Discharge Profiles of Abducens, Accessory Abducens, and Orbicularis Oculi Motoneurons During Reflex and Conditioned Blinks in Alert Cats

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Trigo, Jose´ A., Agnev Gruart, and Jose´ M. Delgado-Garcı´a. Discharge profiles of abducens, accessory abducens, and orbicularis oculi motoneurons during reflex and conditioned blinks in alert cats. J. Neurophysiol. 81: 1666–1684, 1999. The discharge profiles of identified abducens, accessory abducens, and orbicularis oculi motoneurons have been recorded extra- and intracellularly in alert behaving cats during spontaneous, reflexively evoked, and classically conditioned eyelid responses. The movement of the upper lid and the electromyographic activity of the orbicularis oculi muscle also were recorded. Animals were conditioned by short, weak air puffs or 350-ms tones as conditioned stimuli (CS) and long, strong air puffs as unconditioned stimulus (US) using both trace and delayed conditioning paradigms. Motoneurons were identified by antidromic activation from their respective cranial nerves. Orbicularis oculi and accessory abducens motoneurons fired an early, double burst of action potentials (at 4–6 and 10–16 ms) in response to air puffs or to the electrical stimulation of the supraorbital nerve. Orbicularis oculi, but not accessory abducens, motoneurons fired in response to flash and tone presentations. Only 10–15% of recorded abducens motoneurons fired a late, weak burst after air puff, supraorbital nerve, and flash stimulations. Spontaneous fasciculations of the orbicularis oculi muscle and the activity of some orbicularis oculi motoneurons that generated them also were recorded. The activation of orbicularis oculi motoneurons during the acquisition of classically conditioned eyelid responses happened in a gradual, sequential manner. Initially, some putative excitatory synaptic potentials were observed in the time window corresponding to the CS-US interval; by the second to the fourth conditioning session, some isolated action potentials appeared that increased in number until the CS-US interval; by the second to the fourth conditioning session, some small movements were noticed in eyelid position traces. No accessory abducens motoneuron fired and no abducens motoneuron modified their discharge rate for conditioned eyelid responses. The firing of orbicularis oculi motoneurons was related linearly to lid velocity during reflex blinks but to lid position during conditioned responses, a fact indicating the different neural origin and coding of both types of motor commands. The power spectra of both reflex and conditioned lid responses showed a dominant peak at ≈20 Hz. The wavy appearance of both reflex and conditioned eyelid responses was clearly the result of the high phasic activity of orbicularis oculi motor units. Orbicularis oculi motoneuron membrane potentials oscillated at ≈20 Hz after supraorbital nerve stimulation and during other reflex and conditioned eyelid movements. The oscillation seemed to be the result of both intrinsic (spike afterhyperpolarization lasting ≈50 ms, and late depolarizations) and extrinsic properties of the motoneuronal pool and of the circuits involved in eye blinks.

INTRODUCTION

The motor system controlling eyelid responses is an excellent experimental model to study how relatively simple, although diverse, movements are generated by central neural circuits (see references in Evinger 1995; Gruart et al. 1995). The eyelid motor system is load free, has an almost negligible mass, and, according to recent anatomic and functional data, is free of proprioceptors (Porter et al. 1989; Trigo et al. 1997, 1999). Indeed, in spite of the system’s apparent simplicity, many different movements are accomplished by the lids in diverse behavioral situations; in fact, different motor systems are involved in blink responses. Essentially, a blink is a reflex eyelid response to the mechanical activation of the cornea and periocular skin or to the electrical stimulation of the supraorbital nerve (Baker et al. 1980; Crucu et al. 1987; Evinger et al. 1991; Gordon 1951; Gruart et al. 1995; Hiraoka and Shimamura 1977; Kugelberg 1952). However, blinks also can be evoked by strong visual and acoustic stimuli (Evinger and Manning 1993; Gruart et al. 1995; Manning and Evinger 1986). The kinematics of a reflex blink is the result of the brief, fast contraction of both eyelids produced by the phasic activation of the orbicularis oculi muscle, but it also involves the cocontraction of most of the extraocular muscles, the relaxation of the levator palpebrae muscle, and, in those species with a nictitating membrane, the activation of the retractor bulbi muscle (Baker et al. 1980; Evinger 1995; Evinger and Manning 1993). Apart from reflexively evoked responses related to corneal wetting and protection, lids move (mostly passively) accompanying rotational eye movements in the orbit, in such a way that the pupil is not covered during voluntary and reflex eye saccades and fixations (Becker and Fuchs 1988).

In addition to their participation in massive reflex blinks, lids play an active role in complex and precise motor displays involved in behavioral responses such as smiling and winking in humans (Evinger 1995; Gordon 1951) and during grimacing and friendly displays in felines ( Bateson and Turner 1988; Darwin 1872). Another example of graded and precisely elaborated lid movement is the generation of classically conditioned eyelid responses (CR). For the past 60 yr, the nictitating membrane/eyelid response has been employed in the study of neural mechanisms underlying motor learning (Gormezano et al. 1983; Kim and Thompson 1997; Marquis and Porter 1939; McCormick et al. 1982; Welsh and Harvey 1992; Woody 1986). The profile and kinematics of CRs are different from those of reflex blinks, suggesting a different origin and/or neural generation ( Domingo et al. 1997; Gruart et al. 1995; Rescorla 1988). The neural site where learning of eyelid response occurs (Aou et al. 1992; Bloedel 1992; Kim and Thompson 1997; MeEchron and Disterhoft 1997), as well as the putative subcellular mechanisms involved (Bliss and Col-
lingridge 1993; Coulter et al. 1989; Malenka 1995; Woody et al. 1989) are currently a subject of intensive research.

Studies about the functional involvement of neural centers and circuits in reflex and conditioned eyelid responses have followed different approaches. Thus while there are many studies on the firing profiles of trigeminal, red nucleus, cerebellar cortex and nuclei, and cerebral cortex neurons in relation to the acquisition and/or performance of new motor skills (Aou et al. 1992; Bloedel 1992; Kim and Thompson 1997; McEchron and Disterhoft 1997; Richards et al. 1991; Woody 1986), less attention has been paid to the functional properties of those brain stem motoneurons that represent the final common pathway for learned lid responses—namely abducens, accessory abducens, and orbicularis oculi motoneurons (Keifer et al. 1995; Matsumura and Woody 1986). In contrast, there are good descriptions of the electrophysiological properties of these three types of blink-related motoneurons in both acute and in vitro preparations (Aghajanian and Rasmussen 1989; Baker et al. 1980; Fanardjian et al. 1983b; Grantyn and Grantyn 1978; Gueritaud 1988; McCall and Aghajanian 1979; Nishimura 1985; Nishimura et al. 1989). The firing profiles of abducens (Delgado-García et al. 1986), accessory abducens (Delgado-García et al. 1990), and orbicularis oculi (Hall and Hicks 1973) motoneurons have been recorded in cats or rats during reflex blinks but not during the acquisition and performance of CRs.

It should be remembered that the eyelid CR is an adaptive response that has to be accomplished by three different motor systems not equally suited for this motor task. In two previous papers, we have described the kinematics of spontaneous, reflexively induced, and classically conditioned eyelid responses in alert behaving cats (Gruart et al. 1995) and the frequency-domain and time-domain properties of these facial movements (Domingo et al. 1997). It was found that both reflex and conditioned eyelid responses present movement discontinuities at a dominant frequency of ~20 Hz. Thus lid movements seem to be constructed from a basic 50-ms downward wave or sag. Moreover reflex blinks seem to be the result of the massive, synchronous activation of many orbicularis oculi motor units, whereas CRs are elaborated in a succession of small downward sags in a staircase-like manner. In this context, it seems necessary to study the particular contribution of each blink-related motoneuronal pool to the generation of these peculiar motor responses. The present experiments were aimed at recording the firing activity of identified abducens, accessory abducens, and orbicularis oculi motoneurons in the alert behaving cat during the presentation of stimuli able to evoke reflex blinks (puffs of air, flashes of light, tones, and electrical stimulation of the supraorbital nerve). Recorded motoneurons were identified by antidromic activation from their corresponding cranial nerves. The discharge rate of these motoneurons was recorded during two different (trace and delayed) conditioning paradigms. The unconditioned stimulus (US) was always a long, strong air puff. As conditioned stimulus (CS), either a short, weak air puff or a tone was used. Animals were provided with eyelid search coils and with electromyographic (EMG) recording electrodes. Results proved the different involvement of each motoneuronal type in reflex and conditioned eyelid responses and suggested some insights into the peculiar way CRs are generated at the motoneuronal level.

METHODS

Subjects

The present experiments were carried out on six adult cats weighing 2–2.6 kg obtained from an authorized supplier (Iffa-Credo). Animals were prepared for the chronic recording of upper eyelid movements, the EMG activity of the orbicularis oculi muscle, and the electrical activity of identified abducens, accessory abducens and orbicularis oculi motoneurons. All experimental procedures were carried out in accordance with the guidelines of the European Union Council Directive (86/609/EU) and following Spanish legislation (BOE 67/ 8509-12 1988) concerning the use of laboratory animals in chronic experiments.

Preexperimental surgical procedures

Each animal was anesthetized with pentobarbital sodium (Nembutal, 35 mg/kg) following a protective injection of atropine sulfate (0.5 mg/kg) to avoid unwanted vagal reflexes. A five-turn coil (3-mm diam) was implanted into the center of the upper left lid at ~2 mm from the lid margin. Coils were made from Teflon-coated multi-stranded stainless steel wire with an external diameter of 50 μm. Because of its low weight (12.5 mg, i.e., <1.5% of the cat’s lid weight), the implanted coil did not produce any noticeable lid drooping or impairment of movement when compared with the nonimplanted eyelid. Two hook electrodes, made of the same wire as the coils and bared ~1 mm at their tips, were implanted in the left orbicularis oculi muscle. The two hook electrodes were aimed at the zygomatic subdivision of the facial nerve, 1–2 mm posterior to the external canthus, and at the tarsal portion of the upper lid. 1 mm medial to the coil, respectively. Other two hook electrodes were implanted on the ipsilateral supraorbital branch of the trigeminal nerve. A silver electrode (1-mm diam) was attached to the skull as ground. A silver bipolar stimulating electrode was implanted on the left VIth nerve at its exit from the brain stem (stereotaxic coordinates L = 3.5 and P = 1). The position of the electrode was adjusted to produce an abducting movement of the ipsilateral eye with a 50-μs single pulse of <0.2 mA (Delgado-García et al. 1986). To allow transerebellar access to selected brain stem motor nuclei during recording sessions, a square (6 × 6 mm) window was drilled in the occipital bone. The dura mater was removed, and an acrylic resin chamber was constructed around the window. The cerebellar surface was protected with a piece of silicone sheet, and the chamber was filled with sterile gauze and capped with a plastic cover. A needle tip was implanted stereotaxically in a corner of the chamber as reference point for the location of brain stem motor nuclei. Finally, a head-holding system was built in to give stability to unitary recordings and to have a coil reference during the experimental sessions. Three bolts were anchored to the skull in the vertical stereotaxic plane with the aid of six small, self-tapping screws fastened to the bone. The bolts were cemented to the skull with acrylic resin. Eyelid coil, EMG, stimulating, and grounding wire terminals were soldered to a nine-pin socket cemented to the holding system. Further details of this chronic preparation have been described elsewhere (Delgado-García et al. 1986; Gruart et al. 1995).

General conditions of recording sessions

Recording sessions started 15 days after surgery and lasted ≤3 h/day. For each session, the animal was introduced into a fabric bag, lightly wrapped with an elastic bandage, and placed in a foam-coated Perspex box on the recording table. The animal’s head was immobilized by attaching the holding system to a bar affixed to the table. Data illustrated in Figs. 1–11, corresponding to spontaneous and reflex blink responses, were obtained during the first five recording sessions. After these sessions, each animal was assigned randomly to one of the two (A, B) conditioning paradigms described later in METHODS. Conditioning sessions lasted ≤15 days, and were preceded by two habit-
Unitary recordings were carried out during all of the sessions in which reflex or conditioned eyelid responses were evoked in the experimental subjects. Special care was taken during recording sessions to avoid any discomfort to the animals. In fact, the continuous monitoring of heart and respiratory rhythms with noninvasive procedures during the recording sessions yielded values not significantly different from those obtained while the cat was resting in the arms of one of us (Gruart et al. 1995). Figure 1 illustrates recording and stimulating sites as well as other experimental procedures.

**Unitary recordings**

The recording chamber was uncovered to allow electrode access to blink-related brain stem motor nuclei. Unitary and field electrical activities were recorded with glass electrodes filled with 1.5–2 M NaCl. Intracellular recordings were carried out using pipettes filled with 3 M potassium acetate. Field potentials were recorded with low-impedance electrodes (1–3 MΩ), while extra- and intracellular unitary activity was recorded with smaller tips (3–8 and 8–12 MΩ, respectively). Neuronal activity was recorded with the aid of a high-impedance circuit located in a head-stage close to the animal’s head. Electrical signals were amplified and filtered with a bandwidth of 1 Hz to 10 kHz for display and digitization purposes.

Micropipettes were advanced with a three-axis micromanipulator through the intact cerebellum to reach the brain stem. The abducens and accessory abducens nuclei were approached with the help of stereotaxic coordinates and with the guidance of the patterns of the electrical activity generated in the surrounding neuronal structures (Delgado-García et al. 1986, 1990). In the case of orbicularis oculi motoneurons, the electrode was aimed at the dorsolateral subdivision of the facial nucleus, where those motoneurons are located (Shaw and Baker 1985). The abducens and the accessory abducens nuclei were located finally with the aid of the antidromic field potential produced in their electrophysiological limits by the electrical stimulation of the ipsilateral VIth nerve. The center of each nucleus was considered to be the point of maximum antidromic negativity. The VIth nerve was stimulated with 50-μs, 1- to 20-Hz cathodal square pulses with current intensities small enough (<0.2 mA) to prevent its spreading to the Vth nerve. The accessory abducens nucleus usually was located 2–2.5 mm lateral to the center of the main abducens nucleus. The orbicularis oculi subdivision of the facial nucleus was located by electrical stimulation of the zygomatic nerve with electrical pulses similar to
those applied to the VIth nerve. The center of the dorsolateral subdivision of the facial nucleus was found 3–4 mm lateral to the center of the main abducens nucleus (Fig. 1, A–C) (see Delgado-García et al. 1986, 1990; Shaw and Baker 1985 for details).

Given the high density of blink-related units located in the neighborhood of blink-related brain stem motor centers (Gruart et al. 1993), the collision test between orthodromic and antidromic action potentials was used systematically to identify recorded units. Only the recordings of properly identified motoneurons were stored and analyzed. Recorded units remained well isolated up to a maximum of 2 h. Surprisingly, some accessory abducens and most facial motoneurons could be recorded for a long time (≤20 min) during intracellular impalements. We decided to take advantage of this fact, given the additional information offered by the intracellular recording of neuronal electrical activity. In those cases, experimental recordings started after membrane potential stabilization. Once stable resting potentials (range: −67 to −45 mV) were reached, no significant differences were found between the patterns of activity recorded intracellularly or extracellularly (see Aou et al. 1992; Terzuolo and Araki 1961). For technical reasons, no DC recording was available during activation of the eyelid search coil recording system. Averages and/or quantifications from intracellular records (such as those illustrated in the insets of Figs. 11 and 15) were carried out only for spikes >45 mV (Kitai et al. 1972). Intracellular records with spikes <20 mV were discarded (Aou et al. 1992).

At the end of every recording session, the recording chamber was cleaned aseptically and instilled with antibiotics. The scar tissue was bathed with lidocaine and carefully removed. Finally, the chamber was sealed as previously described until the next recording session.

Recordings of eyelid movements and electromyographic activity of the orbicularis oculi muscle

Eyelid movements were recorded using the magnetic field search-coil technique (Fuchs and Robinson 1966). The coils were calibrated with the cat still under the effects of the anesthetic. A transparent protractor was placed sagittally to the head and centered at the external canthus of the lids (Gruart et al. 1995). For the sake of homogeneity, the gain of the recording system was always set at 1 V = 10°. Lid opening ranged from 37–43° for the six animals. The EMG activity of the orbicularis oculi muscle was recorded with differential amplifiers with a bandwidth of 10 Hz to 10 kHz.

Stimuli evoking reflex eyelid responses

 Reflex blinks were evoked with air puffs, light flashes, and tones and by electrical stimulation of the supraorbital nerve. Air puffs directed to the cornea and tarsal skin were applied through the opening of a plastic pipette (2.5-mm diam) located 1 cm away from the eye, at a lateral angle of 45° with respect to the central direction of gaze. Air puff duration ranged from 20 to 100 ms, and air pressure at the source was set at 1–3 kg/cm². The onset of air puff stimuli to skin mechanoreceptors was determined with a microphone located at the same distance as the animal’s cornea. The signal recorded by the microphone was rectified, integrated, and fed into the computer as a 1-V square pulses for latency measurements. Visual blinks were evoked with full-field flashes lasting ~1 ms. Flashes were produced by a xenon arc lamp located 1 m in front of the animal. Tones of 600 or 6,000 Hz, 90 dB, applied for 10–100 ms were used as acoustic stimuli. The loudspeaker was located 80 cm below the animal’s head. After the isolation of a motoneuron, each stimulus was presented in blocks of 10 stimuli at variable intervals (5 ± 1 s), to avoid interactions between subsequent blinks (Powers et al. 1997). A minimum of 30 s was allowed between successive presentations of stimuli of different modalities. The electrical stimulation of the supraorbital nerve consisted of single or double (1- to 2-ms interval), 50-μs cathodal square pulses with current intensities <0.2 mA.

No experimental procedure was used to evoke spontaneous eye blinks or ‘‘eyelid friendly displays’’ (Gruart et al. 1995). These eyelid motor responses were collected off-line from tape recordings with the help of a window discriminator set to the detection of the initial fast increase in velocity of these eyelid responses.

Classical conditioning paradigms

The classical conditioning of lid responses was achieved by the use of trace (A) or delayed (B) conditioning paradigms. For trace air puff–air puff (ap–ap) paradigm, the animal was presented with a short (20 ms), weak (1 kg/cm²) air puff, directed to the left cornea as CS. The CS was followed 250 ms later by a 100-ms, 3-kg/cm² air puff directed to the ipsilateral cornea as US. For delayed trace air puff (T-AP) paradigm, the animal was presented with a 350-ms, 600-Hz, 90-dB tone as CS. The tone was followed 250 ms from its onset by a 100-ms, 3-kg/cm² air puff stimulus directed to the left cornea as US. The tone and the air puff finished simultaneously.

Each of the two (A, B) conditioning paradigms was used to train three animals. An animal was considered to be conditioned when it could produce 95% of CRs per session to the CS-US paired presentation. Although this criterion was reached by the fifth conditioning session for the six animals, recordings were obtained up to the 15th session to obtain the maximum number of recorded motoneurons. Conditioning sessions were preceded by two habituation sessions.

Each conditioning session consisted of 12 blocks separated by a variable time interval (range of 4–6 min). These intervals were used to locate and identify recorded units. Each block consisted of 10 trials separated at random by intervals ranging from 20 to 40 s. In a trial within each block, the CS was presented alone, that is, was not followed by the US. A complete conditioning session lasted ~2 h. Only the stimulus selected as CS was presented during habituation sessions with the same number of blocks per session and trials per block and with a similar random distribution of interblock and intertrial intervals (see details in Gruart et al. 1995).

Data collection and analysis

Vertical and horizontal position of left upper eyelid, unrectified EMG activity of the ipsilateral orbicularis oculi muscle, neuronal activity, 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 at a sampling frequency of 22 kHz for biopotentials and 11 kHz for the other signals. Data were transferred later through an analog digital converter (CED 1401 Plus) to a computer for off-line analysis. Most data were sampled at 1–4 kHz with an amplitude resolution of 12 bits. Selected unitary records were sampled at 22 kHz for representation purposes and precise analysis of spike profiles. Additionally, action potentials were fed into a window discriminator and the resulting Schmidt trigger pulses were stored on the computer, along with the analog signals, using the same A/D conversion card.

Commercial computer programs (Spike 2 and SIGAVG from CED and MATLAB) were modified, and new programs were developed to display single, overlapping, averaged, and raster representation of eyelid position, velocity, and acceleration and of EMG and neuronal activities. Velocity and acceleration traces were computed digitally as the first and second derivative of lid position records after low-pass filtering of the data (~3 dB cutoff at 50 Hz and a 0 gain at ~100 Hz). The single and averaged histograms of instantaneous firing rate profiles of the neuronal discharge were displayed in relation with the corresponding EMG and eyelid activities. The instantaneous firing rate was calculated as the inverse of the interspike intervals for segments of ~1 s (see Domingo et al. 1997 for details).

These computer programs also allowed the quantification, with the aid of cursors, of lid position, EMG, and neuronal parameters, such as onset latency, amplitude, duration, and peak and mean values of recorded responses. Relationships between neuronal firing rate and lid position and velocity were obtained by linear regression analysis to
calculate their respective slopes, i.e., the neuronal sensitivity to lid position (in spikes per second per degree) and to lid velocity (in spikes per second per degree per second). Because lid position was sampled at ≈1 kHz, bin size for linear correlation was set at 1 ms. The power of the spectral density function (i.e., the power spectrum) of selected eye acceleration recordings was calculated using a fast Fourier transform to define the relative strength of the different frequencies present in lid responses. Analysis were carried out according to procedures described in detail elsewhere (Bendat and Piersol 1986; Domingo et al. 1997; Wessberg and Vallbo 1995). In short, acceleration recordings were divided in 1.024-s segments, starting 100 ms in advance of the presentation of the reflex- or CR-evoking stimuli. Illustrated power spectra (Figs. 11 and 15) correspond either to a single acceleration segment (Fig. 11) or to the mean value of power spectra computed from 10 different acceleration segments (Fig. 15). Data were processed for statistical analysis with the SPSS/PC + package, for two-tailed tests with a statistical significance level of $P = 0.05$. Mean values are followed, when necessary, by the standard deviation (SD). Statistical differences of mean values were determined with the help of the Student’s $t$-test for variables of two categories or with the ANOVA for variables of more than two categories. Rate-position and rate-velocity relationships for spike activity and eyelid movements were calculated by linear regression analysis and nonlinear curve fits with the SigmaStat/SigmaPlot software. For this, lid position or lid velocity was plotted versus the instantaneous firing rate, in a point to point procedure. Peaks of power spectra were tested with the $\chi^2$-distributed test for spectral density functions (see Delgado-García et al. 1986; Domingo et al. 1997; Gruart et al. 1995 for details).

**RESULTS**

**General properties of recorded motoneurons**

The number of motoneurons recorded, identified, and analyzed in the present experiments was 317, subdivided as 61 abducens, 99 accessory abducens, and 157 orbicularis oculi motoneurons. Of those motoneurons, 163 units were recorded during conditioned eyelid responses (11 abducens, 22 accessory abducens, and 120 orbicularis oculi motoneurons). Motoneurons always were identified by their antidromic activation from the VIth nerve (abducens and accessory abducens) or from the zygomatic branch of the facial nerve (orbicularis oculi). Extracellular somatic spikes were identified by their characteristic positive-negative-positive or negative-positive shapes (see Fig. 1, A and B). Abducens motoneurons were activated antidromically at a mean latency of 0.66 ± 0.14 (SD) ms ($n = 30$; range, 0.4–0.8 ms), measured at the first negative peak of the spike. Accessory abducens motoneurons were activated antidromically at slightly shorter latencies: 0.51 ± 0.12 (SD) ms ($n = 50$; range, 0.4–0.7 ms). Considering the distance between the recording site and the position of the stimulating electrode, the mean conduction velocity of abducens motoneurons was 43.5 ± 15.3 m/s ($n = 30$; range, 20–60 m/s), whereas that of accessory abducens motoneurons was 47.5 ± 13.1 m/s ($n = 50$; range, 28–70 m/s). The mean latency for antidromic activation of orbicularis oculi motoneurons was 2.13 ± 0.23 ms ($n = 100$; range, 1.6–2.8 ms; Fig. 2C), with a mean conduction velocity of 46.9 ± 10.2 m/s ($n = 100$, range 27–75 m/s). These results indicate similar ranges of axonal conduction velocities for the three populations of blink-related brain stem motoneurons. The three motoneuron pools presented similar values for minimum intervals during double shock activation. Thus the mean minimum interval computed from well-isolated spikes ($n > 15$ for each group) was 1.2 ± 0.05 ms for abducens, 1.5 ± 0.9 ms for accessory abducens, and 1.7 ± 0.9 for orbicularis oculi motoneurons.

All identified abducens motoneurons displayed a continuous, modulated firing rate in relation to eye movements (Delgado-García et al. 1986). In contrast, accessory abducens motoneurons recorded here seemed to lack a tonic firing rate. Instead they fired almost exclusively during the early phase of
EMG activity of the orbicularis oculi muscle was 7.2 ± 6 ms for blink initiation was 11.8 ± 6 ms and muscle displacement by 2 ms, and muscle electrical activity preceded actual lid movement by 5.1 ± 6 ms. The mean latency of the activation latency for orbicularis oculi motoneurons was 5.1 ± 6 ms. Long (100 ms), strong (3 kg/cm²) air puffs evoked a brief burst of action potentials in orbicularis oculi motoneurons followed by a large-amplitude (range 0.5–7.2 mV) afterhyperpolarization (Fig. 2A). The amplitude of this afterhyperpolarization seemed to be dependent on the number of spikes in the initial burst (Figs. 2, 8B, 10, and 11). This initial burst of activity usually was followed, with a high degree of synchrony, by late single action potentials, as suggested by the coincidence of each action potential with phasic activities in the innervated orbicularis oculi muscle.

Single pulses applied to the ipsilateral supraorbital nerve evoked a fast downward displacement of the lid followed by a slower upward phase. Stimulation of the supraorbital nerve activated orbicularis oculi motoneurons after a mean latency of 3.7 ± 0.9 ms (n = 30; range, 3–6 ms). Usually, a single action potential was evoked. A second action potential was seen at 9–12 ms from stimulus presentation (see Figs. 1C and 2B). This double activation of orbicularis oculi motoneurons by supraorbital nerve stimulation corresponded quite well with the R1 and R2 responses described in the EMG activity of the orbicularis oculi muscle as evoked by the same stimulus (Kugelberg 1952). Time intervals between neural activity and muscle activation (≈2 ms), and between the latter and lid displacement (≈4–5 ms), were similar to the descriptions above for air-puff-evoked blinks.

Flash-evoked blinks showed a significantly (P < 0.01) longer (n = 40; 49.1 ± 5.3 ms) latency than those evoked by air puffs (Fig. 2C). Flash-evoked blinks were usually of smaller amplitude and more-easily fatigable than air-puff-evoked ones. Orbicularis oculi motoneurons fired a brief burst of action potentials in response to flash presentations, sometimes followed by a second burst (or just a single action potential) at an interval of ≈50 ms. As illustrated in Fig. 2C (D), on many occasions the depolarization corresponding to the late activation of orbicularis oculi motoneurons during flash presentation did not reach the threshold to produce an action potential.

Tones rarely evoked noticeable blinks in our experimental animals. In fact, two animals never blinked in response to tone presentation. When recorded, tone-evoked blinks presented a mean latency of 52.4 ± 10.2 ms (n = 25). Figure 2D illustrates a putative synaptic potential recorded intracellularly in an identified orbicularis oculi motoneuron during tone presentation, indicating that these cells probably are activated by acous-

Discharge profiles of abducens, accessory abducens, and orbicularis oculi motoneurons during reflexively evoked blinks

The three populations of blink-related brain stem neurons considered in this study showed quite different firing profiles during the presentation of air puffs, flashes of light, and tones and during single pulses applied to the ipsilateral supraorbital nerve (see Figs. 2 and 3).

As illustrated in Fig. 2A, an air-puff-evoked blink consisted of a fast downward displacement of the upper lid followed by a longer-lasting, wavy upward phase. Both the initial fast downward movement and the later small sags resulted from the phasic contraction of the orbicularis oculi muscle. For 100-ms, 3-kg/cm² air puffs (n = 60 presentations), the mean latency for blink initiation was 11.8 ± 0.9 ms, the mean latency of the EMG activity of the orbicularis oculi muscle was 7.2 ± 1.3 ms, and the activation latency for orbicularis oculi motoneurons was 5.1 ± 0.7 ms. Thus the initial firing of orbicularis oculi motoneurons led the EMG activity of its innervating muscle by ≈2 ms, and muscle electrical activity preceded actual lid displacement by ≈4.5 ms. Long (100 ms), strong (3 kg/cm²) air puffs evoked an initial burst of action potentials in orbicularis oculi motoneurons followed by a large-amplitude (range 0.5–7.2 mV) afterhyperpolarization (Fig. 2A). The amplitude of this afterhyperpolarization seemed to be dependent on the number of spikes in the initial burst (Figs. 2, 8B, 10, and 11). This initial burst of activity usually was followed, with a high degree of synchrony, by late single action potentials, as suggested by the coincidence of each action potential with phasic activities in the innervated orbicularis oculi muscle.

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Tones rarely evoked noticeable blinks in our experimental animals. In fact, two animals never blinked in response to tone presentation. When recorded, tone-evoked blinks presented a mean latency of 52.4 ± 10.2 ms (n = 25). Figure 2D illustrates a putative synaptic potential recorded intracellularly in an identified orbicularis oculi motoneuron during tone presentation, indicating that these cells probably are activated by acous-

![Figure 3](http://jn.physiology.org/content/167/1/1611/F2.large.jpg)
tic inputs but only occasionally reach the threshold to evoke full spikes.

Figure 3 summarizes the more representative firing profiles of brain stem, blink-related motoneurons during reflexively evoked blinks. Only 12% of the recorded abducens motoneurons (6 of 50) modified their firing during reflex blinks, presenting a late and weak increase in their discharge rate. For example, their latency of activation to supraorbital nerve stimulation was 15 ms. Although eye movements were not measured in the present experiments, the response of abducens motoneurons was noticeably more related to eye rotational than to lid downward blinking movements. This fact could explain the weakness and variability in abducens motoneuron responses to reflexively evoked blinks, depending probably on the adducted or abducted eye position in the orbit before stimulus presentation (see Delgado-García et al. 1986, 1990).

No response to tone presentation could be obtained from abducens motoneurons. Accessory abducens motoneurons presented a fast and brief increase in their firing rates in response to air puff and supraorbital nerve stimulations. However, the late components of air-puff-evoked lid responses were not accompanied by any noticeable activity in accessory abducens motoneurons (Fig. 3A). None of the recorded accessory abducens motoneurons (n = 40) fired in response to flash or tone presentations even though synaptic potentials were recorded both extra- and intracellularly in the accessory abducens nucleus at the expected latencies (i.e., ≈40–45 ms after stimulus presentation, not illustrated). As shown in Fig. 4, accessory abducens motoneurons restricted their firing in the alert behaving cat to highly demanding eyelid (and, supposedly, nictitating membrane) responses. Thus air puffs presented to the same animal on different occasions evoked smaller or larger lid downward displacements depending on the participation of accessory abducens motor units. In fact, a displacement of the nictitating membrane in the cat was observed only during eye retractions accompanying large eyelid blinks.

Quantitative relationships between the firing rate of abducens, accessory abducens and orbicularis oculi motoneurons and reflexively evoked and spontaneous eye blinks

The possible linear relationships between the firing rate of selected abducens (n = 10), accessory abducens (n = 10), and orbicularis oculi (n = 10) motoneurons and eyelid position and/or velocity during air-puff-evoked blinks were analyzed (see Figs. 5 and 6). Separate rate-position and rate-velocity plots were made for the early (I) and late (II) components of eye blinks (Fig. 5A). The firing rate of orbicularis oculi motoneurons was not linearly related to either early (I, Fig. 5B) or late (II, not illustrated) phases of eye blinks. Also the firing rate of abducens and accessory abducens motoneurons did not seem to be linearly related to lid position (not illustrated). The coefficients of correlation (r) for rate-position plots during both early and late phases of the blink calculated for a total of 30 motoneurons (10 of each type) yielded values of r ≤ 0.41 (P ≤ 0.05). Conversely, orbicularis oculi motoneurons firing was related linearly to lid velocity during both early and late phases of eye blinks (see I and II in Fig. 5A). Figure 5, C and D, illustrates the linear relationships between the firing rate of an
orbicularis oculi motoneuron and lid velocity during the early (Fig. 5C) and late (Fig. 5D) phases of air-puff-evoked blinks. It should be pointed out that the slope of these linear relationships was larger for the same motoneuron during the late (2.34 spikes/s per °/s) phase of the blink than for the early one (0.39 spikes/s per °/s). As illustrated in Fig. 6, C and D, the mean slope of the linear relationship between the firing rate of 10 orbicularis oculi motoneurons and the late component of air-puff-evoked blinks (1.99 ± 0.32 spikes/s per °/s; range, 1.57–2.46) was about four times larger (P < 0.01) than the corresponding value for the early component of the same set of blinks (n = 10; 0.41 ± 0.09 spikes/s per °/s; range, 0.27–0.57).

The firing rate of accessory abducens motoneurons was linearly related with lid velocity during the early downward phase of the blink (Fig. 6B). The mean slope of the linear relationship was 0.16 ± 0.03 spikes/s per °/s (n = 10; range, 0.12–0.22). Because accessory abducens motoneurons were rarely active during the late phase of eye blinks, no numerical relationship could be established between the two variables.

The firing rate of abducens motoneurons (n = 10) was not

FIG. 5. Relationship between the firing rate of an identified orbicularis oculi motoneuron and eyelid position and velocity. A, top to bottom: illustrations of mean firing rate (FR) and eyelid velocity (EV) and position (EP) during the repeated (n = 50) presentation of a 100-ms, 3 kg/cm² corneal air puff. B: relationship obtained between firing rate and lid position for the neuron illustrated in A for the time window indicated by I. C: same as B but for lid velocity. D: same as C but for lid velocity during the time window indicated by II. Regression lines in C and D showed a P < 0.01. Note the different velocity signals carried out by the same orbicularis oculi motoneuron during the 2 different time windows (I in C and II in D).

FIG. 6. Relationship between the firing rate of 10 representative abducens (A), accessory abducens (B), and orbicularis oculi (C and D) motoneurons and eyelid velocity during air puff-evoked blinks. A–C: illustrated data correspond to the initial, fast downward movement of the lid (i.e., period I in Fig. 5A) evoked by corneal air puffs. D: illustrated data were collected during the late, slow movement of the lid evoked by the same stimulus (i.e., period II in Fig. 5A). Ten orbicularis oculi motoneurons analyzed in D are the same as in C. Each regression line was calculated from >200 measurements collected from n = 50 air puff presentations. Coefficients of correlation (r) for data illustrated in A were r = 0.45, whereas those for data illustrated in B–D were r ≥ 0.8. All regression lines showed a P < 0.01. Mean slopes and ranges of illustrated regression lines are indicated in the text.
significantly related to lid velocity during either early (I) or late (II) phases of the blink (see Fig. 6A).

For purposes of comparison, Fig. 7 illustrates an air-puff-evoked eye blink and a spontaneous downward lid movement with the kinetic characteristics of an “eyelid friendly display” (Gruart et al. 1995). As opposed to reflex blinks, eyelid friendly displays have a symmetric bilateral presentation and consist of a slow, long-lasting downward lid displacement followed by a slow upward phase. These spontaneous lid movements were made by the animal from time to time (0.5–2/min), usually when viewing the experimenters moving around in the recording room. The profile of eyelid friendly responses was similar to that of the late component of air-puff-evoked blinks (Figs. 5A and 7). Indeed, the firing rate of orbicularis oculi motoneurons during eyelid friendly displays was related linearly to lid velocity but not to lid position. The mean slope of the relationship between neuronal firing rate and eyelid velocity during “eyelid friendly displays” was 2.31 ± 0.43 spikes/s per °/s (n = 10; range, 1.57–2.46; r = 0.73; P ≤ 0.01), that is, similar to the high values (1.99 spikes/s per °/s) obtained during the late phase (II in Fig. 5A) of air-puff-evoked blinks, and five times larger than those (0.41 spikes/s per °/s) obtained for the same set of orbicularis oculi motoneurons during the early phase (I in Fig. 5A) of reflex blinks. The long-lasting record shown in Fig. 7 illustrates the lack of tonic activity in most of the identified orbicularis oculi motoneurons recorded in the present study. Only when the lids were maintained actively closed for a few seconds was a tonic activity in ≈10% of recorded neurons observed.

Probable origin of spontaneous lid fasciculations

The spontaneous firing activity of isolated (i.e., single) orbicularis oculi motoneurons was observed on a few occasions (n = 9 motoneurons) during which the animal did not make any overt spontaneous, reflex, or conditioned eyelid movements, and only minute eyelid displacements could be observed on the ipsilateral side. Sometimes, a small-amplitude, although evident, fasciculation of the upper or lower subdivision of the orbicularis oculi muscle was observed under the orbital skin. No stimulus was being applied during those recordings, although all of the observations were made during the late recording sessions in the two animals showing this spontaneous activity. The low amplitude of the evoked lid response (0.1–1°), the absence of any EMG activity not accompanied by the corresponding spike in the neuronal recording trace (see Fig. 8A), and the lack of any noticeable lid movement on the contralateral side further suggest that we were recording the spontaneous activity of a single motor unit. Accordingly, we concluded that fasciculations recorded in lid position traces and in the EMG activity of the orbicularis oculi muscle were the result of the sole action of the recorded motoneuron (see Fig. 8A and B). Interestingly, when firing single action potentials, these motoneurons presented a spike afterhyperpolarization lasting for ≈40–60 ms (Fig. 8A, inset). Moreover, the units tended to fire in doublets, the second spike evoking a larger displacement than the preceding one, as a function of the interspike interval (Fig. 8C). Available data indicated that the optimum interval for this potentiating effect was ≈10 ms, i.e., close to the time-interval values between R1 and R2 components of blinks evoked by the electrical stimulation of the supraorbital nerve (Gruart et al. 1996; Kugelberg 1952). This optimum interval was one-fifth and one-eighth the values described for fast (49.4 ms) and slow (86.9 ms) gastrocnemius muscle units also in cats (Burke et al. 1976).

Spike-triggered averaging of lid movement and of the electromyographic activity of the orbicularis oculi muscle from abducens, accessory abducens, and orbicularis oculi motoneurons

The degree of synchrony between spikes produced by identified blink-related motoneurons, the EMG activity of the or-
bicularis oculi muscle, and the actual lid displacement was checked in a few \( (n = 5) \) selected motoneurons from each of the three motor groups considered in the present study. As shown in Fig. 9C, orbicularis oculi motoneurons preceded by \( \approx 2 \) ms the activation of the innervated muscle, and the latter also preceded by \( \approx 4 \) ms lid downward displacement (see Woody and Brozek 1969). Although accessory abducens motoneurons do not innervate the orbicularis oculi muscle and, accordingly, do not act directly on lid displacement, their action potentials also were synchronized with muscle activity and lid reflex responses (Fig. 9B). These results suggested a high degree of synchrony for the activation of both populations of motoneurons in response to strong (3 kg/cm\(^2\)) air puffs presented to the cornea and tarsal skin. Because accessory abducens motoneurons almost exclusively fired slightly preceding (\( \approx 5-6 \) ms) the initial, fast downward component of the reflex blink, their apparent effect on lid movement was larger than that evoked by orbicularis oculi motoneurons (compare the two sets of records in Fig. 9, B and C). On the other hand, we averaged orbicularis oculi motoneuron spikes produced through both the early and late phases of the blink, which probably contributed to a decrease in the amplitude of the averaged lid displacement. Finally the firing of abducens motoneuron action potentials was related poorly to lid movement and, in fact, lagged behind orbicularis oculi muscle activation by \( \approx 5-7 \) ms (Fig. 9A).
Oscillatory properties of orbicularis oculi motoneurons

As illustrated in Figs. 2, 8, and 9C, the firing activities of orbicularis oculi motoneurons were correlated highly with even minute changes in the EMG activity of the orbicularis oculi muscle and with the smaller changes in eyelid position. This fact is further illustrated in Fig. 10 during a reflex lid response evoked by the electrical stimulation of the supraorbital nerve (Fig. 10B) and during spontaneous blinks (Fig. 10, A and C). In all of these intracellular records, it could be observed that a noticeable afterhyperpolarization followed each orbicularis oculi spike, with a mean duration of 47.3 ± 7.4 ms (n = 30; range 37–56 ms). As already indicated, the spike afterhyperpolarization depended in amplitude on the number of spikes present in the preceding burst (Figs. 2 and 10, A and B). Moreover, the afterhyperpolarization usually was followed by a late depolarization or rebound potential (Figs. 2C and 10, A–C). In Fig. 10B three intracellular record traces corresponding to the same orbicularis oculi motoneuron are overlapped, each evoked by a single electrical stimulus applied to the ipsilateral supraorbital nerve. As shown in Fig. 10, every single stimulation of the supraorbital nerve evoked the synaptic burst activation of the motoneuron as well as an evident oscillation of its membrane potential. Obviously subsequent spikes appeared with a higher probability riding on the top of each wave, as if it were a time window when the neuron was depolarized more easily to reach threshold for a full action potential (see Fig. 10, B and D).

A further attempt was made to correlate the oscillatory properties of orbicularis oculi motoneurons and the wavy profile of air-puff-evoked blinks. As already described (Domingo et al. 1997; Gruart et al. 1995), and as illustrated in Fig. 11, the succession of downward sags after the initial down phase of air-puff-evoked blinks outlasted the duration of the stimulus, and when measured by hand from peaks in velocity profiles, those sags yielded a mean duration of 40–60 ms. The power spectra of the acceleration profile of the eye blink illustrated in Fig. 11 presented a significant peak (P < 0.01) at 20 Hz, over a broad band of frequencies between 15–25 Hz. The figure also illustrates the high degree of coincidence between the firing profile of the orbicularis oculi motoneuron and the wavy appearance of the lid position record. Interestingly, the average (n = 200) of single spikes corresponding to the same neuron showed a spike afterhyperpolarization lasting ≈ 50 ms (see inset in Fig. 11). In consequence, the oscillation observed in lid acceleration profiles could be the result of the intrinsic membrane properties of innervating orbicularis oculi motoneurons. However, the participation of some premotor circuits cannot be ruled out as depolarizing rebound potentials also could be of synaptic origin (Fig. 10B).

Discharge profiles of abducens, accessory abducens, and orbicularis oculi motoneurons during conditioned eyelid responses

Representative profiles of eyelid CRs obtained through different conditioning sessions are illustrated in Figs. 12–15. Latency, maximum amplitude, peak velocity, and profile of these CRs were very different depending on the animal and on the modality of the sensory cue used as CS. Because this paper is devoted mostly to the study of the firing properties of blink-related brain stem neurons, no further mention will be made of the analysis of latency and profiles of eyelid learned movements, which has been reported in two recent publications (Domingo et al. 1997; Gruart et al. 1995).

Abducens, accessory abducens, and orbicularis oculi motoneurons were recorded systematically during the successive conditioning sessions in the six animals. As illustrated in Fig. 12, the firing rate of identified orbicularis oculi motoneurons changed dramatically from stages preceding the appearance of a CR (Fig. 12A) to those during which the CR was being formed (Fig. 12B). Being highly phasic cells, orbicularis oculi motoneurons only rarely fired spontaneously, unless excited experimentally by blink-evoking stimuli, or during spontane-
ous eyelid movements (see preceding text). In this situation, and during the first (1–3) conditioning sessions, the ap-AP trace conditioning paradigm activated the firing of these motoneurons only during air puff presentations but not during the CS-US time interval. However, before a CR started to be formed, some synaptic potentials were observed during the CS-US interval (n = 2–5 per CS-US interval; see Figs. 12A and 13), some of them large enough to evoke a single action potential (Fig. 12A). Moreover, the membrane potential background activity increased immediately after CS presentation. By the fourth conditioning session (Fig. 12B), the CS-US interval appeared full of action potentials (≥12–15 per CS-US interval) distributed at more-or-less fixed intervals (see averages in Fig. 14, B and D). The progressive increase in the number of synaptic potentials as well as in the number of full spikes is better illustrated in Fig. 13, together with the subsequent appearance and consolidation of the CR. Obviously, the continuous increase in the number of action potentials during the time window represented by the CS-US interval evoked an increase in the EMG activity of the orbicularis oculi muscle and a ramp-like displacement of the upper lid in the downward direction (Figs. 12 and 13). This ramp-like profile of lid movement radiated from the CS until producing the almost complete closing of the lids at the time of the US presentation (see later set of intracellular records in Fig. 13). The latency between CS presentation and the initiation of action potentials from identified orbicularis oculi motoneurons recorded in the CS-US interval during trace ap-AP conditioning decreased from a mean value of 180 ± 17 ms (n = 10, range 158–205 ms), during the 4th conditioning session, to 52 ± 6 ms (n = 10, range 45–61 ms), during the 15th one (if the early alpha response, clearly separated from the true CR by a noticeable spike afterhyperpolarization is not taken into account; see Fig. 13, left). The ramp movement of the lid was thus the result of the low-rate, repetitive, and tonic-like firing of orbicularis oculi motoneurons, a fact also noticed in the smaller amplitude of muscle complex action potentials, mostly when compared with the EMG activity of the muscle during reflex blinks (Figs. 2A and 12). It was concluded that the ramp-like profile of CRs, as well as its wavy aspect, were the result of the low-rate and sustained activity of orbicularis oculi motoneurons (at a dominant oscillatory frequency of ≈20 Hz). Similar results were obtained when recording the activity of identified orbicularis oculi motoneurons during the T-AP delayed conditioning paradigm; that is, synaptic potentials were observed during the first conditioning sessions, well in advance of the appearance of action potentials and, obviously, of any slight sign of a CR (not illustrated). In the case of the T-AP conditioning paradigm, the latency between CS presentation and the initiation of action potentials from identified orbicularis oculi motoneurons recorded in the CS-US interval decreased from 137 ± 16 ms (n = 10, range 118–151 ms) during the 4th conditioning session to 55 ± 8 ms (n = 10, range 45–64 ms) during the 15th one.

Figure 14 summarizes the activity of identified abducens, accessory abducens, and orbicularis oculi motoneurons during
the classical conditioning of eyelid responses. To our surprise, none of the units recorded and identified as an accessory abducens motoneuron (n = 22) fired during the CS-US interval, i.e., during the time of CR presentation (see Fig. 14, middle set of records). However, all of these motoneurons fired during US presentations, indicating that their activity still was recorded by the micropipette when firing. From the 11 abducens motoneurons recorded during the classical conditioning of lid responses, only 1 (Fig. 14A) seemed to fire in relation to US presentation, but not even 1 of them fired in response to the presentation of the two different CSs (ap and T) used in the present study. It should be pointed out that the ≈20-Hz, repetitive response of orbicularis oculi motoneurons triggered by CS presentation was still noticeable in the averages (n = 10) illustrated in Fig. 14, B and D (arrows), corresponding to the sole CS presentation.

The frequency-domain properties of CRs were analyzed quantitatively and compared with the firing properties of recorded orbicularis oculi motoneurons. As illustrated in Fig. 15, CRs after CS presentation in well-conditioned animals had a power spectrum in their acceleration profiles with a significant peak \( P < 0.01 \) at ≈20 Hz over a broadband of frequencies between 10–30 Hz. There was also a high degree of coincidence between the power spectra of acceleration profiles, those
corresponding to the EMG activity of the orbicularis oculi muscle, and those to the firing rate profiles of simultaneously recorded neurons (not illustrated). Thus the oscillation observed in lid acceleration profiles during CRs could be the result of the tendency of orbicularis oculi motoneurons to fire at a dominant frequency of \( \approx 20 \) Hz.

Quantitative relationships between conditioned eyelid responses and the firing rate of abducens, accessory abducens, and orbicularis oculi motoneurons

The firing rate of orbicularis oculi motoneurons was plotted against eyelid position and velocity during CRs obtained after the sole presentation of the CS (Fig. 16). As shown, motoneuronal activity seemed to be linearly related to eyelid position, but not to eyelid velocity, during CRs. These results were opposite to those mentioned in the preceding text above and illustrated in Figs. 5 and 6, suggesting that orbicularis oculi motoneurons encode eyelid velocity during reflexively evoked blinks. In fact, no orbicularis oculi motoneuron \((n = 10)\) presented a firing rate linearly related to lid velocity during CRs. The mean slope of rate-position plots was 7.18 spikes \( \cdot s^{-1} \cdot \text{deg}^{-1} \) \((n = 10); \text{range } 4.11-10.5)\). Accordingly, the firing rate of orbicularis oculi motoneurons seemed highly synchronized during reflex blinks (Fig. 2), a finding related to the fact that single neurons used to fired in doublets (Figs. 2, 4, 7, and 8). This latter circumstance favors a fast lid downward displacement for optimal interspike intervals of \( \approx 10 \) ms. Conversely, orbicularis oculi motoneurons changed to a low-rate, tonic firing during CRs, a fact that explains the ramp-like profile of these lid displacements (Figs. 12–15). This tonic firing also explains the low peak and mean velocity of CRs (about \( \frac{1}{10} \) that of reflex blinks).

DISCUSSION

Motor roles of blink-related brain stem motoneuronal pools during spontaneous and reflexively evoked blinks

The present work is an attempt to compare the firing characteristics of identified abducens, accessory abducens, and orbicularis oculi motoneurons during the performance of spontaneous, reflexively induced and classically conditioned eyelid responses in the alert behaving cat. As shown here, each motoneuronal pool contributed in a specific manner to the generation of the different types of eyelid response. Air-puff-evoked blinks seemed to be produced by the cooperative participation of the three motoneuronal pools. Abducens motoneurons presented a weak and variable relationship with upper lid blinking responses. As already reported (Delgado-García et al. 1986, 1990), abducens motoneurons mostly are related to rotational eye movements. Only 10–15% of abducens motoneurons seemed to have some trigeminal input signals, but even...
those motoneurons fired during eyelid blinks, depending on the position of the eye in the orbit, making their firing not linearly related to either eyelid position or velocity. Their contribution to eye retraction and, as a consequence, to the passive downward displacement of the upper lid during blink responses had necessarily to depend on the synchronous cocontraction of the other three recti muscles, a fact not confirmed in the present experiments, but already described in the goldfish (Pastor et al. 1991) and in the rabbit (Evinger and Manning 1993).

It has been described in cats that accessory abducens motoneurons fired a fast burst of action potentials in response to long, strong air puffs presented to the cornea and periorbital skin (Delgado-García et al. 1990). Their firing in response to electrical stimulation of the supraorbital nerve consisted of a double activation, usually reduced to a couple of spikes separated by a 10-ms interval. This double activation corresponds to the R1 and R2 responses recorded in the EMG activity of the orbicularis oculi muscle in humans (Kugelberg 1952) and also was observed directly in eye retractional movements during air-puff-evoked blinks (Delgado-García et al. 1990). The firing rate of accessory abducens motoneurons was found to be linearly related to lid velocity, but not to lid position, during reflex blinks, which further indicates the contribution of this motoneuron pool to a fast closing of the eye during protective eyelid responses. Accessory abducens motoneurons recorded here did not respond to flash or tone presentations.

**FIG. 15.** Frequency-domain properties of conditioned eyelid responses. Top to bottom: illustrations of a presentation of CS alone (a 350-ms, 600-Hz, 90-dB tone), the firing activity of an identified orbicularis oculi motoneuron, the acceleration of the lid response, and lid position. - - -, expected time for US (a strong, long air puff). This set of records was obtained during the 6th conditioning session of a T-AP delayed conditioning paradigm. Histogram at the bottom represents the power spectrum profile [in (deg/s²)] of acceleration profiles averaged during 10 presentations of the CS alone from the same conditioning session. *Inset:* average (n = 25 unpaired action potentials, with > 100 ms of interspike interval) of the intracellular (Intra) record to illustrate the mean duration of the spike afterhyperpolarization.

FIG. 16. Relationships between the firing rate of identified orbicularis oculi motoneurons and lid position and velocity during eyelid conditioned responses. **A**–**C:** illustrated data correspond to firing profiles (in spikes/seconds) and eyelid position (in degrees) and velocity (in degrees/second) for a selected orbicularis oculi motoneuron. Data were collected from n = 20 CS-alone presentations. **C:** illustration of the relationship between firing rate and eyelid position for 10 selected orbicularis oculi motoneurons. For each regression line, data were collected from n = 20 CS-alone presentations. Coefficients of correlation (r) for data illustrated in **C** were r < 0.85. All regression lines showed a P < 0.01. Mean slope and range of illustrated regression lines are indicated in the text.
The firing rate of identified orbicularis oculi motoneurons followed the profile of lid displacements during blinks evoked by air puffs, flashes of light, and tones, and during the electrical stimulation of the supraorbital nerve. The EMG activity of the orbicularis oculi muscle showed an extremely high degree of synchrony with the presence of spikes in the neural recording, suggesting a phasic, synchronous firing of orbicularis oculi motoneurons activated during a given eyelid reflex response. The firing of identified orbicularis oculi motoneurons was organized in an early double burst of spikes that evoked the R1 and R2 responses in the innervated muscle (Cruccu et al. 1987; Hiraoka and Shimamura 1977; Kugelberg 1952), and a fast downward displacement of the upper lid. This burst usually was followed by a late response involving a sustained activation of the orbicularis oculi muscle and a slow displacement of the lid further in the downward direction. The firing of orbicularis oculi motoneurons could not be related linearly to eyelid position, but it was related significantly to eyelid velocity during both the early and late phases of air-puff-evoked blinks. This finding confirms that orbicularis oculi motoneurons are exclusively involved in the fast downward displacement of the lid. Thus the maintenance of lid position during the intervals between successive blinks is achieved by tonic position signals present in the levator palpebrae muscle (Evinger 1995; Fuchs et al. 1992; Gruart et al. 1995; Trigo et al. 1999).

Involvement of abducens, accessory abducens, and orbicularis oculi motoneurons in eyelid classically conditioned responses

A CR consists of a ramp-like downward lid displacement that seems to be generated in a quantal manner, from a basic 50-ms downward wave or sag (Domingo et al. 1997). As shown here, even a single motor unit can move the lid. Although it is more probable that ‘quantal’ lid movements observed during the acquisition of CRs are the result of the synchronous activation of more than one motor unit, the perfect time coordination between different motoneurons inside the orbicularis oculi subdivision of the facial nucleus suggests a repetitive mechanism intrinsic to the motoneuronal membrane, i.e., an oscillation triggered by CS presentation. The present results further confirm that the wavy aspect of CRs is the result of the peculiar firing pattern of orbicularis oculi motoneurons during the learning process. The finding that motoneuronal firing was correlated with eyelid position during CRs, but with eyelid velocity during reflex responses, suggests a putative mechanism involving the functional peculiarities of the motoneuronal pool. For example, it could be expected that tonic signals typical of CRs were generated at a motoneuronal level by inputs arriving at distal dendrites, whereas phasic firing characterizing the activity of the motoneuron during reflex responses was produced by a strong input impinging on the soma and proximal dendrites.

The electrical activity of identified orbicularis oculi motoneurons was modified by the classical conditioning process well in advance (i.e., days) of the appearance of the first single action potential, and even more in advance if correlated to the appearance of a noticeable downward wave in the lid position records during the CS-US interval. This finding indicates a slow building up of the CR that remains unnoticed unless recorded directly from motoneuronal membrane activity. In this sense, further experiments in a more stable, and better-controlled paradigm are necessary to unravel the early stages of the appearance of the CR at the motoneuronal level.

The lack of involvement of accessory abducens motoneurons in CRs was rather a surprise. Nevertheless, given the high-threshold of this motoneuronal pool in cats, as reported in both acute (Baker et al. 1980) and chronic (Delgado-García et al. 1990; present work) experiments, their behavior during conditioned eyelid responses is understandable. In cats, CRs have a peak velocity about one order of magnitude lower than that observed during air-puff-evoked blinks (Domingo et al. 1997; Gruart et al. 1995), a fact further confirmed here. In this sense, CRs are generated, at least in cats, from a neural site unable to provide enough synaptic excitation to generate full spikes in accessory abducens motoneurons. The peculiar morphology and passive and active membrane properties of accessory abducens motoneurons make them suitable for burst firing during fast and huge depolarizing potentials, mostly from corneal origin (Baker et al. 1980; Grant and Horcholle-Bossavit 1983), but apparently unable to fire in a low, stable rate during CRs.

Origin of the 20-Hz oscillation underlying reflex and conditioned eyelid responses

Brain stem and cortical projections to the facial nucleus have been described in detail based on histological (Courville 1966; Holstege et al. 1986a,b; May et al. 1996; Mizuno and Shimamura 1971; Pozo and Cerveró 1993; van Ham and Yeo 1996) and electrophysiological experiments (Fanardjian and Manvelyan 1984, 1987a,b; Fanardjian et al. 1983a,b; Hiraoka and Shimamura 1977; Vidal et al. 1988) carried out in rats, rabbits, cats, and monkeys. Briefly, the principal and trigeminal nuclei, the dorsal red nucleus, the superior colliculus, specific regions of the mesencephalic, pontine and medullary reticular formation, and different cortical areas were described as projecting directly (i.e., monosynaptically) or indirectly (di- or polysynaptically) to blink-related brain stem motoneuronal pools, mainly to the accessory abducens nucleus and to the orbicularis oculi subdivision of the facial complex. Most of those descriptions indicated that trigeminal signals arriving at the main abducens nucleus follow a polysynaptic pathway. Taken together, these hodological and electrophysiological descriptions are in agreement with data reported here on the firing peculiarities of abducens, accessory abducens, and orbicularis oculi motoneurons. However, some comments should be made about discrepancies between the described pathways and the functional findings. For example, a similar pathway, originating at the olivary pretectal nucleus and at the nucleus of the optic tract, has been reported to arrive at both the accessory abducens and orbicularis oculi motoneuronal pools carrying signals related to light-evoked blinks (Holstege et al. 1986a,b). Because, as shown here, accessory abducens motoneurons are not activated by light flashes, we must accept that the actual function of a given pool of neurons is not determined exclusively by the presence of a given neural pathway or circuit and that other factors, such as intrinsic neuronal properties and particular behavioral situations, also can play an important role. A similar comment could be made regarding the lack of involvement of accessory abducens motoneurons in CRs, when this motoneuronal pool has been reported to be one of the two final common pathways involved in CRs (Holstege et al. 1986a,b; Kim and Thompson 1997). A parsimonious explana-
These membrane potential oscillations were blocked by TTX, described in rats in vitro (Magarin˜os-Ascone et al. 1997). To a membrane depolarization as reported here and as recently suggested by reported here and described in more precise recordings in extraocular motoneurons (Guertitaud 1988). Recently, the presence of specific membrane ionic conductances able to produce rebound responses after their hyperpolarization has been described in rat facial motoneurons (Magari˜nos-Ascone et al. 1997). These rat facial motoneurons are susceptible to change to a stable, tonic, repetitive firing after the application of carbachol (Magari˜nos-Ascone et al. 1997). Nevertheless, it cannot be ruled out completely that these late-depolarizing rebound responses could have a synaptic origin because, as reported in acute experiments (Baker et al. 1980; Grant and Horcholle-Bossavit 1983), accessory abducens motoneurons present a 10- to 140-ms depression during conditioning-test stimulations, suggesting the presence of local (trigemino-facial) circuits able to facilitate repetitive firing at selected time intervals. Indeed, the high degree of synchronization between different muscles (extraocular recti, retractor bulb and orbicularis oculi) during reflex and conditioned eyelid responses (Berthier 1992; McCormick et al. 1982) also suggests the presence of higher-level centers triggering the activity of the set of motoneurons involved. Some cortical areas and the cerebellar interpositus nucleus could be involved in this synchronizing mechanism because both structures have been reported to fire at dominant frequencies of ~20 Hz in alert behaving cats during reflex and conditioned eyelid responses (Aou et al. 1992; Gruart and Delgado-García 1994).

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