Supporting Information

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SI Methods

Stages of Estrus. Each day, vaginal smears were collected to assess the stage of estrus, as described (1). Cotton Q-tips were immersed in saline and then guided along the vaginal track to pick up cells. The cells were placed onto slides and stained with 1% Toluidine blue. Stages of the estrous cycle were verified by visualization under a light microscope with 10× magnification. Only adult females that demonstrated two 4- to 5-day cycles with all stages of estrus were included in the study.

Eyeblink Conditioning. Eyeblinks that occurred during the trace interval were considered CRs and were detected by changes in eyelid EMG activity. The electrodes were connected to a differential amplifier with a 300- to 500-Hz band pass filter (amplified 10K and digitized at 1 kHz). Changes in EMG activity during the trace interval were compared to baseline recordings 250 ms before CS onset. If the activity exceeded a minimum of 0.5 mV and a maximum amplitude of the baseline by >4 standard deviations and persisted for >10 ms, it was considered as a CR (2, 3).

Immunohistochemistry for BrdU. Twenty-four hours after the last day of training (12 days after the BrdU injection), rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and intracardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were extracted and postfixed in 4% paraformaldehyde for up to 48 h, and were later transferred to 0.1M phosphate buffer. Coronal sections (40 μm) were cut through the entire dentate gyrus of one hemisphere of the brain with an oscillating tissue slicer. For BrdU peroxidase staining, a 1:12 series of sections were mounted onto glass slides and pretreated by heating in 0.1 M citric acid (pH 6.0). Tissue was then incubated in trypsin (Sigma-Aldrich, Inc.), followed by 2N HCl and overnight in primary mouse anti-BrdU (1:200; Becton Dickinson ImmunoSys) and 0.5% Tween 20 (Sigma-Aldrich, Inc.). The next day, tissue was incubated for 1 h in biotinylated anti-mouse antibody (1:200; Vector Laboratories, Inc.), then in avidin-biotin-horseradish peroxidase (1:100; Vector Laboratories, Inc.), and lastly in diaminobenzidine (Sigma-Aldrich, Inc.). After rinsing in phosphate buffer, slides were counterstained with cresyl violet and cover-slipped with Permount (2–4).

Volume. The volume of the dentate gyrus was calculated using Cavalieri's principle (5): Volume = T X ($\Sigma A_{1\text{-}m}$). In this formula, A represents the area of each reference section (sections 1-m), $\Sigma A_{1\text{-}m}$ is the sum of the areas of sections 1 to m, and T is the distance between each reference section (in this case 40 μm × 12). The final number was expressed in cubic μm, multiplied by 2 (the number of hemispheres) and converted to mm^3. BrdU data were expressed as density of BrdU-labeled cells per volume of the total DG or GCL/SGZ alone (number of BrdU-labeled cells/mm^3). The numbers of BrdU-labeled cells were examined in the rostral (interaural 3.70 to 6.88 mm) and caudal (interaural 2.28 to 3.70 mm) hippocampus, as described elsewhere (6). The rostral region contains the DG in most of the dorsal hippocampus, whereas the caudal region contains a part of the DG in the dorsal hippocampus and the entire DG in the ventral hippocampus. Because the number of tissue sections in the dorsal versus hippocampus was different, cell numbers were presented as density of new cells rather than as absolute numbers.

Double Labeling: BrdU with DCX. For double labeling, free-floating sections were rinsed with Tris-buffered saline (0.1 M TBS at pH 7.5). DNA was denatured with 2N HCl in TBS. Sections were incubated in primary antibodies, goat anti-doublecortin (1:100, Santa Cruz Biotechnology), and mouse anti-BrdU (1:200, Becton-Dickinson ImmunoSys) with 0.5% Tween-20 (Sigma-Aldrich, Inc.) in TBS for 48 h at 4 °C. Sections were incubated in secondary antibodies, Rhodamine Red-X anti-goat (1:200, Jackson Immunoresearch) and Fluro 488 anti-mouse (1:200, Molecular Probes) in TBS for 30 min. Tissue was mounted onto slides and coverslipped with glycerol:TBS (1:3). The number of colocalized cells was determined with a Zeiss (Oberkochen) LSM 510 confocal laser scanning microscope. Sections were scanned using a Plan-Neofluar 40x water-immersion objective and dual channel excitation with argon (488 nm) and helium-neon (543 nm) (7). Twenty cells per subject ($n = 4$) were counted on random sections throughout the hippocampus. The coexpression of BrdU and DCX was inspected using the size and shape of the cell throughout a z-stack, orthogonal plane, including a profile of excitation intensity.

Statistical Analyses. The percentage of CRs across training trials was analyzed using repeated measures ANOVA with sex (male versus female) as the independent variable. Blocks of training trials were used as the repeated measures (blocks of 20 trials for the first 100 and blocks of 100 for the remaining 700 trials). Spontaneous blinks, sensitized responses, and responding during trial 1 were analyzed with 1-way ANOVA, with sex as the independent variable. Cell counts, volume, and density were analyzed with 2-way ANOVAs, with training and sex (males versus female) as independent variables. For significant interactions, Tukey's posthoc tests were performed. All results are presented as means ± one standard error. The alpha level of significance was set to 0.05.

The conditioned responses expressed by each animal across training were analyzed. Some animals did not display a gradual acquisition curve but rather increased their responding abruptly, after a period of low responding. To identify where changes in responding occurred, we used an algorithm developed by Gallistel and colleagues. This algorithm detects changes in the slope of the cumulative record (8–10), capitalizing on Skinner's insight that the slope of a cumulative record indicates the momentary rate of responding (11). Abrupt changes in performance are indicated by similarly abrupt changes in slope. The algorithm was applied to an animal's cumulative record of eyeblinks on all CS-alone and paired trials, as described previously (10). Briefly, the algorithm steps through the cumulative record point by point. At each point (datum), it considers whether an earlier change in the slope has occurred: that is, whether the record up to this point consists of 2 segments with different slopes. To do so, it first finds a putative change point, which is the trial of the datum that maximizes the difference in the slopes between the 2 segments. The algorithm uses a binomial test to compare the distribution up to that putative change point, with the distribution following it. If the log of the odds against the null hypothesis (that there is no difference) exceeds a criterion of 2 (which is almost equivalent to a traditional P value of 0.01), the difference in the slopes is considered significant and the putative change point is taken as a true change point. Then, the record up to that change point is truncated and the algorithm begins anew, taking the trial after the change point as its first datum.

Using this approach to characterize acquisition, we defined 2

measures of learning: First, the estimate of the "asymptote" for each rat is the mean percentage of CRs during the last 2 sessions of training (last 360 paired and CS-alone trials). Second, the "asymptotic trial" is the trial of the change point that led to a slope of 95% or more of the asymptote. This number reflects the number of trials that an animal used to reliably express an eyeblink during the trace interval and in anticipation of the US.

To have a more traditional estimate of the asymptotic trial, we also used another learning criterion: the trial, in which a learning criterion of 8 CRs on 9 consecutive trials was satisfied. This trial was strongly correlated with the asymptotic trial ($r = 0.95$; $P < 0.001$).

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