Supplementary Methods

Allen Gene Expression Atlas annotation, analysis, and mapping

The Allen Institute provides an online open-access resource of thousands of mouse *in situ* hybridization experiments each visualizing gene expression patterns of a single gene (www.brain-map.org), methodology can be found online in the Documentation section[51]. The archive of mouse gene expression patterns can be searched either directly by name or by gross anatomical structure as listed in the Allen Reference Atlas (ARA) [20]. The Allen Gene Expression Atlas (AGEA) allows for a point-to-point correlation comparison of two anatomical locations in the mouse brain. To discover gene expression patterns within the hippocampal formation, we applied all of these methods to manually search for genes that were located within the major cell layers of the mouse hippocampus and subiculum. As described by Thompson et al., gene expression patterns were observed to be a nested mosaic, such that one gene could be expressed in an entire structure while another gene's expression could be limited to various parts of that structure[10]. To identify hippocampal subregions, we examined combinatorial gene expression patterns of hundreds of hippocampal-expressed genes that displayed restricted expression patterns until we could determine the least common pattern that defines a unique subregion. For some areas, a single gene clearly demarcates a subregion whereas other subregions are interpreted based on the presence or absence of multiple genes. Overall, we manually annotated the expression pattern of over 250 genes across the entire hippocampal formation (**Supplementary Table 2**).

A major consideration when observing and annotating Allen Brain Atlas gene expression data is the highly varied histological tissue sectioning. Different sectioning angles create inaccuracies when comparing individual sections across animals and matching and registering whole tissue sections directly on to Allen Reference Atlas levels. Point-to-point comparisons

across different brains can be inaccurate as one part of the tissue can align well while another part is mismatched. For the hippocampus, this problem is particularly relevant to the dorsal/ventral axis and may be the reason for previous observations of many small subdomains in the ventral CA3 [10] and ventral CA1 [9] as well as the interpretation of gene expression gradients. To accurately map rostrocaudal HGEA boundaries, we used local tissue landmarks at multiple points throughout the tissue section to estimate the sectioning angle, treat each section as a composite gradient of multiple rostrocaudal ARA levels, and appropriately match the data to the HGEA (**Supplementary Figure 3a**). Notably, tissue sections from the same brain are cut at the same angle while tissue sections from other brains are often cut at different angles (**Supplementary Figure 3b-c**). Oblique sectioning angles can also create both medial/lateral and dorsal/ventral gradients.

Final determinations of HGEA boundaries are accomplished by examining the positioning of gene expression across multiple tissue sections and interpreting the changes that occur between. For example, the caudal boundary of the CA2 with the CA1i has been of recent debate [15]. The rostrocaudal progression of the CA2 'marker gene' *Amigo2* can be observed in both the coronal and sagittal sections (**Supplementary Figure 3e-f**). At level HGEA 80, dense *Amigo2* expression is located within the CA2, with lighter expression within the CA3v and CA3vv (**Supplementary Figure 3e**). At HGEA 81, the dense *Amigo2* CA2 expression is now present in a large continuous area between the CA1d and CA1v (caudal to the CA3 in HGEA 80). Finally, at HGEA 82, *Amigo2* CA2 expression is limited to a sparse lamina of neurons deep to the CA1i. A similar progression from medial to lateral can be observed in the sagittal tissue sections (**Supplementary Figure 3f**). In sagittal HGEA 4, Amigo2 expression is present in dorsal and ventral parts of the hippocampus separated by CA3. At sagittal HGEA 2, Amigo2 CA2 expression becomes continuous between the CA1d and CA1v. Finally, at sagittal HGEA 1, Amigo2 expression is limited to a superficial lamina with CA1i located more deeply (CA2 rostral

to CA1i). Considered together, the coronal and sagittal Amigo2 expression show that the caudal extension of the CA2 is interposed between the CA1d and CA1v in the dorsoventral direction and the CA3 and CA1i in the rostrocaudal direction. The boundary between the CA2 and CA1i is non-parallel to the coronal sectioning plane resulting in CA2 neurons located deep to CA1i neurons at HGEA 81. This progression is similar across all CA2-expressed genes.

Comparison of HGEA gene expression annotation with single-cell RNAseq database

To relate our qualitative gene expression annotation approach to more quantitative single-cell RNAseq methods, we compared the binary HGEA gene expression annotation to the Dropviz drop-seq database[21] (www.dropviz.org). The Dropviz database contains single-cell gene expression from 113,000 hippocampal cells and over 32,000 genes and clustering analysis reveals that the hippocampal neurons can be divided into 7 'global clusters' (Interneuron_Gad2 vs. Cajal-Retzius vs. Dentate_C1ql2 vs. CA3CA3_Pvrl3-Rgs15-Calb2 vs. CA1_Subiculum_Postsubiculum_Entorhinal_Fibcd1-Dcn-Cbln1-Ptgfr-Fezf2 vs. Subiculum_Entorhinal_Nxph3 vs. Subiculum_Slc17a6) that contains multiple 'subcluster' cell-types (27 interneurons and 31 principal neurons, note the subiculum neurons are divided among 3 different clusters). DropViz data distribution was assumed to be normal but this was not formally tested.

First, to compare the relative correspondence of our HGEA gene expression annotation to the presence of positive gene expression within a hippocampal region, we used the DropViz query feature to input HGEA annotated genes for each region and compared their expression within a corresponding DropViz global cluster (i.e. DGd-expressed genes within Dentate_C1ql2, CA3dd-expressed genes within CA3CA3_Pvrl3-Rgs15-Calb2; see **Supplementary Information 3**). Comparisons for each HGEA SUB layer were performed for each of the 3 global SUB-related global clusters. In addition, we compared the significance of this expression within a

global cluster vs. the rest of the hippocampus although only the ‘marker genes’ we identified would be expected to be different (all data exported from DropViz website is included in **Supplementary Information 3**). For each set of annotated genes within a HGEA region, most of the genes were found to have positive expression within the DropViz ‘global cluster’. Notably, many of the same ‘marker genes’ identified in our annotation as unique to a single HGEA region had significant positive expression within the global cluster and 0 expression in the rest of the hippocampus (highlighted green in **Supplementary Information 3**). HGEA genes that were annotated as having positive expression, but showed 0 expression within the DropViz dataset, were queried for their expression within the Interneuron_Gad2 ‘global cluster’. Genes which were found to be expressed within other relevant global clusters that could account for positive expression within an HGEA region (Interneuron_Gad2 or one of the other SUB-containing global clusters) were highlighted orange. All other genes which were annotated as having expression in the HGEA annotation, but 0 expression in any relevant ‘global clusters’ were highlighted red (note, much of the discrepancy comes from repetition of a few genes that DropViz reported as not expressed anywhere in the hippocampus, but were annotated as expressed in multiple HGEA regions). In total, the number and proportion of genes with positive expression (non-red highlighted) and 0 expression (red-highlighted) were calculated for each comparison to evaluate the relative similarity at the ‘global cluster’ scale (for each SUB layer, the three comparisons were averaged to create one percentage value). Overall, the percentage of similar positive expression between each HGEA region vs. DropViz ‘global cluster’ averaged 91.7±0.8%.

To further analyze similarities between the HGEA annotation and DropViz data, we performed a principal components analysis (PCA) using DropViz’s ‘meta-cell’ data. According to Dropviz’s website, a ‘meta-cell’ contains the aggregate unique molecular identifier (UMI) counts for all single-cells that belong to a ‘subcluster’ so that there is one ‘meta-cell’ per subcluster. Using the UMI count data from DropViz, we performed two separate PCA clustering analyses

on the ‘meta-cells’ using all 32,307 DropViz gene set and compared it to the more limited set of 248 HGEA annotated genes. We found that PCA clustering from the 248 HGEA genes performed remarkably similar to the clustering with all 32,307 DropViz genes, suggesting that the more limited HGEA annotated gene set can still recapitulate the overall differences in hippocampal cell type gene expression (see **Supplementary Fig. 5**).

Finally, we reasoned that the annotated gene expression patterns that define HGEA subregions may reflect the distinct gene expression profiles of the individual ‘meta-cells’ if each HGEA region is also composed of different cell-types. To compare the Dropviz ‘meta-cell’ data to the binary HGEA gene annotation, we first binarized the UMI count data for the HGEA genes within the DropViz ‘meta-cells’ after thresholding out UMI counts less than 30. Then, we calculated a Rogers-Tanimoto dissimilarity matrix (SciPy Python library) for the HGEA annotated genes between where they were expressed (HGEA subregion) and which meta-cell they were expressed in (**Supplementary Table 3**.; all coefficient values between 0 and 1, lower value means more similar and vice versa). As expected, the dissimilarity matrix showed that ‘meta-cells’ were more similar to corresponding HGEA subregion and more dissimilar to other HGEA regions (for example, CA1 meta-cells had lower dissimilarity values in HGEA CA1 regions, etc.). Notably, our annotated ‘Putative Interneurons’ were highly similar to the DropViz interneuron ‘meta-cells’. Some HGEA subregions were notably similar to a specific ‘meta-cell’. For example, the Neuron.Slc17a7.Calb2-Vgll3 ‘meta-cell’ had a low dissimilarity value for the CA3vv region. Consistent with this similarity, DropViz query feature reports the CA3vv ‘marker gene’ *Coch* as being significantly expressed in the Neuron.Slc17a7.Calb2-Vgll3 subcluster (2.77, p=7.44 x 10^{-192}) and almost 0 expression in all other CA3 subclusters.

Mouse Connectome Project methodology

Anatomical tracer data was generated as part of the Mouse Connectome Project (MCP) within the Center for Integrative Connectomics (CIC) at the University of Southern California

Mark and Mary Stevens Neuroimaging and Informatics Institute (formerly the Laboratory of Neuro Imaging at the University of California, Los Angeles). Some MCP experimental procedures for data generation and online publication have been described previously[18,52,53].

We systematically and carefully mapped neuronal connectivity of every molecular domain of the hippocampus and SUB to determine their connectivity (for injection site list, see **Supplementary Table 4**). We used multiple fluorescent tracing strategies with a combination of classic tract-tracing and viral tracing methods. First, we used a double coinjection approach that injects two different tracer cocktails each containing one anterograde and one retrograde tracer to simultaneously visualize two sets of input/output connectivity [18]. To investigate the convergence or divergence of axonal fiber pathways either into or out of the hippocampus, we used a triple anterograde tracing approach with individual injections of PHAL and EGFP- and tdTomato- expressing adeno-associated viruses (AAV). Finally, to compare neuronal projection cell types and fiber pathway origin, we used a quadruple retrograde tracing method with individual injections of 4 different retrograde tracers (cholera toxin subunit B (CTb) conjugated with 488, 555, or 647, as well as FG) into 4 different hippocampal projection targets.

Subjects

All tracer experimental data was generated using 2-6 month-old male C57BL/6J mice (Jackson Laboratories). Mice were pair-housed within a room that was controlled for temperature (21-22°C), humidity (51%), and light (12 hr light:12 hr dark cycle with lights on at 6:00 am and off at 6:00 pm). Subjects had ad libitum access to tap water and mouse chow throughout the experiments. Rabies injection surgeries were performed in a BSL-2 level environment and performed by individuals who had been rabies-vaccinated. Following surgery, rabies-infected animals were individually housed in a separate BSL-2 level facility. All experiments were conducted according to the regulatory standards set by the National Institutes

of Health Guide for the Care and Use of Laboratory Animals and by the institutional guidelines set by the Institutional Animal Care and Use Committee at USC and the Animal Research Committee at UCLA. This study's protocol was approved by the Institutional Animal Care and Use Committee at USC and the Animal Research Committee at UCLA.

Tracer Injection Experiments

The Mouse Connectome Project's standard experimental approach is dual coinjections of anterograde and retrograde tracers into different brain areas within the same mouse. Each coinjection contained an anterograde (*phaseolus vulgaris* leucoagglutinin [PHAL] or biotinylated dextran amine [BDA]) and a retrograde (cholera toxin subunit b [CTb] or Fluorogold [FG]) tracer. PHAL (2.5%; Vector Laboratories) and CTb conjugated to Alexa Fluor 647 (CTB-647, 0.25%; Invitrogen) were coinjected, while BDA (biotinylated dextran amine, 5%; Invitrogen) or AAV1.CAG.RFP (Penn Vector Core, originally created at Allen Institute for Brain Sciences) was injected in combination with FG (1%; Fluorochrome, LLC).

To provide further details on specific connectivity patterns, we also performed quadruple retrograde tracer, triple anterograde tracer, and rabies/PHAL experiments. Quadruple retrograde tracer experiments involved four different injections sites receiving a unique injection of either 0.25% CTB-647, CTB conjugated to Alexa Fluor 555 (CTB-555, 0.25%; Invitrogen), CTB conjugated to AlexaFluor 488 (CTB-488, 0.25%; Invitrogen), or 1% FG. Triple anterograde tracing experiments involved three separate injections of 2.5% PHAL, AAV1.CAG.RFP, and AAV1.hSyn.GFP (Penn Vector Core, originally created at Allen Institute for Brain Sciences). For rabies/PHAL experiments, we used G-deleted rabies-GFP (Salk Institute vector core) at the same or separate injection site as PHAL. G-deleted rabies virion constructs are pseudotyped with the normal rabies glycoprotein (G)-containing viral envelope but are unable to incorporate rabies glycoprotein in new virions, working similar to classic retrograde tracers. However, while

most retrograde tracers only label cell bodies, G-deleted rabies-GFP functions as a retrograde tracer that produces bright fluorescent labeling of both the cell body and dendrites. Together with PHAL axonal labeling, G-deleted rabies is a useful tool for examining anterogradely-labeled axons and their approximate location adjacent to retrogradely-labeled dendrites and cell bodies. All cases used in this study are listed in **Supplementary Table 4** and are available online at www.MouseConnectome.org. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications[18,52]. In most cases, anterograde tracing results are cross-validated by retrograde labeling injections at anterograde fiber terminal fields and vice versa. No data has been excluded from this study and all image data generated is published online as part of the Mouse Connectome Project (www.MouseConnectome.org).

Stereotaxic surgeries

Mice were anesthetized in an induction chamber primed with isoflurane (Hospira) and subsequently mounted to a Kopf stereotaxic apparatus where they were maintained under anesthetic state via a vaporizer (Datex-Ohmeda). For dual coinjections, tracer cocktails were delivered iontophoretically via glass micropipettes (outer tip diameter of 15–20 μm) using alternating 7 s pulsed positive 5 μA current for 5 (BDA or AAV/FG) or 10 min (PHAL-CTB-647) generated by a current source (Stoelting Co.). Triple anterograde tracing experiments were performed using similar iontophoretic parameters although the duration was different (5 min for PHAL, 1.5 min for AAVs). In quadruple retrograde tracing experiments, retrograde tracers were loaded into glass micropipettes that were connected to a picoPump pressure injector. At each injection site, 50nl of retrograde tracer was pressure injected at a rate of 10nl/min. For PHAL/rabies experiments, PHAL was delivered iontophoretically and 50nl of G-deleted Rabies-GFP was pressure injected following the same parameters described above. In all experiments, pipettes were left in place for an additional 5 min following delivery to avoid diffusion of tracers

along the needle track. Animals survived for 7 days prior to being sacrificed except animals injected with AAV1.hSyn.GFP or AAV1.CAG.RFP were sacrificed 3 weeks following surgeries to ensure fluorescent labeling had sufficiently labeled distant axon terminals.

Histology and Immunohistochemical Processing

Each animal was deeply anesthetized with an overdose injection of sodium pentobarbital and trans-cardially perfused with approximately 50 ml of 0.9% saline solution followed by 50 ml of 4% paraformaldehyde (PFA; pH 9.5). The brains were post-fixed in 4% PFA for 24-48 hr at 4°C.

For tissue sectioning, fixed brains were embedded in 3% Type I-B agarose (Sigma-Aldrich) and sectioned into four series of 50 µm thick coronal sections with a Compresstome (VF-700, Precisionary Instruments, Greenville, NC). For all experiments, one series of sections was stained for NeuroTrace 435/455 (NT; 1:1000; Invitrogen, #N21479) and PHAL (if necessary) immunofluorescence using the free-floating method and the other three series were stored in cryopreservant under -20°C if additional staining was needed. For PHAL immunostaining, sections were placed in a blocking solution containing normal donkey serum (Vector Laboratories) and Triton X (VWR) for 1 hr. Following 3-5 min rinses, sections were incubated in PHAL primary antiserum (KPBS solution comprised of donkey serum, Triton, and 1:1000 rabbit anti-PHAL antibody (Vector Laboratories, #AS-2300; see our previous study for validation of this antibody [48,52])) for 48-72 hr at 4°C. Sections were rinsed 3 times in KPBS and then soaked for 3 hr in the secondary antibody solution (donkey serum, Triton and either 1:500 anti-rabbit IgG conjugated with Alexa Fluor 488 for dual coinjection experiments or 1:500 anti-rabbit IgG conjugated with Alexa Fluor 647 for triple anterograde and rabies tracing experiments (Invitrogen, 488:#A-21206, 647: #A-31573). After processing, sections were mounted and coverslipped using 65% glycerol.

Imaging and Post-acquisition Processing

All tissue sections were scanned as high-resolution virtual slide image (VSI) files using an Olympus VS110 high-throughput microscope fitted with a 10X objective lens. Images were captured tile by tile using appropriately-matched fluorescent filters and then assembled together as whole-brain images. The number of color channels in each image depends on the number of tracers used in each experiment. For dual coinjection experiments, each image contains 5 color channels (green=PHAL, magenta=CTB-647, red=BDA, yellow=FG, and blue=NT). Images from quadruple retrograde tracer experiments contain 5 channels (green=CTB-488, magenta=CTB-647, red-CTB-555, yellow-FG, blue=NT), images from triple anterograde tracing data contain 4 channels (green= AAV1.hSyn.GFP, red=AAV1.CAG.RFP, magenta=PHAL), and images from rabies experiments contain 3 channels (green=G-deleted Rabies-GFP, magenta=PHAL, blue=NT). Before online publication, all images are aligned to the correct left-right orientation, matched to the nearest ARA atlas level, and converted to tiff format prior to being registered (detailed below). Following registration and registration refinement, the NT blue channel was converted to a bright-field image. Next, each channel for every image was adjusted for brightness and contrast to maximize labeling visibility and quality in iConnectome. Following final modifications (i.e., skewness, angles) and JPEG2000 file format conversions, all images were published to iConnectome (www.MouseConnectome.org). For publication, some tracer colors have been changed from the raw images for better multicolor visualization.

Tracer Experiment Reproducibility

All injection experiments shown in this manuscript are listed in **Supplementary Table 4,** although additional supporting data and relevant injection sites can be found at www.MouseConnectome.org (currently, 553 total experiments, 1194 injection sites labeling 1872 pathways across the mouse brain). For each figure panel with tracer experiments, we list

the number of times the experiment has been repeated and the number of cases which cross-validate the result using the data in **Supplementary Table 4**:

Fig. 2a,b. Retrograde injections were repeated with similar results [CA3d (2), CA3ic (4), CA3v (2), CA1dr (4), CA1i (5), CA1v (4), CA1vv (3), CA1dc (4), SUBdd (8), ProSUB (3)] and are cross-validated by anterograde injections [CA3dd (2), CA3d (2), CA3id (2), CA2 (3)].

Fig. 2c. Some CA3 anterograde injections were repeated with similar results [CA3dd (2), CA3d (2), CA3id (2), CA3ic (2), CA3v (1), CA3vv (1) and are cross-validated by retrograde CA1 injections (CA1dr (4), CA1dc (4), CA1i (5), CA1v (4), CA1vv (3)].

Fig. 3a. Retrograde injections were repeated with similar results [MM (9), SM/PH (3), RE/AMd (5)] and cross-validated by SUB anterograde injections (SUBdd (8) and ProSUB (2)].

Fig. 3b. Some retrograde injections were repeated with similar results [AONm/TTd (1), MEAad/CEA (3), RE (5), PVT (2), PT (2)] and cross-validated by SUB anterograde injections [SUBv (2) and SUBvv (3)].

Fig. 3d. Retrograde injections were repeated with similar results [CA1i (5), CA1v (4), CA1vv (3), SUBvv (1)] and cross-validated by SUB anterograde injections [SUBv (2) and SUBvv (3)]. Anterograde injections were repeated with similar results [CA1vv (1), SUBv (2)) and cross-validated by retrograde injections (CA1vv (3) and SUBv (3)].

Fig. 3e. Retrograde injections were repeated with similar results [AD (1), RE (5), PVT (2), PT (2)] and cross-validated by SUB anterograde injections [SUBv (2) and SUBvv (3)]. Anterograde injections were cross-validated by retrograde injections [SUBv (2) and SUBvv(1)].

Fig. 5a. Anterograde injections were repeated with similar results [SUBdd (9), SUBdv (4)] and cross-validated by retrograde injections [RSPv (3), POST (2), PRE (2), PAR(2)].

Retrograde injections were repeated with similar results [SUBdd (8), SUBdv (3)] and cross-validated by anterograde injections [CA1dc (2) and CA1i (1)].

Fig. 5b. Retrograde injections were repeated with similar results [SUBdd (8), SUBdv (3), POST (2), PRE (2), PAR (2)] and cross-validated by anterograde injections [LD (2)]. Anterograde injections were repeated with similar results [SUBdd (9), SUBdv (4), LD (2)] and cross-validated by retrograde injections [POST (2), PRE (2), PAR (2)].

Fig.5c. Some retrograde injections were repeated with similar results [MM (9), RE/AMd (5), PT (3), AD (1), PVT (2), AV (4)] and cross-validated by anterograde injections [SUBdd (9) and SUBdv (4)].

Fig. 5d. Some retrograde injections were repeated with similar results [MM (9), RE/AMd (5), AV (4), RSPv (3), LM (1)] and cross-validated by anterograde injections [SUBdd (9) and SUBdv (4)].

Fig. 6a. Anterograde injections were repeated with similar results (SUBdd (9), ProSUB (2), SUBv (2)) and cross-validated by retrograde injections [RSPv (3), POST (2), PAR (2), ILA (1)].

Fig. 6c. Anterograde injections were repeated with similar results [SUBdd (9), ProSUB (2), SUBv (2), SUBvv (3)] and cross-validated by 21 hypothalamus retrograde injections.

Fig. 7a. Some retrograde injections were repeated with similar results [RSPd (1), RSPv (3), ACAd (1), ACAv (1), SUBdd (8), ProSUB (3)]. Anterograde injections were repeated with similar results [RSPd (1), RSPv (1), ACAd (1), ACAv (1), SUBdd (9), ProSUB (2), SUBv (2)].

Fig. 7b. Some retrograde injections were repeated with similar results [CA3v (2), PL (1), ILA (1), ProSUB (3), CA1i (5), CA1v (4)].

Fig. 7c. Retrograde injections were repeated with similar results [MM(9) and are cross-validated by anterograde injections (SUBdd (9), SUBdv (4), ProSUB (2), SUBv (2), SUBvv(3)].

Fig. 8a. These experiments were not repeated but are cross-validated by anterograde injections [CA1v (2), CA1vv (1), SUBv (2), and SUBvv (3)].

Supplementary Fig. 6c. DGd anterograde injections were repeated with similar results [DGd(5)] and cross-validated by CA3 retrograde injections [CA3dd(1), CA3d (2), and CA3id (1)].

Supplementary Fig. 6b,d,e,f were not repeated.

Supplementary Fig. 7a. Anterograde injections were repeated with similar results [CAdd (2) and CA3d (2)] and cross-validated by CA1 retrograde injections [CA1dr(4), CA1dc (4), and CA1i (5)].

Supplementary Fig. 7b. Anterograde injections were repeated with similar results [CA3dd (2) and CA3d (2)] and cross-validated by CA1 retrograde injections [CA1dr(4), CA1dc (4), CA1i (5)].

Supplementary Fig. 7c. Retrograde injections were repeated with similar results [SUBdd (8) and ProSUB (3)] and cross-validated by CA1 anterograde injections [CA1dr(1), CA1dc (2), and CA2 (3)].

Supplementary Fig. 7d. Anterograde injections were repeated with similar results [CA1dr (1) and CA1dc (2)] and cross-validated by SUB retrograde injections [SUBdd (8) and ProSUB (3)].

Supplementary Fig. 8. Anterograde injections were repeated with similar results [CA3dd (2), CA3d (2), CA3id(2), CA3ic (2), CA3v(1), and CA3vv(1)] and cross-validated by CA1 retrograde injections [CA1dr(4), CA1dc (4), CA1i (5), CA1v(4), CA1vv(3), SUBv(2), and SUBvv(1)].

Supplementary Fig. 9a. Anterograde injections were not repeated but are cross-validated by SUB retrograde injections [SUBv (2) and SUBvv (1)]. Retrograde injections were repeated with similar results [CEA/MEA (3), LA (2), and mBLAa (2)] and are cross-validated by SUB anterograde injections [SUBv (2) and SUBvv (3)].

Supplementary Fig. 9b. Anterograde injections were repeated with similar results [SUBv (2) and SUBvv (3)] and are cross-validated by 6 amygdala retrograde injections.

Supplementary Fig. 9c. Anterograde injections were repeated with similar results [CA1v (2), ProSUB (2), SUBv (2) and SUBvv (3)].

Supplementary Fig. 9d. Anterograde injections were repeated with similar results [CA1v (2), ProSUB (2), SUBv (2) and SUBvv (3)].

Construction and Analysis of Intrahippocampal Connectivity Matrix

Data collection was not randomized and analysis were not performed blind to the conditions of the experiments, as injection site location is apparent in tissue sections when annotating connectivity data. For intrahippocampal connectivity, the presence or absence of anterograde or retrograde labeling within all hippocampal regions was manually annotated with both directionality and labeling density weight (**Supplementary Table 6**; weighting scale=0-3; 0=none to sparse labeling, 1=minor labeling, 2=moderate labeling, 3=robust labeling). The annotated data was used to construct a directed, weighted connectivity matrix using the gene expression-defined and additional anatomically-defined hippocampus regions as the major network nodes (**Fig. 4b**). The connectivity matrix can be read in two ways. For each node listed on the vertical axis, the output connections are listed across the row. Alternatively, the inputs to each node can be interpreted by reading down the columns on the horizontal axis.

Separately, all hippocampal region inputs and outputs were manually annotated from exemplar injection cases and listed in **Supplementary Table 5** (non-weighted). Data from **Supplementary Table 5** was used for extrahippocampal analysis and wiring diagram construction (**Fig. 4c-d**).

Hippocampal Network Analysis and Visualization

We applied a multi-scale strategy [54] to explore the network organization of the intrahippocampal anatomical data. First, we executed a parallel Louvain modularity maximization analysis using the Brain Connectivity Toolbox for Python (bctpy; https://github.com/aestrivex/bctpy, https://sites.google.com/site/bctnet/) on USC Stevens Neuroimaging and Informatics Institute computational resources[55]. The algorithm was executed from gamma values of 0.01 to 20.00, at increments of 0.01. To account for variability in the Louvain results, the algorithm maximized modularity over 1000 runs per iteration (2,000,000 total runs across all gamma values). We calculated a Mean Partition Similarity (MPS) metric to identify the most relevant of the 2000 scales[56]. Finally, we computed a consensus partition from the maximization runs calculated at each scale[57].

Noteworthy high MPS value peaks were recorded at gamma 0.15, 1.36, and 2.04. Gamma 0.15 is the lowest gamma value featuring an MPS peak (4.23). The corresponding consensus partition exhibits a distinct bifurcation between ‘dorsal hippocampus’ and ‘ventral hippocampus’ regions. Gamma 2.04 exhibits the highest MPS value (6.57) of all scales, whereas gamma 1.36 contained the second highest MPS value (6.44). For a multi-scale representation of the intrahippocampal data, we therefore assigned each community in the consensus partition of gamma 2.04 as a subset of the larger communities ("subnetwork modules") defined by the consensus partition at 1.36 and 0.15 (connectivity matrix in Fig. 4b). Additionally, we constructed a representative network connectivity graph using pydot (https://github.com/erocarrera/pydot) and matplotlib Python libraries[58]. The network graph contains three levels of decreasing edge weights corresponding to intra-community, inter-community, and inter-module connectivity with the densest weights referring to intra-community connections at gammas 0.15, 1.36, and 2.04. All related code and data are available at https://git.ini.usc.edu/ibowman/HGEA. In addition, we have created several informatics and

visualization tools to view the data that are openly available on our Mouse Connectome Project website (www.MouseConnectome.org/Analytics/page/matrix).

For extrahippocampal analysis, we employed a similar multiscale approach using the non-weighted annotated connectivity data in **Supplementary Table 5**. For this dataset, we found the highest MPS peak at gamma 9.74 with a corresponding consensus partition featuring 46 different communities:

[['AAA'], ['ACAv', 'AONd', 'COApm', 'SUBvv', 'TMd', 'TMv'], ['ACB', 'AONl', 'ARH', 'BMAp', 'BSTtr', 'CA2'], ['AD'], ['ADP', 'CLA', 'CM'], ['AHN', 'BMAa', 'BSTdm'], ['AMd', 'IAD', 'PH', 'SUBdv'], ['AMv', 'BSTif', 'IMD', 'LPO', 'MEPO', 'ProSUB'], ['AONe', 'BLAa', 'BSTmg', 'CA1v', 'PL'], ['AONm', 'BSTal'], ['AONpv', 'BSTam', 'CA1dc', 'ORBm', 'SUM'], ['AV'], ['AVP', 'BSTv', 'DGd', 'ENTl', 'ENTm'], ['AVPV'], ['BLAp', 'BLAv', 'EPd'], ['BSTpr', 'CSl', 'CSm'], ['CA1dr', 'LA', 'POST', 'PRE'], ['CA1i', 'DP', 'SUBv'], ['CA1vv', 'LSv', 'MEApv', 'MM'], ['CA3d', 'CA3dd', 'CA3id', 'LSc'], ['CA3ic', 'caudal BLAa'], ['CA3v', 'CA3vv'], ['CEAc', 'TTd', 'TTv', 'TU', 'VLPO'], ['COAa', 'PAA', 'PIR', 'TR'], ['COApl', 'CP', 'ECT', 'PA'], ['DGi'], ['DGpod'], ['DGpov'], ['DGv'], ['DMH'], ['Epv', 'PERI'], ['FS', 'MPN', 'MPO', 'OT'], ['IAM', 'NLOT', 'ORBvl'], ['ILA', 'LHA'], ['LC'], ['LD'], ['LSr', 'PS'], ['MEAad', 'MEAav', 'MEApd', 'PAR', 'RCH', 'RE'], ['MS/NDB'], ['PMv', 'PT', 'PVT', 'PVp'], ['PVpo'], ['RSPv'], ['SBPV'], ['SI'], ['SUBdd'], ['VMH']]","[['AAA', 'BSTpr', 'CLA', 'EPd', 'FS', 'LA', 'NLOT', 'ORBvl', 'PAA', 'SI', 'TTv', 'VLPO'], ['ACAv', 'AONe', 'BLAa', 'CA1dr', 'CA1v', 'DGv', 'PL', 'POST', 'PRE', 'TMv'], ['ACB', 'ARH', 'BSTv', 'CA3ic', 'TU', 'caudal BLAa'], ['AD', 'AV', 'AVPV', 'BMAp', 'CA2', 'ENTl', 'LD', 'RSPv', 'SUBdd'], ['ADP', 'DGi', 'DGpod', 'DGpov', 'LC', 'MS/NDB', 'PMv', 'PS', 'PVp', 'PVpo', 'SBPV', 'SUBvv', 'TMd', 'VMH'], ['AHN', 'CA3id', 'COApl', 'DGd', 'DMH', 'PH'], ['AMd', 'COAa', 'IAD', 'PERI', 'PIR', 'PT', 'RE', 'SUBdv'], ['AMv', 'AONl', 'BMAa', 'CA3d', 'ENTm', 'IMD', 'LPO', 'MEPO', 'PAR', 'ProSUB'], ['AONd', 'BSTdm'], ['AONm', 'SUBv'], ['AONpv', 'CA1dc', 'ORBm', 'SUM'], ['AVP', 'BSTtr', 'CA1i', 'DP'], ['BLAp'], ['BLAv', 'CA3dd', 'LSc'], ['BSTal', 'CEAc', 'LSr', 'MEApd'], ['BSTam', 'ECT', 'PA', 'PVT'], ['BSTif', 'TTd'], ['BSTmg', 'IAM'], ['CA1vv', 'LSv',

'MEApv'], ['CA3v', 'CA3vv', 'MEAad', 'MEAav', 'RCH'], ['CM', 'TR'], ['COApm'], ['CP'], ['CSl', 'CSm', 'MM'], ['Epv', 'MPN'], ['ILA', 'LHA'], ['MPO'], ['OT']]"

We manually arranged each of these 46 different communities into five subnetworks based on the similarity of their extrinsic connectivity with the intrahippocampal communities found in the previous analysis. The resulting community structure was graphed using pydot (https://github.com/erocarrera/pydot) and matplotlib Python libraries [58] with three levels of decreasing edge weights corresponding to intra-community, inter-community, and inter-module connectivity (employing a similar process as the intrahippocampal analysis). All related code and data are available at https://git.ini.usc.edu/ibowman/HGEA.

Reporting Summary

Additional information is available in the "Life Sciences Reporting Summary" linked to this article.

Data Availability

All images of mouse *in situ* hybridization gene expression data are available at the Allen Brain Atlas website (www.mouse.brain-map.org). Gene accession codes for all genes and the *in situ* hybridization probe sequences can be found by querying the gene name through the Allen Brain Atlas website (www.mouse.brain-map.org). All anatomical tracer image data is available through our iConnectome viewer as part of the Mouse Connectome Project at USC (www.MouseConnectome.org). The HGEA stereotaxic coordinate atlas, 3-D atlas viewer, and annotation data are available for download at www.MouseConnectome.org/MCP/papers. Additional informatics and visualization tools are available online at www.MouseConnectome.org/Analytics/page/matrix.

Code Availability

All related code and data are available at https://git.ini.usc.edu/ibowman/HGEA and are included in the Supplementary Software file.

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