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# One-Trial Associative Odor Learning in Neonatal Mice

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## Abstract

Behavior genetics studies in mice demand efficient training protocols for rapid phenotypic screening. However, the capacity of neonatal mice to form and retain associative memories has been difficult to study due to their limited sensorimotor capacities. The present study describes a method for robust, naturalistic associative learning in neonatal mice as young as 3 days old. After removal of the dam from the home cage for 2 h, preweanling CD-1 mice of ages 3, 5, and 10 days postnatal were conditioned to associate an arbitrary odorant with the suckling and milk delivery that ensued upon her return to the home cage. After a second maternal deprivation, neonates were tested on their acquired preference for that odorant. Neonates exhibited a learned preference for the conditioned odorant over a novel control odorant. No learning was observed without deprivation, that is, when the dam was removed only briefly for scenting. One-trial learning sufficed to show clear preferences for the conditioned odorant, although repeated training (three sessions over 8 days) significantly increased the expression of preference. The development of neonatal associative learning protocols requiring minimal human intervention is important for the behavioral phenotyping of mutant and transgenic strains, particularly those modeling developmental disorders.

**Key words:** appetitive conditioning, behavioral phenotyping, memory, mouse, olfactory bulb, unblocking

## Introduction

Behavioral phenotyping is a vital tool for the realization of the scientific promise of genetically modified animals. The influence of genetic variables on cognitive and neural processes in particular is often subtle and complex, requiring well-chosen behavioral test batteries in order to elucidate their fundamental effects. Many adult behavioral tests designed in rats and other species have been modified for the phenotyping of mouse models, both in order to better suit the ethology and capabilities of mice (Wolfer *et al.*, 1998; Gerlai and Clayton, 1999; Gerlai *et al.*, 2002) and to address the particular requirements and limitations of behavior genetics studies. However, there remains a pressing need to develop learning and memory tasks suitable for the phenotyping of neonatal mice. The neural circuitry underlying neonatal learning and memory can be qualitatively different from its adult counterpart (Moriceau and Sullivan, 2004, 2005; Roth and Sullivan, 2005; Sullivan, 2005), and many developmental disorders that may be studied using mutant/transgenic mouse models are initially afflictions of the developing nervous system that may or may not be understandable solely via research on adults. While there are substantial limitations on neonatal studies in altricial species—vision and hearing in mice are not functional before roughly 12 days of age in mice, and neonates' motor capabilities are very limited—successful learning protocols have been developed in some species pairing olfactory conditioned stimuli (CSs) along with stroking or nutritive unconditioned stimuli (USs).

Positive-reinforcement neonatal olfactory learning paradigms have been well developed in rats (Sullivan and Leon, 1987; Sullivan and Hall, 1988; Sullivan and Wilson, 2003; McLean and Harley, 2004). Rat pups develop a behavioral attraction for maternal odors (Sullivan *et al.*, 1990) and also exhibit learned preferences for arbitrary odorants paired with a 10-min reinforcing tactile stimulation comparable to that received from the dam (Sullivan and Leon, 1987) via a norepinephrine- and serotonin-dependent learning mechanism (Sullivan *et al.*, 1989; Sullivan *et al.*, 1991; Price *et al.*, 1998; McLean and Harley, 2004). This protocol has been adapted for neonatal mice (Bouslama *et al.*, 2005), using a 20-trial acquisition protocol during which the odorant CS+ was paired with repetitive tactile stimulation. An earlier study of associative learning in neonatal mice relied on aversive conditioning, in which odor presentations were

paired with sickness-inducing LiCl injections (Alleva and Calamandrei, 1986). While both of these latter studies demonstrated robust conditioned odor preferences in neonatal mice, the training protocols are relatively time and labor intensive and hence difficult to adapt for high-throughput phenotypic screening. We sought to replace this training method with a naturalistic protocol enabling robust and efficient training with minimal human intervention.

A neonatal mammal's ability to efficiently locate and attach to the dam's nipple is crucial to its survival (Coureaud *et al.*, 2000), particularly in species in which several littermates must compete with each other for access to milk. While tactile, thermal, and olfactory cues all can play substantial roles in directing suckling (Distel and Hudson, 1985; Koffman *et al.*, 1998), olfaction seems to play a singular role in this process (Bruno *et al.*, 1980; Hudson and Distel, 1995; Hongo *et al.*, 2000). Indeed, transgenic mice with anosmic or hyposmic phenotypes will often die of starvation as neonates unless special efforts are made to raise them until they are weaned (Brunet *et al.*, 1996; Baker *et al.*, 1999; Contos *et al.*, 2000; Hongo *et al.*, 2000; Wong *et al.*, 2000; Lin *et al.*, 2004). Odor learning also clearly contributes to successful nipple location and attachment (Hudson *et al.*, 1992; Cheslock *et al.*, 2004). Even in rabbit pups, in which suckling is initially elicited by mammary pheromone (Hudson, 1985; Hudson and Distel, 1995; Coureaud *et al.*, 2001; Schaal *et al.*, 2003), contextual olfactory cues present at the first nursing experience are subsequently able to substitute for this pheromonal cue, even up to 5 days after the initial pairing (Hudson, 1985; Hudson and Distel, 1995). Both suckling *per se* as well as milk delivery can serve as USs for odor learning. While they are most potent when paired (Brake, 1981), milk alone has been shown to be an effective US in neonatal rats even in the absence of the dam (Johanson and Hall, 1979; Johanson and Teicher, 1980; Johanson and Hall, 1982; Sullivan and Hall, 1988). Prenatal odor learning may also contribute to successful suckling (Coureaud *et al.*, 2002), as has been suggested in human studies as well (Marlier *et al.*, 1998).

In this study, we present a protocol for measuring associative learning and memory capabilities in neonatal mice that is both based on their natural behaviors and motivations (Gerlai and Clayton, 1999) and amenable to reasonably high-throughput training (Brunner *et al.*, 2002). We positively condition neonatal mice to associate arbitrary odorant CSs with a suckling/milk US after a brief deprivation period (removal of the dam from the home cage) and show that one-trial learning by this method results in conditioned odor preferences for these odorants. Prior deprivation is required to obtain measurable learning, and repeated training trials produce increased learning. We show that appetitive conditioning to odor stimuli can be measured reliably in mice as young as 3 days old, facilitating behavioral assessment of the development of olfactory learning and memory in mutant and transgenic model strains.

## Materials and methods

### Subjects

Neonatal mouse pups of the outbred CD-1 strain were used in the three experiments of this study at three postnatal ages: 3 days (P3), 5 days (P5), and 10 days (P10). Deliveries were recorded once daily in the morning; the day of delivery was recorded as postnatal day 1 (P1). Litter size varied from 7 to 16, with an average of 13 pups. In keeping with the desire for efficiency in training that motivated this study, all the pups in a given litter were tested as a cohort; both male and female pups were included in the study. However, some of the pups in the first experiment of the present study were cross-fostered on the day of delivery (P1) in order to screen for any prominent artifacts potentially deriving from the correlation between litter of origin and odor contingency learning.

### Odor stimuli

Two odorants, *n*-hexanoic acid and citronellal, were used as stimuli. Each odorant was used as the training odorant for half of the cohorts in each experiment to counterbalance any possible innate preferences for the odors. The odorants were selected both for their substantial differences in quality and their similar vapor pressures, the latter estimated with the Hass–Newton equation as implemented in ACD/Boiling Point & Vapor Pressure Calculator (Version 4.5; Advanced Chemistry Development, Toronto, Ontario, Canada). All odorant stimuli, whether used for scenting dams' nipples or for placement under the test arena, were diluted in mineral oil to liquid-phase concentrations that theoretically emitted vapor-phase partial pressures of 1.0 Pa. Corresponding volume/volume dilutions in mineral oil were as follows: *n*-hexanoic acid, 148 μl/ml; citronellal, 166 μl/ml. A formula weight of 335 g/mol for mineral oil (Jefo Nutrition, St Hyacinthe, Quebec, Canada) was used for mole fraction calculations. Solvent surface effects and other nonlinearities were neglected. These dilutions should be considered a reduction in the variance of odor concentrations rather than true vapor-phase concentration matching as could be achieved by gas chromatographic measurements. Odorants were diluted at least 1 day in advance of testing to ensure an even distribution of odorant within the mineral oil solvent.

### Training procedures

The standard training procedure in these experiments consisted of removing the dam from her litter in the home cage for 2 h in the morning. The home cage contained substantial bedding and was maintained on a slide warmer set to 37°C to prevent hypothermia in the pups. Immediately before returning the dam to the home cage, the diluted training odorant was applied to each of her nipples using a saturated Q-tip. Pups typically began to suckle shortly after the dam's replacement, thus putatively associating the training odorant with the reward value of the dam's replacement (suckling,

milk delivery) after a period of deprivation. If a test trial was scheduled for that day, the pups were allowed to nurse for 1 h, after which the dam was again removed from the home cage for 2 h before testing. In order to minimize the potential effect of innate preferences, *n*-hexanoic acid and citronellal were each used as the training odorant for half of the litters employed in each experiment. Other training procedures varied among the three experiments and are described below. Unless otherwise specified, this training procedure was only conducted once for any given mouse.

### Testing procedures

Testing was performed on the same day as training, beginning 3 h after training was completed (see Training Procedures). If multiple training trials were performed, testing was performed on the same day as the last training session. Neonatal mice were tested for place preference in a 32 × 19–cm arena (13-cm wall height) with a wire mesh floor placed atop two 12 × 19 × 7–cm deep compartments (Alleva and Calamandrei, 1986; Bouslama *et al.*, 2005). One of these compartments contained a Kimwipe scented with 500 µl of diluted training odorant and the other contained a Kimwipe scented with 500 µl of the other test odorant, to which the mouse pup had never been exposed. The two scented compartments were separated by 0.7 cm (wall thickness) where the two compartments met under the center of the arena. The orientation of the two compartments was varied with respect to both odor identity and odor contingency between test trials; furthermore, the spatial orientation of the scented compartments was also varied with respect to the room to control for differences in external cues (e.g., light, sound). The odor compartments were cleaned thoroughly with ethanol and allowed to dry fully between testing sessions.

Each pup was observed and scored for 180 s, measured by a countdown timer. At the start of each trial, pups were placed with their muzzle on the centerline between the two scented compartments such that their right limbs were over one compartment and their left limbs were over the other compartment. Odor preferences were recorded based on the movement of neonates off of the midline into the region above one of the scented compartments. Specifically, whenever a pup moved its muzzle completely off the center divide and directly over a particular compartment, the pup was scored as being on that side. Pups were replaced upon the midline when the following criteria were met so that their relative immobility would not dominate the assessment of preference: if a pup fell over so as to be unable to regulate its movements, reached the external wall of the arena, froze for 3 s without head movements, or began rotating in place (more than one full circle while further than one body length from the midline), it was replaced on the midline in the opposite orientation. The total accumulated time spent over each of the two compartments was recorded by stopwatch.

### Screening for innate odor preferences

One hundred neonatal mice from nine litters were trained once as described above, including deprivation periods before both training and testing, except that only unscented mineral oil was applied to dams' nipples before replacement. Hence, neonates were naïve to both odorants when tested for odor preference. Neonates of 3-, 5-, and 10-days' age were grouped for analysis. Odor preference testing was performed normally as described above.

### One-trial odor preference learning

A total of 440 neonatal mice from 34 litters were divided by age into three cohorts (3, 5, and 10 days postnatal) and subjected to the standard training and testing protocols described above. Specifically, 204 neonates from 15 litters were trained and tested at P3, 155 neonates from 11 litters at P5, and 81 neonates from eight litters at P10.

### Dependence on maternal deprivation during training

A total of 147 neonatal mice from 12 litters, all 10 days of age, were divided into two cohorts. The "control" cohort was subjected to the standard training and testing protocols described above. The "nondeprived" cohort was subjected to identical procedures, except that the period of maternal deprivation before training was only several seconds, just long enough to enable the scenting of the dam. The 2-h deprivation period before testing was the same for both cohorts.

### Dependence on amount of training

A total of 95 neonatal mice from eight litters, all 10 days of age, were divided into two cohorts. The control cohort was subjected to the standard training and testing protocols described above, receiving a single training session on P10. The "overtrained" cohort was subjected to three training sessions: once on P3, once on P5, and once on P10. Testing was performed normally on P10.

## Results

### Screening for innate odor preferences

We tested neonatal mice at three preweaning ages, postnatal days 3, 5, and 10, in order to assess the effectiveness of our training paradigm across the period of neonatal development. First, we tested whether the mice would exhibit an innate preference for either odor used: *n*-hexanoic acid or citronellal. One hundred neonatal mice (P3: $n = 27$; P5: $n = 60$; P10: $n = 13$) underwent the standard maternal deprivation protocol (see Materials and Methods ), except that only unscented mineral oil was applied to the mothers' nipples. After a second deprivation period, the neonates were tested in the arena using these two odorants, and the time spent over each odorant was recorded. analysis of variance (ANOVA) testing with "odor identity" and "age" as main effects revealed

no significant effect of either odor identity [$F(1,194) = 2.400$, $P > 0.05$] or age [$F(2,194) = 0.738$, $P > 0.05$]. These two odorants were then used for all subsequent studies.

### One-trial odor preference learning

Neonates of all three ages were separately tested for one-trial odor preference learning (P3: $n = 204$; P5: $n = 155$; P10: $n = 81$) with additional controls. One potential disadvantage of this maternal-deprivation training protocol is that litters must necessarily receive the same training contingency. This limitation creates a potential confound if the litter of origin is a significant contributor to experimental variance, in which correlations among littermates may be falsely attributed to shared contingency rather than to factors dependent on litter of origin. To address this potential confound, we cross-fostered litters on postnatal day 1 such that some litters contained both the dam's own pups along with fostered pups from other dams. Separate analyses of variance were performed on each of the three age cohorts with "conditioning" and "cross-fostering" as main effects. Conditioning was a significant effect at all ages [P3: $F(1,404) = 14.841$, $P < 0.001$; P5: $F(1,306) = 6.323$, $P < 0.02$; P10: $F(1,158) = 17.877$, $P < 0.001$], revealing a significant preference for the training odorant over the novel odorant (Figure 1). The effect of cross-fostering was not significant at P5 or P10, although it was marginally significant at P3 [P3: $F(1,404) = 6.201$, $P < 0.05$; P5: $F(1,306) = 0.005$, $P > 0.05$; P10: $F(1,158) = 0.067$, $P > 0.05$]. Furthermore, cross-fostering did not significantly affect the ability to associate odor with reward at any age [interaction of conditioning × cross-fostering—P3: $F(1,404) = 3.361$, $P > 0.05$; P5: $F(1,306) = 0.585$, $P > 0.05$; P10: $F(1,158) = 2.519$, $P > 0.05$], in agreement with previous studies (Bouslama *et al.*, 2005).

### Dependence on maternal deprivation during training

If the odor preferences expressed by neonatal mice are due to contingency learning, then the learned preferences should be sensitive to variations in US parameters (Rescorla and Wagner, 1972). Specifically, learning should be reduced in the absence of deprivation during training. This is particularly true in the present paradigm: neonates have extensive experience with maternal care and with suckling that are not associated with arbitrary test odorants, such that odor preference learning in this context is essentially superconditioning (unblocking), owing to the increased intensity of reinforcement due to deprivation (Rickert and Lorden, 1983; Holland, 1984; Holland and Kenmuir, 2005). The addition of a new CS without a change in reinforcement value would be predicted to result in blocking (Fanselow, 1998).

Sixty-six 10-day-old neonates were sham deprived (dams were removed for scenting and immediately replaced) and compared with 81 P10 neonates from which the dam was removed for 2 h before scenting and replacement as described above. The dam was removed from both cohorts for 2 h prior to testing in order to render motivation comparable between the two groups. ANOVA testing with "conditioning" and "deprivation" as main effects indicated that deprivation was a highly significant determinant of the effect of reward [Figure 2; interaction of conditioning × deprivation: $F(1,290) = 8.400$, $P < 0.01$]. Conditioning alone was a

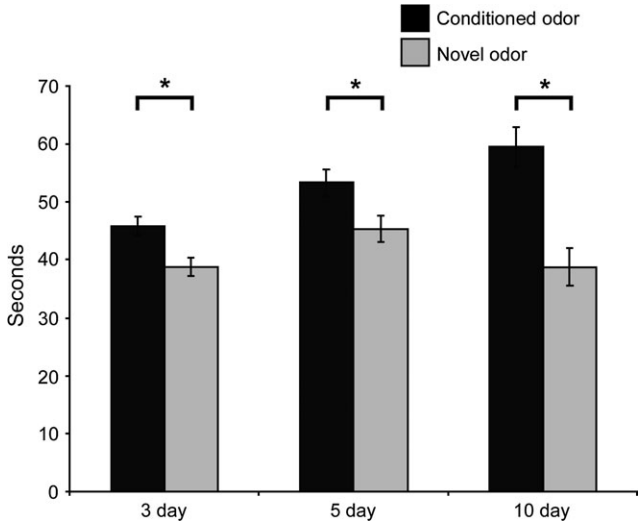


**Figure 1** One-trial odor preference learning in 3-, 5-, and 10-day-old neonatal mice. Neonatal mice exhibited a significant preference for odorants paired once with a naturalistic reward during conditioning (suckling and milk delivery owing to the replacement of the dam after a 2-h deprivation) in comparison with a novel odorant. Ordinate: time spent over the conditioned or novel odorant, in seconds. Asterisks indicate significant differences.

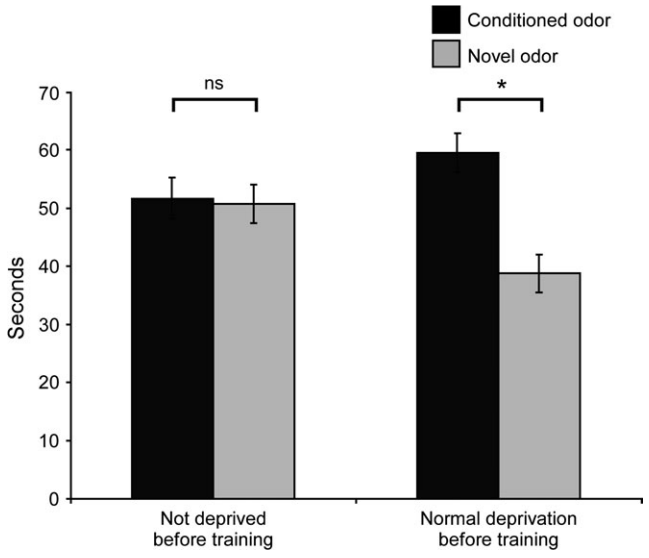


**Figure 2** Odor preference learning requires prior deprivation. Neonatal mice (10-days old) that were trained and tested normally, but experienced no maternal deprivation prior to training, showed no preference for the conditioned odorant over a novel odorant. Removal of the mother from the home cage for 2 h prior to conditioning generated a significant preference for the conditioned odorant. Ordinate: time spent over the conditioned or novel odorant, in seconds. Asterisks indicate significant differences, ns: not significant.

significant main effect [$F(1,290) = 10.148$, $P < 0.01$], replicating the conclusions of the prior experiment, while deprivation irrespective of conditioning was not significant [$F(1,290) = 0.361$, $P > 0.05$]. Separate $t$-tests on the nondeprived and deprived cohorts indicated that the effect of reward on odor preference was significant in the deprived cohort ($P < 0.001$) but not in the nondeprived cohort ($P > 0.05$; Figure 2).

### Dependence on amount of training

Learned odor preferences also should be sensitive to variations in training; specifically, increased numbers of training trials should increase the magnitude of learned preferences (Rescorla and Wagner, 1972), all else being equal and excepting ceiling effects. We compared two cohorts of 10-day-old neonates in this experiment. One cohort (''singly trained''; $n = 49$) was trained and tested on P10 using the one-trial learning paradigm described above. The other (''multiply trained''; $n = 46$) was trained with deprivation three times using the same odorant (on P3 and P5 as well as on P10), but tested only on P10. ANOVA testing with conditioning and ''experience'' as main effects revealed that this increased training had a highly significant influence on the effect of conditioning [Figure 3; interaction of conditioning × experience: $F(1,186) = 24.880$, $P < 0.001$]. Separate $t$-tests on the singly trained and multiply trained cohorts indicated that the effect of reward on odor preference was significant in both cohorts ($P < 0.001$ for each). Hence, while one-trial learning suffices to evoke conditioned odor preferences, it is clear that these learned associations can persist and accumulate over a timescale of days, implicating a contribution of long-term odor memory mechanisms.

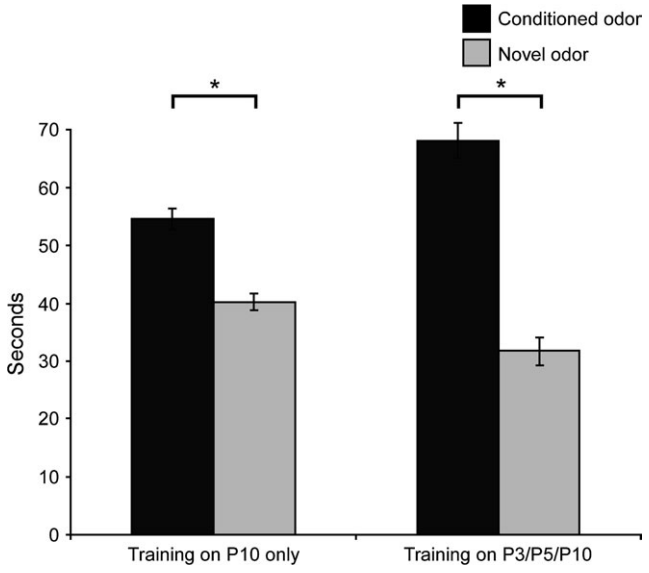


**Figure 3** Increased training produces stronger learning. One-trial learning produces a significant preference for the conditioned odorant. Three training trials over 8 days significantly increase the strength of this learned preference (see text). Ordinate: time spent over the conditioned or novel odorant, in seconds. Asterisks indicate significant differences.

## Discussion

Robust, efficient protocols for the assessment of learning and memory in neonatal mice are critical components of behavioral phenotyping batteries. Learning and memory in neonates rely on neural circuitry and receptor expression profiles that in several cases differ qualitatively from those in adult animals (Watanabe *et al.*, 1992; Monyer *et al.*, 1994; Cao *et al.*, 2000; Johnston *et al.*, 2001; Law *et al.*, 2003; Moriceau and Sullivan, 2004, 2005; Roth and Sullivan, 2005; Sullivan, 2005). Hence, relying solely upon adult behavioral studies may lead to misidentification of the mechanisms underlying the production of certain behavioral phenotypes. This has direct implications for human disorders such as autism (Rutter and Sroufe, 2000; Sigman *et al.*, 2004), borderline personality disorder (Agrawal *et al.*, 2004; Clarkin and Posner, 2005), reactive attachment disorder (Wilson, 2001), and other pathologies (Rutter and Sroufe, 2000; Mathew *et al.*, 2001; Gilmer and McKinney, 2003), the adult symptoms of which may wholly or partially reflect ''normal'' responses to transient disability or deprivation during critical stages of development. Neonatal rodent models are increasingly being used to study these critical issues (Winslow and Insel, 2002; Sullivan, 2003; Parfitt *et al.*, 2004; Moriceau and Sullivan, 2005; Roth and Sullivan, 2005).

We describe an efficient, naturalistic associative learning training protocol for neonatal mice as young as 3 days old that is appropriate for relatively high-throughput behavioral phenotyping screens. The association of arbitrary odorant CSs with a reinforcer (suckling, milk delivery) requires deprivation prior to training to elicit unblocking and is strengthened by repeated training trials, consistent with the predictions of learning theory. In particular, the dependence on prior deprivation indicates that the acquired odor preferences arise via associative learning, rather than reflecting a nonassociative familiarity response as has been observed in spiny mice (Janus, 1989). One particular consequence of this protocol is that litters nursed by the same dam must necessarily receive the same training contingency. This could be a potential confound if the litter of origin is a significant determinant of odor preference; however, in a typical behavioral phenotyping context utilizing littermate controls it could instead be advantageous, improving the uniformity of the training context among littermates of differing genotypes. Nevertheless, in the present study, cross-fostered litters exhibited learning profiles no different from those of noncross-fostered litters.

The present results raise questions about memory consolidation and the duration of conditioned responses in neonatal learning tasks. While mice were only tested 2–3 h after conditioning in the present study, they accumulated learning over several days; mice trained on P3, P5, and P10 learned significantly more than did mice trained only on P10. However, in

another study, the conditioned responses of P1–P7 outbred mice (considering the day of birth as P1) to odorant CSs acquired by association with tactile stimulation (stroking) were only observable immediately after training (0-h delay), becoming nonsignificant in tests conducted 5 and 24 h later (Bouslama *et al.*, 2005). In contrast, 6-day-old rat neonates subjected to a similar conditioning procedure routinely retained the conditioned response for at least 24 h, but less than 48 h (Sullivan and Leon, 1987; McLean *et al.*, 2005). Neonatal mice in which an odor CS was paired with an LiCl injection on P7 learned a conditioned aversion to the odor that persisted for up to 3 days (Alleva and Calamandrei, 1986); while these data demonstrate the capacity of neonatal mice to retain conditioned responses over multiple days, neonatal rats trained on P2 retained a similar odor association with LiCl injection for at least 8 days (Rudy and Cheatle, 1977). Hence, it is likely that outbred (CD-1, Swiss) mice do not retain conditioned odor responses as strongly as do rats under current experimental protocols, though the US modality (nutritive vs. tactile) may also contribute to observed differences among studies (Bouslama *et al.*, 2005), as may the longer (1 h) periods of CS–US pairing utilized by Alleva and Calamandrei (1986) and the present study (Smith *et al.*, 1983).

The present study describes a method for robust, naturalistic associative learning in neonatal mice as young as 3 days old. Litters are trained as a cohort with minimal experimenter intervention—a potential benefit in behavior genetics studies utilizing littermate controls—and the effects of learning persist for hours or days. Exploration of the learning and memory capacities of genetically modified neonatal mice is an essential addition to the mandate of behavioral phenotyping studies, particularly pursuant to the study of psychiatric developmental and personality disorders.

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