thus the metric properties of eyelid blinks made them similar to skeletal ballistic movements, but different from eye saccades, because the latter depend on both the velocity and the duration of eye movements (Evinger et al. 1984). The upward phases of reflex blinks reported here were much slower than the downward phases and presented lower gains in peak velocity for increasing lid displacement, a result that has also been reported previously (Evinger et al. 1991; Gruart et al. 1995).

It seems very important to characterize the effects of stimulus parameters rabbit eyelid responses to provide much-needed background information for plasticity studies of conditioned blinks. For comparative purposes (Gruart et al. 1995; Marshall-Goodell et al. 1992), air puffs of different duration and/or pressure were selected here for a systematic analysis of stimulus parameters on reflex eyelid blinks (Schreurs et al.

1995). Latency of evoked blinks to stimulus onset presented an inverse exponential-like relationship with air puff pressure. Indeed, the eyelid response to short (10 ms) and weak ($1 \text{ k}/\text{cm}^2$) air puffs was both weak and variable, being very much dependent on the attentive state of the animal. Both amplitude and peak velocity of the downward phase of the evoked blinks increased with air puff pressure for stimuli >50 ms in duration. Here again, stimuli ≤ 10 ms were apparently unable to activate corneal receptors in a constant way. Another interesting finding was that the set of activated sensory receptors is a determinant of the metric and profile of the evoked response, because small displacements of the pipette used for air puff presentations significantly modified the evoked reflex (see Fig. 3). Compared with those in cats, air-puff-evoked reflex blinks in rabbits seem to be more dependent on direct application of the stimulus to corneal receptors.

The duration of air puff stimulation did not affect the latency, amplitude, or peak velocity of the initial downward phase of reflex blinks. More importantly, the rise time of evoked blinks did not change for stimuli $>2 \text{ k}/\text{cm}^2$. As a

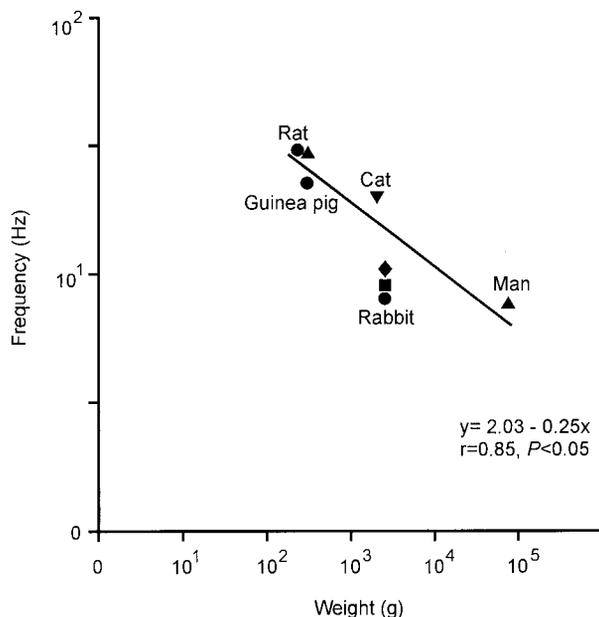


FIG. 11. Relationship between mean body weight and the dominant frequency of eyelid responses for different species. Plot illustrates data obtained from rats (●, present experiments), guinea pigs (●, present experiments; ▲, C. Evinger, personal communication), cats (▼, Domingo et al. 1997), rabbits (●, present experiments; ◆ and ■, data calculated from illustrations in Berthier 1992, and Welsh 1992, respectively), and humans (▲, C. Evinger, personal communication).

consequence, further increases in the duration of eye blinks can be accomplished only by increasing the number of downward waves or sags, as already demonstrated for cats (Gruart et al. 1995).

In agreement with previous reports (Evinger et al. 1991; Gruart et al. 1995), peak EMG amplitude was linearly related with peak downward lid velocity, suggesting that lid velocity depends on the number of motor units simultaneously active. On the other hand, the rectified EMG area was related to lid maximum displacement, that is, an indication that the final lid position is dependent on the total number of activated motor units. A recent study on the firing activities of cat facial motoneurons innervating the orbicularis oculi muscle further supports these contentions (Trigo et al. 1999a).

Metric properties of conditioned eyelid responses

Animals used here for classical conditioning of blink responses reached criterion in a similar number of trails to those previously described (Bartha and Thompson 1992; Gormezano et al. 1962, 1983; Mauk and Ruiz 1992; Smith 1968). The accepted criterion for rabbit nictitating membrane CR is ≈ 0.5 mm (Marshall-Goodell et al. 1992). The 0.5-mm criterion represents 2–3° of eyelid displacement, which is within the range of the small waves or downward sags shown here as the constitutive (or quantum) elements of CRs. The resolution of the eye coil system used here was ≈ 15 min of arc, which allows an excellent signal/noise ratio, even for the smallest eyelid responses.

The profile and metric properties of rabbit CRs were different from those of reflex blinks. The CR showed a slow buildup throughout the successive conditioning sessions and had a wavy shape and smaller amplitude than maximum reflexively

evoked blinks. Moreover, CRs have always a slower building-up than reflex responses. The peak velocity of the CR was only one-fifth of that reached by reflex blinks. The peak velocity of the CR (considered as a single motor process) was shown to be a linear function of CR amplitude, but the gain of this relationship was $\approx 1/10$ that of air-puff-evoked blinks. The gain of the CR peak velocity-amplitude relationship was also related to the conditioning paradigm, with $ap > T$. As observed for reflex blinks, CR duration was not significantly related to CR amplitude, mostly because the CR tended to reach its maximum amplitude at the (already fixed) CS-US interval. All these metric properties are similar (with slight differences) to a previous report on the topography and kinetics of eyelid CRs in the cat (Gruart et al. 1995). However, it should also be stated that CRs were similar to reflex lid responses regarding the dominant frequency of the power spectrum and to the presence of downward sags.

The latency of CR decreased with successive sessions, and by the seventh session was longer than the corresponding value for air-puff-evoked reflex blinks (Berstein 1934; Gormezano et al. 1983; Mauk and Ruiz 1992; Smith 1968). Latency values for T-AP conditioning for lid CRs (≈ 120 ms) were smaller than those reported for a similar conditioning of nictitating membrane CRs (≈ 180 ms) (McCormick et al. 1982). On the other hand, the latency of CRs during ap-AP conditioning was longer than for the T-AP conditioning paradigm. This fact could be explained by the inhibitory postsynaptic potential that follows the early reflex (i.e., alpha) response evoked by CS (short, weak air puff in this case) presentation (Grant and Horcholle-Bossavit 1983; Trigo et al. 1999a).

As reported here, rabbits did not show detectable alpha responses to tones, but did respond to short, weak air puffs used as CS. Alpha responses reported here had latency (≈ 40 ms), magnitude ($\approx 1/8$ of the CR by the 7th conditioning session), and duration (≈ 35 ms) values similar to those reported previously in the same species (Gormezano et al. 1983). Alpha conditioning is easily produced in humans to light CS (Grant and Adams 1944), and in cats to short, weak air puffs and, sometimes, to tones (Gruart et al. 1995; Woody 1986). The main difference between cats and rabbits is that the former are able to integrate the alpha response with the CR, because no temporal gap exists in CR profiles (Gruart et al. 1995), a result not observed in rabbits (Schreurs and Alkon 1990, and the present experiments). In fact, different CR profiles can be induced by different CSs, and different species may solve the same motor learning problem in different ways (Gruart et al. 1995; Rescorla 1988). The different functional organization of neural circuits controlling nictitating membrane/eyelid motor learning in cats and rabbits should not be a surprise if one considers the noticeable differences between species for the related oculomotor system (e.g., Graf and Simpson 1981).

Oscillatory mechanisms underlying reflex and conditioned eyelid responses

The fact that reflex and conditioned blinks have a wavy profile has been noticed even for nictitating membrane recordings (Bartha and Thompson 1992; Berthier 1992; Welsh 1992) and was observed in early human experiments (Marquis and Porter 1939). Illustrations of reflex and conditioned nictitating membrane responses in previous reports (Berthier 1992; Welsh

1992) also present noticeable oscillations at 8–10.5 Hz. The dominant frequency of eyelid responses in the rabbit is about one-third of its resonant frequency (≈ 30 –35 Hz) (see Evinger et al. 1984). A similar finding has been reported for finger movements in humans (Halliday and Redfearn 1956; Wessberg and Vallbo 1995). In agreement with recent reports in cats (Domingo et al. 1997), increases in the duration of reflex blinks were accomplished by the addition of successive downward waves of ≈ 125 ms. Moreover, and as reported for finger movements in humans (Vallbo and Wessberg 1993), larger lid CRs in rabbits were achieved by increasing the amplitude and number of waves composing them, but not by modifying the dominant frequency of the movement. If we follow this line of thought, CRs could be envisioned as the process of reaching a target in a given time with the help of a fixed-frequency neuronal oscillating machinery (Domingo et al. 1997).

The fact that light-evoked blinks presented a dominant oscillation frequency lower than air-puff-evoked ones has also been reported recently in cats (Domingo et al. 1997) and suggests that functional properties of the circuits involved may contribute to determine the dominant frequency of each reflex response. In this sense, the number of synapses involved in each of the circuits and the possibility of recurrent loops could contribute to the differences reported here.

As illustrated in Fig. 11, when the available data on lid dominant oscillation frequencies for different species are plotted against species body weight, an inverse logarithmic relationship is obtained. Indeed, these data suggest that lid biomechanics could be tuned to the lid's weight and to its viscoelastic properties. A similar inverse logarithmic relationship (with exactly the same slope) has been demonstrated to exist between heart rate and body mass for mammals (Stahl 1967). The slope of this relationship (-0.25) indicates that lid oscillation frequency (as shown for heart rate) could be related to oxygen consumption per unit body mass (in l/kg/h), because the latter is also related to body mass (in kg) by a linear relationship of the same slope (i.e., -0.25) (see Figs. 4.9 and 6.10 in Schmidt-Nielsen 1979).

Because the eyelid is load free, and facial motoneurons receive no feedback proprioceptive signals from the orbicularis muscle (Porter et al. 1989; Trigo et al. 1999b), it could be suggested that the oscillatory behavior of the eyelid is the result of the activity of the neuronal mechanisms controlling it. In fact, it has been shown recently in cats and rats that tremor of the lids is an inherent rhythmical property of facial motoneurons innervating the orbicularis oculi muscle (Magariños-Ascone et al. 1999; Trigo et al. 1999a). Moreover, a noticeable oscillatory behavior has been observed in cat pericruciate cortex neurons during the classical conditioning of blink responses (Aou et al. 1992) and in cat cerebellar interpositus neurons during reflexively evoked blinks (Gruart and Delgado-García 1994). Coherent 25- to 35-Hz oscillations have also been reported in the sensorimotor cortex of awake monkeys during exploratory and manipulative movements (Murthy and Fetz 1992). Taken together, these data suggest that motor systems could be controlled by central neural oscillators tuned to the inertial and viscoelastic needs of moving appendages.

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