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Animal Welfare 2009, 18: 257-261
ISSN 0962-7286

Neonate rats demonstrate high levels of retention for odour preference when a substantially reduced training trial of two minutes is used

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Abstract

Neonate rats have played an important and unique role within the memory literature. Specifically, they are relatively naïve to experience, can be trained in an ethologically-valid way, using a single trial, which is not aversive and can demonstrate retention for at least 24 h, post-training. As such, they have provided salient insights into the biological mechanisms underlying the memory trace and brain development. The task of choice for rat pups is one using odour preference requiring the removal of each pup from the nest/dam for a ten-minute training trial. For such young animals this may reasonably lead to undue stress and the potential of subsequent rejection by the dam. Little research has considered whether the training duration could be substantially shortened to provide significant animal welfare benefits while maintaining, if not improving, task efficacy. This issue was addressed in the current paper using six-day old Wistar rats (n = 175) exposed to a single-trial, odour-preference task using either a standard ten-minute training trial or a shorter two-minute training trial. Exposing rat pups to the training odour for two minutes did not compromise the level of discrimination observed at test 24 h later. This finding suggests that significantly shorter training trials can be used without compromising retention levels at test. This not only has obvious welfare benefits, but may reasonably be considered to reduce pups' stress levels which are known to alter both the strength and timing of the memory trace.

Keywords: *animal welfare, memory, neonates, odour preference, olfactory discrimination, rats*

Introduction

The use of neonate animals to study memory processing is both common and useful given that they are naïve to experience, sparing the memory trace from confounds present when training older animals. For example, day-old chicks have been used by a number of laboratories for several decades. These animals are particularly useful in that they are precocious, naïve to experience (Ng *et al* 1991; Rose 2000) and can be trained using an ethologically-appropriate single-trial task, thus generating a single memory trace (Gibbs & Ng 1979; Crowe *et al* 1991a,b). However, while avians share with mammals a number of common molecular processes underlying the trace, certain differences remain. As such, researchers favour mammalian models of memory processing.

Interestingly, unlike the day-old chick (Ng *et al* 1991; Rose 1991), learning in neonate rodents is more dependent upon attachment (Moriceau & Sullivan 2005) than avoidance. Therefore, researchers have designed tasks for the neonate rat which do not use aversive conditioning and thus alleviate many welfare concerns. In particular, researchers have used odour-preference testing whereby an odour is paired with positive tactile stimulation at training and sometime later the same odour is presented at test along with a novel odour (Sullivan *et al* 1991; Sullivan & Wilson 1994). Retention is noted by the preference the neonate rodent shows towards the odour to which they were previously exposed (Sullivan & Leon 1986; Sullivan *et al* 1989; Sullivan & Wilson 2003). Importantly, this task is both ethologically valid and can be designed with a number of controls to demonstrate that an odour preference is due to memory processing. Taken together, the use of neonate rodents in odour preference testing is not only an excellent task but holds a unique place within the literature.

However, this task uses a training trial whereby the pup is removed from the nest/dam for at least ten minutes (eg Sullivan *et al* 1989, 1991) which presents both welfare and scientific problems. For example, separation from the dam increases the risk of rejection at the completion of the training trial (Kaneko *et al* 1996) which leads both to suffering on the part of the pup, as well as a reduction in sample size. In addition, even brief periods of removal are likely to induce stress on the part of both the pup and dam (Kuhn & Schanberg 1998; Roman *et al* 2003). Not only is this a serious welfare concern but stress induced by separation may confound the study both by altering the strength of

the memory trace and the timing of when the trace reaches each memory stage (Yerkes & Dodson 1908; De Vaus *et al* 1980; Beylin & Shors 2003; Quiroz *et al* 2003; Gulpinar & Yegen 2004; Zhang *et al* 2004). Further, the effect of this stress may be so pronounced as to even cause neurobiological changes (Kuhn & Schanberg 1998; Roman *et al* 2003). Although the separation of the pup from the dam for training cannot be avoided, the duration of the separation may be able to be reduced. Therefore, the aim of the present study was to determine if a substantially shorter training trial (two minutes) could elicit equivalent retention levels to the standard ten-minute training trial at test 24 h, post-training.

Materials and methods

Eight female and two male Wistar rats were used to breed rat pups for this study. Two female adults were mated at a time, by different males, and matings were randomised with males to ensure heterogeneity of genotypes. Food and water were provided to adult rats *ad libitum* and the laboratory was run on a 12 h light/dark cycle with lights on at 0800h and off at 2000h.

Neonates (n = 175) were chosen at random for training. Pups were briefly placed under a heat lamp maintained at 30°C prior to training to maintain body heat. The odours used in this experiment were eucalyptus oil (Bosisto's, Oakleigh South, VIC, Australia) and tea tree oil (Thursday Plantation, Ballina, NSW, Australia) and have been demonstrated to be detectable and approached by neonates (Ogeil & Edwards 2007). By random choice, eucalyptus was used as the training odour in this study while tea tree acted as the novel odour during testing.

Each pup was trained in a 1 litre glass beaker under a heat lamp maintained at 30°C before being subjected to one of four conditions, each lasting two or ten minutes (see Sullivan *et al* 1989, 1991): (i) Odour + stroke training condition — the beaker contained cotton wool that had four drops of eucalyptus placed upon it and pups were stroked with a sable brush for 30 s per minute (stroking with a sable brush is equivalent to the positive tactile behaviours of the dam) (Sullivan *et al* 1991); (ii) Odour alone control condition — the beaker contained four drops of eucalyptus on cotton wool to determine whether presentation of an odour alone would influence a pup's performance during testing; (iii) Stroke alone control condition — the beaker contained cotton wool with no odour but pups in this condition were stroked with a sable brush for 30 s per minute to determine whether stroking alone would influence behaviour during testing and (iv) Naïve baseline control condition — pups in this condition were placed in a beaker that contained cotton wool without being subjected to either the eucalyptus odour or stroking with a sable brush.

Testing occurred 24 h, post-training and the experimenter was 'blinded' to the training condition that the pups had been allocated. Retention for the eucalyptus odour was tested by placing pups, one-at-a-time following random selection, into a custom-built test chamber, similar to Sullivan *et al* (1991). It was a black, Perspex chamber open at the top that measured 21 × 15 × 40 cm (length × width × height) (see Figure 1). The floor of the chamber was perforated by two grids with 1 mm holes. The two grids were separated by a 2 cm wide 'neutral zone' which bisected the length of the chamber and was where each rat pup was to be placed during testing. Beneath the floor was a compartment divided by a barrier which bisected its length. Each half of the sub-floor compartment was filled with odourless paper pellets. In one half of the sub-floor compartment, eight drops of eucalyptus were spread onto the pellets, and in the other half, eight drops of tea tree were spread. As such, the eucalyptus acted as the conditioned odour and the tea tree acted as a novel reference odour in the preference test.

During testing, pups were placed, one-at-a-time, in the middle of the neutral zone. By random allocation, half the pups were placed facing towards the experimenter and half facing away from the experimenter to avoid a potential turning bias, which had been found to occur in other rat tasks (Roberts 1979). During each 120 s test trial, the experimenter measured the length of time a pup spent over each side of the neutral zone, be it towards the conditioned odour or towards the novel odour. A choice was considered to have been made when a pup's nose and front paws were placed out of the neutral zone towards one of the sides containing the perforations.

The procedures used in this experiment were approved by the Animal Ethics Committee of the School of Psychology, Psychiatry and Psychological Medicine, Monash University and were performed in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Seventh Edition)* (NHMRC 2004).

Statistical analysis

A discrimination ratio was calculated for each pup by dividing the amount of time each animal spent over the conditioned odour by the test duration (120 s). This value was subsequently divided by the mean discrimination ratio of the naïve group and multiplied by 100 to provide a percentage score relative to the naïve baseline group.

Data were analysed using SPSS for Windows version 14. One-tailed orthogonal *a priori* comparisons using *t*-tests were used to compare the conditioned and control groups. On the basis of previous results (eg Sullivan *et al* 1991) we had hypothesised that only the odour + stroke group would be significantly different from the other control conditions demonstrating the presence of retention. An independent measures *t*-test was then conducted to compare the two- and ten-minute conditioned groups. The assumptions of normality and homogeneity were satisfied for all tests.

Results

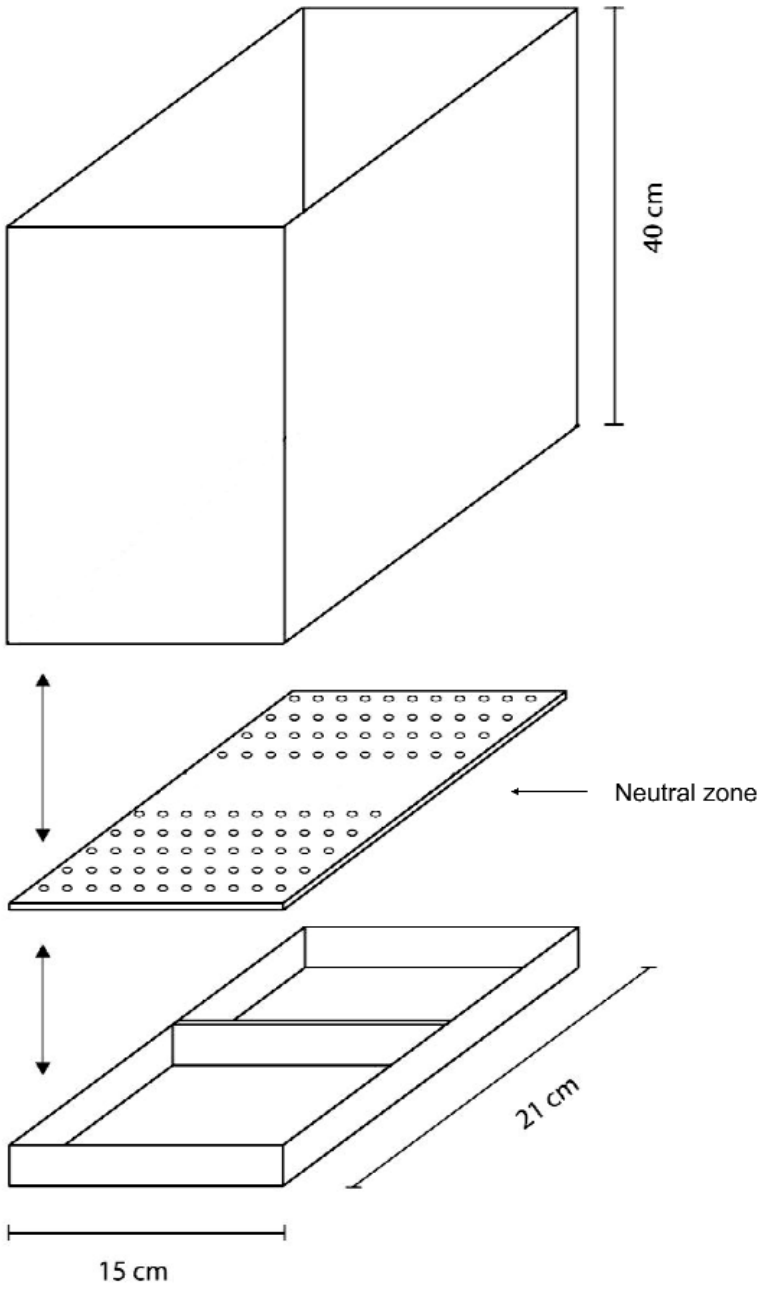
Figure 2 displays the mean discrimination ratios for the odour + stroke conditioned group, the odour alone control group and the stroke alone control group when using a ten-minute (a) and two-minute (b) training trial.

Figure 2 shows that the odour + stroke conditioned groups spent significantly more time over the odour paired with stroking (eucalyptus) compared to the control groups.

https://doi.org/10.1017/S0962728600000506 Published online by Cambridge University Press

Figure 1

The testing apparatus. The floor of the box sat upon a divided compartment in which the paper pellets and odours were placed. A Perspex covering was used to enclose the floor and divided compartment.



It was found that the ten-minute odour + stroke group had a significantly greater mean discrimination ratio compared to the ten-minute stroke alone group $t(73) = 2.15$, $P < 0.05$, $\eta^2 = 0.11$. It was also found that the ten-minute odour + stroke group had a significantly greater mean discrimination ratio compared to the ten-minute odour alone group $t(73) = 2.59$, $P < 0\ .05$, $\eta^2 = 0.15$.

It was found that the two-minute odour + stroke group had a significantly greater mean discrimination ratio when compared to the two-minute stroke alone control group $t(94) = 1.67$, $P < 0.05$, $\eta^2 = 0.06$. It was also found that the two-minute odour + stroke conditioned group had a significantly greater mean discrimination ratio compared to the odour alone group $t(94) = 1.84$, $P < 0.05$, $\eta^2 = 0.07$. When comparing the two- and ten-minute conditioned groups, no significant difference was found $t(43) = 0.13$, $P > 0.05$.

Discussion

This experiment sought to identify whether a substantially shorter training duration could be used in a common odour discrimination task to alleviate important animal welfare concerns and to improve scientific efficacy. In particular,

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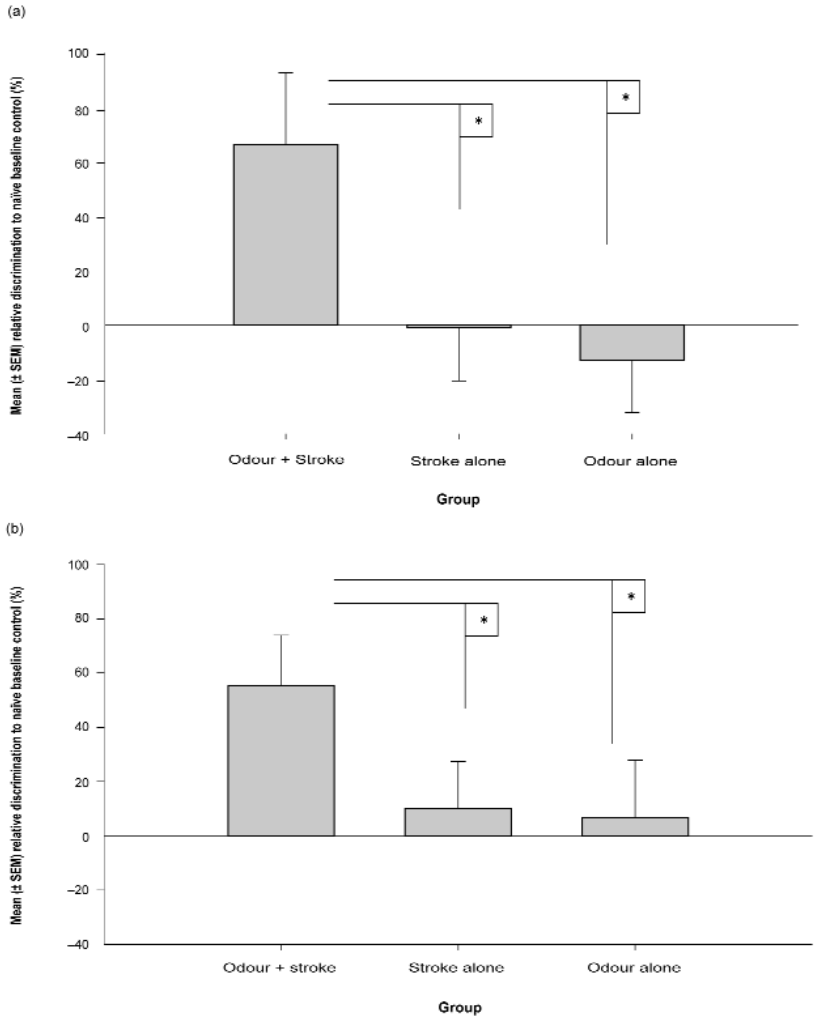
Figure 2



Group mean (+ SEM) percentage discrimination of groups relative to naïve baseline control using an (a) ten-minute or (b) two-minute training trial (*P <0.05). Positive values indicate that the overall group mean favoured the conditioned odour at test, and negative values indicate that the overall group mean favoured the novel odour at test.

these concerns related to stress during training with neonate rats. Such a consideration is important from a neurobiological perspective because increased stress may interfere with the memory trace (De Vaus *et al* 1980), and inhibit researchers who are trying to identify to which stage of memory particular biological processes belong.

In the current experiment, it was demonstrated that when neonate rats were conditioned to eucalyptus odour using either a two- or a ten-minute training trial, they spent significantly more time over the eucalyptus odour, compared to the novel tea tree odour, during testing 24 h later. Pups displayed excellent retention for the eucalyptus when paired with stroking but not when the odour was presented alone, or they were only provided with stroking. In particular, the shorter training duration was found to be adequate for use with this odour preference task (Sullivan *et al* 1989, 1991). This finding suggests that other animal memory tasks may also be amenable to the use of shorter training durations without an appreciable loss of retention.

Animal welfare implications

This study found that a two-minute training trial was sufficient to condition neonate rats using a common odour preference task and demonstrate similar levels of retention to those seen with a ten-minute training trial. This finding demonstrates several potential benefits to welfare. A reduced training duration reduces the likelihood of pups being rejected by the dam once returned to the nest. In addition, a shorter training duration limits the degree of stress suffered by the pups.

Acknowledgements

The authors wish to thank Kat Kallady for designing the figure of the test apparatus (Figure 1). The authors also wish to thank Dr Jim Phillips and Kathleen Bagot for their useful comments on a previous draft of this paper.

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