

Classical Conditioning to Hypoxia Using Odors as Conditioned Stimuli in Rats

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The authors performed a differential conditioning experiment in 30 rats, using 2 odors as the conditioned stimuli (CS+ and CS-) and hypoxia (8% O₂) as the unconditioned stimulus. Vanillin was the CS+ and rose the CS- in half of the rats, and vice versa in the other half. Fifteen paired CS+/hypoxia trials and 15 CS- only trials were performed in random order, followed by 3 CS+ only and 3 CS- only trials to test for conditioning. The increase in ventilation from prestimulus levels averaged $116 \pm 85\%$ in response to CS+ versus $55 \pm 36\%$ in response to CS-. This effect was supported by the significant Pre-Post Stimulus \times CS Type interaction for this variable ($p < .003$). The data confirm the sensitivity of breathing to conditioning processes and also indirectly support the hypothesis that feedforward responses may complement feedback reflex pathways in respiratory homeostasis.

In mammals, arterial partial pressures of O₂ and CO₂ are maintained within normal limits despite large changes in metabolic needs, caused in particular by muscular exercise. This is achieved by means of two control processes, both of which act on the respiratory muscles to produce breathing movements. The first process involves the bulbopontine respiratory centers and the central and peripheral chemoreceptors. It automatically operates as a negative-feedback control loop that adjusts mean ventilation to keep arterial partial pressures of O₂ and CO₂ at baseline levels by using input from the chemoreceptors (Bianchi, Denavit-Saubié, & Champagnat, 1995). The second component involves suprapontine central nervous system centers and acts either on the bulbopontine centers or directly on the respiratory muscles via the corticospinal pathway (Harper, 1996). It operates as a feedforward control mechanism that allows voluntary breathing (e.g., during phonation) and involuntary breathing pattern changes induced by emotions or cognitive activities.

Several arguments militate against the hypothesis that

ventilatory changes ensuring blood gas regulation are driven primarily by chemoreceptor input, including reports that ventilatory changes can anticipate metabolic needs (Tobin, Perez, Guenther, D'Allonzo, & Dantzer, 1986), failure to identify error signals from chemoreceptors at the onset of exercise (Somjen, 1992), and numerous data from animals with lesions (Elridge, 1994). The alternative hypothesis that breathing pattern is primarily controlled by feedforward influences from higher centers has recently received indirect support. Using positron emission tomography scans, researchers have demonstrated that areas in the right and left superolateral primary motor cortices previously shown to be associated with voluntary breathing are also activated during leg exercise (Fink et al., 1995; Guz, 1997). In addition, breathing has been shown to be activated during imagined exercise, without this activation being explained by metabolic changes (see Gallego, Denot-Ledunois, Vardon, & Perruchet, 1996).

Among the various mechanisms suggested for the suprapontine control of breathing (e.g., cortical irradiation or motor preparation; Jeannerod, 1994), classical conditioning has received special attention because it would explain how internal or external cues signaling metabolic changes can trigger anticipatory ventilatory responses. Under the classical conditioning hypothesis, exercise hyperpnea would be mainly a learned response, in which the roles of reflex chemoreceptor feedback would be, first, to provide the experiences without which the central nervous system is unable to learn and, second, to fine-tune the breathing pattern determined by feedforward mechanisms (Houk, 1988; Konradi, 1960; Somjen, 1992).

The hypothesis that respiratory homeostasis is driven by conditioning processes has received indirect support from

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experiments, as yet few in number, demonstrating that breathing is highly sensitive to classical conditioning. Sensory stimuli initially paired with hypercapnia rapidly acquired the ability to affect ventilation when used alone (Nsegebe, Vardon, Perruchet, & Gallego, 1997; Van den Bergh, Kempynck, Van de Woestijne, Baeyens, & Eelen, 1995; Van den Bergh, Stegen, & Van de Woestijne, 1997). In contrast to experience with hypercapnia, the hypothesis that hypoxia may support conditioning has not been convincingly confirmed because studies of this issue were limited by a number of conceptual and methodological flaws. For example, the experiments in human volunteers reported by Voitkevitch (1952) were conducted in only five healthy adults, with no controls, and the participants were told in advance that they would be administered a hypoxic mixture, which probably prompted them to voluntarily control their breathing. Recently, Thomas et al. (Thomas, Austin, Friedman, & Strohl, 1992; Thomas, Friedman, MacKenzie, & Strohl, 1995) reported that conditioning to hypoxia of rats in the neonatal period affected the frequency of sleep apneas in adulthood. However, none of the control groups (stimulus-free group and conditioned stimulus [CS+] only group) received hypoxia. Therefore, it was not clear whether the observed differences in apnea frequency were caused by early conditioning or by long-term unconditional effects of neonatal hypoxia (Soulier, Dalmaz, Cottet-Emard, Lagercrantz, Pequignot, 1997). Finally, a controlled experiment in human volunteers (Gallego & Perruchet, 1991) showed that an auditory stimulus paired eight times with a brief hypoxic stimulus acquired the ability to elicit a ventilatory response when delivered alone. However, only short-term conditioned increases in breath duration were observed.

The aim of this study was to produce conditioning to hypoxia. To do this, we performed a differential conditioning experiment in rats using two odors (vanilla and rose) as the conditioned stimuli (CS+ and CS-) and hypoxia (8% O₂) as the unconditioned stimulus (US). Our choice of odors as the conditioned stimuli was based on the fact that conditioning is generally easier to achieve when the CS and US have some similarity (Hamm, Vaitl, & Lang, 1989; Hollis, 1997; Mackintosh, 1974; Rescorla, 1980) and on recent reports that human volunteers were successfully conditioned using odors as the CS and hypercapnia as the US (Van den Bergh et al., 1995, 1997). In addition, rats preferentially attend to odors rather than lights or sounds (Nigrosh, Slotnick, & Nevin, 1975). In contrast to previous attempts to produce conditioning to hypoxia (Thomas et al., 1992, 1995; Voitkevitch, 1952), our study used a control procedure in which one odor, the CS+, was paired with the hypoxic US, whereas the other odor, the CS-, was never paired with the US. After each rat had received the paired and unpaired stimuli, we tested conditioning by comparing ventilatory responses to the CS+ and CS- during normoxia. We expected that a CS+ paired with a relatively long and intense hypoxic US would stimulate ventilation, thus producing conditioning to hypoxia.

Method

Subjects

Thirty adult male Wistar rats (mean weight = 356 ± 44 g) were used for the experiment. The rats were housed at 24 °C with a 12-hr light-dark cycle and fed ad libitum. They were tested at least 5 days after their arrival at the laboratory, during daytime, between 10 a.m. and 6 p.m.

Apparatus

A built-in whole-body plethysmograph based on Drorbaugh and Fenn's principle (1955) was used. According to this principle, when an animal breathes in a nondistensible chamber, the pressure in the chamber increases during inspiration, owing to addition of water vapor to the inspired gas and to warming of the inspired gas from the temperature in the chamber to that in the alveoles; conversely, the pressure decreases during expiration, owing to condensation of water vapor and cooling of the expired gas. Measurement of these pressure changes can be used to calculate breath duration (T_T), tidal volume (V_T), and ventilation (V_I, calculated as V_T/T_T) of totally unrestrained animals.

The plethysmograph consisted of three superimposed, communicating cylindrical chambers of 0.27, 0.7, and 4.5 L, respectively, from top to bottom. The upper chamber was used for gas mixing, the middle chamber served as a reference for pressure measurements, and the bottom chamber contained the animal. A 2,500 ml/min flow of dry air (MKS airflow stabilizer, Le Bourget, France) was flushed through the admission chamber and then divided into two 1,250-ml/min flows through the reference and measurement chambers. This airflow avoided CO₂ and water accumulation in the animal chamber and maintained the temperature between 23.8 ± 2.3 °C and 25.6 ± 2.0 °C. Pressure changes in the measurement chamber caused by external pressure changes or by airflow fluctuations were equalized between the measurement and the reference chambers. The difference between the pressures in the reference and animal chambers (measured with a Celesco VR pressure transducer, range: ± 2 cm H₂O; Canoga Park) was proportional to V_T according to Drorbaugh and Fenn's equation (Drorbaugh & Fenn, 1955; Epstein & Epstein, 1978). The differential pressure signal was filtered (bandwidth = 0.05–15 Hz) and converted into a digital signal (MacAdios analog-to-digital 14-bit converter; GW-Instruments, Somerville, MA) at a sampling rate of 100 Hz. A homemade software (derived from Superscope II software; GW-Instruments, Somerville, MA) detected the onset of each breath and calculated T_T and V_T. We calibrated the plethysmograph before use by injecting 200 µl of air into the measurement chamber via a syringe. The pressure rise induced by this volume was of the same order of magnitude as that produced by inhalation by the rat of one tidal volume. We introduced the value of this calibration pressure rise into Drorbaugh and Fenn's equation for V_T calculations. We estimated the concentrations of O₂ and CO₂ inside the animal chamber from the outflow value using an infrared CO₂ analyzer and a paramagnetic O₂ analyzer (Arelco, Fontenay-sous-Bois, France), calibrated with known gas mixtures. To avoid restraining the rats, body temperature was not recorded and was assumed to be stable at 37 °C (Gordon, 1990).

Conditioned and Unconditioned Stimuli

The unconditioned stimulus (US) was a hypoxic mixture created by replacing the air flow through the plethysmograph by a constant flow of nitrogen for 3 min. This caused the fraction of O₂ (F_IO₂)

inside the plethysmograph to drop linearly from 21% to 8% in 3 min 30 s and to return to the baseline level in about 10 min (Figure 1). These time characteristics of the US determined the length of the trials (15 min). The slow rise-time of hypoxia is ascribable to the relatively large dead space created by the admission and measurement chambers of the plethysmograph. A hypoxic stimulus delivered through a facial mask to a restrained animal may be more rapid, but the strong effects of restriction in rats (Lai, Tsuya, & Hildebrandt, 1978) might hamper conditioning. The residual fraction of CO₂ inside the chamber was less than 0.5%. The US elicited an approximately threefold increase in V_I (the unconditioned response).

The two conditioned stimuli (CS+ and CS-) consisted of vanilla and rose odors. Assignment of these odors to CS+ or CS- was counterbalanced among animals. We took into consideration the fact that some airborne chemicals stimulate both olfactory sensors and trigeminal nerve endings and therefore odors may either trigger activation of breathing as part of the orienting response or induce inhibitory effects because of their irritant properties. We attempted to minimize possible differences between the unconditioned effects of the CS+ and the CS- by choosing two chemicals—vanillin and phenylethyl alcohol—that are virtually devoid of irritant effects on trigeminal endings (Doty et al., 1975).

The two odors were created by bubbling the inflow to the plethysmograph in one of two bottles containing 50 ml of a solution of vanillin (1 mg/100 ml) or phenylethyl alcohol (1 ml/100 ml) for 3 min. A third bottle was filled with distilled water. A system of electrovalves placed before and after each of the three bottles controlled which gas (air or N₂) was let into the bottles and which odor (vanillin, rose, or none) was associated with the gas.

Design

The design was derived from the one used by Van den Bergh et al. (1995) in humans. To control for possible differences between the odors (e.g. salience, unconditioned effects, affective valence, etc.), vanillin was the CS+ and rose the CS- in half of the rats, and vice versa in the other half. This determined two groups of rats with similar weights and baseline ventilatory variables (Table 1).

The rats in the two groups were tested in alternation. The design of the experiment is summarized in Figure 2. Each rat was tested during two sessions on 2 consecutive days. Each session lasted 6 hr

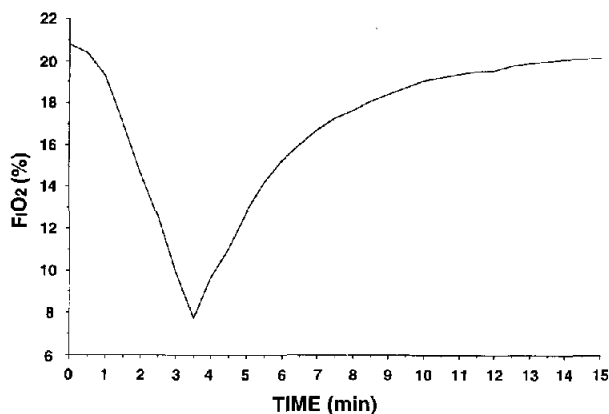


Figure 1. Unconditioned stimulus. The fraction of inspired O₂ inside the plethysmograph is FiO₂. The electrovalve controlling N₂ inflow into the plethysmograph was opened at time = 0 and closed at time = 3 min. Residual FiCO₂ was less than 0.5%.

Table 1

Mean Baseline Ventilatory Data (Plus or Minus Standard Deviations) in the Rose-CS+ and Vanillin-CS+ Groups

	CS+	Weight (g)	T _T (ms)	V _T (ml)	V _I (ml/s)
Rose (n = 15)		357 ± 46	606 ± 152	1.219 ± 433	2.253 ± 1.386
Vanillin (n = 15)		356 ± 44	674 ± 161	1.415 ± 536	2.381 ± 1.881
Total mean (n = 30)		356 ± 44	640 ± 160	1.317 ± 497	2.317 ± 1.653

Note. T_T = breath duration; V_T = tidal volume; V_I = ventilation.

15 min and comprised three series of six trials. Each series was preceded by 15 min of baseline recording. The three series of the 1st day and the first two series of the 2nd day served as acquisition. Each of these series was composed of three CS+/US and of three CS- only trials. The third series of the 2nd day served to test conditioning. It was composed of three CS+ only (i.e., without hypoxia) and of three CS- trials. Responses to CS+ and to CS- were compared to assess conditioning. Thus, each rat underwent 15 acquisition and 3 test trials. The six trials of each series (the five acquisition series and the one test series) were done in random order, with the restriction that no more than two CS+ or two CS- trials could be done consecutively. This resulted in 14 possible combinations (e.g., CS+, CS+, CS-, CS+, CS-, CS-). For each rat, the combinations of each of the six series (five for acquisition and one for the test) were sorted without repetitions.

We did not conduct an analysis of the time course of conditioning during acquisition for several reasons. First, a CS- was about twice as likely as a CS+ to be preceded by a CS+ during acquisition. Because of the arousing effects of hypoxia, this difference would have hampered a comparison of early responses to CS+ versus CS-. In addition, during acquisition, the hypoxic US was never delivered alone, and it would not have been possible to distinguish possible nonassociative changes in the response to hypoxia from conditioned changes in the response to the paired CS-. However, these potential sources of biases did not affect our test results, because no hypoxia was delivered. Our analysis of conditioning was based only on the test, with all acquisition data being excluded.

First, each rat was placed in the plethysmograph for a 1-hr familiarization period. During the five acquisition series, the animals received either paired CS+/US or CS-. Each trial lasted 15 min. During paired CS+/US trials, the electrovalves leading N₂ through the bottle containing the CS+ odor were opened simultaneously at the onset of the trial and were left open for 3 min. Then, the plethysmograph was flushed with fresh humidified air for 12 min to eliminate the odor and to reestablish normoxia (Figure 1). The CS- trials were identical except that air was led through the bottle containing the CS- odor. The odor reached the animals within about 30 s, as shown by the occurrence at that time point of sniffing (a vigorous increase in breathing frequency to 5–8 Hz) characteristic of the orienting response to new odors in awake rats. This time lag was caused by the dead space in the tubing and admission chamber of the plethysmograph. The ventilatory response to hypoxia occurred later, typically about 1 min 30 s to 2 min after the opening of the electrovalves (i.e., when the FiO₂ fell below 15%; Figure 1). This response displayed wide intraindividual differences related to differences in the level of wakefulness.

Data Reduction

Dependent variables were the breath-by-breath values of T_T (ms), V_T (ml), and V_I (ml/min), which were averaged over successive 15-s periods. Prestimulus levels were assessed by

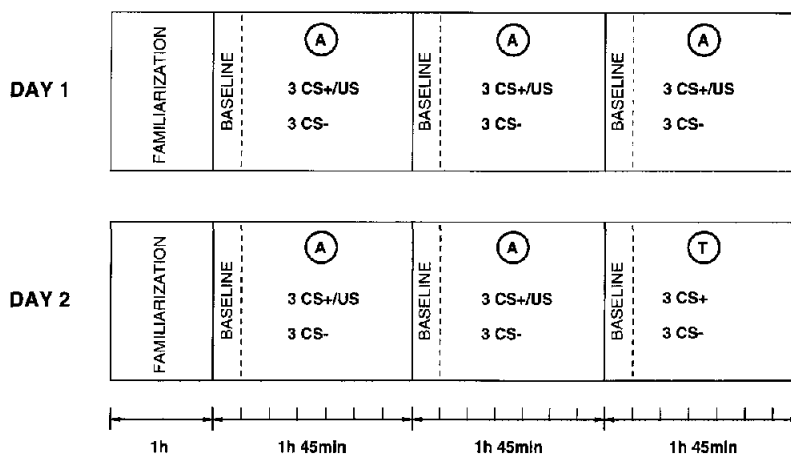


Figure 2. Summary of the design. Each baseline period lasted 15 min. During the test, the conditioned stimulus (CS)+ was presented without the hypoxic unconditioned stimulus (US). A = acquisition series; h = hour; T = test.

averaging these variables over the minute preceding the onset of the stimulus. To take into account interindividual differences in response latency, the ventilatory responses to each CS+ and CS- were considered to be the largest 1-min moving average of V_I from Minute 1 to Minute 15. We used the T_T and V_T values corresponding to this peak V_I (these values can differ from the minimum value of T_T and the peak value of V_T , respectively).

Statistical Analysis

Breathing variables were analyzed separately with repeated measures analyses of variance (ANOVA) (Superanova software; Abacus Concepts, Berkeley, CA), with the group defined as CS+ odor (vanillin vs. rose) as a between-subjects factor and with the CS type (CS+ vs. CS-) and the pre-post effect (pre-CS value vs. post CS peak value) as the within-subject factors. Pre-post changes may have been overestimated because pre-CS levels were assessed over a shorter interval than post-CS levels. However, this bias did not influence comparisons between responses to CS+ and to CS-. Acquisition data were analyzed by a trial-block factor corresponding to Series 1 to 5. To take into account the heterogeneous correlations among the repeated measurements with more than 2 degrees of freedom, we adjusted the degrees of freedom using the Greenhouse and Geisser factor (ϵ). The reported p values are based on these adjusted degrees of freedom (Crowder & Hand, 1990).

Results

Acquisition

Baseline ventilatory data were collected during the initial baseline period preceding the first exposure to CS+ or CS- (Table 1). The two groups (rose CS+ and vanillin CS+) were not significantly different for either variable. Examples of individual data are shown in Figures 3 and 4. These figures illustrate the wide interindividual variability in the magnitude and latency of the conditioned response (CR).

The ventilatory responses to CS- and CS+ during acquisition were consistent throughout acquisition (Figure 5). The V_I response to CS- exhibited small habituation effects, as suggested by the nonsignificant Block \times Pre-Post

interaction for V_I , $F(4, 108) = 2.54$, $p < .08$, $\epsilon = 0.56$; in fact, exploratory analyses revealed that habituation occurred within the first block. Between-group comparisons indicated that V_I responses to rose and to vanillin alone (i.e., when used as CS-) were not significantly different (neither the main effect for group nor interactions of group with other factors was significant). The same analyses done for T_T and V_T produced similar results. The rats responded to paired CS+/hypoxia stimuli by decreasing T_T and increasing V_T (Figure 5). These responses did not exhibit significant changes across trial blocks.

The increase in V_I from prestimulus level shown in Figure 5 was partly due to the bias introduced by calculating the response as the peak moving average over the 15-min trial, as previously noted (Method, *Statistical Analysis*). This bias can be roughly estimated by comparing the mean value of the 15-min baseline period of each block with the peak average (i.e., without any stimulus). We found that the nonspecific increase in V_I between the mean value and the peak value was $73 \pm 62\%$ on average for the entire group of animals, whereas the corresponding increase in response to CS- was $92 \pm 69\%$, suggesting that the actual response to CS- was an approximately 20% increase in V_I .

Test

Ventilatory responses were significantly larger to CS+ than to CS- (Figure 5, Test). For example, the V_I response to CS+, calculated as the percentage increase from prestimulus levels, averaged $116 \pm 85\%$, whereas the V_I response to CS- was only $55 \pm 36\%$. This conditioning effect, which concerned T_T , V_T , and V_I , was supported by the significant Pre-Post \times CS Type interactions for these variables, $F(1, 28) = 11.02$, $p < .002$, $F(1, 28) = 4.29$, $p < .048$, and $F(1, 28) = 11.02$, $p < .003$, respectively. Partial comparisons of post-stimulus values confirmed that V_I was larger (and T_T smaller) during CS+ than during CS-, $F(1, 28) = 6.35$, $p < .018$, and $F(1, 28) = 13.51$, $p < .001$, respectively.

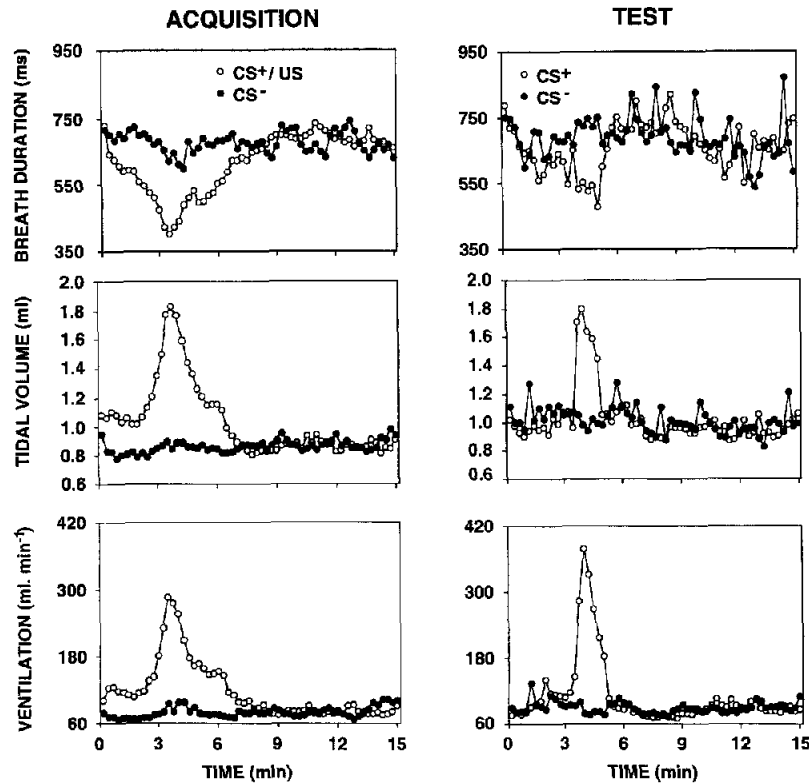


Figure 3. Acquisition and test data from 1 rat in the rose conditioned stimulus (CS)+ group. Acquisition data are averaged over the 15 CS+ and the 15 CS- trials. Test data are averaged over the three CS+ only and the three CS- trials. Each point represents the mean of breath-by-breath values over 15 s. Test data display a conditioned response with similar magnitude and latency as compared with the unconditioned response. US = unconditioned stimulus.

Comparisons of peak- V_T did not reach significance ($p < 0.118$). Prestimulus values were not significantly different for either variable.

Figure 5 also shows that the magnitude of the CR was smaller than that of the unconditioned response to hypoxia. Neither the main effects of group nor the interaction of this factor with the other ANOVA factors, including the Group \times CS Type \times Pre-Post interaction ($F < 1$), were significant. This suggested that conditioning occurred with both odors used as the CS+. Finally, neither main effect of trial nor the interaction of trial by pre-post factor were significant, suggesting that the extinction effects across the three test trials were small.

Discussion

We found that an odor paired 15 times with hypoxia subsequently elicited a conditioned increase in ventilation when presented alone. The control procedure used in our study incorporated both within-subject (CS+ vs. CS-) and between-subjects controls (vanillin as CS+ vs. rose as CS+). This established that the CR was not due to nonassociative effects, such as exposure to the context, or to preexisting differences in the unconditioned effects of each odor.

Conditioned Ventilation or Conditioned Fear?

Our data raise the issue of whether the ventilatory CR was specifically elicited by the CS or rather was mediated (a) by conditioned changes in motor activity or (b) by conditioned defense-arousal responses caused by the aversive effects of hypoxia. Behavior of the rats was not systematically recorded but was observed throughout the experiment. The first possibility is unlikely because the rats generally remained still during the trials. The second possibility deserves closer scrutiny because arousal and fear are generally associated with faster breathing rates, although not necessarily with increased motor activity (freezing can occur). Conditioned arousal or fear could theoretically account for the conditioned increase in breathing frequency seen in our study. This hypothesis is in line with the opinion voiced by Soltysik, Nicholas, and Wilson (1984) that conditioned apneas may not specifically reflect conditioned ventilatory responses, but may rather be related to the ventilatory components of emotional responses, such as those elicited by any aversive US. It is also consistent with the finding by Mongeluzi, Rosellini, Caldarone, Stock, and Abrahamsen (1996) that a single exposure to 100% CO_2 can lead to conditioned freezing. However, two arguments militate against fear and arousal being the only factors responsible

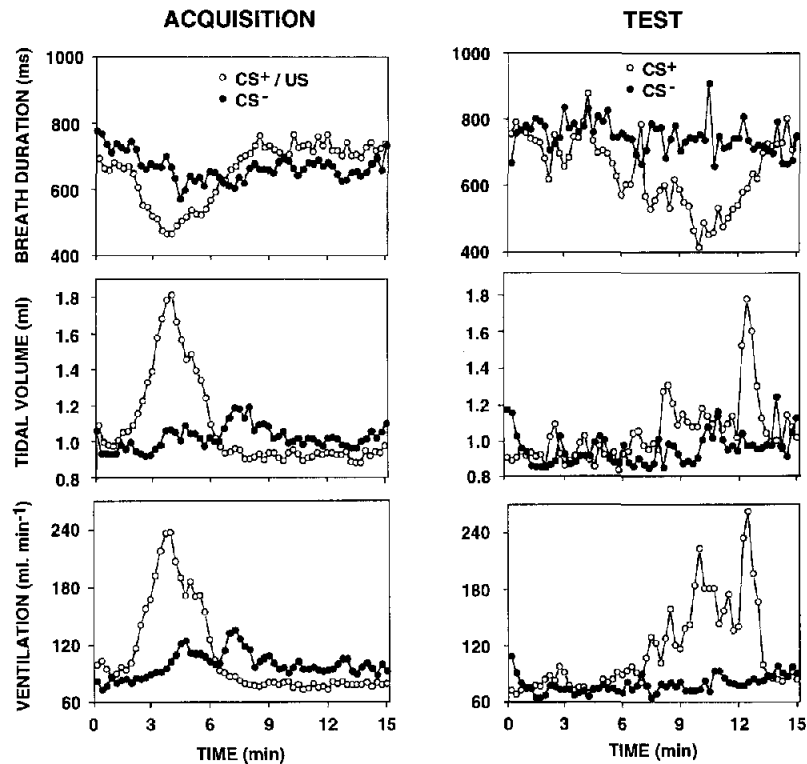


Figure 4. Acquisition and test data from 1 rat in the vanillin conditioned stimulus (CS)+ group, different from that of Figure 4. Variables are the same as in Figure 4. Test data show a conditioned response with similar magnitude but longer latency as compared with the unconditioned response. US = unconditioned stimulus.

for the CR in our study. First, we failed to observe behavioral indices of fear such as escape behavior or freezing in response to the CS during the test trials. Second, the CR consisted not only of a breathing-frequency increase but also of an increase in V_T , which is not a typical ventilatory characteristic of emotion or arousal (e.g., Soltysik et al., 1984). Thus, the ventilatory CR was not merely secondary to conditioned fear response in our study.

Classical or Instrumental Conditioning

Another issue raised by our data is whether the CR was mainly determined by instrumental rather than classical contingencies, that is, whether the rats aimed at reducing the aversiveness of hypoxia (Lc Magnen, Dugas du Villard, Hantz, & MacLeod, 1971) by increasing ventilation. This difficulty of interpretation is common to most classical conditioning experiments characterized by use of an aversive US and by a CR that reduces the aversiveness of the US. It is important to stress that both possibilities are relevant in the context of breathing under normal conditions but point to different theories of ventilatory control. As noted above, classical conditioning would explain how internal or external cues signaling exercise may trigger ventilatory responses before the occurrence of changes in metabolism. On the other hand, the possibility that conditioned breathing-pattern changes may be governed by instrumental contingencies is akin to the theory, proposed by several authors (e.g.,

Chonan, Mulholland, Altose, & Cherniack, 1990), that the spontaneous breathing pattern may be designed to minimize respiratory discomfort. This hypothesis rests on the finding that voluntary changes in breathing frequency or minute ventilation at given levels of partial pressure of CO_2 consistently intensified dyspnea sensations, suggesting that spontaneous patterns normally minimize dyspnea. Thus the conditioned increase in breathing seen in our study may have been designed to avoid the dyspneic sensations associated with hypoxia.

Arousal Effects

Several factors may account for the large interindividual variability in the magnitude and latency of the CR. First, perception of the CS odor may have been affected by the changes in levels of arousal that occurred during the 6-hr recording period. We did not assess arousal, but we observed (based on behavioral criteria) that the rats spent much of the time sleeping, especially at the end of the second experimental session when no hypoxia was delivered (i.e., during the test-trial series). A previous analysis of electroencephalograms of rats placed in a plethysmograph for 5–7 hr (Pappenheimer, 1977) showed that during normoxia the proportion of time spent in slow-wave sleep was 45%; during constant hypoxia with 10% O_2 , the proportion of slow-wave sleep fell to 27% and awakenings were more frequent (Pappenheimer, 1977). In our study, the level of

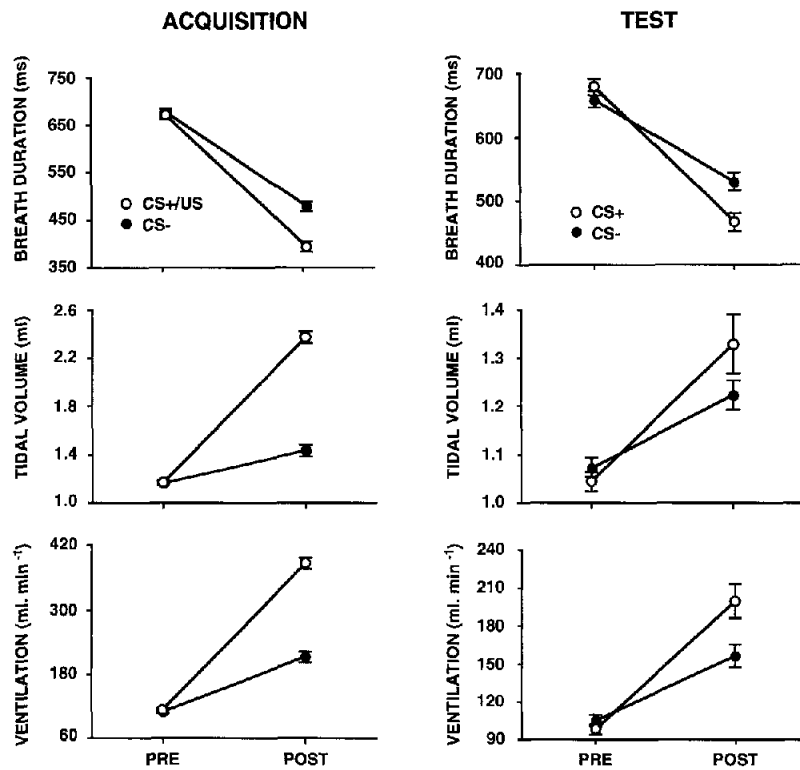


Figure 5. Acquisition: ventilatory responses to conditioned stimulus (CS+; paired with hypoxia) and CS-. Test: ventilatory responses to CS+ only and CS-. Prestimulus (pre) values were calculated as the average of breath-by-breath values over the minute preceding the onset of the stimulus. Poststimulus (post) values were determined as the peak moving average for ventilation (V_I) over 1 min from Minute 1 to Minute 15, and the breath duration (TT) and tidal volume (VT) values corresponding to this peak V_I value. Values were averaged over the 15 CS+ and the 15 CS- trials for acquisition and over the three CS+ and the three CS- for the test. Values in the figure are group means (±SEM) for the entire group (N = 30). The magnitude of the conditioned response was lower than that of the unconditioned response (note the different scales for the acquisition and test data). US = unconditioned stimulus.

arousal at the time of CS delivery during the test trials may have markedly influenced the latency time to CS perception and therefore the latency of the CR.

The fact that the rats slept for long periods of time during the acquisition and test periods does not necessarily mean that they did not undergo conditioning during these periods. Wakefulness is not a prerequisite for conditioning to occur. Delivery of paired CS-US during wakefulness can lead to CRs during sleep (Amzica, Neckelmann, & Steriade, 1997), and conditioning established during non-REM sleep in humans or during halothane anesthesia in mice can result in CRs during sleep or wakefulness (Ikeda & Morotomi, 1996; Pang, Turndorf, & Quatermain, 1996). However, the fact that each rat in our study exhibited virtually all the possible arousal levels during the acquisition and test periods may have strongly affected both the associative process and its expression as the CR, and this may explain the wide interindividual differences in CRs.

Comparison with Conditioning to Hypercapnia

Our finding that an odor paired with hypoxia subsequently elicited a conditioned increase in ventilation when presented

alone contrasts with the results of a recent experiment showing inhibitory conditioning to hypercapnia in rats (Nsegbe et al., 1997). This experiment established conditioning by pairing a 1-min tone with an 8% CO₂ US (different groups were used for paired and unpaired presentations of the CS and the US). The opposite directions of the CRs obtained with hypercapnia and hypoxia may be ascribable to differences in the salience and in the aversiveness of hypercapnic and hypoxic stimuli. Perception of CO₂ may prompt the animal to decrease ventilation to avoid the aversive effects of CO₂ (Nsegbe et al., 1997). In contrast, hypoxia was achieved in our study by adding nitrogen, which has no sensory effects (a rat can, however, discriminate between large differences such as 21% O₂ and 13–17% O₂; Arieli, 1990). We cannot completely rule out the possibility that the compressed nitrogen or the tubing leading the nitrogen to the plethysmograph may have an odor detectable by rats. If this is indeed the case, this odor may have acted as a CS, thereby partially “overshadowing” the intended odor stimulus and making conditioning more difficult (Nsegbe et al., 1997). It remains, however, that even if nitrogen delivery was associated with sensory cues, it did

not trigger the strong aversive effects seen with CO₂. This difference may have contributed to the different directions of the CRs obtained with hypercapnia and hypoxia. Another contributing factor may be the differences in CSs used in the two studies (an auditory CS for hypercapnia versus an odor CS for hypoxia). Previous studies have shown that the nature of the CS influenced the nature of the CR to the same CS (Holland, 1984; Hollis, 1997). Conceivably, different CSs paired with the same US might elicit CRs of opposite directions, although we are not aware that this has ever been demonstrated.

A further difference between our experiments and previous studies is that the conditioned increase in V_I was produced by changes in both V_T and T_T, rather than only in T_T (Gallego & Perruchet, 1991; Nsegbe et al., 1997; Van den Bergh et al., 1995). As noted above, this difference may reflect a smaller contribution of emotional or arousal processes to conditioning. In humans, even at concentrations of 7–8%, CO₂ may cause anxiety and enhanced arousal, possibly because of a belief that a noxious chemical is being inhaled (Van den Bergh et al., 1995). The US used in our study—a very gradual drop in F_IO₂—was less salient and less aversive than the hypercapnic US used in previous studies.

Putative Neurological Substrate

Under the hypothesis that the ventilatory CR is a component of the defense–arousal response, the origin of this CR may be in the hypothalamus and amygdala, which serve as integrative sites between the defense–arousal system and breathing (Waldrop & Porter, 1995; Zhang, Harper, & Ni, 1986). The tonic discharge of central amygdaloid neurons is correlated with respiratory frequency, and the ventilatory component of a CR obtained by pairing a tone with a shock in cats is attenuated by cryogenic blockade of the central nucleus of the amygdala (Zhang, Harper, & Ni, 1986). Both the amygdala and the hypothalamus contribute to the activation of breathing via their descending projections to pontine nuclei (Goldstein, Rasmusson, Bunney, & Roth, 1996; Hilton, 1982). However, our data do not provide evidence that these structures are involved in classically conditioned respiratory responses.

More generally, the neurological basis of the behavioral control of breathing is not known, but several experiments have shown that specialized cells within the brainstem respiratory complex are involved in conditioned respiratory responses (Chang, 1992; Orem & Trotter, 1994). In cats trained to stop breathing in response to a puff of ammonium hydroxide vapor, some cells within the ventral and dorsal ventilatory complex become active during conditioned apneas, whereas other cells in the same structures are inactivated (Orem & Trotter, 1994). Furthermore, the inhibition of breathing concomitant with behaviors such as back scratching in freely moving guinea pigs is accompanied by recruitment of units from the Böttinger complex and by inactivation of units in the nucleus para-ambiguus (Chang, 1992). These data suggest that changes in breathing elicited by different behavioral processes may be dependent on

activation of neuronal activities within the bulbopontine respiratory group. This mechanism could theoretically explain the conditioned ventilation increase observed in our study.

Conclusion

In conclusion, our controlled experiment produced classical conditioning to hypoxia in rats after a relatively small number (15) of paired presentations of hypoxia with an odorous stimulus. Our data confirm the high sensitivity of breathing to previously reported conditioning processes that used hypercapnia as a US and also indirectly support the hypothesis that feedforward responses may complement feedback reflex pathways in respiratory homeostasis.

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