Learning and survival of newly generated neurons: when time matters

A. Mouret, G. Gheusi, M.-M. Gabellec, F. de Chaumont,
J.-C. Olivo-Marin, and P.-M. Lledo

SUPPLEMENTAL METHODS

Mice and housing conditions
Animals (n = 175) were maintained under a 12 h light/12 h dark cycle. At arrival, they were randomly assigned to control and trained groups for the various experiments. Each trained group had its own control group. For partial water deprivation, mice received 1.5 ml of water daily.

Tissue preparation
Under deep anesthesia (sodium pentobarbital, 100 mg/kg, Sanofi, France), mice were killed by the intracardiac perfusion of 40 ml of saline (NaCl 0.9%) supplemented with heparin (5x10^3 units/ml) at 37°C, followed by 200 ml of cold fixative (4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4). Brains were removed, post-fixed in the same fixative at 4°C overnight and stored in 0.1 M PBS at 4°C. Coronal or sagittal 40 µm serial sections were cut with a vibratome (VT100S, Leica, Germany) and collected in PBS (0.1 M, pH 7.3).

Immunohistochemistry
The primary antibodies used were a rat monoclonal anti-BrdU antibody (1:200; Oxford Biotech, Kidlington, UK), a mouse anti-NeuN antibody (1:200; Chemicon,
Temecula, CA, USA) and a rabbit anti-DCX antibody (1:2000; Abcam, Cambridge, UK). BrdU-labeled cells were detected with a peroxidase-based detection system (ABC Systems, Vector Laboratories, Inc., Burlingame, CA, USA), using biotinylated donkey anti-rat IgG antibodies (1:200; Vector Laboratories) and DAB (0.05%, Sigma-Aldrich, St. Louis, MO) as the chromogen. For immunofluorescence detection, the secondary antibodies used were species-specific (1:1000; Alexa-conjugated goat anti-rat 568, Alexa-conjugated goat anti-mouse 488 or Alexa-conjugated goat anti-rabbit 568, Chemicon, Temecula, CA, USA). For DCX labeling, sections were counterstained with DAPI.

**Fluorescence image acquisition**

A microscope (Zeiss, Germany) equipped with an Apotome and Axiovision 4.6 software was used for the acquisition of fluorescence images (BrdU/NeuN and DCX/DAPI staining). A 25x objective was used to reconstruct images of each section and Z-sectioning was performed at 5 µm intervals. The phenotype of BrdU+/NeuN+ cells was confirmed using a 63x objective for Z-sectioning at 1 µm intervals on all double-positive cells. The colocalization of markers was then confirmed by studying merged images for staining in a given cell in each of the xy-, xz-, and yz-planes, in the orthogonal view.

**BrdU+ cell counting by image analysis and DCX+ cell analysis**

BrdU+ cells were counted automatically with a dedicated computer program using a B3 wavelet filtering approach (Quia, de Chaumont et al., 2008). Our method enhances spots (corresponding to BrdU+ cells) while filtering out the background, and the calculations involved are rapid. For each animal, counts were made for one in
every three coronal sections of the OB (120 µm apart). We used anatomical landmarks within the OB to align coronal sections across animals. The rostral landmark, defining the origin of the rostrocaudal axis, contained the first clear mitral cell and external plexiform layers. The accessory olfactory bulb (AOB) was used as the caudal landmark and the last section counted contained the first, clear AOB. The internal and external borders of the glomerular (GL), external plexiform (EPL), granule cell (GCL) layers and the border of the rostral migratory stream of the OB (RMSOB) were drawn interactively. The program then numbered cells detected in the GL, EPL and GCL. Values were given as BrdU+ cell density (number of positive cells per mm²). In some experiments, BrdU+ cell distribution throughout the internal-external axis of the GCL was quantified by dividing the GCL into 10 concentric rings, automatically interpolated using neighbor-interpolation techniques (Olivo-Marin, 2002). The interpolated points were obtained by searching for each point of the external region of interest (ROI), the closest point belonging to the inner ROI. We define O and I as the sets of points corresponding to the external and internal ROIs, respectively. \( O_n \) and \( I_n \) are the \( n \) points of the corresponding ROIs. We define pairs \((O_n, I_m)\) such that the distance \((O_n, I_m)\) is the minimum distance between \( O_n \) and all \( I_v \) in I. We define the \( w \)-th interpolated ROIs as \( \text{INT}_w \). We also define \( k \) as the number of interpolated ROIs desired. The \( n \)-th point of \( \text{INT}_w \) is computed as \( \text{INT}_{wn} = w \times (I_m - O_n) / k \). Ring 1 was the border between the RMSOB and the GCL, and ring 10 was the border with the mitral cell layer.

For sagittal sections, one cerebral hemisphere was analyzed. Counts were made for all consecutive slices containing a clear SVZ and RMS. The SVZ, the RMSv and the RMSh were drawn interactively with the software. The program then numbered the cells detected in the different zones. Values are expressed as BrdU+
cell density (number of positive cells per mm²) for BrdU staining. For DCX staining, we quantified the areas (mm²) covered by DCX⁺ cells for the SVZ, the RMSv and the RMSh.

**Behavioral apparatus**

Mice were trained in computer-controlled eight-channel olfactometers functionally identical to those described in detail by Bisulco and Slotnick (2003). Briefly, solenoid pinch valves controlled air streams, and odors were generated by passing a 50 cc/minute stream of air over the surface of mineral oil-diluted odorants in disposable 50 ml centrifuge tubes. This odorized air was diluted by a factor of 40 in clean air before its introduction into an odor sampling tube in the mouse operant chamber. Before use or the introduction of a new odorant, the olfactometer was washed with 95% ethanol and air-dried. Each odorant and odorant concentration was maintained in its own saturator tube, and the liquid odorant was refreshed daily.

**Odorants**

The odorants used and their rated purities were amyl acetate (99%), anisole (99%), cineole (98%), (+)-limonene (98%), (-)-limonene (99%), (+)-carvone (98.5%), (-)-carvone (99%), linalol (97%), β-ionone (96%) and β-damascenone (97%). Odorants were purchased from Sigma-Aldrich (St. Louis, MO, USA), except β-damascenone, a gift from Philippe Darriet (Onoelogy Faculty, Victor Segalen University, Bordeaux, France). All the odorants used were diluted on a per volume basis with odorless mineral oil to the desired concentration, and 10 ml of solution was used as the odorant source in the odor saturation tubes. Odorant concentrations are given as the liquid dilution of the odorant in the saturator tubes, and the stimuli used in training
were designated by the name of the odorant and its liquid dilution. The 50 cc/minute odorant vapor from the saturator tube was mixed with 1950 cc/minute clean air before its introduction into the sampling port. Thus, the odor concentration delivered to the sampling port was 2.5% that of the headspace above the liquid odorant. We did not check the odorant concentration of the headspace above the liquid solution, but gas chromatographic analyses have shown that the headspace concentrations of various hydrocarbons from mineral oil dilutions are proportional to their liquid dilution (Cometto-Muniz et al., 2003).

**Olfactory discrimination training**

*Pretraining sessions*

Partially water-deprived mice were trained using an operant conditioning go/no-go paradigm, as described by Bodyak and Slotnick (1999). Standard operant conditioning methods were used to train mice to insert their snouts into the odor sampling port and to respond by licking the water delivery tube (located within the odor sampling port) in the presence of mineral oil vapor. The first snout insertion after a 5 s intertrial interval initiated a trial. At the beginning of the trial, the stimulus control valves and a valve directing the air stream away from the sampling tube were functional. This resulted in the odorant vapor being combined with the main air stream and the diversion of the main air stream to an exhaust path. The diversion valve relaxed 1 s later, and the odor stimulus was presented to the odor sampling port. The stimulus valves relaxed 2 s later, thus terminating delivery of the odor. Reward delivery depended on the mouse keeping its snout in the odor sampling port and licking the water delivery tube in seven or more of the last ten 0.2 s periods of the 2 s odor presentation period. Trials in which the mouse did not keep its snout in
the odor sampling port for at least 0.1 s after odor onset were aborted and counted as short sample trials. A 5 µl water reward was delivered if the mouse satisfied the response criterion. All mice underwent two such sessions before being trained on the odor discrimination tasks described below.

**Odorant discrimination**

In each trial, a single stimulus (S+ or S-) was presented. If the response criterion was met in S+ trials, a 5 µl droplet of water was given as a reward and the trial was scored as a hit, whereas failing to meet the response criterion was scored as a miss. Meeting the response criterion in S- trials was scored as a false alarm, and failing to make a criterion response was scored as a correct rejection. S+ and S- trials were presented in a modified random order, such that each block of 20 trials contained equal numbers of each type of trial and no one type of trial was presented more than three times consecutively. The trial procedures were identical to those used in the initial pretraining sessions. The percentage of correct responses was determined for each block of 20 trials \([(\text{hits} + \text{correct rejections})/20 \times 100]\). When mice were trained using enantiomer odors, the (+)-enantiomer served as the S+ and the (-)-enantiomer served as the S-. Scores above 85% implied that mice had correctly learned to assign the reward value of the S+, and the non-reward value of the S-.

The trained mice of the first experiment were given intensive odor discrimination training with multiple odorants for two weeks. They were trained in discrimination tasks in which 1% amyl acetate, 1% anisol, 1% cineol, and 1% linalol were used successively as the S+ and the mineral oil solvent was used as the S-. Mice then learned to discriminate between 1% (+)-limonene and 1% (-)-limonene solutions and between 1% (+)-carvone and 1% (-)-carvone solutions.
Single-odor discrimination

The other groups of mice were trained to recognize a single odorant during one week. They were trained in a discrimination task in which 10% linalol was used as the S+ and the mineral oil solvent was used as the S−, during two days. In each subsequent task, the same procedures were followed, but odorant concentration was sequentially reduced in separate sessions (one per day) to 1, 0.1, 0.01, 0.001 and 0.0001 %. In some experiments, for additional groups, linalol was replaced by amyl acetate or β-ionone.

Statistical analysis

All statistical analyses were performed with Statistica 7.0, with $p<0.05$ considered significant. The density or normalized density of BrdU+ cells surviving on different days after injections and the percentage of BrdU+/NeuN+ cells were compared between groups, using two-tailed Student's t-tests for independent groups. The distribution of BrdU+ cells along the rostrocaudal axis of the GCL was compared between the control and trained groups, by two-way analysis of variance (ANOVA) for repeated measures (group effect and slice effect). The distribution of BrdU+ cells along the entire length of the internal-external axis of the GCL was compared between the control and trained groups, by two-way analysis of variance (ANOVA) for repeated measures (group effect and ring effect- see Fig. 4C). Two-way analysis of variance (ANOVA) for repeated measures was also carried out separately for the population of deep granule cells and the population of superficial granule cells. In all cases, data were expressed as mean values ± SEM.
SUPPLEMENTAL LEGENDS

**Supplemental Figure 1.** Training procedures without odorant in the olfactometer have no effect on neuronal survival in the GCL. A, New cells were labeled with BrdU and their survival was evaluated 30 and 38 days later. Nine days before perfusion, trained mice underwent one week of olfactory training (n = 5). S+ was linalol, and S- was mineral oil. Activity-control mice underwent one week of pretraining sessions whereas naive animals were only handled daily (n = 5). B, BrdU+ cell density in the GCL after training. **p ≤ 0.001 (n = 5). Error bars indicate the SEM.

**Supplemental Figure 2.** Effects of olfactory learning on neuronal survival are distributed along the entire length of the rostrocaudal axis in the GCL. A, New cells were labeled with BrdU and their survival was evaluated 30 and 38 days later. Nine days before perfusion, trained mice underwent one week of olfactory training (n = 6). S+ was linalol, β-damascenone or amyl acetate and S- was mineral oil. B, BrdU+ cell density in the GCL after training. **p < 0.001 (n = 6). C, Distribution of BrdU+ cell density along the rostrocaudal axis in the GCL after training, for animals perfused 30 days after BrdU administration (n = 6). D, Distribution of BrdU+ cell density along the rostrocaudal axis in the GCL after training, for animals perfused 38 days after BrdU administration (n = 6). Error bars indicate the SEM.
SUPPLEMENTAL DATA 1

We controlled for the putative influence of stress, general attentiveness and motor activity associated with the olfactometer procedures on newborn neurons survival. For this purpose, in separate experiments, animals trained to recognize odorants in the olfactometer were compared to two different control groups: naive and activity-control groups. Naive animals were handled daily whereas activity-control animals were submitted to exactly the same procedure as the trained animals, except for odorant exposure. When trained animals underwent daily olfactory discrimination sessions, activity-control animals underwent daily pretraining sessions (see Supplemental Methods and Fig. S1A). Thus, they were conditioned to get a reward when licking a water delivery tube, in the presence of mineral oil vapor, in the olfactometer. At the end of the different experiments, the neuronal survival of naive and activity-control animals was similar. Only learning (trained animals) had significant effects on the survival of newly generated GCs (Fig. S1B). We conclude that the changes induced in neuronal survival following olfactory training in the olfactometer can be attributed only to olfactory learning.
SUPPLEMENTAL DATA 2

Our first one-week olfactory training experiments were carried out with a single odorant (linalol). Then, we checked that training-induced changes were not odorant-specific (Fig. S2A). Regardless of the odorants used, our findings confirmed that learning had marked effects on the survival (group effect for 30-day-old neurons: Fig. S2B, $F_{(2,12)} = 14.65, p < 0.001$) and elimination (group effect for 38-day-old neurons: Fig. S2B, $F_{(2,12)} = 11.51, p = 0.002$) of newly generated GCs. We also observed that changes in the number of newly generated GCs could be observed along the entire rostrocaudal axis of the OB. The density of GCs decreased along the rostrocaudal axis for control and trained animals (slice effect for 30-day-old neurons: Fig. S2C, $F_{(11,165)} = 228.78, p < 0.001$; for 38-day-old neurons: Fig. S2D, $F_{(11,165)} = 77.19, p < 0.001$). We conclude that, in adult mice, GCs throughout the OB are sensitive to learning, even if sensory inputs arrive at different glomeruli. The spatial and temporal coincidence of sensory entry (i.e., the ascending pathway) and centrifugal afferents (i.e., the descending pathway) to the OB may be the conditions needed for triggering modulation of the bulbar neurogenesis.
SUPPLEMENTAL REFERENCES


