Brief Communication

Classical conditioning of eyelid and mystacial vibrissae responses in conscious mice

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The murine vibrissae sensorimotor system has been scrutinized as a target of motor learning through trace classical conditioning. Conditioned eyelid responses were acquired by using weak electrical whisker-pad stimulation as conditioned stimulus (CS) and strong electrical periorbital stimulation as unconditioned stimulus (US). In addition, conditioned vibrissal protraction was obtained pairing either weak electrical whisker-pad stimulation or a tone as CS, with a strong electric shock delivered in the whisker-pad as US. This finding suggests that evolutionary pressure has selected a sensorimotor system capable of constructing conditioned responses on the basis of temporal relationships of stimuli, independently of any putative functional purpose.

Classical conditioning of blink responses is widely used to explore the neural mechanisms underlying associative learning and memory storage in diverse species (Gormezano et al. 1983; Gruart et al. 1995; Bao et al. 1998; Weiss et al. 1999; Zhao et al. 1999). This conditioning is acquired after repetitive and contingent paired presentations of a neutral, conditioned stimulus (CS) and a blink-evoking, unconditioned stimulus (US).

Eyeblink conditioning has been attained by using auditory, somatosensory, or visual CSs paired with an air-puff to the eye or a strong periorbital shock as USs (Gormezano et al. 1983; Paczkowski et al. 1999; Gruart et al. 2000). Das and colleagues (2001) obtained eyeblink conditioning in rabbits by using vibratory stimulation of the mystacial vibrissae as CS.

The vibrissal sensorimotor system is used to scan object surfaces, extracting detailed information from the environment (Woolsey and Van der Loos 1970; Carvell et al. 1991). Both behavioral (Bermejo et al. 1996) and experience-induced cortical plasticity (Fox 2002) have been demonstrated as a result of adult whisker manipulations. Here, we examined the possibility of using stimulation of its sensory branch as CS and/or its motor branch as the generator of conditioned responses (CRs). Thus, we evaluated the acquisition of eyeblink conditioning by using electrical stimulation of the mystacial vibrissae as CS, and the acquisition of conditioned vibrissal protraction by using either electrical stimulation of the mystacial vibrissae or auditory stimulation, also as CS.

Whisker-pad electrical stimulation as CS during blink conditioning

An isolated weak electrical stimulus in the whisker-pad occasionally elicited a variable-latency (~25 to 35 msec), brief (<10 msec), low-voltage (<20 μ V) blink response (Fig. 1C). When this CS was consistently paired with a strong electric shock in periorbital region (US), using a trace paradigm (w-E), the subjects gradually displayed CRs (%CR: $F_{(9,90)} = 57.321$, $P < 0.001$; amplitude: $F_{(9,90)} = 19.635$, $P < 0.001$) (Fig. 1A,B). Initially, these responses were brief and had variable latency (Fig. 1C); however, as the training advanced, the CRs acquired long latency and an in crescendo profile (Fig. 1C). During extinction, CR frequency progressively decreased ($F_{(3,36)} = 25.901$, $P < 0.001$); however, the amplitude decreased abruptly after the first session (C_{10} versus E_1 : $t_{(18)} = 6.552$, $P < 0.001$) and remained stable thereafter (Fig. 1). In

contrast, the blink response to a weak whisker-pad electrical stimulation did not change (%CR: $F_{(9,30)} = 1.03$, $P = 0.48$; amplitude: $F_{(9,30)} = 0.86$, $P = 0.58$) during pseudoconditioning (Fig. 1A,B). Moreover, in the w-E conditioned group, there were no changes in the vibrissal musculature activity during the 50- to 250-msec interval after CS presentation (%CR: $F_{(9,90)} = 1.655$; $P = 0.112$; amplitude: $F_{(9,90)} = 0.98$; $P = 0.462$; data not shown).

Vibrissal protraction as conditioned response to whisker-pad electrical stimulation

An isolated weak electrical stimulus in the whisker-pad evoked a short-latency (~9.5 msec), small (<30 μ V), brief (<40 msec) vibrissal protraction response (Fig. 2C). After repeated paired presentations of this CS followed by a strong electric shock in the same site (US), using a trace paradigm (w-W), the subjects rapidly displayed CRs ($F_{(9,90)} = 28.837$, $P < 0.001$) (Fig. 2A). These CRs had long latency (>50 msec) and appeared after the above-mentioned short-latency responses (Fig. 2C). However, the growth in amplitude of the long-latency responses was slow (Fig. 2B). These vibrissal protraction CRs rapidly diminished in frequency ($F_{(3,36)} = 32.372$, $P < 0.001$) and amplitude ($F_{(3,36)} = 2.903$, $P < 0.05$) during extinction (Fig. 2A–C). Contrarily, the vibrissal response to a weak electrical shock did not change (%CR: $F_{(9,30)} = 0.493$, $P = 0.849$; amplitude: $F_{(9,30)} = 0.841$, $P = 0.598$) during pseudoconditioning (Fig. 2A,B). In addition, in the w-W conditioned group, there were no changes in the orbicularis oculi activity during the 50- to 250-msec interval after CS presentation (%CR: $F_{(9,90)} = 1.576$; $P = 0.103$; amplitude: $F_{(9,90)} = 0.992$; $P = 0.387$; data not shown).

Vibrissal protraction as conditioned response to an acoustic stimulus

An isolated tone elicited a clear-cut, short-latency (~11 msec), moderately intense (<200 μ V), brief (<20 msec) vibrissal protraction response (Fig. 3C). When this tone (CS) was paired with a strong electric shock in the whisker-pad (US), using a trace paradigm (t-W), all subjects rapidly acquired CRs ($F_{(9,90)} = 15.93$, $P < 0.001$) (Fig. 3A). The CRs appeared shortly after the reflex response, displaying a characteristic in crescendo profile (Fig. 3C). The amplitude of the CRs increased stably during conditioning ($F_{(9,90)} = 19.377$, $P < 0.001$), reaching its maximum in the last three sessions (Fig. 3B). During extinction, these CRs diminished in frequency ($F_{(3,36)} = 4.032$, $P < 0.05$) but not so clearly in amplitude, although it was significantly lower after the fourth session (C_{10} versus E_4 : $t_{(18)} = 5.052$, $P < 0.001$) (Fig. 3A–C). In contrast, the vibrissal response to tone did not change (%CR:

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Figure 1. Learning evolution and representative EMG records during w-E trace paradigm. Progress of the percentage of CRs (A) and maximum CR relative amplitude (B) in conditioned (Cond) and pseudoconditioned (Pse I) groups. Representative single-trial records of the orbicularis oculi EMG (C) during different training sessions obtained from one conditioned subject. Hab indicates habituation; Cond, conditioning; and Ext, extinction.

$F_{(9,30)} = 0.705$, $P = 0.695$; amplitude: $F_{(9,30)} = 0.534$, $P = 0.821$) during pseudoconditioning (Fig. 3A,B). Furthermore, in the t-W conditioned group, there were no changes in the orbicularis oculi activity during the 50- to 250-msec interval after CS presentation (%CR: $F_{(9,90)} = 1.812$; $P = 0.097$; amplitude: $F_{(9,90)} = 1.231$; $P = 0.267$; data not illustrated).

Our results suggest that not only is electrical stimulation in the whisker-pad useful as CS for facial motor responses (either blinking or vibrissal protraction), but also the whisker motor system is able to display CRs to somatosensory as well as acoustic stimuli.

Agreeing with findings in rabbits (Das et al. 2001), the acquisition of eyeblink conditioning in mice, using electrical stimulation of the mystacial vibrissae as CS, was relatively rapid and robust. This, conjointly with the CR diminishment during extinction and the absence of CR changes during pseudoconditioning, clearly attests associative learning. Accounting the former, and given that orbicularis oculi electromyographic (EMG) records are devoid of reflex responses, researchers can take advantage of electrical stimulation in the whisker-pad as CS to study the neural underpinnings of conditioned blink generation.

The acquisition of vibrissal protraction conditioning in mice was relatively quick and prominent using either the somatosensory or the acoustic CS. In addition, the lack of CR frequency change during pseudoconditioning discards any irritant effect of electrical vibrissal stimulation. Therefore, a strong electric shock in the whisker-pad can be used as US in order to motivate the acquisition of vibrissal protraction conditioning.

The contrast between CR frequency and amplitude evolution suggests that it is difficult for the facial motor system to

generate tonic and in crescendo activity in the intrinsic whisker-pad musculature. In fact, the active movements of the mystacial vibrissae in rodents are bursting and rhythmic in nature (Gao et al. 2001). Whatever the type of whisking (exploratory or discriminative), the activity of the intrinsic musculature is phasic and periodic (Berg and Kleinfeld 2003). The slow development of sustained and growing CRs may be due to a progressive and time-consuming change in the firing pattern of the intrinsic musculature motoneurons from a bursting to a tonic mode, as has been demonstrated in the orbicularis oculi motoneurons in cats (Trigo et al. 1999). A substantial portion of the facial nucleus is devoted to controlling the vibrissal musculature. This, added to the slow nature of the process, can make it a profitable model to study how and under what inputs do facial motoneurons change their firing mode during motor learning.

The absence of generalization of CRs between two divisions of the facial nucleus (the ventrolateral, controlling the vibrissal musculature, and the dorsolateral, controlling the orbicularis oculi muscle) suggests that the different components of the facial

motor system are independently regulated.

By no means does the sustained protraction of the mystacial vibrissae prevent or minimize the effects of a strong electric shock in the whisker-pad. Thus, even though the vibrissal sensorimotor system builds up complete CRs based on a contingent temporal association of stimuli, these CRs are devoid of obvious functional sense. This suggests that evolutionary pressure would have selected a sensorimotor system capable of constructing CRs on the basis of temporal relationships of stimuli, independently of any putative functional purpose.

Figure 2. Learning evolution and representative EMG records during w-W trace paradigm. Progress of the percentage of CRs (A) and maximum CR relative amplitude (B) in conditioned and pseudoconditioned groups. Representative single-trial records of the intrinsic vibrissal musculature EMG (C) during different training sessions obtained from one conditioned subject. Abbreviations as in Figure 1.

Figure 3. Learning evolution and representative EMG records during t-W trace paradigm. Progress of the percentage of CRs (A) and maximum CR relative amplitude (B) in conditioned and pseudoconditioned groups. Representative single-trial records of the intrinsic vibrissal musculature EMG (C) during different training sessions obtained from one conditioned subject as in Figure 1.

Subjects were 42 adult male Swiss-Webster mice, weighing 35–40 g. All procedures were carried out following European Union regulations for the use of laboratory animals in chronic experiments. Animals were anesthetized with ketamine (100 mg/kg, ip) and xylazine (10 mg/kg, ip), and bipolar recording and stimulating electrodes were implanted in the right upper lid and in the right whisker-pad. An eight-pin socket, to which the wire terminals were soldered, was cemented to the skull.

Conditioning consisted of 16 daily sessions of 60 trials: two habituation (CS-only trials; H_1 – H_2), 10 conditioning (paired CS–US trials; C_1 – C_{10}), and four extinction (CS-only trials; E_1 – E_4). In all paradigms, the interstimulus interval was 250 msec. Eyeblink conditioning was achieved by using a w-E (whisker-eyelid) trace paradigm ($n = 10$). The CS was a 50- μ sec electric shock in the whisker-pad, with an intensity $1.5 \times$ threshold to evoke whisker protraction responses. The US was a 500- μ sec electric shock in the upper lid, with an intensity $2.5 \times$ threshold to evoke reflex eyeblink responses. Vibrissal protraction conditioning was achieved by using two trace paradigms: w-W (whisker-whisker, $n = 10$), and t-W (tone-whisker, $n = 10$). In both paradigms, the US was a 500- μ sec electric shock in the whisker-pad, with a $2.5 \times$ threshold intensity. In the w-W paradigm, the CS was a 50- μ sec electric shock in the whisker-pad, with a $1.5 \times$ threshold intensity. In the t-W paradigm, the CS was a binaural tone (20 msec, 2415 Hz, 90 dB).

To be considered as a CR, each increase in the EMG amplitude of the respective muscle (orbicularis oculi, in w-E paradigm, or the intrinsic vibrissal musculature, in w-W and t-W paradigms) had to fulfill the following criteria: (1) onset between 50 and 249 msec after the CS; (2) amplitude greater than the mean plus 3 SD of the baseline activity (100 msec before CS onset); and (3) duration >5 msec. Responses beginning during the first 50 msec after CS onset were considered reflex α responses and were not included in the analysis.

Pseudoconditioning consisted of 10 sessions with 60 CS and 60 US unpaired, random presentations. Three pseudoconditioned groups served as control of each conditioned group: Pse I ($n = 4$), Pse II ($n = 4$), and Pse III ($n = 4$), corresponding to w-E, w-W, and t-W, respectively.

For recordings, subjects were lightly restrained by using a cylindrical plastic box, and its socket was connected to the recording and stimulating systems. The stimulus dispensers were commanded by a computer-controlled pulse generator (EMDPP, Cibertec). The EMG signal was magnified ($1000 \times$) and filtered (1 Hz–10 kHz) with a differential amplifier (P511, Grass Instrument), and digitized at 10 kHz (1401-Plus, CED, UK). Records were analyzed by using Signal 2.06 (CED). CR amplitude was normalized by expressing it as relative amplitude (maximum CR amplitude/mean amplitude during the 100 msec before CS). Statistical analyses were made by using Statistica 5.0 (Stat Software). A $P \leq 0.05$ was used as statistical significance criterion.

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