Survival of olfactory memory through metamorphosis in the fly *Musca domestica*

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Abstract

Metamorphosis in the fly (*Musca domestica*) involves extensive lysis, neurogenesis and reorganization of neural tissue. Despite this, two experiments are reported which show aspects of larval memory persist into the adult form. Experimental larvae were reared in sawdust scented with aversive odours (mint or geraniol). Control larvae were reared on plain sawdust. In blind post-metamorphosis testing using a Y maze, adult flies showed a preference for the specific odour to which the larvae had been exposed. Control flies found both test odours aversive. A second experiment explores the possible role of cell survival through metamorphosis as a mechanism of the persistence of memory. Cells from trained donor flies when grafted into untrained recipients produced specific changes in recipient odour preference reflecting the donor larvae training. © 1999 Elsevier Science Ireland Ltd. All rights reserved

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Sporadic reports have appeared in the literature intimating that memory acquired during insect larval stages can survive the profound neural changes associated with metamorphosis and can be appropriately expressed in the adult [1,5,7,12,17]. Indeed, such studies have not been confined to the invertebrate kingdom, similar results have been reported in amphibians [11]. Other researchers have failed to find survival of memory [8]. While most of these studies are now of some antiquity, there remains little attempt to validate these findings or explore the possible mechanism of this interesting phenomenon.

The most popular hypothesis to explain such survival of memory is the persistence of larval neurones through metamorphosis and their subsequent integration to the adult nervous system [16], how this may happen remains unaddressed. The adult insect brain is formed after the lysis of most larval neurones and a period of neurogenesis, migration and differentiation of new cells resulting in an increased neuropile in the complex adult brain with cells derived from both neuroblasts and ganglion cells.

Two studies are reported to firstly confirm post-metamorphosis survival of memory in the fly, and secondly to explore the role of persisting larval cells as a possible mechanism of such survival by grafting tissue from trained larvae into untrained recipients.

An initial experiment was conducted to establish olfactory learning in house fly larvae, and confirmed earlier reports that larval exposure to aversive odours in the rearing environment (mint or geraniol) made these odours attractive in subsequent testing. One hundred and fifty larvae from the same stock were reared in identical green circular plastic containers (12 cm diameter) in one of three conditions. Group one was reared on sawdust scented with mint (1 ml of mint oil in 500 ml of sawdust), a second group was reared in sawdust scented with geraniol oil and a final group were reared on plain sawdust with no odour. Odour exposure training commenced 72 h prior to expected pupation and larvae remained in these conditions for 48 h. After exposure, 10 larvae were randomly selected from each group, washed in distilled water and placed in sterile glass vivaria containing unodoured sawdust and maintained at 20°C with a relative humidity of 60% on a 12 h light/dark schedule, through pupation. After emergence as adult flies, flies remained in
dark room with temperature and humidity maintained at 20°C at the centre of the maze. Testing was conducted in a dark room with temperature and humidity maintained at 20°C and 65%, respectively.

Flies were tested individually for 5 min. The amount of time each fly spent in each odour arm was recorded. A second experimenter conducted all testing of adult flies with no knowledge of the original larval training.

Pre-metamorphosis training to an aversive odour has a clear and highly specific effect on the odour preference of adult flies (Fig. 1). In both the mint and geraniol trained groups; flies showed a strong preference for their trained odour and an avoidance of the other odour. Interestingly, both odour trained groups showed a preference for the arm containing their trained odour compared to the no odour arm. Control flies showed a strong aversion to both mint and geraniol and remained for most of the test in the odourless arm, thus confirming these odours to be aversive in untrained flies [17]. These data clearly illustrate the survival of olfactory memory acquired in the larval stage through the extensive cellular lysis, remodelling and proliferation of metamorphosis in the fly.

How such memory survival is mediated remains to be elucidated. One possibility is that the memory is the product of surviving cells, which remain largely intact throughout metamorphosis but acquire new arborizations. While it is well established that metamorphosis is accompanied by extensive destruction of the larval nervous system, it is also known that cellular destruction in the insect brain occurs simultaneously with neurogenesis [16]. This had led several laboratories to suggest that the surviving larval cells make connections with new cells produced during neurogenesis. Unfortunately, this suggestion has received little empirical scrutiny thus precluding further speculation as to the mechanism(s) of such transfer or induction of behaviour.

The development of neural grafting techniques in insects [13,14] finally enables a direct study of the influence of cells from trained larvae on subsequent development and behaviour of recipients and it is to this that the second study is addressed.

Ten donor larvae were trained as in experiment one, five trained to mint odour and five trained to geraniol. After 48-h exposure to the training odour, larvae were washed with distilled water and larval brains were dissected out using a stereo dissecting microscope in a laminar flow hood. Larvae were anaesthetized with hypothenalia for 30 min at 0°C, then immersed in insect saline. Brain tissue was carefully dissected from the head and aspirated by glass pipette. Dissected tissue from each group was pooled for cellular dissociation in 1 ml of insect saline and immediately dissociated by gentle trituration through a 21 g needle followed by further trituration through a 26 g needle attached to a 5 ml disposable syringe. Double trituration was found to be necessary to produce a homogenous cell suspension and minimize cellular aggregation of donor tissues. Larval brain tissue was found not to be amenable to dissociation in trypsin, this caused extensive destruction of larval brain cells. Donor cells were pelleted by a 3 min spin in a microcentrifuge at 1000 rev./min. The pelleted cells were then resuspended in 0.3 ml of Schneider’s medium supplemented with 2 mM glutamine and 10% FBS for subsequent injection. An identical cell suspension was prepared from untrained larvae for injection to control recipients.

Graft injections were conducted approximately 24 h prior to pupation and were conducted under a dissecting microscope using a micro-manipulator driven Drummond Micro-pipette injector, with a pulled glass needle with a final internal diameter of 35 μm with a 45° bevel at the injection tip. Fresh needles were used for each injection. Needles were back filled with paraffin oil and cell suspensions were then aspirated into the needles for injection. The injected cell suspension volume was 0.2 μl delivered over 60 s. The needle remained in situ for a further 2 min to minimize leakage of injected tissue. Injections were made into the recipient larvae head directly above the larval brain with a needle penetration of 0.1 mm. No attempt was made to localize injected tissue in the recipient cerebral ganglion.

Twenty naive recipient larvae received injections of cell suspension derived from mint trained larvae, 20 received an equivalent graft of cells from geraniol trained donors, and a further 20 were injected with an identical suspension prepared from untrained donors. Mortality rates were three, five and four larvae in the mint, geraniol and control groups.
respectively, so all groups were randomly reduced to 15 animals.

After graft injection a small patch of liquid skin (Opsite; Smith & Nephew) was applied to the head to prevent any further leakage of the cell suspension, in latter studies this was found to be unnecessary. After the procedure, each group of recipient larvae was separately placed on fresh sawdust to pupate and housed as reported above. Adult flies were tested 18–29 h post-emergence in the Y maze apparatus reported above for 5 min. Total time in each odour arm was again recorded for each animal in blind testing.

Flies surviving the initial injection showed no obvious deleterious effects of the surgical procedure. Results of behavioural olfactory testing in the maze are presented in Fig. 2. Control animals receiving untrained brain tissue showed a strong aversion to both mint and geraniol, further confirming the aversive properties of these odours. These flies preferentially localized in the unodoured arm showed great agitation when they briefly explored the other two odours. However, recipients of both mint and geraniol trained grafts both showed a strong and highly specific preference to the odours to which the donor larvae had initially been trained.

These results show that grafting of cells from trained larvae into the head of naive recipients can influence subsequent recipient nervous system development and, ultimately the behaviour of the adult fly. Further experiments, not reported here, show these effects are reproducible and can be achieved with other aversive odours. It is also interesting to note that the behavioural changes mediated by the cell grafts do not require any recipient training to the donor odour prior to testing, yet produce a strong and specific change in recipient behaviour.

The transplantation of cells from trained larvae and their subsequent effects on recipient post-metamorphosis behaviour provide strong support for the role of surviving cells as mediators of memory survival through metamorphosis. The ease of training and stability of learning over time and life cycle reported in the above experiments offer advantages to laboratories interested in fly learning. Currently, such work is dominated by studies of Drosophila learning which has made a major contribution to our understanding of the molecular basis of learning [4]. Many genes have now been isolated which affect olfactory learning in flies; similarly, mutant strains have been raised which show anatomical abnormalities including mushroom body abnormalities [10]. The mushroom bodies of insects are known to play a key role in learning and memory. Studies of such gene activity, both during metamorphosis and following transplantation, in normal and mutant flies would enhance our understanding of the role of the genome in the establishment and maintenance of behavioural plasticity.

That transplanted cells are capable of altering recipient behaviour is not new, tissue grafts of suprachiasmatic nucleus have been shown to determine circadian period in recipient rats [15]. Similarly, grafts of quail brain tissue into embryonic chicks subsequently influence the calls of the birds post-hatching [2]. To our knowledge the experiments reported here are the first demonstrating that similar grafts in flies can be achieved with modification of recipient fly behaviour concomitant with donor training. Little is currently known of the fate of dissociated tissue when subsequently grafted into a host larval or adult insect. In the above studies no attempt was made to follow the grafts histologically, so we cannot determine if the grafts integrate with the host or mediate their behavioural effects chemically. However, an expanding literature shows both the viability of such insect neurones in tissue culture [3], yet transplantation studies in the adult honeybee report that integration of surgically grafted mushroom bodies was not necessary for the grafts to confer on the recipient bees a donor-learned time signal [13]. Further research is necessary to delineate between these possibilities.

Pertaining to this issue in the current fly experiments, the direct placement of the tissue graft into the brain substance of the recipient was not necessary. It is possible that host metamorphosis may facilitate the survival and possibly the integration of grafted cells, this awaits further investigation. Grafting of cell suspensions into the vertebrate nervous system is now a well established technique [9] and shows cell suspensions carrying various markers; when injected to the embryonic vertebrate recipients are found to colonize appropriate brain regions of the host nervous system [6]. Unfortunately similar studies are lacking in the invertebrate literature.

A further hypothesis currently under investigation in our metamorphosis research, is that the grafted cells fail to integrate into the host nervous system but add humoral factors, which subsequently influence the connectivity in the re-organizing host nervous system. Given the profound effects of brain derived extracts on neural tissues both in vitro and in vivo, and the importance of chemical signalling in the developing brain, a chemical basis to the survival of learn-
ing through metamorphosis is not beyond the bounds of possibility. Such a possibility has already been suggested to explain a related phenomenon, the survival of memory through planaria regeneration, generating a prolific if controversial literature [16].

In conclusion, the survival of olfactory memory through metamorphosis is confirmed by the above results. Furthermore, the transplantation study suggests that tissues derived from trained larvae can profoundly influence subsequent recipient behaviour. Exploration of this interesting phenomenon offers a complementary technique to the established molecular approach to fly learning, and offers a unique opportunity to study the substrates of learning and memory at the physiological and pharmacological level.