Perirhinal input to neocortical layer 1 controls learning

One Sentence Summary: *Perirhinal cortex gates memory formation in neocortical layer 1*

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Abstract

Signals sent back to the neocortex from the hippocampus control the long-term storage of memories in the neocortex^{1,2}, but the cellular mechanisms underlying this process remain elusive. Here, we show that learning is controlled by specific medial-temporal input to neocortical layer 1. To show this we used direct cortical microstimulation detection task that allowed the precise region of learning to be examined and manipulated. Chemogenetically suppressing the last stage of the medial temporal loop, i.e. perirhinal cortex input to neocortical layer 1, profoundly disrupted early memory formation but had no effect on behavior in trained animals. The learning involved the emergence of a small population of layer 5 pyramidal neurons (~10%) with significantly increased firing involving high-frequency bursts of action potentials that were also blocked by suppression of perirhinal input. Moreover, we found that dendritic excitability was correspondingly enhanced in a similarly-sized population of pyramidal neurons and suppression of dendritic activity via optogenetic activation of dendrite-targeting inhibitory neurons also suppressed learning. Finally, single-cell stimulation of cortical layer 5 pyramidal neurons showed that burst but not regular firing retrieved previously learned behavior. We conclude that the medial temporal input to the neocortex controls learning through a process in L1 that elevates dendritic calcium and promotes burst firing.

1 Results

2 The distributed nature of long-term memory formation in the cortex has
 3 challenged research into the underlying mechanisms. For hippocampal-
 4 independent learning paradigms there is converging evidence to suggest that
 5 cortical layer 1 (L1) is a locus for plasticity³⁻⁶ involving activity in the distal tuft
 6 dendrites of pyramidal neurons that innervate L1^{4,7-9}. Far less is known about
 7 the mechanisms underlying hippocampal-dependent memory formation in the
 8 cortex. Application of the retrograde tracer, Fast Blue, to L1 of primary
 9 somatosensory cortex (S1), revealed labeled cells in the deep layers of the
 10 perirhinal cortex (Fig. 1a, bottom left). Conversely, expression of ChR2-EYFP via
 11 a viral vector (AAV) injected into the deep layers of the perirhinal cortex densely
 12 labeled axons in L1 of S1 (Fig. 1a, bottom right), confirming that the perirhinal
 13 cortex is the last station in the medial temporal loop before the primary
 14 somatosensory neocortex in rodents (Fig. 1a, right and Extended Fig. 1)^{2,10,11}.

15 In order to examine the influence of perirhinal cortex on memory
 16 formation in neocortex, we adapted a fast-learning, associative and cortex-
 17 dependent task¹². Rodents were trained to report short (200 ms) trains of direct
 18 electrical microstimulation (μ Stim) pulses in layer 5 (L5) of S1 (Fig. 1b) where
 19 μ Stim detection threshold is lowest¹². Animals initially received a block (5
 20 repetitions) of μ Stim paired with the reward (sweetened water) regardless of
 21 their licking responses. Following a brief pairing period (1-2 blocks), the reward
 22 became available if the animal actively licked within a response window of 100–
 23 1200 ms following μ Stim onset (Fig. 1b). Animals learned this task extremely
 24 quickly during the first training session and became experts after about 3
 25 training sessions (see Methods). Ipsilateral injections of lidocaine in the
 26 hippocampus showed that this task is hippocampus-dependent (Extended Fig.
 27 2). Making behavior contingent on μ Stim of S1 allowed us to precisely define the
 28 area of interest and the temporal window in order to examine the underlying
 29 neuronal mechanisms of memory formation. Moreover, it allowed us to precisely
 30 target the perirhinal projection to L1 of S1. We chose a chemogenetic approach
 31 to down-regulate synaptic transmission¹³ at the axon terminals of perirhinal
 32 long-range projecting neurons without influencing the hippocampus and

33 parahippocampal regions. Here, we expressed hM4Di receptors (inhibitory
34 designer receptors exclusively activated by a designer drug, DREADD¹⁴) in the
35 perirhinal cortex of mice (Fig. 1c). The axon terminals in S1 were inhibited by
36 application of clozapine-N-oxide (CNO, 10 μ M), injected in to L1 above the
37 stimulated region (Fig. 1c), 20 mins before training (see Methods).

38 Specifically blocking perirhinal cortex input to L1 of S1 severely reduced
39 learning during the first training session (Fig. 1d&e). We quantified learning as
40 the cumulative difference between the number of successful and failed licking
41 responses to μ Stim ($\Sigma[hits-misses]$). By this criterion, mice in which the influence
42 of perirhinal axons on L1 of neocortex was suppressed could not associate the
43 water reward with the μ Stim over the first training session but rather licked in
44 approximately 50% of the trials (average learning score 0.48 ± 0.06 normalized to
45 the total number of trials, $n=6$ in ctrl versus -0.03 ± 0.08 , $n=7$ in hM4Di/CNO-
46 treated mice; Wilcoxon rank-sum test, $p=0.0047$). Note, CNO alone¹⁵, i.e. without
47 expression of hM4Di, had no effect on learning ($n = 3$, Wilcoxon rank-sum test,
48 $p=0.4$; Extended Fig. 2). In contrast to control animals, untrained animals rarely
49 responded to μ Stim (-0.54 ± 0.05 , $n=5$ in untrained mice, Wilcoxon rank-sum
50 test, $p=0.0043$; Fig. 1d&e). After 3 sessions, trained animals had improved
51 learning scores (0.87 ± 0.04 at session 3, Wilcoxon rank-sum test, $p=0.02$) and
52 this was not affected by suppression of the perirhinal influence on S1 (0.84 ± 0.06 ,
53 $n=3$ in CNO-treated trained mice, Wilcoxon sign-rank test, $p=1$; Fig. 1e and
54 Extended Fig. 2). This suggests that perirhinal cortex is involved in early memory
55 formation but does not affect perception of the μ Stim per se. The second order
56 somatosensory thalamic area, POM, also projects to L1 in S1 and has been
57 implicated in different learning paradigms^{3-6,16}. To examine the influence of POM
58 input in μ Stim task, this time we expressed hM4Di receptors in POM in Gpr26-
59 cre transgenic mice¹⁷. Suppression of this projection from POM slightly affected
60 learning, however the effect was not significant (0.25 ± 0.05 , $n=7$, Wilcoxon rank-
61 sum test, $p=0.18$; Extended Fig. 2). Taken together, these results show that the
62 influence of the perirhinal cortex on L1 of the neocortex is crucial for learning
63 the μ Stim detection task.

64 The results of the chemogenetic experiments (Fig. 1) imply that activity in
65 perirhinal cortex influences activity in S1. Since the μ Stim electrode was most
66 effective when placed in L5¹², we reasoned that the stimulation at least affected
67 L5 neurons. Furthermore, L5 neurons have been implicated in perceptual
68 detection tasks and it has been recently shown that the output of these neurons
69 depends partly on the activation of their apical dendrites that project into L1¹⁸
70 where the perirhinal inputs arrive. We confirmed *ex vivo* that perirhinal inputs
71 arriving in L1 synapse on to the tuft dendrites of L5 pyramids (Extended Fig. 3).
72 To investigate the influence of PRh input in S1 activity we made juxtacellular
73 recordings from L5 in S1 in the same region as the μ Stim (Fig. 2a, left). We
74 recorded activity from S1 during learning with and without chemogenetic
75 suppression of perirhinal input to the cortical L1. As in the purely behavioral
76 experiments (see Fig. 1), hM4Di/CNO-treated animals also did not learn the task
77 over the first session (0.1 ± 0.04 , $n=4$, Wilcoxon rank-sum test against control,
78 $p=0.04$). Both the baseline (1 s before μ Stim) and post-stimulus (0.5-2.5 s after
79 μ Stim) AP firing rates in L5 pyramidal neurons of these animals ($n=4$, 52 cells,
80 826 trials) was significantly reduced in comparison to control animals ($n=2$, 28
81 cells, 706 trials) treated with CNO only (Wilcoxon rank-sum test, $p<0.001$ for
82 both baseline and post-stimulus; Fig. 2b-d). These results refer to 'Hit' trials
83 where the animals responded correctly to μ Stim although we found analogous
84 results in 'Miss' trials (Extended Fig. 3). There was no significant difference in
85 firing rate in control animals between baseline and post-stimulus activity and a
86 slight but significant reduction in hM4Di/CNO-treated animals (see also
87 Extended Fig. 3).

88 Previously, we found that animals are biased to respond to irregular firing
89 patterns in animals trained on a μ Stim task¹⁹ and that burst firing correlates with
90 perceptual detection¹⁸. We therefore examined burst firing of the same cells
91 during learning in control and hM4Di/CNO-treated animals where learning was
92 blocked. Here, we found that blocking of learning via suppression of perirhinal
93 input significantly decreased both baseline and post-stimulus burst rate
94 (Wilcoxon rank-sum test, $p<0.001$). Interestingly, the burst rate following μ Stim
95 compared to baseline was greatly increased in control animals (Wilcoxon sign-

rank test, $p=0.001$) but not in hM4Di/CNO-treated animals and only in 'Hit' trials (see Extended Fig. 3). We conclude that perirhinal input to L1 mediates learning-related increases in excitability and burst firing in neocortical L5 neurons.

What information does perirhinal cortex convey during μ Stim learning? To investigate this, we examined the firing of deep layer neurons in perirhinal cortex (Fig. 2a, right). We found that perirhinal neurons responded robustly to hit trials but not to miss trials after μ Stim in S1 (Fig. 2f&g; $n=6$ animals, $n=287$ trials in 28 neurons; firing rate: miss $-8\pm 11.2\%$ versus hit $30.9\pm 11.2\%$, $p<0.001$, Wilcoxon rank-sum test). Moreover, the responses in perirhinal cortex included an increase in burst rate compared to baseline only during hit trials (Wilcoxon sign-rank test, $p<0.001$). However, the relative change of burst rate between miss and hit trials was not significant (Fig. 2f&g; burst rate miss $14 \pm 21\%$ versus hit $55.9\pm 22.3\%$, $p=0.1$, Wilcoxon rank-sum test). This shows that the perirhinal cortex signals information related to 'Hit' trials primarily via increased AP firing during learning.

It has recently been shown that memory formation is accompanied by an increase in slow cortical oscillations²⁰⁻²³. We therefore also analyzed the local field potential (LFP) signals, taken from the same recordings in S1 and perirhinal cortex to assess cortical oscillations during learning. Theta power (4 - 8 Hz) in S1 was significantly higher in trained versus untrained animals (Extended Fig. 4; Wilcoxon rank-sum test, $p<0.0001$). Analogously, in perirhinal cortex, we found a significant increase in the theta power in 'Hit' compared to 'Miss' trials during learning (Extended Fig. 4; Wilcoxon rank-sum test, $p=0.002$). These results suggest that elevated theta power in perirhinal cortex correlates to a transition to elevated theta in response to μ Stim in S1 in expert animals.

Depolarization of the apical dendrites in L5 pyramidal neurons is shown to reliably lead burst firing behaviour²⁴⁻²⁶. Since learning correlated with an increase in burst firing in these neurons (Fig. 2e) that was dependent on perirhinal input to L1, we hypothesized that the mechanism of learning-induced bursting might involve an enhancement of synaptic influence to the tuft dendrites. We therefore examined Ca^{2+} -dependent activity in the apical

dendrites of L5 neurons in S1 using 2-photon microscopy in trained animals. To do this, we expressed GCaMP6f in Rbp4-Cre transgenic mice²⁷ and imaged at a depth of ~200 μm , the region of the apical dendrite known for initiation of dendritic Ca^{2+} activity⁷ (Fig. 3a-c). Calcium transients measured from 1 s before the μStim until 3 s after the μStim in 318 dendrites (Fig. 3d; $n = 4$ mice), revealed three populations with distinct fluorescence profiles (Fig. 3e). A small population (10%, “ON” dendrites) of dendrites showed substantial increases in fluorescence following μStim with another population (37%, “OFF” dendrites) of dendrites showing reduced Ca^{2+} fluorescence. The rest were not responsive to μStim (53%, “NR” dendrites).

We found similarly distinct and stereotypical output firing patterns in L5 neurons using juxtacellular recordings from trained animals (Fig. 3f; see Extended Fig. 5 for examples). In 11% of cells we saw a sudden and marked increase in firing (21.44 ± 42.16 Hz) briefly following μStim (Fig. 3f; L5 “ON” cells). In another population consisting of 40% of neurons, there was a decrease in firing (-6.39 ± 4.93 Hz) immediately following the μStim (Fig. 3f; L5 “OFF” cells). In most of the cells (49%), we observed no response to μStim (L5 “NR” cells). Interestingly, the baseline firing rate in L5 ON and L5 OFF cells was significantly higher than in NR cells (Wilcoxon rank-sum test, NR vs. ON: $p < 0.0001$, NR vs. OFF: $p < 0.0001$). In contrast to expert animals, we observed low firing rates over all neurons in untrained animals (Extended Fig. 6). Most L5 neurons in untrained animals did not respond to μStim at all (95%, $n=63/66$ cells) with a small population (5%, $n=3/66$ cells) responding with a small increase (6.9 ± 6.30 Hz) briefly after μStim . Taken together with the 2-photon dendritic recordings, we conclude that learning enhances the responsiveness of a small population of L5 pyramidal neurons to apical dendritic input.

To test whether dendritic activity influences learning, we optogenetically activated dendrite-targeting inhibitory neurons during the μStim training. Previous studies have implicated somatostatin (SST) positive interneurons in suppressing plasticity and learning via dendritic inhibition^{5,6,8,28,29}. We reasoned that if the same circuitry is activated during the μStim detection task, activating SST neurons with channelrhodopsin2 (ChR2) should also impair learning. We

159 activated SST neurons during training in SST::ChR2 mice using a 500 ms light
160 pulse starting 300 ms before μ Stim (Fig. 3g). This abolished learning in a manner
161 almost identical to removing the influence of perirhinal input to L1 (SST: $-0.16 \pm$
162 0.05 , Wilcoxon rank-sum test, $p=0.002$; Fig. 3h&i). Notably, continued activation
163 of SST neurons through subsequent training sessions prevented learning over
164 several sessions, unlike block of perirhinal input in which the animals eventually
165 became experts (Extended Fig. 7). Altogether these results suggest that the
166 emergence of a population of neurons underlying learned behavior in the μ Stim
167 task depends on a dendritic mechanism.

168 The correlation between both bursting and dendritic activity with
169 learning suggests that bursting might underlie memory retrieval in cortical
170 neurons. In order to test this hypothesis we devised another learning paradigm
171 in which we first trained animals to respond expertly to μ Stim and then
172 manipulated the firing of single neurons in S1 using single-cell stimulation
173 (“nanostimulation”^{12,19,30}) via a juxtacellular electrode (Fig. 4a). Expert animals
174 were significantly more likely to lick for reward if bursts of APs (80–120 Hz)
175 were elicited in a single L5 pyramidal neuron of S1 compared to false-positive
176 trials where no current was injected. However, response rate to a train of
177 regularly spiking APs (30–50 Hz) was not significantly different from false-
178 positive rate (Fig. 4b&c; Hit rate; false-positive: $25.94 \pm 3.6\%$, regular:
179 $28.11 \pm 4.07\%$, burst: $31.47 \pm 4.24\%$, $n=27$ cells, one-sided paired t-test, $p=0.03$).
180 This indicated that burst firing increased the downstream readout of the firing of
181 a single L5 pyramidal neuron leading to successful behavior. Since the learned
182 behavior could be recovered by burst firing in single pyramidal neurons, these
183 data suggest that burst firing observed in L5^{ON} neurons might be extremely
184 effective in memory recall.

185 Overall, we have shown that the perirhinal connection to L1 of neocortex
186 is crucial for learning a μ Stim detection task and involves the conversion of
187 neurons in neocortex to high-firing, burst mode correlated with an increase in
188 dendritic activity (Fig. 4d). This implies that the apical dendrites of L5 neurons
189 are the locus of plasticity related to memory consolidation. This idea is
190 corroborated by our previous study where we showed that stimulus detection in

191 S1 was dependent on dendritic activity in trained animals¹⁸ and was disrupted
 192 by inhibiting this activity. In addition, dendritic activity is shown to be generated
 193 by feedback signals from other cortical areas^{7,9} and is enhanced during
 194 learning³¹, suggesting that perirhinal input to L1 might serve as a gating signal
 195 for the enhancement of cortico-cortical feedback inputs (Fig. 4d). We conclude
 196 that the medial temporal input to the neocortex controls learning through a
 197 process in L1 that is encoded by dendritic calcium promoting burst firing as the
 198 neural signature of memory recall.

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320 **Methods**

321 **Animals.** All experiments and procedures were approved and conducted in
322 accordance with the guidelines given by Landesamt für Gesundheit und Soziales
323 Berlin. The following animal lines were used in this study: C57BL/6J wild-type
324 mice, Gpr26-cre transgenic mice¹⁷, SST::Chr2 transgenic mice (SST-IRES-Cre
325 mice (JAX stock #018973) were crossed with Ai32 mice (JAX stock #024109))³²
326 Rbp4-cre transgenic mice²⁷ and Wistar rats (Charles River). Male animals were
327 used except for 2 Rbp4-cre mice. The animals were housed in reversed 12 h
328 light/dark cycle (light on between 21:00 and 09:00) and all the behavioral
329 experiments were performed during dark period of the cycle.

330

331 **Retrograde and anterograde tracing.** For the retrograde labeling of S1
332 projecting perirhinal neurons, Fast Blue (25% in dH₂O, Polysciences) soaked in a
333 sterile piece of tissue was applied onto the surface of S1 for 10 min. Incubation
334 time was 7 days before transcardial perfusion. For anterograde tracing and
335 optogenetic *ex-vivo* experiments AAV-hSyn-hChr2(H134R)-EYFP (Penn Vector
336 Core) was injected in the PRh of > 2 weeks old C57/BL6 mice. Anesthesia was
337 induced and maintained with isoflurane at 5% and 2%, respectively. Mice were
338 placed in a stereotaxic frame and craniotomies were performed using stereotaxic
339 coordinates: anterior-posterior axis (AP) -1.8 mm, medial-lateral axis (ML) $\pm$ 4.1
340 mm, DV -4.2 mm from bregma. Injections were carried out using graduated
341 pipettes broken back to a tip diameter of 10-15 μ m, at a rate of $\sim$ 0.025 μ l/min
342 for a total volume of 0.05-0.07 μ l. Incubation time was at least 3 weeks before
343 transcardial perfusion or *ex-vivo* experiment.

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345 **YFP fluorescence analysis.** AAV-hSyn-hChR2(H134R)-EYFP containing acute
346 brain sections were imaged using an Olympus BX51 Microscope with a 4x
347 objective. Fluorescence intensity was quantified with ImageJ software by
348 plotting a line profile across the cortical layers that calculates the brightness
349 value. The average gray value of all images was then normalized to the negative
350 SEM of the lowest grey value across the average line profile.

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352 **Ex-vivo electrophysiology.** After 3-4 weeks of virus expression, sagittal or
353 coronal slices (300 μ m thick) were prepared from 35-50 day old C57/BL6 mice.
354 Whole-cell patch-clamp recordings were performed from visually identified
355 layer-5 pyramidal neurons using infrared (IR) Dodt-gradient contrast video
356 microscopy. The extracellular solution contained 125 mM NaCl, 25 mM NaHCO₃,
357 25 mM Glucose, 3 mM KCl, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, 1 mM MgCl₂, pH 7.4
358 at $\sim 33^{\circ}$ C. The intracellular solution contained 115 mM K⁺-gluconate, 20 mM
359 KCl, 2 mM Mg-ATP, 2 mM Na₂-ATP, 10 mM Na₂-phosphocreatine, 0.3 mM GTP,
360 10 mM HEPES, 0.05 mM Alexa 594 and biocytin (0.2%), pH 7.2. Whole-cell
361 voltage recordings were performed from the soma (4-6 M Ω) using a Multiclamp
362 700b (Molecular devices) amplifier. Data was acquired with an ITC-18 board and
363 analyzed using Igor software. Optogenetic synaptic stimulation was performed
364 via an LED (470 nm) (2 ms pulses) located in L1 around the tuft dendrite. To
365 activate hM4D_i receptor, Clozapine-N-Oxide (CNO) (Tocris Bioscience) was bath
366 applied (final concentration 10 μ M).

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368 **Chemogenetic manipulation of PRh axonal activity.** Mice (> 4 weeks) were
369 anesthetized with ketamine/xylazine (13 mg kg⁻¹/1mg kg⁻¹) by intraperitoneal

370 injection (i.p.). Animals were kept on a thermal blanket during entire surgery
371 and recovery. Lidocaine (1%, wt/vol, AlleMan Pharma) was injected around the
372 surgical site before the scalp incision. The periosteum was removed and small
373 craniotomy was made on the injection sites. Injection coordinates for PRh were
374 AP -1.8 mm, ML $\pm$ 4.2 mm, DV -4.2 mm and for POm were AP -2 mm, ML +1.2
375 mm, DV -3.0 mm from bregma. AAV1/2-hSyn1-hM4D(Gi)-mCherry-WPRE-
376 hGHP(A) (Viral Vector Facility of the University of Zurich) was injected either
377 bilaterally (n=16 for PRh and n=7 for Pom) or unilaterally (n=3 for PRh) (0.15 –
378 0.20 μ l per side). Further experiments were performed after 3 weeks of
379 expression.

380 In order to activate hM4Di receptor, CNO dissolved in extracellular
381 solution (10 μ M) was applied into superficial layers of S1 (initial depth at 150
382 μ m and over the day injection pipette was advanced (up to 300 μ m) in order to
383 compensate tissue growth on the craniotomy) at least 20 min before the
384 microstimulation training. CNO was applied into two adjacent sites (150 μ l each)
385 of the craniotomy to maximize the CNO diffusion area.

386

387 **Headpost implant and head-restraint habituation.** A lightweight aluminum
388 head-post (mouse) or a metal bolt (rat) was implanted on the skull of the animal
389 under ketamine/xylazine anaesthesia (13 mg kg⁻¹/1mg kg⁻¹ for mice, 100 mg kg⁻¹
390 1/ 5 mg kg⁻¹ for rats, i.p.). For mice used in chemogenetic experiments, the
391 implantation was performed > 10 days after viral injection. After the scalp and
392 periosteum were removed, a thin layer of light curing adhesives (OptiBond, Kerr
393 and Charisma, Kulzer) was applied to the skull. A head-post was fixed on the
394 skull on the left hemisphere with a dental cement (Paladur, Heraeus Kulzer).

395 Head-restraint habituation began > 3 days after the head-post
396 implantation. Habituation time at the first day was 5 min and then gradually
397 increased each day until the animal sat calmly for 1 h. Animals were water
398 restricted from the second day (1 ml/day) of the habituation and then trained to
399 receive the saccharin (Sigma-Aldrich) water (0.5% for mice and 0.1% for rats)
400 from the licking port. Licking was monitored using a piezo-based sensor attached
401 to the licking port. Weight and health of the animal were monitored daily.
402 Habituation for head restraint and licking typically took 5 days.

403 Two to three days before the microstimulation training or/and
404 juxtacellular recording, 1.5 mm x 1.5 mm craniotomy was made on the right
405 barrel cortex centered at AP 1.25 mm and ML 3.75 mm from bregma for mice
406 and AP 2.5 mm and ML 5.5 mm from bregma for rats. For perirhinal cortex
407 recording in rats, craniotomy was made on AP 4.5 mm and ML 5.0 mm from
408 bregma. Then a recording chamber was implanted for chronic access to this
409 region. The dura was left intact and the craniotomy was covered with silicon
410 (Kwik-Cast, World Precision Instruments).

411

412 **Optogenetic manipulations.** For optogenetic activation of SST neurons,
413 SST::ChR2 transgenic mice that express ChR2 in SST-positive cells were used.
414 Photostimulation light (465 nm, 2 mW, 500-ms pulse starting at 300 ms before
415 stimulus onset) was delivered via the optic fiber placed above the craniotomy.
416 To prevent the mice from distinguishing photostimulation trials from control
417 trials using visual cues, the recording chamber was covered with a black rubber
418 to prevent light leakage from photostimulation into the animals' eyes.

419

420 **Microstimulation detection task.** Animals were trained to perform
 421 microstimulation task as described elsewhere^{12,19,33}. Briefly, animals were
 422 trained to respond with tongue lick to a 200 ms train of microstimulation pulses
 423 applied to barrel cortex (40 cathodal pulses at 200Hz, 0.3 ms pulse duration)
 424 through a tungsten microelectrode (Microprobes) in depth of ~700 μm (mice) or
 425 ~1500 μm (rats) from pia and presented at random intervals. In the first session,
 426 initial intensity of 160 μA pulses were injected into the cortex and paired with a
 427 drop of water reward (pairing period). After 5 pairings, testing began where
 428 animals were rewarded only if they lick the licking port within 100 to 1,200 ms
 429 after stimulus onset. Tongue lick responses were detected with piezo-based
 430 sensor (mice) or beam breaker (rats). The time of the first lick after stimulus
 431 onset was taken as the reaction time. To encourage animals to use a
 432 nonconservative response criterion, we only mildly punished licks in the
 433 interstimulus interval with an additional 1.5 s delay to the next stimulus
 434 presentation. Once animals reached 80% hit rate, pulse intensity was gradually
 435 decreased during and over the sessions until it reached 10 μA (mice) or 5 μA
 436 (rats). Control mice reached to 10 μA within 3-5 days of training. Expert in this
 437 study means animals who performed the task with >80% hit rate at the 10 μA
 438 (mice) or 5 μA (rats).

439
 440 ***In vivo* juxtacellular recording.** Following head-restraint habituation,
 441 juxtacellular recordings were performed from deep layer neurons from S1 and
 442 PRh in awake head-fixed animals during μStim detection task. The glass pipette
 443 (4–8 M Ω) for juxtacellular recording during microstimulation task was filled
 444 with extracellular solution containing: 135 mM NaCl, 5.4 mM KCl, 1.0 mM MgCl₂,

445 1.8 mM CaCl₂ and 5 mM HEPES (pH 7.2). The juxtacellular signal was amplified
446 and low-pass filtered at 3 kHz by a patch-clamp amplifier (NPI) and sampled at
447 25 kHz by a Power1401 data acquisition interface under the control of Spike2
448 software (CED). For PRh recording in rats, the pipette was inserted with 17°
449 toward lateral and 50° toward anterior. The mean depth in juxtacellular
450 recording of S1 in mice was 1156.8±25.56 mm and of PRh in rats was 6339.64
451 ±122.07 mm, which is likely an overestimate of the true depth due to oblique
452 penetrations and dimpling.

453

454 **Two-photon Ca²⁺ imaging.** For *in vivo* two-photon calcium imaging, AAV2/1-
455 Syn-Flex-GCaMP6f-WPRE (Penn Vector Core) was injected through a glass
456 pipette (tip diameter, 5–10 μm) into the left S1 barrel cortex on the basis of
457 stereotaxic coordinates (AP -1.5 mm and ML 3.2 mm from bregma). A single
458 injection (100 nl) was made at 700 μm deep from the pial surface. Three weeks
459 after the injection, a 3-mm craniotomy was made over the injection site and
460 sealed with a 3-mm glass coverslip (#1) with cyanoacrylate glue. A light-weight
461 head-post was fixed on the skull in the right hemisphere with light-curing
462 adhesives and a dental cement. Habituation of mice to head restraint and
463 following imaging experiment begin 4 weeks after the virus injection.

464 Imaging from behaving mice was performed with a resonant-scanning
465 two-photon microscope (Thorlabs) equipped with GaAsP photomultiplier tubes
466 (Hamamatsu Photonics). GCaMP6f was excited at 940 nm (typically 30–40 mW
467 at the sample) with a Ti:Sapphire laser (Mai Tai eHP Deep See, Spectra-Physics)
468 and imaged through a 16×, 0.8 NA water immersion objective (Nikon). Full-
469 frame images (256 × 256 pixels) were acquired from apical dendrites of L5

neurons expressing GCaMP6s at a depth of 150–200 μ m at 58.6 Hz using ScanImage 4.1 software (Vidrio Technologies). Tungsten electrodes for microstimulation was inserted through the access port on the chronic glass window.

Histology. Animals were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were removed and post-fixed for > 24 h. Coronal sections (150 μ m thick) were collected using a vibratome. For DAPI staining, NucBlue (Invitrogen) was applied to sections in PBS for 10 min. The sections were imaged with an epifluorescence microscope or a confocal microscope.

Single-Neuron Stimulation Detection Task. Once rats performed at current intensities below 5 μ A on 2 consecutive days, we switched to single-cell stimulation experiments, as previously described^{12,33}. Briefly, the animals were head fixed during the task, and waited for the microstimulation/nanostimulation detection task to begin, which it did when a neuron was found. The glass pipette for juxtacellular single-cell stimulation and recording was glued to a tungsten microelectrode used for microstimulation at a distance of ~70 μ m, as described elsewhere^{12,33}. The glass pipette was filled with intracellular solution containing: 135 mM K-gluconate; 10 mM HEPES; 10 mM Na₂-phosphocreatine; 4 mM KCl; 4 mM MgATP; and 0.3 mM Na₃GTP (pH 7.2). Recording depth was 1902±60.73 μ m, which is likely an overestimate of the true depth due to oblique penetrations and dimpling.

494 During single-cell stimulation trials, a fixed duration square-wave current pulse
 495 was injected into a neuron through a glass pipette. Every stimulation sequence
 496 contained each step exactly once, while their order was varied pseudo-randomly
 497 from trial to trial. To induce a regular spike pattern, we used a single 100 ms DC
 498 current step. To elicit burst like spike pattern, brief stimulation duration of 25
 499 ms was used, followed by 1175 ms inhibition at current intensities of 50% used
 500 in the nanostimulation, to prevent any further spikes during the stimulation trial.
 501 Single-cell stimulation trials, catch trials without current injection and
 502 microstimulation trials were pseudo randomly interleaved in series of 6 trials
 503 including 3 microstimulation trials, 2 single-cell stimulation trials (each of
 504 different duration) and 1 catch trial. All trials were presented at random
 505 intervals (Poisson process, mean 3 s). Microstimulation currents were adjusted
 506 (range 3-8 μ A, mean 4.2 ± 1.1 μ A (s.d.)) such that animals performed close to the
 507 detection threshold, resulting in an average microstimulation hit rate of 90%.

508

509 **Data analysis and statistics.** Recorded neurons were separated into putative
 510 fast-spiking (FS) interneurons and regular-spiking (RS) pyramidal neurons
 511 based on spike half-width and firing rate. Cells with spike half-width lower than
 512 0.5 ms and firing rate higher than 8 Hz were classified to FS. Only RS were used
 513 for further analysis.

514 All the cells and trials recorded over days were pooled together for
 515 comparing activity (firing rate or burst rate) changes during miss and hit trials.
 516 Bursts were identified as at least two spikes with an inter-spike interval of ≤ 15
 517 ms. Time window between 1 and 0 s before the stimulus $[-1 \ 0]$ s) was used to
 518 calculate the baseline activity and 0.5 – 2.5 s $[+0.5 \ +2.5]$ s) after stimulus was

519 used to calculate post-stimulus activity. For firing rate and burst rate change
520 analysis, difference between pre-stimulus frequency and post-stimulus
521 frequency was divided by average pre-stimulus frequency.

522 All analysis for Ca^{2+} imaging was performed using imageJ and custom
523 written codes in Matlab. Horizontal and vertical drifts of imaging frames due to
524 animal motion were corrected by registering each frame to a reference image
525 based on whole-frame cross-correlation. The reference image was generated by
526 averaging any given consecutive 100 frames in which motion drifts were
527 minimal. Regions of interest (ROIs) for apical dendrites of L5 neurons were
528 manually selected with the help of average intensity and standard deviation
529 projections across movie frames. For each ROI, pixel values inside the ROI were
530 averaged to obtain the time series of Ca^{2+} fluorescence. The extracted signals
531 were corrected for neuropil contamination by subtracting the local, peri-
532 dendritic neuropil signals. Fluorescence change ($\Delta F/F_0$) was calculated as
533 $(F - F_0)/F_0$, where F_0 was the baseline fluorescence value in the ROI throughout
534 the whole imaging session.

535 For local field potential (LFP) analysis, juxtacellularly recorded voltages
536 were band-pass filtered at 4-30 Hz and a power spectrum was calculated using
537 the Stockwell Transform^{34,35}, over a 2 s period before stimulus onset and 5 s
538 period afterwards. In order to avoid potential artifacts caused by stimulation, the
539 analysis was restricted to microstimulation or nanostimulation trials where the
540 following trial occurred more than 5 s after stimulus onset. The power spectrum
541 was calculated separately for individual trials and its absolute magnitude
542 averaged within the different response categories (hits and misses). To obtain

the population spectra for the different response categories the power spectra of individual trials were then averaged.

For the classification of cells, peri-stimulus time-histograms (PSTHs) were calculated for each cell by averaging spikes in time bins of 100 ms for times within 2 seconds of hit-trials. For each cell, the stationary rate and standard deviation were computed based on the PSTHs in the period [-2,0] s. Cells were classified to ON cell or OFF cell if PSTHs in the period [0.3,0.4] s was either more than 3*standard deviation (SD) above the stationary rate, or less than 3*SD below, respectively. Other cells were classified as NR cells. Similarly, dendrites were classified into ON, OFF and NR dendrites.

Unless otherwise stated, all values are indicated as mean $\pm$ SEM. Shapiro-Wilk test was performed to test normality of the data. For non-parametric test, significance was determined using Wilcoxon signed-rank test within group and Wilcoxon rank-sum test between groups at a significance level of 0.05. No statistical tests were run to predetermine sample size, and blinding and randomization were not performed.

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594 Main figures

595
596 **Figure 1 | Perirhinal projection to neocortical L1 is necessary for learning a**
597 **μ Stim task.** **a**, Left, sagittal view of the rodent brain showing perirhinal
598 projection to primary somatosensory cortex (S1). Lower left, retrograde tracing.
599 Deep layer neurons in perirhinal cortex (PRh) were labeled with Fast Blue after
600 application of dye to L1 of S1. Lower right, anterograde tracing. Chr2/EYFP
601 labeled axons of PRh project strongly to L1 of S1. Right, simplified connectivity
602 map between the neocortex, perirhinal cortex, entorhinal cortex and
603 hippocampus. Investigated connection is highlighted in green. Note that not all
604 the connections are shown here. **b**, Schematic of μ Stim detection task. Tungsten
605 electrode was placed in L5 of S1. Animals learned to respond by licking within a
606 1.1-s window following μ Stim. **c**, Schematic of chemogenetic silencing of PRh
607 axons in L1 of S1 during μ Stim task. AAV.hM4Di was injected to PRh and CNO
608 was applied in superficial layer of S1 before μ Stim task (see Methods). Inset,
609 enlarged view of superficial layer of S1. Red shade represents CNO effective area.
610 **d**, Cumulative learning curve of control mice (black), mice with PRh axonal

611 suppression (hM4Di; red) and untrained mice (grey) during 150 trials in the first
612 session. Light lines represent individual mouse. Bold lines with error bars
613 represent the mean and SEM, respectively, of each group. **e**, Last value of
614 cumulative learning curve normalized by total number of trials (Norm. learning
615 score) during learning and after learning (Trained). Wilcoxon rank-sum test
616 against control, * $p < 0.05$, ** $p < 0.01$.
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Figure 2 | Learning-induced burst firing in layer 5 pyramidal neurons in S1 is PRh-dependent. **a**, Schematic of juxtacellular recording of L5 pyramidal neurons in S1 (left) and deep layer pyramidal neurons in PRh (right). Red shade: CNO targeted area, rf: rhinal fissure. **b, c**, Representative raster plot (upper) and PSTH (lower) during hit trials in L5 pyramidal neurons in control S1 and hM4Di S1, respectively. Bursts are marked by yellow ticks in the raster plot. Gray box: μ Stim. Note that y-axis scales differ for visibility. **d, e** Firing rate and burst rate during hit trials in control S1 and in hM4Di S1, respectively. **f**, Representative raster plot (upper) and PSTH (lower) during hit trials in perirhinal neuron. **g**, Relative change of firing rate and burst rate during miss and hit trials from PRh neurons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3 | Dendritic activity-dependent emergence of distinct L5 subpopulations after learning. a, Two-photon Ca^{2+} imaging from the apical dendrites of L5 pyramidal neurons in Rbp4-cre mice during μStim task. DiI shows the location of μStim electrode. **b**, Z-stack image of recorded dendrites and μStim electrode in L5. **c**, Horizontal imaging plane (upper) ($\sim 200 \mu\text{m}$ from pia) and average Ca^{2+} responses (lower) for all trials from a dendrite marked with a yellow arrow. **d**, Ca^{2+} responses in an apical dendrite marked in **c**. during 180 trials of μStim task. **e**, Left, Average peri-stimulus time Ca^{2+} responses in ON, OFF and NR dendrites (total $n=318$ dendrites) during hit trials (See Methods for classification criteria). Gray box: μStim . Right, the fraction of ON, OFF and NS dendrites. **f**, Left, Average PSTH of L5^{ON} , L5^{OFF} and L5^{NR} neurons (total $n=272$ cells) during hit trials (See Methods for classification criteria). Gray box: μStim . Right, the fraction of L5^{ON} , L5^{OFF} and L5^{NR} neurons. **g**, Schematic of optogenetic activation of SST+ interneurons during μStim task in SST::ChR2 mice. Blue light (465 nm) was shed on the surface of the craniotomy (See Methods). Inset, magnified view of the targeted circuit in S1. **h**, Cumulative learning curve of control ($n=6$, black) and SST::ChR2 mice ($n=6$, dark blue) during the first session. **i**, Normalized learning score of control and SST::ChR2 mice at the first session. Wilcoxon rank-sum test, ** $p<0.01$.

Figure 4 | Burst firing in single L5 pyramidal neurons can retrieve learned behavior. **a**, Schematic of single-cell stimulation. Inset, μStim stimulates a population of neurons surrounding electrode (red shade) and glass electrode stimulates a single neuron (triangle). **b**, Current injection protocol to induce either regular AP firing (30 Hz, black) or high frequency (80-120 Hz, yellow) bursts in single cells. **c**, Response rates (hits) for regular AP firing (black) or burst firing (yellow) trials versus false-positive trials (n=27 cells). One-sided paired t-test. **d**, Gating theory of memory formation in cortex. PRh inputs to L1 gate long-range inputs that modulate the firing mode of L5 pyramidal neurons in S1 and learning.