1 **Title**

2 Telencephalic outputs from the medial entorhinal cortex are copied directly to the
3 hippocampus

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28 **Abstract**

29 Complementary actions of the neocortex and the hippocampus enable encoding and long-
30 term storage of experience dependent memories. Standard models for memory storage
31 assume that sensory signals reach the hippocampus from superficial layers of the entorhinal
32 cortex (EC). Deep layers of the EC on the other hand relay hippocampal outputs to the
33 telencephalic structures including many parts of the neocortex. Here we show that cells in
34 Layer 5a of the medial EC send a copy of their telencephalic outputs back to the CA1 region
35 of the hippocampus. Combining cell-type specific anatomical tracing with high-throughput
36 RNA-sequencing based projection mapping and optogenetics aided circuit mapping, we
37 show that in the mouse brain these projections have a unique topography and target
38 hippocampal pyramidal cells and interneurons. Our results suggest that projections of deep
39 medial EC neurons are anatomically configured to influence the hippocampus and neocortex
40 simultaneously and therefore lead to novel hypotheses on the functional role of the deep EC.

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52 **Introduction**

53 Interplay between the hippocampus and the neocortex is a central tenet of theories for
54 systems memory consolidation (Marr, 1971; McClelland et al., 1995). The entorhinal cortex
55 (EC) mediates the two-way communication between the neocortex and the hippocampus. Its
56 superficial and deep layers respectively channel inputs to and outputs from the
57 hippocampus. During a new experience the superficial layers relay convergent inputs from
58 the cortex to the hippocampus where initial conjunctive representations of everyday
59 experiences are generated. Subsequently during rest or sleep, hippocampal output is
60 redistributed to the brain (Squire et al., 2015; Frankland and Bontempi 2005). Leading
61 theoretical models consider the deep layers of EC as a relay of hippocampal output that
62 reinstates neocortical activation patterns representing memories, enabling neocortex-wide
63 synaptic changes required for long-term storage (Koster et al., 2018; Kumaran et al., 2016;
64 McClelland et al., 1995; Schapiro et al., 2017).

65

66 Recent discoveries revealed complex and distinct input-output interactions of molecularly
67 defined subtypes of neurons in the deep layers of medial EC (MEC), suggesting their
68 functions extend beyond passing on hippocampal outputs. Layers 5a (L5a) and 5b (L5b) of
69 the EC are genetically distinct with differential input-output organisations. L5a but not L5b is
70 the sole origin of the long-range telencephalic outputs of the EC (Sürmeli et al., 2015),
71 whereas L5b, but not L5a, is directly influenced by superficial layers of EC (Beed et al.,
72 2020; Sürmeli et al., 2015). Inputs from the dorsal hippocampus preferentially target L5b
73 over L5a (Rozov et al., 2020; Sürmeli et al., 2015; Wozny et al., 2018) whereas ventral
74 hippocampus preferentially target L5a (Rozov et al. 2020). Moreover, the capacity of deep
75 layers to integrate neocortical inputs with hippocampal output (Czajkowski et al. 2013; Beed
76 et al. 2020; Sürmeli et al. 2015), suggest more complex roles.

77

78 An intriguing possibility, noted in experiments with classic retrograde tracers, is that deep
79 layers of MEC might also project to the hippocampal CA1 region, which provides the deep
80 EC with hippocampal output (Köhler, 1985; Witter and Amaral, 1991). Such recurrent
81 connectivity between CA1 and the deep MEC could be important in coordinating
82 hippocampal-entorhinal activity during memory consolidation (Ólafsdóttir et al. 2016),
83 provide a shortcut for hippocampal outputs to re-enter the hippocampus (Kumaran and
84 McClelland 2012) or have a more global coordination function encompassing the
85 hippocampus and the neocortex (Khodagholy et al., 2017). Despite its potential functional
86 significance, the key properties of this projection pathway including whether it shares the
87 same source with the telencephalon projections and which neurons it targets in the
88 hippocampus remain unknown.

89

90 Here we reveal that, neurons in L5a, but not L5b, of the MEC project to hippocampal CA1.
91 We establish that the telencephalon-projecting neurons copy their outputs directly to
92 pyramidal and subclasses of interneuron populations in CA1. Our data define a novel
93 anatomical framework for Layer 5a of the MEC to coordinate neocortical and hippocampal
94 networks.

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96

97 **Results**

98 To find out whether neurons in either L5a or L5b project to the hippocampus we injected
99 retrograde adeno-associated viral vectors (AAVs) expressing green fluorescent protein
100 (GFP) or mCherry into intermediate hippocampus (Figure 1A,B; Figure 1- figure supplement
101 1A,B). As well as finding retrogradely-labelled cell bodies in layer 3 of MEC, which contains
102 neurons that are well-established as a critical input to stratum lacunosum/moleculare of CA1
103 (Naber et al., 2001; Kitamura et al., 2014), we also found labelled cell bodies in L5a, which

104 we identified by immunostaining against the ETS variant transcription factor-1 (Etv1) protein.
105 Retrogradely-labelled neurons were not found in L5b.

106 To further investigate this projection, we identified and validated a transgenic mouse line that
107 enables specific genetic access to L5a. In this mouse line, Cre expression is driven by the
108 promoter of the retinol binding protein 4 gene (*Rbp4-Cre*; Gerfen et al., 2013). Following
109 injection of a Cre-dependent AAV expressing GFP or mCherry into the deep MEC of *Rbp4-*
110 *Cre* mice, fluorescent signal was detected mainly in L5a (Figure 1C,D and Figure 1- figure
111 supplement 1C). At the centre of the injection site, where labelling was densest, the majority
112 of neurons in L5a (71.3 ± 2.2 %, $n = 3$ mice, 180 neurons) were found to be fluorescently
113 labelled (Figure 1D). Labelled neurons were non-GABAergic (99.5 ± 0.3 %, $n = 3$ mice, 1137
114 cells; Figure1- figure supplement 1D), most of which expressed Etv1 (88.6 ± 3.1 %, $n = 3$
115 mice, 1866 cells; Figure 1D). This is similar to the proportion of Etv1-expression in all L5a
116 neurons (Sürmeli et al., 2015) and also in retrogradely labelled subpopulations of L5a
117 neurons from the hippocampus and from cortical and subcortical target areas (Figure 1-
118 figure supplement 1E,F,G,H). Moreover, axons of L5a neurons labelled in the *Rbp4:Cre* line
119 were found in the telencephalon (Figure1-figure supplement 2). Thus, we established that
120 the *Rbp4-Cre* line provides unbiased genetic access to excitatory projection neurons in L5a.
121

122 To what extent does the projection from L5a to the hippocampus differ from previously-
123 identified projections arising from superficial layers of MEC? In contrast to projections from
124 superficial layers 2 and 3, which respectively target stratum lacunosum (SL) and stratum
125 moleculare (SM) of CA1 (Kitamura et al., 2014), axons from L5a neurons were primarily
126 found in deep stratum pyramidale (SP) and stratum lacunosum (SL), but were sparse in
127 stratum moleculare (SM; Figure 2A,B,C,D and Figure 2- figure supplement 1A). Axons from
128 L5a also differed in their proximo-distal organisation within CA1. Whereas projections from
129 superficial layers of MEC favour proximal CA1 (Tamamaki and Nojyo, 1995), axons from L5a

130 had highest density in distal CA1 (paired one-tailed Wilcoxon signed rank test, $p = 0.0312$, n
131 $= 5$ mice; Figure 2E,F,G,H and Figure 2- figure supplement 1B,C,D,E,F) and the subiculum
132 (Figure 2I and Figure 2- figure supplement 1G). We observed the same topography when
133 axons and axon terminals of L5a neurons were labelled with membrane-bound GFP and
134 fluorescently-tagged presynaptic proteins (synaptophysin), confirming that the topography is
135 not a result of labelling of passing axons (Figure 2J,K,L,M,N,O). This distinct topography
136 suggests a unique functional role for the projections from L5a of MEC to CA1.

137

138 Our findings suggest two intriguing possibilities for the organisation of output neurons in L5a
139 of the MEC. The hippocampus-projecting neurons in L5a may be distinct from the
140 telencephalon-projecting neurons, implying separate processing within the deep MEC layers
141 of signals to the hippocampus and telencephalon. Alternatively, the same neurons in L5a
142 may project to both the telencephalon and the hippocampus, implying that telencephalic
143 outputs from MEC are copied back to the hippocampus. To distinguish between these
144 possibilities, we used a combinatorial viral labelling strategy. We restricted reporter gene
145 expression to subpopulations of L5a cells that project to specific targets by injecting a
146 retrograde AAV expressing Cre recombinase in either the retrosplenial cortex (RSC) or
147 nucleus accumbens (NucAcb), and a Cre-dependent fluorescent reporter virus into the MEC
148 (Figure 3A,B and Figure 3- figure supplement 1). With this approach we detected in CA1
149 fluorescently-labelled axons originating from both RSC-projecting and NucAcb-projecting
150 L5a neurons (Figure 3C,D). The axon distribution across layers was similar to when L5a
151 neurons were labelled in bulk using the *Rbp4-Cre* mouse line (Figure 3E,F). These results
152 establish the principle that axon projections from L5a of MEC to the hippocampus are
153 collaterals of projections to telencephalic targets. However, it is unclear from these
154 experiments whether this principle applies to projections from L5a to all of its many
155 telencephalic targets.

156

157 Entorhinal cortex outputs have diverse targets, including the entire cortical mantle as well as
158 parts of basal ganglia and amygdala (Swanson and Köhler, 1986, Sürmeli et al., 2015). To
159 test whether axon collateralisation to the hippocampus is a general feature of all
160 telencephalon-targeting neurons, we used Multiplex Analysis of Projections by Sequencing
161 (MAPseq; Kébschull et al., 2016). MAPseq is a high-throughput alternative to single neuron
162 anterograde tracing. In MAPseq, instead of multiple copies of a virus carrying the same
163 genetic material, a virus library is utilised where each virus facilitates expression of a unique
164 30-nucleotide RNA particle. The vast diversity of unique RNA sequences and relatively small
165 number of neurons infected allows each neuron to be marked with a unique RNA barcode
166 (Kébschull et al., 2016). Because the barcodes are actively transported to axon terminals,
167 their detection can be used to establish connectivity to putative target structures; if a neuron
168 projects to multiple target structures then its barcode should be detected in each one.

169

170 We injected the MAPseq barcode RNA virus library into the full dorso-ventral extent of the
171 deep MEC (Figure 4- figure supplement 1A) and quantified barcode RNA expression in
172 tissue collected from dorsal and ventral MEC, dorsal and ventral hippocampus, isocortex,
173 cerebral nuclei (CNU), olfactory areas and cortical subplate (Olf/Ctxsp) as well as brainstem
174 and spinal cord as negative controls (see methods for detailed description and controls,
175 Figure 4A). The majority of barcodes that were detected in any one of the three target
176 divisions (isocortex, CNU or Olf/Ctxsp) were also detected in the hippocampus, suggesting
177 that collateral axons to the hippocampus are a common feature of telencephalon-projecting
178 neurons (Figure 4B). The results were similar regardless of whether neurons were located in
179 dorsal or ventral MEC (Figure 4- figure supplement 1B), suggesting that collateralisation
180 does not depend on the neuron's position in the dorso-ventral axis of the MEC. Using
181 barcode counts as a measure of projection strength (Kébschull et al., 2016) we further found
182 that dorsal MEC neurons preferentially project to dorsal aspects of the hippocampus and
183 ventral MEC neurons to the ventral aspects, indicating that the back-projections to the
184 hippocampus are topographically organised (Figure 4C). Together these results reveal

185 general organising principles by which all projections from MEC to the telencephalon are
186 copied back to the hippocampus.

187

188 Which cell types within the hippocampus receive signals from L5a of MEC? While the
189 compartmentalised arrangement of axons from L5a of MEC suggests selective targeting of
190 CA1 layers, axonal topography does not necessarily reflect functional connectivity.

191 Therefore, we targeted viral vectors expressing a channelrhodopsin2-mCherry conjugate
192 to L5a neurons in MEC and tested for connectivity using whole-cell ex-vivo patch clamp
193 recordings from CA1 neurons.

194

195 We focused initially on responses of pyramidal cells. Brief light pulses evoked depolarising
196 sub-threshold postsynaptic potentials (PSPs) in 64% of SP pyramidal neurons recorded at
197 their resting membrane potential (n = 64 cells, 27 mice, Figure 5A,B,C and Figure 5- figure
198 supplement 1E). The response probability of neurons in distal CA1 was not significantly
199 different than neurons in proximal CA1 (Figure 5D). Although the vast majority of axons
200 occupy the deep portion of the SP we found no significant difference in the response
201 probability of neurons in deep versus superficial SP (Fig. 5d). Thus, rather than selecting
202 subtypes of pyramidal neurons axons might target distinct compartments of neurons
203 according to their depth in SP.

204

205 The majority of PSPs (20 out of 26) maintained their polarity when the membrane potential
206 was adjusted from rest ($V_m = -66.7 \pm 0.6$ mV) to -50 mV, indicating that they were
207 glutamatergic. In these neurons EPSPs were maintained when GABA_A receptors were
208 blocked with Gabazine, but were abolished by the AMPA receptor antagonist NBQX,
209 confirming that they are glutamatergic (Figure 5E). The responses had short latencies (3.04
210 ± 0.26 ms, n = 20 cells, 15 mice) that were relatively invariant from trial to trial (Figure 5F)
211 and were independent of the response's position within a train of stimuli, indicating that they

212 are monosynaptic (Figure 5- figure supplement 1D). Consistent with this, responses were
213 sensitive to bath application of tetrodotoxin (TTX) but recoverable after application of 4-
214 Aminopyridine (4-AP; Figure 5G; Petreanu et al., 2007). Together, these data demonstrate
215 that projections from L5a of the MEC provide glutamatergic excitatory inputs directly to
216 pyramidal neurons in proximal and distal CA1.

217

218 A smaller population of pyramidal neurons showed PSPs with characteristics of indirect
219 inhibitory connections (6 out of 26). These responses either reversed polarity when the cell's
220 membrane potential was held above the chloride reversal potential (Figure 5H) or had an
221 early depolarising and a late hyperpolarising component (Figure 5- figure supplement 1B).
222 Application of Gabazine either completely blocked these PSPs (Figure 5I) or revealed a
223 larger excitatory component that was sensitive to application of NBQX (Figure 5- figure
224 supplement 1B). Thus, inputs from L5a of the MEC also recruit local interneurons that
225 provide inhibitory input to SP pyramidal neurons.

226

227 To identify which interneurons in CA1 were targets of L5a projections, we tested responses
228 of interneurons in all layers. In SP we distinguished interneurons from principal cells either
229 by classifying them based on biophysical properties (fast-spiking: SP_{FS} and non-fast spiking:
230 SP_{NFS}; Figure 5- Figure supplement 1a and see methods), or by recording from
231 fluorescently-labelled GABAergic or parvalbumin-expressing (PV+) inhibitory neurons in
232 double transgenic mice (Figure 6A,B and Figure 5- figure supplement 1C). Stimulation of
233 L5a axons elicited both sub-threshold and supra-threshold responses primarily in SP, SR
234 and SL interneurons (Figure 6C,D and Figure 5- figure supplement 1E). Depolarising PSPs
235 were observed in 64% of the SP_{FS} including PV+ interneurons, 46% of the SP_{NFS}, 64% of SR
236 and lower proportions of recorded neurons in SL (31%), SM (23%) and SO (16%). Except for
237 interneurons in SO, PSPs in all layers showed characteristics of monosynaptic connectivity
238 (Figure 6G,H,I,J and Figure 5- figure supplement 1G,H) and AMPA receptor-mediated

239 glutamatergic synaptic transmission (Figure 6E,F and Figure 5- figure supplement 1F).
240 Therefore, projections from L5a of MEC recruit CA1 interneurons through monosynaptic
241 inputs driven by excitatory glutamatergic synapses.

242

243 **Discussion**

244 Together our results suggest a substantial revision to the idea that the entorhinal deep layers
245 unidirectionally convey hippocampal output messages to the neocortex. Using two lines of
246 evidence, combinatorial viral labelling and MAPseq, we show that L5a axons bifurcate to
247 target both the telencephalon and the hippocampus. Our results imply that nearly all
248 telencephalic output is copied onto the hippocampus regardless of its destination in the
249 telencephalon. There is a topographical register between the dorso-ventral origin of the
250 entorhinal cells and their target zone within the hippocampus. This is similar to the dorso-
251 ventral topography of perforant-path projections of the superficial EC targeting the dentate
252 gyrus, supporting the view that dorso-ventral compartments of the hippocampus operate
253 independently (Ruth et al., 1982; Dolorfo and Amaral 1998). Interestingly in the transverse
254 axis, the density of projections has a gradient descending towards proximal CA1 (towards
255 CA2). This is in contrast to previous reports where labelling of MEC projections using
256 traditional tracers revealed dense projections in proximal but not in distal CA1 (Tamamaki
257 and Nojyo, 1995; Naber et al., 2001). Instead, distal CA1 is thought to be primarily targeted
258 by the lateral EC. The distinct organization of projections originating in L5a of the MEC that
259 we reveal here suggests that they serve functions distinct from the projections targeting CA1
260 from superficial layers instead of merely supplementing them (Witter et al., 2017). This also
261 opens a possibility for the lateral and medial entorhinal cortex input streams to converge in
262 CA1.

263

264 Although cells in deep entorhinal layers have been previously observed when retrograde
265 dyes were placed in the CA1-subiculum border (Köhler, 1985; Witter and Amaral, 1991) the

tools we introduced in this study made it possible to investigate the layer origin of these projections and their targets within CA1 with cell type specific anterograde labeling. Our observation that L5b does not participate in this pathway reinforces the distinct input-output organisation of the deep MEC. While L5b is a point of integration for local and neocortical input with hippocampal output and distributes its signals locally within MEC, based on our anatomical observations L5a appears to be suited to coordinate the wider hippocampal-neocortical loop by providing a copy of entorhinal output back to CA1.

Ideas and Speculation

Our findings raise new questions about the functional roles of outputs from the entorhinal cortex, and about the nature of interactions between the entorhinal cortex and the hippocampus. Telencephalic projections of L5a cells have been shown to be necessary for long-term memory formation and retrieval (Kitamura et al., 2017), likely by distributing compressed hippocampal outputs to the neocortex (McClelland et al., 1995). Returning a copy of these outputs back to CA1 might create a self-reinforcing or a self-correcting loop. They might also provide a gating signal to modulate the impact in CA1 of inputs from CA3 (Dudman et al., 2007) and/or neocortical return inputs via L3 of EC. It is also conceivable that they influence mechanisms of learning and memory by affecting rhythmic network activity through the inputs on the pyramidal cells and interneuron populations that we described here (Colgin 2016; Ognjanovski et al. 2017; Xia et al. 2017). While our study brings new anatomical insight, more experiments are required to reveal the function of this pathway and the role it plays in spatial cognition, learning and memory.

Methods

Mice

292 All animal experiments were approved by the University of Edinburgh animal welfare
293 committee and were performed under a UK Home Office project license. The *Rbp4-Cre*
294 mouse line was generated by GenSat and obtained from MMRRC (*Tg(Rbp4-*
295 *cre)KL100Gsat/Mmucd*). The *Vgat-Venus* mouse line was generated by Dr. Atsushi
296 Miyawaki at RIKEN, Wako, Japan (Wang et al., 2009). The *Pvalb-Flp* mouse line was
297 obtained from Jackson Laboratories (*B6.Cg-Pvalb-tm4.1(FLPo)Hze/J; Jax 022730*). Wild-type
298 mice were obtained from Charles River Laboratories C57Bl6J stock. Double transgenics
299 were generated by crossing the *Rbp4-Cre* line to the *Vgat-Venus* or the *Pvalb-Flp* lines.
300 *Rbp4-Cre* and *Pvalb-Flp* lines were maintained as heterozygous and all mice were on
301 C57Bl/6 background.

302 Viral constructs and injection strategy

303 8 to 14 week-old male and female mice were used in all experiments. For targeting deep
304 MEC a craniotomy was made 3.4 to 3.65 mm lateral to the bregma (X) between the
305 transverse sinus and lambdoid suture (Y = 4.5 to 5 mm caudal to bregma). The injection
306 pipette was at a 9 degree angle towards the caudal end of the brain. 100 nl of virus was
307 slowly released at four Z-depths 3.0, 2.8, 2.6 and 2.4 mm from the surface of the brain. 3
308 mins after delivering the virus the injection needle was retracted to the next injection depth
309 until the most dorsal location where 10 mins past before the needle was fully retracted. For
310 anterograde tracing of L5a axons and terminals *AAV-EF1a-DIO-mCherry* (serotypes 2 and
311 5, titer 5.3×10^{12} , lot #AV4375H, K., Deisseroth, UNC Vector Core), *AAV-CAG-FLEX-GFP*
312 (titer 3.7×10^{12} , lot #AV4530B, E. Boyden, UNC Vector Core) and *AAV-hSyn-FLEX-mGFP-*
313 *2A-Synaptophysin-mRuby* (Addgene:71760) were used.

314 For injections in CA1 a craniotomy was made 3.5 mm lateral and 3.30 mm caudal to the
315 bregma in C57BL6J mice. 100nl virus was delivered at 3.0, 2.8, 2.6, 2.4 and 2.2 mm from
316 the surface. For retrograde labelling of hippocampus-projecting EC neurons, 100nl of a

317 *rAAV2retro-CAG-GFP* (titre 3.5×10^{12} , lot #AV7493D, E. Boyden, UNC Vector Core) or
318 *rAAV2retro-hSyn-mCherry* (E. Boyden, UNC Vector Core) was injected at each depth.

319 To assess whether telencephalic projection neurons in L5a of MEC co-express Etv1 (Figure
320 1- figure supplement 1D), fluorophore coupled cholera toxin beta subunit (CTB-Alexa 488
321 and CTB-Alexa 555) was injected at various sites in wild-type mice as previously described
322 (Sürmeli et al., 2015) and coexpression of Etv1 and Alexa dyes were examined in confocal
323 images.

324 To achieve Cre expression in target specific subpopulation of L5a neurons in the MEC
325 (Figure 3) a *rAAVretro-EF1a-Cre-WPRE* (viral construct was a gift from Karl Deisseroth, titre
326 2.4×10^{12}) was made using an AAV-retro helper plasmid (Addgene plasmid ID 81070) as
327 described previously (McClure et al., 2011). A cocktail of *rAAVretro-EF1a-Cre-WPRE* and
328 *AAV-CAG-GFP* (titer 4.3×10^{12} , lot #AV6951B, Boyden, UNC Vector Core) viruses were
329 injected either in NucAcb (X: +1.0 mm; Y: +1.2 mm; Z: -3.8, -4.0 mm) or RSC (X: +0.4 mm;
330 Y: -2.8, -3.2 mm; Z: -0.9 mm) in wild-type mice. A Cre inducible reporter virus was also
331 injected in the MEC as described above. GFP expression driven by the CAG promoter at the
332 target site was used for verification of injection location.

333 To achieve expression of channelrhodopsin-2 in L5a axons, we injected *AAV2-EF1a-DIO-*
334 *hChR2(H134R)-mCherry-WPRE-pA* (titer 5.1×10^{12} , lot #AV4319J, Deisseroth) in the MEC
335 of the *Rbp4-Cre* or the *Rbp4-Cre X Vgat-Venus* or the *Rbp4-Cre X Pvalb-FLP* double
336 transgenic mice. Additionally, in order to fluorescently label parvalbumin expressing
337 interneurons in CA1 a Flp recombinase-dependent AAV vector was constructed in house
338 and injected in the hippocampus (X: +3.0 mm; Y: -3.5 mm; Z: -2.0, -3.0 mm, 200 nl virus was
339 injected at each Z-depth). To produce the virus, the viral construct pAM-FLEX-GFP (Murray
340 et al., 2011) (a gift from Peer Wulff) was used as the viral backbone. The eGFP, loxP and
341 lox2272 sites were removed and replaced with a synthesised cassette (GenScript)
342 containing a multiple cloning site flanked by two sets of heterotypic (FRT and F3),

343 antiparallel FRT sites to produce pAM FLPX. For pAM FLPX eGFP and pAM FLPX mCherry,
344 the fluorescent proteins were amplified from pAM FLEX eGFP and pmCherry-C1 (Clontech)
345 respectively, with EcoRI and SacI overhangs and cloned into pAM FLPX. All AAV preps
346 were generated in house as previously described (McClure et al., 2011) and were titered by
347 qPCR following expression in HEK cells ($> 10^{12}$ genome copies (GC)/ml).

348 Tissue processing and immunohistochemistry

349 3 to 4 weeks after the virus injection surgery, mice were transcardially perfused with 4%
350 paraformaldehyde as previously described (Sürmeli et al., 2015). Brains were sectioned at 60
351 μ m using a cryostat (Leica CM3050 S) either coronally or horizontally and sections were
352 stored in PBS at 4°C. All subsequent incubation and washing steps were carried out at 4°C.
353 Prior to antibody staining, sections were blocked with either 5% normal goat serum (NGS;
354 Abcam: ab13970) or 2% bovine serum albumin (BSA; Sigma: A9418) in 0.3% PBST (PBS
355 with 0.3% Triton-X100) for 2hrs. Sections were then transferred to primary antibody solutions
356 made with either 5% NGS or 2% BSA in 0.3% PBST and incubated overnight. After 3
357 washes, each 20 min, in 0.3% PBST, sections were transferred to secondary antibody
358 solutions made with 0.3% PBST and, if required, NeuroTrace 640/660 (1:800; Life
359 Technologies: N21483) and incubated overnight. After 3 washes, sections were incubated in
360 DAPI (1:2,000; Sigma Aldrich: D9542) solution made in PBS for 20 min at room temperature,
361 where required, and then mounted on microscope slides using Mowiol® 4-88 (Aldrich:
362 81381). Slides were covered with glass coverslips and left to dry overnight at 4°C in the dark.
363 The following primary antibodies were used: rat anti-mCherry (1:2,000; ThermoFisher:
364 M11217), chicken anti-GFP (1:10,000; Abcam: ab13970), rabbit anti-Etv1 (Arber et al., 2000)
365 (1:1,000), chicken anti-NeuN (1:1,000; Sigma Aldrich: ABN91). The following secondary
366 antibodies from Thermo Fisher Scientific were used at a concentration of 1:800: goat anti-
367 rabbit A488 (A11034), goat anti-chicken A488 (A11039), goat anti-rat A555 (A21434) and
368 goat anti-rabbit (A21244).

369 Cell counting

370 Every 4th brain section was imaged as a Z-stack (1 μ m step size) using either the Zeiss
371 LSM 800 (Zen, v2.6.76) or Leica SP8 confocal microscope (Leica Application Suite X,
372 v3.5.6.21594) at 20x magnification. Regions of interest were drawn around L5a and cell
373 counting was carried out either manually using Fiji software or using cell-counting tools in
374 Vision4D (Arivis, v3.2.0).

375
376 The boundaries for L5a were determined using either a DAPI or Neurotrace counterstain.
377 The border between medial and lateral divisions of EC was determined in each section using
378 layer 2 as a guide – in lateral EC, layer 2 is separated into two distinct layers, while this
379 separation is not seen in medial EC(Cappaert et al., 2015).

380 Quantification of the distribution of fluorescent signal

381 *Quantification of fluorescence across the radial axis of the CA1*

382 The distribution of axons of L5a neurons in the radial axis of the hippocampal CA1 was
383 quantified in slide-scanner images (Figure 2) or confocal images (Figure 3) using Fiji
384 software. Coronal brain sections between 3.28 to 3.58 mm posterior to Bregma, which
385 contain distal CA1, were selected for analysis. 2 sections from each brain in which the
386 fluorescent labeling was representative of all the sections were used. DAPI was used as a
387 counterstain. In each section, a 400 μ m-wide rectangular ROI was drawn across the radial
388 axes, covering all layers of a randomly selected region in intermediate CA1 (Figure 2B). This
389 ROI was then divided into 20 equal bins and the mean fluorescence intensity was calculated
390 for each bin. The mean intensity values were then normalised in each brain by scaling the
391 values such that the highest value is 1.

392 *Quantification of fluorescence in the transverse axis of the CA1*

393 A Zeiss Axioscan slidescanner was used to image every second brain section at 10x
394 magnification. The projection strengths of MEC L5a neurons to proximal and distal halves of
395 CA1 were quantified in horizontal sections located at depths between 2.56 mm to 4.12 mm
396 from the surface of the brain, with injection sites located across medial, mid and lateral MEC
397 (15-18 brain sections per mouse, n = 5 mice). GFP and mCherry fluorescence signals were
398 amplified with immunostaining as described previously. The borders of CA1 were drawn on
399 brain section images using a customisable digital microscopy analysis platform (Visiopharm),
400 and the proximo-distal border was defined as the border equidistant from the proximal and
401 distal ends of CA1. The mean fluorescence density, defined as the total number of pixels
402 above a set threshold in an area divided by the total number of pixels in the area, was
403 measured in proximal and distal CA1. The threshold was determined manually by ensuring
404 that only pixels representing axons were detected as signal. A median unsharp background
405 correction was used to remove background noise from axons outside of the focus plane of
406 the image. The mean fluorescence density values were then normalised within each brain by
407 scaling the values such that the total fluorescence density value (proximal + distal) in each
408 brain is equal to 1.

409 The spread of viral expression in MEC was assessed from the fluorescent signal in all
410 sections for each brain. Brains in which labelled neurons were found outside L5 were
411 excluded; this was a result of occasional and sparse labelling in L2 or L3 or the
412 parasubiculum in the *Rbp4-Cre* line.

413 MAPseq

414 5 C57Bl6J adult male and female mice were injected in the deep MEC with the MAPseq
415 Sindbis viral barcode library (3×10^{10} GC/ml) provided by the MAPseq facility (Cold Spring
416 Harbor Laboratories). To cover the whole mediolateral and dorsoventral extent of the MEC
417 virus was injected in two locations: 3.4 mm and 3.6 mm lateral to bregma. 100 nl virus was
418 delivered to 3.0, 2.8, 2.6, 2.4 mm below the surface of the brain. After 44 hrs, mice were

419 sacrificed, brains were extracted and immediately immersed in oxygenated cold ACSF
420 prepared in ultrapure water. All surgical tools and surfaces were treated with RNaseZAP
421 (Invitrogen) prior to the start of the experiment and in-between samples. 400 μ m fresh
422 sagittal brain sections were cut using a vibratome (Pastoll et al., 2012). Immediately after
423 this, GFP expression in the deep EC was verified under a fluorescent microscope and tissue
424 was dissected in cold oxygenated ACSF using microdissection blades on sylgard covered
425 petri dishes which were kept on ice. Each slice was dissected on a previously unused
426 surface of the plate with fresh ACSF and a dedicated microblade was used for the dissection
427 of each brain division to prevent contamination. Tissue pieces were collected into bead
428 containing (Qiagen 69989) collection tubes (Qiagen 19560) on dry ice. After tissue
429 collection, 400 μ l Trizol (Thermo Fisher Scientific #15596026) was added to each collection
430 tube. The procedure was repeated for all 5 brains. 3 brains with the largest coverage of MEC
431 deep layers were selected to proceed to the next steps. Tissue was stored at -80 degrees
432 before it was shipped on dry ice to CSHL MAPseq core facility for further processing. RNA
433 extraction, production of double stranded cDNA, library preparation and Illumina sequencing
434 and preprocessing of sequencing data was performed by the MAPseq core facility as
435 described in Kebschull et al., 2016.

436 For the tissue dissections, identification of brain areas was done by using Allen Brain
437 Reference Atlas (<https://mouse.brain-map.org/>). The isocortex division included the
438 somatosensory, motor, visual, posterior parietal, anterior cingulate and retrosplenial areas
439 combined. The cerebral nuclei (CNU) division was restricted to striatum. The
440 olfactory/cortical subplate division (Olf/Ctxsp) was a combination of olfactory areas and
441 cortical subplate including amygdalar nuclei. The remaining two divisions were dorsal (dHip)
442 and ventral hippocampus (vHip) including subiculum. Some brain areas were excluded from
443 the study because of the difficulty in dissecting or identifying brain areas in the sagittal plane.
444 All sections > 3.7 mm lateral to bregma are not annotated in Allen Brain Reference Atlas and
445 were excluded. Therefore, neocortical areas in the most lateral sections such as perirhinal

cortex, ectorhinal cortex, temporal association areas were not included in the study. The claustrum and adjacent neocortical areas (visceral, agranular insular, gustatory) were excluded as it was not possible to separate these areas precisely to prevent contamination between the assigned divisions. Since borders between the brain divisions CNU and OLF/Ctxsp were not always clear, dissections avoided these areas hence the brain areas in these divisions are partially included. White matter between the hippocampus and the neocortex carrying axon tracts were also excluded. Brainstem and cervical spinal cord tissue were used as control. When L5a neurons in the MEC were labelled using strategies explained in Figure 2, no axonal projections to these areas were observed (unpublished experiments). Consistent with this, a low number of barcodes were identified in the negative control samples (on average $1.0\% \pm 0.3\%$ of barcodes had counts in control areas, $n = 3$).

A limitation of the barcode-based single-cell tracing method comes from the possibility of multiple neurons being represented by the same barcode. This is prevented by adjusting the barcode diversity and the size of the target population as explained in detail in (Kebeschull et al., 2016). In order to assess the expected fraction of uniquely labelled neurons in our study we counted the total number of neurons in L5a of the MEC. A total of 2314 ± 131 , ($n = 2$ mice) NeuN-labelled neurons were counted. Using the formula $F = (1 - (1/N))^{k-1}$ where N is the barcode diversity (2×10^6) (Kebeschull et al., 2016) and k is the number of infected neurons the predicted ratio of uniquely labelled neurons is 99.9%. Multiple representation of neurons due to a neuron being infected by several different barcode RNA-expressing viral particles was not corrected for since the projection patterns are not affected by overrepresentation of neurons (Kebeschull et al., 2016).

MAPseq data analysis

Barcode counts were first normalised in each area by the relative number of spike-in RNAs for each sample. Orphan barcodes, barcodes which did not have counts in the injection site, (dMEC or vMEC) were removed. We then calculated the 90th percentile of the barcode

counts in our negative controls and based on this set all barcode counts of 1 to 0. A small number of barcodes had a higher count in any target area compared to the injection site which might be a result of incomplete dissection of the injection site or viral expression in areas that the virus spilled into as we retracted the pipette. These barcodes were removed. Since our goal was to find whether neurons projecting to the telencephalon also project to the hippocampus we removed the barcodes that had no counts in any of the telencephalic target areas. For the same reason, barcodes that were detected only in the hippocampus were also removed. Finally, we excluded all barcodes with counts of less than 400 in the injection site to minimise the possibility of incomplete transport of RNA barcodes to axons in target areas due to weak expression at the cell bodies or low counts due to PCR or polymerase errors. Barcodes with low counts in all target areas (< 10) were also excluded to account for potential false positives.

To quantify the proportion of barcodes that were present in both a target division (Figure 4B; Isocortex, CNU or Olf/Ctxsp) and the hippocampus, the following formula was used: (total number of barcodes with counts detected in both hippocampus and target division / total number of barcodes with counts detected in target division). To classify the barcodes as originating from dMEC vs vMEC, the barcodes were divided into two groups – “dorsal MEC”, where counts in dMEC were higher than in vMEC, and vice versa for “ventral MEC”.

For a visual display of the relative barcode counts in dHip and vHip of barcodes originating from “dorsal MEC” or “ventral MEC”, we generated heat maps showing normalised projection strengths between dHip and vHip for all barcodes (Figure 4C). The normalised projection strengths were calculated by the formula: $\text{projection strength}_{\text{dHip}} = (\text{counts}_{\text{dHip}}) / (\text{counts}_{\text{dHip+vHip}}) * 100$, and likewise for vHip. Barcodes were sorted by maximum projection site.

Ex-vivo electrophysiology

499 *Slice preparation*

500 Slice preparation and subsequent data acquisition was done as previously described
501 (Sürmeli et al., 2015). 3 to 4 weeks after the injection of the viral vectors ex-vivo brain slices
502 were prepared. Following decapitation, brains were immersed for 2 mins in 4°C artificial
503 cerebrospinal fluid (ACSF) of the following composition (mM): 86 NaCl, 1.2 NaH₂PO₄, 2.5
504 KCl, 25 NaHCO₃, 25 Glucose, 75 Sucrose, 0.5 CaCl₂, 7 MgCl₂, bubbled with 95% O₂ / 5%
505 CO₂. They were then sectioned horizontally (400 µm) using a vibratome (Leica VT1200)
506 ACSF. Tissue was collected and maintained in extracellular solution of the following
507 composition (mM): 124 NaCl, 1.2 NaH₂PO₄, 2.5 KCl, 25 NaHCO₃, 20 Glucose, 2 CaCl₂ and 1
508 MgCl₂, continuously supplied with 95% O₂ / 5% CO₂. Slices were allowed to rest for 15 mins
509 at 35°C followed by a minimum of 30 mins recovery time at room temperature before the
510 start of the experiment.

511 *Electrophysiological recordings*

512 Whole-cell patch-clamp recordings were made in pyramidal neurons and interneurons in all
513 layers of hippocampal CA1. Typically, 2 to 3 slices from the intermediate hippocampus were
514 used where morphologies of the pyramidal cells and interneurons were confirmed to be
515 intact with post hoc staining and imaging of biocytin filled neurons. Data was collected using
516 AxoGraph (v1.7.6) software.

517 Pipettes with 4 to 6 MΩ resistance were pulled from borosilicate glass (Sutter Instruments)
518 and filled with an intracellular solution of following composition (mM): 130 K Gluconate, 10
519 KCl, 10 HEPES, 2 MgCl₂, 0.1 EGTA, 2 Na₂ATP, 0.3 Na₂GTP, 10 NaPhosphocreatine, 5.4
520 Biocytin. The intracellular solution was adjusted to a pH of 7.2 to 7.4 with KOH and
521 osmolarity 290-300 mOsm. All recordings were made in current clamp mode with pipette
522 capacitance neutralisation and bridge balance applied. Subthreshold membrane properties
523 were measured from the changes in membrane potential upon depolarising and
524 hyperpolarising current injections (typically -40 pA to +40 pA, in 20 pA). Rheobase was

525 established from responses to a depolarizing current ramp (50 pA/sec, maximum current
526 400 pA).

527 For optogenetic stimulation of ChR2, an LED of wavelength 470 nm (ThorLabs) was
528 attached to the epifluorescence port of the microscope. Where necessary, the irradiance of
529 the LED (max 9 mW) was controlled by voltage commands. Pharmacological tests were
530 done by bath application of the following reagents with the indicated final concentrations in
531 standard extracellular solution: Gabazine (10 μ M, Hello Bio, Cat. No. HB0901), NBQX (10
532 μ M, Tocris, Cat. No. 0373), D-AP5 (50 μ M, Tocris, Cat. No. 0106), 4-AP (200 μ M, Tocris,
533 Cat. No. 0940), TTX (1 μ M, Hello Bio). To allow for morphological reconstructions cells were
534 filled with Biocytin (5.4 mM in intracellular solution, Sigma, Cat. No. B4261) during
535 electrophysiological recordings.

536 *Analysis of electrophysiological recordings*

537 Electrophysiological properties were analysed using built-in and custom routines in
538 Igorpro8 (WaveMetrics) and Matlab (MathWorks). All basic properties were established
539 from the I-V protocol described above. Input resistance was determined from the largest
540 depolarising current step injected. Sag ratio was calculated as $V_{\text{steady state}} / V_{\text{min}}$ from the largest
541 hyperpolarising current step. Rheobase and action potential (AP) threshold were determined
542 from the first AP during injection of steadily increasing current in a ramp. From the same
543 protocol, after-hyperpolarisation (AHP) was calculated as the difference between AP
544 threshold and the most negative peak of the hyperpolarisation following the first spike. Half-
545 width was measured as the width of the AP at half its maximum spike amplitude. Firing
546 frequency was measured from a 1 s current injection of 200 pA. Maximum firing frequency
547 was measured between the first two APs, and base frequency between the last two APs in a
548 train.

549 For optogenetic stimulations, responsiveness was confirmed for each cell by a significant
550 difference between the detected peak of change in membrane potential after light stimulation

551 and the average baseline using a 2-tailed, type 1 student's t-test. The PSP amplitude and
552 latency were measured using the Neuromatic toolbox in IGORPro8.

553 Cell types were determined based on biophysical properties extracted from
554 electrophysiology data. Pyramidal cells were identified from a max firing frequency of < 50
555 Hz, AHP < 10 mV, AP half-width ≥ 0.7 ms and sag ≤ 0.9 , with descending hierarchical order.
556 Fast spiking interneurons were categorised from a max firing frequency ≥ 100 Hz, AHP ≥ 12
557 mV, AP half-width < 0.5 ms and sag > 0.9. Non-fast spiking interneurons were classified
558 from a max firing frequency 50-100 Hz, AHP ≥ 12 mV, AP half-width 0.5 to 0.9 ms, sag ratio
559 > 0.9. and max firing frequency 50-100 Hz.

560 Neurons with a resting membrane potential less negative than -50 mV or a bridge balance
561 higher than 40 M Ω were excluded from the analysis, as were neurons that did not fit within a
562 class from above criteria.

563 The proximo-distal position and sublayer identity of neurons filled with biotin during patch-
564 clamp recordings were determined post-hoc on immunostained slices after streptavidin
565 staining. CA1 was divided into two halves by drawing a line equidistant to CA1/CA2 and
566 CA1/Sub border. Only neurons that were clearly located in the proximal and distal halves
567 were included in the analysis. Neurons from the first two cell layers of SP were assigned a
568 superficial, neurons in deeper parts of SP towards SO were assigned a deep sublayer
569 profile. Neurons in intermediate positions were excluded from the analysis since it was not
570 possible to unequivocally determine whether they belong to deep or superficial SP.

571 *Immunostaining of electrophysiology slices*

572 For identification of cells following recordings, tissue was fixed in 4% PFA overnight at 4°C.
573 Slices were washed with PBS three times, 20 min each, and incubated in Streptavidin-Alexa
574 488 (S11223) or Alexa 647 (1:500, S32357, Invitrogen) and DAPI (1:1000, in 0.3% PBST)
575 overnight at 4°C. Slices were washed in 0.3% PBST four times and mounted on glass slides

576 with Mowiol® 4-88. Mcherry fluorescence in the MEC was examined with confocal imaging
577 and those animals where virus injection missed MEC L5 were excluded.

578 For staining of the interneuron marker parvalbumin (PV), slices were prepared as described
579 above, then incubated in primary antibody solution containing mouse anti-PV (1:1000, PV
580 235, Swant) and 5% NGS in 0.3% PBST for 48 hrs at 4°C. Slices were then washed and
581 incubated in secondary antibody solution with Alexa-conjugated Streptavidin and DAPI
582 prepared in 0.3% PBST and mounted on glass slides after overnight incubation.

583 A Zeiss LSM800 microscope was used for image acquisition of the slices. Pinhole was set to
584 1 Airy Unit. Objectives used include x10 (air), x20 (air) and x40 (oil) to image the
585 hippocampal formation, morphology of biocytin filled cells, and immunolabelling of
586 interneurons, respectively.

587 **Image processing and generation**

588 Experimental design sketches were generated using Brainrender (Claudi et al., 2021) or
589 Affinity Designer. Where necessary images were cropped and size-adjusted using
590 Photoshop, Pixelmator and ImageJ softwares.

591 **Statistics**

592 Statistical tests (Student's t-test, Chi-squared test, Wilcoxon signed rank test) were
593 performed using R (www.r-project.org) 3.6.0, 4.0.0 and 4.0.3. Normality was tested for using
594 both the Shapiro-Wilk test and QQ plots. All data is presented in the format of mean ± SEM
595 unless otherwise stated. A p-value < 0.05 was regarded as significant.

596 **Data availability**

597 On publication data and analysis scripts will be made publicly available via University of
598 Edinburgh's Datashare service (<http://datashare.is.ed.ac.uk/>). This is an online data

599 repository maintained by the University. MAPseq data will be made available at NLM
600 Sequence Read Archive BioProject.

601 **Code availability**

602 Analysis script will be made publicly available upon publication via University of Edinburgh's
603 Datashare service

604

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613

614 **References**

- 615 Arber, S., Ladle, D.R., Lin, J.H., Frank, E., and Jessell, T.M. (2000). ETS gene Er81 controls
616 the formation of functional connections between group Ia sensory afferents and motor
617 neurons. *Cell* 101, 485–498.
- 618 Beed, P., de Filippo, R., Holman, C., Jochenning, F.W., Leibold, C., Caputi, A., Monyer, H.,
619 and Schmitz, D. (2020). Layer 3 Pyramidal Cells in the Medial Entorhinal Cortex Orchestrate
620 Up-Down States and Entrain the Deep Layers Differentially. *Cell Rep.* 33, 108470.
- 621 Cappaert, N.L.M., Van Strien, N.M., and Witter, M.P. (2015). Chapter 20 - Hippocampal
622 Formation. In *The Rat Nervous System (Fourth Edition)*, G. Paxinos, ed. (San Diego:
623 Academic Press), pp. 511–573.
- 624 Claudi, F., Tyson, A.L., Petrucco, L., Margrie, T.W., Portugues, R., and Branco, T. (2021).
625 Visualizing anatomically registered data with brainrender. *Elife* 10.
626

627 Colgin, L.L. (2016). Rhythms of the hippocampal network. *Nature Reviews Neuroscience* 17,
628 239–249.

629 Czajkowski, R., Sugar, J., Zhang, S.-J., Couey, J.J., Ye, J., and Witter, M.P. (2013).
630 Superficially projecting principal neurons in layer V of medial entorhinal cortex in the rat
631 receive excitatory retrosplenial input. *J. Neurosci.* 33, 15779–15792.

632 Dolorfo, C. L., and D. G. Amaral. 1998. Entorhinal Cortex of the Rat: Topographic
633 Organization of the Cells of Origin of the Perforant Path Projection to the Dentate Gyrus. *The*
634 *Journal of Comparative Neurology* 398 (1): 25–48.
635

636 Dudman, J.T., Tsay, D., and Siegelbaum, S.A. (2007). A role for synaptic inputs at distal
637 dendrites: instructive signals for hippocampal long-term plasticity. *Neuron* 56, 866–879.

638 Frankland, P. W. & Bontempi, B. The organization of recent and remote memories. *Nat. Rev.*
639 *Neurosci.* 6, 119–130 (2005).
640

641 Gerfen, C.R., Paletzki, R., and Heintz, N. (2013). GENSAT BAC cre-recombinase driver
642 lines to study the functional organization of cerebral cortical and basal ganglia circuits.
643 *Neuron* 80, 1368–1383.

644 Kebschull, J.M., da Silva, P.G., Reid, A.P., Peikon, I.D., Albeanu, D.F., and Zador, A.M.
645 (2016). High-Throughput Mapping of Single-Neuron Projections by Sequencing of Barcoded
646 RNA. *Neuron* 91, 975–987.

647 Ketz, N., Morkonda, S.G., and O'Reilly, R.C. (2013). Theta coordinated error-driven learning
648 in the hippocampus. *PLoS Comput. Biol.* 9, e1003067.

649 Khodagholy, D., Gelinás, J.N., and Buzsáki, G. (2017). Learning-enhanced coupling
650 between ripple oscillations in association cortices and hippocampus. *Science* 358, 369–372.

651 Kitamura, T., Pignatelli, M., Suh, J., Kohara, K., Yoshiki, A., Abe, K., and Tonegawa, S.
652 (2014). Island Cells Control Temporal Association Memory. *Science* 343, 896–901.

653 Kitamura, T., Ogawa, S.K., Roy, D.S., Okuyama, T., Morrissey, M.D., Smith, L.M., Redondo,
654 R.L., and Tonegawa, S. (2017). Engrams and circuits crucial for systems consolidation of a
655 memory. *Science* 356, 73–78.

656 Köhler, C. (1985). A projection from the deep layers of the entorhinal area to the
657 hippocampal formation in the rat brain. *Neurosci. Lett.* 56, 13–19.

658 Koster, R., Chadwick, M.J., Chen, Y., Berron, D., Banino, A., Düzel, E., Hassabis, D., and
659 Kumaran, D. (2018). Big-Loop Recurrence within the Hippocampal System Supports
660 Integration of Information across Episodes. *Neuron* 99, 1342–1354.e6.

661 Kumaran, D., Hassabis, D., and McClelland, J.L. (2016). What Learning Systems do
662 Intelligent Agents Need? Complementary Learning Systems Theory Updated. *Trends Cogn.*
663 *Sci.* 20, 512–534.

664 Marr, D. (1971). Simple memory: a theory for archicortex. *Philos. Trans. R. Soc. Lond. B*
665 *Biol. Sci.* 262, 23–81.

666 McClelland, J.L., McNaughton, B.L., and O'Reilly, R.C. (1995). Why there are
667 complementary learning systems in the hippocampus and neocortex: insights from the
668 successes and failures of connectionist models of learning and memory. *Psychol. Rev.* 102,
669 419.

670 McClure, C., Cole, K.L.H., Wulff, P., Klugmann, M., and Murray, A.J. (2011). Production and
671 titrating of recombinant adeno-associated viral vectors. *J. Vis. Exp.* e3348.

672 Murray, A.J., Sauer, J.-F., Riedel, G., McClure, C., Ansel, L., Cheyne, L., Bartos, M.,
673 Wisden, W., and Wulff, P. (2011). Parvalbumin-positive CA1 interneurons are required for
674 spatial working but not for reference memory. *Nat. Neurosci.* *14*, 297–299.

675 Naber, P.A., Lopes da Silva, F.H., and Witter, M.P. (2001). Reciprocal connections between
676 the entorhinal cortex and hippocampal fields CA1 and the subiculum are in register with the
677 projections from CA1 to the subiculum. *Hippocampus* *11*, 99–104.

678 Ognjanovski, N., Schaeffer, S., Wu, J., Mofakham, S., Maruyama, D., Zochowski, M., and
679 Aton, S.J. (2017). Parvalbumin-expressing interneurons coordinate hippocampal network
680 dynamics required for memory consolidation. *Nat. Commun.* *8*, 15039.

681 Pastoll, H., White, M., and Nolan, M. (2012). Preparation of parasagittal slices for the
682 investigation of dorsal-ventral organization of the rodent medial entorhinal cortex. *J. Vis.*
683 *Exp.*

684 Petreanu, L., Huber, D., Sobczyk, A., and Svoboda, K. (2007). Channelrhodopsin-2–assisted
685 circuit mapping of long-range callosal projections. *Nat. Neurosci.* *10*, 663–668.

686 Rozov, A., Rannap, M., Lorenz, F., Nasretdinov, A., Draguhn, A., and Egorov, A.V. (2020).
687 Processing of Hippocampal Network Activity in the Receiver Network of the Medial
688 Entorhinal Cortex Layer V. *The Journal of Neuroscience* *40*, 8413–8425.

689 Ruth, R. E., Collier, T. J. & Routtenberg, A. Topography between the entorhinal cortex and
690 the dentate septotemporal axis in rats: I. Medial and intermediate entorhinal projecting cells.
691 *J. Comp. Neurol.* **209**, 69–78 (1982).
692

693 Schapiro, A.C., Turk-Browne, N.B., Botvinick, M.M., and Norman, K.A. (2017).
694 Complementary learning systems within the hippocampus: a neural network modelling
695 approach to reconciling episodic memory with statistical learning. *Philos. Trans. R. Soc.*
696 *Lond. B Biol. Sci.* *372*.

697 Sürmeli, G., Marcu, D.C., McClure, C., Garden, D.L.F., Pastoll, H., and Nolan, M.F. (2015).
698 Molecularly Defined Circuitry Reveals Input-Output Segregation in Deep Layers of the
699 Medial Entorhinal Cortex. *Neuron* *88*, 1040–1053.

700 Squire, L. R., Genzel, L., Wixted, J. T. & Morris, R. G. Memory consolidation. *Cold Spring*
701 *Harb. Perspect. Biol.* *7*, a021766 (2015).
702

703 Swanson, L.W., and Köhler, C. (1986). Anatomical evidence for direct projections from the
704 entorhinal area to the entire cortical mantle in the rat. *J. Neurosci.* *6*, 3010–3023.

705 Tamamaki, N., and Nojyo, Y. (1995). Preservation of topography in the connections between
706 the subiculum, field CA1, and the entorhinal cortex in rats. *J. Comp. Neurol.* *353*, 379–390.

707 Wang, Y., Kakizaki, T., Sakagami, H., Saito, K., Ebihara, S., Kato, M., Hirabayashi, M.,
708 Saito, Y., Furuya, N., and Yanagawa, Y. (2009). Fluorescent labeling of both GABAergic and
709 glycinergic neurons in vesicular GABA transporter (VGAT)–Venus transgenic mouse.
710 *Neuroscience* *164*, 1031–1043.

711 Witter, M.P., and Amaral, D.G. (1991). Entorhinal cortex of the monkey: V. Projections to the
712 dentate gyrus, hippocampus, and subicular complex. *J. Comp. Neurol.* *307*, 437–459.

Witter, M.P., Doan, T.P., Jacobsen, B., Nilssen, E.S., and Ohara, S. (2017). Architecture of the Entorhinal Cortex A Review of Entorhinal Anatomy in Rodents with Some Comparative Notes. *Front. Syst. Neurosci.* 11, 46.

Wozny, C., Beed, P., Nitzan, N., Pössnecker, Y., Rost, B.R., and Schmitz, D. (2018). VGLUT2 Functions as a Differential Marker for Hippocampal Output Neurons. *Front. Cell. Neurosci.* 12, 337.

Figure legends

Figure 1. L5a but not layer 5b of the MEC projects to the hippocampus

A, Schematic showing the injection site of a retrograde AAV in the intermediate hippocampus (light grey) of wild type mice. The black outline in the horizontal plane roughly corresponds to the dorso-ventral position of the horizontal section shown in B. Corresponding injection site image is shown in Figure 1- figure supplement 1A. **B**, Immunostained brain section in the horizontal plane showing retrogradely-labelled neurons in L3 and L5a of the MEC. Etv1 expression marks L5a (Scale bar: 250 μ m). Inset: high-magnification image of boxed region in left panel showing co-expression of GFP and Etv1 (Scale bar: 50 μ m). Abbreviations: PaS, parasubiculum; P, posterior; A, anterior; M, medial; L, lateral. **C**, Schematic showing the injection site of an AAV in the deep MEC (dark grey) of *Rbp4-Cre* mice for Cre dependent expression of GFP. The black outline in the horizontal plane roughly corresponds to the dorso-ventral position of the slice shown in D. **D**, Entorhinal area on an immunostained brain section in the horizontal plane showing the overlapping expression of GFP with Etv1 (Scale bar: 250 μ m). Inset: high-magnification of boxed region in middle panel showing GFP and Etv1 expression confined mainly to L5a in MEC (Scale bar: 100 μ m).

Figure 2. Projections to CA1 of L5a neurons of the MEC have distinct topography

A, Schematic showing the injection strategy of a Cre-dependent virus in the deep MEC of *Rbp4-Cre* mice. **B**, Immunostained coronal brain section showing the distribution of axons of MEC L5a cells in CA1 (Scale bar: 500 μ m). Inset: high-magnification image of boxed region in left panel (Scale bar: 100 μ m). Bottom-left schematic indicates the antero-posterior position of the slice. Corresponding injection site image is shown in Figure 2- figure supplement 1A. Abbreviations: SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SL, stratum lacunosum; SM, stratum moleculare; D, dorsal; V, ventral; M, medial; L, lateral. **C**, Quantification of fluorescence intensity of GFP across the layers of CA1. The area between 0 and 20 in (B) was divided into 20 equally-sized bins, and the mean fluorescence intensity was calculated for each bin. Values shown are normalised mean fluorescence intensity values ($n = 6$ mice, 2 sections per brain). **D**, Normalised mean fluorescence density values in deep versus superficial SP ($n = 6$ mice). Within SP, axon density in deep SP was significantly higher than density in superficial SP (paired one-tailed Wilcoxon signed rank test, $p = 0.0156$, $n = 6$). **E**, Schematic showing the injection strategy of a Cre-dependent virus in the deep MEC of *Rbp4-Cre* mice. The black outline in the horizontal plane roughly corresponds to the dorso-ventral position of the horizontal slice shown in (F). **F**, Immunostaining of a horizontal brain section showing the distribution of axons of MEC L5a cells in the subiculum (Sub), distal CA1 (dCA1) and proximal CA1 (pCA1; Scale bar: 200 μ m). Corresponding injection site image is shown in Figure 2- figure supplement 1B. **G**, High-magnification images of the pyramidal cell layer in distal (top) and proximal (bottom) CA1 showing axonal labelling (Scale bar: 20 μ m). **H**, Normalised mean fluorescence density values in distal versus proximal CA1 ($n = 5$ mice). **I**, High-magnification image of the subiculum showing axonal labelling (Scale bar: 20 μ m). **J**, Schematic showing the injection strategy of an AAV for Cre-dependent expression of a membrane-bound form of GFP and a synaptophysin-mRuby conjugate in L5a of the MEC in the *Rbp4-Cre* mice. The black outline in the horizontal plane roughly corresponds to the dorso-ventral position of the slice shown in (K). **K**, Immunostained brain section in the horizontal plane showing the distribution of axons (green) and synaptic terminals (red) in the hippocampus as well as the parasubiculum (PaS) and perirhinal cortex (PRh). Injection site in the MEC is distinguishable by the strong fluorescent labelling of L5a cell bodies (Scale bar: 400 μ m). **L**, High-magnification image of the injection site in the MEC showing cell membrane and synaptic labelling specifically in L5a (Scale bar: 20 μ m). **M**, High-magnification image of the subiculum showing axonal and synaptic labelling (Scale bar: 20 μ m).

N,O, High-magnification images of the pyramidal cell layer in distal (N) and proximal (O) CA1 showing axonal and synaptic labelling (Scale bar: 20 μ m).

Figure 3. Retrosplenial cortex and nucleus accumbens outputs of the MEC are copied to CA1

A, B, Experimental strategy. In wild-type mice, a retrogradely-transported virus expressing Cre was injected in either the retrosplenial cortex (RSC; A) or nucleus accumbens (NucAcb; B), while a Cre-dependent reporter virus was injected in the deep MEC.

C, D, Hippocampal axon collaterals of RSC-projecting (C) or NucAcb-projecting MEC neurons (D; Scale bar: 250 μ m). Insets: high-magnification images from CA1 showing distribution of axons across CA1 layers (Scale bar: 100 μ m). Bottom-left schematic indicates the antero-posterior position of the slice. Corresponding injection site images are shown in Figure 3- figure supplement 1A and B.

E, F, Quantification of mean fluorescence intensity across the layers of CA1 when only RSC-projecting (E) or NucAcb-projecting (F) MEC neurons were labelled (n = 3 mice for each group). Axon topography is similar to when L5a neurons are marked globally in the *Rbp4-Cre* mice (Figure 2C).

Figure 4. Telencephalic outputs are widely copied onto the hippocampus

A, Experimental strategy. A MAPseq virus library was injected into deep MEC (n = 3). 44 hours later, mice were sacrificed and brains were serially sectioned in the sagittal plane at 400 μ m thickness. Tissue from 5 major divisions (Isocortex, CNU, Olf/Ctxsp, dHip and vHip), as well as dorsal and ventral MEC were then dissected and collected in tubes. Brainstem tissue was collected as a negative control. Tissue was further processed for RNA extraction and next generation sequencing.

B, Nearly all barcodes detected in CNU, Olf/Ctxsp and Isocortex were also detected in the hippocampus (Isocortex: 98.6% $\pm$ 0.06%; CNU: 99.2% $\pm$ 0.08%; Olf/Ctxsp: 99.0% $\pm$ 0.4%; n = 3 mice, 1603, 3493, 1677 barcodes from brains 1,2 and 3 respectively)

C, Dorso-ventral topography of projections revealed by relative barcode counts. For each barcode, counts were normalised to show the relative projection strength of each neuron to dorsal and ventral hippocampus (n = 3 mice).

Figure 5. L5a neurons of the MEC provide direct excitatory and indirect inhibitory inputs to pyramidal cells in CA1

A, Experimental design showing viral expression, placement of patch-clamp electrode and light delivery over CA1.

B, Examples of biocytin filled pyramidal neurons recorded in CA1.

C, An example electrical recording of a pyramidal neuron at rest (-67 mV) showing depolarizing responses upon 3 ms light stimulation (top) (blue line, Scale bar: 1 mV, 10 ms). Train of action potentials upon 200pA step-current injection (bottom). (Scale bar: 20 mV, 100 ms)

D, Proportion of responsive pyramidal neurons located in the distal and proximal halves of CA1(top). The difference in proportions was not significant (X-squared = 1.9071, df = 1, p-value = 0.167291, Chi-squared test, n = 12 cells in proximal and n = 39 cells in distal CA1). Proportion of responsive pyramidal neurons located in the deep versus superficial locations of CA1 pyramidal cell layer (bottom) (X-squared = 0.2969, df = 1, p-value = 0.585804, Chi-squared test, n = 8 cells in deep SP and n = 13 cells in superficial SP).

E, Effects of bath application of Gabazine (orange, n = 10 cells, 9 mice) and NBQX (green, n = 5 cells) on PSPs recorded from a pyramidal neuron (Scale bar: 0.5 mV, 10 ms) and a summary plot of PSP amplitude measurements for all tested pyramidal neurons. Note that some neurons were only treated with Gabazine which did not cause a significant change in amplitudes (p = 0.97, 2-tailed Student's t-test, n = 10 cells, 9 mice).

F, An example of ten consecutive PSP responses recorded from a single pyramidal neuron illustrates the short and invariant latency of PSPs (Scale bar: 0.5 mV, 10 ms) and a cumulative probability plot of standard deviation of latencies for neurons with PSP responses that were > 1 mV in amplitude (n = 20 cells).

G, Effects of bath application of TTX (orange) and 4-AP (green) on PSPs recorded from a pyramidal neuron (Scale bar: 0.5 mV, 10 ms) and a summary plot of changes in PSP amplitudes for all tested pyramidal neurons. TTX application abolished responses (n = 5 cells, 5 mice, p = 0.01, 2-tailed Student's t-test).

H, An example inhibitory PSP response recorded from a pyramidal neuron upon 10 Hz light stimulation (blue bars). Response polarity reversed when the neuron's membrane potential was adjusted to -50 mV and was abolished after application of Gabazine (Scale bars: 0.2 mV, 100 ms). Inset shows the long latency (> 10 ms) of PSP onset indicating polysynaptic connectivity.

Figure 6. L5a neurons of the MEC provide direct excitatory inputs to interneurons in CA1.

A, Experimental design showing viral expression, patch pipette and light stimulation. Interneurons are marked by the expression of venus fluorescent protein driven by the vesicular GABA transporter (*Vgat*) gene's promoter.

B, Biocytin filled neurons in the *Rbp4-Cre X Vgat-Venus* double transgenic mouse line. Fluorescent labelling facilitated patch-clamp recordings made from GABAergic interneurons and distinguishing SR, SL and SM layers.

C, Representative examples of depolarising responses recorded at resting membrane potential following 3 ms blue light stimulation (blue line) of L5a axons in CA1 (left) (Scale bar: 1 mV, 10 ms). Neurons in SO and SM were typically not responsive (< 30%). Responses from SR neurons were on average larger than the responses from neurons in other layers (Scale bar: 1 mV, 10 ms). Neurons' spiking response to injecting 200 pA current (right) (Scale bar: 10 mV, 100 ms).

D, Proportion of responsive interneurons in all layers of CA1 recorded at resting membrane potential. Green highlighted segment corresponds to the proportion of cells with depolarising membrane potentials; grey highlighted segment corresponds to neurons with no change in their membrane potential.

SO: n = 18 cells, 10 mice;
 SP_{FSint} : n = 33 cells, 8 *Rbp4-Cre X Pvalb-Flip* mice, 5 *Rbp4-Cre X Vgat-Venus* mice, 8 *Rbp4-Cre* mice;
 SP_{NSFint} : n = 13 cells, 11 *Rbp4-Cre* mice, 1 *Rbp4-Cre X Vgat-Venus* mouse;
 SR: n = 14 cells, 6 mice; SL: n = 60 cells, 15 mice; SM: n = 21 cells, 10 mice

E,F, Effects of bath application of Gabazine (orange) and NBQX (green) on PSPs recorded from a fast-spiking pyramidal layer interneuron (left) (Scale bar: 1 mV, 5 ms). Summary quantification of PSP amplitudes for multiple cells (right). The PSP amplitudes were largely unaffected by application of Gabazine (SP_{int} : n = 6 cells, p = 0.99 2-tailed Student's t test) but were largely blocked by NBQX (SP_{int} : n = 5 cells, p = 0.03, 2-tailed Student's t test) indicating AMPA receptor mediated glutamatergic synaptic transmission.

G,H, Effects of bath application of TTX (orange) and 4-AP (green) on the response amplitude PSPs recorded from pyramidal layer interneurons. Example traces from a fast-spiking pyramidal layer interneuron (Scale bar: 1 mV, 5 ms). Summary plots of response amplitude measurements from multiple recordings (SP_{int} : n = 4 cells).

I, J, An example of ten consecutive PSP responses recorded from a single fast-spiking interneuron in SP illustrates the short and invariant latency of PSPs. Cumulative probability plots of standard deviation of latencies for neurons with PSP responses that were > 1 mV in amplitude (mean latency SP: 2.08 ± 0.08 ms, n = 21 cells).

Figure 1, figure supplement 1. Injection site images, quantification of co-localisation between retrograde markers and Etv1.

A, Immunostained horizontal sections showing the injection site in the intermediate hippocampus of a retrograde AAV virus. L3 and L5a cells in the MEC were retrogradely-labelled via GFP expression driven by the CAG (CMV early enhancer/chicken β actin) promoter (Scale bar: 200 μ m).

B, Results in (A) were replicated with an AAV virus that enables retrograde labelling of cells via expression of mCherry driven by the human synapsin (hSyn) promoter (Scale bar: 200 μ m).

C, Immunostained brain sections in horizontal plane from a single brain showing the dorso-ventral spread of virus-mediated reporter gene expression in the *Rbp4-Cre* mice (Scale bar: 500 μ m).

D, Schematic of a Cre-dependent mCherry expressing virus injection in the deep MEC of *Rbp4-Cre X Vgat-Venus* mouse line (left). Immunostained brain sections in the MEC showing absence of overlap between mCherry and Venus which marks GABAergic interneurons (right). Scale bar: 100 μ m, Inset scale bar: 30 μ m)

E, Immunostained brain sections in the sagittal plane showing injection sites of fluorophore-coupled CTB (Cholera toxin beta subunit; Scale bar: 500 μ m).

F, Schematic of a two-colour retrograde dye injection in the cingulate and retrosplenial cortices of wild-type mice (left). Immunostaining in the MEC shows overlap between Etv1 expression and retrograde dyes (right; Scale bar: 50 μ m)

G,H, Quantification of overlap between retrograde labelling from cortical target areas and Etv1 expression in L5a of the MEC. The number of mice, mean proportion and total cells counted for each area are shown in the table.

Hip, hippocampus; Amg, amygdala; AOM, medial anterior olfactory area; Cg, cingulate area; NucAcb, nucleus accumbens; V1, primary visual cortex; V2, secondary visual areas; RSC, retrosplenial cortex.

Figure 1, figure supplement 2. L5a neurons of the MEC innervate neocortical and subcortical areas.

A. Schematic showing the injection site of a Cre-dependent virus in the deep MEC of *Rbp4-Cre* mice.

B. Confocal image of a coronal brain slice showing axons originating from entorhinal L5a in the anterior telencephalon. Insets show higher magnification images of boxed areas.

C. Confocal image of a coronal brain slice showing axons originating from entorhinal L5a in the mid-telencephalon. Inset shows higher magnification image of boxed area.

D. Confocal image of a coronal brain slice showing axons originating from entorhinal L5a in the posterior telencephalon. Inset shows higher magnification image of boxed area.

Cg, cingulate area; M2, secondary motor area; V1, primary visual cortex; V2M, secondary visual area (medial); RSC, retrosplenial cortex; Hip, hippocampus; NucAcb, nucleus accumbens.

Figure 2, figure supplement 1. Injection site images and additional examples of axon labelling in CA1.

A, Immunostained brain section in the coronal plane showing cell body labelling at the injection site in MEC (Scale bar: 500 μ m). Inset shows a higher magnification view of the boxed area (Scale bar: 50 μ m). Schematic of a coronal brain section indicates the antero-posterior position of the section.

B, Immunostained brain section in the horizontal plane showing cell body labelling at the injection site in MEC (Scale bar: 200 μ m).

C, Schematic showing the injection site of a Cre-dependent virus in the deep MEC of *Rbp4-Cre* mice. The black outline in the horizontal plane roughly corresponds to the dorso-ventral position of the slice shown in (D).

D, Immunostained brain section in the horizontal plane showing cell body labelling at the injection site in MEC (Scale bar: 200 μ m).

E, Immunostained brain section in the horizontal plane showing the distribution of axons of MEC L5a cells in the subiculum (Sub), distal CA1 (dCA1) and proximal CA1 (pCA1) (Scale bar: 200 μ m).

F, High-magnification images of the pyramidal cell layer in dCA1 and pCA1 showing axonal and synaptic labelling (Scale bar: 20 μ m).

G, High-magnification image of the subiculum showing axonal labelling (Scale bar: 20 μ m).

Prh, Perirhinal cortex; ECT, Ectorhinal cortex; V1, primary visual cortex; V2, secondary visual cortex; RSC, retrosplenial cortex; PaS, parasubiculum.

Figure 3, figure supplement 1. Combinatorial viral strategy effectively labels L5a cells of the MEC and their cortical projections.

A, B, Coronal brain sections showing labelled neurons in MEC with the combinatorial viral strategy (Scale bar: 500 μ m). Note that the conventional layer organization is distorted in coronal sections due to the curved geometry of the MEC. For comparison with results of the same experiment in sagittal plane please refer to Surmeli et al., 2015. Inset: high-magnification confocal image of boxed region in left panel showing cell bodies in MEC expressing mCherry. Schematic of a coronal brain section indicates the antero-posterior position of the sections.

C, D, Coronal brain sections showing GFP expression and mCherry-labelled axon terminals at the injection sites in retrosplenial cortex (**D**) and nucleus accumbens (**E**) (Scale bar: 500 μ m). Inset: high-magnification of boxed region in left panel.

RSC, retrosplenial cortex; NucAcb, nucleus accumbens; DS, dorsal subiculum; aca, anterior commissure anterior part; V2, secondary visual area medial part

Figure 4, figure supplement 1. Verification of viral expression and further MAPseq data visualisation.

A, Brightfield and epifluorescence microscope images of the injection site on a sagittal brain section showing the spread of neurons infected by the MAPseq virus library in wild type mice.

B, A neuron's position in the dorso-ventral axis does not affect the likelihood of collateralisation of its axons to the hippocampus (Paired two-tailed Wilcoxon signed rank test, $p = 1$, median proportion of collateralizing barcodes in dEC = 99.3%, vEC = 99.8%, $n = 3$).

Figure 5, figure supplement 1. Experimental design and properties of responses to optogenetic stimulation of axons originating from L5a of the MEC.

A, Summary table for biophysical properties of neurons recorded in the study.

B, An example of a biphasic response recorded from a pyramidal neuron upon 3ms stimulation of axons of MEC L5a neurons in CA1. A late hyperpolarising (red) response was abolished by application of Gabazine to reveal a larger depolarising PSP (black) which was abolished by application of NBQX (grey) (Scale bar: 10 ms, 0.5 mV).

C, Experimental strategy for fluorescence guided patch-clamp recordings made from Parvalbumin expressing neurons. Left: A FlpX-dependent GFP expressing virus was injected in the hippocampus in *Rbp4-CreX Pvalb-Flp* mice. Right: Confocal image of horizontal slice showing labelled interneurons in the hippocampus. Bottom: An example fast-spiking interneuron marked as described in **c**, that showed depolarising PSP upon light stimulation. During recording, the neuron was filled with biocytin and later stained with Streptavidin and Anti-Parvalbumin antibody.

D, (i) Traces of responses to a train of 10 light stimulation (blue lines), showing relatively stable EPSP amplitude across simulations. (Scale bar: 30 ms, 1 mV) (ii) Magnified images of 1st and 10th pulse illustrate the invariant latency in one example cell (Scale bars: 0.5 ms, 0.5 mV) (iii) Quantification of latency across the train in all cells. Error bars represent SEM.

E, Plots showing the frequency distribution of PSP amplitudes in pyramidal neurons and interneurons. PSPs over 12.5 mV were detected in interneurons and often resulted in spiking responses. (SP_{Pyr} : $n = 19$, SP_{int} : $n = 19$, SR: $n = 9$, SL&SM: $n = 22$ cells).

957 **F**, Effects of bath application of Gabazine and NBQX on PSPs recorded from interneurons in SR and SL. The
958 PSP amplitudes were largely unaffected by application of Gabazine (SR: n = 2 cells & SL n = 2 cells, p = 0.28, 2-
959 tailed Student's t test) but were largely blocked by NBQX (SR: n = 2 cells & SL n = 2 cells, p = 0.04, 2-tailed
960 Student's t test) indicating AMPA receptor mediated glutamatergic synaptic transmission.

961 **G**, Effects of bath application of TTX and 4-AP on the response amplitude PSPs recorded from interneurons in
962 SR, SL and SM (SR: n = 2, SL: n = 3, SM: n = 1 cells).

963 **H**, Cumulative probability plots of standard deviation of latencies for neurons with PSP responses that were > 1
964 mV in amplitude (SR, n = 6 cells, SL, n = 15 cells, SM, n = 4 cells). EPSPs of interneurons in all layers except SO
965 had a short latency after light stimulation (mean latency SR: 2.00 ± 0.08 ms, n = 6 cells; SL & SM: 2.12 ± 0.21
966 ms, n = 23 cells). In SO 2 out of 3 of the evoked PSPs had a larger latency of onset (mean latency = 8.5 ± 0.63
967 ms, n = 2 cells, 2 mice, data not shown).

968

969 **Source Data File 1. MAPseq barcode count matrix.** Source data used to generate all plots in Figure 4 and
970 Figure 4 figure supplement 1. Barcode counts were filtered and normalised by spike-in counts as described in
971 methods. Column names are abbreviations of brain area names as explained in figure legends.

A

B

Inset

Figure 1

C

D

Inset

A **B** **C**

A

AAV-EF1a-DIO-mCherry

Rbp4-Cre

B

DAPI mCherry

C

DAPI mCherry

D

DAPI mCherry

A**GFP DAPI** **inset** **B****mCherry DAPI** **C***AAV-CAG-FLEX-GFP* **D****GFP DAPI** **E****GFP DAPI** **GFP** **F****dCA1** **pCA1** **G****Subiculum**

A

RSC-projecting L5a

mCherry DAPI

Inset

B

NucAcb-projecting L5a

mCherry DAPI

Inset

C

mCherry GFP DAPI

Inset

D

mCherry GFP DAPI

Inset

A

Brightfield

Sindbis-barcodeRNA-GFP

B

Proportion of barcodes (%)

100
90

All targets

All targets

Hip

dorsal
MEC

Hip

ventral
MEC

A

	SO (n = 18)	SP Pyr (n = 64)	SP FSint (n = 34)	SP NFSint (n = 15)	SR (n = 14)
Vm rest (mV)	-63 ± 1.48	-66.7 ± 0.6	-63.7 ± 0.7	-63.1 ± 0.9	-60.3 ± 1.3
IR (M Ω)	339 ± 64.1	146.9 ± 6.5	178.2 ± 29.4	214.1 ± 18.3	287.3 ± 48.7
Tm (ms)	22.3 ± 6.8	23.4 ± 2.1	14.7 ± 1.7	21.2 ± 3.7	25.2 ± 4.4
Rheobase (pA)	65.8 ± 17.8	112.8 ± 8.5	128.2 ± 15.1	83.9 ± 12.3	62.8 ± 8.8
Sag coefficient	0.85 ± 0.2	0.85 ± 0.01	0.86 ± 0.02	0.87 ± 0.01	0.85 ± 0.02

B **C** **D** **E** **F** **G** **H**