Preferences of newborn mice for odours indicating closer genetic relatedness: is experience necessary?

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Evidence from studies with adult rodents indicates that individual recognition enables distinctions between familiar individuals irrespective of relatedness (but including close kin) and a separate mechanism enables discriminations based on genetic relatedness without prior familiarity. For example, adult mice could assess the extent of their genetic relatedness to unfamiliar individuals using perceptual similarities between their individual odours. The ontogeny of this genetic relatedness assessment mechanism, however, had not been investigated. Here, in two-choice tests, newborn mice differentially preferred odours of more genetically similar lactating females (paternal aunts to unrelated conspecific and conspecific to heterospecific) even without prior direct exposure to adults with the tested genotypes. The results provide a direct demonstration of genetic relatedness assessment abilities in newborns and show that experience with parental odours is not necessary for genetic relatedness distinctions. Future studies will be necessary to determine whether exposure to odours of other foetuses in the womb or littermates shortly after birth affects this genetic relatedness assessment process.

**Keywords:** genetic relatedness; kin recognition; odour preferences; newborn mice

1. INTRODUCTION

Differential responses to other individuals based on different genetic relatedness are thought to provide adaptive benefits, including optimal mate choice to promote heterozygosity/genetic variability and to avoid the detrimental fitness consequences of inbreeding and outbreeding depression (Batson 1983; Thornhill 1993; Tregenza & Wedell 2000; Edmands 2002) and enhancing inclusive fitness (Hamilton 1964). This has led to investigations of kin recognition in juveniles and adults in a wide array of animal species, including insects, frogs, birds, and primates as well as rodents (Fletcher & Michener 1987; Hepper 1991; Sherman et al. 1997). Distinctions among individuals that lead to differential responses require both the expression of individually distinctive phenotypic characteristics and neurological processes to perceive these characteristics. It is generally assumed that kin recognition mechanisms underlying such discriminative responses depend on both some sort of ‘learning’ and some sort of ‘matching’ (Tang-Martinez 2001). In these mechanisms, ‘recognition by association’ involves learning characteristics of familiar individuals and matching remembered characteristics to specific individuals on subsequent encounters, and ‘phenotype matching’ is thought to involve incorporating learned kin characteristics into a composite template and matching characteristics of unfamiliar individuals against that template (Tang-Martinez 2001). Even self-referencing (using one’s own phenotype as the referent) ostensibly involves learning and matching one’s own phenotypic characteristics (Sherman et al. 1997; Hauber & Sherman 2001). This inclination to interpret data in terms of learning is understandable given the remarkable range of associations that rodents are able to make. A recent review of kin recognition data (Todrank & Heth 2003), however, raised new hypotheses about genetic relatedness distinctions (not only between kin and non-kin) that do not involve postnatal learning and presented a new way of thinking about recognition mechanisms that contrasts with the traditional views and theoretical approaches (restated, for example, in Holmes 2004; Mateo 2004; Thom & Hurst 2004).

Although rodents can recognize familiar individuals from their odours, in several species tested, learning these individual odours did not influence their genetic relatedness discriminations. For example, golden hamsters (*Mesocricetus auratus*) raised in mixed litters (see Todrank & Heth 2001, 2003 for an explanation of the cross-fostering procedure) recognized the individual odours of their biological and foster siblings (Todrank et al. 1998, 1999), but they did not incorporate these odours (or that of the female that nursed them) into a composite categorical template for discriminating between unfamiliar kin and non-kin (Heth et al. 1998). As a result, hamsters responded to odours of conspecifics, whether familiar or unfamiliar, in a graded fashion based on the degree of similarity with their own phenotype (Heth et al. 1998). Subsequent studies have also explored the simultaneous use of individual recognition and self-referencing in hamsters (Mateo & Johnston 2000). In another cross-fostering study in which pups from two species of wild mice (*Mus spicilegus* and *Mus musculus*)
were raised (by a laboratory mouse) in mixed litters from within hours of birth, adults’ odour preferences across a range of genetic relatedness from conspecifics through heterospecifics were graded based on similarity to their own phenotypes and were not influenced by being reared with pups from another species or by a foster mother (Heth et al. 2003). What juvenile rats reared in mixed litters (Hepper 1983) learned about their foster-siblings did not interfere with their genetic relatedness preferences. It was also found, as in the studies mentioned above with hamsters and mice, that differential responses of rats to conspecific odours were graded based on genetic relatedness (Hepper 1987a). Given that those cross-fostered rodents did not incorporate odours of the mother and littersmates into a composite template during postnatal development, they must have been using a separate mechanism to discriminate degrees of genetic relatedness from the mechanism they used to learn templates to recognise familiar individuals (Todrank & Heth 2003). Evidence consistent with separate familiarity and genetic relatedness mechanisms was also found in ground squirrels (Spermophilus beldingi; Holmes & Sherman 1982).

In addition, this genetic relatedness assessment mechanism enables subtle discriminative responses across a wide range of genetic relatedness from distinctions between siblings and half-siblings to distinctions between closer as opposed to more genetically distant heterospecifics (reviewed in Todrank & Heth 2003). Recent studies of the subtlety of the differential responses (Mateo 2002; Koeninger Ryan & Lacy 2003; Busquet & Baudoin in press) also suggested that the discriminations were more fine-tuned than categorical distinctions between kin and non-kin or conspecifics and heterospecifics. It has been found in several rodent species and for many degrees of genetic relatedness (Todrank & Heth 2003; Busquet & Baudoin in press) that similarities in the qualities of individual odours covary with the degree of genetic relatedness between individuals, a phenomenon termed ‘odour–genes covariance’ (Heth & Todrank 2000). This covariance between individual odour phenotypes and their underlying genotypes provides a basis for assessing one’s relatedness to another individual by comparing the degree of similarity between the other individual’s odour and one’s own (Heth et al. 2003).

The current study was conducted to investigate the ontogeny of this genetic relatedness assessment mechanism in two discrimination contexts: kin versus non-kin and conspecifics versus heterospecifics. It has been suggested that prenatal learning primes kin recognition because newborn rats preferred the odour of amniotic fluid from their mother to that from another parturient female (Hepper 1987b); however, it was not possible to determine from that study whether familiarity with one’s own amniotic fluid was the important factor or whether the pups were discriminating between the two samples on the basis of differential genetic relatedness. The study reported here was designed to test abilities of newborns to discriminate between unfamiliar odours on the basis of differential genetic relatedness. It may not be possible to isolate foetal mice from exposure to the phenotypic characteristics of their mother or other foetuses during gestation. It is possible, however, to create an early postnatal environment that precludes the possibility of learning associations that could facilitate genetic relatedness assessments. To this end, we presented newborn wild mice with a choice between the ventral body odours of two lactating females that were comparably unrelated to the mother but differentially related to the pups: a paternal aunt and an unrelated female. We hypothesized that if these newborn mice (even without direct exposure to the father) prefer the odour of their father’s sister to the odour of an unrelated female, they would have to be responding to the degree of overlap with their own phenotype. We also investigated whether newborns can discriminate between the odours of conspecific and heterospecific lactating females.

2. MATERIAL AND METHODS

(a) Animals

We tested three species of mice from the Mus species complex. The M. spicilegus mice were fifth generation born in the lab, from wild progenitors caught in Hungary and were maintained under standard conditions (Heth et al. 2001; Busquet & Baudoin in press) at University of Paris XIII. The M. musculus and M. domesticus mice were sixth generation born in the lab, from wild progenitors caught in Denmark and were maintained under standard conditions (Smadja et al. 2004) at University of Montpellier II. In both colonies, the genealogy was carefully monitored from the time of capture, and breeding couples were comprised of animals selected to maintain maximal genetic diversity across the particular population.

For the test with 69 pups from 11 litters of M. spicilegus, 12 new couples were established at the same time (from a pool of 12 pairs of brothers and sisters) in the hope of synchronising births. In some cases, however, the paternal aunt gave birth up to 3 days later so the pups were tested between the first and third days of life depending on when the paternal aunt gave birth. Pups tested on their first day of life were at least 2 h but not more than 16 h old. For the ‘father-removed’ condition with 66 pups from 8 litters of M. spicilegus, the pups were from established couples that had had several previous litters so that the time of birth was predictable. Expectant mothers were moved to clean cages without the father or his odours at least 2 days before the anticipated birth, because in contrast with M. musculus and M. domesticus, in natural conditions the father is an important participant in parental care in M. spicilegus (Patris & Baudoin 2000). For the test with 39 pups from 6 litters of M. musculus and M. domesticus the colonies were small and the birth rates sufficiently irregular that it was not possible to determine whether a paternal aunt would be giving birth within 3 days, thus the couples were not disrupted by removing the father. For the conspecific–heterospecific condition, 50 pups from 7 litters of M. musculus and M. domesticus were tested on the first or second day of life. An additional 40 pups from 4 litters of M. musculus and M. domesticus were tested in the control condition on their first day of life.

(b) Procedure

We assessed differential interest of newborn mice in two-choice tests using odours of adult lactating females. We used odours of lactating females because results from pilot tests indicated that although newborn laboratory mice preferred the odours of unfamiliar males and virgin females from their own strain to those of males and virgin females from another
strain, they responded more quickly during choices between odours of lactating females than during choices between either males or virgin females. These unpublished findings corroborate those in which mouse pups preferred the body odours of lactating females to those of virgin females, although previous tests investigating responses of mouse pups to maternal odours were conducted with 3–20 day old mice and in apparatus that required the pups to move at least 6.5 cm to contact the odorous stimuli (Breen & Leshner 1977; Hennessy et al. 1980; Koski et al. 1977). Odours of lactating females would also be more biologically relevant to newborns than other body odours. The two odour donors for each test were selected based on their genealogy to be comparably related to the pups’ mother and to have given birth as closely together as possible, usually within 1 day but never more than 3 days apart. Odours were collected by rubbing one edge of a small (3 cm diameter) plastic Petri dish back and forth 8 times along both sides of the mouse’s ventrum including the nipples but avoiding the ano-genital area. The odorous edges of the two dishes (randomly coded as A or B) were placed 2.5 cm apart equidistant from a line drawn on clean tissue paper. Pups were tested in warm conditions (around 30 °C, comparable to the nest temperature). Subjects, which were approximately the size of a kidney bean, were positioned on the line 4 cm from the odours, slid gently (with their hindquarters stabilized in the small triangle between the tips of the experimenter’s thumb and index finger) up the line until the nose was in the centre between the dishes, and held until their head and shoulders moved enough that their nose touched one of the dishes, constituting the choice. This technique was adopted after extensive pilot testing with unsuccessful methods that permitted the pups to move freely, which resulted in their moving randomly about, lying curled up, or, if held only until they were in position, rolling over so their backs rather than their noses touched the stimulus. These free movement techniques also required that the pups be removed from their nests for longer periods, increasing the risk of parental predation on their return.

Choosing times varied between a few seconds and less than 2 min. Half of the pups from each litter were removed (along with nest material from the home cage) to a holding cage and tested individually; then the position of the odours was reversed and the second half of the litter was removed from the home cage and tested. Switching the sides on which the odours were presented for the second half of the tests also controlled for any effects of being held by the experimenter. This procedure also ensured that mothers were never left without young in the nest and pups were not left without other pups near them. The experimenters wore latex gloves during preparation of the stimuli and testing and were blind to the position of the odours during the test. All experiments followed the rules of French Ministries (Agriculture; Research and Technology).

(c) Data analysis

Each litter was treated as a statistical unit (after it was determined that there was no difference in the performance of pups tested immediately after removal from the nest and those that spent a few minutes in the holding cage prior to testing). The proportion of litters in which pups chose the more closely related female was tested for significance with binomial tests. (No preferential choice was shown in control tests when the odour of the same unfamiliar and unrelated lactating female was presented on both sides: 21 left versus 19 right.)

3. RESULTS AND DISCUSSION

In this investigation of odour-based genetic relatedness discrimination in newborn mice, more pups chose the odour of the more genetically similar female in all the litters tested and in all types of test. Mouse pups preferred the odour of their lactating paternal aunt to the odour of an unrelated lactating female (table 1; M. spicilegus: \( n=11 \) litters, \( p=0.001 \), corresponding to 54 out of 69 pups; \( M. musculus \) complex: \( n=6, p=0.031, \) and 31out of 39 pups), indicating that pups discriminate between odours of adults on the basis of genetic relatedness. It was possible in these tests, however, that the pups learned enough about their father’s odour during the first few hours of life to facilitate the discrimination between the father’s sister and a female that was not related to either of the parents. In a key test, however, \( M. spicilegus \) newborns (less than 16 h old) from litters that were born to mothers that had been moved to clean cages without the father at least 2 days prior to their birth also preferred odours from their lactating paternal aunts (table 1; \( n=8, p=0.008, \) and 56 out of 66 pups). Thus the same pattern was found

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**Table 1. Preferences of newborn mice in two-choice odour tests.**

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<th>Species</th>
<th>Day of Life</th>
<th>Litter Size</th>
<th>Choice</th>
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<td><strong>Condition: conspecific versus heterospecific</strong></td>
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whether the father was present or not. Although the pups may have learned about their father’s odour when he was present, this learning did not mediate the discrimination. Aspects of the father’s odour are evident in the odours of foetuses because the father contributes 50% of his genes to each of them, but this is not the same as direct exposure to the father’s actual odour during gestation. Neither prenatal nor postnatal exposure to the mother’s odour could have helped pups to discriminate between odours of lactating females that were comparably unrelated to their mother. These results provide the first direct and non-confounded demonstration of the ability of newborns to discriminate between odours of kin and non-kin without the necessity of learning about their parents’ odours. It is a clear demonstration of the remarkable ability of newborns to make genetic relatedness discriminations on the basis of odours.

These odour-based genetic relatedness distinctions were not restricted to kinship discrimination, however. In tests using odour donors that were conspecifics and heterospecifics, newborn M. musculus and M. domesticus mice preferred the odour of a lactating female from a different population of conspecifics to that of a heterospecific lactating female (table 1; \(n=7, p<0.016\), and 39 out of 50 pups), demonstrating that a wide range of genetic relatedness distinctions is possible from birth. Although using the mother’s odour as a referent could have helped the newborns make the discrimination between conspecifics and heterospecifics, it is interesting to note that in previous tests with adults raised in mixed litters of M. musculus and M. spicilegus, the odour of the rearing lactating female did not affect subjects’ preference for the odour of the more genetically similar individual (Heth et al. 2003). Given the greater differences between the donors’ odours in this test, it is noteworthy that the effect was just as strong for discrimination between aunt and unrelated conspecific as for conspecific as opposed to heterospecific.

With the experimental design used in this study, it is not possible to rule out effects of environmental odours, including odours of littermates, but we have shown in previous tests with mice from these species that were cross-fostered within a few hours of birth that environmental odours, including odours of fostered littermates, did not affect their genetic relatedness assessments (Heth et al. 2003), thus it seems unlikely that environmental odours affected the discriminative abilities or choices of newborns.

In previous self-referencing studies (reviewed in Todrank & Heth 2003) that involved adult rodent subjects, the discrimination tasks used odours from other adults. In those studies, subjects were making comparisons between odours of animals that were all the same age. In the current study, the subject’s own odour was that of a newborn while the test odours were from lactating adult females, probably posing a more challenging task for the subjects. Nonetheless, newborn mice completed the discrimination task successfully. When the findings from the odour–genes covariance studies were combined with the results of the self-referencing studies, it became clear that the genetic relatedness assessment mechanism being used was different from the traditionally conceived kin recognition mechanisms (Todrank & Heth 2003). First, because more closely related individuals produce odours that have ‘relatively’ more similar qualities (whether they are double cousins compared with cousins (Busquet & Baudoin in press) or conspecifics from different populations compared with heterospecifics (Heth et al. 2001) or closer as opposed to more distant heterospecifics (Heth & Todrank 2000)), the distinctions could be quite subtle. Second, the range across which rodents are able to make subtle genetic relatedness distinctions precludes simple categorical distinctions such as between kin and non-kin or conspecifics and heterospecifics. To distinguish this process from self-referent phenotype matching, we called the mechanism based on odour–genes covariance ‘genetic relatedness assessment through individual odour similarities’ and abbreviated it ‘G-ratios’ (Heth et al. 2003). Previous evidence indicates that the G-ratios mechanism in adult rodents is not affected by learning the individual odours of nest mates (Todrank & Heth 2003), but the ontogeny of this process had not been explored previously. The current results extend the G-ratios findings, which had previously been demonstrated only in adult rodents, to include newborns as well. Having established that genetic relatedness assessments are possible from birth, future research will be necessary to investigate, among other questions, the functions of such assessments and how they change across the lifespan from newborns through juveniles to adults.

It will also be important in future studies to determine how soon after birth pups are able to recognize familiar individuals from their odours. It is known from previous studies that young adult hamsters do not remember enough from the interactions with their littermates during the first week of life to recognize their littermates from their individual odours but that interactions during the second through fourth weeks of life enable individual recognition even following nine months of separation (Todrank et al. 1999). It is clear from the results reported here that mouse pups can make genetic relatedness assessments from shortly after birth, thus if they are not able to remember and identify conspecifics in the first hours or days after birth, this will provide further clarification of the separateness of the individual recognition and genetic relatedness assessment mechanisms.

Exposure to odours during gestation is known to influence postnatal behaviour in some rodent species (e.g. Hepper 1987b, 1988; Beauchamp et al. 1994; Hudson et al. 1999; Gruest et al. 2004). Determining whether exposure to littermates (which, like the individual’s own odour, provide partial representation of both the father’s and mother’s odour) during gestation or immediately after birth influences or is necessary for successful genetic relatedness distinctions in these species would require embryo-transplant experiments. Such transplants would also be necessary to determine whether preferences of newborns are subject to “parent-of-origin” effects (Isles et al. 2001). Nonetheless, it is clear from the evidence presented here that prenatal and postnatal experience with parental odours are not necessary for newborns to make genetic relatedness discriminations at the levels of kinship and species. A more natural way to test this question would be to investigate a rodent species that routinely produces mixed paternity litters, such as ground squirrels. Yearling ground squirrels are known to play less aggressively with full siblings as opposed to paternal half-siblings.
If newborn ground squirrels prefer the odour of their own paternal aunt to the odour of their half-sibling’s paternal aunt, this would indicate that exposure to half-siblings (with a different father) during gestation did not disrupt the pup’s ability to discriminate genetic relatedness using its own phenotype as a referent. At the same time, such an investigation would extend the evidence concerning the ability of making genetic relatedness assessments at birth to another species.

These findings extend the recent breakthroughs in understanding several aspects of recognition mechanisms. It has previously been shown that individual odours in animal excretions and secretions express each individual’s genotype in a distinctive odour phenotype that is perceived as a composite gestalt (Todrank & Heth 2003). Perception of these odour gestalts enables individual recognition of familiar individuals apart from relatedness and, through a separate mechanism, differential responses to individuals of varying degrees of genetic relatedness apart from familiarity or learning from parents or nest mates (Todrank & Heth 2003). The first direct demonstration of genetic relatedness assessment abilities in newborns without the necessity of experience with parental odours reported here casts additional doubts on recognition mechanisms depending on ‘learning’ and ‘matching’ as they have traditionally been and are currently typically conceived (Sherman et al. 1997; Hauber & Sherman 2001; Tang-Martinez 2001; Holmes 2004; Mateo 2004) and adds urgency to the call to re-examine the theoretical and empirical underpinnings of recognition systems.

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2088 J. Todrank and others  Preferences of newborn mice for odours