Target-selective GABAergic control of entorhinal cortex output

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Supplementary Figure 1. VGLUT3 is an alternative marker for CCK/CB1R positive boutons in layer II of the MEC. CCK immunoreactivity strongly labels cell bodies but displays variable staining intensity in terminals (single arrows point to weakly labeled, double arrows point to strongly labeled CCK boutons). Note that the CB1R and VGLUT3 (see Refs. 7, 10 in main text) immunoreactivity intensely labels most putative CCK boutons. The added advantage of VGLUT3 over CB1R labeling is that VGLUT3 marks the terminals especially strongly, with virtually no labeling of the inter-bouton axons, therefore, we used VGLUT3 immunoreactivity in the immunocytochemical experiments related to Fig. 1d in the main paper to quantify the number of terminals on calbindin⁺ versus reelin⁺ cells in layer II.
Supplementary Figure 2. VGLUT3⁺ axon terminals form synaptic contacts on the somata of calbindin⁺ cells in MEClayer⁺. Left: Low magnification electron micrograph (scale bar: 2 μm) of three VGLUT3⁺ boutons (red boxes) surrounding a calbindin⁺ neuron (outlined in yellow). Note that the VGLUT3 positivity is indicated by the strong, dark DAB-Nickel immunoreaction, while the calbindin positivity in the cell body is visualized by the lighter DAB precipitate. Right: High magnification of the three VGLUT3⁺ terminals (scale bar: 0.5 μm); arrows indicate the synaptic junctions.
Supplementary Figure 3. Paired recordings show functional DSI expressing synaptic inputs from local interneurons. (a) Top traces show action potentials evoked in a presynaptic cell; bottom traces show 20 individual unitary IPSCs in the postsynaptic cell from each time point (gray) as well the averaged unitary IPSC (black; including failures). The time points were as follows, with the zero time point being the start of the depolarizing pulse: Baseline or pre-DSI period: -2s to 0s; DSI: 1s to 3s; Recovery: 4s to 6s. The illustrated traces are consecutive responses obtained by triggering action potentials in the presynaptic interneuron at 10 Hz, as described previously (Földy et al., 2006). Recordings were performed in control ACSF and DSI was evoked by a 500ms pulse to 0mV from -60mV in the postsynaptic cell. (b) The presynaptic DSI expressing cell in panel (a) was VGLUT3 positive (b1; asterisks indicate cell body; BIO: biocytin); b2: the dendrite of the postsynaptic cell in panel (a) was calbindin positive. In addition, the axons of the presynaptic cell in panel (a) formed axosomatic VGLUT3⁺ synapses (b3; arrows) on other (non-recorded) calbindin⁺ cells (asterisks) in MEClayer1. Scale bars: b1 & b3: 5 µm; b2: 2 µm).
Supplementary Table 1. Intrinsic properties of intracellularly recorded, post-hoc immunocytochemically identified reelin$^+$ and calbindin$^+$ cells in MEC$_{layer II}$. See Supplementary Methods for details on measurements of electrophysiological parameters (n=7 cells for each group). Note that the reelin$^+$ and calbindin$^+$ cells show similar differences in electrophysiological parameters that have traditionally differentiated between the “stellate” and “non-stellate” cell groups (see Alonso and Klink, 1993; Nolan et al., 2007; Giocomo and Hasselmo, 2008). $V_{rest}$ = Resting membrane potential; $R_{in}$ = Input resistance; $\tau_{sag}$ = Time constant of sag potential.

|                  | reelin$^+$          | calbindin$^+$         | P      |
|------------------|---------------------|-----------------------|--------|
| $V_{rest}$ (mV)  | -55.51 ± 0.88       | -55.63 ± 0.5          | 0.924  |
| $R_{in}$ (MΩ)    | 51.03 ± 4.54        | 56.62 ± 5.02          | 0.382  |
| $\tau_{sag}$ (ms)| 36.33 ± 2.66        | 54.17 ± 6.11          | 0.020  |
| Rebound depolarization (mV) | 8.56 ± 4.3        | 5.28 ± 0.44          | 0.001  |
| Adaptation ratio | 0.97 ± 0.12         | 1.36 ± 0.06          | 0.018  |
| Spike duration (ms) | 1.72 ± 0.04      | 1.97 ± 0.09          | 0.039  |
Supplementary Methods

Tracing and immunohistochemical experiments

Adult male Wistar rats (P60-P120; 250-450g; Charles River Laboratories, Boston, MA) were anesthetized with ketamine-xylazine (60mg/Kg ketamine; 10mg/Kg xylazine) and placed in a stereotaxic frame. Biotinylated dextran amine (BDA, MW10000, Invitrogen; 50-300nl, 20% BDA in saline) was injected into the molecular layer of the dentate gyrus (lateral: 2.5mm, anterior-posterior:-4.2mm, ventral:-3.5mm from Bregma). Fluorogold (Fluorochrome; 100nl, 4% in saline) was injected into the hippocampal commissure / fimbria-fornix region (lateral: 0mm, anterior-posterior: -0.5mm, ventral: -5mm) or the medial entorhinal cortex (lateral: 4.5mm, anterior-posterior: -9mm, ventral: -4.5mm). After 5-7 days, the animals were anesthetized and perfused with a fixative containing 2% paraformaldehyde (PFA) dissolved in 0.1M phosphate buffer. The brains were removed, post-fixed, immersed in 30% sucrose in 0.1M phosphate buffer for 48 hours, and 60µm horizontal sections were cut with a freezing microtome. Immunoreactions were carried out with the following antibodies raised against reelin (Chemicon clone G10; 1:1000, mouse), calbindin (Swant CB-38; 1:5000, rabbit; and Swant 300; 1:5000, mouse), CCK (Dr. G. Ohning, CURE/Digestive Diseases Research Center, Antibody/RIA Core, Los Angeles, CA; 1:5000, mouse), VGLUT3 (Chemicon AB5421; 1:5000, guinea pig), CB1R (Frontiers Science Co CB1-GP-Af530-1; 1:5000, guinea pig; and Dr. K.Mackie, University of Indiana, Bloomington; 1:5000, rabbit), PV (Swant PVG-214; 1:1000, goat), GluR2/3 (Chemicon AB1506; 1:500, rabbit), GABA (Sigma clone GB-69; 1:500, mouse), flurogold (Fluorochrome; 1:5000, rabbit). Secondary antibodies conjugated to DyLight488/549/649, raised in donkey against mouse/rabbit/guinea pig (Jackson Immunoresearch) were used to detect the location of the primary antibodies. Note that glutamatergic principal cells show strong immunoreactivity to GluR2/3 in their cell bodies (see Toth et al., 1997, Leranth et al., 1996). All primary and secondary antibodies were diluted in 0.1M phosphate buffer containing 2% normal donkey serum, 0.3% Triton-X100 and 0.05% Sodium-azide. The sections were incubated overnight in the primary, then in the secondary antibody cocktails overnight at room temperature. The BDA retrograde tracer was visualized with the tyramide signal amplification (TSA) kit (Invitrogen). For confocal imaging of the fluorogold labeled cells, slices were processed with rabbit anti-fluorogold and then biotinylated donkey anti-rabbit antibodies, and visualized with the TSA kit.

For electron microscopy, rats were transcardially perfused with 4% PFA and 0.1% glutaraldehyde in 0.1M phosphate buffer. Horizontal 60µm sections of the entorhinal cortex were prepared with a vibratome (Leica VT1000S). Sections were cryoprotected overnight in 30% sucrose, then freeze-thawed 3 times over liquid nitrogen. The sections were incubated 24 hours at room temperature in primary antibody cocktail containing mouse anti-calbindin (Swant CB-38; 1:10000) and guinea pig anti-VGLUT3 (Chemicon AB5421; 1:10000) in 0.1M phosphate buffer. Next, the sections were incubated in biotinylated donkey anti-guinea pig antibody overnight (Jackson Immunoresearch, 1:1000, in 0.1M phosphate buffer), then in Vectastain peroxidase ABC (Vector Laboratories PK-6100, 1:500, in 0.1M phosphate buffer, 4
hours). The peroxidase reaction was carried out using DAB-Nickel as chromogen. After terminating the reaction, the sections were incubated in biotinylated donkey anti-mouse antibody (Jackson Immunoresearch, 1:1000, in 0.1M phosphate buffer, overnight), then in ABC (1:500, in 0.1M phosphate buffer, 4 hours). For visualization of the calbindin cells, DAB was used as chromogen. The sections were osmicated with 1% osmium tetroxide, contrasted with 1% uranyl acetate (Electron Microscopy Sciences), and dehydrated in an ascending series of ethanol. After treating the sections with propylene oxide, the sections were soaked in durecupan overnight, then flat embedded. Serial ultrathin (70nm) sections were cut with an ultramicrotome, and were analyzed on a Jeol transmission electron microscope (JEM-1400) and pictures were taken with a Gatan SC1000 ORIUS CCD camera.

The VGLUT3$^+$ boutons surrounding the somata of calbindin$^+$ or reelin$^+$ neurons in layer II of the medial entorhinal cortex were counted from single confocal images (Olympus FV1000 microscope; objectives: UPlanS Apo 20X/0.75 and PlanApo N 60X/1.42). After images were collected, samples were analyzed using ImageJ (US National Institutes of Health). For illustration in Fig. 1, up to three consecutive confocal images were Z-stacked. The density of innervation is given as the ratio of VGLUT3 positive boutons per 1mm of somatic membrane of the calbindin$^+$ versus reelin$^+$ cell bodies. Retrogradely labeled layer II neurons were counted for colocalization with reelin or calbindin. The BDA labeled cells were counted from images taken with a confocal microscope. The fluorogold labeled cells were counted from images taken with a conventional epifluorescence microscope (Zeiss Axioskop). Only those cells in which the nucleus was clearly visible were counted. For electron microscopy, postsynaptic targets of VGLUT3$^+$ terminals were randomly sampled in MEC$_{layerII}$.

Electrophysiology and analysis

Transverse entorhinal-hippocampal slices (350 µm) were prepared from P21-P40 Wistar rats. Because there is a dorso-ventral gradient of cellular properties (see Nolan et al., 2007; Giocomo and Hasselmo, 2008), the slices were prepared from the middle of the medial entorhinal cortex. Slices were incubated for an hour at 33°C in sucrose-containing artificial cerebrospinal fluid (ACSF; 85 mM NaCl, 75 mM sucrose, 2.5 mM KCl, 25 mM glucose, 1.25 mM NaH$_2$PO$_4$, 4 mM MgCl$_2$, 0.5 mM CaCl$_2$ and 24 mM NaHCO$_3$), then transferred to an oxygenated standard ACSF solution (126 mM NaCl, 2.5 mM KCl, 26 mM NaHCO$_3$, 2 mM CaCl$_2$, 2mM MgCl$_2$, 1.25 mM NaH$_2$PO$_4$, and 10 mM glucose) for electrophysiological recordings performed at 33°C. For DSI experiments, 5 µM NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide), 10 µM APV (D-2-amino-5phosphovaleric acid), and 5µM carbachol were added to the recording ACSF (note that DSI experiments were also performed without the addition of carbachol; or in 10 µM AM251); all drugs were from Tocris. Slices were visualized with an upright microscope (Olympus, BX-50) with infrared differential interference contrast (IR-DIC) optics. Recordings were made using MultiClamp700A amplifier (Axon Instruments, Union City, CA). Signals were filtered at 4 kHz using a Bessel filter and digitized at 10 kHz with a Digidata 1320A analog–digital interface (Axon Instruments).
For examining intrinsic properties, whole-cell current clamp recordings were performed with an internal solution containing 126 mM K-Gluconate, 4 mM KCl, 10 mM HEPES, 4 mM ATP-Mg, 0.3 mM GTP-Na, 10 mM Phosphocreatine and 0.2% biocytin (pH 7.2, 270 - 290 mOsm). For DSI experiments, cells were recorded in voltage clamp mode, with internal solution containing 40mM CsCl, 90mM K-Gluconate, 1.8 mM NaCl, 1.7 mM MgCl$_2$, 3.5 mM KCl, 0.05 mM EGTA, 10 mM HEPES, 2 mM MgATP, 0.4 mM Na$_2$GTP, 10 mM Phosphocreatine, and 0.2% biocytin (pH 7.2, 270-290 mOsm). The depolarizing pulse used to evoke DSI was a 500 ms step to 0 mV from a holding potential of -60 mV.

For paired recordings, whole-cell recordings were obtained from presynaptic interneurons with patch pipettes (internal solution: 126 mM K-Gluconate, 4 mM KCl, 10 mM HEPES, 4 mM ATP-Mg, 0.3 mM GTP-Na, 10 mM Phosphocreatine and 0.2% biocytin; pH 7.2, 270 - 290 mOsm). The interneurons included in the study were all DSI-sensitive that evoked fast unitary IPSCs (Földy et al., 2006), and they were located near the border of Layer II and Layer III of MEC. The synaptically coupled postsynaptic cells were recorded with internal solution also used for the DSI experiments in Fig. 2a of the main text (40mM CsCl, 90mM K-Gluconate, 1.8 mM NaCl, 1.7 mM MgCl$_2$, 3.5 mM KCl, 0.05 mM EGTA, 10 mM HEPES, 2 mM MgATP, 0.4 mM Na$_2$GTP, 10 mM Phosphocreatine, and 0.2% biocytin; pH 7.2, 270-290 mOsm). DSI was elicited with a 500 ms depolarizing pulse to 0mV from a holding potential of -60mV. Unitary IPSCs were individually inspected and included in analysis based on their onset latency after the presynaptic action potential.

After recordings (typically lasting 15-20min), the slices were immersion fixed in 4% PFA dissolved in 0.1M phosphate buffer overnight at 4°C. After cryoprotection in 30% sucrose dissolved in 0.1M phosphate buffer, the slices were resectioned at 60µm and processed for calbindin, reelin and VGLUT3 immunoreaction. Subsequently, the sections were processed with the standard ABC method using DAB-Nickel as chromogen (Vector Laboratories). Only cells with confirmed immunopositivity for either calbindin or reelin were used for data analysis. The recorded traces were analyzed using the Clampfit software (Axon Instruments), MiniAnalysis (Synaptosoft, Chapel Hill, NC), and SigmaPlot (Systat Software, San Jose, CA). For DSI experiments from single cells (Fig. 2a), MiniAnalysis was used for detection of the sIPSCs and calculation of the area underlying the IPSCs. DSI was measured by the percentage of charge transfer following the depolarizing pulse (2-5 sec post-pulse), with respect to a prepulse period (3 sec). Data are presented as mean ± s.e.m, and statistical significance was determined using a two-sided Student's t-test with a level of significance of P<0.05. Resting membrane potential was taken after break-in. Input resistance was calculated from the steady-state voltage responses evoked by intracellular current injections (starting from -240pA, in 10pA steps). Time constant for sag potential ($\tau_{sag}$) was defined (see Giocomo and Hasselmo, 2008) from the hyperpolarizing voltage responses (current pulse: -240pA) as the time to reach 63% of the steady-state voltage after the trough. Rebound depolarization was measured as the peak amplitude of the depolarizing voltage deflection following the offset of the test hyperpolarizing pulse (-240pA). Adaptation
ratio was calculated as the ratio of the last interspike interval to the first interspike interval. Spike duration was measured at threshold (see Alonso and Klink, 1993).

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