SUPPLEMENTAL MATERIAL

Supplemental Methods

Immunohistochemistry

Sections were washed in 0.1 M Tris buffer, and incubated in 1% hydrogen peroxide (all chemicals and reagents were obtained from Sigma-Aldrich, St. Louis, MO, unless otherwise stated) in 0.1 M Tris buffer for 2 min, to block endogenous peroxides. All incubations were carried out at room temperature with continuous agitation on a rotator, and were followed by 3 washes in 0.1 M Tris buffer. After washes, sections were incubated for 10 minutes, first in Tris A (0.25% Triton X-100 in 0.1 M Tris buffer) and then Tris B (0.25% Triton X-100 and 0.005% bovine serum albumin in 0.1 M Tris buffer). Sections were blocked in serum (Table S1) for 1 hour, to minimize non-specific binding of immunoreagents during the process of immunolabeling.

The sections were incubated for 24 hours in the primary antiserum (Table S1), followed by one 10 minute wash in Tris A and then one 10 minute wash in Tris B. The sections were then incubated in the secondary antibody (Table S1) for 1 hour and subsequently washed for 10 minutes in Tris A followed by 10 minutes in Tris D (0.25% Triton X-100 and 0.05% bovine serum albumin in 0.5 M Tris buffer). Then the avidin-

biotin–horseradish peroxidase complex (ABC) method for visualization with immunoperoxidase was used (Hsu et al., 1981). The ABC solution (Vectastain Elite Kit, Vector Laboratories) was diluted 1:100 in Tris D, and the sections were incubated in the diluted ABC solution for 2 hours. Following washes in 0.1 M Tris buffer, immunoreactivity (ir) was visualized using 3,3-diaminobenzidine (DAB) with $NiCl_2$ intensification. Sections were incubated in a solution containing 0.022% DAB (in 0.1 M Tris), 1 mM $NiCl_2$, 0.2 % ammonium chloride (in distilled H_20, dH_20), 0.1% glucose oxidase (in dH_20), and 0.8% D(+)-glucose (in dH_20) in 0.1 M Tris buffer. The reaction was stopped by washing sections in 0.1 M Tris buffer. Sections were then washed in 0.1 M Tris buffer and mounted on subbed slides. Slides were allowed to dry overnight, and then were dehydrated in a graded series of ethanol (5 minutes 70% ethanol, 5 minutes 90%, 15 minutes 95%, 15 minutes 100%), cleared in xylene for 15 minutes, and then coverslipped with Permount (Fisher).

All slides were analyzed using a brightfield microscope (BX51, Olympus, Hauppauge, NY), photographed using a digital camera (Retiga 2000R, Q Imaging, Surrey, BC, Canada) and acquired using ImagePro (Media Cybernetics, Bethesda, MD). All settings for the camera (e.g., exposure time) and microscope settings (e.g., lighting) remained the same when photographing sections for the figures.

Double Labeling

Double labeling methods for light microscopy were based on those previously described (Scharfman et al., 2002), with some modifications described below. For double-labeling with antibodies to c-fos and GluR2/3, sections were first incubated with

the goat polyclonal c-fos antibody (Table S1) and visualized using DAB with $NiCl_2$ intensification, as described above. Following washes and blocking in serum (Table S2), sections were subsequently incubated for 24 hours with a rabbit polyclonal antibody to GluR2/3 (Table S2). The sections were then incubated in the secondary antibody, biotinylated goat anti-rabbit IgG diluted in Tris B (1:400; Vector Laboratories, Burlingame, CA) for 1 hour. Immunoreactivity was visualized with the water soluble chromagen, NovaRED (per manufacturer's instructions, Vector Laboratories).

Double-labeling was also conducted with immunofluorescence using methods described previously (Barouk et al., 2011). Briefly, all sections were washed in 0.1 M phosphate buffer (PB) and blocked in 10% donkey serum (Sigma, St. Louis MO) in 0.25% Triton and 0.005% BSA in 0.1 M PB for 1 hour. The sections were then incubated in goat polyclonal c-fos antibody (Table S1) and either the antibody to GluR2/3, paravalbumin (PV) or neuropeptide Y (NPY; for details see Table S2) in 1% donkey serum and 0.25% Triton X-100 in 0.1 M PB for 24 hours. Following primary antibody incubation and three 5 minute washes in 0.1 M PB, sections were incubated for 2 hours in the secondary antibodies (Table S2). Subsequent to rinses in 0.1 M PB, sections were mounted on glass slides and coverslipped with Prolong Gold antifade reagent (Invitrogen, Carlsbad, CA). Confocal microscopy was performed using a LSM 510 Meta (Zeiss, Carl Zeiss Microimaging, Thornwood, NY), equipped with Ar and HeNe lasers. The 488 and 546 mm laser lines were used with a 20X and a 40X objective. Imaging parameters were optimized using LSM 510 Meta Software (Zeiss) and the same parameters were used for each tissue section.

Quantification and Data Analysis

Hilar area was quantified using Bioquant software (Bioquant Image Analysis, Nashville, TN). The hilus was outlined by drawing a line along the granule cell (GC) layer (GCL)/hilar border and then connecting the ends (at the lateral tips of the upper and lower blades) to the end of the PCL by two straight lines (Fig. S2A1). All GluR2/3 positive cells and c-fos labeled cells within this region were quantified. Cells that crossed the lines were not counted.

Data are presented as mean ± standard error of the mean (SEM) and $p < 0.05$. Statistical comparisons were made using one-way and two-way repeated measures analysis of variance (RMANOVA) followed by Bonferroni multiple comparisons tests or ANCOVA (GraphPad Prism, v.5.0, San Diego, CA). Where ANOVA comparisons showed no effect of factors or an interaction between factors they are not reported. Prior to ANOVA, Bartlett's test (Snedecor and Cochran, 1989) was used to test for homoscedasticity of variance, and when significant departure from homoscedasticity was detected, it was corrected by log transformation of the data.

Primary Antibody	Generation and characterization	Blocking solution	Secondary antibody
C-fos Goat polyclonal (Santa-Cruz) Brightfield: 1:10,000 Confocal: 1:100	Raised against the N-terminus peptide of human c-fos (amino acids 3–16). Western blot was used to show that the antibody recognized a 62 kD protein corresponding to the molecular weight of c-fos (manufacturer's datasheet). Specificity has also been confirmed by immunoblot using a Syrian hamster embryonic cell line (Preston et al., 1996) and subsequently verified with Western blot of human T cells (Whisler et al., 1997).	Brightfield: 1.5% normal horse serum (Vector) in Tris blocking solution***** Confocal: 10% donkey serum (Sigma) in 0.1 M PB blocking solution******	Brightfield: Horse anti-goat (1:400, Vector) Confocal: Donkey anti-goat, Alexa fluor 488 (1:400; Invitrogen, Carlsbad, CA)
C-fos Rabbit polyclonal (Santa Cruz) Brightfield: 1:10,000 Confocal: 1:100	This antibody was raised to the N-terminus of human c-fos as described above. Western blot was used to show that the antibody recognized a 62 kD protein corresponding to the molecular weight of c-fos (manufacturer's datasheet). Preabsorption studies result in the complete blocking of staining (Howorth et al., 2009).	Brightfield: 1.5% normal goat serum (Vector) in 0.1M Tris blocking solution. Confocal: 10% donkey serum (Sigma) in 0.1 M PB blocking solution.	Brightfield: Goat anti-rabbit (1:400, Vector). Confocal: Donkey anti-rabbit, Alexa fluor 560 (1:400, Invitrogen)
Fos B Rabbit polyclonal (Santa Cruz) Brightfield 1:300	This antibody was raised against against recombinant human fos B (amino acids 75-150). Western blot was used to show that the antibody recognizes a 45 kD and 35-37 kD protein corresponding to the molecular weight of fos B and a truncated splice variant, ΔfosB (manufacturer's datasheet). A similar labeling pattern in neuronal tissue was observed with another fos B antibody, which also recognizes bands corresponding to fosB and ΔfosB in Western blot (Ruud and Blomqvist, 2007).	Brightfield: 1.5% normal goat serum (Vector) in 0.1M Tris blocking solution.	Brightfield: Goat anti-rabbit (1:400, Vector).

Supplemental Table 1. Information about antibodies to c-fos and fos B.

Information about the c-fos and fos B antibodies that were used is presented in tabular format. In the two columns on the left, information about the antibody is provided; in the two columns on the right, the blocking sera and secondary antibodies are described, ***** Tris blocking solution = 0.25% Triton X-100 and 0.005% bovine serum albumin in 0.1 M Tris buffer. ****** 0.1 M PB blocking solution = 0.25% Triton X-100 and 0.005% bovine serum albumin in 0.1 M PB.

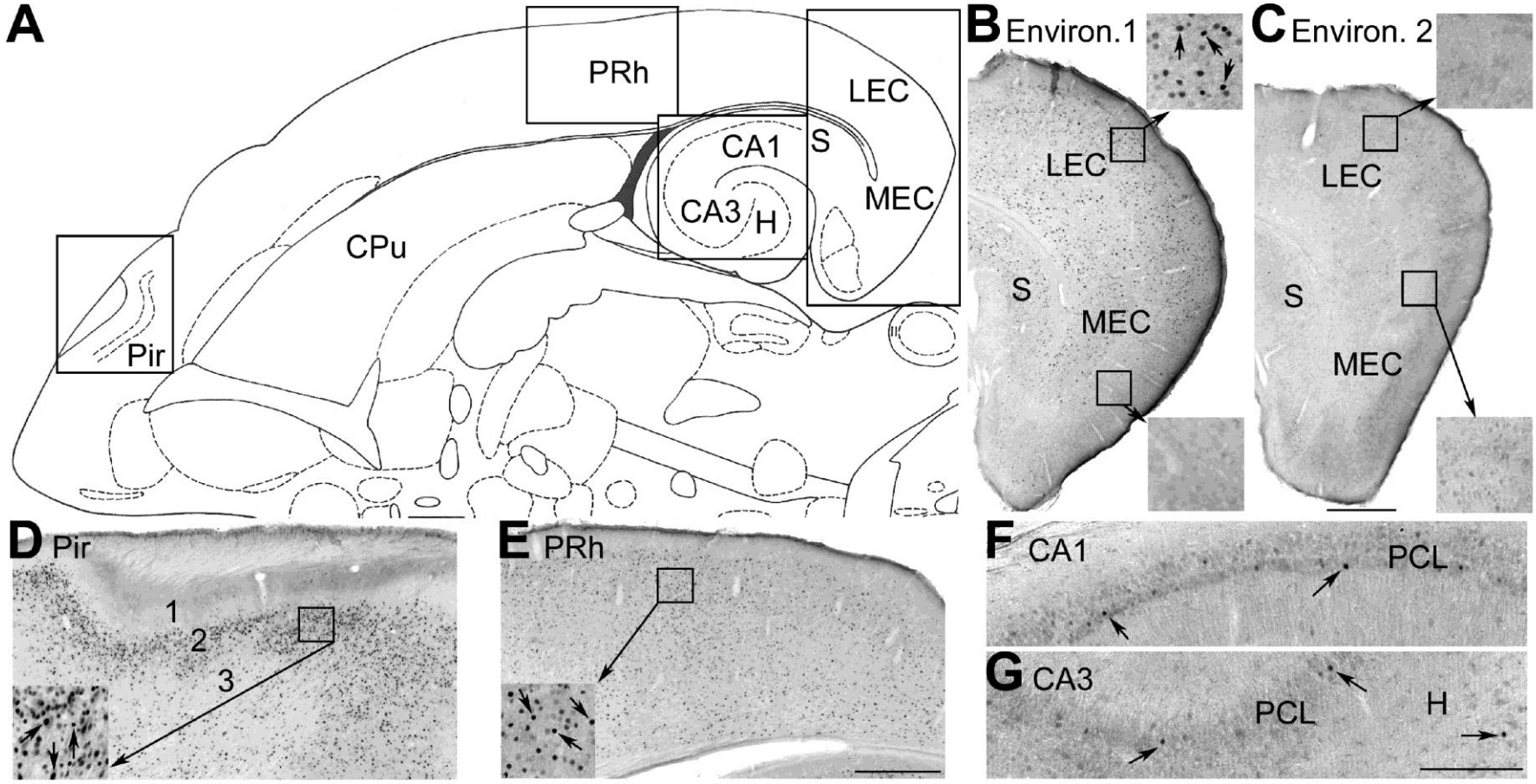
Primary Antibody	Generation and characterization	Blocking solution	Secondary antibody
GluR2/3 Rabbit polyclonal (Millipore) Brightfield: 1:100 Confocal: 1:100	Raised against a synthetic peptide corresponding to the C-terminus (amino acids 864-883) of rat GluR2, this antibody recognized a 110 kDa protein on Western blot corresponding to the molecular weight of GluR2/3 (manufacturer's datasheet and Tse et al., 2008).	Brightfield: 1.5% normal goat serum (Vector) in 0.1M Tris blocking solution. Confocal: 10% donkey serum (Sigma) in 0.1 M PB blocking solution.	Brightfield: Goat anti-rabbit (1:400, Vector). Confocal: Donkey anti-rabbit, Alexa fluor 560 (1:400, Invitrogen)
PV Mouse monoclonal (Millipore) Confocal: 1:100	Raised against frog muscle PV. Western blot was used to show that the antibody recognizes a single band of 12 kDa corresponding to NPY (manufacturer's datasheet). We observed a similar pattern of labeling as described in previous reports where the antibody was characterized (Celio, 1990).	Confocal: 10% donkey serum (Sigma) in 0.1 M PB blocking solution.	Confocal: Donkey anti-mouse, Alexa fluor 488 (1:400; Invitrogen)
NPY Rabbit polyclonal (Immunostar) Confocal: 1:100	Raised against a synthetic porcine NPY. Preabsorption of the diluted antiserum with excess NPY blocked all staining, whereas related peptides (e.g. peptide YY and avian pancreatic peptide) and somatostatin, which is colocalized in some interneurons with NPY) had no effect (manufacturer's datasheet).	Confocal: 10% donkey serum (Sigma) in 0.1 M PB blocking solution.	Confocal: Donkey anti-rabbit, Alexa fluor 560 (1:400, Invitrogen)

Supplemental Table 2. Information about antibodies to GluR2/3, parvalbumin (PV) and neuropeptide Y (NPY).

Information about antibodies other than c-fos and fosB are shown, with the same format as Table S1. ***** Tris blocking solution = 0.25% Triton X-100 and 0.005% bovine serum albumin in Tris. ****** 0.1 M PB blocking solution = 0.25% Triton X-100 and 0.005% bovine serum albumin in 0.1 M PB.

Supplemental Results

To estimate the percentage of c-fos positive GCs, we assumed that there are 1 million GCs/hippocampus in the rat based on previous studies (Gaarskjaer, 1978; Boss et al., 1985; Amaral et al., 1990; Patton and McNaughton, 1995; Rapp and Gallagher, 1996). Given that our highest value for GCs that were c-fos-ir was 27/section, and our mean value for the number of sections/hippocampus was 200, we estimated that - at the most - there were <0.54% of GCs that were c-fos-ir [(27 x 200)/1,000,000=0.0054 or 0.54%].


A
PRh
LEC
CA1
S
MEC
CA3
H
CPu
Pir
B Environ.1
LEC
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C Environ. 2
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D Pir
1
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E PRh
F CA1
PCL
G CA3
PCL
H

Supplemental Figure 1. C-fos-ir in hippocampal and extrahippocampal areas in rats housed in environments 1 and 2.

A. A schematic illustrates the areas of the brain where photomicrographs in B-G were taken. Modified from Paxinos and Watson (1982). Pir = piriform cortex; Cpu= caudate/putamen; H= hilus; S= subiculum; MEC= medial entorhinal cortex; LEC = lateral entorhinal cortex; PRh= perirhinal cortex.

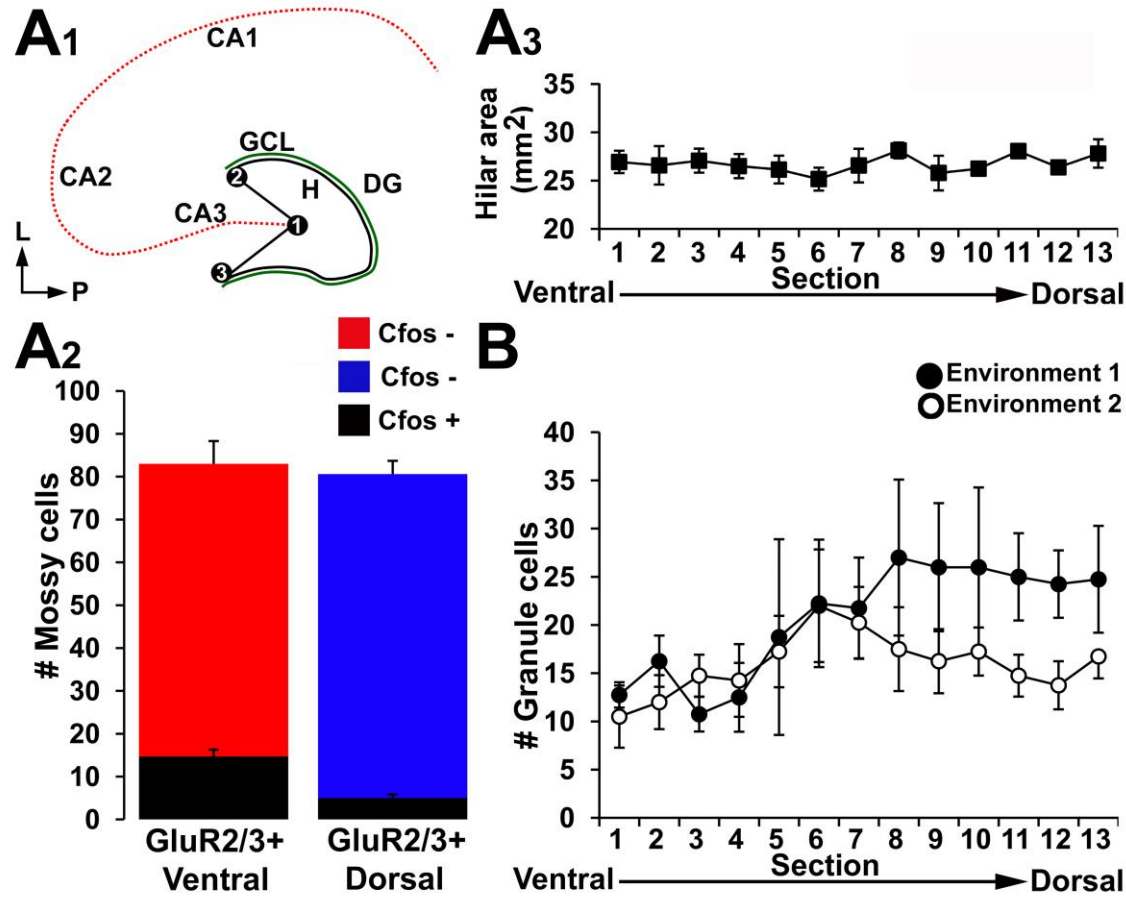
B. The EC from a rat housed in environment 1 (Environ.1) is shown, illustrating numerous c-fos-immunoreactive cells in the superficial layers of the LEC (upper right inset; arrows) but not the MEC (lower right inset). Calibration for B, C = 500 μm (shown in C). Calibration for all insets = 100 μm (shown in G).

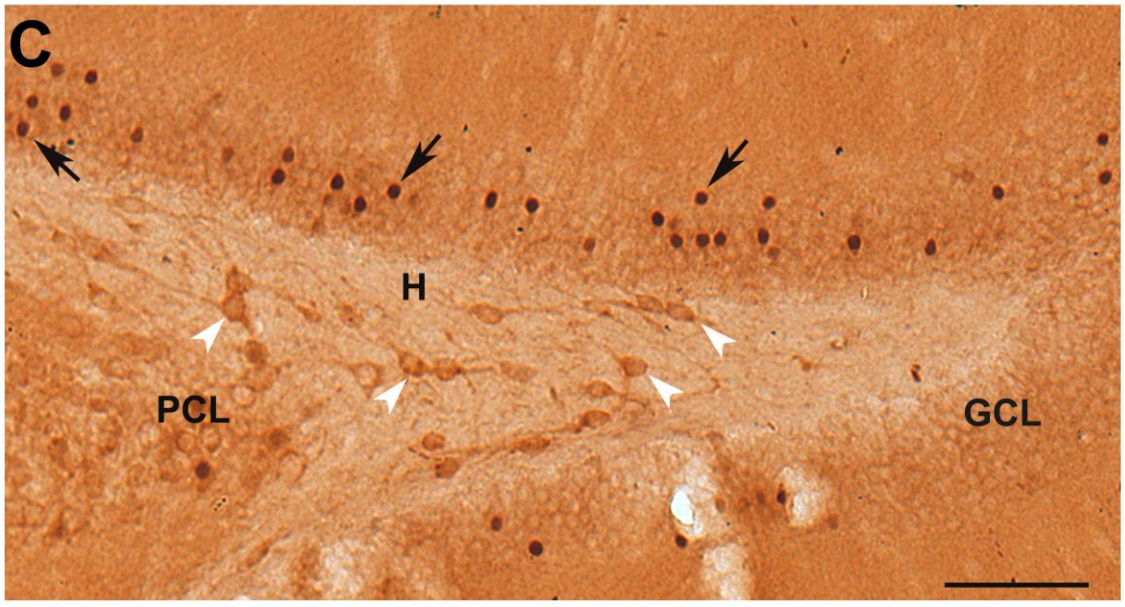
C. The EC from a rat housed in environment 2 (Environ. 2) is shown, illustrating the scarcity of c-fos-immunoreactive cells in the superficial layers of the LEC (upper right inset) and MEC (lower right inset). This scarcity of c-fos-ir was observed in all 6 environment 2 animals labeled with c-fos.

D. A portion of the piriform cortex is shown to illustrate the density of c-fos-immunoreactive cells in this area, which was present regardless of the housing environment. Layers are labeled 1-3. Calibration for D, E = 500 μm (shown in E).

E. A portion of the perirhinal cortex is shown to illustrate the density of c-fos-immunoreactive cells in this area, which was robust in both A and NA rats.

F-G. A portion of area CA1 (F) and CA3 (G) is shown to illustrate the scarcity of cells in this area that were immunoreactive for c-fos. The same animal was used for parts F-G. PCL= pyramidal cell layer. Calibration for D, E = 100 μm (shown in G.).


A1
CA1
CA2
GCL
H
DG
CA3
L
P
A3
Hilar area (mm2)
35
30
25
20
1 2 3 4 5 6 7 8 9 10 11 12 13
Section
Ventral
Dorsal
A2
Cfos -
Cfos -
Cfos +
Mossy cells
100
90
80
70
60
50
40
30
20
10
0
GluR2/3+
Ventral
GluR2/3+
Dorsal
B
Environment 1
Environment 2
Granule cells
40
35
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13
Section
Ventral
Dorsal


C
H
PCL
GCL

Supplemental Figure 2. Quantification of GluR2/3-labeled cells, hilar area and c-fos-ir in GCs.

A

1. A schematic diagram in which the definition of hilar area is illustrated (black line). To calculate hilar area a line (black line) was drawn along the inner side of the GC layer (GCL; green line). A straight line was extended from the tip of the CA3 pyramidal cell layer (PCL; point 1) to the tip of upper (also called superior or enclosed) blade of the GCL (point 2). Another line was drawn from point 1 to the tip of the lower (also called inferior or exposed) blade of the GCL (point 3). L= lateral; P= posterior.

2. The number of GluR2/3 immunoreactive cells (mossy cells; MCs) in ventral (red) compared to dorsal (blue) sections. GluR2/3-labeled cells that were double-labeled with c-fos are represented by the black bars. There was no difference in the number of GluR2/3 immunoreactive cells in ventral versus dorsal levels (n=4; p=0.741), whereas there was a difference in ventral and dorsal c-fos-labeled cells (p=0.00009).

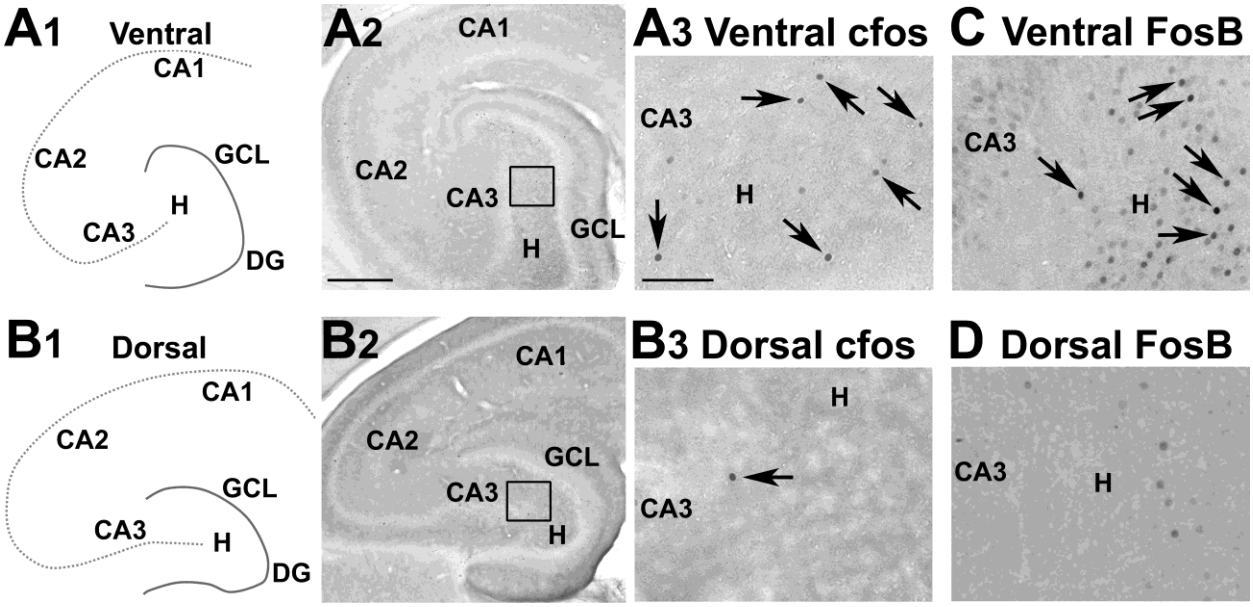
3. The hilar area is shown across the dorsoventral axis (n=3). There was no difference in hilar area along the dorsoventral axis (one-way RMANOVA, $F(12,39)=0.98$; $p=0.480$).

B.

The mean number of c-fos-labeled GCs/section are shown for animals housed in environment 1 (black circles; n=4 rats) or environment 2 (white circles; n=4 rats).

C.

A representative septal section cut in the coronal plane, from a rat that was housed in its normal housing quarters (environment 1). Arrows point to GCs labeled with c-fos. Mossy cells (MCs) are labeled with GluR2/3 (arrowheads). Calibration=100 μm.



Supplemental Figure 3. Robust c-fos-ir in ventral hilar mossy cells (MCs) observed with c-fos rabbit polyclonal antibody and an antibody to fosB.

A. 1. A schematic diagram of a ventral section in the horizontal plane. Dotted red line = pyramidal cell layer; green line = granule cell layer (GCL); DG = dentate gyrus; H = hilus. A = anterior, M = medial.

2. A low power image of a representative ventral DG horizontal section from a rat that was housed in environment 1. The boxed area is shown in the sections depicted in A3. A high power image is shown in A3. Calibration = 500 µm.

3. Arrows point to numerous hilar cells with c-fos-ir, labeled using a rabbit polyclonal antibody. There were relatively few c-fos-ir cells in the GCL. The c-fos-ir labeling is a similar pattern to that observed in Figure 1 (3 sections/animal labeled with the c-fos anti-rabbit antibody; n=7 rats). Calibration=100 µm for A-B3 and C-D.

B. 1. A schematic diagram of a horizontal dorsal section.

2. A low power image of a representative dorsal DG horizontal section from a rat housed in environment 2. A high power image is shown in B3. Calibration (shown in A2) = 500 µm.

3. The area in the box in B2 is shown for a representative dorsal section from a rat housed in environment 2. Hilar c-fos-ir is uncommon, similar to that observed in Figure 1.

C. A ventral section from an animal housed in environment 1 labeled with fosB, which labels numerous hilar cells (arrows).

D. A dorsal section from a rat housed in environment 1 labeled with fosB. Few hilar cells show light labeling.

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