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MEMBRANE EXCITABILITY AND FEAR CONDITIONING IN CEREBELLAR PURKINJE CELL

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Abstract—In a previous study it has been demonstrated that fear conditioning is associated with a long-lasting potentiation of parallel fiber to Purkinje cell synaptic transmission in vermal lobules V and VI. Since modifications of intrinsic membrane properties have been suggested to mediate some forms of memory processes, we investigated possible changes of Purkinje cell intrinsic properties following the same learning paradigm and in the same cerebellar region. By means of the patch clamp technique, Purkinje cell passive and active membrane properties were evaluated in slices prepared from rats 10 min or 24 h after fear conditioning and in slices from control naïve animals. None of the evaluated parameters (input resistance, inward rectification, maximal firing frequency and the first inter-spike interval, post-burst afterhyperpolarization, action potential threshold and amplitude, action potential afterhyperpolarization) was significantly different between the three studied groups also in those cells where parallel fiber–Purkinje cell synapse was potentiated. Our results show that fear learning does not affect the intrinsic membrane properties involved in Purkinje cell firing. Therefore, at the level of Purkinje cell the plastic change associated with fear conditioning is specifically restricted to synaptic efficacy. **© 2006 Published by Elsevier Ltd on behalf of IBRO.**

Key words: aversive conditioning, cerebellum, firing properties, activity-dependent plasticity.

Fear conditioning is considered a model to study brain mechanisms underlying emotional learning. Here, a neutral stimulus, usually a tone acting as a conditioned stimulus (CS), is repeatedly paired with an aversive foot shock, acting as an unconditioned stimulus (US). As a result of this pairing, the CS comes to elicit a set of defensive behavioral and somatic responses that include freezing, increases in heart rate and startle (Fendt and Fanselow, 1999; Maren, 2001; Sacchetti et al., 2005). Observations on human (Maschke et al., 2002; Frings et al., 2002) and animals (Supple and Leaton, 1990; Supple and Kapp, 1993; Sacchetti et al., 2002a, 2005) suggest that the cerebellum is involved in the acquisition and expression of fear conditioning. Recently, it has been shown that fear conditioning elicited by acoustic and noxious stimuli is accompanied by a long-term potentiation (LTP) of the synapses between parallel fibers (PFs) and Purkinje cells (PCs). Such a plasticity has been shown to occur in the vermal lobules V and VI of the cerebellar cortex (Sacchetti et al., 2004), a region where these two sensory modalities converge (Snider and Stowell, 1944; Huang et al., 1982).

The functional characteristics of a neuron are the outcome of a complex interaction between synaptic strength and intrinsic membrane properties. Recent evidence indicates that memory processes may also involve an activity-dependent change in the intrinsic excitability of neurons (Marder et al., 1996; Moyer et al., 1996; Thompson et al., 1996; Barkai and Saar, 2001; Hansel et al., 2001; Daoudal and Debanne, 2003; Cudmore and Turrigiano, 2004). In fact, changes in membrane excitability have been reported both after learning tasks (Moyer et al., 1996; Barkai and Saar, 2001) and following electrically induced LTP *in vitro* (Frick et al., 2004; Cudmore and Turrigiano, 2004; Xu et al., 2005).

An increase in the intrinsic excitability of PC dendrites has been documented following the classical conditioning of nictitating membrane responses, assessed by the decreased threshold for eliciting dendritic spikes (Schreurs et al., 1997, 1998). A similar effect was also seen in the consolidation process of trace eye blink conditioning in hippocampal pyramidal neurons, where a reduced post-burst afterhyperpolarization (PB-AHP) and reduced spike frequency adaptation were found (Moyer et al., 1996; Thompson et al., 1996). Accordingly, a reduction of the PB-AHP has been observed in neurons of the piriform cortex after odor-discrimination training (Saar et al., 1998).

Therefore it is possible that fear learning can affect both PF–PC synapse strength and PC intrinsic membrane properties. PC action potential (AP) is generated at the level of the soma–axon hillock (Stuart and Hausser, 1994). This is the region where all input signals converge and are integrated, determining the final output signaling leaving the cerebellar cortex. In such an integration, the intrinsic membrane properties of the axosomatic region play a crucial role.

In the present paper we have submitted this PC region to an analysis to reveal possible changes of intrinsic membrane properties related to fear learning in the same cerebellar area where LTP at PF–PC synapse was found (Sacchetti et al., 2004).

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Abbreviations: AP, action potential; AP-AHP, action potential afterhyperpolarization; CS, conditioned stimulus; Ih, inward rectifying cationic current; ISI, inter-spike interval; LTP, long-term potentiation; MFF, maximal firing frequency; PB-AHP, post-burst afterhyperpolarization; PC, Purkinje cell; PF, parallel fiber; PF-EPSC, parallel fiber-evoked excitatory postsynaptic current; PPR, paired-pulse ratio; US, unconditioned stimulus.

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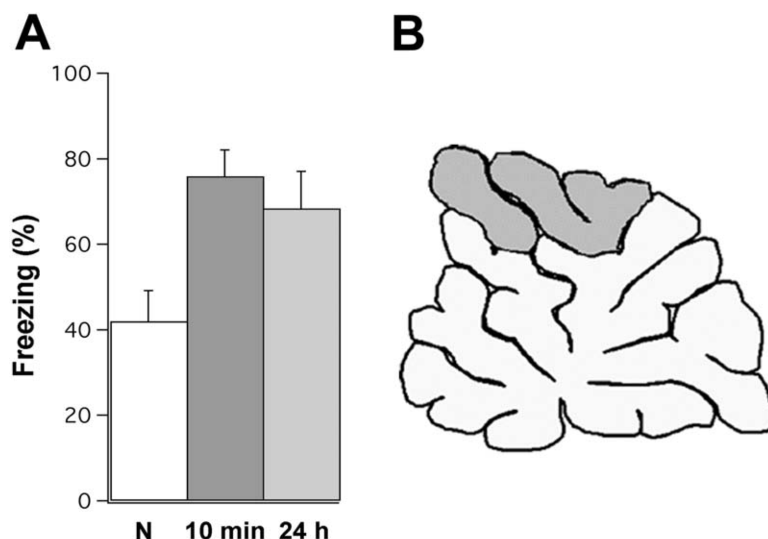

Fig. 1. Cerebellum and fear conditioning. (A) Fear responses were evaluated 10 min and 24 h after the acquisition session by measuring freezing behavior. Mean±S.E. freezing was expressed as percentage of the immobility during the retrieval phase in the naïve (N) and conditioned groups, respectively, at 10 min and 24 h after the conditioning session. (B) The passive and active membrane properties of PCs were studied in vermal lobules V and VI (indicated in shadow) where it has been previously found an LTP of PF–PC synaptic transmission (Sacchetti et al., 2004).

EXPERIMENTAL PROCEDURES

Behavior

Young Wistar rats (postnatal days 14–16) (Harlan Italy, Verona, Italy) were randomly divided into three groups. Two groups of animals underwent fear conditioning training ("fear conditioned" groups). The rats were placed in a basic Skinner box module (Modular Operant Cage, Coulbourn Instruments, Lehigh Valley, PA, USA) and left undisturbed for 2 min and 24 s. Then a training session consisted of eight presentations of a 6 s, 1000 Hz, 70 dB tone (CS) that coterminated with a 2 s, 1 mA electrical foot shock (US) was delivered to the rat at intervals of 30 s. The third group of rats ("naïve") was kept always in their home cage before surgery. Fear retention was evaluated by measuring freezing, the expression of fear behavior (Sacchetti et al., 1999, 2002a,b, 2004), during the administration of the CS alone at 10 min and 24 h after the acquisition session or after the same time lapse in the home cage for the naïve animals (Fig. 1A). Freezing (immobility) was defined as the complete absence of somatic mobility except for the respiratory movements. The subjects were placed inside the conditioning apparatus and were left for 2 min and 24 s. Thereafter, a series of eight acoustic stimuli (CS) was administered, identical to those used during the acquisition session.

Slice preparation

Cerebellar slices were prepared 10 min or 24 h after the acquisition session from the two conditioned groups (T 10 min and T 24 h) and from the naïve group. The rats were anesthetized by inhalation of halothane and decapitated. The cerebellar vermis was removed and chilled rapidly in 95% O_2 and 5% CO_2 saturated ice-cold Ringer's solution (in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$, 20 glucose). Parasagittal slices (200 μm thick) were cut on a vibratome (Vibroslice 752, Campden Instruments, Loughborough, UK) and left to recover for 60 min in the 95% O_2 and 5% CO_2 bubbled Ringer's solution at 25 °C. One slice at a time was transferred to a recording chamber and constantly perfused with 95% O_2 and 5% CO_2 bubbled Ringer's solution at room temperature.

Electrophysiological recordings

The experimental setup consisted of an upward microscope (Axioskope, Zeiss, Germany) with a 40× water immersion objective, an EPC9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Recordings were filtered at 3 kHz and the sampling rate was 40 kHz. The whole-cell current-clamp recordings performed on the PC soma were obtained in lobules V–VI (Fig. 1B) using borosilicate glass microelectrodes (2–3 MΩ) filled with intracellular solution containing (in mM): 136.5 KCl, 1 $CaCl_2$, 10 EGTA, 10 HEPES, 4 Na_2ATP, 0.4 Na_3GTP, 2 $MgCl_2$, pH 7.3 adjusted with KOH. In a first set of experiments 20 μM bicuculline methochloride and 1 mM kynurenic acid (Tocris Cookson, Bristol, UK) were added to the perfusate to block the GABAergic and ionotropic glutamatergic synaptic inputs to the PCs. In a second set of control experiments bicuculline methochloride was replaced by 20 μM gabazine (SR95531) (Sigma-Aldrich Inc., St. Louis, MO, USA) in order to assess possible effects on Ca^{2+}-dependent K^+-channels reported to be related to the use of bicuculline methiodide (Johnson and Seutin, 1997). Moreover, in the latter set of experiments, in the same PC we estimated both the synaptic drive of the PF input and the membrane excitability in naïve and conditioned animals to assess the presence of the fear related potentiation of the PF–PC synapse as previously reported (Sacchetti et al., 2004).

Responses to PF stimulation were recorded in the presence of $GABA_A$ blockers in a group of cells before applying glutamate receptors blockers by placing a stimulating electrode in the middle of the molecular layer above the recorded PC. Two current pulses were delivered at a time interval of 100 ms with increasing intensity from 20 μA to 80 μA and the identification of the PF response was based on the graded development with stimulus intensity and on the paired pulse facilitation as previously reported (Konnerth et al., 1990).

Data measurement and analysis

Data were acquired by Pulse software (HEKA Elektronik) and analyzed offline with the program Igor Pro (Wavemetrics, Lake Oswego, OR, USA). Neuronal responses were studied at membrane potential of -70 mV to minimize the influence of voltage-

dependent changes on membrane conductance. Neurones in which the holding current was greater than 400 pA were discarded from analysis. A series of current steps, each lasting 500 ms, was delivered to the PCs at intensities from −600 to +1000 pA in increments of 100 pA with a step interval of 5 s and each series was repeated for five trials. Different parameters were analyzed to evaluate the PC excitability. (1) Input resistance was determined by measuring the maximal voltage deflection (the difference between the baseline before the current step and the maximal negative voltage reached during the hyperpolarizing current injection) (Llinás et al., 1980a) (Fig. 2A). Values from five trials with current steps to intensities between −600 pA and −300 pA were averaged. (2) As shown in Fig. 2A, the amount of the contribution of the inward rectifying cationic current (Ih) was calculated as the difference between the maximal negative voltage and the plateau voltage deflection (Williams et al., 2002). Measurements were taken from the same hyperpolarizing steps as in (1). (3) The maximal firing frequency (MFF) was determined by measuring the spike frequency at the highest depolarizing current injection at which the PC fired throughout the step duration. From the same step, the first inter-spike interval (ISI) was measured. The values of the above parameters in each cell were collected and averaged across the five trials (Fig. 3A). For the following parameters, mean values for each cell were taken from the first three trials. (4) To measure PB-AHP, we considered those traces where the spiking ended at least 50 ms before the offset of the depolarizing steps, to avoid possible influences of spiking on the membrane conductance. The PB-AHP was measured as the voltage difference between the baseline before the onset and the peak after the offset of the depolarizing current steps (Fig. 3A). (5) The threshold for APs was measured from the first spike of the step to −100 pA. On the rising phase we determined as the threshold for firing APs the first point where the velocity entered within the range 30–60 mV/ms. The AP amplitude was measured from the same spike as the difference between the peak and the threshold voltage. The AP AHP was measured as the difference between the AP threshold and the negative peak of AHP (Fig. 4A).

Statistical evaluations were performed using one-way ANOVA and Newman-Keuls post hoc for multiple comparison tests and using the *t*-test for the second set of experiments where only two groups were compared.

The experimental plan was designed according to the European Communities Council Directive of 24 November 1986 (86/609/EEC), the U.S. National Institutes of Health guidelines and the Italian law for care and use of experimental animals (DL116/92), and approved by the Italian Ministry of Health and the Bioethical Committee of the University of Turin. All efforts were made to minimize the number of animals used and their suffering.

RESULTS

Behavioral procedures

Passive and active membrane properties of PCs were studied in three experimental groups of rats including a control group that did not undergo fear conditioning paradigm (naïve) and two experimental groups that had been conditioned to exhibit a fear-conditioned response. The two groups of fear-conditioned animals differed in the time interval between conditioning and slice preparation. Such interval was either 10 min (T 10 min), to test changes related to short-term learning, or 24 h (T 24 h), to test changes related to long-term memory. Freezing response was tested 10 min and 24 h after the acquisition session or after the same time lapse in the home cage for the naïve animals. The periods of immobility during the retrieval phase were significantly higher in the T 10 min (mean±S.E: 168.42±13.65 s) and in T 24 h (151.85±19.21 s) fear-conditioned groups relative to naïve (93.14±15.9 s) group [$F(2,39)=5.804$, $P<0.05$, one-way ANOVA and Newman-Keuls post hoc test] (Fig. 1A).

PC responses to hyperpolarizing currents

A first assessment of the main passive properties was obtained by evaluating input resistance and inward rectification through negative current injections. PCs are known to respond in a complex fashion to such stimulation, mainly because they express a large amount of the inward rectifier non-selective cationic current, Ih (Crepel and Penit-Soria 1986). The voltage deflection evoked by negative current injection consists of a negative bump followed by a short rising phase and a plateau (Fig. 2A). The initial negative deflection is due to the passive response of the membrane and it peaks and changes direction when the inward rectifier current recovers from inactivation and starts to depolarize the cell. Thus, the peak of the negative voltage deflection was used to measure the input resistance, since at this time the contribution of Ih is still minimal (Crepel and Penit-Soria, 1986). The amplitude of the shift from the peak to the steady level of potential was used to measure the inward rectification. To better estimate the input resistance (negative peak of the voltage deflection), the measures were taken with four different intensities of current injection and were repeated for five trials (Fig. 2A–B). The slope of the best-fitting line was considered as an index of input resistance (Fig. 2B). One-way ANOVA test showed no significant difference of such input resistance index among the three experimental groups of animals, [$F(2,56)=0.73$, $P>0.05$] (Fig. 2D). Also for the inward rectification the measures were taken with four different intensities of current injection and repeated for five trials. The slope of the best-fitting line was considered as an index of inward rectification (Fig. 2C). One-way ANOVA showed no significant difference among the three groups [$F(2,25)=2.40$, $P>0.05$] (Fig. 2E).

PC responses to depolarizing currents

Active firing properties were studied by delivering steps of depolarizing current. Fig. 3A shows some representative traces of a PC response to a series of depolarizing current steps. The amplitude of each step was set relative to the zero level. With this protocol, the cell capability to fire APs was measured by determining the MFF. This parameter was considered as the spiking frequency at the highest depolarizing current injection that evoked a PC discharge throughout the current step, without inactivation. One-way ANOVA test showed no statistically significant difference in MFF among the three groups [$F(2,48)=0.37$, $P>0.05$] (Fig. 3B). Since in most PCs the firing frequency was not constant during each train of APs, we also analyzed the first ISI of each train, as a measure of the excitability of the cell at the beginning of evoked discharge (Fig. 3A inset). Again ANOVA test of ISI showed no significant difference between naïve and conditioned groups [$F(2,48)=2.31$, $P>0.05$] (Fig. 3C).

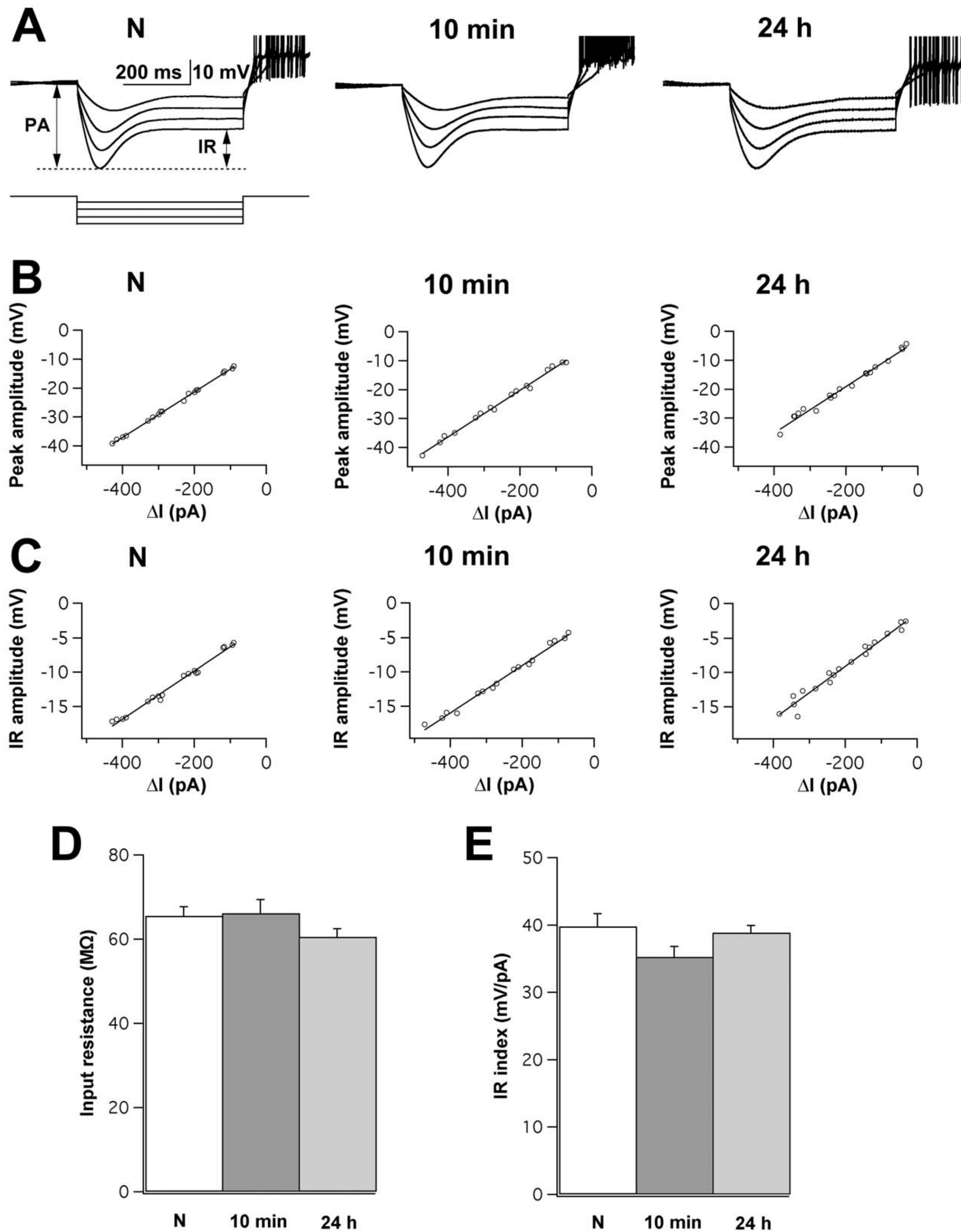

Fig. 2. Responses to hyperpolarizing currents are not changed in fear conditioned animals. N (naïve), 10 min (10 min after conditioning) and 24 h (24 h after conditioning). (A) Representative traces for each group of animals of the voltage deflections evoked by different current steps (−300, −400, −500 and −600 pA). IR, inward rectification; PA, peak amplitude. (B) Plots of peak amplitudes versus current as described in A. Each point is the result of one trial; data were well fitted by a linear slope. (C) Plot of IR amplitude as a function of current. The data points were gathered from five trials in one PC. (D) Input resistance in the three groups of animals. No significant difference was present between naïve (N) (n=16), 10 min after conditioning (10 min) (n=16) and 24 h after conditioning (24 h) (n=26). (E) Inward rectification index±S.E. in the three experimental groups. No significant difference was present between N (n=16), 10 min (n=16) and 24 h (n=26) groups.

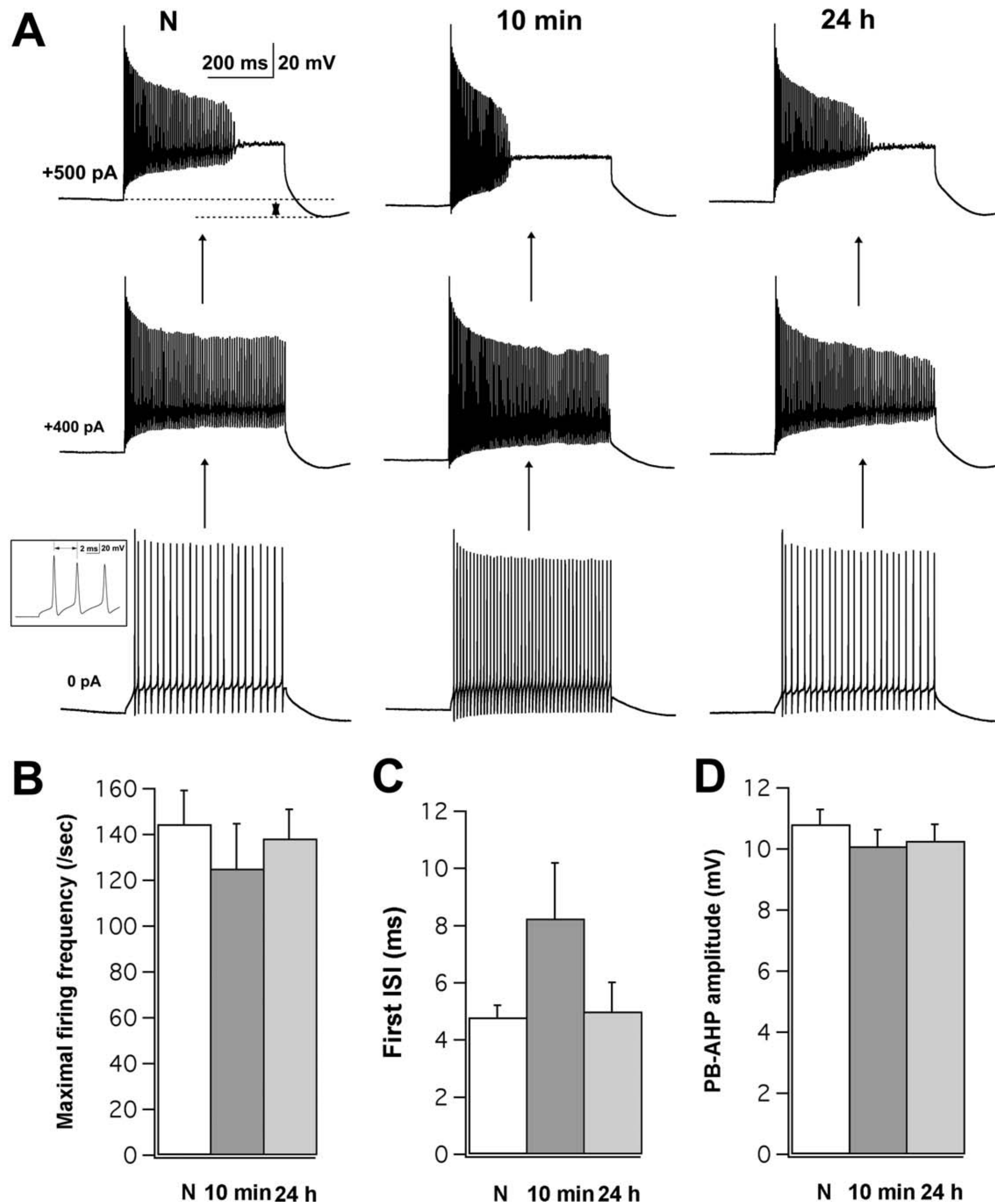

Fig. 3. MFF, first ISI and post-pulse AHP are not significantly altered by fear conditioning. (A) Representative traces of a PC response to depolarizing current steps (0, + 400 and +500 pA). For the three cells shown in A, the MFF and first ISI (shown in inset) were measured from the middle row where firing extends throughout the step duration. An example of first ISI measurement is shown in the inset. (B) MFF for PCs from naïve ($n=16$), 10 min ($n=14$) and 24 h after conditioning ($n=21$) is not significantly different. (C) First ISIs for PCs from the three groups (naïve, $n=16$; 10 min, $n=14$ and 24 h, $n=21$) do not significantly differ. (D) Post-pulse AHP is not significantly different across the three groups (naïve, $n=16$; 10 min, $n=15$ and 24 h, $n=25$).

Immediately after the end of an evoked burst of firing activity, the potential constantly showed a hyperpolarization (PB-AHP) (Fig. 3A). This property was studied by measuring the amplitude of the PB-AHP in the traces where AP firing inactivated at least 50 ms before the end of the current step, as illustrated in Fig. 3A (top traces). Also this parameter was not significantly changed following fear conditioning [$F(2.53)=0.40$, $P>0.05$] (Fig. 3D).

A change in intrinsic excitability is likely to affect the threshold voltage at which APs are generated. Therefore, we analyzed this parameter, using a velocity criterion. The threshold was defined as the membrane potential at which the rate of depolarization entered within the range 30–60 mV/ms (Fig. 4A). The mean threshold potential for each group was not significantly different [$F(2,43)=0.16$, $P>0.05$] (Fig. 4B). In addition, the amplitude of the AP was

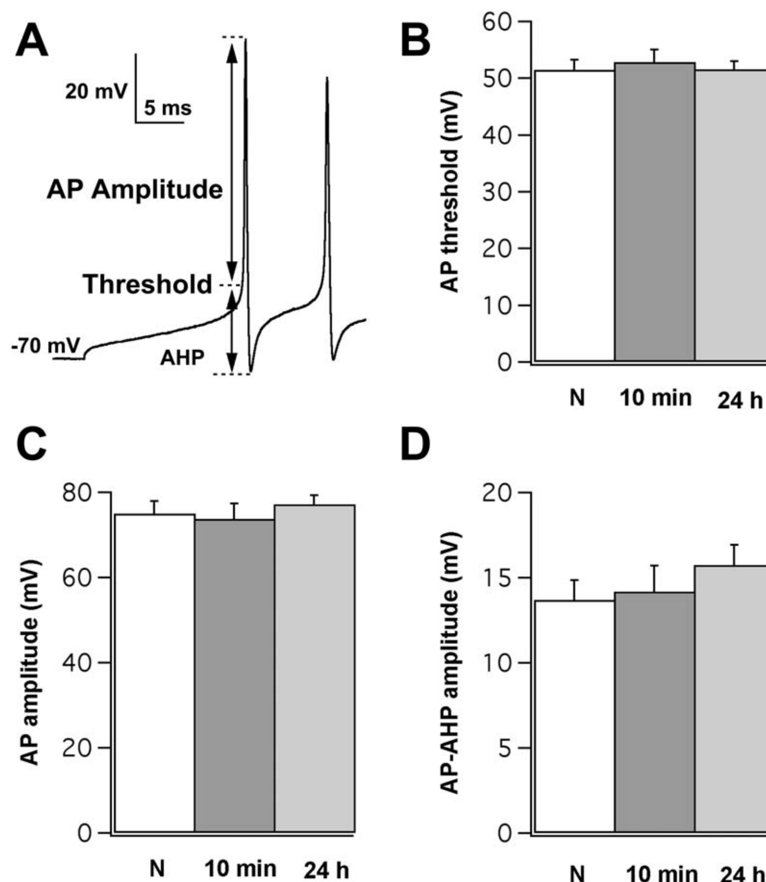

Fig. 4. AP threshold, AP amplitude and AHP are not significantly different in naïve and conditioned rats. (A) Measurements for AP threshold (velocity is around 50 mV/ms, range 30–60 mV/ms), amplitude and AHP, taken from the first spike to current step −100 pA. (B) AP threshold is not significantly changed 10 min (n=12) and 24 h after fear conditioning (n=20) compared with naïve group (n=14). (C) AP amplitude measured from the peak to the threshold is not significantly different for PCs in three groups (naïve, n=14; 10 min, n=12 and 2 h, n=21). (D) AP AHP measured from the AP threshold to AHP peak is not significantly different for PCs in naïve (n=14) and conditioned rats (10 min, n=12 and 24 h, n=21).

measured (Fig. 4A), but also this parameter showed no significant difference between the groups [$F(2,44)=0.40$, $P>0.05$] (Fig. 4C). Finally, the amplitude of the afterhyperpolarization following each action potential (AP-AHP) was measured. Also the AP-AHP was not significantly different in conditioned groups relative to the naïve group [$F(2,44)=0.77$, $P>0.05$] (Fig. 4D).

In another set of experiments parallel fiber-evoked excitatory postsynaptic current (PF-EPSC) and the intrinsic membrane properties were recorded in the same cells in order to test whether the lack of changes in excitability was accompanied by an increase in synaptic gain of the PF to PC synapses previously reported as a consequence of the associative fear learning (Sacchetti et al., 2004). To this aim, we recorded 14 and 10 cells respectively from naïve animals and from animals conditioned 10 min before kill. In the first part of each experiment we performed voltage-clamp recordings with the aim of measuring the PF-EPSC amplitudes at increasing stimulus intensities and the paired-pulse ratio (PPR) for the two groups. The stimulus intensity ranged between 20 and 80 μA, the slopes of the linear fittings performed on the input–output curve of PF responses from naïve and 10 min conditioned animals were respectively (mean±S.E.): $(7.80\pm1.42)\times10^{-6}$ and $(12.26\pm0.88)\times10^{-6}$ which were significantly different (t-test, $t=-2.44$, $P=0.02$) (Fig. 5A–B). The PPR calculated as the amplitude ratio of the second PF-EPSC versus the first PF-EPSC in response to the paired stimuli was (mean±S.E.) 146.00±2.97% for the naïve and 143.70±3.91% for the 10 min conditioned group which were not significantly different from each other (t-test, $t=0.48$, $P>0.05$, Fig. 5C). These results are similar to those previously reported (Sacchetti et al., 2004) and provide evidence that the evaluation of intrinsic membrane properties was done in a model where the fear conditioning paradigm was effective in inducing LTP at the PF–PC synapse.

Recently it has been shown that bicuculline methiodide, in addition to its antagonist effects on $GABA_A$ receptors, also reduces the slow afterhyperpolarization that follows AP due to a direct effect on Ca^{2+}-dependent K^+-channels (Johnson and Seutin, 1997; Strobaek et al., 2000). A blockade of these channels affects the burst generation itself (Hill et al., 1992; El Manira et al., 1994). Although a similar effect was never reported to be induced by bicuculline methochloride, in this second set of experiments we evaluated the same intrinsic membrane proper-

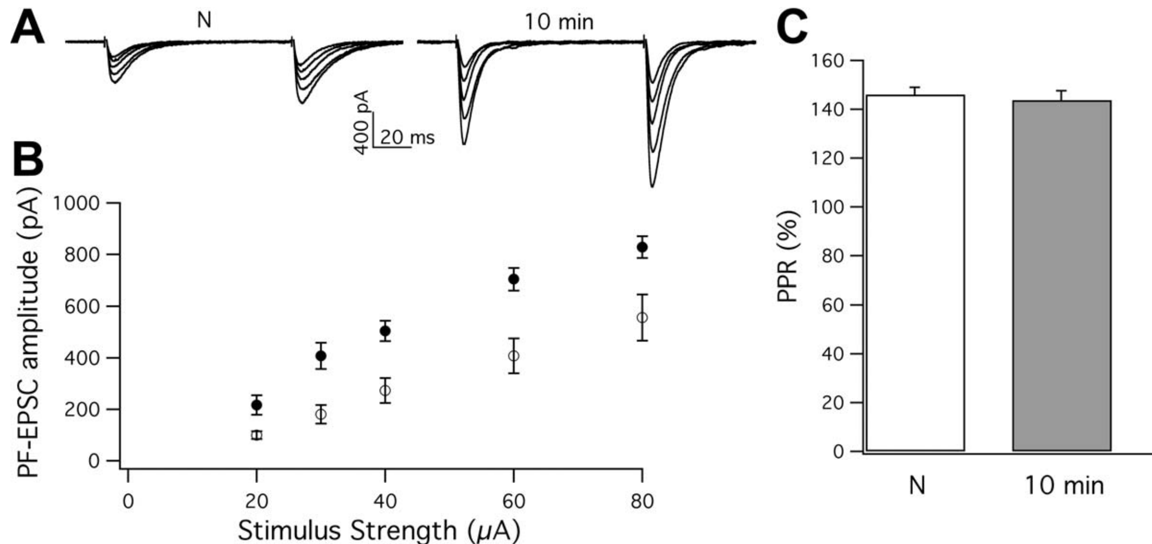

Fig. 5. PF synaptic drive onto PCs in conditioned versus naïve animals. (A) Electrophysiological recordings of PF-EPSC respectively from a naïve animal (N) and animals conditioned 10 min before being killed (10 min) at different stimulus intensities. (B) Amplitude of PF-EPSC as a function of stimulus intensity expressed as mean±S.E. in naïve group (n=14, open circles) and conditioned group (n=10, filled circles). The slope of the linear fitting for the conditioned group was significantly larger compared with the naïve group, indicating an increase in PF–PC synaptic transmission following fear conditioning. (C) PPR in naïve (N) versus conditioned (10 min) group. The PPR was not significantly different in the two groups indicating that the PF–PC synaptic increase was postsynaptically expressed.

ties and verified that the results obtained with bicuculline were consistent with those observed when using a different $GABA_A$ antagonist. Thus, after the assessment of PF–PC synaptic drive, we switched to current-clamp and performed the recordings in the presence of kynurenic acid and gabazine in the external medium. We repeated the measurements as described before and results obtained were consistent with those observed with bicuculline. By comparing all parameters from the two groups with the *t*-test, we found no difference between naïve and 10 min conditioned animals (see Table 1).

Table 1. Intrinsic membrane excitability parameters assessed in the presence of gabazine

Parameter	Mean±S.E.	*n*	*t, P*
R input	91.75±2.88 MΩ	14	−0.97, 0.34
	95.90±3.06 MΩ	10	
IR slope	30.70±2.64 mV/pA	14	−1.07, 0.30
	34.43±1.81 mV/pA	10	
AP threshold	−54.68±1.34 mV	13	−0.46, 0.65
	−53.31±3.15 mV	8	
AP-AHP	17.36±1.11 mV	13	−0.14, 0.89
	17.70±2.58 mV	8	
AP amplitude	77.11±4.64 mV	13	1.33, 0.20
	67.57±5.19 mV	8	
PB-AHP	−7.41±0.86 mV	9	1.60, 0.13
	−9.25±0.74 mV	8	
MFF	37.07±2.83 Hz	14	−0.002, 0.1
	37.08±3.30 Hz	10	
ISI	24.65±2.38 ms	14	0.60, 0.55
	22.64±2.09 ms	10	

The table reports the value of each parameter for the naïve and 10 min conditioned groups, respectively. These values were obtained from recordings performed in the presence of gabazine and the two groups were compared using Student's *t*-test. The last column reports the values of *t* and the corresponding level of significance *P*.

DISCUSSION

In a previous study it has been shown that shortly and 24 h after fear conditioning there is a LTP of the synapses between PF and PC of the vermis (Sacchetti et al., 2004). This plastic change was postsynaptic, mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors present in the PC synaptic domain. In this paper we show that in the same vermal area and also in the same PC population membrane excitability remains unchanged. To test membrane excitability in these cells, we evaluated many parameters that have been previously reported to be modified by activity-dependent processes.

Input resistance is a passive membrane property related to neuronal size, shape and resting membrane conductance. It can be modified by synaptic activity (Armano et al., 2000) and after associative learning (Farley et al., 1983).

Hyperpolarization-activated cationic current Ih can influence the pattern of PC spontaneous burst generation (Crepel and Penit-Soria, 1986; Chang et al., 1993). A recent study has suggested an important role of Ih in maintaining the membrane potential of PC within the range necessary for tonic AP firing in brain slice (Williams et al., 2002). A learning-related reduction of the PB-AHP has been observed in a variety of preparations (Saar and Barkai, 2003). Such PB-AHP reduction was due to a decrease of distinct types of K^+ current, e.g. Ca^{2+}-dependent K^+ current in hippocampal (Coulter et al., 1989) and piriform cortex (Saar et al., 2001) neurons or I_A like current in cerebellar PC dendrites (Schreurs et al., 1997, 1998).

In neurons I_A is involved in the regulation of membrane excitability and the control of the firing pattern (for review see Hille, 2001). In cerebellar PCs, I_A is also involved in the modulation of dendritic $[Ca^{2+}]_i$ transients (Midtgaard et al., 1993) and in the generation of the PB-AHP (Sacco and Tempia, 2002).

In addition, we did not observe any change in somatic spike threshold or AP amplitude, indicating that also the fast Na^+ conductance (Llinás and Sugimori, 1980a) was not altered by fear conditioning, in line with the previously observed absence of change in this parameter after conditioning of nictitating membrane response (Schreurs et al., 1991). AP AHP in PCs is due to the contribution of two types of K^+ channels, voltage-dependent K^+ channels of Kv3 type and large conductance Ca^{2+}-activated K^+ channels (Raman and Bean, 1999; Womack and Khodakhah, 2002a; Edgerton and Reinhart, 2003). The lack of changes in the AP-AHP implies that these types of K^+ channels are not modified by fear conditioning. Furthermore, it also suggests that Ca^{2+} influx was unchanged since this BK channel is modulated by Ca^{2+} influx (Llinás and Sugimori, 1980b; Edgerton and Reinhart, 2003).

Recently it has been reported that bicuculline methiodide potentiates burst firing by blocking an apamin-sensitive Ca^{2+}-activated K^+ current (Johnson and Seutin, 1997; Strobaek et al., 2000). In our experiments we did not observe a similar effect with bicuculline since control experiments with gabazine led to similar results.

The dendrites of PC have a high density of voltage-gated Ca^{2+} channels (Llinás and Sugimori, 1980b; Tank et al., 1988; Usowicz et al., 1992) and exhibit prominent dendritic Ca^{2+} spikes (Llinás and Sugimori, 1980b). Following eyeblink conditioning, intradendritic recordings showed changes in membrane excitability related to the generation of Ca^{2+} spikes (Schreurs et al., 1991). Therefore, it can be speculated that our recordings, obtained with somatic patch-clamp, would not allow detecting possible changes in dendritic membrane properties. However, this is unlikely, because extracellular recordings at the cell body near the axon hillock have shown that dendritic conductances contribute significantly to PC excitability (Womack and Khodakhah, 2002b). In fact, perfusion with an ion-free solution or with Cd^{2+} (a voltage-gated Ca^{2+} channels blockers) to the distal or proximal portion of PC dendrites resulted in a clear change of the firing frequency and firing pattern (Womack and Khodakhah, 2002b). This observation suggests that any change in dendritic excitability does not merely exert a local effect in this cellular region, but likely it affects PC excitability by controlling the capability to generate APs at the axon hillock trigger zone.

However, our experiments cannot exclude subtle changes in dendritic conductances that do not directly affect the cell spiking properties, but they influence the propagation of synaptic signals from the dendrites to the soma. Generally speaking, changes in neuronal intrinsic properties have been proposed to be involved in brain information storage (Hansel et al., 2001; Daoudal and Debanne, 2003; Saar and Barkai, 2003) and recent reports present evidence that synaptic LTP and associative learning can change the intrinsic excitability of neurons (Marder et al., 1996; Moyer et al., 1996; Thompson et al., 1996; Barkai and Saar, 2001; Hansel et al., 2001; Daoudal and Debanne, 2003; Cudmore and Turrigiano, 2004; Frick et al., 2004; Xu et al., 2005). Changes in neuronal excitability make the neuron likely to fire more or less AP in response to the excitatory synaptic stimuli. In addition, in hippocampal pyramidal neurons, voltage-gated channels endow dendrites with the capability to support active back-propagation of AP and even to generate local spikes, both of which can trigger an increase in intracellular Ca^{2+} by activating voltage-gated Ca^{2+} channels (Frick et al., 2004). This type of plasticity may represent an additional storage mechanism, providing the neuron with many additional degrees of freedom for plasticity and enhancing the storage capacity of the brain. Despite the important role of this type of plasticity, in our study we did not observe any change in PC intrinsic properties after fear conditioning. This means that such associative learning paradigm may not require this kind of plasticity, at least in the PCs of the cerebellar vermis. In fact, the same learning protocol induced in this locus a change in synaptic efficacy between PF and PC (Sacchetti et al., 2004). Such a change consists of an LTP of excitatory synaptic transmission, similar to what was previously found in amygdala (Rogan et al., 1997) and hippocampus (Sacchetti et al., 2002b). To date, in both these structures, there are no reports showing a change in neuronal intrinsic properties following fear conditioning. The essential physiological changes underlying classical conditioning involve the convergence of inputs from CS and US pathways. Therefore, it may be speculated that, at least in the cerebellum, synaptic changes in the pathway carrying CS information are sufficient to mediate learned fear, without requiring any further change in the intrinsic properties of the neurons. Moreover one has to consider that eye-blink conditioning, another form of cerebellar associative learning, involves the intermediate part of the cerebellar cortex (Kim and Thompson, 1997) while for fear conditioning the vermal part seems to be crucial. Thus it cannot be excluded that different learning paradigms (like fear and eye-blink conditioning) involving activation of different sensorial projections and different responses may also rely on different physiological substrates for memory codification. An alternative view can be that changes in the excitability related to associative fear processes can be localized to other elements of the cerebellar circuit. For instance, it has been recently reported that the intrinsic properties of the granule cell (Armano et al., 2000) and of cerebellar nuclei neurons (Aizenman and Linden, 2000) may be changed after an increase in synaptic activity. Granule cells influence PC by means of PF and cerebellar nuclei represent the final destination of the PC axon. Therefore, a change in the intrinsic excitability at these sites can regulate either PC activity or its participation to memory processes. In any case, the present results support a synaptic specificity of the plastic change previously found at the PF–PC synapse after learned fear.

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