

Ontogeny of Olfaction: Development of the Rats' Sensitivity to Urine and Amyl Acetate

JEFFREY R. ALBERTS AND BRAD MAY

Department of Psychology, Indiana University Bloomington, IN 47405

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ALBERTS, J. R. AND B. MAY. *Ontogeny of olfaction: Development of the rats' sensitivity to urine and amyl acetate.* *PHYSIOL. BEHAV.* 24(5)965-970, 1980.—Rat pups, 1 to 17 days of age, were tested for sensitivity to two olfactants, amyl acetate and adult rat urine. Biological and non-biological olfactory stimuli were generated by sparging and delivered to subjects via a dilution olfactometer. Unconditioned respiratory responses, odor-induced polypnea and sniffing, were used to measure detection of an odorant injected into a background stream of filtered air. Amyl acetate was presented in an ascending series of concentrations. Pups of all ages detected amyl acetate; chemosensitivity increased with age to all 3 concentrations of this nonbiological stimulus. Two concentrations of adult rat urine odor were equated to the strength of amyl acetate for 9-day-olds. Each normalized urine stimulus was then tested across the full range of age groups. Again, there was a dramatic age-related increase in chemosensitivity. Moreover, there was no indication of differential sensitivity to these biological and non-biological olfactants. These data were discussed within methodological and conceptual frameworks related to analyses of early olfactory and behavioral ontogenesis.

Amyl acetate Olfactometry Olfactory development Olfactory sensitivity Sniffing Urine odors

OLFACTORY stimuli play numerous vital roles in sustaining, directing, and shaping the early life of infant rodents. Within minutes of birth the blind and deaf neonates use specific chemical cues to locate nipples [15, 25, 26]. Proximity to the homenest and to littermates during the vulnerable early period depends on olfactory input [3, 9, 14]. Initial adaptation to the complex world beyond the nest is accomplished by utilizing chemical cues [2, 12, 17]. In view of the crucial importance of olfactorily-guided infant behavior, it is surprising to find that the olfactory system of altricial rodents is anatomically and physiologically incomplete. Many neural groups and synapses prominent in the adult olfactory bulb are partially formed or absent in the pup, implying that olfactory-perceptual functioning must also be incomplete during early life.

There is, in fact, indirect behavioral corroboration for the idea of early developmental improvement in olfactory-perceptual function. The range of odors that affect tonic motor activity and orienting movements of neonatal rats broadens with age [13, 21, 27], suggesting that only some olfactory stimuli are detected by newborns. Infant rodents readily orient to and approach the odors of a homenest, but they do not discriminate between familiar and novel nest odors until somewhat later in life [14]. Rat pups use olfactory cues for social contact behavior as early as Day 5 but they do not accurately recognize the scent of their species and prefer it over another species' scent until about Day 15 [4], the stage at which pups begin to approach the odors of a lactating dam [19]. These studies, however, were primarily inves-

tigations of behavioral development and the experiments were not intended to address directly questions of olfactory ontogenesis.

Nevertheless, all available evidence implies that behavioral development in rodents is linked to, or perhaps even determined by olfactory development [2]. To understand further the relationship between behavioral and olfactory ontogenesis we must evaluate the development of olfaction with tests that better separate sensory and behavioral maturation.

Unfortunately, there are no quantitative or systematic studies of the development of olfactory perception in rodents (e.g., detection, adaptation, discrimination, recognition), despite the fundamental importance of this sensory channel. One cause for this gap in knowledge is the need for measures of odor perception in neonatal rodents; their limited strength, endurance and behavioral repertoire preclude use of most conventional olfactometric techniques, which typically require extensive training and fairly rigorous performance demands.

We describe a method for measuring the detection of controlled quantities of odors by rat pups from 1 to 17 days of age. We used this method to ask some basic questions about olfactory development: Does olfactory acuity improve (or decline) during early life? Is there an enhanced degree of chemosensitivity for biological substances compared to non-biological, "chemical" olfactants? Is there a difference in the development of chemosensitivity for a biological versus a chemical odor of equivalent strength? In this prelimi-

nary study we examined the ontogeny of chemosensitivity to the nonbiological odorant, amyl acetate, and to the odor of conspecific urine.

EXPERIMENT 1: SENSITIVITY TO AMYL ACETATE

Experiment 1 was an olfactometric study of the development of chemosensitivity to a standard olfactant, amyl acetate. Strain gage plethysmography was used to measure odor-induced respiratory change. We recently described an extensive developmental investigation of respiratory rate and sniffing in the rat pup, using this non-invasive method for recording thoracic movements [5]. We found that repeated presentations of suprathreshold olfactory stimulation induced polypnea (accelerated nasal respiration) and sniffing in 1- to 20-day-old rats. Odor-induced polypnea should therefore be an excellent measure of odor sensitivity in neonates since it requires no training and involves a basic, organismic response to a perceived change in olfactory environment. This technique was used to construct odor sensitivity curves for the developing pup.

METHOD

Subjects

A total of 320 Sprague-Dawley rat pups were used as subjects in this experiment. Each pup was tested once, at 1, 3, 5, 7, 9, 11, 13, or 17 days of age (day of Birth=Day 0). Each age group was comprised of pups from at least 3 different litters.

All subjects were bred and born in the Indiana University colony, outbred from stock originally purchased from Laboratory Supply, Inc., Indianapolis. Litters resided in polypropylene maternity cages (48×20×26 cm) on a substrate of pine shavings. The colony was maintained on a 16:8 light/dark cycle. Testing occurred during the light phase. Purina Rat Chow and water were available ad lib.

Apparatus for Odor Delivery and Olfactometry

Our odor delivery system is illustrated schematically in Fig. 1. Room air was driven by a diaphragm compressor through a series of columns of activated charcoal (about 14 kg) and then through a column of indicating calcium sulfate (Drierite). The filtered, dried air then passed through a final column of activated charcoal before being re-humidified by bubbling through 100 ml of distilled water in a gas washing bottle. The total flow line was then divided into 2 streams. The upper stream in Fig. 1, regulated by a teflon reducing valve (Mace Co., El Monte, CA) and calibrated flowmeter (Manostat, N.Y., NY), was used as a clean air "carrier" stream to which odor streams were added. The remaining stream was divided into four independently regulated substreams (teflon reducing valves and Manostat flowmeters). Each of these substreams was bubbled through separate gas washing bottles containing measured amounts of olfactant. By varying the concentration of olfactant in solution, odorant concentration was varied in each odor stream. Glass wool traps were used to capture aerosols in the effluents from the odorizing bottles. Odor streams then passed onto a system of parallel teflon Skinner valves, arranged so that each line could flow into a common port and then to the outdoors, under negative pressure. A remote set of electronic switches permitted any of the odor streams to be quickly re-directed away from the outgoing port and into the nearby manifold, where it mixed with the carrier stream. The com-

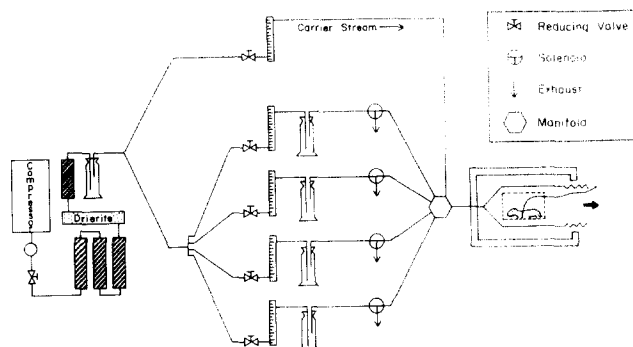


FIG. 1. Odor-delivery system. Substreams from any of 4 washing bottles could be directed into manifold to join the clean air carrier stream and pass through the subject's compartment.

bined carrier and odor stream then passed through the subject's compartment which was contained in a glass tunnel, fabricated by fusing a pyrex funnel to a 10.2 cm diameter glass cylinder (35 cm long). The subject's compartment was a cylindrical cage (10.2×7.6 mm) made of 6 mm hardware cloth, hinged to open along its length, and provided with a solid floor and upturned border, so that the pup rested on a shallow substrate of wood shavings. The tunnel and compartment were housed within an insulated, temperature-regulated chamber with transparent front panel; tunnel temperature was 28° C. At the distal end of the tunnel a connection was made to a plastic exhaust hose (adapted from 4 in commercial dryer hose) and fan that assisted removal of the airstream to the outdoors by gentle negative pressure.

The design of this continuous-flow odor delivery system has several noteworthy features. Because odor streams flowed constantly at a regulated rate, and were switched very rapidly (14 msec) into the manifold, there was little backpressure to cause a sudden, initial surge in concentration with entry of the odor stream. Similarly, the ratio of carrier stream airflow to odor stream flow was 16:1, so injection of the odor stream into carrier stream did not cause a large change in net flow.

Maintenance of Odor-Delivery System

The glass wool filters were removed and replaced after every 7 hr of use. All olfactometer components downstream from the sparging vessels were made of glass or teflon. The entire system, from the gas-washing bottles to the animal chamber was dismantled, washed, and dried every 4 working days. At these intervals the charcoal was reactivated by baking overnight at 300° F. The Drierite was restored by baking when the color indicator showed hydration.

Evaluation of Odor-Delivery System

Production of olfactory stimuli and measurement of responses in a chemosensitive recipient are notoriously complex problems that have traditionally dominated the practice of olfactometry. It is important to evaluate the design and limits of our apparatus and procedure in a framework used to describe other methodologies [10].

The method of odor-delivery used in the present experiment was designed to be a reliable, flexible, and replicable system for delivering a controlled series of concentrations of chemical and biological odors at constant flowrates, and to measure an unconditioned response in subjects varying in

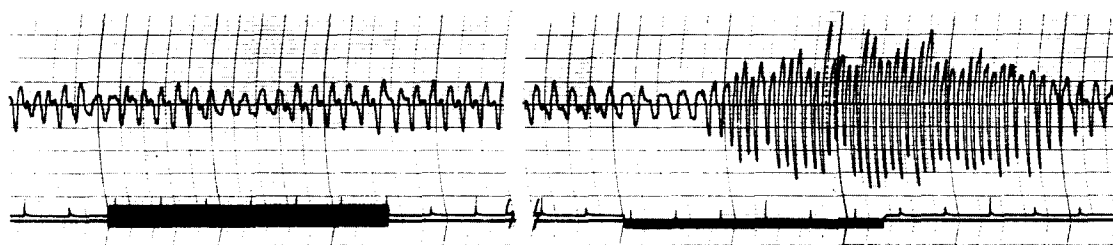


FIG. 2. Respiratory record of a 10-day-old presented with a clean air control injection (double deflection) that did not significantly alter respiration and, later, a 6 sec pulse of an odorant injection that induced polypnea.

size and behavioral repertoire. Odorant stimuli were generated by sparging, and concentration was determined by a solvent dilution method. Stimulus concentration was thus determined, in part, by the concentration of odorant in solution as well as in the air above the solvent. Concentration of odorant in headspace is proportional to the concentration of odorant in the solution by a dynamic "activity coefficient" that varies for each substance, solvent, concentration, and temperature (see [10], pp. 7-9; 12-15). These factors, along with the unknown contribution of odorants attached to aerosols trapped in the glass wool filters, prevent accurate specification of the molecular concentration of our stimuli without direct assessment by chromatographic measures and we have avoided such inaccuracies. Nevertheless, the standardization used in our odor delivery method, together with the log-unit concentration differences in our stimuli, meet Dravnieck's criteria for undertaking a preliminary analysis of chemosensitivity.

Odor-Induced Respiratory Responses

Respiratory change (acceleration) was measured by strain gage plethysmography. The strain gages were mercury-filled silastic loops matched to the size of the pups [5]. Output of the plethysmograph was displayed on a Grass polygraph. The switches that controlled odor injection were wired to activate the event channel on the polygraph for the duration of odor injection. Figure 2 is a tracing from a typical record. Respiratory rate is seen to be stable, at 3 cps. The double deflection of the time-event channel denotes a period during which a "blank" odor stream was injected into the carrier. There was no reaction. The downwards deflection on the event channel shows a 6 sec pulse of odorant injection. About 3 sec after odor injection the pup responded, as can be seen by the large deflection and burst of accelerated respiration (polypnea) that reached 6 cps and extended beyond the duration of odor injection, probably until the odorant molecules had passed. Transient accelerations (<1 sec) or movements without polypnea were not scored as a response. Interobserver reliability judging positive responses was 0.99.

Our previous work on the development of odor-induced sniffing in rat pups indicated that respiratory reactions (polypnea and patterned sniffing) can be reliably and repeatedly elicited from rat pups [5]. Although the sniffing reaction is a reliable measure of odor detection it is probably not a powerful measure of threshold. Nevertheless, the present method has the advantage of using an untrained, natural reaction of the animal so that it can be used with newborns as well as with pups in more advanced stages of development.

Parameters and Testing Protocol

After temperature and flowrates were calibrated in the

olfactometer, pups were taken directly from the maternal nest, quickly fitted with a strain gage, and gently placed in the wire test chamber. Thus contained, the subject was inserted into the glass tunnel (Fig. 1) which was then sealed for ventilation.

There were age differences in reactions to the novel test compartment. It was therefore standard procedure to wait 1-min, 3-min and 5-min before beginning to test 1- to 5-day-olds, 7- to 13-day-olds, and pups more than 13-days respectively, so that a stable pre-stimulus baseline could be recorded. Certain characteristics of the subjects' compartment were essential for reliable performance by the pups. If the "comfort" qualities of the test compartment were sub-optimal, pups acted agitated and did not orient or react to stimuli. If conditions were made "too comfortable" pups appeared to fall asleep readily and failed to react. Our pilot tests indicated that a moderately warm, 28° C ambient temperature, and the presence of a soft substrate of pine shavings in the test compartment were both necessary characteristics for reliable testing.

Amyl acetate was prepared in log-units of concentrations: 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} parts of amyl acetate to distilled water. A "blank" stimulus was filtered air passed through pure distilled water. The sparging vessels (250 ml gas washing bottles) each contained 100 ml of solution. The carrier stream was regulated at 4 liters/min. Each odor stream was maintained at 250 ml/min so that the net flow rate during stimulus presentations was 4.25 liters/min. Increasing net flow decreased response latency, indicating that respiratory reactions were related to characteristics of the air stream, rather than switching noises. We refer to the strength of amyl acetate stimuli in terms of solvent concentrations: 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} since the concentration of olfactant in the air stream was not known to the same degree of accuracy.

Pups were tested with a series of amyl acetate presentations in order of ascending concentration. Following habituation the pups' respiration was monitored until a stable respiratory rate was established for a minimum of 10 sec. The solenoid valves were activated to switch in the "blank", i.e., the airstream traveling through the plain distilled water solution (see Fig. 2). Typically, pups did not respond to this event, i.e., the small pressure transients, and switching noises were below threshold for a discernible response. If pups showed a reaction correlated with presentation of the "blank", the test series would not begin until at least two consecutive presentations were made during which no response was shown. This precaution was only necessary, on occasion, with the older pups.

Each amyl acetate presentation was made for 20 sec, beginning with the most dilute stimulus. The polygraph record was used to monitor and score respiratory reactions. If there

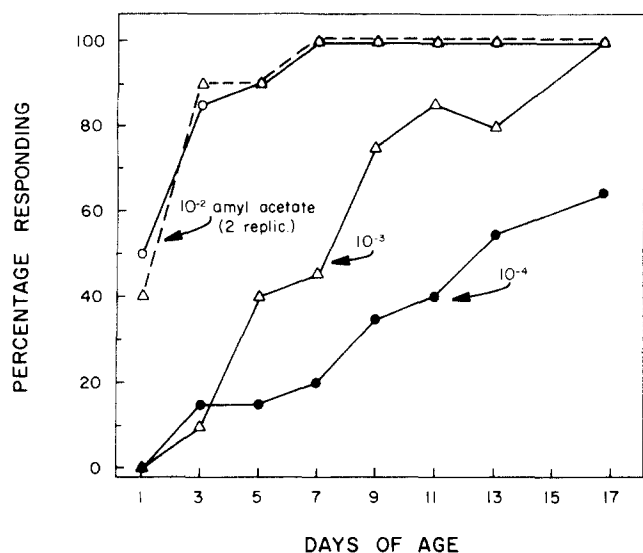


FIG. 3. Development of sensitivity to three concentrations of amyl acetate presented in ascending order of concentration. Independent groups of pups were tested with the 10^{-4} and 10^{-2} dilutions (circles) and the 10^{-3} and 10^{-2} dilutions (triangles). $N_s=20$ per age for both replicates.

was no response to the first presentation the same stimulus was presented again after a 30 sec interval. After two negative trials the next higher concentration was similarly presented.

In the first test battery, pups ($n=160$) were tested with the "blank" stream and then odor streams created by passing filtered air through solutions of 10^{-4} and 10^{-2} parts amyl acetate in distilled water, in ascending order of concentration. The second test battery, using naive pups ($n=160$), was identical to the first, except that a mid-concentration of amyl acetate, 10^{-3} , was used in place of the most dilute stimulus (10^{-4}); the 10^{-2} was retained in the series. By combining the data of all 320 pups, developmental sensitivity curves were constructed for the 10^{-2} (two replicates), 10^{-3} , and 10^{-4} amyl acetate stimuli.

We wanted to avoid "false negatives", i.e., failure to react due to some competing activity. It was possible that a pup might fall asleep or display persistent agitated movements and therefore fail to emit a measurable response. To eliminate recalcitrant "non-responders", a final odor injection containing 10^{-1} amyl acetate (intense stimulus) was presented to pups that showed no response on any of the standard trials. Pups that failed to respond to the 10^{-1} amyl acetate were not included in the data analysis (but are reported below). Thus, all pups included in the computed results were responding to a concentration of 10^{-1} amyl acetate or a more dilute stimulus.

RESULTS

The results of the present experiment indicated that sensitivity to the odor of amyl acetate increases dramatically during early postnatal development. Figure 3 summarizes the findings. Although the weaker stimuli (10^{-4} and 10^{-3} concentrations) failed to elicit detection responses from the neonates, the same pups responded to the stronger concentrations (10^{-2} and 10^{-1}). Each age group displayed more re-

sponding to higher concentrations of amyl acetate until about 11–13 days when asymptotic performance imposed a ceiling effect on the series. It was necessary to reject 13, 1-day-olds to accumulate 20 suitable subjects in the first ascending series; in the other age groups no more than 4 pups had to be rejected.

For each concentration of amyl acetate there was a clear, age-related increase in the percentage of pups responding. Kruskal-Wallis analysis of variance revealed significant age effects for all test concentrations ($p_s < 0.01$). The reliability of the age-sensitivity relationship is emphasized by the orderly pattern of results across concentrations and the replicability of 10^{-2} test, shown in Fig. 3.

EXPERIMENT 2: SENSITIVITY TO URINE ODORS

The present experiment was designed to measure the development of olfactory sensitivity to a naturally-occurring biological stimulus, viz, the odor of rat urine. We were particularly interested in comparing the development of chemosensitivity to a natural, species-relevant cue with sensitivity to the "arbitrary" olfactory stimulus used in the previous experiment. There are many examples of perceptual biases in the animal world, such as perception of polarized light by bees [11], high frequency acoustic patterns by bats [23], and the dramatic effects of specialized olfactory stimuli called pheromones [6,22]. In this experiment we were asking, in effect, whether the rat pups' olfactory system is "tuned" or "biased" during development to be differentially sensitive to a rat odor.

Unfortunately, it is not yet possible to equate chemical and biological substances for their intensity as olfactants on the basis of their physical properties. The active constituents of complex biological products lack adequate identification and the functional dimensions of olfactory cues are not known. To solve the problem of comparing stimuli of unknown intensities, we employed a method to "normalize" the strength of urine and amyl acetate with respect to each other for a single age group, and then compared the course of development of sensitivity to these cues. Specifically, we established concentrations of rat urine odor that elicited responses from 9-day-old rat pups equal to the response levels to 10^{-4} and 10^{-3} concentration of amyl acetate found in Experiment 1. These age-concentration combinations provided mid-range baseline levels that could reveal either potentiated or attenuated performance. Thus, the question was whether pups younger and older than 9-days would be differentially sensitive to these normalized concentrations of urine odors in comparison to amyl acetate.

METHOD

Subjects

Twenty rat pups were used as subjects in each of the 8 age groups tested in the two replicates of this experiment ($N=320$). Each pup was tested only once, at 1, 3, 5, 7, 9, 11, 13, or 17 days of age. Animals were reared under the conditions described in Experiment 1.

Procedure

Independent empirical tests indicated that filtered air (500 ml/min) bubbled through 100 ml of 10% and 25% solutions of adult rat urine (vol/vol in distilled water) and mixed with 7.5 l/min of clean air elicited polypnea in 35% and 70% of the 9-day-olds tested. Urine was collected overnight from pairs

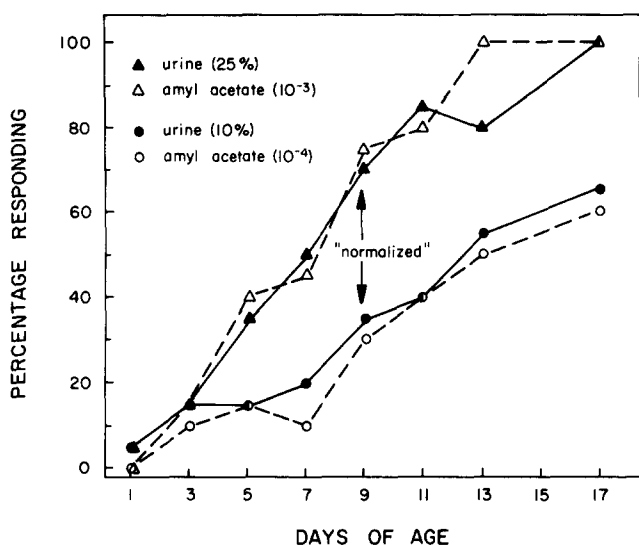


FIG. 4. Development of sensitivity to 2 concentrations of conspecific urine odors (solid lines), compared to the development of chemosensitivity to amyl acetate. The urine stimuli were adjusted to match the strength of the amyl acetate dilutions for the 9-day age group. $N_s=20$ per age for each concentration of both stimuli.

of nulliparous adult, female Sprague-Dawley rats housed in stainless steel metabolism cages. No attempt was made to control for the donors' estrus conditions because urine samples were pooled and used randomly for testing. The 10% and 25% odor streams were injected into the manifold for 6 sec and 20 sec, respectively in these tests. Following statistical confirmation that the 9-day-olds' responses to urine were equivalent to their amyl acetate sensitivity, the full cross-sectional age series was completed with pups 1- to 17-days of age.

The test protocol was a simplified version of the previous method. Each pup was tested with the "blank" stimulus (clean air through distilled water), then the urine stimulus (either 10% or 25% solution in distilled water) and, if necessary the 10^{-1} amyl acetate test to determine if the pup was a responder. As in the first experiment, subjects that failed to emit a discernible response to any stimulus including the 10^{-1} amyl acetate cue were omitted from the study. Experiment 2 involved two replicates in which 1- to 17-day groups ($n=20$ per group) were tested with either the 10% or 25% urine solution effluent. In these series only 7 and 3 newborns were rejected. In one group of 9-day-olds, ten pups failed to respond before 20 suitable subjects were tested.

RESULTS AND DISCUSSION

There was significant, age-related increases in sensitivity to both concentrations of urine odors (Kruskal-Wallis tests). Separate chi-square tests indicated that the stronger urine stimulus elicited greater response levels beginning in Day 5. The solid lines in Fig. 4 shows the full sensitivity curves to urine odors. Figure 4 also shows the outcome of the intended comparison between the development of sensitivity to amyl acetate and urine odors normalized for the 9-day-old age group. It is readily apparent from the graph that the ontogenesis of sensitivity to these substances is identical for both dilutions tested. Hollander's test for parallelism [16] and chi-square tests confirmed this statistically. We must

therefore conclude that we have found no evidence that the olfactory system of the developing rat is either specially tuned or biased to detect biological odors more readily than an arbitrary, non-biological chemical scent. Naturally, the generality of this conclusion cannot be assessed solely on the basis of data from only 2 substances. Amyl acetate was chosen as the olfactant for the initial experiment because it is widely recognized as an exceptionally efficacious, broad-spectrum olfactory stimulus. It remains possible that rat pups are, in general, more sensitive to biological scents than to chemical odors, but that amyl acetate is an unusually potent chemical cue.

GENERAL DISCUSSION

We have described the early postnatal appearance and development of chemosensitivity in the rat. Chemosensitivity was assessed by measuring respiratory acceleration, polypnea, induced by a detectable airborne chemical stimuli delivered to pups in a dilution olfactometer. Newborns (1- to 5-days) reliably detected a relatively strong concentration of amyl acetate in the olfactometer but they displayed no reaction to more dilute concentrations of the same substance to which their older counterparts responded. Using two ascending series of amyl acetate concentrations, we found consistent, monotonic age-related increases in sensitivity. The development of chemosensitivity to a species-typical biological product, conspecific urine odors, followed the same developmental pattern found with the pure chemical stimulus. Moreover, when the relative strengths of the two odorant substances were "normalized" by equating their efficacy on 9-day-olds, the levels of sensitivity were identical through the developmental period. There was no perceptual bias in sensitivity. Previous research has established that the rat pups, including neonates, possess some degree of chemosensitivity [2], but the present paper is, to our knowledge, the first attempt to make a cross-section comparison from newborns to weanlings and to establish quantitative measures of sensitivity across these ages.

The present experiments successfully employed odor-induced polypnea as a measure of detection [5]. Again, it is important to emphasize that the method of odor-delivery, range of stimulus concentrations, and the response measure were not intended to define true olfactory "thresholds", that is, lower limits of odor detection. We sought instead to measure possible developmental changes in chemosensitivity, using a measure that would, hopefully, be an accurate, relevant, and reliable index of the degree to which there is a molar, organismic response to an airborne chemical signal.

In this paper, the pups' response to chemical stimuli has been defined as a measure of chemosensitivity, rather than olfactory sensitivity, to acknowledge the uncertainty regarding which chemosensitive receptor system(s) are at work during early life. There are multiple, afferent systems to transduce airborne chemical stimuli. These include the olfactory nerve (CN I), and various branches of the trigeminal nerve (CN V) [1,28]. Alberts [2] has reviewed evidence for heterochrony in anatomical development of the central nervous system structures associated with these receptors and he suggested that, during early development, olfaction in altricial rodents may be mediated by combinations and arrays of inputs different from those used by adults. The picture of olfactory anatomical development is not well resolved but it is quite clear that each of the various subsystems of the olfactory system is differentially mature at birth

and that they proceed to develop at different rates [2]. In view of the possibility that the reactions we measured were mediated by the trigeminal nerve rather than a receptor system with synapses in the olfactory bulbs, we chose to describe "chemosensitivity" rather than "olfactory sensitivity" in these studies. It should be possible to adapt the approach used in the present to investigations involving additional odorants and analyses of the afferent systems subserving the sense of smell during development.

Work from this laboratory and from others suggests that the rats' species-typical olfactory perceptual preferences and olfaction-guided behaviors are derived from experience with species-typical odors during early life [2, 7, 8, 18]. Are pups more sensitive to biological than non-biological substances?

Preliminary investigation did not reveal differential sensitivities. A broader range of stimuli must be tested, however. In addition, we should consider the possibility that the neonates' chemosensitive apparatus may be tuned or biased for species' odors by mechanisms other than differential sensitivity.

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