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Expression of C-fos in Hilar Mossy Cells of the Dentate Gyrus *In Vivo*

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Abstract

Granule cells (GCs) of the dentate gyrus (DG) are considered to be quiescent - they rarely fire action potentials. In contrast, the other glutamatergic cell type in the DG, hilar mossy cells (MCs) often have a high level of spontaneous activity based on recordings in hippocampal slices. MCs project to GCs, so activity in MCs could play an important role in activating GCs. Therefore, we asked if MCs were active under basal conditions *in vivo*, using the immediate early gene c-fos as a tool. We hypothesized that MCs would exhibit c-fos expression even if rats were examined randomly, under normal housing conditions. Therefore, adult male rats were perfused shortly after removal from their home cage and transfer to the laboratory. Remarkably, most c-fos immunoreactivity (ir) was in the hilus, especially temporal hippocampus. C-fos-ir hilar cells co-expressed GluR2/3, suggesting that they were MCs. C-fos-ir MCs were robust even when the animal was habituated to the investigator and laboratory where they were euthanized. However, c-fos-ir in dorsal MCs was reduced under these circumstances, suggesting that ventral and dorsal MCs are functionally distinct. Interestingly, there was an inverse relationship between MC and GC layer c-fos expression, with little c-fos expression in the GC layer in ventral sections where MC expression was strong, and the opposite in dorsal hippocampus. The results support the hypothesis that a subset of hilar MCs are spontaneously active *in vivo* and provide other DG neurons with tonic depolarizing input.

Keywords

CA4; GluR2/3; hilus; hippocampus; immediate early gene; novelty

The dentate gyrus (DG) plays an important role in spatial navigation (Derrick, 2007; Kesner, 2007) pattern separation (Leutgeb et al., 2007; McHugh et al., 2007; Clelland et al., 2009), and other functions related to context (Lee and Kesner, 2004; Hernandez-Rabaza et al., 2008). Because the DG appears to be involved in functions which require discrimination of changing environments, it has been hard to explain why the cells which are thought to be central to DG functions, the granule cells (GCs), appear to be relatively quiet under most conditions. GCs in hippocampal slices have relatively hyperpolarized resting potentials, rarely discharge spontaneously, and have other characteristics that limit their discharge (Mody et al., 1992; Scharfman, 1992; Lubke et al., 1998; Williamson and Patrylo, 2007).

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Extracellular recordings of GCs *in vivo*, or studies of the immediate early gene Arc, suggest that most GCs are relatively unresponsive, even in a novel environment (Jung and McNaughton, 1993; Chawla et al., 2005; Leutgeb et al., 2007).

These observations suggest that other DG neurons besides GCs may play an important role in DG functions. DG cells that could be important in this respect are hilar mossy cells (MCs, Henze and Buzsáki, 2007; Scharfman and Myers, 2013), because MCs are a substantial population of hilar neurons (Fujise et al., 1998; Buckmaster and Jongen-Relo, 1999), responsible for most of the proximal glutamatergic input to GC apical dendrites, and target GCs throughout the ipsilateral and contralateral DG (West et al., 1979; Ribak et al., 1985; Buckmaster et al., 1996). MCs receive strong afferent input from GCs, and a subset of MCs can also be activated at short latency by stimulation of the perforant path (Scharfman, 1991). MCs receive input from ascending brainstem noradrenergic (Bijak and Misgeld, 1995; Harley, 2007), serotoninergic (Ghadimi et al., 1994; Bijak and Misgeld, 1997), cholinergic systems (Brunner and Misgeld, 1994; Deller et al., 1999) and additional extrinsic afferents (Leranth and Hajszan, 2007). MCs are also innervated by the 'backprojecting' axon collaterals of CA3 pyramidal cells (Scharfman, 2007). Recordings of MCs in hippocampal slices suggest that they typically have frequent depolarizing input (EPSPs), are relatively depolarized, and fire spontaneously, in contrast to GCs (Scharfman and Schwartzkroin, 1988; Scharfman, 1993; Larimer and Strowbridge, 2008). For a subset of MCs, threshold for synaptic activation appears to be low, because stimulation of the molecular layer in hippocampal slices can readily activate MCs even when adjacent GCs do not reach threshold (Scharfman, 1991).

A great deal of information about MC physiology has been based on recordings in hippocampal slices. The data predict that MCs would be spontaneously active *in vivo*, but data from the anesthetized rat, where mossy cells have been recorded intracellularly, do not necessarily show that this is true (Soltesz et al., 1993; Buckmaster and Schwartzkroin, 1995). Some studies *in vitro* also suggest that MCs are primarily activated disynaptically, by GCs (Uchigashima et al., 2011), and therefore would not be likely to reach threshold very often. However, it is difficult to infer MC activity in awake behaving animals based on recordings in slices or anesthetized animals.

To address this issue, we used the immediate early gene c-fos as a marker of recent neuronal activity. C-fos protein expression reflects neuronal activity occurring within the preceding hours (Dragunow and Robertson, 1987; Morgan et al., 1987). In dorsal root ganglion cells in culture, c-fos protein expression depends on sodium-dependent action potential generation (Sheng et al., 1993; Fields et al., 1997). Therefore, we hypothesized that c-fos-ir could be used as a marker of action potential generation in MCs, and if MCs were c-fos-ir in rats removed from their home cage, the results would support the hypothesis that MCs generate action potentials regularly and spontaneously. Therefore, we perfused animals shortly after removal from their home cages. An antibody to glutamate receptor subunits 2/3 (GluR2/3) was used to distinguish MCs from other hilar neurons (Leranth et al., 1996).

Adult Sprague-Dawley rats (75–100 days old; Charles River, Kingston, NY) were housed 2–3/cage with food and water *ad libitum* and a 12-hour light/dark cycle. Experiments were conducted in accordance with the guidelines of the NIH. Animals (n=12) were deeply anesthetized in the laboratory (isoflurane, Baxter, Deerfield, IL, followed by urethane 2.5g/kg, i.p.; Sigma-Aldrich, St. Louis, MO) and transcardially-perfused as described elsewhere (Barouk et al., 2011). Prior to perfusion, the animals used in the experiments were housed in 2 different locations. The first group of animals was housed in the animal facility, or "environment 1". They were brought to the laboratory and perfused within 10 minutes following removal from the facility. To eliminate c-fos expression related to moving the

animals to the laboratory, a second group, "environment 2," were housed in the laboratory where they were to be perfused, and were handled daily (Monday-Friday) by the investigator who would conduct perfusion-fixation. This second group, a control for any c-fos expression induced by the novelty experienced during the transfer to the laboratory, might seem unnecessary because it is generally assumed that c-fos protein requires more than 10 min for expression. However, no study has evaluated the time course in MCs specifically, and in other cell types, c-fos protein expression has not been studied very often during the first 30 min after a stimulus (Dragunow and Robertson, 1987; Morgan et al., 1987). Sections (50 μm-thick) were cut horizontally and immunostained using a goat polyclonal antibody to c-fos. In some animals, a second c-fos antibody was also used to be sure results were independent of the antibody. An antibody to another member of the Fos family of transcription factors, fosB/ΔfosB, was also used because it labels neurons that are active during a longer period of time prior to perfusion-fixation compared to c-fos (McClung et al., 2004). Details are provided in the Supplemental Material.

Quantification of c-fos-labeled cells was performed using digital thresholding of c-fos-ir nuclei (Bioquant Image Analysis, Nashville, TN) described elsewhere (Duffy et al., 2011). The threshold for detection was set at a level where dark c-fos-ir nuclei were counted, but nuclei with light labeling, similar to the background staining, were not (see Fig. 6 in Lee et al., 2012). Results from thresholding were confirmed by counting c-fos-ir nuclei manually in a subset of experiments. For each animal, 13 sections were selected at 150 μm intervals, starting ventrally at the first section where both blades of the DG were evident (Fig. 1A, approximately 2.50 mm above the interaural line, Paxinos and Watson, 2007), and ending at the dorsal extreme where cell layers became difficult to define (Fig. 1A; 5.40mm above the interaural line). The borders of the hilus were defined by established criteria (Amaral, 1978). In statistical comparisons below, interactions between factors are reported only where they are significant.

Figure 1 shows that, remarkably, hilar cells were some of the only hippocampal neurons that expressed c-fos protein (Fig. 1; Fig. S1). When septotemporal levels from rats that were exposed to either environment 1 or 2 were compared, ventral levels showed the most hilar c-fos-ir in both groups (two-way RMANOVA, effect of dorsoventral level; $F(12,39)=3.92$; $p=0.0006$; Fig. 1D).

Interestingly, there was greater c-fos-ir in environment 1 than environment 2 (two-way RMANOVA, effect of environment; $F(1,39)=22.37$; $p<0.0001$ followed by Bonferroni's tests, $p<0.05$; Fig. 1D). The difference was primarily in dorsal levels; in environment 2, c-fos-ir hilar cells were more numerous in ventral than dorsal levels (Fig. 1, one-way RMANOVA; F (12, 39) 3.54; $p=0.0013$) but in environment 1, there was no difference in c-fos-ir along the septotemporal axis (one-way RMANOVA; F (12,39) 0.663; $p=0.775$).

The results for numbers of cells were similar to results for percentages, because the numbers of MCs and hilar area were consistent across sections (Fig. S2). Results with the rabbit c-fos antibody were similar to these using the goat c-fos antibody (Fig. S3).

Remarkably, every c-fos-ir hilar cell co-expressed GluR2/3, suggesting that they were all one cell type - MCs. Results were the same whether brightfield (Fig. 1E) or confocal (Fig. 1F) microscopy was used. The mean value for double-labeled cells (c-fos$^+$/GluR2/3$^+$) for all sections (dorsal and ventral) in both environments was 9.22 ± 1.31 cells/section (n=4 rats/group), which was approximately 11.29% of the total number of GluR2/3$^+$ hilar cells (based on the mean, 81.65 ± 3.22 GluR2/3$^+$ hilar cells/section; Fig. S2). Hilar GABAergic neurons expressing parvalbumin (PV) or neuropeptide Y (NPY) did not co-express c-fos (c-fos$^+$/

PV^+: 0/28 cells, n=4 sections, sampled at dorsal and ventral levels in 2 rats; c-fos^+/NPY^+: 0/23 cells, adjacent sections to those used for PV; Fig. 1G–H).

C-fos-ir cells in the GC layer were presumably GCs because they were labeled with GluR2/3 and not PV or NPY (data not shown). The number of c-fos-ir GCs may seem numerous from the Figures, but they represent a small percentage of all GCs (see Supplemental Results) consistent with the quiescence of GCs in the normal rat brain (Jung and McNaughton, 1993; Chawla et al., 2005; Leutgeb et al., 2007).

Analysis of c-fos-ir GCs (Fig. S2) by two-way RMANOVA also showed there was an effect of dorsoventral level ($F(12,72)=3.54$; $p=0.0004$), with most GCs labeled dorsally. The effect of septotemporal level on c-fos-ir in GCs occurred regardless of the environment ($F(1,6)=0.06$; $P=0.448$).

As one might gather from the greater number of ventral MCs expressing c-fos protein (relative to dorsal MCs) and greater number of dorsal GCs that were c-fos-ir (relative to ventral GCs), there was a reciprocal relationship between MC c-fos-ir and GC c-fos-ir (Fig. 2). To determine if the inverse correlation between MC c-fos-ir and GC c-fos-ir was significant, numbers of MCs and GCs were analyzed in the same sections. The correlation was significant (environment 1; $r^2=0.552$; $p=0.0036$; environment 2; $r^2=0.318$; $p=0.0446$; Fig. 2C–D), and. was greater for environment 1 than environment 2 (ANCOVA; $F(1,23)=28.73$; $p<0.0001$). One reason for the greater correlation in environment 1 might be that in environment 1 there was more inhibition of dorsal GCs by dorsal MCs, because MCs innervate interneurons in the vicinity of their somata, which inhibit GCs (Scharfman, 1995; Larimer and Strowbridge, 2008). Another explanation is that there was increased lateral EC (LEC) input to GCs in environment 1, because indeed, there was more LEC c-fos expression in rats exposed to environment 1 than 2 (Fig. S1).

In summary, the results suggest that a subset of MCs are active *in vivo*, and these MCs are primarily in ventral hippocampus. Presumably these MCs are close to threshold normally, making them likely to reach threshold by even a minor additional excitatory input. For example, MCs may be readily activated by small stimuli in the home cage such as intermittent noises or odors. That interpretation is consistent with the observations that extrahippocampal areas exhibited strong c-fos-ir in the same rats, and the extrahippocampal areas that expressed c-fos were some of the regions that process sensory input and project to the DG, such as superficial layers of LEC (Fig. S1, Witter and Wouterlood, 2002; Kerr et al., 2007).

The small subset of MCs that appear to be active *in vivo* could correspond to the subset that has dendrites in the molecular layer, because these neurons are very sensitive to perforant path stimulation (Scharfman, 1991). MCs could also be innervated by deep layer EC neurons which project to the inner molecular, GC layer and subgranular zone; they target both dendritic spines and shafts, but most of the targeted neurons are GABAergic (Deller et al., 1996). Other inputs that could facilitate MC activity are subcortical, such as the septal input, which primarily targets GABAergic DG neurons and could disinhibit MCs (Freund and Gulyas, 1997). Disinhibition of MCs could also occur in response to endocannabinoids (Hofmann et al., 2006).

It was unexpected that c-fos expression in hilar MCs was different in the two environments. The difference could be due to the fact that the novelty and/or stress of transfer to the laboratory stimulated dorsal MCs more in the environment 1 than environment 2 group. However, fosB/ΔfosB-ir was similar to c-fos-ir, suggesting that c-fos expression reflected activity long before the transfer to the laboratory (Fig. S1). Therefore, we suggest that the differences between environments were related to the distinct types of stimuli in the two

environments, which included sporadic odors, noises, and visual input that differed. Distinctions may also have been due to handling in only one of the groups, which could decrease tonic stress, or stress responses to human voices.

Ventral MCs were active independent of the environment, whereas dorsal MCs were influenced by the environment - they appeared to be active to a different degree depending on the environment. These dorsal-ventral differences support previous reports that there are dorsal-ventral differences in MCs: 1) ventral MCs exhibit intrinsic burst discharges (Jinno et al., 2003), and 2) calretinin is only expressed in ventral MCs in the mouse (Blasco-Ibáñez and Freund, 1997; Fujise et al., 1998; Fujise and Kosaka, 1999). The long part of the MC axon - i.e., the part of the axon that projects primarily to GCs far away - is also different, with a shorter axon projection from dorsal MCs relative to ventral MCs (West et al., 1979; Buckmaster et al., 1996; Scharfman and Myers, 2013). The results suggest that different environments will lead to activation of different subsets of MCs and therefore distinct subpopulations of GCs.

The small subset of ventral MCs that were c-fos-ir in this study could have a considerable effect despite their small numbers, because MC axons are divergent, with potential to depolarize numerous GCs. MCs also innervate local interneurons (Scharfman, 1995; Larimer and Strowbridge, 2008; Scharfman and Myers, 2013). However, there was no evidence of c-fos-ir in DG interneurons. There could be several reasons: 1) DG interneurons may require more action potential firing to induce c-fos relative to MCs, or 2) interneurons may require different frequencies of action potential firing than MCs to induce c-fos protein. Indeed, there is evidence that DG interneurons have differences in c-fos expression compared to GCs: in response to seizures, DG interneuron c-fos-ir is delayed relative to c-fos-ir in GCs (Peng and Houser, 2005). There also is heterogeneity among interneurons in c-fos protein expression, with hilar somatostatin-expressing neurons exhibiting c-fos protein under conditions that only induce c-fos-ir in a subset of parvalbumin-expressing neurons (Dragunow et al., 1992).

In conclusion, the results suggest that a subset of hilar MCs are active *in vivo*. The data support the findings of *in vitro* electrophysiological experiments showing that MCs typically exhibit a high level of spontaneous activity relative to other cell types. Therefore, MCs – especially a subset - are likely to provide ongoing basal excitatory tone to the DG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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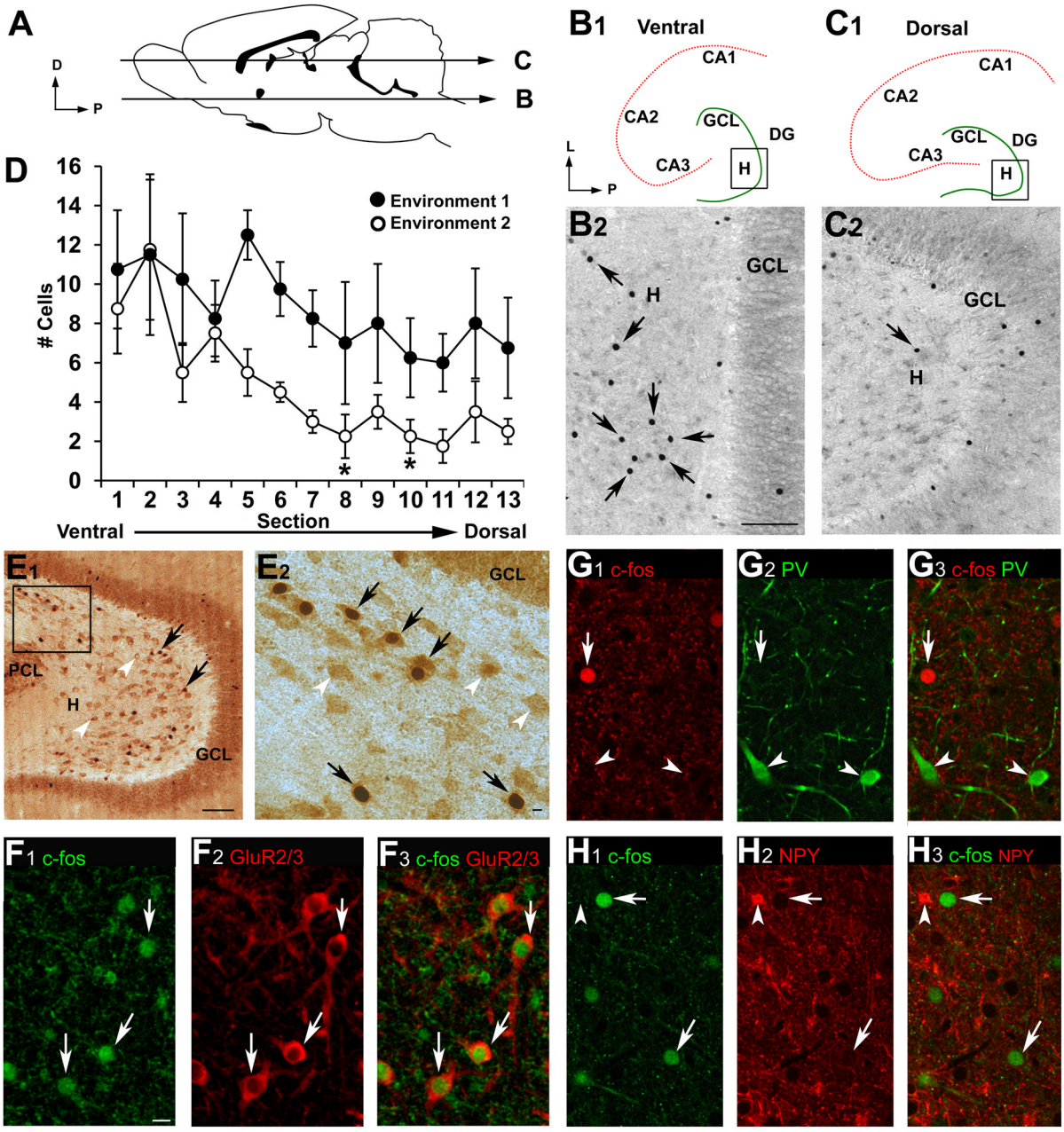

Figure 1. Robust c-fos-ir in ventral hilar mossy cells (MCs)

A.

A schematic of the hippocampus in the sagittal plane (adapted from Paxinos and Watson, 2007). Arrows indicate the location of the horizontal sections in parts B–C. D=dorsal, P=posterior.

B.

1. An illustration of a ventral horizontal section. The boxed area corresponds to B2. Dotted red line=pyramidal cell layer; green line=granule cell layer (GCL); DG=dentate gyrus; H=hilus. L=lateral.

2. A representative ventral horizontal section from a rat that was housed in its normal housing quarters (environment 1). Arrows point to hilar cells with c-fos-ir. There were relatively few c-fos-ir cells in the GCL relative to the hilus. Calibration=100 μm.

C.

1. An illustration of a dorsal horizontal section. The boxed area corresponds to C2.

2. A section from a rat housed in environment 2 where there was habituation to the laboratory and investigator who perfused the rat. Hilar c-fos-ir is rare but there is robust labeling in the GCL. Calibration=100 μm (shown in B2).

D.

The total number of c-fos-labeled hilar cells are shown (environment 1: black circles; environment 2: white circles; n=4 rats/group). Asterisks indicate $p<0.05$.

E.

1. Examples of c-fos-ir hilar cells (arrows) that co-express GluR2/3 (arrowheads). Calibration=100 μm (shown in B2).

2. The boxed area in E1 is shown at higher power. C-fos-labeled nuclei exhibited cytoplasmic immunoreactivity for GluR2/3 (arrows). GluR2/3-ir hilar cells without c-fos labeling are also present (arrowheads). Calibration=10 μm.

F.

1–3. Examples of c-fos-ir cells (F1, arrows), GluR2/3-labeled cells (F2; arrows) and double-labeled cells (F3, arrows) are shown. Calibration for F–H=10 μm (shown in F1).

G.

1–3. Examples of c-fos-expressing hilar cells are shown (1; arrows) to illustrate their lack of PV co-expression (2; arrowheads; merged image in 3).

H.

1–3. Examples of c-fos-immunofluorescent hilar cells are shown (1; arrows) demonstrate that they did not co-express NPY (2, arrowheads; merged image in 3).

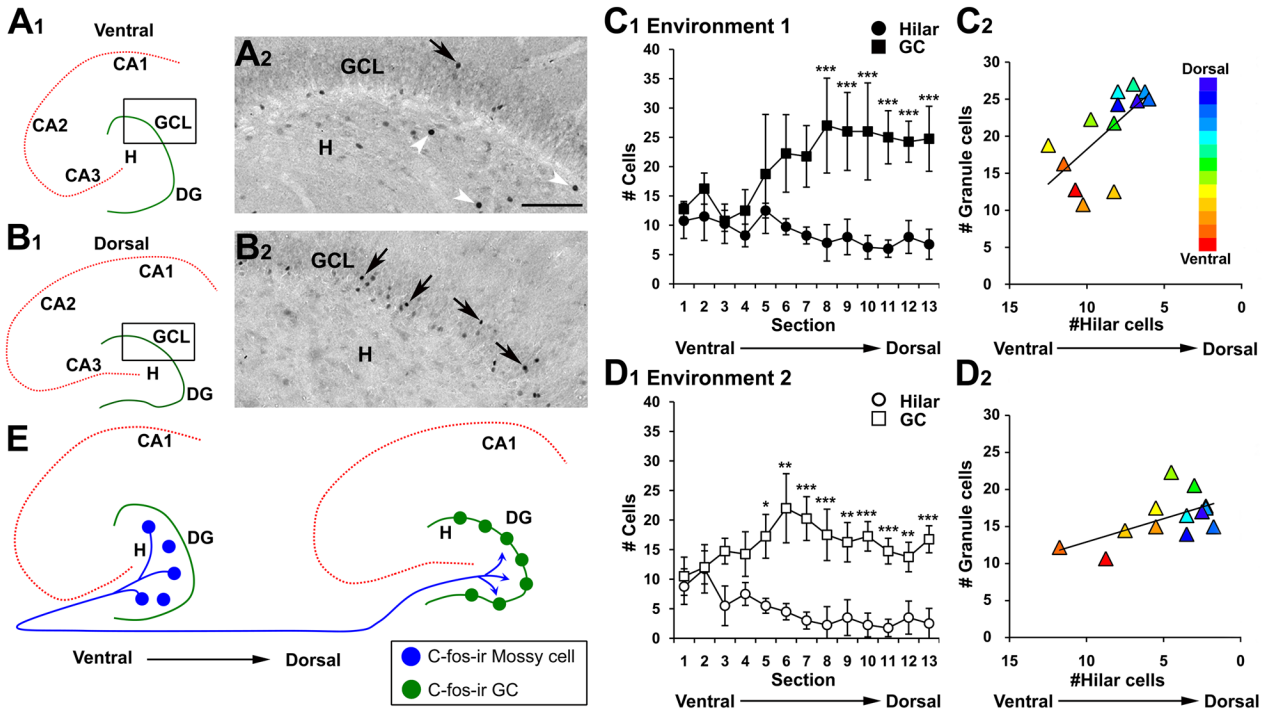

Figure 2. Inverse relationship in c-fos labeling of MCs and the GCL along the dorsal-ventral axis

A.

1. A schematic of ventral hippocampus (horizontal plane). Same abbreviations as Figure 1.

2. The boxed area in A1 is shown at higher power for a representative section from a rat housed in environment 1. There were few c-fos-ir cells in the GCL (arrows), and many c-fos-ir hilar cells (arrowheads). Calibration=100 μm.

B.

1. A schematic of the dorsal rat hippocampus (horizontal plane).

2. The boxed area in B1 is shown at higher power for a representative dorsal horizontal section from a rat housed in environment 1. There were many c-fos-ir cells in the GCL (arrows) but not the hilus. Calibration=100 μm (shown in A2).

C.

1. The number of cells in the GCL that were c-fos-ir in animals that were housed in environment 1 (black square; n=4 rats) are compared to the number of hilar c-fos-ir in the same animals (black circle). Triple asterisks indicate $p<0.001$.

2. The relationship between the numbers of hilar and GCL c-fos-ir cells are shown for rats housed in environment 1.

D.

1–2. The number of hilar and GCL c-fos-ir cells are plotted for animals from environment 1 (1) and environment 2 (2). Single asterisks indicate $p<0.05$, double asterisks indicate $p<0.001$.

E.

A schematic of the septotemporal axis is shown, with ventral (left) and dorsal (right) hippocampal levels. Ventral MCs excite dorsal GCs (blue line) because of their ipsilateral projection to the distal inner molecular layer (Ribak et al., 1985; Frotscher et al., 1991; Buckmaster et al., 1996).