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Research report

Error signals as powerful stimuli for the operant conditioning-like process of the fictive respiratory output in a brainstem–spinal cord preparation from rats

Alessandro Formenti*, Luciano Zocchi

DEPT—Department of Pathophysiology and Transplantation, University of Milan, Via Mangiagalli, 32, 20133 Milano, Italy

HIGHLIGHTS

- Proprioceptive input induced operant conditioning-like process on respiratory activity in vitro.
- Afferences contingent on respiratory bursts increase their frequency and amplitude.
- Long and short term effects are described.
- Diaphragmatic proprioceptive inputs represent unconditioned stimuli with hedonic features.
- A forward model is assumed to interpret the results.

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ABSTRACT

Respiratory neuromuscular activity needs to adapt to physiologic and pathologic conditions. We studied the conditioning effects of sensory fiber (putative Ia and II type from neuromuscular spindles) stimulation on the fictive respiratory output to the diaphragm, recorded from C4 phrenic ventral root, of in-vitro brainstem–spinal cord preparations from rats. The respiratory burst frequency in these preparations decreased gradually (from 0.26 ± 0.02 to 0.09 ± 0.003 burst $s^{-1} \pm$ SEM) as the age of the donor rats increased from zero to 4 days. The frequency greatly increased when the pH of the bath was lowered, and was significantly reduced by amiloride.

C4 low threshold, sensory fiber stimulation, mimicking a stretched muscle, induced a short-term facilitation of the phrenic output increasing burst amplitude and frequency. When the same stimulus was applied contingently on the motor bursts, in an operant conditioning paradigm (a 500 ms pulse train with a delay of 700 ms from the beginning of the burst) a strong and persistent (>1 h) increase in burst frequency was observed (from 0.10 ± 0.007 to 0.20 ± 0.018 burst s^{-1}). Conversely, with random stimulation burst frequency increased only slightly and declined again within minutes to control levels after stopping stimulation.

A forward model is assumed to interpret the data, and the notion of error signal, i.e. the sensory fiber activation indicating an unexpected stretched muscle, is re-considered in terms of the reward/punishment value. The signal, gaining hedonic value, is reviewed as a powerful unconditioned stimulus suitable in establishing a long-term operant conditioning-like process.

1. Introduction

1.1. Learning is a process that produces adaptive changes in the organism

Operant or instrumental conditioning [1] has been proposed as a fundamental type of associative learning[1] in which it is assumed

* Corresponding author. Tel.: +39 02 50315464; fax: +39 02 503 15455.
E-mail address: Alessandro.Formenti@unimi.it (A. Formenti).

[1] Here the term "associative learning" refers to the empirical phenomenon that animals adapt their behavior to the presence of significant events in the

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that either a spontaneous or induced behavior, i.e. the operant behavior, will adapt in form, frequency, or strength in relation to the hedonic value of its consequences, i.e. the reinforcement [2]. The contingency between the operant behavior and the reinforcement has been recognized as crucial in determining their association [3].

1.2. Respiratory activity adapts to changed physiological and pathological conditions

The components of the nervous system which control respiratory neuromuscular activity also generate the modifications required to adapt to changes in respiratory mechanics during development, pregnancy, or disease which involve dyspnea. In addition, these parts of the brain modify the respiratory activity coordinating it with voluntary and emotional behaviors such as speech, singing, laughing or crying [4]. While some effort has been made to clarify the mechanisms that underlie operant conditioning of the respiratory rhythm in simple animal models [5] little is known about this function in mammals. In the rat a rhythmic electrical activity recorded from the root of the fourth cervical motor nerve (a branch of the phrenic) has always been described comprehensively, as the motor output to the diaphragm [6]. The function of this motor output is to control respiratory movements. Changes in motor output directly affect ventilation. The respiratory-related bursts recorded from the 4th cervical motor spinal nerve (C4) appear to be correlated with an increase of activity in the preBötC nucleus [7]. This nucleus is hypothesized to generate the inspiratory rhythm [8,9] driven by a subset of neurons exhibiting bursting or pacemaker properties [10,11]. The respiratory rhythm is modulated by descending and ascending signals [4]. Extracellular recordings of neuronal populations within the pre-BotC of perinatal medullary slice preparations from rats demonstrated that the rhythmical respiratory discharge commences approximately at embryonic day E17 [12].

1.3. The brainstem–spinal cord preparation in vitro

The brainstem–spinal cord preparation from the rat is a well-known model and has recently been reviewed [13]. It seems very promising in studying the operant conditioning-like process in the mammalian nervous system. In fact, in addition to the general advantages offered by an in vitro preparation, such as easy access to electrophysiological techniques, and almost full biochemical and pharmacological control of the extracellular medium bypassing the blood-brain barrier, this preparation also retains a rhythmic burst discharge identified as fictive respiratory activity on the phrenic motor roots. This putative respiratory behavior is quite well characterized and represents the motor command to the diaphragm. In addition, sensory input from the periphery can be simulated by activating the dorsal root stumps by means of electrical stimulation.

1.4. May the spontaneous fictive respiratory activity be changed by operant conditioning in vitro?

We addressed the question if the plasticity necessary for operant conditioning of respiratory circuits was retained in brainstem–spinal cord preparations. This in vitro model seems particularly interesting since it permits the exclusion of the unavoidable effects derived from the stimulation of the peripheral structures (e.g. muscle fatigue, receptor habituation etc.) that affect whole animal experiments, and presents spontaneous, fictive operant behavior. The aim of this research has been to study the conditioning effects induced by activating the sensory fibers from the diaphragmatic proprioceptors on the fictive respiratory discharge.

1.5. Operant conditioning paradigm vs. forward model theory

From the literature we know that operant conditioning behavior will change depending on the hedonic value of its consequences. So the question arises: what is the hedonic value, if any, of the proprioceptive afferences?

A forward model theory has been proposed to explain certain features of how movement is controlled. With some analogy to the operant conditioning paradigm, in which the consequences of a certain behavior will change it, in a forward model it is postulated that the error signal, i.e. the difference between the expected and the actual situation, will cause an adjustment of movement. Assuming that the afferences from the proprioceptors represent an error signal to the CNS, indicating an unexpected length and tension of the muscles, we can hypothesize that the operant conditioning paradigm and the forward model may coexist in one coherent and more general theory helpful in this specific context to better understand the pathophysiology of respiratory neural control.

2. Methods

2.1. Ethical approval

All experiments were carried out in accordance with the accepted standards of humane animal care under the guidelines of the ethic committee of the University of Milan.

2.2. In vitro isolated brainstem–spinal cord preparation

The brainstem–spinal cord was prepared, with some minor changes, using the methods already described in the literature [6]. Sprague-Dawley rats 0–4 days (total: $n = 43$) were deeply anaesthetized by means of equitensine (4 ml/kg) intra peritoneum, dissected, and immediately placed into an artificial cerebrospinal fluid (aCSF) at 4 °C with high [Mg^{2+}] to decrease neuronal excitability during poor oxygenating conditions. The composition in mM was: $MgSO_4$ 2, $NaHCO_3$ 26, NaH_2PO_4 1.25, KCl 3, Glucose 30, and Sucrose 210. The rostral end of the en bloc preparation was cut at the level of the caudal cerebellar artery. Once meninges were removed, the brainstem–spinal cord preparation (Fig. 1A) was put into a recording chamber made of silicone elastomer, and perfused at the rate of 6 ml/min with a second aCSF recording solution. The composition in mM of the aCSF recording solution was: NaCl 130, KCl 8, $CaCl_2$ 1.3, $MgSO_4$ 1.3, NaH_2PO_4 0.58, $NaHCO_3$ 25, and Glucose 30. The perfusion solution was continuously bubbled with a carbogen mixture 95% O_2 and 5% CO_2. Temperature was maintained at 27 °C ± 1 and pH at 7.4 unless otherwise indicated. All substances were supplied from Sigma.

2.3. In vitro electrophysiology

Electrical recordings and stimulations were performed utilizing fire polished borosilicate micropipettes (tip diameter 100–150 μm) placed on homolateral ventral and dorsal roots, as indicated in diagrams in Figs. 1 and 2. Signals were amplified, digitized, recorded on a pc using a Digidata 1200 (Axon Instruments) and analyzed utilizing the software pClamp (Axon Instruments). Stimulation was delivered by means of a SD9 stimulator (Grass) and timed utilizing a Pulsmaster A300 (WPI).

environment, and considers behavioral and physiological changes as the ultimate criterion to determine whether learning has taken place.

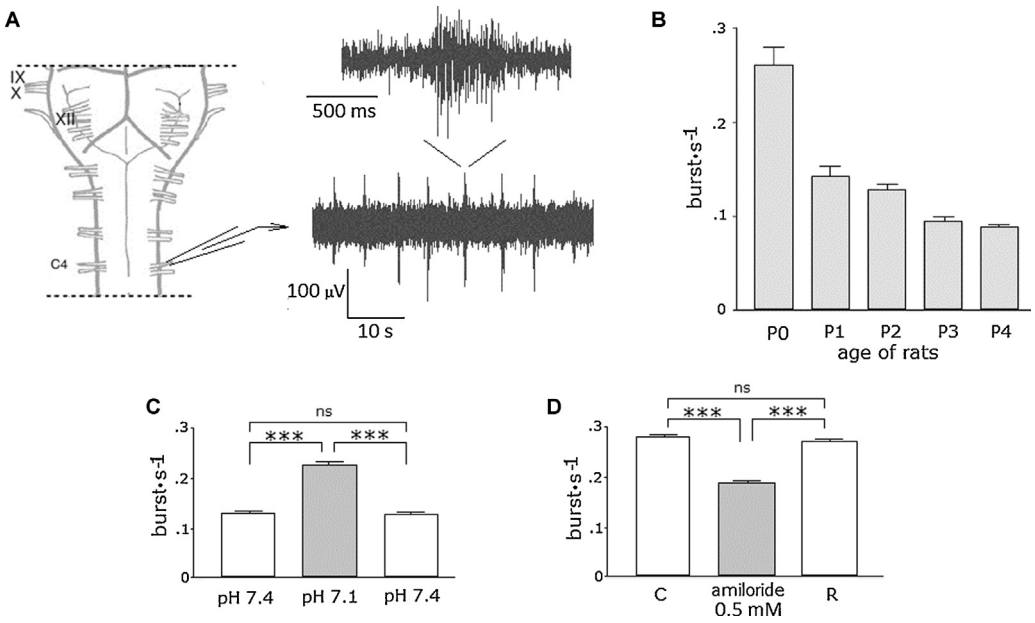

Fig. 1. In vitro newborn-rat brainstem–spinal cord preparations presented a fictive respiratory activity that was modulated varying physiological and pharmacological conditions. (A) Schematic representation of the brainstem–spinal cord preparation from a ventral view indicating the site of placement of the ventral root electrode. On the right a typical recording from the IV cervical anterior root is shown. In the upper trace a burst is expanded on the time scale. The mean frequency of burst discharge was a function of the age of the rat. The bar chart in (B) shows the mean frequency of burst discharge at 27 ± 1 °C from 43 brainstem–spinal cord preparations dissected between postnatal (P) days 0 to 4 and recorded in control conditions. (C) A pH variation from 7.4 to 7.1 modulated the motor output recorded from root C4 nearly doubling the rate in P3 rat preparations ($n = 4$) as expected. (D) Amiloride (0.5 mM) reduced the burst rate. The inhibitory effect of amiloride was completely removed upon washing. This graph refers to the data recorded from repeated perfusions and washings from 3 P0-only rat preparations. In all panels, C stands for control, and R for the recovery after removing the drug from the bath. Each bar represents the mean (vertical lines = SEM). Asterisks indicate a significant difference: *** $P < 0.0001$; *ns* means no significant difference: $P > 0.05$.

A recording electrode on the phrenic motor root was utilized to monitor the spontaneous fictive respiratory activity. In the experiments in which electrical stimulation was utilized, the stimulus voltage was adjusted to elicit only the activation of low threshold, large diameter, sensory fibers (see also [14–16]). The effectiveness of stimuli was evaluated by recording both the fiber discharge from the same activated sensory nerve stump and the related H reflex on the homologous ventral root (Fig. 2, diagram above). The threshold of the H reflex (T) was determined and the pulse voltage was defined as 3 times the threshold potential. The experiments were carried out exclusively on brainstem–spinal cord preparations that presented a stable fictive respiratory activity and H reflex from the fourth cervical (C4) ventral root (Figs. 1A and 2A).

Data is expressed as mean ± SEM. Statistical analysis was performed by means of the GraphPad Prism 4 program (GraphPad Software, Inc.) using one-way analysis of variance (ANOVA) with Bonferroni's post-test. Fitting analysis with exponential equations was conducted with the Clampfit 10 (MDS Analytical Technologies).

3. Results

3.1. Brainstem–spinal cord viability and characterization

Some preliminary experiments were carried out in order to check the functional integrity of the model. At the beginning of each experimental session the frequency of the fictive

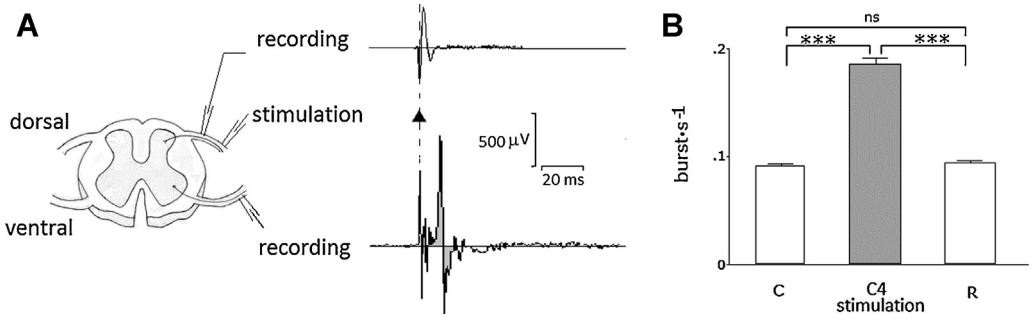

Fig. 2. *Low threshold, large diameter, fiber activation induced an increase in phrenic burst frequency.* (A) on the left a diagram of a transverse section of the spinal cord at the fourth cervical neuromere (C4) is shown, indicating the sites of placement of the dorsal root stimulating and recording electrodes, and the recording electrode on the motor root of the phrenic nerve. On the right, the upper trace indicates a typical dorsal root recording of the afferent volley induced by means of a low voltage pulse on the same root stump (black triangle); the amplitude of the afferent volley was constant during the whole experiment. The lower trace indicates the H reflex from the ventral root. The shaded area of the voltage oscillations within 20 ms after the dorsal stimulus was determined as a measure of the amount of reflex vs. the stimulus intensity. (B) Histogram bar chart indicating that the burst frequency in control conditions (C) increased during electrical stimulation of the dorsal root at level C4, utilizing 500 ms trains of voltage pulses 50 μs each, repeated every 50 ms. The pulse trains were repeated, one pulse train every 3 min for 5 repetitions. The burst frequency dropped to control levels during the recovery, at the end of stimulation (R) ($n = 6$). Each bar represents the mean (vertical lines = SEM). Asterisks indicate a significant difference: *** $P < 0.0001$; *ns* means no significant difference: $P > 0.05$.

respiratory activity was evaluated in each preparation. The data was plotted against the different ages of the donor animals. The respiratory burst frequency changed depending on the age of the rats, i.e. as the rats grew older (from 0 to 4 days) the burst frequency was reduced (Fig. 1B). Some experimental sessions were devoted to test the response of the brainstem–spinal cord preparations to specific biochemical and pharmacological conditions. In particular, it was checked to see if they could retain the capability to respond to different pH, for instance, correlated to altered respiratory function. Eight brainstem–spinal cord preparations from rats between postnatal (P) day 0 and 4 were used to assess the effects of pH variation on burst frequency. Lowering the pH from 7.4 to 7.1 induced a marked acceleration of the fictive respiratory activity in all the preparations tested (Fig. 1C). To further explore the characteristics of the respiratory pacemaker cells, amiloride, a drug known to block ionic channels involved in the generation of the rhythmic discharge in other pacemaker cells in the CNS, was used. Amiloride (0.5 mM) when added to the aCSF recording solution reduced the burst frequency (6 rat preparations; P 0 to 4). All the effects were completely reversed in a few minutes after washing out the drug. Fig. 1D shows the data recorded during repeated perfusions and washings, from 3 P0 preparations.

3.2. Fourth cervical dorsal root stimulation

In a series of ten experiments the possibility to modulate the motor output from the C4 nerve by means of stimulation of low threshold sensory fibers of the homologous and homolateral posterior root (putative sensory fibers from the phrenic proprioceptors) was evaluated. Trains of stimuli of 500 ms duration were delivered onto the dorsal root. Within the train, 50 μs voltage pulses were repeated every 50 ms. The pulse voltage was previously determined as no more than 3 times the threshold voltage necessary to evoke the H-reflex on the homologous ventral root. At this voltage, the monosynaptic ventral reflex achieved about 95% of its maximum intensity (which was evaluated measuring the area of the voltage oscillation within 20 ms after the dorsal stimulus; Fig. 2A) and, on the dorsal root, only the peak indicating the activation of low threshold, large diameter, sensory fibers, putative I and possibly II fibers, was present (Fig. 2A). Utilizing this stimulation paradigm, a short term facilitation of the phrenic motorneurons was observed. Since, it is reasonable to assume that the integrated burst amplitude is proportional to the phrenic nerve activity and the tidal volume [17], in some experiments the area of the bursts (i.e. the area defined by the potential profile and the baseline; V s) was measured in controlled conditions, and after low threshold, fiber activation. The stimulation of the fibers of large diameter, inside the dorsal root, increased the phrenic burst discharge. The facilitation lasted only for a few seconds with a significant effect only evident on the first burst. Then, the discharge dropped to control levels as shown in Fig. 3. In another series of experiments, pulse trains were repeated, one pulse every 3 min for a total of five repetitions. The large diameter, low threshold, fiber activation induced a strong increase in burst frequency immediately after the first pulse train, compared to that of control. However, at times, it increased slightly following the second and third pulse trains, but then tended to stabilize. The high burst frequency persisted for about 10–15 min after the end of stimulation, then returned to control levels (Fig. 2B).

3.3. Operant conditioning

In order to examine how sensory inputs, either contingent or non-contingent on the bursts in C4 ventral roots may affect their rhythm of discharge, an operant conditioning protocol was defined for the fictive respiratory activity. To allow a better comparison

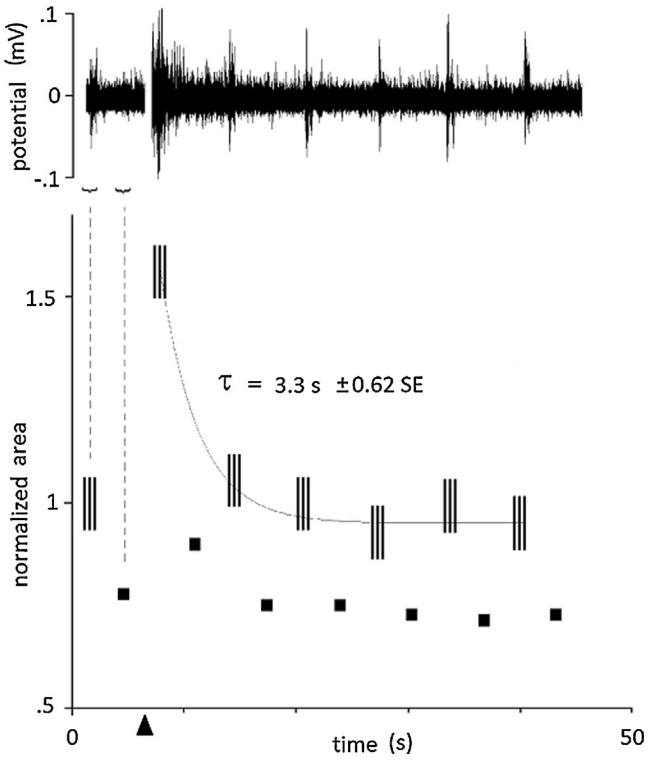

Fig. 3. *Low threshold, large diameter, fiber activation induced a short term facilitation of the phrenic motorneurons.* The upper diagram shows a recording from the IV cervical anterior root before and after stimulation of the homologous dorsal root. For the *X*-axis refer to the lower graph, the stimulation is indicated by black triangle. The stimulus artifact has been blanked out for clarity. The stimulus protocol consisted of a 500 ms train of pulses. The area of the bursts [V s] i.e. the area defined by the positive and negative potential oscillations and the baseline, comprised in a arbitrarily fixed time interval of 1 s has been measured in control conditions and after sensory fiber activation. The normalized values (vertical bars) are plotted on the graph below. On the same graph, the basal discharge recorded during the interburst intervals has been plotted for comparison (filled squares). The stimulation induced a transient increase in the area of the bursts that returned to control values within a few seconds. The decay of facilitation has been fitted with a monotonic function with a time constant $\tau = 3.3 \pm 0.62$ s SE (thin line).

between the effects of contingent and non-contingent operant stimulation it was necessary to minimize the differences in spontaneous fictive respiration frequency. Therefore, the experiments were carried out on preparations from rats divided into two sets firing almost at the same frequency. To meet these criteria, they were chosen from the same postnatal day (P3), and had been selected after a period of control during which the frequency of burst discharge was assessed. After that, the training period followed on the first set of preparations and the contingent protocol was applied. A 500 ms pulse train activating low threshold fibers in C4 dorsal root was set every time that a burst was detected in the homologous ventral root. The train was set with an interval of 700 ms from the beginning of each burst. The protocol was repeated for four sessions, each of 200 s (separated by intervals of about 4 min). This protocol of stimulation induced a strong increase in burst discharge and, unlike the experiments presented in Fig. 2B, the frequency of discharge did not return to control levels after stopping stimulation, even after more than 1 h (Fig. 4A). To evaluate the contribution of operant-stimulation contingency in determining the long term effects observed, another experiment was carried out, this time on the second set of preparations, in which the same number of stimuli was set, however, without contingency. In this second set of experiments, the burst frequency increased only moderately, and returned to control levels within a few minutes after stimulation was ended (Fig. 4B).

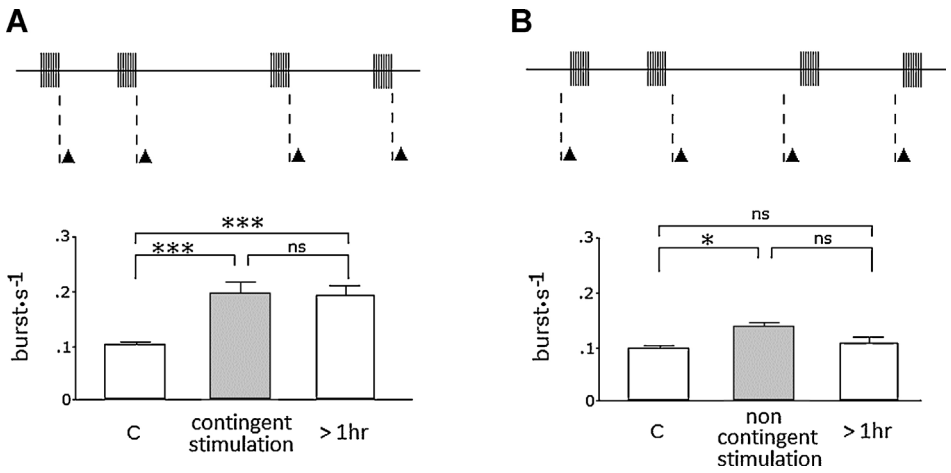

Fig. 4. The fictive respiratory activity was studied by means of an operant conditioning paradigm to test how sensory inputs, both contingent and non-contingent, on the bursts detected in phrenic motor root may affect burst frequency. (A) The upper diagram shows the experimental protocol, in which contingent train stimuli (triangles) activating Ia and II fibers in C4 dorsal root were set every time that a burst in the homologous ventral root (vertical bars in the trace above) was detected. The bar chart below summarizes the results of these experiments. The protocol was repeated in 4 sessions of 200 s duration each, and separated by intervals of about 4 min. This protocol of stimulation induced a long term increase in burst discharge that persisted for the entire duration of observation, which was more than 1 hour ($n = 10$). (B) Control experiments, in which brainstem–spinal cord preparations of the same postnatal day (P3) and also the same fictive respiratory frequency as those utilized in A, were subjected to the same number of train stimuli set at regular intervals of time and non-contingent on the bursts recorded from the phrenic root. Stimulation only moderately increased the burst frequency and the effect persisted for only a few minutes after cessation of stimulation indicating that contingency was essential in consolidating the memory trace ($n = 9$). Each bar represents the mean (vertical lines = SEM). The asterisk indicates a significant difference, * $P < 0.05$; *** indicates $P < 0.0001$; *ns* means no significant difference $P > 0.05$.

4. Discussion

4.1. The brainstem-spinal cord preparation is a suitable in vitro model for the study of respiratory rhythm conditioning

Brainstem–spinal cord preparation from newborn rats was introduced as an experimental model to study fictive respiratory activity almost 30 years ago [6]. The characterization of the preparations showed parameters in agreement with those reported in the literature. The relatively low frequency of the respiratory bursts, compared to the frequency of ventilation in vivo in newborn rats (1.5 Hz), may be explained by the lack of sensory input, as already suggested [6,18] and confirmed from the data presented here. The lack of descending excitatory drive to respiratory nuclei must also be taken into account [4,19]. The respiratory control network in rapid development, with its ongoing myelination, could be the main reason for the striking age-dependent decrease in motor burst frequency shown in Fig. 1B. In fact, an increase in myelinated fibers and a parallel decrease in unmyelinated fibers has been described in the phrenic nerve of the rat between P0 and P4, with large axons that myelinate earlier compared to smaller axons [20,21].

As expected, the fictive respiratory frequency promptly responded when the pH of the aCSF was altered (Fig. 1C) [6,22] confirming the viability of the preparations.

The fictive respiratory frequency was significantly reduced by the diuretic amiloride (Fig. 1D), a drug known as a nonspecific blocker of acid-sensing ion channels ASIC [23–25] and of low voltage activated calcium currents I_{CaLVA} [26–28]. Both channels exert an excitatory action. Thus, we should expect that the blocking effects of amiloride would reduce the bursting activity of the pacemaker neurons and the fictive respiratory frequency. In particular, I_{CaLVA}, which is found in preBötC neurons from newborn rats [29] and known already to contribute to the bursting pacemaker activity in other parts of the central nervous system [30,31], may play a role in bursting pacemaker neurons, by influencing the fictive respiratory output at this early stage in synergy with other rhythmogenic mechanisms [10,32,33]. However, other drug actions cannot be excluded.

4.2. The respiratory circuits in the preparation retain plastic properties

The data presented suggests that the activation of the low threshold afferent fibers in the dorsal C4 stump increases ventilation through both the number and amplitude of inspiratory acts, although the latter effect is very brief compared with the former one. On the whole, these results show that the necessary plasticity, for operant conditioning protocols to be effective in shaping the functions of respiratory circuits, is retained in in vitro brainstem–spinal cord preparation. They confirm that the structures above the facial motor nucleus and the parafacial respiratory group are not necessary to establish an operant conditioning-like process in the spinal cord [34] and extend this notion to the circuits responsible for respiratory control. Moreover, this preparation, excluding any peripheral structure (e.g. muscles, receptors, etc.) allows the study of the neural mechanism of conditioning and excludes the unavoidable effects of stimulation of these structures that typically affect whole animal experiments.

If the preBötC nucleus generates the inspiratory rhythm [8], then we can infer that sensory fiber activation up-regulates the pacemaker activity of the preBötC nucleus, and vice versa, in agreement with the observations that bilateral sensory deafferentation and vagotomy strongly reduced the respiratory frequency in the rat [18]. The method adopted to define the intensity of stimulation is based on both the monitoring of the afferent volley on the dorsal root, and on the ventral motor output which had to be limited to the H-reflex without any evident polysynaptic activity (Fig. 2A). This guarantees with reasonable confidence that the effects observed were derived from the specific stimulation of low threshold fibers, putative proprioceptive type I and possibly II. On the other hand, an increase in voltage of the stimulation to recruit medium threshold sensory fibers induced the opposite effect

decreasing burst frequency compared with the control (unpublished results).

4.3. Low threshold proprioceptive sensory afferents modulate respiratory rhythm

In general, the low threshold fibers in the IV cervical dorsal root are known to convey the signals from the phrenic muscle spindles (Ia) and the Golgi tendon organs (Ib) to the spinal cord. However, other origins of the fibers cannot be completely ruled out. It is known that, to a small extent, the phrenic fibers may include afferents from proprioceptive receptors located outside the phrenic muscle [35,36]. Their small number, if any, makes it unlikely that these afferents play a significant role in the operant conditioning-like effects. Furthermore, these effects were only seen when stimulation was made contingent on the fictive respiratory output.

Direct proprioceptive stimulation of the limbs and the low threshold fiber activation in cervical dorsal roots have been indicated to induce synchronization of the respiratory rhythm in the newborn rat [37]. Also, this mechanism does not seem to be involved in the phenomena shown here for at least two reasons. One is that limb movements or stimulation of the limb proprioceptive afferents induce a locomotor–respiratory coupling but not an increase in respiratory frequency in newborn rats. The second, even more stringent, is that the effects induced by limb proprioception on the respiratory rhythm are mediated by the pontine PB/KF nuclei [37], but these nuclei were not present in the brainstem–spinal cord preparation. In addition, the observation that the physical removal of the pons increased the respiratory frequency is quite interesting [20].

On the other hand, it seems unlikely that the diaphragmatic Golgi tendon organ afferents [21,38,39], are responsible for the increase in burst amplitude or frequency. As emphasized by a viewpoint, the activation of these fibers has inhibitory effects on the motor neurons innervating the same muscle. Thus, they do not seem good candidates to explain the excitatory phenomena described here. However, this view has been questioned, as stated below.

Although the role of muscle spindle afferents from the diaphragm has been controversial, in the literature there is data showing that during respiration at least part of the diaphragmatic muscle spindles fire in phase with the inspiratory contraction of the diaphragm, and intense firing is present also during cough reflexes. These observations configure the interesting possibility of a regulatory mechanism of the diaphragmatic contractions through the fusimotor activation [39]. This mechanism may also extend its influence even on the Golgi tendon organs. Indeed, two important facts must be taken into account. (1) The Golgi tendon afferents from one muscle contribute to the functional control of muscle groups as a whole and, vice versa, the afferent information forwarded to individual motorneurons is affected by the length and tension of many muscles. This is in contrast with the view that the function of Golgi tendon organs is to contribute to a feed-back system controlling the homonymous muscle, since muscles are not normally activated in isolation and the afferents from several muscles are fused together before reaching specific motorneurons [40,41]. (2) Although these organs do not have a specific centrifugal system that control their ability to respond, they are frequently anatomically associated with muscle spindles to form dyads, in parallel [42,43] or in series with Golgi receptors attached to intrafusal fibers [43,44]. Interestingly, it has been suggested that, for their close association, they work together under the fusimotor control, sensing both the muscle length and tension [41,45]. If the inhibitory action of the Golgi tendon organs from one muscle act on other muscles, namely the antagonists, and these receptors work under the fusimotor control, than, they may act coherently with the muscle spindles in a neural network organizing the pattern of activity in muscle regions.

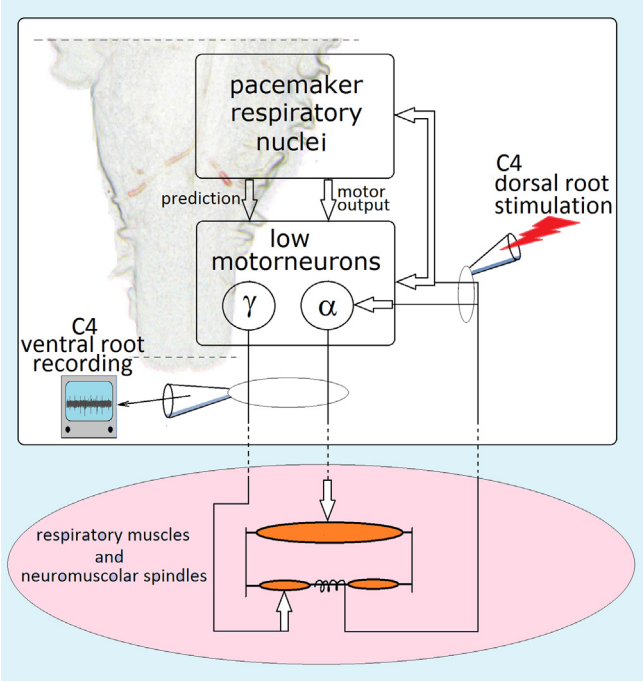

Fig. 5. *Scheme for a possible interpretation of the main experimental results.* The upper white square represents the brainstem–spinal cord preparation and the experimental layout, while the oval below shows the missing peripheral structures, i.e. a muscle with a muscle spindle. Assuming that the output recorded from the ventral root represents both the motor command for the respiratory muscles (α motorneurons) and the prediction of the shortening of the muscles themselves sent to neuromuscular spindles (γ motorneurons), it follows that the stimulation of large nerve fibers in the homologous dorsal root represents the signal from the spindles that indicates the discrepancy between the desired and the actual shortening, i.e. the error. This signal, conveyed to the central nervous system, changes the excitability the low α motorneurons, which may affect the respiratory burst intensity, and the pacemaker which in turn adjusts the burst frequency.

4.4. A theoretical frame for interpreting the experimental results: operant conditioning vs. forward model

A scheme for a possible interpretation of the main experimental results is shown in Fig. 5. The inspiratory responses are mediated by both a direct effect on the α motorneurons, and also by a low threshold mechanism, represented by the γ neurons and the spindle loop. While the α motorneurons induce direct contraction of muscles, the spindle loop activation determines the magnitude of the α motorneurons activation [46] in order to obtain the necessary tension to reach desired muscle length. If we assume a forward model system [47,48] to describe the sensory-motor system involved in the respiratory control, then the γ neuron activation may be regarded as the effect of a 'corollary discharge' i.e. a signal that, in parallel with the efference signal, results in the α and γ motorneurons co-activation. The corollary discharge is supposed to represent the prediction of the respiratory movements. If the movement is correct, then the intrafusal and extrafusal muscle fibers will shorten to the same extent, and no error is detected. The error signal here should be represented by a change in frequency of the discharge of the neuromuscular spindle sensory afferents. This signal, in the whole animal, is fed back and conveyed to the CNS through the dorsal root sensory nerves, whereas in the

brainstem–spinal cord preparation this afference and related muscle spindles are absent. However, in this in vitro preparation it is possible to artificially produce an error signal activating directly the spindle afferents by direct stimulation of the low threshold fibers present in the dorsal root stump. According to the model, the meaning (salience) of this signal is that the extent of the inspiratory movement is less than expected; i.e. the intrafusal muscle fibers shorten more than the extrafusal fibers, and this difference is detected by the stretch sensor.

As predicted by the forward model, and in analogy to the 'length-tension inappropriateness' [49] proposed to explain the pathophysiology of dyspnea (for review see [50]), proprioceptor sensory fiber activation contingent on burst discharge, represents an error signal from the muscle spindles that, in this way, inform the CNS about an unexpected muscle length. In the model outlined here, the neuromuscular spindle itself is the postulated "difference" calculator, at the lowest hierarchical level of motor control. The signal is fed back onto the motor neurons (Fig. 5) to modify coherently the motor pattern. The data presented suggests that adjustments are involved for an increase in both the efferent burst strength and frequency.

In addition, in support for a forward model hypothesis is the data obtained utilizing an operant conditioning paradigm. Only when proprioceptor sensory fiber activation was induced contingent on the respiratory bursts, it was effective in establishing a long term increase in spontaneous fictive respiratory frequency (>1 h). However, this effect was not observed when the same number of stimuli was delivered randomly, without temporal correlation with the respiratory burst discharge. Confirming proprioceptor sensory fiber activation as a strong reinforcement of the pacemaker function, this suggests that proprioceptor activation represents a powerful unconditioned stimulus suitable in establishing a long-term operant conditioning-like process (i.e. it acquires the necessary salience) only when correlated with the respiratory rhythm, providing a mechanism by which the forward model may be updated over both short and long term time-scales [47]. On the other hand, no long term increase in burst area was observed in operant conditioning experiments, suggesting that this effect perhaps requires other nerve structures which were not included in the preparation.

In operant conditioning, the reward/punishment value is a fundamental characteristic of the conditioning stimulus. Pleasure or displeasure depends on the quality of the stimuli and on the internal state of the subject. These qualities correlate to the benefits or dangers of the stimuli with reference to the homeostasis, motivating useful behavior [2]. Thus, a question arises about the reward/punishment value of proprioceptor fiber activation. Since the internal state of the subject is not easily known, a common assumption is that whether a stimulus is or is not rewarding or punishing is only recognized by its effects on the behavior on which the stimulus is contingent: a reward increasing the rate and vice versa.

In the present experiments low threshold fiber stimulation, the error signal, contingent on the respiratory bursts, indicating that the inspiratory muscles have shortened less than expected, it should assume the salience of an insufficient inspiration, likely responsible, at least in part, for the sensation of dyspnea [51,52]. An apparent contradiction arises considering the operant conditioning paradigm, in which an unpleasant stimulus, the error signal, as low threshold fiber activation is supposed to be in this case, is expected to decrease the likelihood of a certain behavior, namely the bursts. A reasonable explanation, that reconciles both the forward model hypothesis and the operant conditioning paradigm, may be that the proprioceptors activation is not interpreted by the nervous system as a consequence of the burst per se, but by the effect of an unexpected insufficient respiratory command that results in a poor inspiratory act. With this perspective in mind, the observation that the bursts result strengthened, and also their frequency increased, as a result of an error signal, gain consistency.

4.5. Conclusions

In conclusion, the data presented suggests that the rhythmic discharge of the respiratory pacemakers undergoes long term adaptation following contingent proprioceptive input. This occurrence is generally interpreted as operant conditioning, since it presents all the paradigmatic elements of this type of learning. This associative learning model seems of particular interest because, in a simplified context in vitro, it offers the rare possibility to study an operant conditioning-like process of a spontaneous behavior of medical relevance at a high mammalian phylogenetic level.

Although we are aware that the theoretical frame suggested needs further investigations to be validated, if we assume that the afference from the neuromuscular spindles may represent an error signal as it has been proposed in a forward model for the control of movement, then, we can hypothesize that the operant conditioning paradigm proposed by the behaviorists, and the old and evergreen forward model may coexist in one coherent theory helpful to better understand the mechanisms of motor learning and the pathophysiology of respiratory neural control.

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