Cerebellar Posterior Interpositus Nucleus as an Enhancer of Classically Conditioned Eyelid Responses in Alert Cats

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1División de Neurociencias, Laboratorio Andaluz de Biología, Universidad Pablo de Olavide, 41013 Sevilla; 2Departament de Psicobiologia i de Metodologia de les Ciències de la Salut, Facultat de Psicologia, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain; and 3División de Neurociencias, Instituto Mexicano de Psiquiatría, San Lorenzo Huipulco, 14370 México, DF, Mexico

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Gruart, A., G. Guillazo-Blanch, R. Fernández-Mas, L. Jiménez-Díaz, and J. M. Delgado-García. Cerebellar posterior interpositus nucleus as an enhancer of classically conditioned eyelid responses in alert cats. J Neurophysiol 84: 2680–2690, 2000. Cerebellar posterior interpositus neurons were recorded in cats during delayed and trace conditioning of eyeblinks. Type A neurons increased their firing in the time interval between conditioned and unconditioned stimulus presentations for both paradigms, while type B neurons decreased it. The discharge of different type A neurons recorded across successive conditioning sessions increased, with slopes of 0.061–0.078 spikes/s/trial. Both types of neurons modified their firing several trials in advance of the appearance of eyelid conditioned responses, but for each conditioned stimulus presentation their response started after conditioned response onset. Interpositus microstimulation evoked eyelid responses similar in amplitude and profiles to conditioned responses, and microinjection of muscimol decreased conditioned response amplitude. It is proposed that the interpositus nucleus is an enhancer, but not the initiator, of eyelid conditioned responses.

INTRODUCTION

The eyelid motor system is an excellent experimental model for the study of how central neural circuits generate motor responses (Evinger 1995; Gormezano et al. 1983). This motor system is peculiar, because eyelid movements are load-free and have a minute mass. Also, lid displacements depend on the exclusive action of a few extraocular and facial muscles, apparently free of proprioceptors (Evinger 1995; Evinger et al. 1991; Gruart et al. 1995; Trigo et al. 1999). The kinetic, frequency-domain, and time-domain properties of reflex and conditioned eyelid blinks, as well as the functional properties of innervating brain stem motoneurons, have been described recently for both cats and rabbits (Domingo et al. 1997; Evinger 1995; Evinger et al. 1991; Gruart et al. 1995; Trigo et al. 1999; Welsh 1992).

Besides this apparent simplicity in their sensorimotor organization, eyelid motor responses are involved in spontaneous, reflex, emotional, and eye-related movements. Moreover, since its popularization in the 1960s, the nictitating membrane/eyelid response has been repeatedly employed in the study of the neural mechanisms underlying motor learning (Gormezano et al. 1983; Kim and Thompson 1997; McCormick et al. 1982; Welsh 1992; Woody 1986).

It is now well known that the profile and kinetics of eyelid conditioned responses (CRs) are different from those of reflex blinks, a fact suggesting a distinct origin and/or neural generation process (Domingo et al. 1997; Evinger 1995; Gruart et al. 1995; Trigo et al. 1999; Welsh 1992). For example, in contrast to the fast downward upper lid displacement characterizing reflex blinks, CRs are usually ramp-like, reaching peak velocities that never surpass 1/6 of those reached during air puff-evoked blinks (Gruart et al. 1995). Moreover, CRs seem to be built up over an underlying oscillatory mechanism, already described in cats for reflex and conditioned eyeblinks. This underlying oscillatory mechanism has also been determined in the electromyographic (EMG) activity of the orbicularis oculi muscle, and in the activity of identified facial motoneurons both in vivo and in vitro (Domingo et al. 1997; Magarín-Ascone et al. 1999; Trigo et al. 1999).

The neural site where learning of eyelid CRs occurs, as well as the putative subcellular mechanisms involved, are currently a matter of intensive research (Bliss and Collingridge 1993; Kim and Thompson 1997; Malenka 1995; Mauk 1997; Woody 1986). One of the suggested sites of motor learning is the deep cerebellar nuclei, particularly some not yet well-defined regions of the anterior and/or posterior interpositus nuclei (Bracha et al. 1999; García and Mauk 1998; Krupa et al. 1993; Mauk 1997; Schreurs et al. 1998). The role of interpositus nuclei in eyeblink motor learning has been extended to other classically conditioned withdrawal reflexes (Bracha et al. 1999). These proposals have been supported mostly on lesion and pharmacological studies (Bracha et al. 1999; Krupa et al. 1993; García and Mauk 1998; Mauk 1997; Yeo et al. 1985).

Nevertheless, specific areas of the interpositus nucleus are indeed involved in eyelid responses, as recently confirmed by retrograde transneuronal tracing with rabies virus (Ugolini et al. 1999).

Although very valuable evidences have been accumulated until now regarding the firing activities and functional proper-
ties of interpositus neurons during the acquisition and performance of eyelid CRs (Berthier and Moore 1990; Freeman and Nicholson 1999; Gruart et al. 1997), more specific information is still needed. For example, to accomplish their putative role in motor learning, interpositus neurons should fire a few milliseconds in advance of the initiation of the CR, and, perhaps, their firing should be time-locked to CR profiles. Moreover, interpositus neurons should start increasing their firing monotonically in the time window between conditioning (CS) and unconditioning (US) stimuli some trials in advance of the appearance of the CR. Finally, microstimulation of the learning-related zone of the interpositus nuclei should evoke an eyelid response resembling the profile and kinetic properties of CRs, while the chemical inactivation of interpositus neurons should remove (or at least modify) the normal performance of CRs.

To assess those fundamental questions in a suitable experimental model, cats were prepared for the chronic recording of eyelid movements and of the EMG activity of the ipsilateral orbicularis oculi muscle. The unitary activity of antidromically identified posterior interpositus neurons was recorded during controls and during classic conditioning of eyelinks. Both trace and delayed conditioning paradigms were used. The US was always a long, strong air puff. The CS was either a short, weak air puff or a tone. Electrical microstimulation and microinjection of muscimol [α-aminobutyric acid-A (GABA_A) receptor agonist; see Krupa et al. 1993] were carried out at selected recording sites. Present results suggest that although posterior interpositus neurons are probably not involved in the initiation of eyelid CRs, they do contribute to the reinforcement of motor commands of a yet unknown origin.

**METHODS**

**Subjects**

The present experiments were carried out on eight adult cats weighing 2.5–3 kg obtained from an authorized supplier (Iffa-Credo). Experimental procedures were performed in accordance with the guidelines of the European Union Council regulations (86/609/EU) and following Spanish legislation (B.O.E., 67: 8509–8512, 1988) for the use of laboratory animals in chronic experiments.

**Preexperimental surgical procedures**

Animals were anesthetized with pentobarbital sodium (Nembutal, 35 mg/kg, ip) following a protective injection of atropine sulfate (0.5 mg/kg) to prevent vagal reflexes. As illustrated in Fig. 1A, a five-turn coil (3 mm in diameter) was implanted into the center of the upper left lid at 2 mm from the lid margin. Coils were made from Teflon-coated multistranded stainless steel wire with an external diameter of 50 μm. Two hook electrodes, made of the same wire and bared 1 mm at their tips, were implanted in the ipsilateral orbicularis oculi muscle. A bipolar stimulating electrode (200 μm in diameter, enamel-coated silver wire) was implanted in the vicinity of the magnocellular division of the red nucleus following stereotoxic coordinates (Berman 1968) to activate axons located in the ascending limb of the superior peduncle. A bare silver electrode (1 mm in diameter) was attached to the skull as a ground. To allow a transcortical access to deep cerebellar nuclei, a recording window of 5 × 5 mm was drilled in the occipital bone. The dura mater was removed, and an acrylic recording 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...
recording chamber was opened and a microelectrode advanced toward the interpositus nucleus. The correct location of the micropipette was confirmed by the antidromic identification of recorded units and by the characteristic firing of interpositus neurons to blink-evoking stimuli (see Gruart and Delgado-García 1994). After these sessions, each animal was assigned randomly to one of the two (trace: 3, delayed: 5) conditioning paradigms. Conditioning sessions lasted for ≤10 days, were preceded by two habituation sessions, and followed by ≤4 extinction sessions. Muscimol injection was carried out in two animals conditioned with a delayed paradigm. A microstimulation session was carried out in all the animals, but in two of them (1 trace, 1 delayed) the session took place before extinction sessions.

**Unitary recordings and neuron identification**

Neuronal electrical activity was recorded with glass micropipettes filled with 2 M NaCl. Field potentials were recorded with low-resistance electrodes (1–3 MΩ), while the unitary activity was recorded with microelectrodes of 3–6 MΩ of resistance. Neuronal activity was filtered in a bandwidth of 10 to 10 kHz. The recording electrode was tilted anteriorly by 30 deg and moved in the sagittal and coronal planes in 0.2-mm steps. The recording area was approached with the help of stereotaxic coordinates (Berman 1968) and the field potentials were induced by electrical stimulation of the red nucleus. Electrical stimuli consisted of single- or double- (1- to 2-ms interval) cathodal 50-μs square pulses with current intensities <0.2 mA. The recording of single-unit activity was restricted to those neurons identified by their antidromic activation. The collision test was used to determine whether the recorded and the activated neuron were the same (Fig. 1B). Criteria for the discrimination of somatic versus axonic recording were systematically followed (see Gruart and Delgado-García 1994). Data corresponding to identified orbicularis oculi facial motoneurons illustrated in Fig. 6 were collected from unpublished records from a recent work from our group (Trigo et al. 1999).

**Recordings of eyelid movements and electromyograms**

Eyelid movements were recorded using the search coil in a magnetic field technique (Gruart et al. 1995). Eyelid movements were calibrated with the help of a transparent protractor placed sagittally to the head and with its center located at the lid external canthus. Lid opening ranged 36–42 deg in the eight animals. The EMG activity of the orbicularis oculi muscle was recorded with differential amplifiers at a bandwidth of 10 Hz to 10 kHz.

**Stimuli-evoking reflex blinks**

Air puffs directed to the cornea and periorbital region were applied through the opening of a plastic pipette (3 mm in diameter) located 2 cm from the eye. Air pressure was regulated at the source from 1–3 kg/cm² and lasted 20–100 ms. Bright full-field xenon flashes were used as visual stimulus. Tones (600 or 6,000 Hz) were presented for 10–100 ms at ≤90 dB. During control sessions, stimuli were presented at random, with intervals >5 s (Fig. 1A).

**Electrical stimulation of cerebellar nuclear sites**

Selected sites of the interpositus nucleus were stimulated for 1 s with trains of cathodal 50 μs square pulses, at 5, 10, 20, 30, 40, and 60 Hz. Stimuli were delivered with tungsten electrodes of 1–5 MΩ. Unitary recordings in the aimed area were always carried out before the start of electrical microstimulation sessions.

**Injection experiments**

A guide tube consisting of a 26-gauge stainless steel needle was implanted up to 1.5 mm over the desired injection sites in two of the animals 1 day before classical conditioning. The guide tube was maintained sterile and protected by a removable 33-gauge stainless steel rod. A total of 2.5 μg of muscimol (Sigma) dissolved in 1 μl artificial cerebrospinal fluid (pH = 7.4) was injected 20 min before selected conditioning sessions. Injection was carried out at a rate of 0.25 μl/min with the help of a 1-μl Hamilton syringe connected by a calibrated plastic tubing to a 33-gauge stainless steel needle inserted inside the guide tube. The internal needle projected 1.2 mm beyond the guide tube tip.

**Classical conditioning paradigms**

The classical conditioning of eyelid responses was achieved by the use of delayed and trace conditioning paradigms (Fig. 1C). For delayed (T-AP) conditioning, a 370-ms, 600-Hz, 90-dB tone (T) was used as CS. The tone was followed 270 ms from its onset by a 100-ms, 3-kg/cm² air puff (AP) directed at the left cornea as US. Thus the tone and the air puff terminated simultaneously. For trace (ap-AP) conditioning, the animal was presented with a short (20 ms), weak (1 kg/cm²) air puff (ap) directed at the left cornea as CS. The CS was followed 250 ms later by a 100-ms, 3-kg/cm² AP directed at the same eye as US.

Each conditioning session consisted of 12 blocks separated by a variable (4–6 min) interval. 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. The CS was presented alone in the first trial of each block. A complete conditioning session lasted for ~2 h. The CS was presented during habituation and extinction sessions for the same number of blocks/session and trials/block and with similar random interblock and intertrial distribution (see Gruart et al. 1995). Although conditioned criterion (95% of CRs/session) was reached in the six unit-recording animals by the fourth to sixth conditioning session, conditioning was maintained for 10 sessions to obtain the maximum number of recorded neurons.

**Histology**

At the end of recording sessions, animals were deeply anesthetized (pentobarbital sodium, 50 mg/kg, ip). Electrolytic marks were placed at selected recording sites with a tungsten microelectrode and at stimulating sites with the help of the implanted electrode (1 mA for 10 s). Animals were then perfused transcardially with saline and phosphate-buffered formalin. Serial 50-μm sections of the cerebellum and brain stem were mounted on glass slides and stained with toluidine blue or neutral red.

**Data collection and analysis**

Eyelid position, EMG and neuronal activity, and 1-V rectangular pulses corresponding to blink-evoking stimuli or to CS and US presentations were stored digitally on a videotape recording system at a sampling frequency of 22 kHz for biopotentials and 11 kHz for the other signals. Data were transferred 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, but selected unitary records were sampled at 22 kHz for representation purposes. In addition, action potentials were fed into a window discriminator and the resulting Schmidt trigger pulses were stored on the computer 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 representations of eyelid position, velocity and acceleration, and EMG and neuronal activities. The color raster shown in Fig. 5 was made with the help of a representation program written in Java language by one of us (R.F.-M.). Velocity and acceleration profiles were computed digitally as the first and second derivative of lid position records after low-pass filtering. No significant differences were observed when data were analyzed with smooth or filtered signals.
filtering of the data (−3 dB cutoff at 50 Hz and a 0 gain at −100 Hz). The instantaneous firing rate was calculated as the inverse of the interspike intervals (see Trigo et al. 1999 for details).

These computer programs also allowed the quantification of lid position, EMG, and neuronal parameters. Data were processed for statistical analysis with the SPSS for Windows package, for two-tailed tests with a statistical significance level of \( P = 0.05 \). Unless otherwise indicated, mean values are followed by the standard deviation (SD). The possible relationship between neuronal firing rate and lid position and velocity was checked by linear regression analysis. For this, eye acceleration recordings was calculated using a fast Fourier transform to define the relative strength of the different frequencies present in eyelid responses. Significance of power spectra peaks was tested with the \( \chi^2 \)-distributed test for spectral density functions. Statistical differences of mean values were determined with the help of Student’s \( t \)-test for variables of two categories, or with the analysis of variance (ANOVA) for variables of more than two categories, followed by a contrast analysis when needed (see Domingo et al. 1997, and Trigo et al. 1999 for details).

**RESULTS**

**Neuronal response types**

The recording area was selected according to available morphological and neurophysiological reports indicating its putative involvement in the neuronal control of facial muscles for both cats (Gruart et al. 1995, 1997) and monkeys (van Kan et al. 1993). Thus the posterior interpositus nucleus was explored during the random presentation of blink-evoking stimuli (Fig. 1A). This procedure took 4–9 recording sessions. For all the animals (\( n = 8 \)), this area was found to contain neurons related to eyeblinks. Recorded neurons could be classified as type A and B (Gruart et al. 1994). Type A neurons were activated antidromically from the red nucleus at latencies of 0.76 ± 0.11 ms (measured from the stimulus artifact to the first negative peak, \( n = 50 \)). Type B were activated at significantly longer latencies (0.98 ± 0.08, \( n = 50 \), \( P < 0.01 \), Fig. 1B). The firing rate for type A neurons was rather irregular, with mean values ranging from 10–60 spikes/s (\( n = 50 \)) and peak firing reaching 400 spikes/s. The spontaneous firing of type B neurons was more regular, ranging between 30 and 80 spikes/s (\( n = 50 \)) and reaching peak values of 300 spikes/s. The main functional difference between type A and type B neurons was that type A cells increased their firing during air puff, flash, and (on occasions) tone presentations, while type B cells paused or even stopped firing during the presentation of similar blink-evoking stimuli. No attempt was made here to quantify the response of type A and B neurons to blink-evoking stimuli, as these functional properties have been described elsewhere (Gruart et al. 1994). Once the selected recording area was found, six of the animals were assigned at random to a trace (\( n = 3 \)) or a delayed (\( n = 3 \)) classical conditioning paradigm (Fig. 1C). In the other two animals, a guide tube was implanted over the recording site and no further recordings of neuronal activity was carried out.

**Neuronal activities during classical conditioning of eyelid responses**

The precise location area where neurons used in this study were recorded is shown in Fig. 2. Recorded neurons (\( n = 320 \)) were concentrated in the dorsal part of the anterior pole of the posterior interpositus nucleus. Of this, 192 neurons were classified as type A and 128 as type B. Since a total of 96 conditioning sessions were carried out (2 habituation, 10 conditioning, and 4 extinction sessions in six animals), on average, 3.5 neurons per session were recorded. In fact, many recorded neurons were held for the whole conditioning session.

Recordings carried out outside the shaded area illustrated in Fig. 2 (but within the stereotaxic coordinates of both anterior and posterior interpositus nucleus, Berman 1968) did not show unitary activity related to lid responses. Neurons with firing profiles qualitatively related to ear and/or mouth movements were found in the immediate vicinity of the area selected for this study. Those neurons were not further considered in this study.

As illustrated in Figs. 3, A–D and 4, A–C, type A neurons discharged during CRs similarly to the way already described for blink-evoking stimuli (Gruart et al. 1994). They fired a burst of action potentials following initiation of the eyelid CR. This burst of activity could be individualized for the successive CS and US presentations (Figs. 3A and 4A) or could be present through the whole CR (Fig. 4C). Some intermediate responses of type A neurons could also be observed (Fig. 4B). The increase in firing rate of type A neurons during the CS–US interval over control values ranged from 2–4.5 times when calculated from averaged records (\( n \geq 20 \)) for \( n = 50 \) neurons. In contrast, type B neurons decreased their firing slightly after initiation of CRs. The decrease in firing rate of type B neurons remained until US presentation (Fig. 4D).

As already reported for cats (Gruart et al. 1995), the kine-
matics of eyelid CRs was very different during delayed (Figs. 3A and 4, A, C, and D) versus trace (Fig. 4B) conditioning paradigms. Nevertheless, for present experiments, no significant difference was noticed between type A firing rates during eyelid CRs evoked by delayed versus trace conditioning paradigms (see Figs. 3A and 4, A and C, for delayed and 4B for trace conditioning).

Single (Figs. 3A and 5A) and averaged (Figs. 3D and 4, A–C) firing profiles of type A neurons sometimes seemed to reproduce eyelid position records, i.e., type A neuron firing was apparently related to eyelid position and/or velocity profiles. Linear regression analysis of firing rate versus eyelid position and velocity yielded slopes of 2–9 spikes/s/deg and 0.02–0.13 spikes/s/deg/s (quantified from n = 100 records per neuron; n = 30 neurons). However, the low coefficients of correlation obtained for these linear relationships (r ≤ 0.42, P ≤ 0.05 for firing rate/eyelid position and r ≤ 0.37, P ≤ 0.01 for firing rate/eyelid velocity, n = 30 neurons) precluded us from proposing that type A neurons were encoding eyelid position and/or velocity signals. Similar negative results were obtained for type B neurons when analyzed for eyelid position and/or velocity signals (n = 30, r ≤ 0.31, P ≤ 0.05 for firing rate/eyelid position relationships). Finally, cross-correlation analyses of neuronal firing versus the EMG activity of the orbicularis oculi muscle or lid position profiles did not show any significant relationship (not illustrated).

It was evident from the simple observation of firing records obtained across successive conditioning and neuronal recording sessions that the discharge rate profiles were increasing in their averaged values. Figure 5 illustrates the time-course in both firing rate and in acceleration profiles of CRs across conditioning in a representative animal. As shown, the firing rate for different neurons across conditioning was evident trials in advance of the appearance of a noticeable CR (Fig. 5, B–D). Linear regression analyses of data illustrated in Fig. 5D indicated that recorded interpositus neurons increased their firing at a rate of 0.078 spikes/s per trial (r = 0.71, P < 0.001, Fig. 5B), while eyelid acceleration during the first downward movement of the CR (Fig. 5C) increased at 6.99 degrees/s² per trial (r = 0.8, P < 0.01). The same analysis carried out in two other animals conditioned with a delayed paradigm yielded values for the increase in the firing rate of identified posterior interpositus neurons of 0.06 and 0.072 spikes/s per trial (r ≥ 0.65; P <
analyze the temporal relationships between the neuronal groups involved in blinks. Figure 6, A–C, illustrates the EMG activity of the orbicularis oculi muscle and eyelid displacement evoked by electrical stimulation of the posterior interpositus nucleus (A), the red nucleus (B), and the dorsomedial division of the facial nucleus (C). The electrical stimulation of the interpositus nucleus evoked lid movements at a mean latency of \(10.6 \pm 0.9\) ms \((n = 10)\). The latency of lid response to red nucleus stimulation was \(8.2 \pm 0.6\) ms \((n = 10)\). Finally, lid displacement triggered by orbicularis oculi motoneurons \((n = 5)\) was \(5.9 \pm 0.6\) ms. Accordingly, an interpositus neuron should start firing \(\approx 4\)–\(5\) ms in advance of facial motoneurons to be accepted as directly premotor to them. On the contrary, as illustrated in Fig. 6, D–F; recorded interpositus neurons fired following the burst of activity characterizing the orbicularis oculi motoneurons (Trigo et al. 1999). Thus antidromically identified orbicularis oculi motoneurons fired at \(53.1 \pm 9.1\) ms \((range 46–64, n = 100)\) with respect to CS presentation during the delayed conditioning paradigm. The mean latency for the beginning of EMG activity in the orbicularis oculi muscle was \(55.2 \pm 7\) ms \((range 48–69; n = 100)\). Eyelid CRs started at \(59 \pm 9\) ms \((range 51–70, n = 100)\) following CS presentation. Finally, mean latency with respect to CS presentation for type A posterior interpositus neurons was significantly larger \((P < 0.001)\) than values for eyelid CRs: \(71.5 \pm 13.9\) ms \((range 54–144, n = 100)\).

Since CR latency with respect to CS presentation has some variability, perimovement histograms were also carried out by triggering spike data after CR onset. In this case, the mean delay between CR initiation and type A neuron firing during delayed conditioning was \(22.5 \pm 9.1\) ms \((range 3–75, n = 100)\), with the neuron lagging CR initiation. Significant values \((P < 0.01)\) were obtained for type A neurons during trace conditioning \((35\) ms, \(n = 100)\) and for type B neurons during delayed \((29.2\) ms, \(n = 100)\) and trace \((31\) ms, \(n = 100)\) conditioning paradigms. Accordingly, these data confirmed latency analyses of neuronal activity carried out with respect to CS presentation, as illustrated in Fig. 6F.

**Eyelid movements evoked by microstimulation of the interpositus nucleus**

A further attempt was made to determine the exact contribution of posterior interpositus neurons to eyelid CRs. For this, microstimulation sessions at selected recording sites were carried out following or preceding extinction sessions in four and two animals, respectively. The two animals subjected to muscimol injections were stimulated at the end of the eighth conditioning session. Several stimulation patterns were tried: 5, 10, 20, 30, 40, and 60 Hz trains lasting for 1 s. The 20-Hz train proved to be the most efficient to evoke a ramp-like downward lid movement with the minimum of current applied \((\leq 45\) \(\mu A)\). As illustrated in Fig. 7B, microstimulation of the posterior interpositus nucleus evoked an eyelid response more similar in its kinetics to a CR than to a reflex blink (see Gruart et al. 1995). Moreover, microstimulation of this area did not produce any observable motor response (in mouth, nose, whiskers, or ears) in addition to eyelid movement. When compared with CR responses evoked in the same animal by CS-alone presentations in a delayed conditioning (Fig. 7A), the eyelid response evoked by microstimulation was significantly \((P < 0.001)\)
smaller in amplitude than the natural CR (it was less than one half). However, the simultaneous presentation of the CS and the train of electrical impulses significantly \((P < 0.001)\) increased the size of the evoked eyelid response (Fig. 7C). It should be noted that this pairing of stimuli increased the amplitude of the evoked eyelid response, but did not significantly modify its latency or duration, even considering that the train of electrical stimuli started \(\approx 50\) ms before and finished \(\approx 200\) ms after the CR.

Effects of muscimol injections in the posterior interpositus nucleus

Two of the animals were conditioned following the implantation of a guide tube to allow the injection of muscimol in a site of the posterior interpositus nucleus selected after recording blink-related neuronal activity in it (Fig. 8). In control trials, it was checked that injections of 1-\(\mu l\) artificial cerebrospinal fluid or of 2.5 \(\mu g\) muscimol dissolved in the same volume of fluid did not produce any significant modification of air puff- (100 ms, 3 kg/cm\(^2\)) and flash-evoked blinks. Following controls, both animals were conditioned using a delayed paradigm. Mean values of CR maximum amplitude increased exponentially through the first four conditioning sessions. However, CR maximum amplitude decreased significantly \((P < 0.05\) at least) with respect to the fourth session for the two conditioning sessions (fifth and sixth) during which the muscimol was injected 20 min in advance of session start. The
The mean decrease was 35.1% for the fifth and 18.2% for the sixth conditioning sessions. The next two conditioning sessions (seventh and eighth) showed mean values in peak amplitude of the evoked CRs corresponding to the expected evolution of the learning acquisition curve (Fig. 8B). A significant decrease in CR peak velocity (30.3% for the fifth and 21.1% for the sixth conditioning sessions, \( P < 0.05 \) at least) during the two muscimol-injection sessions was also observed. Nevertheless, no change was observed in CR latency during the two muscimol sessions.

**Discussion**

**Posterior interpositus as a site for motor learning**

Different sorts of irreversible (Welsh 1992; Yeo et al. 1985) and reversible (Bracha et al. 1999; Garcia and Mauk 1998; Krupa et al. 1993) inactivation procedures of the cerebellar interpositus nucleus in both rabbits and cats have addressed its possible participation in the acquisition of motor abilities, mainly that of the classical conditioning of the nictitating membrane/eyelid motor response. Although there is general agreement regarding the involvement of the cerebellar interpositus nucleus in the generation of eyelid CRs (Bracha et al. 1999; Garcia and Mauk 1998; Kim and Thompson 1997; Mauk 1997), some controversy remains regarding its preferred role in the acquisition of new eyeblink responses (Garcia and Mauk 1998; Krupa et al. 1993; Mauk 1997) or in their performance (Welsh 1992; Welsh and Harvey 1992). Available information about unitary recordings of putative deep cerebellar nuclei neurons also indicates their involvement in eyelid CRs (Berthier and Moore 1990; Freeman and Nicholson 1999, 2000; Gruart et al. 1997).

Present results indicate that a very definite area of the posterior interpositus nucleus is involved in the genesis and control of newly acquired eyelid CRs in cats. Indeed, the activity of these neurons fulfilled some of the conditions required for this role. First, they increased their firing in the CS–US time interval across conditioning several trials in advance of the appearance of the CR. In this regard, it has been shown that the amplitude of field potentials recorded in dorsal posterior interpositus by contralateral inferior olive stimulation increases across conditioning sessions in advance of the appearance of eyelid CRs (Gruart et al. 1997). This fact indicates that some facilitation for the firing of posterior interpositus neurons takes place in this area in the CS–US time interval during successive conditioning trials. Second, the microstimulation of the same recording area evoked ramp-like eyelid movement profiles similar in their kinetic characteristics to cat eyelid CRs (Gruart et al. 1995). Third, the microinjection of muscimol in the recording area decreased the amplitude of
eyelid CRs across conditioning sessions, although did not completely abolish it (see Krupa et al. 1993). However, although the discharge rate profiles of these posterior interpositus neurons were apparently related to the profiles of eyelid CRs, their firing could not be proved to encode eyelid position and/or velocity signals. Moreover, the rapid decrease in neuronal activity in parallel with CR extinction suggests a tight relationship with the learned motor response, since the extinction process should be considered a new form of learning during which the meaning of the CS is reinterpreted. For all of those reasons, and because firing at the CS–US interval was initiated after CR onset, it cannot be proposed that this region is the place were eyelid CRs are initiated. Nevertheless, the interpositus nucleus certainly seems to be a site were CRs are facilitated and/or enhanced for an appropriate motor expression. The present results fit well with some proposed models regarding cerebellar functions on motor control and learning (see Houk et al. 1996).

This study has also shown that posterior interpositus neurons are equally involved in both delayed and trace conditioning paradigms and that the two functional types of neuron (A and B) described here could be related to the antagonist roles of the orbicularis oculi muscle (for closing the lids) and of the levator palpebrae muscle (for opening them). The same types of interpositus neuron were described previously in a study restricted to reflexively evoked eyelid blinks (Gruart and Delgado-García 1994).

A damping role for posterior interpositus neurons

As confirmed here with the Fourier analysis of eyelid acceleration profiles, a significant oscillation at ≈20 Hz was observed during CRs (Domingo et al. 1997). A surprising fact regarding the activity of these posterior interpositus neurons was the absence of oscillation, i.e., of a repetitive bursting firing in their discharge rate. Thus, their interspike time intervals were unimodal, and their autocorrelation functions showed no sinusoidal profiles. The absence of any noticeable oscillation in the firing rate of eyelid-related posterior interpositus neurons is in evident contrast with reports regarding the wavy form of eyelid CRs, formed in the cat by a succession of small waves at a dominant frequency of ≈20 Hz (Domingo et al. 1997). This oscillatory behavior is by no means an exclusive property of cat eyelid CRs, since, according to recent data, oscillation underlying reflex and/or conditioned responses has been recorded in up to five different species (Gruart et al. 2000). Thus we have to assume that this population of posterior interpositus neurons might play a role more related to the damping of the oscillation already observed in eyelid motor responses than to its generation. A role in this direction, i.e., the predominant involvement of interpositus neurons in the control of terminal tremor, has already been proposed from monkey studies (Thach et al. 1992) and apparently confirmed recently in dystonic rats (LeDoux et al. 1998). In fact, a neuronal oscillation at the same (≈20 Hz) dominant frequency
has been recorded in the facial nucleus (Magariños-Ascone et al. 1999; Trigo et al. 1999), motor cortex (Aou et al. 1992), and dentate nucleus (Gruart et al. 1993) of alert cats. Accordingly, it could be necessary to have a neural site taking charge of canceling an overt oscillation of the lids, mostly during the acquisition of a new motor ability. In this sense, the irregular firing and the wide range of latencies at which these neurons are recruited could contribute to their role as a neuronal damping device (Thach et al. 1992). According to the present results, muscimol injections did not produce an increase in eyelid oscillations, a fact that contradicts the putative role of interpositus nucleus as a damping device (Thach et al. 1992). Perhaps the low dose of muscimol used here did not allow us to evoke a significant eyelid oscillation during CRs (see Mason et al. 1998).

Functional properties of type A and B neurons

This study has supplied evidence indicating that the activity of posterior interpositus neurons is related to some aspects of the acquisition and/or performance of eyelid CRs, but not to the modality and/or properties of the sensory stimuli. Thus two different sensory modalities were used as a CS, but no functional difference was observed in the response of recorded neurons, other than their different activation time, probably related to the different latencies presented by air puff- and tone-evoked eyelid CRs (Gruart et al. 1995). Furthermore, Berthier and Moore (1990) have reported that deep cerebellar nuclear neurons do not contain specific frequency information regarding auditory stimuli, a fact confirmed for tone-evoked blinks in cats (Gruart and Delgado-García 1994). Moreover, the origin of signals present in posterior interpositus neurons cannot be a feedback from external cues, as it has been demonstrated recently that muscles involved in eyelid responses are devoid of typical proprioceptors and of a stretch reflex (see Trigo et al. 1999 for references). In addition, the response of these neurons was not strictly time-locked to CS presentation or to CR performance, suggesting a more diffuse generation system for the motor-learning signals present in their firing. In this sense, their activation could be related to attentive activation processes mainly present in advance of well-defined eyelid CRs (Allen et al. 1997).

Type A neurons increased their activity during the CR, but not when the cat kept its eyes open. This could be related to the facilitating role of interpositus neurons in flexor activities during movement involving inhibition of antigravitatory muscles (Armstrong and Edgley 1988; Bracha et al. 1999; Orlovsky 1972). Flexor activity is accomplished for the upper lid by the orbicularis oculi muscle, and interpositus nucleus neural signals could reach facial motoneurons by the red nucleus (Gonzalo-Ruiz and Leichnetz 1990). In the same line of thought, type B neurons decreased their firing during eyelid CRs, as already described for this type of neuron during reflexively evoked blinks (Gruart and Delgado-García 1994). Their putative role could be to disfacilitate the continuous antigravitatory action of the levator palpebrae muscle. It has been described that interpositus neurons project by the ascending limb of the posterior peduncle not only to the red nucleus, but also, among other structures, to the periculomotor area (Gonzalo-Ruiz and Leichnetz 1990). Accordingly, the role of type A neurons could be to facilitate the acquisition of an eyelid-closing response, and that of type B cells to disfacilitate the antagionic action of the neural mechanism active during alertness to maintain the eyes open. This opposing activity of type A and B posterior interpositus neurons raises an interesting question about the possible antagonistic organization of overlying Purkinje cell pools, a problem addressed recently using in vitro procedures (Schreurs et al. 1998).

Microstimulation and chemical inactivation of the posterior interpositus nucleus

The (opposite) effects of microstimulation and drug depression of the posterior interpositus nucleus reported here are further complementary proofs of the involvement of the posterior interpositus nucleus in eyelid motor learning. It is accepted generally that the chemical inactivation of the interpositus neurons with muscimol prevents the acquisition of eyelid CRs (see Kim and Thompson 1997 and Mauk 1997 for details). In present experiments, the CRs were already acquired when muscimol was applied. In this situation, a significant decrease (but not a complete abolition) of the CR was observed. A different susceptibility between rabbits (Garcia and Mauk 1998; Krupa et al. 1993) and cats (Bracha et al. 1999) to muscimol injections in cerebellar structures has recently been proposed to explain the different intensity in the evoked responses observed in the two species (Bracha et al. 1999). On the other hand, microstimulation of this deep cerebellar area after conditioning evoked an eyelid response resembling the profile of a CR, but which did not reach the amplitude of a normal CR. Nevertheless, the combined presentation of CS and microstimulation produced a significant increase in the amplitude of the evoked eyelid CR.

In summary, results obtained with microstimulation and muscimol infusions in the dorsal posterior interpositus nucleus further support data collected with unitary recordings in the same area, demonstrating a reinforcing role of interpositus neurons in the acquisition and performance of eyelid learned movements, but probably not in its initiation.

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