



Use of ultrasonic vocalizations to assess olfactory detection in mouse pups treated with 3-methylindole

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Abstract

Altricial mammals use olfaction long before the olfactory bulb has reached its anatomically mature state. Indeed, while audition and vision are still not functional, the olfactory system of newborn animals can clearly process distinct odorant molecules. Although several previous studies have emphasized the important role that olfaction plays in early critical functions, it has been difficult to develop a sensitive and reliable test to precisely quantify olfactory ability in pups. One difficulty in determining early sensory capabilities is the rather limited behavioral repertoire of neonates. The present study examines the use of ultrasonic vocalizations emitted by isolated rodent pups as a potential index of odor detection in newborn mice. As early as postnatal day 2, mice reliably decrease their emission of ultrasonic calls in response to odor exposure to the bedding of adult male mice but not in response to clean bedding odors or to non-social odorant molecules. A toxin known to damage the olfactory epithelium in adult, the 3-methylindole, impairs the ultrasonic call responses triggered by exposure to male bedding, thus confirming the efficiency of this olfactotoxin on mice pups. The administration of 3-methylindole severely reduced the life expectancy of the majority of subjects. This result is discussed according to the critical role of olfaction in nipple-seeking behavior in mouse pups. © 2004 Elsevier B.V. All rights reserved.

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1. Introduction

The olfactory system allows animals to discriminate and remember odorant chemicals that bring information from their environment. Olfactory-guided be-

havior already appears in utero (Stickrod et al., 1982; Pedersen and Blass, 1982; Coppola and Millar, 1997) and plays a critical role in survival and growth of rodent neonates when other senses are not yet functional. After birth, the immediate maternal environment constitutes the major source of odorant molecules (Doty, 1986). Olfactory cues from the mother, siblings and nest associated-odors are crucial for early behaviors such as nipple localization and attachment (Blass

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and Teicher, 1980; Hongo et al., 2000), home orientation (Gregory and Pfaff, 1971; Sczerzenie and Hsiao, 1977), arousal (Schapiro and Salas, 1970) and huddling (Brunjes and Alberts, 1979). Furthermore, olfactory learning influences several genres of adult behaviors, including sexual (Marr and Gardner, 1965; Fillion and Blass, 1986), maternal (Shah et al., 2002), aggressive behaviors (Wuensch and Cooper, 1981) and food preferences (Galef and Henderson, 1972; Galef, 1992).

Experimental impairment of this system is a useful research tool for revealing functions depending on olfaction (Brunjes, 1994; Leon, 1998). However, the difficulty of assessing sensory capacity of immature animals lies in adapting functional tests that can be repeatedly applied throughout the postnatal period and are appropriate to subjects that have rather limited behavioral responses. Despite these caveats, exploration of olfactory perception and learning has been attempted in young rodents (e.g., Cheal, 1975; Leon, 1992a; Wilson and Sullivan, 1994) using various methods based on specific behavioral patterns (e.g., nipple attachment, odor-choice and classical conditioning) and physiological parameters, such as the heart rate-orienting response (Martin and Alberts, 1982; Fletcher and Wilson, 2002). Since previous studies have reported odor-induced modulation of ultrasonic vocalizations (USV) (Oswalt and Meier, 1975; Conely and Bell, 1978; Takahashi, 1992a,b; Shair et al., 1999), we carried out such a methodological approach to assess early olfactory detection.

Immediately after birth, rodent pups that have been separated from their mother or from their littermates produce ultrasonic calls that promote mother-offspring interaction (Noirot, 1968; Hofer, 1996; Branchi et al., 2001). As a result, emission of USV elicits maternal care (Noirot, 1972; Brouette-Lahlou et al., 1992; D'Amato, 1987) and acts as a location guide for maternal retrieval (Smotherman et al., 1974). On one hand, investigating olfactory function by USV analysis presents several advantages: (i) modulation of USV can be elicited by olfactory stimuli and, therefore, offers the possibility of investigating odor processes; (ii) ultrasonic calls are produced as early as P1; (iii) USV emission does not require any specific motor skill; (iv) the emission rate of USV is relatively easy to quantify. On the other hand, USV emission has been shown to be sensitive to many other non-olfactory parameters such as strain (Bell et al., 1972; Hahn et al., 1998),

age (Elwood and Keeling, 1982; Hahn et al., 1998), temperature (Okon, 1970; Allin and Blanks, 1971; Sokoloff and Blumberg, 1997; Blumberg and Sokoloff, 1998), tactile stimulations (Oswalt and Meier, 1975) and time (Shair et al., 2003). Therefore, in the first part of this study, we developed an experimental approach designed to specifically analyze odor-induced USV in mice pups, i.e. by minimizing as much as possible the influence of non-olfactory sensory cues.

Early olfactory deprivation is a very interesting approach to investigate the role of postnatal olfactory experiences on the development of behavioral phenotypes. Anosmia or hyposmia in preweaning rodents are generally induced by bulbectomy or intra-nasal administration of zinc sulfate (see, for instance, Singh and Tobach, 1975; Singh et al., 1976; Risser and Slotnick, 1987). However, due to side effects of surgery (Alberts, 1974; van Rijzingen et al., 1995; Holmes et al., 1998) and the limitation of zinc sulfate in triggering anosmia (Slotnick et al., 2000), we used an olfactotoxicant 3-methylindole (3-MI), previously found to induce extensive damage in the olfactory mucosa (Turk et al., 1987; Peele et al., 1991) and produce a dose-dependent deafferentation of the olfactory bulb in adult rats (Setzer and Slotnick, 1998a). Additionally, 3-MI has been reported to decrease olfactory performance during a conditioned odor preference test (Peele et al., 1991) and during an odor-detection task (Setzer and Slotnick, 1998b), without totally eliminating odor discrimination (Slotnick and Bodyak, 2002). Nevertheless, the effects of 3-MI in young animals have not yet been explored. Therefore, in the second part of this study, we examined the effects of the administration of 3-MI in mouse pups. These effects were investigated by measuring the emission rate of USV from 3-MI treated pups, using the olfactometric method developed in the previous set of experiments.

2. Materials and methods

2.1. Subjects

Twenty-seven C57/Bl6 female mice were bred with males from the same strain. Pregnant females were housed individually on wood-shavings in polypropylene cages under a 12:12 h light–dark cycle; all animal rooms were maintained at 20 ± 1 °C. Food and water were available ad libitum. The date of birth was desig-

nated as P0. All litters were left undisturbed until the first test day.

2.2. Behavioral procedure

Experiments were performed between 14:00 p.m. and 16:00 p.m. Each animal was randomly chosen, removed from the nest and gently placed in the transparent chamber of an eight-channel olfactometer (Slotnick and Bodyak, 2002). Fifty liters of air per min, supplied by a 6 psi aquarium pump (Hagen), was filtered through an activated charcoal canister-type filter (Cole-Parmer) and a fritted glass particle filter, and divided into a main airflow and an odor channel flow. The main airflow was connected to the odor sampling tube of the transparent chamber via a glass manifold, a 25 mm diameter glass odor mixing tube and normally open ports of a 3-way pinch valve (Angar). Sixty millilitre glass bottles (odor saturator bottles) were used to hold approximately 10 g of soiled bedding or 10 ml of liquid odorant. Separate pinch valves (Neptune Research) controlled airflow. Operating the pair of pinch valves allowed air from the odor flow meter line, via the glass manifold, to pass over the surface of the odorant material and into the main air stream. Operation of odor control pinch valves resulted in a 50 ml/min flow of headspace air from the odor saturator container into the 1950 ml/min main air stream. This produced a 2.5% air dilution of the odorant headspace. A fan on the back wall brought room air into the transparent chamber. The use of an olfactometric method presented several advantages: (i) it allowed us to expose a same pup successively to clean and odorized air without disturbing it; (ii) air flow intensity can be precisely controlled. After testing, pups were marked and put back with their mother. The temperature of the test chamber was checked before and after every test (420 Digital thermometer, Rapid Electronics) and maintained at 20 ± 1 °C. The test chamber was cleaned and dried before submitting each new pup to testing conditions.

In a first set of experiments, we analyzed the time course of the USV emission of pups one week following their birth, a period when they substantially emit USV (Branchi et al., 2001). To do this, animals were separated from their mother and siblings for 90 s and the mean number of USV during each bins of 10 s was recorded. During this period, only clean air was presented to the subjects. We thus established the ontogenetic

profile of USV production of isolated pups from P1 to P14 (n ranging from 40 to 62).

In a second set of experiments, we studied the responses of 8-day-old mice to different odors. A test session consisted of an initial isolation period of 90 s for baseline recording of USV, followed by 60 s of odor exposure. In this experiment, the rate of USV emitted during the last 30 s of isolation was compared with the rate of USV produced during the first 30 s of odor exposure. Odorant stimuli consisted either of vapor from soiled bedding from the subject's own cage ($n = 25$), soiled bedding from unfamiliar adult males ($n = 22$), a 10% mineral oil dilution of (+) carvone (Fluka) ($n = 14$) or a 10% mineral oil dilution of citral (Aldrich) ($n = 7$). As a control, USV were recorded during a total period of 150 s (90 plus 60 s) with clean air only ($n = 17$). Later, the dynamic of the male odor-induced USV response was analyzed by recording the emission of the ultrasonic calls for each 30 s block of the test session. Finally, the ontogenetic profile of this response was studied in mice pups from P1 to P14 (n ranging from 15 to 31).

In a third set of experiments using the same protocol (90 s of isolation followed by 60 s of odor exposure), only male odor was used to examine the olfactory response of pups from P6 (D1, first day after injection) to P10 (D5, fifth day after injection), following 3-MI (100 mg/kg; $n = 57$) or corn oil ($n = 52$) injection (see below).

2.3. Recording and analysis of ultrasonic vocalizations

The recording of ultrasonic vocalizations began 10 s after placing the pups in the test chamber in order to avoid measuring calls induced by handling during the transfer of the animals from the homecage to the olfactometer. USV were detected using an ultrasonic microphone (SM2 Microphone, Ultra Sound Advice) placed 10 cm above each pup and connected to a bat detector (S25 Bat Detector, Ultra Sound Advice; frequency range 10–180 kHz), which converts ultrasonic sounds into the audible frequency range. Using the broadband output of the detector with the frequency division of 1:16, ultrasonic vocalizations were sampled and recorded on a computer for further analyses.

Sound files were analyzed using an homemade software which extracts USV for automatic count-

ing. Signals were band-pass filtered between 2.5 and 7.5 kHz, thus keeping ultrasonic frequencies from 40 to 120 kHz, which corresponds to the range of emission of rodent pups. USV were extracted using an endpoint detector algorithm (Fig. 1A). This algorithm continuously compares the root mean squared (RMS) energy of a signal with the average energy of a noisy part of this signal. The reference noise is used to compute lower and upper energy thresholds. Averaged

noise is dynamically updated. Under these conditions, the time resolution was ± 5 ms. Extracted USV were then stored using the SDIF file format (IRCAM, see <http://www.ircam.fr/equipes/analyse-synthese/sdif> for further details). The mean rate of USV (USV/min) was computed for each 30 s time block. Due to technical problems, such as power supply failures which corrupted signals, some data were discarded following analysis.

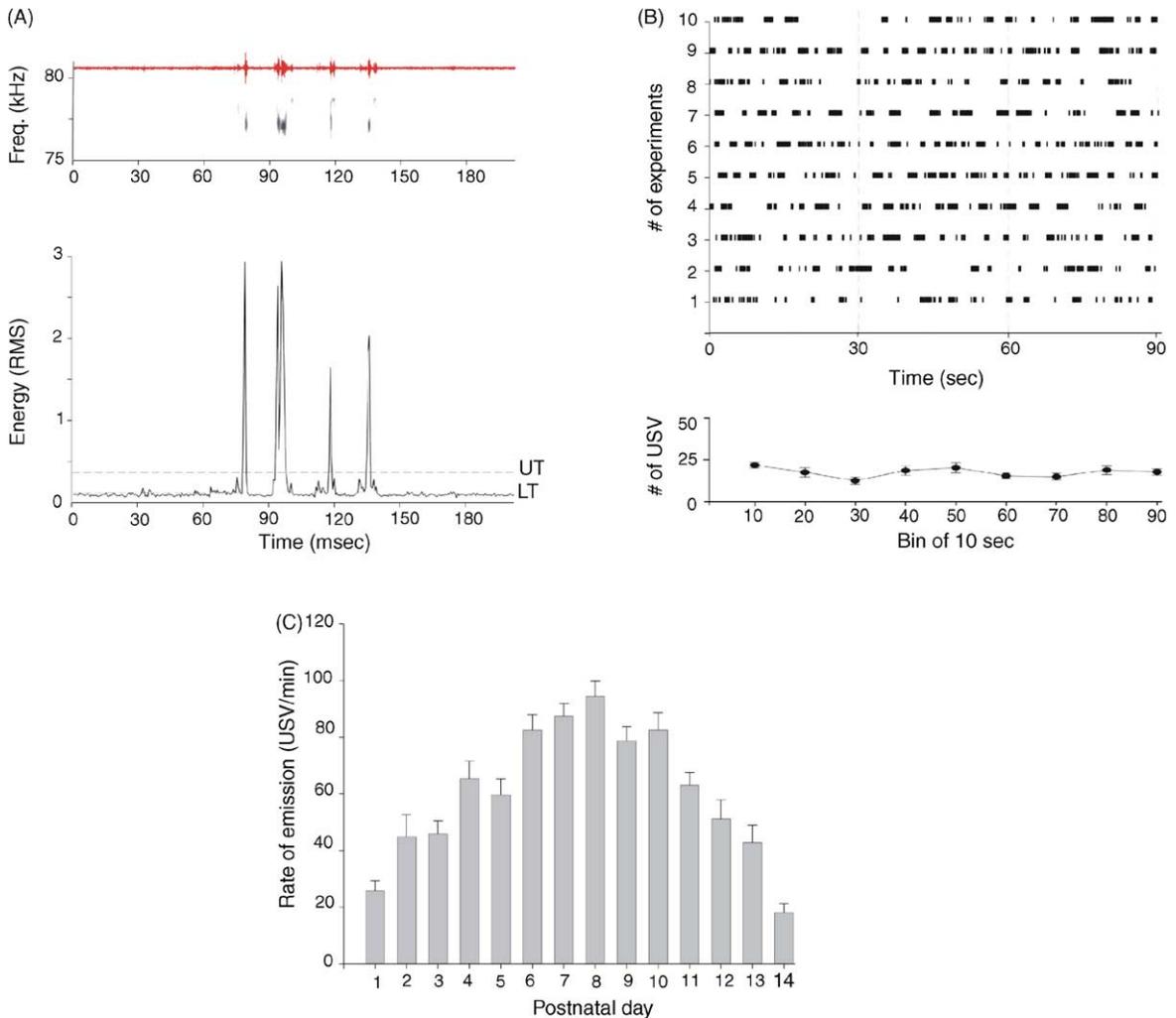


Fig. 1. Emission and quantification of USV. (A) A typical spectrogram with the corresponding USV record (top) and energy measure (below), of 8-day-old mouse, are displayed for four short ultrasonic calls. Dotted lines show upper (UT) and lower (LT) thresholds used to discriminate sounds in a noisy environment. (B; top) Time course of USV emitted by 8-day-old mice during 90 s of isolation. Each row represents recordings of USV from individual 8-day-old animals ($n = 10$) and vertical bars correspond to a single call. (B; below) Mean USV rate for each 10 s of isolation (same recordings as in B top). (C) Ontogeny of USV emitted, during the last 30 s of the isolation period, by animals tested daily from P1 to P14 (n ranging from 40 to 62).

2.4. 3-MI treatment

In the absence of studies reporting the effects of 3-MI on neonates, preliminary experiments were performed to set the 3-MI doses. Pups from a same litter were injected IP with 20 μ l of a solution containing 3-MI (99% purity, Sigma) dissolved in corn oil (Sigma) at one of three different concentrations: 100 mg/kg ($n=57$), 200 mg/kg ($n=12$) or 300 mg/kg ($n=14$). Controls ($n=52$) were injected with vehicle (20 μ l). To minimize the possible differences of USV emission between litters, an equivalent number of pups from the same litter received either corn oil or 3-MI injection. The 3-MI and corn oil administrations were done at P5, which corresponds to the day before the emission of USV reaches a maximum (see below). 3-MI was prepared immediately prior to use. After injection, animals were individually marked and returned to their mother and littermates in a clean home cage. Since mice injected with 3-MI carried a strong aroma after treatment, all litters were kept under a vented hood overnight. Pups were then weighed daily and tested for their response to odors from D1 to D5.

2.5. Data analysis

Differences in USV rates and body weights were assessed using either independent or paired Student's *t*-test as required. Differences among independent groups were tested with an analysis of variance (ANOVA). Levels of significance were set at $P < 0.05$.

3. Results

3.1. Dynamic and ontogenetic profile of USV rate during isolation

Separation of mouse neonates from their mother constitutes a permissive condition for emission of ultrasonic calls. As mentioned earlier, the emission rate of USV highly depends upon physical (temperature, tactile stimulation and duration of the test session) and social parameters (isolation, age, strain). For this reason, we recorded ultrasonic calls from C57/B16 mice daily under controlled conditions during the first two postnatal weeks and then measured the changes in

USV rate following acute exposure to odorants during a short test using an olfactometer and at constant temperature.

First, we analyzed the dynamics of USV emission during 90 s following the separation of the pups from their mother (Fig. 1). Results showed that the production of ultrasonic calls by 8-day-old mice remained constant under these conditions (Fig. 1B; top). Since the mean rate of USV did not differ between the successive 10 s bins ($F(8, 72) = 1.44$, $P = 0.19$; Fig. 1B, bottom graph), only the last 30 s of the isolation period were considered in the following experiments. Second, we established the ontogenetic profile of USV production in C57/B16 pups (Fig. 1C) by quantifying their daily calling rate during the last 30 s of the isolation period. In accordance with previous studies using other strains of mice (Bell et al., 1972; Elwood and Keeling, 1982), the ontogenetic profile of C57/B16 pups' USV rate displays an inverted U-shape curve, with a maximum between P6 and P10 ($F(13, 195) = 5.74$, $P < 0.001$).

3.2. Modulation of USV rate by odorant exposure

To determine whether recording of USV can be considered to be a sensitive method of evaluating olfactory detection, we exposed mouse pups to social and non-social odors. The odor-evoked responses of 8-day-old mice were first analyzed by comparing the mean rate of USV emitted during the last 30 s of isolation before odor presentation with that of USV emitted during the first 30 s-period of odor exposure. ANOVA analysis revealed that responses differed according to the nature of the test odor ($F(4, 80) = 3.48$, $P < 0.05$). No significant change appeared during exposure to carvone ($t(29) = -0.58$, $P > 0.05$) or to home-cage odor cues ($t(40) = 0.18$, $P > 0.05$) compared to blank conditions (Fig. 2A). In contrast, when animals were exposed to citral or shavings taken from the cage of unfamiliar adult male mice, a decrease in USV rate was observed (respectively, $57.87 \pm 12.83\% - t(22) = 2.33$, $P < 0.05$ and $36.18 \pm 10.9\% - t(37) = 2.07$, $P < 0.05$; Fig. 2A). Because unfamiliar male shavings is the most natural odor which induced a variation in the USV emission, we have used this olfactory stimulus for further experiments. Analysis of the time course of this response showed a dramatic change in vocalizations emitted by pups immediately following male odor exposure ($F(4,$

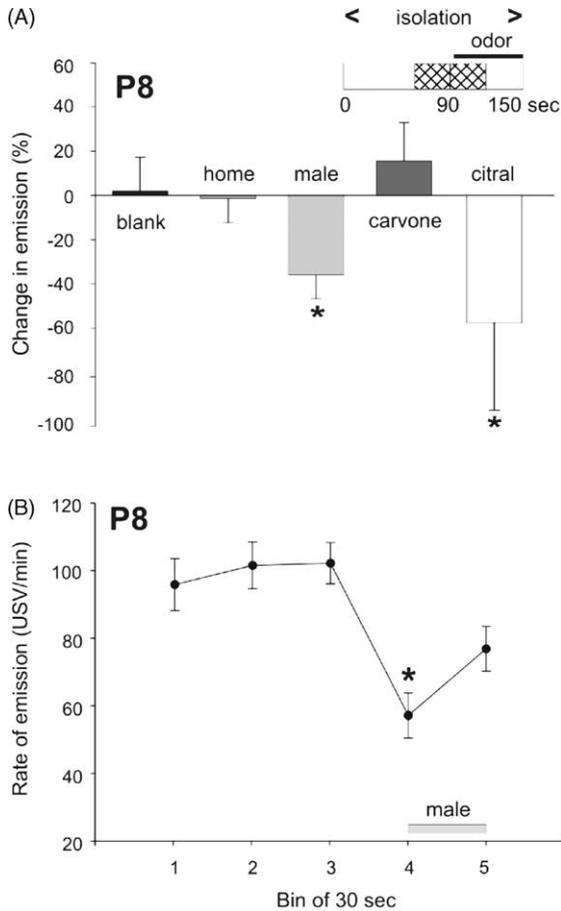


Fig. 2. Changes of USV emission following odor exposures. (A) Mean differences in the rate of USV (in percents), of 8-day-old mice, between the last 30 s of isolation (reference) and the first 30 s of odor exposure. Inset illustrates the protocol with hatching representing the test period. * $P < 0.05$ with the Student's t -test. (B) Time course of USV emitted by 8-day-old mice during isolation (periods 1–3) and male odor exposure (periods 4–5). * $P < 0.001$. Blank: empty bottle; home: shavings from the home cage; male: shavings from the cage of an adult male mouse; carvone: carvone dissolved in mineral oil (10%); citral: citral dissolved in mineral oil (10%).

84) = 9.39, $P < 0.001$; Fig. 2B). Interestingly, we also found that the male odor-induced response was transient since, at the end of the first 30 s of odor exposure, the rate of calling tends to return to the baseline, probably due to a fast habituation or adaptation process (Dalton, 2000).

Analysis of the ontogenetic profile of male odor-induced USV response (Fig. 3) clearly demonstrate that P2 pups are able to detect an odorant, and reduce their

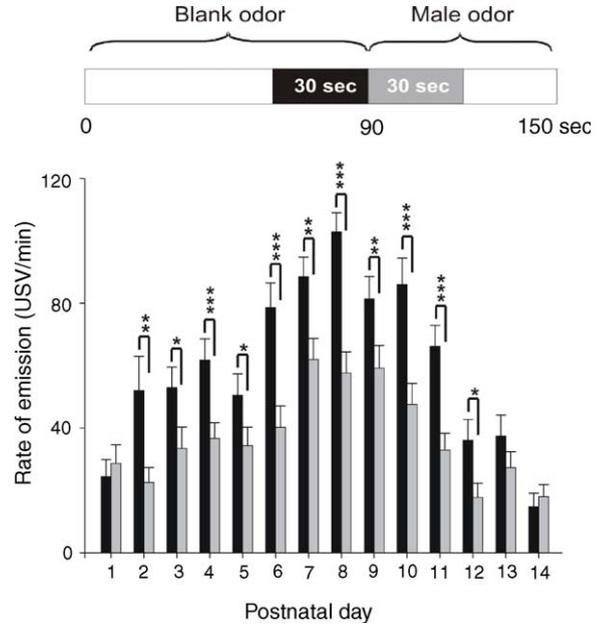


Fig. 3. Ontogeny of male odor-induced responses. In the top diagram, filled areas indicate the time frames during which the number of USV are counted. The histograms show the response of pups during the test sequence from P1 to P14 (n ranging from 15 to 31). Each bar represents the mean rate (\pm S.E.M.) of USV emission during the isolation period (black bars) and male odor exposure (gray bars). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

calling rate in response to male odor exposure from P2 to P12 ($2.12 < t$'s < 4.44 , $0.05 < P$'s < 0.001 ; Fig. 3).

3.3. Effects of 3-MI on development and olfactory abilities of mouse pups

Fig. 4A illustrates the dose-dependent effect of 3-MI on neonate survival. The survival rate was 7% ($n = 14$) with 300 mg/kg, 25% with 200 mg/kg ($n = 12$) and nearly 77% with 100 mg/kg ($n = 57$). Therefore, we used 3-MI at 100 mg/kg in further experiments. Fig. 4B illustrates the changes in body weight on successive days after the administration of 3-MI or vehicle only. The 3-MI treated pups which survived the injection weighed significantly less than control pups (treatment \times effect, $F(1, 93) = 116.17$, $P < 0.001$). Weight gain differed between the two groups over the first 5 post-injection days (treatment \times days, $F(4, 372) = 50.45$, $P < 0.001$).

We next studied male odor-induced responses in corn oil and 3-MI treated pups, to assess whether ol-

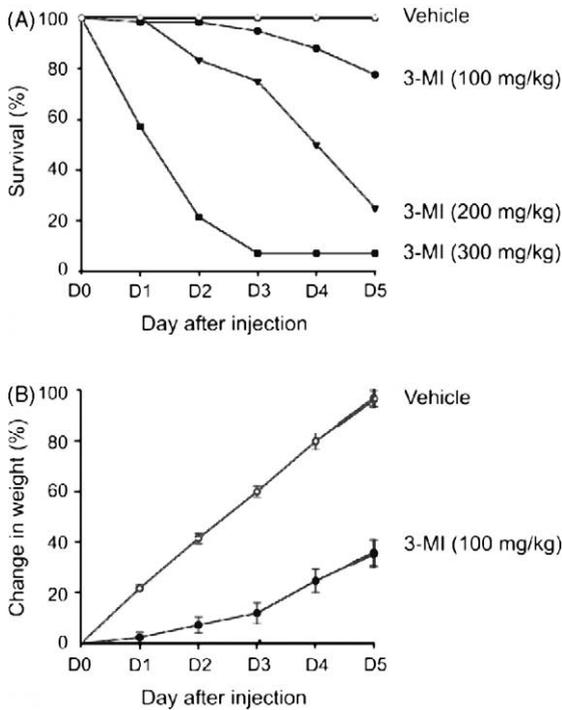


Fig. 4. Effects of 3-MI. (A) Percentage of pups surviving after treatment with 100, 200 or 300 mg/kg of 3-MI or corn oil only (vehicle). (B) Changes in body weight of 3-MI (100 mg/kg) and corn oil treated pups. The daily mean weight is calculated from the weights of all animals still alive on that day.

faction is impaired in 3-MI treated pups. Since some animals died several days following the 3-MI injection (Fig. 4A), separate analyses were performed with subjects that did not survive the treatment (lethal injection) and with those that did (non-lethal injection). Except for the first post-injection day (Fig. 5C), no significant difference was found between the two groups regarding their baseline emission rate ($F(1, 42) = 1.64, P > 0.05$). On the day following the administration of 3-MI (D1), the USV rate was significantly lower in animals receiving a lethal dose ($F(1, 43) = 11.87, P < 0.01$) than in those surviving or in control animals. As shown in Fig. 5A and B, both control and non-lethally injected animals responded significantly to male odor exposure on all test days ($2.43 < t's < -4.79, 0.05 < P's < 0.001$ and $2.12 < t's < -3.76, 0.05 < P's < 0.001$, respectively). In contrast, no male odor-induced response was observed in the lethal group ($0.04 < t's < 1.24, P's > 0.05$;

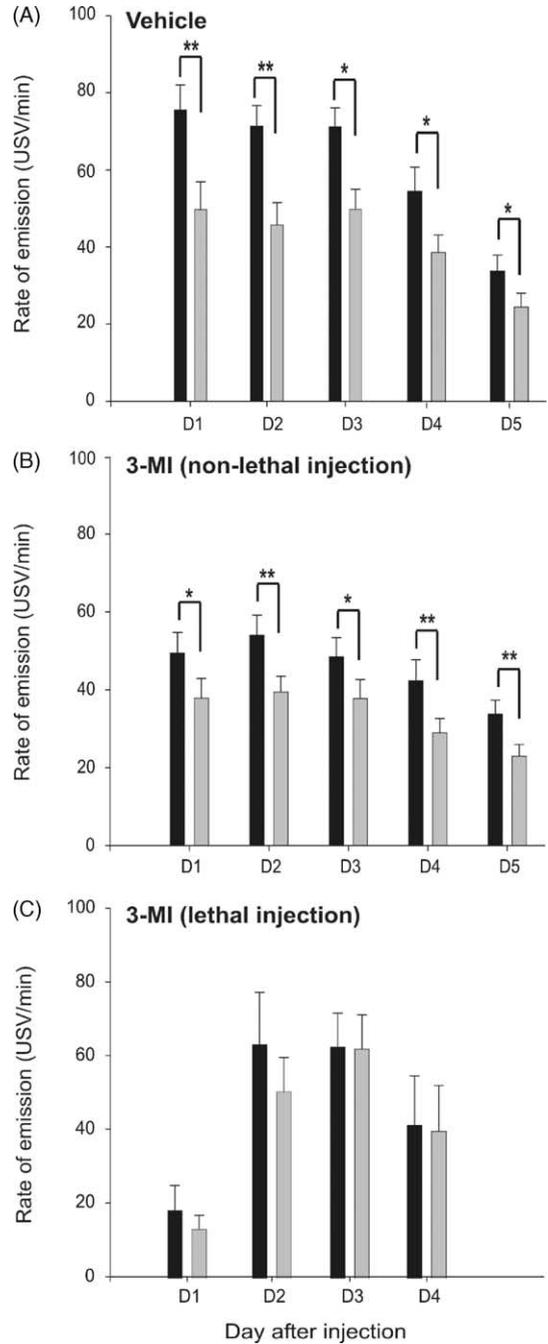


Fig. 5. Effects of 3-MI (100 mg/kg) on the response of pups to male odor. Rate of emission during the last 30 s of isolation (black bars) compared to the first 30 s of odor exposure (gray bars) for corn oil (A), non-lethally 3-MI (B) and lethally 3-MI (C) treated mice. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ —Student's *t*-test.

Fig. 5C). It is noteworthy that the number of mice tested in this group decreased daily because of mortality ($n=14$ on D2, 12 on D3, and only 8 on D4).

4. Discussion

Our results indicate that the analysis of USV constitutes a sensitive tool to explore olfactory detection capacity in neonates as well as to reveal olfactory deficits. Using analysis of the rate of USV, it appears that 3-MI is effective in inducing olfactory deficits in some mouse pups and confirms the critical role of early olfaction in pups' survival.

4.1. Odor-induced changes in the rate of USV

We report here that pre-weaning pups emit ultrasonic vocalizations following isolation and that the rate of their calls can be modified by acute exposure to odors. In agreement with previous studies (Conely and Bell, 1978; Elwood et al., 1990), we found that modulation of the production of USV depends on the nature of the odorant used. Carvone and home nest odors did not modify the rate of USV emitted by neonates, whereas citral and the odors of unfamiliar males immediately decreased their USV rate.

It has already been observed that rodent pups show specific approach behaviors depending on the given odor. They are attracted to familiar odors, such as maternal and home nest odors (Gregory and Pfaff, 1971; Cheal, 1975; Cornwell-Jones and Sobrian, 1977; Brown, 1982; Polan and Hofer, 1997; Leon, 1992b), or to synthetic odors associated with rearing environment or reinforcement (Rodriguez Echandia et al., 1982; Duveau and Godinot, 1988; Leon, 1992b; Wilson and Sullivan, 1994). Pups are spontaneously repelled by the majority of artificial odors (Cornwell-Jones and Sobrian, 1977; Brown and Willner, 1983; Amiri et al., 1998), such as citral (unpublished results). Our results strongly suggest that the rate of USV responses illustrates the preference of mice pups for the odor used. Aversive odors may elicit a reduction in the rate of USV emission whereas attractive ones may have no effect on this rate.

In line with this, Takahashi (1992a, 1994) showed that the exposure of isolated pups to an anesthetized

adult male not only suppresses their calls but also elicits behavioral freezing and elevates corticosterone and ACTH levels in preweaning rats. These results suggest that pups decrease their USV emission to prevent their localization by predators such as adult males (Elwood et al., 1990) and this strengthens our hypothesis that USV responses reflect olfactory preferences.

Interestingly, Conely and Bell (1978) reported that P3 rats already respond to odors taken from shavings soiled by adult males. In contrast, Takahashi (1992b) found that the ability of rat pups to respond to an anesthetized adult male emerges only at 12 days of age. This discrepancy may result from the type of stimuli used, i.e. soiled bedding and unfamiliar male in Conely and Bell's and in Takahashi's studies, respectively. In the present investigation based on the use of soiled bedding, we found that mouse pups respond to male odors as soon as P2 and that this response persists until P12.

The present results argue that pups specifically respond to olfactory cues. Indeed, it is unlikely that the change in the emission of USV during the testing session results from non-olfactory cues since the emission of USV during a 150 s-isolation period in clean air remained constant (see the blank in Fig. 2A). Furthermore, the USV response to a variety of odors is ethologically relevant since it does not require any conditioning or initial training and can be completed within less than 150 s.

4.2. Importance of olfaction for mouse pup survival

Our results showed that 3-MI (100 mg/kg) treatment induces a lack of male odor-induced USV response in some mouse pups, confirming that the treated mice display impaired olfactory-mediated behavior.

Interestingly, the 3-MI treated animals which did not survive the injection had no milk in their stomach. Moreover, the time course of the body weight loss in the lethal group was comparable to that found in pups housed with non-lactating females (data not shown). This strongly suggests that 3-MI treated animals that did not respond to the exposure of the male odor might have experienced anosmia or hyposmia and failed to thrive due to an impaired nipple-seeking behavior (Setzer and Slotnick, 1998b). Hence, it is well established that in neonatal rabbits, rats and mice, nip-

ple searching and suckling behaviors are critically dependent upon olfactory cues (McClelland and Cowley, 1972; Singh and Tobach, 1975; Singh et al., 1976; Distel and Hudson, 1985; Risser and Slotnick, 1987). In line with this, transgenic mice showing impaired olfaction have been reported to have a high death rate (e.g., Brunet et al., 1996; Hongo et al., 2000).

It has been demonstrated that high concentrations of 3-MI induce peripheral injury such as pulmonary mucosal lesions in adult mice (Turk et al., 1986). However, the ability of the pups in the lethal group to spontaneously emit USV during the baseline isolation period at the same rate as pups in the non-lethal group, as well as in the vehicle group, from D2 (P7) to D4 (P9) weakly supports a primary 3-MI-induced sickness behavior in those pups. Further studies aimed at examining the degree of damage of the olfactory mucosa, as well as peripheral injury, need to be performed.

Sensory deprivation is an extensively used procedure to investigate the role of epigenetic factors, such as experience-induced activity, on the structural and functional development of sensory systems. By suggesting that olfactory deprivation impairs suckling in neonates, leading to a high death rate, the present study clearly indicates that such a procedure still remains a challenge when applied to newborn rodents. However, this study demonstrates that a careful analysis of USV responses, combined with the use of olfactometric methods, represents an adequate means to investigate olfactory detection in transgenic or treated young mice. Additionally, since the present results show that pups also respond to artificial odors by changing the rate of USV, one could use USV analysis for odor discrimination and threshold detection paradigm.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.beproc.2004.09.001](https://doi.org/10.1016/j.beproc.2004.09.001)

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