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The Use of Alert Behaving Mice in the Study of Learning and Memory Processes

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The availability of transgenic mice that mimic human neurodegenerative processes has made it necessary to develop new recording and stimulating techniques capable of being applied in this species. We have studied here the motor learning and memory capabilities of wild-type and transgenic mice with deficits in cognitive functions, using classical conditioning procedures. We have developed an electrical shock/SHOCK paradigm corresponding to a *trace* classical conditioning; that is, a learning task involving the cerebral cortex, including the hippocampus. The conditioning procedure is a modification of the air-puff/AIR-PUFF conditioning (Gruart *et al.*, *J. Neurophysiol.* 74:226, 1995). Animals were implanted with stimulating electrodes in the supraorbitary branch of the trigeminal nerve and with recording electrodes in the orbicularis oculi muscle. Computer programs were developed to quantify the appearance and evolution of eyelid conditioned responses. According to the present results, the classical conditioning of eyelid responses appears to be a suitable (associative) learning procedure to study learning capabilities in genetically-modified mice.

Keywords: Classical conditioning; Mice; Orbicularis oculi muscle; Trace conditioning

INTRODUCTION

Functions and properties of the central nervous system (mostly with reference to learning and memory capabilities) should be studied in awake, freely moving animals; i.e., animals in true physiological conditions (Gruart *et al.*, 2000c; Delgado-García and Gruart, 2002). Since 1990, our group has developed stimulating and recording techniques allowing the study of neural processes underlying associative learning in conscious mammals. These techniques include original procedures for the recording of reflex and learned eyelid responses, for classical conditioning of alert behaving cats and rabbits (Marshall-Goodell *et al.*, 1992), and for *in vivo* electrophysiological recordings of neuronal activity during the learning process (see Delgado-García and Gruart, 2002).

Nevertheless, the availability of transgenic mice that mimic human neurodegenerative processes, as in Alzheimer's disease (AD) (see Moechars *et al.*, 1999; Selkoe, 2002) has made it necessary to develop new recording and stimulating techniques capable of being applied in wild-type, knock-out, and transgenic mice (Vogel *et al.*, 2002; Chen A *et al.*, 2003). It is important to point out here that classical conditioning of eyelid responses appears to be a more suitable (associative) learning procedure for mice than does the usual water maze test (see, for example, Chen G *et al.*, 2000), above all because mice are terrestrial animals. Specifically, we have studied here the motor learning and memory capabilities of wild-type and of transgenic mice with deficits in cognitive functions, using classical conditioning procedures. For the implementation of this aim, the classical conditioning of the eyelid response, a well-known conditioning technique widely used in rabbits (Gruart *et al.*, 2000b,c) and cats (Gruart *et al.*, 1995; Domingo *et al.*, 1997; Trigo *et al.*, 1999; Delgado-García and Gruart, 2002), was adapted to mice.

For these experiments, we developed an "electrical shock/SHOCK" conditioning paradigm corresponding to a *trace* classical conditioning; that is, a learning task

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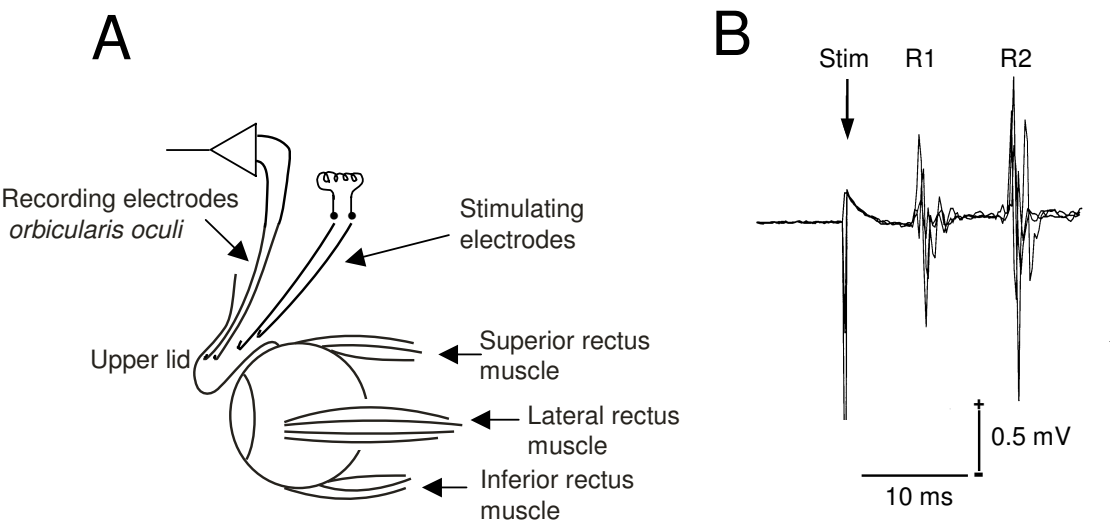

Figure 1 Diagrams illustrating the experimental design. **A.** Location of recording and stimulating electrodes. A pair of 50-µm Teflon-insulated stainless steel wires was implanted in the upper eyelid to record the electromyographic (EMG) activity of the orbicularis oculi muscle. Another pair of the same wire electrodes was implanted near the supraorbitary branch of the trigeminal nerve for stimulation purposes. **B.** Example of a reflex eyelid response evoked by the electrical stimulation (Stim.) of the (ipsilateral) supraorbitary branch of the trigeminal nerve. The stimulus consisted of a 50 µs, square cathodal pulse presented at a rate of 0.1 per second. Three traces are superimposed to illustrate the latency stability of the evoked EMG responses. Note the early (R1) and late (R2) components of the evoked reflex eyelid response. Calibrations as indicated.

involving the cerebral cortex, including the hippocampus (Múnera *et al.*, 2000; 2001). The "electrical shock/SHOCK" is a modification of a method also developed by our group namely, the "air-puff/AIR-PUFF" conditioning (Gruart *et al.*, 1995; 2000a). In both procedures the conditioned stimulus (CS) consists of a weak puff of air applied to the cornea/eyelid surface (or a mild electrical shock applied to the supraorbitary branch of the trigeminal nerve), and is followed 250 ms later by a strong puff of air (or a supra-threshold electrical shock also applied to the trigeminal nerve), as unconditioned stimulus (US). For comparative purposes, we have also developed a delay paradigm, in which the CS consists of a tone presented for 250 ms. The tone co-terminates with the electric shock (as described above) used as US. The main difference between trace and delay paradigms is that in the former the CS finishes before US presentation, while in the latter the CS co-terminates with the US, obviously trace conditioning can be achieved using stimuli of the same sensory modality (as those shock/SHOCK or air-puff/AIR-PUFF described here), while for delay conditioning it is necessary to use stimulus of different sensory modality.

To study learning evolution across conditioning sessions we recorded the electromyographic (EMG) activity of the orbicularis oculi muscle. This is also a standard procedure in classical conditioning of eyelid responses (Gruart *et al.*, 1995; 2000c).

The aims of this study were as follows: i) To develop classical conditioning techniques for application in wild-type and transgenic mice; and, ii) To study the learning capabilities of wild-type mice using the classical conditioning of eyelid responses and to compare the results with those obtained from transgenic mice over-expressing the human amyloid precursor protein (APP695). Transgenic mice bear a mutation found in familial forms of AD (V642I, "London" mutation) driven by the Thy-1 promoter in a C57Bl/6 background (hereafter C57 APPLd2 mice).

DETAILS OF THE EXPERIMENTAL PREPARATION

Animals

Male 3-month-old C57BL/6 and Swiss mice were obtained from the Animal House of Granada University (Granada, Spain). Male 3-, 10-, and 12-month-old C57 APPLd2 transgenic mice were obtained from Aventis Pharma Recherche-Developpement, France. Animals were divided in five groups: i) 3-month-old, wild-type Swiss mice (n = 10); ii) 3-month-old, wild-type C57BL/6 mice (n = 14); iii) 3-month-old C57 APPLd2 mice (n = 10); and iv) 10-month-old C57 APPLd2 mice (n = 9).

After their arrival, the animals were kept in the Animal House facilities of the Pablo de Olavide University for a week before surgery, in collective

cages ($n \leq 20$ per cage) with free access to regular rat diet (Panlab, S.L., Cornellá, Barcelona, Spain) and water. The animal house was maintained at an ambient temperature of 23 ± 2ºC, with a humidity of 50 ± 7%.

Behavioral studies were conducted in accordance with the guidelines of the European Union Council (86/609/EU) and following Spanish regulations (BOE 67/8509-12, 1988) for the use of laboratory animals in chronic experiments. All the experiments described here, were previously approved by the local Institutional Committee, for animal care and handling.

Surgery

A week after their arrival, animals were anesthetized with a mixture of Ketolar (ketamine, 35 mg/kg) and Rompum (xylazine, 2 mg/kg, i.p.). They were implanted with bipolar stimulating electrodes on the left supraorbitary branch of the trigeminal nerve and with bipolar recording electrodes in the ipsilateral orbicularis oculi muscle. Electrodes were made of 50 µm, Teflon-insulated, annealed stainless steel wire (A-M Systems, Carlsborg, WA-98324, USA). Tips of both stimulating and recording electrodes were cleansed of the isolating cover by ~1 mm. Electrode tips were bent as a hook to facilitate their permanent implantation in the upper eyelid. Wires were connected to a 4-pin socket (RS-Amidata, Madrid, Spain). As a final step, the skin over the cranium was dissected out and the socket was fixed to the skull with the help of two small screws and dental cement (see FIG. 1A).

Conditioning Sessions

For recordings, the animal was placed in a small (5 x 5 x 10 cm) plastic chamber situated inside a Faraday box. The animal was not further immobilized inside the plastic chamber. A small hole in the chamber top allowed the passage of a 4-strand cable connecting the animal to the recording/stimulating system.

Classical conditioning was achieved using trace and delay paradigms. The trace conditioning procedure consisted of a single, square, cathodal pulse (50 µs; 1.5 x threshold) as CS, followed 250 ms later by a second longer, stronger pulse (500 µs; 2-3 x threshold) as US. Both stimuli were applied to the supraorbitary branch of the trigeminal nerve. For delay conditioning, a 2400 Hz, 70-90 dB, 250 ms tone was presented as a CS. At the end of the tone, a long (500 µs), strong (2-3 threshold), square, cathodal pulse was presented as US. The electrical shock used as US co-terminated with the tone used as a CS.

Electrical stimulation was carried out with the help of a CS-20 stimulator across an isolation unit (Cibertec, S.A., Madrid, Spain). Acoustic stimulation was delivered from a loudspeaker located 20 cm in front of the experimental animal. Tones were generated with the help of a SG503 sine-wave generator (Tektronix, Beaverton, OR 97077, USA.).

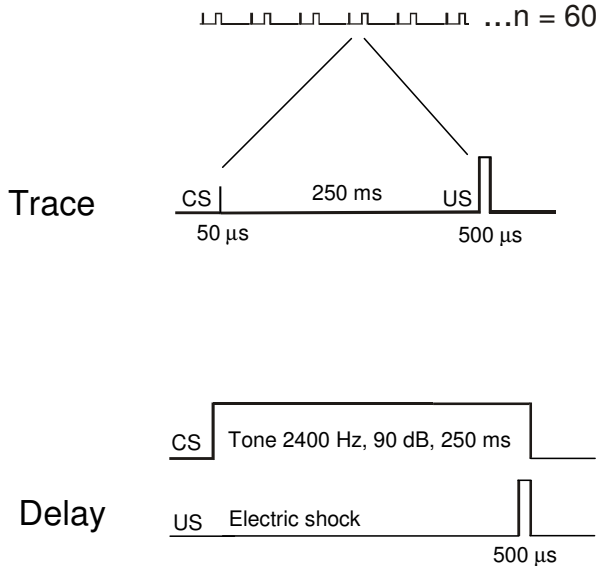

Figure 2 Experimental design for the classical conditioning of eyelid responses. Conditioning sessions lasted for ~30 min and consisted of the repeated presentation ($n = 60$) of pairs of conditioned (CS) and unconditioned (US) stimulus (see top part of the figure). The two different conditioning paradigms used here are illustrated in the bottom part of the figure. For trace conditioning, a short (50 µs), weak (1.5 x threshold), square, cathodal pulse was presented as a CS, followed 250 ms later by a long (500 µs), strong (2-3 x threshold), square, cathodal pulse presented as a US. For delay conditioning a 2400 Hz, 70-90 dB, 250 ms tone was presented as a CS. At the end of the tone, a long (500 µs), strong (2-3 x threshold), square, cathodal pulse was presented as a US. The electrical shock used as US co-terminated with the tone used as CS.

A total of two habituation, 10 conditioning, and 4 extinction sessions were carried out per animal. A conditioning session consisted of the paired CS-US presentation, for 60 times, and lasted ~ 30 min. CS-US presentations were separated at random by 30 ± 5 s. For habituation and extinction sessions, only the CS was presented, also for 60 times per session and at intervals of 30 ± 5s. The recording set-up allowed the simultaneous conditioning of up to 4 animals (see FIGs. 2, 3A).

Recording Procedures

The EMG activity of the orbicularis oculi muscle was recorded with GRASS P511 differential amplifiers with a bandwidth of 1 Hz to 10 kHz (Grass-Telefactor, West Warwick, RI 02893, USA). Data were stored directly on the computer with the help of the Signal Average Program of Cambridge Instruments (Cambridge,

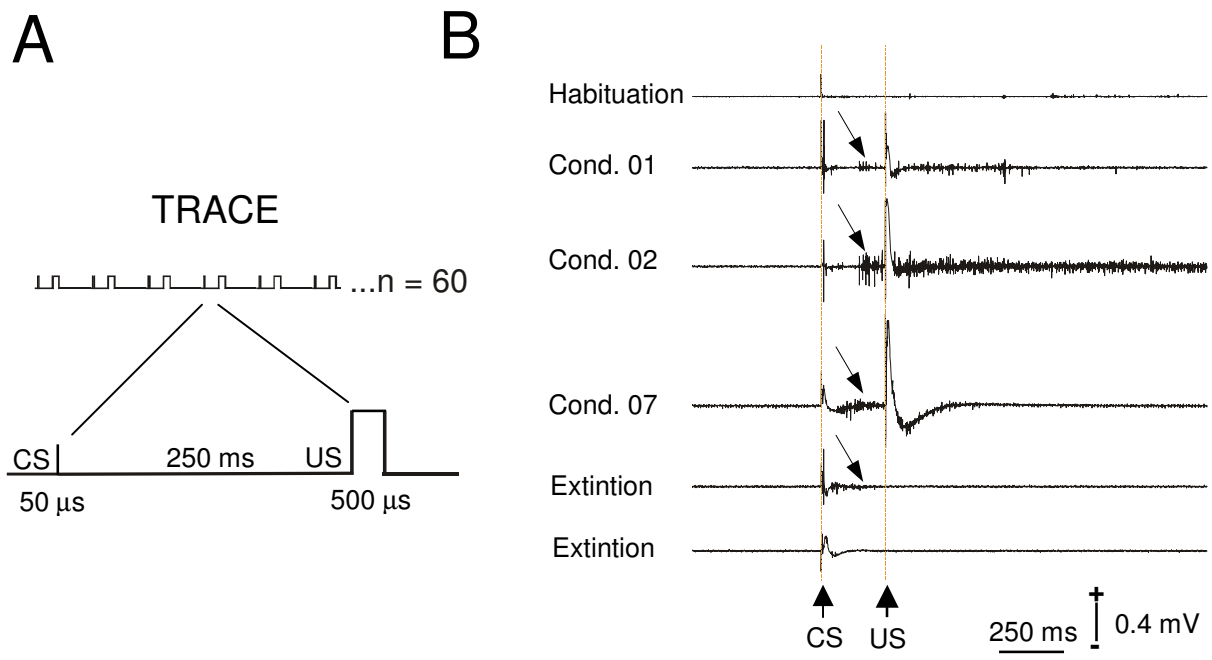

Figure 3 Example of classical conditioning of eyelid responses in a wild-type C57 mouse, using a trace-conditioning paradigm. **A.** Conditioning consisted of the repeated presentation ($n = 60$) of pairs of conditioned (CS) and unconditioned (US) stimulus with a trace paradigm. For conditioning, a short (50 µs), weak (1.5 threshold), square, cathodal pulse was presented as a CS, followed 250 ms later by a long (500 µs), strong (2-3 threshold), square, cathodal pulse presented as a US. A total of two habituation, 10 conditioning, and four extinction sessions were carried out. For habituation and extinction, only the stimulus used as a CS was presented. **B.** Samples of recordings made of the electromyographic (EMG) activity of the orbicularis oculi muscle. Note the appearance of identifiable conditioned responses (arrows) in response to CS presentation during the conditioning (Cond.) sessions, and their disappearance during the extinction sessions. The number of the conditioning session is indicated. Arrowheads indicate the presentation of the CS and US, respectively. Calibration as indicated.

England). Data were analyzed off-line for quantification of conditioned responses. We considered a "conditioned response" the presence of EMG activity during the CS-US period with the following conditions: i) the EMG activity lasted > 10 ms; ii) the EMG was not preceded by any spontaneous activity; iii) the EMG activity was initiated > 30 ms after CS onset; and iv) the EMG activity was as least 2.5 times larger than the activity recorded immediately before CS presentation.

Data Presentation and Analysis

Data were quantified as percentage of conditioned responses per session. Mean values and their Standard Error of the Mean (S.E.M.) are indicated in figures 5 and 7 for each habituation, conditioning, and extinction day. Significance of the results is indicated in the corresponding figure legend. Data were compared day by day for the three different experimental groups using Student's *t*-test.

CLASSICAL CONDITIONING OF ALERT BEHAVING MICE

The electrical stimulation of the supraorbitary branch of the trigeminal nerve was able to evoke a true reflex blink when presented at intensities > 1-1.5 threshold (see FIG. 1B). The evoked EMG response consisted of two components (or bursts of activity), denominated R1 and R2 following the usual convention (Kugelberg, 1952). The latency for the R1 response was ~ 7-7.5 ms, and that for R2 was ~ 15-17 ms. The amplitudes presented by R2 components were generally twice those of R1. According to available information, R1 corresponds to the activation of low-threshold mechanoreceptors of the orbital skin, innervated by Aδ fibers, while the R2 component corresponds to the activation of mechanoreceptors innervated by type C fibers (see Gruart *et al.*, 1995 for references). Reflex eyelid responses evoked in transgenic Alzheimer mice showed values equal to those obtained in controls (both Swiss and C57 strains), indicating that reflex blink circuits are not noticeably affected in transgenic animals, at least at the ages (up to 12 months old) tested.

Classical conditioning of eyelid responses was obtained in control Swiss and C57 mice by the 1st-2nd conditioning session (FIGs. 3-5). Electromyographic records corresponding to the activity of the orbicularis oculi muscle during the CS-US interval showed a typical activity corresponding to eyelid conditioned responses (see FIGs. 3B and 4). Although not yet quan-

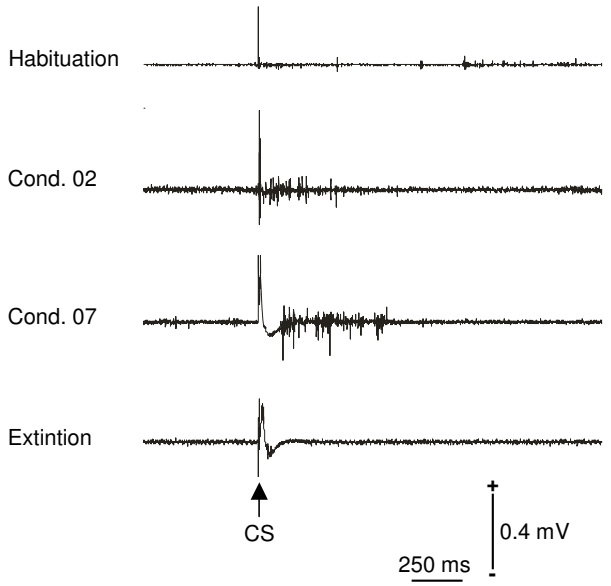

Figure 4 Example of classical conditioning of eyelid conditioned responses (CRs) in a wild-type C57 mouse, using a trace-conditioning paradigm. These CRs were collected in response to the sole presentation of the conditioning stimulus (CS) during habituation (top), conditioning (Cond., middle), and extinction (bottom) sessions. The number of the conditioning session is indicated. Calibration as indicated.

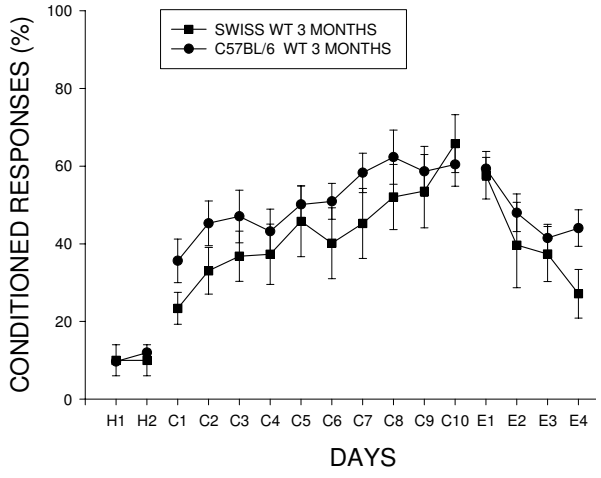

Figure 5 Learning curves for wild-type Swiss and C57 mice. Data collected are from 3-month-old, wild-type Swiss mice (n = 10, squares) and from 3-month-old, wild-type C57BL/6 mice (n = 10, circles). H1, H2 represent data from habituation sessions, C1-C10 represent conditioning sessions, and E1-E4 represent data from extinction sessions. The percentage of conditioned responses (plus its Standard Error) is indicated. No significant difference was observed for any habituation, conditioning, or extinction session for data collected from these two mouse strains.

titatively analyzed, conditioned eyelid responses (determined by the EMG activity of the orbicularis oculi muscle) increased in amplitude and decreased in latency across conditioning in wild-type animals. Criteria were set to determine whether the EMG recorded in the CS-US interval corresponded to a true conditioned response (see Methods). Following those criteria, the curves illustrated in figure 5 (data collected from Swiss and C57 wild-type animals) were constructed. As shown, there was no significant difference between the learning curves of the two strains of animals, although C57 mice seemed to learn more quickly than Swiss. In contrast to early descriptions in both rabbits and cats (see Marshall-Goodell *et al.*, 1992; Gruart *et al.*, 1995, 2000c for references), the learning curves in mice showed a faster and earlier rise, but did not usually reach asymptotic values > 80% of conditioned responses.

A COMPARISON BETWEEN WILD-TYPE AND TRANSGENIC MODELS OF AD

A comparison was made here between the learning capabilities of wild-type mice and transgenic mice that model AD. Those animals over-express the human amyloid precursor protein (APP) and presented plaque deposits when they were > 12-months old (Moechars *et al.*, 1999). Groups of APPLd2 mice were checked at 3, 10, and 12 months of age; i.e., nearly the temporal limit for plaque expression. As illustrated by the EMG records shown in figure 6, 3-month-old C57 APPLd2 mice learned the classical conditioning of eyelid responses in the same way as controls. However, 10-month-old C57 APPLd2 mice presented a very poor learning performance (see FIG. 6 right set of records, and FIG. 7). They presented normal reflex blinks in response to electrical stimulation of the supraorbitary branch of the trigeminal nerve, but were unable to develop noticeable eyelid conditioned responses. These results indicate that the lack of conditioned eyelid responses was not due to any technical problem with the EMG recording system. Thus, the percentages of conditioned eyelid responses collected from 3-month-old wild-type and APPLd2 mice were significantly different ($P < 0.01$, Student's t-test) from values corresponding to 10-month-old C57 APPLd2 mice. These results indicate that the learning deficit was age-related, and that it can be noticed with the experimental procedures used here, even before the appearance of plaque deposits in cortical structures.

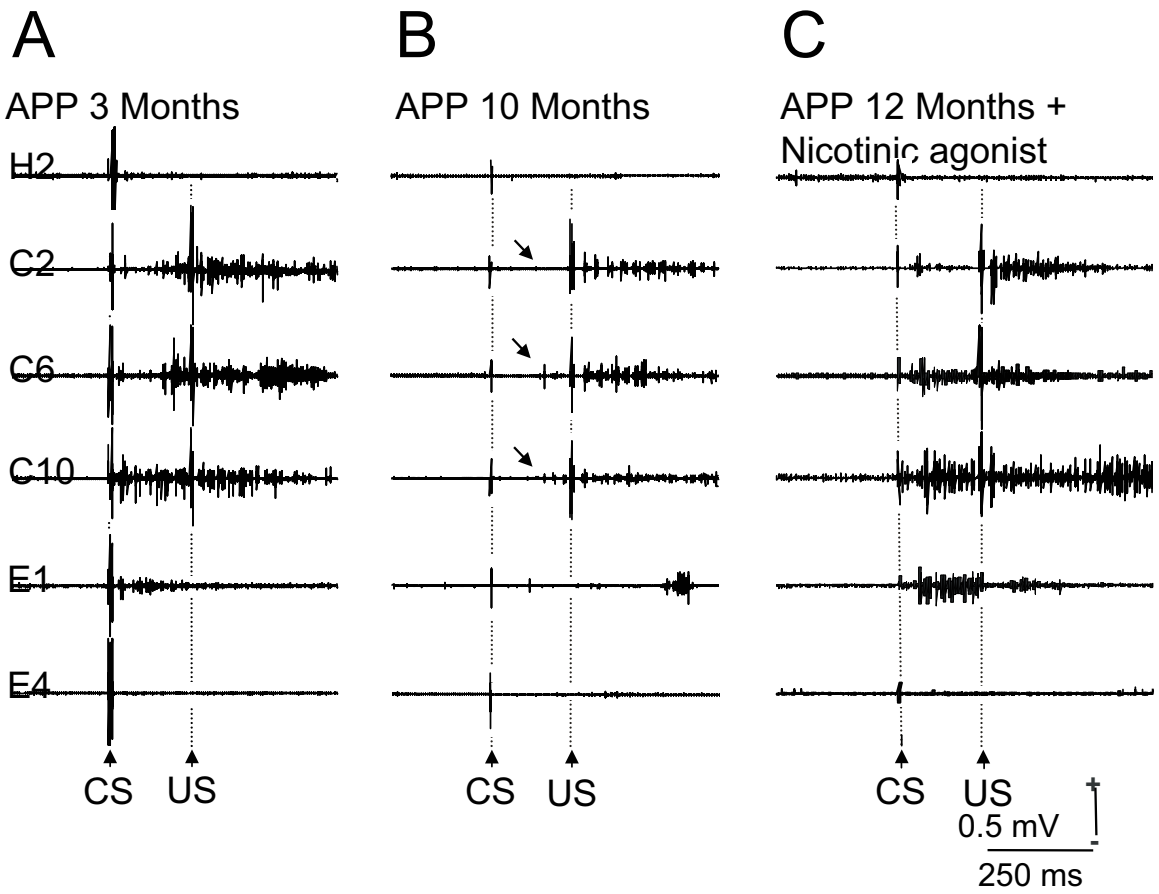

Figure 6 Examples of the electromyographic (EMG) activity recorded from the orbicularis oculi muscle during classical conditioning of transgenic Alzheimer mice. Illustrated EMG records were collected from a 3-month-old C57 APPLd2 mouse (**A**), and 10-month-old C57 APPLd2 mouse (**B**). The number of the habituation (H), conditioning (C), or extinction (E) sessions from where EMG records were collected is indicated. Note the almost complete absence of conditioned responses to CS presentation for the 10-month-old mouse. Calibrations in C are also for A-B.

ADVANTAGES OF THE PROPOSED MODEL FOR PHARMACOLOGICAL STUDIES

The experimental model developed here is also suitable for pharmacological studies. We are currently developing quantitative procedures to make a more precise account of reflex and conditioned eyelid responses collected from wild-type and transgenic animals. Thus, an automatic procedure is being developed to determine, without subjective bias, the presence, as well as other parametric properties (latency, peak amplitude, maximum area) of conditioned eyelid responses. This analytical procedure will complete the analysis presented here based on the determination of the presence (Yes/No) of conditioned responses following previously determined criteria (Marshall-Goodell *et al.*, 1992; Gruart *et al.*, 2000c).

Some preliminary results obtained in our laboratory (Múnera *et al.*, 2000) with scopolamine (a muscarinic antagonist) suggest the involvement of cholinergic neurons in cortical processes underlying associative learning. Ongoing experiments in mice are checking the palliative effect of some cholinergic drugs on learning deficits observed in mice models of AD (Domíngez del Toro, Rodríguez-Moreno, Porras-García and Delgado-García, in preparation).

CONCLUDING REMARKS

Our group has been working for more than a decade on the description of neural mechanisms involved in the acquisition of classically conditioned eyelid responses (Delgado-García and Gruart, 2002). This hippocampus-dependent motor learning is disturbed in patients with AD, possibly because of impairment of the cholinergic septo-hippocampal projection (see references in Múnera *et al.*, 2000, 2001). Recently, we demonstrated in alert behaving cats that, during the acquisition of eyelid conditioned responses, the activity of identified hippocampal CA1 pyramidal cells signals the salience or predictive value of the CS, in linear relationship with the number of its paired presentations with the US

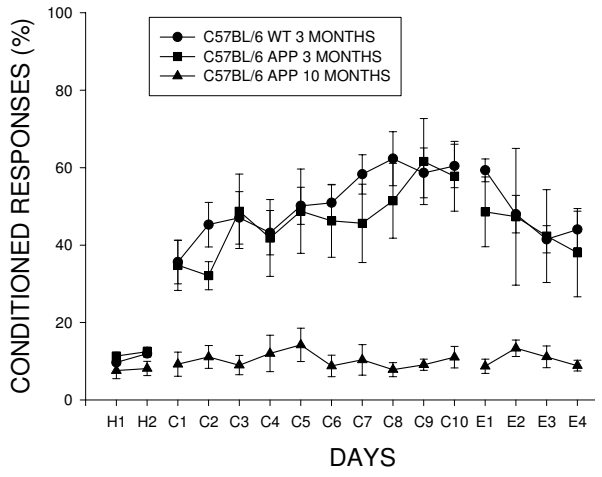

Figure 7 Learning curves for wild-type and transgenic Alzheimer mice. Data collected are from 3-month-old wild-type C57BL/6 mice (n = 14, black circles), 3-month-old C57 APPLd2 mice (n = 10, gray squares), and 10-month-old APPLd2 mice (n = 9, black triangles). H1, H2 represent data from habituation sessions. C1-C10 represent conditioning sessions, and E1-E4 represent data from extinction sessions. The percentage of conditioned responses (plus S.E.M.) is indicated. Differences between 3-month-old wild-type C57BL/6 and 3-month-old APPLd2 mice compared with 10-month-old APPLd2 mice were statistically significant ($P < 0.01$, Student's t-test) for conditioning (C1-C10) and extinction (E1-E4) sessions, but not for habituation (H1-H2) sessions.

(Múnera *et al.*, 2001). This pyramidal cell activity in response to CS-US presentations occurs, specifically, when electroencephalographic oscillatory activity of the hippocampus shifts from low (*theta*) towards high (*beta, gamma*) frequencies (Múnera *et al.*, 2000). Interestingly, the excitability of CA1 neurons is enhanced only during the short CS-US time window. We have further demonstrated in cats that a cholinergic blockade with scopolamine not only inhibits the preferential CS processing, the frequency shift, and the enhanced excitability, but also impairs the performance of previously acquired conditioned responses, without changing the performance of reflex blink responses (Múnera *et al.*, 2000). These results have been further reinforced here, reviving the *cholinergic hypothesis* in relation with AD (see Terry and Buccafusco, 2003 for references). According to our preliminary results, some cholinergic drugs are able to recover lost learning capabilities in mice models of AD. Moreover, it is well known that Alzheimer's patients have difficulties in learning classical conditioning of eyelid responses (Woodruff-Pak *et al.*, 1990), and that most available drugs for palliative treatment of Alzheimer's patients are also related to the cholinergic system (Trinh *et al.*, 2003).

These proposals are based on evidences that molecular changes associated with both AD and deprivation of cholinergic inputs impair long-term potentiation (LTP) in the hippocampus. Despite the fact that the best approach to coping with a given disease is prevention, it is still necessary to develop therapeutic approaches to minimize the negative consequences for the ongoing disease. The reported effects of selected cholinergic drugs in AD patients support, at least initially, our contentions.

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