Adult neurogenesis in a moth brain

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Adult neurogenesis in a moth brain

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ABSTRACT

In both vertebrates and invertebrates, neurogenesis occurs not only during development, but persists throughout adult life. So far, adult neurogenesis has been detected in a few insect orders but not in Lepidoptera. In the moth, *Agrotis ipsilon*, the sensitivity of antennal lobe interneurons of males to sex pheromone is age- and juvenile hormone-dependent in accordance with changes in the behavioral response. As a first step to understand this neuronal plasticity, we tested the hypothesis that adult neurogenesis could occur in the moth brain using 5-bromo-2’-deoxyuridine treatments on newly born and sexually mature moths. Our results show that persistent neurogenesis occurs in mushroom bodies of adult males and females of *A. ipsilon*. Two clusters of 1-4 neuroblasts in each brain hemisphere continue to divide in adult moths and give rise to small clusters of Kenyon cells in the cortex of the mushroom body calyces. Neurogenesis was observed both in newly born and sexually mature males. There was a clear increase in the number of newly born cells in brains as the time increased after the treatment that was performed soon after emergence. When treatments were performed in mature 3-day-old adults, neurogenesis was still detected in brains dissected 3 hours after treatment but was hardly visible two days later. Adult neurogenesis was also detected in the optic lobes, but not in the antennal lobes. We hypothesize that the newly born neurons could play a role in the central nervous plasticity of olfactory processing in the adult moth *A. ipsilon*.

**Indexing terms:** BrdU labeling- mushroom bodies- *Agrotis ipsilon*- Lepidoptera- neuronal plasticity- olfaction
INTRODUCTION

It is now clearly established that new neurons are being added to specific brain structures involved in different kinds of plasticity including learning and memory processes throughout adult life of a wide range of both vertebrates and invertebrates (review in Cayre et al., 2002). Since the pioneer work of Kaplan and Hinds (1977) showing newborn neurons in the dentate gyrus and in the olfactory bulb of the rat, several studies have revealed that neurogenesis in adult vertebrates occurs in different non-mammalian and mammalian species including primates (Gould et al., 1999) and humans (Eriksson et al., 1998). In invertebrates, neurogenesis was detected in the higher brain centers (hemi-ellipsoid bodies) of the adult shore crab, Carcinus maenas (Schmidt, 1997).

In insects, it was long believed that adult brains were unable to generate new neurons. The neuroblasts (NBs) that proliferate during post-embryogenesis were found to stop dividing and to degenerate prior to adult eclosion (Edwards, 1969; Truman and Bate, 1988; Ganeshina et al., 2000). However, some studies described late-dividing NBs in mushroom bodies (MBs) of adults of a few species, but the fate of these new neurons had not been determined (Panov, 1957, 1963). MBs are a paired structure in the protocerebrum that play a key role in the integration of multisensory inputs from the visual and olfactory centers (Kenyon, 1896; Mobbs, 1982; Li and Strausfeld, 1997). The MBs contain densely packed, very small cell bodies of a large number of neurons, the Kenyon cells (KCs), and neuropil forming a peduncle and two to three lobes.

Reconsidering the fact of late-dividing NBs, Cayre et al. (1994) clearly demonstrated, using 5-bromo-2’-deoxyuridine (BrdU) incorporations, that persistent neurogenesis was occurring in MBs of the house cricket, Acheta domesticus. Neurogenesis in the MBs of the house cricket has then been studied in detail (reviews in Strambi et al., 1999; Cayre et al., 2002). Briefly, a cluster of densely packed NBs located at the apex of the cortex of the MBs
continues to divide and produces new KCs throughout adult life. As the delay between BrdU injection and brain dissection increases, labeled cells are then observed within the MB cortex. Detailed studies show the birth and fate of new neurons in the house cricket (Cayre et al., 2000). Moreover, the dividing process of the NBs in MBs of the house cricket was consistent with the accepted paradigm for insects: NBs divide to produce daughter cells, the ganglion mother cells (GMCs), which further divide, giving birth to KCs.

Persistent neurogenesis was further detected in some and lacking in other insect species from various orders (Cayre et al., 1996). In Lepidoptera, although Panov observed NB divisions in three species of Lepidoptera, neurogenesis was not detected in MBs of the adult butterfly, *Danaus plexippus* (Nordlander and Edwards, 1970).

In the house cricket, neurogenesis is controlled by juvenile hormone (JH), a crucial insect hormone produced by a pair of retrocerebral glands, the corpora allata (CA) (Cayre et al., 1994). Cayre et al. (1994) first suggested that adult neurogenesis could be the neuroanatomical basis for the JH-controlled oviposition behavior of *A. domesticus* females. However, suppression of neurogenesis using γ-irradiation showed that the female egg laying behavior was not altered (Scoto-Lomassese et al., 2003). The detailed studies on insect neurogenesis led us to reconsider the possibility that new neurons could be added in brains of adult Lepidoptera as one of the hypotheses to explain our recent results on olfactory-based neuronal plasticity in the moth, *Agrotis ipsilon*. In males of this moth, sex pheromone responsiveness and processing in the antennal lobe (AL) is age- and JH-dependent (Gadenne et al., 1993, Anton and Gadenne, 1999, Gadenne and Anton, 2000). The sensitivity of AL neurons for the sex pheromone increases with age and JH biosynthesis and can be inhibited using surgical removal of the CA, thus revealing an olfactory-based neuronal plasticity. Moreover, this JH-linked plasticity is restricted to the processing of sex pheromone within the macroglomerular complex of the AL and does not affect the processing of plant odors in other
parts of the AL (Greiner et al., 2002). This plasticity could be the result of physiological or anatomical changes occurring during adult maturation.

In this study, we tested the hypothesis that neurogenesis could occur in the brain of the migrant moth, *A. ipsilon*, with newly born neurons participating in the increase of sex pheromone sensitivity with age. Using BrdU treatments in males and females, we show that persistent neurogenesis occurs in the MBs, but not in the antennal lobe of both male and female moths.

**MATERIALS AND METHODS**

**Insects**

*A. ipsilon* larvae originating from a laboratory colony in Bordeaux, France, were reared on an artificial diet (Poitout and Buès 1974), in individual plastic cups until pupation at 21°C ± 1°C under a 16-hour light, 8-hour dark regime. Adults from the field were introduced into the colony each spring. Pupae were sexed and held separately under the same light and temperature conditions with 70% relative humidity. Pupae were checked each day for newly emerged adults (day-0). Adults were held in plastic boxes and had access to 20% sucrose solution throughout adult life.

**Histology**

Brains (supra- and suboesophageal ganglions) were removed and fixed either in Bouin’s fixative or in Carnoy overnight at room temperature, rinsed in distilled water, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Tissues were sectioned at 7 μm and stained with hematoxylin and eosin (Martoya and Martoya, 1967).

**BrdU labeling**
We used the protocol by Cayre et al. (1994, 1996). DNA replication was monitored using BrdU (Sigma, St-Louis, MO, USA), a thymidine analogue incorporated during DNA synthesis, and detected immunohistochemically by using a monoclonal antibody against BrdU. Newly emerged (day-0, within two hours after emergence), and 3-day-old insects received one single abdominal injection of 10 µl of a solution of BrdU using a Hamilton syringe. BrdU was dissolved in Ringer solution to a final concentration of 40 mg/ml and warmed to 50°C until it was completely dissolved.

Survival time is defined as the time delay between BrdU injection and brain dissection. Brains of the injected insects were dissected 3 hours to 8 days after BrdU injection.

**Immunohistochemical staining of BrdU labelled cells**

To reveal in vivo incorporation, we adapted the procedure by Cayre et al. (1994, 1996). Brains were removed and fixed over night in Carnoy’s fixative at room temperature. Alcoholic Bouin’s fixative was also tested (Fahrbach et al., 1995), but, although good tissue preservation was obtained, no positive staining was found in these preparations. Tissues were rehydrated and permeabilized in 0.1M phosphate buffered saline (PBS) for 5 minutes and twice 20 minutes in PBS solution containing 0.3 % Triton-X100 (PBST). DNA hydrolysis proceeded for 30 minutes in 2N HCl in PBST at room temperature followed by four times 15 minutes rinses in PBST to eliminate the acid and 15 minutes in PBS to eliminate Triton excess. Brains were then treated with trypsin warmed 25 to 30 minutes at 40°C prior to treatment (10 mg in 100 ml PBS; Sigma), rinsed three times 15 minutes in PBS and briefly in distilled water. Brains were then dehydrated, cleared and embedded in paraffin. Seven µm sections were mounted on poly-L lysine coated slides. Deparaffined and rehydrated sections were rinsed in PBS. Background staining was reduced by applying 5% normal goat serum (Sigma) for 20 minutes, at room temperature prior to
incubation in the primary antibody serum. Sections were incubated in a 1/100 dilution of a monoclonal anti-BrdU antiserum (Calbiochem, La Jolla, USA) in PBS overnight at 4°C. After careful washing 4 times 15 minutes in PBS, sections were incubated in a goat anti-mouse IgG antibody conjugated with peroxidase (1/1,000 in PBS-T, Calbiochem, La Jolla, USA) for 4 hours at 4°C. Slides were transferred after washing 4 times 5 minutes in PBS into a solution containing 10 mg 3,3’-diaminobenzidine (Sigma), 8 mg Nickel Chloride (Sigma) and 0.03% H₂O₂ in 20 ml PBS for 30 minutes in the dark. Sections were dehydrated, rapidly counterstained with 0.4% picro-indigo-carmin, cleared and mounted in DPX (Fluka, Steinheim, Germany). Control treatments were performed by replacing the primary antibody by PBS.

**Feulgen-Rossenbeck nuclear staining of DNA**

This nuclear staining allows visualizing mitotic cells. Tissues were fixed and treated as described above, embedded in paraffin, serially sectioned, then deparaffined, rehydrated, and treated according to the method of Feulgen-Rossenbeck (Martoja and Martoja, 1967). DNA hydrolysis was achieved using 1N HCl for 8 minutes at 60°C. Sections were rinsed with distilled water and incubated in Schiff’s reagent for 1 hour. Sections were rinsed 3 times in a solution containing 1% Sodium metabisulfite (Sigma) in distilled water, then in distilled water. Sections were counterstained in 0.5 % Light Green (Sigma) for 10 minutes. Sections were washed again, dehydrated, cleared and mounted with DPX.

**Data analysis**

All brain sections were viewed on a Zeiss Axiophot microscope equipped with a spot digital camera. For each treated brain, we localized, counted and measured the diameter of the labeled cells. For each labeled cell, we measured the diameter of its nucleus.
RESULTS

Structure of adult mushroom bodies

In *A. ipsilon*, mushroom bodies consist of a calyx neuropil, situated postero-dorsally in the protocerebrum (Fig. 1A). The cell bodies of the intrinsic neurons, the KCs, are densely packed around the calyces. KCs send their axons into the calyx and through the peduncle to the α, β and γ lobes (Fig. 1A). Using the Feulgen-Rossenbeck nuclear staining of DNA on young immature male and female adults, we observed two proliferative areas (lateral and medial) containing NBs in the apex of the cortex of the mushroom bodies on each side of the brain (Fig. 1B, C) in which cells were found in division (Fig. 1D, E).

Neurogenesis in newly born adults

Using BrdU as a marker of DNA synthesis, injected in newly born adults (within two hours after emergence), we show that neurogenesis occurs in the mushroom bodies of *A. ipsilon* adult brains. Specific BrdU labeling was also detected in optic lobes, but will not be further described in the present paper.

Labeled cells were found in 16 brains dissected 3 hours after BrdU injection (Fig. 2). In vivo incorporation of BrdU revealed two clusters of cells (1-3 cells in the lateral cluster and 1-3 cells in the medial cluster) for each mushroom body that are located in the cortex of the Kenyon cell bodies. These cells are located within the two proliferative areas described earlier. In each cluster, two cell types were found. The first cell type (1-2 cells in each cluster, 12-14 µm diameter) was found in all brains for the medial cluster and only in 7 brains for the lateral cluster. This cell type is lightly stained, has an ovoid nucleus (mean nucleus size: 8.1 ± 0.7 µm diameter, n=17 and 7.1 ± 0.6 µm diameter, n=9, in the medial cluster and lateral clusters respectively), resembling characteristic NBs. The other cell type (1 cell in each cluster) was found in all 16 brains for the medial cluster and only in 7 brains for the lateral
cluster. This second cell type is densely labeled, smaller (8-10 µm diameter) with round nuclei (mean nucleus size: 5.3 ± 0.3 µm diameter, n=14 and 5.9 ± 0.1 µm diameter, n=4 in the medial and lateral clusters respectively), characterizing GMCs and probably the first new KCs. Labeled cells were observed in both female (Fig. 2 A) and male brains (Fig. 2 B).

In the 6 brains of A. ipsilon that were dissected 2 days after BrdU injection, the number of labeled cells forming the two clusters increased, thus showing cell proliferation (Fig. 3 A-D). The lateral and medial cell clusters contain 8-9 cells and 8-12 cells respectively of a uniform size (8-10 µm diameter) (Fig. 3 A-D). As for the 3-hour survival time, the labeling frequency observed was higher in the medial than in the lateral cluster. NBs (1 cell in each cluster) and GMCs (2-3 cells in the medial cluster 1-2 cells in the lateral cluster) were observed. A third type of cells (7-8 cells in each cluster) was observed with nuclei between 3 and 5 µm in diameter (medial cluster: 4.1 ± 0.5 µm diameter, n=46; lateral cluster: 4.0 ± 0.6 µm, n=31). They resemble the KC cells. When brains were dissected four days (4 brains) and eight days (4 brains) after BrdU injection, the labeling of the proliferative area in the dissected brains was hardly visible, but the number and location of the new KC cells was similar as that found in brains dissected two days post-treatment (Fig. 3 E, F).

Neurogenesis in 3-day-old adults

To check if neurogenesis was still occurring in sexually mature A. ipsilon adults, BrdU was injected in sexually mature 3-day-old males and females. After a 3-hour survival time, 8 to 10 BrdU positive nuclei were observed in 3 brains (Fig. 4). Although the three cell types were found, showing the same morphology as described previously, most observed cells were of the KC type. After a 2-day survival time (5-day-old adults), the number of cells was not different from that of adult brains dissected 3 hours post-treatment (data not shown). This
shows that neurogenesis still occurs in the early sexually mature stage but probably stops soon after.

**DISCUSSION**

Our data give evidence that adult neurogenesis occurs in the moth brain. Using BrdU injections, newly born cells were detected in the mushroom bodies of both adult males and females of the noctuid migrant moth, *A. ipsilon*. Persistent NBs located within the mass of the cell bodies of the mushroom bodies were shown to divide in early adult life to give rise to new neurons. Moreover, neurogenesis was also shown to occur in 3-day-old adult insects. In each mushroom body, two groups of 1-5 labeled cells were observed, giving rise to 8-12 cells two days after BrdU incorporation. Our results are similar to those found in Coleopteran species, in which very few dividing NBs were detected, forming two different groups of cells within the mushroom bodies, that gave rise to a few newly born neurons after 17 hours of BrdU treatment (14, 7 and 3 new neurons in *Zophobas sp*, *Tenebrio molitor*, and *Harmonia axyridis* respectively) (Cayre et al., 1996).

The observed labeling of newly born neurons obtained after BrdU injection of adult *A. ipsilon* follows the rule that NBs divide to produce GMCs, giving rise to new KCs in the mushroom bodies. As in detailed studies by Cayre et al. (review in Cayre et al., 2002), the big NBs giving rise to the GMCs were easily visible in our preparations, the labeling in the NBs fading as the delay between BrdU and brain dissection increased. Although the number of cells increased and the size of cells decreased with time, the fate of the newly born neurons was hard to follow in 3-day-old insects. As in the house cricket (Cayre et al., 1996), neurogenesis was also detected in the optic lobes.
Adult neurogenesis in holometabolous insects was so far only detected in species of Coleoptera (Bieber and Fuldner, 1979; Cayre et al., 1996). In hemimetabolous insects, adult neurogenesis is more widespread: it has been detected in Gryllidae (Cayre et al., 1994; 1996) and in the cockroach *Diploptera punctata* (Dictyoptera, Blaberidae) (Gu et al., 1999). It was not found in another Dictyoptera, *Periplaneta americana* and Acrididae (Cayre et al., 1996), in *D. melanogaster* (Ito and Hotta, 1992), or in the honeybee (Fahrbach et al., 1995).

Although Panov (1957, 1963) observed neuroblast divisions in *T. viridissima* and in three lepidopteran species, investigations in *D. plexippus* failed to show neurogenesis in the adult mushroom bodies of this Lepidopteran species (Nordlander and Edwards, 1970). According to Panov (1957), NBs in the corpora pedunculata of larval insects occur either as one aggregate per calyx (e.g. Orthoptera, Hymenoptera, Coleoptera), or as individual NBs, 1-20 per calyx (e.g. Neuroptera, Diptera, Lepidoptera). Aggregated NBs are close to each other and divide both symmetrically and asymmetrically. Individual NBs are separated from each other and divide only asymmetrically like scattered NBs (Nordlander and Edwards, 1970). In general, complex adult mushroom bodies derive from aggregated NBs. Of the Lepidoptera studied by Panov (1957), all show scattered-type NBs, in general two per calyx. However, in *D. plexippus*, the NBs resemble more closely the aggregated-type group. According to Nordlander and Edwards (1970), this is possibly because the monarch adult is long-lived with complex behavior. Like the monarch, *A. ipsilon* adults are migrant insects that travel long distances (review in Showers, 1997) and therefore could be considered as long-lived insects. Indeed, the proliferative area in the adult mushroom bodies is of the aggregate type, two clusters of cells per calyx, like in *D. plexippus*. Altogether these data show that although *D. plexippus* and *A. ipsilon* are both migrant lepidopteran species with long-lived adults, and show the same morphological organization of the proliferative area in the mushroom bodies, they differ by their ability to produce new neurons. One explanation for this striking
difference could be that *D. plexippus* is a butterfly that relies mainly on vision for its reproduction, contrary to *A. ipsilon* that is a night-active moth with a fully developed pheromonal system.

Evidence is now accumulating that neuronal plasticity linked with olfactory behavior and learning is occurring in the adult insect brain. Previous studies showed that age and experience were acting on the morphology and number of mushroom body fibers in peduncles of a coleopteran and *D. melanogaster* (Bieber and Fuldner, 1979; Technau and Heisenberg, 1982; Technau, 1984). Moreover, structural changes in mushroom bodies and olfactory brain centers were associated with foraging experience of honeybees (Withers et al., 1993).

Recently, it was shown that adult neurogenesis in the house cricket, *A. domesticus*, was involved in olfactory learning and memory (Scotto-Lomassese et al., 2003). In particular, it has been shown that suppression of adult neurogenesis using γ-irradiation impaired the processing of olfactory information required for complex odor conditioning. Early irradiation in adult life drastically suppressed NBs and their progeny. Using an escape paradigm involving olfactory cues, visual cues or both, Scotto-Lomassese et al. (2003) showed that irradiated crickets lacking adult neurogenesis exhibited strongly impaired learning only when olfactory cues alone were used.

In moths, as in the honeybee, appetitive learning of flower odors has been shown, using proboscis extension conditioning experiments (Hartlieb, 1996; Hartlieb et al., 1999). However, MB ablation using hydroxyurea did not prevent this elementary learning in the honeybee (Malun et al., 2002). Adult neurogenesis in *A. ipsilon* could nevertheless also be involved in some form of olfactory learning, although learning ability remains to be tested for this specific species.

In *A. ipsilon*, sex pheromone responsiveness is age- and juvenile hormone (JH)-dependent (Gadenne et al., 1993). Newly emerged or JH-deprived mature males do not respond to sex
pheromone in wind tunnel experiments. Further experiments showed that JH does not act at
the peripheral level, but on the processing of sex pheromone in olfactory antennal lobe
neurons (Anton and Gadenne, 1999; Gadenne and Anton, 2000). The sensitivity of antennal
lobe neurons increases with age and JH levels. It is therefore tempting to hypothesize that, at
least in the males, the observed neurogenesis in *A. ipsilon* brains could play a role in the
maturation of the adult central olfactory system by increasing the number of neurons involved
in the processing of the sex pheromone at high brain levels. Behavioral changes could be a
result of summed effects on different levels in the olfactory pathway.

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FIGURE LEGENDS

Fig 1  Proliferative areas in horizontal brain sections of the adult moth *Agrotis ipsilon*. A: General view of the brain (eosine hemalaun staining). Scale bar: 100 µm. Note that α-lobe and γ-lobe of the mushroom bodies are not visible on this section. B: Feulgen Rossenbeck staining of proliferative areas (arrows) in the mushroom bodies. Scale bar: 50 µm. C: Detailed view showing the lateral proliferative area within the cortex of the mushroom bodies. Scale bar: 20 µm. D-E: Mitotic phases (arrows) in Feulgen-Rossenbeck stained horizontal sections of a female brain. Scale bar: 10 µm. D: Telophase; E: Metaphase. AL, antennal lobe; βL, β-lobe; Ca, mushroom body calyces; CB, central body; G, glomerulus; KC, Kenyon cells.

Fig. 2. Neurogenesis in frontal brain sections of newly born *A. ipsilon* adults (BrdU injection two hours after emergence and brain dissected after a 3-hour survival time). A: BrdU immunolabelling in mushroom bodies of an adult female showing active DNA replication in neuroblasts and ganglion mother cells. Scale bar: 20 µm. B: BrdU immunolabelling in mushroom bodies of an adult male. Scale bar: 20 µm. Ca, mushroom body calyces; GMC, ganglion mother cells; NB, neuroblasts.

Fig. 3. Neurogenesis in frontal brain sections of adult *A. ipsilon* (BrdU injection two hours after emergence and brain dissected at various times after BrdU injection). A-D: Brains dissected after a 2-day survival time. A: General view of the brain. Scale bar: 50 µm. B: Detailed view of the medial cluster shows cells with uniform size. Scale bar: 10 µm. C: Detailed view of the lateral cluster shows cells of similar size. Scale bar: 10 µm. D: Next section showing new Kenyon cells not visible in B. Scale bar: 10
μm. E: Brains dissected after a 4-day survival time. Scale bar: 10 μm. F: Brains dissected after a 8-day survival time. Scale bar: 10 μm. Ca, mushroom body calyces; GMC, ganglion mother cells; KC, Kenyon cells; NB, neuroblasts.

Fig. 4. Neurogenesis in frontal brain sections of 3-day-old adult *A. ipsilon* (BrdU was injected three days after emergence and brains were dissected after a 3-hour survival time). A: Lateral cluster of cells. B: Medial cluster of cells. Scale bars: 10 μm. Ca, mushroom body calyces; GMC, ganglion mother cells; KC, Kenyon cells.