IN Volvement of cerebr al cortical structures in the classical conditioning of eyelid responses in rabbits

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Abstract—The classical conditioning of the eyelid motor system in alert behaving rabbits has been used to study the expression of Fos in the hippocampus, and in the occipital, parietal, piriform and temporal cortices. Animals were classically conditioned with both delay and trace conditioning paradigms. As conditioned stimulus, both short and long (20 and 100 ms) tones (600 Hz, 90 dB) or short, weak (20 ms, 1 kg/cm²) air puffs were used. The unconditioned stimulus was always a long, strong (100 ms, 3 kg/cm²) air puff that started 250–270 ms after the onset of the conditioned stimulus. The expression of Fos was significantly increased after both delayed and trace conditioning in the hippocampus, and in the parietal and piriform cortices contralateral to the unconditioned stimulus presentation side, compared with equivalent ipsilateral structures in conditioned animals, or with Fos production in the same contralateral structures in pseudo-conditioned and control animals. Fos expression in some cortical sites was specific to tone versus air puff stimuli when used as conditioned stimulus. Thus, Fos expression was significantly increased in the contralateral temporal lobe when tones were used as conditioned stimulus, for both delayed and trace conditioning paradigms, but not when animals were conditioned to short, weak air puffs.

The present results indicate a specific Fos activation in several cerebral cortical structures during associative eyelid conditioning.

Key words: Fos, hippocampus, nictitating membrane/eyelid responses, parietal cortex, piriform cortex, temporal cortex.

The nictitating membrane/eyelid motor response has been used for many years as a suitable experimental model for the study of the neuronal mechanisms involved in the acquisition of new motor abilities. 16,17,48,51,53,55 Nevertheless, the neural site where the learning of new eyelid conditioned responses (CRs) is taking place is currently a subject of intensive research and controversy. 3,6,26,33 Both cortical and deep nuclear cerebellar structures have been reported as the site where eyelid CRs are generated and stored. 8,26,28 Some particular differences have also been reported: for example, while delayed eyelid conditioning could take place in cerebellar structures, trace conditioning is apparently restricted to the hippocampus. 25,33 On the contrary, there is only fragmentary information about the involvement of large cerebral cortical areas in the acquisition of eyelid CRs, although some electrophysiological studies suggest the participation of, for example, the sensorimotor cortex in their generation. 3

The expression of the protein product of the proto-oncogene c-fos (Fos) was selected here as a useful tool to obtain a general survey of the involvement of several cerebral cortical structures in the acquisition of classically conditioned eyelid motor responses. The induction of c-fos in neural tissue is probably a necessary step for the acquisition of new motor abilities. 22,27,36,50 Thus, an increase in Fos production has been reported in the motor cortex, 27 cerebellum, 21 inferior olive 46 and other brainstem structures 8,24 during different sorts of motor learning, including the generation of new eyelid CRs. 21,24 In addition, the involvement of Fos-producing neurons has been extended to classical fear conditioning. 45

However, a similar description regarding Fos expression in cerebral cortical areas during classical conditioning of eyelid responses is still lacking.

Experiments were carried out on alert behaving rabbits. Eyelid displacements were recorded with the magnetic field technique. 9 The electromyographic (EMG) activity of the ipsilateral orbicularis oculi muscle was also recorded. Fos expression was studied during both delay and trace classical conditioning paradigms. Different sensory modalities (tone, air puff) of conditioning stimulus (CS) were used. The unconditioned stimulus (US) was always a long, strong air puff. The relative involvement of cerebral cortical structures in the acquisition of classically conditioned eyelid motor responses depending on the conditioning paradigm (delay, trace), the CS modality (tone, air puff), and the profile and kinematics of evoked CRs was compared.

EXPERIMENTAL PROCEDURES

Subjects

Experiments were carried out on 26 adult rabbits (New Zealand White albino) weighing 2.2–2.8 kg obtained from an authorized supplier (Iffa–Credo, France). Animals were prepared for the chronic recording of upper eyelid movements and of the EMG activity of the orbicularis oculi muscle. Experiments were carried out in accordance with European Union Council (86/609/EU) guidelines and following Spanish regulations (BOE 67/8509-12, 1998) for the use of laboratory animals in chronic experiments.

Pre-experimental surgical procedures

The procedures used have been described in detail elsewhere. 18,19 In brief, animals were anesthetized with a cocktail of ketamine (35–50 mg/kg), acepromazine (0.3 mg/kg) and xylazine (5 mg/kg) i.m., following an injection of atropine sulfate (0.4 mg/kg, i.m.) to prevent vagal reflexes. As an additional protection, mepivacaine hydrochloride (2%) was injected into wound margins. A five-turn coil (3 mm in diameter) was implanted in the center of the left upper eyelid at...
about 2 mm from the lid margin. Coils were made of 50 μm, Teflon-coated stainless steel wire (A-M Systems). Animals were also implanted with bipolar hook electrodes in the left orbicularis oculi muscle. Hook electrodes were made of the same wire as the coils, and bared about 1 mm at their tips. A bare silver electrode (1 mm in diameter) was anchored to the skull as a ground. The terminals of eyelid coils and EMG and ground electrodes were soldered to a socket. The whole system was attached to the skull with the aid of surgical screws fastened to the bone and covered with acrylic resin.

**Recording of eyelid movements and electromyographic activity of the orbicularis oculi muscle**

Eyelid movements were chronically recorded with the magnetic search coil technique. Maximum angular displacement of the upper eyelid ranged from 35° to 46° for the 26 animals. For the sake of homogeneity, the gain of the coils was set at 1 V  \hat{=}  720 μV, the EMG of homogeneity, the gain of the coils was set at 1 V  \hat{=}  10 kHz. The EMG activity of the orbicularis oculi muscle was recorded with the help of differential amplifiers, at a bandwidth of 10 Hz to 10 kHz.

**Conditioning paradigms**

Classical conditioning of eyelid responses was achieved using either delay or trace conditioning paradigms. As illustrated in Fig. 1C, three different experimental groups were used. (a) For Delayed Tone–Air Puff conditioning (n  =  5 animals), a 350 ms, 600 Hz, 90 dB tone was presented to the animal as CS. The tone was followed 250 ms from its onset by a 100 ms, 3 kg/cm² puff of air directed at the left cornea as US. Thus, the tone and the air puff co-terminated. (b) For Trace Tone–Air Puff conditioning (n  =  5), a 20 ms, 600 Hz, 90 dB tone was presented to the animal as CS. This tone was followed 250 ms from its end by the same US. (c) For Trace Air Puff–Air Puff conditioning (n  =  5), a 20 ms, 1 kg/cm² air puff was presented as CS to the left cornea. The CS was followed 250 ms from its end by the US indicated above.

Conditioning sessions always consisted of 66 trials. Successive trials were separated at random by intervals of 50–70 s. Six of the 66 trials were test trials in which the CS was presented alone. The conditioning session lasted for about 80 min, and each animal was trained for six successive days. Criterion for an eyelid movement to be considered a CR was set at 2° of eyelid downward movement that started in a time window within 50–300 ms from start of the CS (Fig. 1B). The two preceding habituation sessions consisted of the sole presentation of the corresponding stimulus selected as CS. Pseudo-conditioning sessions consisted of 66 trials separated at random by intervals of 50–70 s. For each trial, the corresponding CS (d: the same tone as for a; e: the same tone as for b; and f: the same air puff as for c) was presented unpaired in relation to the US (the same as for a–c), with the restriction that no more than two CS or US trials occurred sequentially.

For pseudo-conditioning, three other groups of animals (labeled d, e and f; see Fig. 1D) were made with three animals/group. These animals were pseudo-conditioned with the unpaired presentations of CS and US used for classical conditioning of eyelid responses in groups a, b and c, respectively, receiving the same total number of stimuli. The unpaired stimulus presentations were carried out with a random delay, with the restriction that no more than two stimuli of the same modality and strength could be presented successively. The total training time for pseudo-conditioning was the same as for conditioning.
followed. Thirty minutes after the end of the sixth conditioning session, all the animals conditioned with this paradigm reached criterion. Kinematics of eyelid conditioned responses

For co-expression of the Fos and calretinin, sections were first processed for Fos immunoreactivity following the described protocol. The color reaction was developed with the addition of nickel ammonium sulfate (0.15%) to the diaminobenzidine/hydrogen peroxide solution. The Fos-positive nuclei appeared filled with black precipitate. Then, the second immunohistochemistry with anti-calretinin antibody (1:8000; SWant, Bellinzona, Switzerland) was developed following the same protocol, but using only 0.01% diaminobenzidine as color-developing solution, obtaining a brown stained cytoplasm in calretinin-positive neurons (Fig. 4d, e).

Serial sections of cortical structures were examined under a light microscope by an observer who was blind to the different experimental conditions (control, conditioned, pseudo-conditioned and US presentation side). Fos-like immunoreactive cells were mapped with camera lucida drawings and selected sections were photographed (Fig. 2C). Immunoreactive cells located inside a 1 mm x 1 mm grid applied over the selected cerebral cortical sites were counted up to 20 times in the different animals from each experimental group. The grid was divided into 100 μm x 100 μm squares to facilitate cell counting (see Fig. 2).

Quantification and data analysis

The horizontal and vertical positions of the upper eyelid, the EMG activity of the orbicularis oculi muscle and 1 V rectangular pulses corresponding to CSs and USs presented during conditioning and pseudo-conditioning sessions were stored digitally on a videotape recording system. Data were transferred to a CED 1401-plus analog digital converter for quantitative off-line analysis. Data were sampled at 1000–2000 Hz, with an amplitude resolution of 12 bits.

Commercial computer programs (SIGAVG, Corel Draw) were used to display single representations of eyelid position and velocity, and of the EMG activity of the orbicularis oculi muscle. Velocity traces were computed digitally as the first derivative of eyelid position records, following low-pass filtering of the data (~3 dB cut-off at 50 Hz and zero gain at 100 Hz).

Statistical analyses were carried out with the SPSS for Windows package. Statistical differences between parametric values of conditioned responses were determined by repeated measures of ANOVA. Mean values for onset latency, duration, maximum amplitude and peak velocity of CRs were calculated from 100 measurements collected from the five animals of each group. Mean values for the number of Fos-positive nuclei per mm² were compared for conditioned (both ipsi- and contralateral cerebral cortex sides) and pseudo-conditioned (only contralateral side) animals. For each group, mean and standard deviation (mean ± S.D.) values were obtained from 20 different measurements from all the selected areas of the experimental subjects: n = 15 conditioned, n = 9 pseudo-conditioned and n = 2 controls. The statistical differences between groups were compared using a two-way (conditioning paradigm by side) analysis of variance (MANOVA) and contrast analyses. The level of significance established was P = 0.01.

RESULTS

Kinematics of eyelid conditioned responses

Learning curves for conditioned and pseudo-conditioned animals for the three conditioning paradigms used in this study are illustrated in Fig. 3. In the five animals conditioned with the Delayed Tone–Air Puff paradigm (Fig. 3A), conditioned responses reaching criterion appeared by the second conditioning session. A typical eyelid CR consisted of a wavy, ramp-like downward eyelid movement that reached its maximum amplitude by US presentation. By the sixth conditioning session, all the animals conditioned with this paradigm had reached criterion, set in this case at 95%
of CRs per session (Fig. 1B). Once the animals were consistently conditioned, they showed eyelid CRs integrated with the subsequent unconditioned response (Fig. 1B). The three pseudo-conditioned animals reached only 1–2% of eyelid CRs by the sixth conditioning session (Fig. 3A). Results obtained for the Trace Tone–Air Puff paradigm were similar, although in this case only one animal (no. 25) reached 85% of eyelid CRs by the sixth conditioning session (Fig. 3B). Pseudo-conditioned animals (n = 5) trained for this paradigm reached 0–3% of eyelid CRs during the sixth session (Fig. 3B). Finally, animals (n = 5) trained with the trace Air Puff–Air Puff paradigm reached criterion by the fifth or sixth conditioning sessions (Fig. 3C). Pseudo-conditioned animals (n = 3) trained for this paradigm reached 3–5% of eyelid CRs during the sixth pseudo-conditioning session (Fig. 3C). For the three paradigms used here, a significant difference (P < 0.001 at least) was observed in the percentage of eyelid CRs obtained during conditioned versus pseudo-conditioned animals, for all training sessions except the first.

The only significant difference in the parametric properties of eyelid CRs evoked during the three conditioning paradigms was related to their latency and duration. Mean latency for CR onset across the conditioning sessions decreased from 211 ± 15 to 118 ± 16 ms (n = 5 animals) for the Delayed Tone–Air Puff conditioning paradigm. A similar evolution followed the latency of eyelid CRs evoked during the Trace Tone–Air Puff paradigm. However, although mean latency of eyelid CRs evoked during Trace Air Puff–Air Puff decreased from 233 ± 31 ms during the first session to 205 ± 13 ms during the sixth, their values were significantly larger (P < 0.01 at the least) for sessions 4–6 than those obtained for delayed Tone–Air Puff and Trace Tone–Air Puff conditioning paradigms. The duration of eyelid CRs increased significantly (≥310%, P < 0.01) across conditioning sessions. However, in this case again, the mean duration of CRs for Delayed Tone–Air Puff (150 ± 20 ms) and Trace Tone–Air Puff (141 ± 18 ms) conditioning paradigms during the sixth conditioning session were significantly larger (P < 0.01 at least) than that obtained during the Trace Air Puff–Air Puff paradigm (53 ± 6 ms). Both maximum amplitude (≥1.800%) and peak velocity (≥255%) of eyelid CRs increased significantly (P < 0.01) across sessions for the three conditioning paradigms used here, but no significant differences were observed for CR values obtained with each of them.

**Fos expression in selected cerebral cortical sites after classical conditioning of eyelid blinks**

In Fig. 4 is illustrated a significant example of Fos production in the parietal cortex after conditioning with a Trace Air Puff–Air Puff paradigm. It is clear that more Fos-producing neurons were labeled in the contralateral parietal cortex than on the ipsilateral side (Fig. 4a, b). For comparison, the contralateral parietal cortex of an animal pseudo-conditioned with the same paradigm is also shown (Fig. 4c), indicating that almost no Fos expression was produced by the unpaired presentation of stimuli used as CS and US for this paradigm. Double immunohistochemistry for Fos and calretinin is illustrated in Fig. 4d. According to quantitative analyses, a total of 42.3% of the labeled neurons co-expressed both proteins, while 24.7% expressed Fos and 33.0% expressed calretinin. From the shapes of double-labeled neurons and from their location, according to Cresyl Violet and calretinin stainings (Fig. 4e), Fos-expressing neurons seemed to be non-pyramidal neurons located preferentially in layers II and III of the parietal cortex. In this regard, it has been reported that the calcium-binding protein calretinin labels specific subpopulations of non-spiny non-pyramidal cells.

Fos expression in selected cerebral cortical structures during the Delayed Tone–Air Puff paradigm is illustrated in Fig. 5. After conditioning, Fos production was observed to be increased by 2.5–6 times in the contralateral parietal, temporal and piriform cortices (Fig. 5a, d, g) compared with the corresponding cortical structures of the ipsilateral side (Fig. 5b, e, h). In contrast, the occipital cortex was scarcely labeled on both sides (not illustrated). Most of the Fos-producing neurons were located in layers II and III, as illustrated by Cresyl Violet staining of corresponding areas of the contralateral cortices (Fig. 5c, f, i). Fos expression in the contralateral hippocampus was also greater but, in this case, neurons were located in the pyramidal layer (Fig. 5j, k, l).

For quantitative purposes, definite areas of parietal, temporal, piriform, occipital and hippocampal cortices were selected. The selected parietal area was the lateral ventral part
of somatic sensory area I (Fig. 2A), corresponding to the area postcentralis. The selected temporal area was the lateral surface of the temporal cortex caudal to the genu of the rhinal sulcus, corresponding to the area temporalis. The piriform cortex where Fos production was quantified corresponded to the regio praepiriformis rostral to the fissura rinalis (Fig. 2), in the vicinity of the lateral olfactory tract. Neurons producing Fos were also quantified in the visual area I, corresponding to the area occipitalis mono- and binocular zones. Finally, cell counts were also carried out in the dorsal and posterior hippocampus.

The quantitative analysis of results obtained for Fos expression during the Delayed Tone–Air Puff paradigm is shown in Fig. 8A. Fos production was significantly increased (P < 0.01 at least) in contralateral parietal, temporal and piriform cortices, and in the hippocampus, with respect to the number of Fos-expressing neurons located in the corresponding areas of the ipsilateral cortices, and to the same areas of the contralateral cortices in pseudo-conditioned animals. The number of Fos-expressing neurons in the hippocampus was significantly lower (P < 0.001) than in the parietal, temporal and piriform cortices.

The number of neurons expressing Fos in the selected cerebral cortical areas during the Trace Tone–Air Puff paradigm is illustrated in Fig. 6. After six days of conditioning, Fos production was observed to be increased in the contralateral parietal, temporal and piriform cortices (Fig. 6a, d, g) compared with the corresponding cortical structures on the ipsilateral side (Fig. 6b, e, h). In contrast, the occipital cortex was nearly unlabeled (not illustrated). For this paradigm, again most of the Fos-producing neurons were located in layers II and III (Fig. 6c, f, i). Fos expression in the contralateral hippocampus was restricted to pyramidal cells and was significantly greater (P < 0.01) than on the ipsilateral side (Fig. 6j, k, l). A quantitative analysis of results obtained for Fos expression during the Trace Tone–Air Puff paradigm is shown in Fig. 8B. Fos production was significantly increased (P < 0.01 at least) in contralateral parietal, temporal and piriform cortices with respect to the number of Fos-expressing neurons located in the corresponding areas of the ipsilateral cortices, and to the same areas of the contralateral cortices in pseudo-conditioned animals. The occipital cortex was almost unlabeled for Fos-expressing neurons on either the ipsi- or contralateral side, for both conditioned and pseudo-conditioned animals.

The expression of Fos in selected cerebral cortical structures during the Trace Air Puff–Air Puff paradigm is illustrated in Fig. 7. After conditioning, Fos production was observed to be significantly increased (P < 0.01 at least) in the contralateral parietal and piriform cortices (Figs 7a, d, g, 8C) compared with the corresponding cortical structures of the ipsilateral side (Figs 7b, e, h, 8C). In contrast, no significant difference was observed between the ipsi- and contralateral sides in the temporal and occipital cortices. However, Fos production on both temporal sides in conditioned animals was significantly (P < 0.01) larger than corresponding values.
in pseudo-conditioned animals. Fos-producing neurons were also preferentially located in layers II and III (Fig. 7c, f, i). Hippocampal pyramidal cells produced a greater Fos expression on the contralateral side compared with the ipsilateral side in conditioned animals and the contralateral side in pseudo-conditioned ones (Figs 7j, k, l, 8C).
Fig. 6. Fos expression in the parietal cortex (PCx; a–c), temporal cortex (TCx; d–f), piriform cortex (PfCx; g–i) and hippocampus (HCx; j–l) following Trace Tone–Air Puff conditioning in a representative animal (no. 17). Note that a, d, g and j correspond to contralateral (c) sections, and b, e, h and k to ipsilateral (i) ones with respect to the US presentation side. Also note that c, f, i and l correspond to contralateral counterstained sections. Abbreviations: fi, fimbria; lo, lateral olfactory tract; ps, pial surface; PY, pyramidal layer; I–IV, cortical layers. Scale bar = 100 μm.
A contrast analysis applied to quantitative data obtained for the three conditioning paradigms (Delayed Tone–Air Puff, Trace Tone–Air Puff and Trace Air Puff–Air Puff) showed no significant difference in the number of Fos-positive neurons in the parietal, occipital, piriform and hippocampal cortices contralateral to the US presentation side. Moreover,
no significant difference was observed in the number of neurons producing Fos in ipsilateral structures for the three conditioning paradigms. Those animals conditioned with the Delayed Tone–Air Puff (n = 5) and Trace Tone–Air Puff (n = 5) paradigms also showed the same number of neurons expressing Fos in the temporal cortex, but in both cases these numbers were significantly (P < 0.01) greater than values obtained from those animals (n = 5) conditioned with the Trace Air Puff–Air Puff paradigm (see Fig. 8).

No significant relationship could be established between the number of Fos-producing neurons and eyelid CR parameters. Thus, Fos production in the contralateral cortex was equal for the three conditioning paradigms, even considering that there were significant differences in the latency and duration of eyelid CRs. These differences were noticed when values obtained for the Delayed Tone–Air Puff and Trace Tone–Air Puff paradigms were compared with the corresponding values obtained during the Trace Air Puff–Air Puff paradigms. No significant relationship could be established between the percentage of eyelid CRs reached by each animal during the sixth conditioning session and the number of Fos-producing neurons located in any of the cortical areas included in this study. For example, animal no. 25 was slightly below criterion (85%) by the sixth conditioning session of the Trace Tone–Air Puff paradigm, but no statistically significant difference was noticed in Fos production in any structure compared with the corresponding values obtained for the other animals (n = 4) of its group.

**DISCUSSION**

The main result presented here confirms the involvement of large areas of somatosensory, motor, auditory and piriform cortices, and of the hippocampus, in both delay and trace conditioning of eyelid motor responses. The fact that contralateral (right side) cerebral structures presented higher levels of Fos expression is in good agreement with the very noticeable CR performance on the side (left) of US presentation.17–19 Early studies have reported an increase in the expression of either *c-fos* or Fos during the acquisition of classically conditioned nictitating membrane responses in trigeminal and reticular structures,24 the locus coeruleus,8 cerebellum21 and medial geniculate nucleus.33 The present results extend the participation of this early gene in the cellular mechanisms of
cerebral cortical structures activated during the expression of eyelid CRs. The involvement of cortical structures in eyelid CRs has previously been substantiated during electrophysiological recordings of unitary activity in the cat motor cortex and with positron emission tomography scanning in humans. These results further confirm the distributed nature of this type of motor learning, in which other structures, such as the brainstem reticular formation or the cerebellum, have been classically involved. Nevertheless, it should be pointed out that the present results cannot be considered as the secondary effect of cerebellar cortex and/or nuclei activation, since cerebellar projections to the cerebral cortex involve different areas than the Fos-expressing areas reported here.

Fos expression in some cortical sites was specific to tone versus air puff stimuli when used as a CS. Thus, the temporal cortex was significantly labeled with Fos-immunoreactive product for tone, but not for air puff, presentations as CS. Moreover, Fos expression was significantly increased in the contralateral parietal cortex for the three paradigms used here, suggesting an important role of the somatosensory and motor cortices in the formation of the eyelid CR regardless of the sensory modality of the stimulus used as a CS. No significant differences were observed in any of the structures included in this study for delay and trace classical conditioning paradigms. Although it is well known that the sensory cortex is required for acquisition of new motor skills, no differences could be established between Fos expression in the motor and/or somatosensory cortex and the actual performance (regarding different latency and profiles) of delayed and trace CRs.

The availability of data for control cortical structures in conditioned animals reinforces the significance of the results. Thus, no noticeable Fos expression was observed in the occipital cortex in conditioned animals. This negative expression was not the result of the inability of occipital cortical neurons to express Fos, as they have been shown to express this protein to light presentation in rats during conditioned and unconditioned aversive stimuli. Except for the piriform cortex, the basal expression of Fos in the other studied structures seemed to be rather low. Moreover, even the long, strong air puff used here as US was unable to increase the expression of Fos in pseudo-conditioned animals. As reported already, very strong peripheral stimuli are needed to evoke Fos expression in cortical structures.

**Increase in Fos expression during classical conditioning of eyelid responses**

The expression of the proto-oncogene *c-fos* in neurons is related to neuronal stimulation by many different sorts of natural and experimental stimuli, including growth factors and neurotransmitters. The *c-fos* product (Fos) is a nuclear phosphoprotein with non-specific and sequence-specific DNA binding properties. Fos apparently acts as a third messenger phosphoprotein with non-specific and sequence-specific DNA binding properties. As reported already, very strong peripheral stimuli are needed to evoke Fos expression in cortical structures.

In order to avoid unwanted Fos-like immunoreactivity, the antibody used here was very specific, as proved with results obtained in naïve and pseudo-conditioned animals.

However, neural activity per se may not be a sufficient stimulus for Fos activation to occur. As shown here, unpaired CS and US presentation during pseudo-conditioning should modify the neuronal activity in related cerebral cortical areas, but a significant increase in Fos production was not observed. It has also been shown that neural depolarization is not always able to activate *c-fos* transcription, and that the activity of hippocampal pyramidal and non-pyramidal cells during classical conditioning of the eyelid response is increased only during CS–US presentation. Since CS–US pairs were presented only 66 times/day, and CS–US intervals lasted only for a few tenths of a second, increased firing activity could not be the trigger for *c-fos* expression. Indeed, long-term potentiation can be induced in granule cells of the dentate gyrus without further induction of *c-fos*. Accordingly, Fos production could be related to more specific neural signals, such as coincident Hebbian interactions between the CS and the US.

Fos production was checked here after two days of habituation and six days of conditioning. In this way, the effects of novelty on the somatosensory and auditory cortices were avoided. Moreover, since no significant Fos expression was observed in pseudo-conditioned animals, the present results cannot be ascribed to the stress produced by the experimental situation.

The *c-fos* gene and/or its protein product have been related to many different behavioral situations, such as aversive and fear conditioning, self-stimulation, intracerebral injection of vasopressin, maternal behavior and aging. Such a diversity of roles, including the one described here related to the acquisition of new motor skills, makes Fos an excellent tool for studying the involvement of large populations of neurons in their phenotypic adaptation to new environmental situations. In particular, the cortical lesion increases Fos production, suggesting that regeneration processes and plastic mechanisms involved in motor learning could share some cellular mechanisms.

**Production of Fos in specific cortical sites**

In agreement with the data reported here, Fos expression has been shown to increase in the motor and/or somatosensory cortices during different experimental situations, such as repeated whisker stimulation, performance of an escape task, motor skill learning and aversive conditioning. The opposite, i.e., a decrease in Fos expression, has been reported in the same areas following trigeminal nerve section. As shown here during the acquisition of eyelid CRs, labeled neurons have been reported to be located in layers II and III, mostly involving stellate cells. This proposal is supported here by the fact that calretinin-positive neurons are specifically non-spiny, non-pyramidal cells.

Fos expression in the piriform cortex during the acquisition of eyelid CRs was rather surprising. Indeed, besides its well-known role in the processing of olfactory information, the piriform cortex has been related to different learning and behavioral situations, such as memory processing, fear conditioning and maternal care. The results reported...
here cannot be ascribed to non-specific Fos-like immunoreactivity, or to olfactory signals, as the expression was greater on the side contralateral to the US presentation, and to the novelty of the stimulus used as CS. In this sense, a temporal involvement of the hippocampal cortex across learning could be expected.

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