Transgenic Inhibition of Synaptic Transmission Reveals Role of CA3 Output in Hippocampal Learning

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The hippocampus is an area of the brain involved in learning and memory. It contains parallel excitatory pathways referred to as the trisynaptic pathway (which carries information from the entorhinal cortex → dentate gyrus → CA3 → CA1 → entorhinal cortex) and the monosynaptic pathway (which connects entorhinal cortex → CA1 → entorhinal cortex). We developed a generally applicable tetanus toxin-based method for transgenic mice that permits inducible and reversible inhibition of synaptic transmission and applied it to the trisynaptic pathway while preserving transmission in the monosynaptic pathway. We found that synaptic output from CA3 in the trisynaptic pathway is dispensable and the short monosynaptic pathway is sufficient for incremental spatial learning. In contrast, the full trisynaptic pathway containing CA3 is required for rapid, one-trial contextual learning, for pattern completion-based memory recall and for spatial tuning of CA1 cells.

The medial temporal lobes of the brain, including the hippocampus, are crucial for learning and memory of events and space across species (1–3). The hippocampus receives input from virtually all associative areas of the neocortex via the entorhinal cortex (EC). In the main excitatory hippocampal network (Fig. 1A) information flows from the superficial layer (layer II) of EC to the dentate gyrus (DG) to CA3 to CA1, and finally to the deep layers of EC directly or indirectly through the subiculum (Sub). This loop is referred to as the “trisynaptic pathway (TSP)”. The hippocampus also contains a parallel excitatory “monosynaptic pathway (MSP)” [EC (layer III) → CA1 → EC (layer V)] as well as other excitatory and inhibitory circuits.

The prevailing view of the contribution of these circuits to hippocampal function (4–7) is that synaptic transmission and plasticity in the feed-forward pathway from EC → DG → CA3, a part of TSP, is primarily responsible for pattern separation, while those in a recurrent network within CA3 is crucial for the rapid association of diverse sets of information and pattern completion. Furthermore, CA1 may be instrumental in recognizing novelty of an event or context (8, 9).

Some of these ideas have been tested by lesioning (10) portions of the hippocampus or EC, although it is difficult to restrict damage to specific subregions and cell types in a quantitative and reproducible manner (11, 12). These difficulties have in part been addressed by deleting the N-methyl-D-aspartate (NMDA) receptor gene, NR1, in specific hippocampal subregions with Cre-lox P recombination technology. These studies found that NMDA receptor-dependent synaptic plasticity in postnatal excitatory neurons of each of several hippocampal subregions is required for specific aspects of hippocampal learning and memory (13–16). In order to completely analyze hippocampal function, we developed a method to block neural transmission rather than synaptic plasticity and used it to assess the differential role of CA3 and EC outputs into area CA1 in hippocampus-dependent learning and memory.

We generated a triple transgenic mouse (Fig. 1B) by doxycycline-inhibited circuit exocytosis-knock down (DICE-K) in which synaptic transmission is blocked by cell type-restricted and temporally controlled expression of the tetanus toxin (TeTX) light chain (17). TeTX is an endopeptidase specific for VAMP2 (18), which is essential for activity-dependent neurotransmitter release from presynaptic terminals (19). The rationale for this general method is described fully in SOM.

We employed the KA1 promoter (14) and α-CaMKII promoter (20) for the transgenic1 (Tg1) and transgenic2 (Tg2) mice, respectively, to block CA3 output in the TSP while keeping EC output in the MSP intact (Fig. 1B). Prior to generating the triple transgenic TeTX mouse line, we investigated several parameters of the DICE-K method by crossing the Tg1xTg2 double transgenic mouse with a Tg3-GFP reporter line (CA3-GFP) (Fig. 1B). Immunohistology (Fig. 1, C to H) indicated that GFP expression was restricted to CA3 and DG in mice maintained on a Dox-free diet (Fig. 1, C to E). There is no expression of GFP in the CA1

[Diagram of hippocampal network not included in text]
pyramidal cell layer (s. pyramidale) or TA pathway (s. lacunosum moleculare), but abundant expression in the SC pathway (s. radiatum and s. oriens) (Fig. 1E). In Tg1xTg2 mouse, the spatial restriction was much greater than in Tg1 (fig. S1). GFP expression is repressed in the Dox-on state (Fig. 1F), de-repressed in Dox-on-off state (Fig. 1G), and repressed in the Dox-on-off-on state (Fig. 1H).

We crossed Tg1xTg2 double transgenic mice with Tg3-TeTX mice to produce a triple transgenic mouse, CA3-TeTX. In hippocampal slices from control double transgenic mice (Tg1xTg3-TeTX), VAMP2 immunoreactivity (IR) was observed where axonal terminals are known to exist (Fig. 1I and 1M). Hippocampal VAMP2-IR patterns were indistinguishable between repressed CA3-TeTX and control mice (Fig. 1J). In hippocampal slices from CA3-TeTX mice that had been on Dox followed by four weeks of Dox withdrawal, there was a striking reduction of VAMP2-IR in s. radiatum and s. oriens of CA1 and CA3 and in the inner one-third of the molecular layer (ML) of DG, but not in other strata (Fig. 1K).

Similar patterns of VAMP2-IR were observed in hippocampal slices throughout the dorso-ventral axis. The CA3-SC innervates CA1 in the s. radiatum and s. oriens, while CA3-RC innervates CA3 in these strata. The inner one-third of ML is where mossy cells (MC) innervate DG granule cells (21). Although the triple transgenic GFP mice showed moderate GFP-IR in DG granule cells (Fig. 1G), there was no significant reduction of VAMP2-IR in s. lucidum of CA3-TeTX mice where MF from DG granule cells innervate CA3 (Fig. 1K). These results indicate that, in the hippocampus of de-repressed CA3-TeTX mice, synaptic transmission should be impaired at SC-CA1 synapses, at the CA3-RC synapses, and possibly at MC-DG granule cell synapses, but not at MF-CA3 synapses. There was no indication of VAMP2-IR reduction in the s. lacunosum moleculare where the TA axons synapse onto CA1 neurons, suggesting that TA synaptic transmission remains intact (Fig. 1K). In CA3-TeTX mice that underwent three weeks of Dox withdrawal followed by seven weeks of Dox re-administration, the VAMP2-IR distribution was similar to that of repressed CA3-TeTX mice (Fig. 1J), indicating that TeTX-mediated blockade of synaptic transmission is reversible (Fig. 1L).

We characterized the input-output relationship of SC and TA inputs to a common population of postsynaptic CA1 neurons using extracellular field recordings. We found no significant genotype-specific effect on SC or TA inputs in repressed mice kept chronically on Dox (Fig. 2A). CA3-TeTX mice raised on Dox and shifted to Dox-off for one, two, three, four, or six weeks, showed a sharp drop-off in synaptic transmission at SC inputs between two and three weeks after Dox withdrawal (fig. S2). At four weeks post-Dox withdrawal, synaptic transmission was impaired at SC inputs, but remained intact at TA inputs (Fig. 2B). Residual synaptic transmission at SC inputs in these mice failed to elicit population spikes in the field excitatory postsynaptic potential (fEPSP) at any stimulation intensity (Fig. 2, B and C insets and table S2) and in response to high-frequency stimulation (data not shown). Synaptic transmission at SC inputs was restored by a re-administration of Dox for six weeks (Fig. 2C) confirming the reversibility of the DICE-K method. Based on these results, we used four weeks of Dox withdrawal (de-repressed mice) in most behavioral and in vivo electrophysiological studies. De-repressed CA3-TeTX mice exhibited no detectable abnormalities in the hippocampal cytoarchitecture (fig. S3) (22), or in locomotor activity, anxiety, motor coordination or pain sensitivity (figs. S4 and S5).

We subjected de-repressed CA3-TeTX mice to the Morris water maze (MWM) task (16). The latency curves of these and control mice were indistinguishable (Fig. 3A) (see SOM for statistics of this and following experiments). Memory recall was tested by probe trials on days 6 and 11. On day 6, there was only a slight preference for the target quadrant (Fig. 3B) and the target platform location (Fig. 3, C and D) in both CA3-TeTX and control animals, and no difference between the two genotypes. On day 11, the preference was robust in both genotypes for both criteria (Fig. 3, B to D), but again no robust difference between genotypes.

To test a possible role of CA3 output in rapidly forming representation of a novel context in the hippocampus, we subjected CA3-TeTX mice to a contextual fear conditioning (CFC) task using a novel context. De-repressed CA3-TeTX mice exhibited less freezing than control littermates (Fig. 3, E and F). The context specificity of conditioning was comparable between genotypes (fig. S6A), as was the level of tone fear conditioning (fig. S6B). The freezing deficit observed while the mice were in the Dox-on-off state (fig. S7C and D) was absent when the same mice were reconditioned in another chamber and tested after six weeks of Dox re-administration (Fig. 3, G and H), demonstrating the reversibility of the DICE-K method at the behavioral level. When the de-repressed CA3-TeTX mice habituated to the chamber prior to receiving a footshock, they still tended to freeze less than the control littermates, but the difference between the two genotypes was not significant (Fig. 3, I and J).

Our earlier study implicated NMDA receptor-dependent synaptic plasticity in CA3 pyramidal cells in pattern completion-based recall (14). To examine whether CA3 output in TSP is crucial for this form of recall we subjected CA3-TeTX mice to the pre-exposure mediated contextual fear conditioning (PECFC) paradigm (23, 24). De-repressed CA3-TeTX mice exhibited less freezing than control littermates, unlike repressed CA3-TeTX mice (fig. S8). To
test whether de-repressed mice are defective in the recall phase, we habituated CA3-TeTX mice to the chamber under Dox-on conditions to ensure the formation of a contextual representation and then switched them to Dox-off conditions. Four weeks later, the animals were returned to the chamber for a 10-s exposure followed by a footshock. CA3-TeTX mice displayed a deficit in freezing when tested on the following day (Fig. 3K), indicating that CA3 output in TSP is crucial for pattern completion-based recall.

To address a possible role of CA3 output in TSP in the detection and encoding of novel space, we recorded CA1 ensemble activity using multi-tetrode recordings (25) as freely moving mice completed 10 laps on a novel linear track (day 1). During this first experience, we observed a significant increase in average firing rate of CA1 pyramidal cells in de-repressed CA3-TeTX mice, which accompanied a significant decrease in spatial tuning of these cells and spatial information (Fig. 4, A to E, and table S1). There were no differences in peak firing rate, bursting properties of these cells, or spike width (table S1). No differences were found in average firing rates of inhibitory interneurons recorded from CA3-TeTX and control littermates (table S1), suggesting that coding deficits are not due to a loss of feed-forward inhibition from CA3. The mice were then returned to the same linear track 24 (day 2) and 48 (day 3) hours following the initial exposure. Place fields remained larger and spatial information was less in CA3-TeTX mice compared to controls (Fig. 4, A to E), indicating that CA3 output is crucial for spatial tuning not only if a novel but also familiar track. Earlier work with CA3-lesioned rats reported a much milder impairment, if any, in a familiar environment (12). We found a decrease in place field size and average firing rate along with an increase in spatial information between days 1 and 2 in CA3-TeTX mice, while no difference was found between days in control mice (Fig. 4, A to E).

The spatial restriction and temporal control over the expression of the transgenic tetanus toxin gene of the DICE-K method permits a greater degree of specificity in silencing neural pathways than possible with traditional lesion or pharmacological methods. Several new genetic methods allow inactivation or activation of specific neurons by manipulation of ligand- or light-activated cell surface receptors or channels to permit rapid inactivation or activation of cells on the subsecond-to-minute time scale. Hence, these are useful for studying relatively fast processes like perception and short-term memory (26–30). In contrast, the kinetics of the DICE-K system are too slow for studying fast cognitive processes. Instead, this method can dissect the contribution of specific synaptic inputs to processes occurring over hours to weeks, such as intermediate- to long-term explicit memories, and skill and habit learning. Because doxycycline can cross the blood brain barrier, the DICE-K method can be used without complications from direct, continuous injections of impermeable ligands into the brain or invasive deep brain light delivery.

Our data show that CA3 output in TSP is dispensable for both acquisition and recall of incremental spatial learning and memory recall in the MWM task. The nearly identical latency curves and probe trial behaviors of CA3-TeTX and control mice, along with the lack of thigmotactic behavior, indicate that CA3-TeTX mice indeed employed an allocentric, spatial strategy to locate the platform (Fig. 3D). Thus, it is likely that direct EC input to CA1 in the MSP can support these mnemonic processes. Our data contradict those of earlier studies conducted with rats with chemical or physical lesions in which the integrity of CA3 or CA3 output was crucial for acquisition and/or recall in the MWM task (11, 12). Although this contradiction may be due to species or protocol differences, it is more likely a result of the greater specificity of our DICE-K method. Tuned spatial and navigational information is present in the superficial layers of EC, which may provide CA1 the necessary information for spatial learning (31, 32). Additionally, although CA1 neurons are poorly connected by recurrent collaterals, MSP does form a closed loop ECIII → CA1 → ECV → ECIII (21) that may associate diverse spatial information, albeit less rapidly and less efficiently than the direct and robust recurrent network of CA3. Further, plasticity at MSP synapses of CA3-TeTX mice may mediate experience-dependent improvement in the MWM.

Our data also show that CA3 output is crucial for rapid one-trial learning in a novel context. It is thought that, for CFC to occur, a representation of various contextual features must first be formed in the hippocampus and then must be conveyed to the amygdala via CA1 and/or subiculum to be associated with the footshock representation (33). Evidently the MSP cannot provide these functions. Interestingly, the CFC deficit is reduced when animals habituate to the context prior to delivery of the footshock. Thus, with sufficient experience MSP seem to be able to fulfill a representation-forming and -conveying function.

Our in vivo recordings also illustrate a dichotomy between the ability of TSP and MSP to support learning in novel and familiar space. The greatly reduced spatial tuning of CA1 pyramidal cells in CA3-TeTX mice on a novel track suggests that information contained in CA3 output is critical for rapid formation of a high-quality spatial representation and is consistent with the CFC deficit in a novel context. Further, the unexpectedly higher firing rates in CA3-TeTX mice under novel conditions suggests that in addition to providing spatial information, CA3 output may also help maintain appropriate levels of network excitability during novelty. On the other hand, the reduced deficit of spatial tuning during days 2 and 3 visits to the same track indicates that the MSP alone can
improve CA1 spatial tuning by experience, which is consistent with the reduced CFC deficit in a familiar context (Fig. 3, I and J) and the normal MWM performance (Fig. 3, A to D).

Thus application of the DICE-K method to CA3 pyramidal cells directly demonstrates that the MSP (which bypasses CA3) can support slow incremental learning in familiar environments but that the CA3 output of the TSP is needed for rapid acquisition of memories in novel environments and for pattern completion-based recall.

References and Notes
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Supporting Online Material
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Materials and Methods
SOM Text
Figs. S1 to S8
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References

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**Fig. 1.** Excitatory hippocampal-EC pathways and the DICE-K method applied to TSP. (A) Excitatory pathways in hippocampal formation and entorhinal cortex. MC: mossy cells. Green and red arrows designate trisynaptic (TSP) and monosynaptic (MSP) pathways, respectively. (B) Tg1; a Cre transgenic line under control of the transcriptional regulator from kainate receptor 1 (KA-1) gene. Tg2; a tTA transgenic line under control of the αCaMKII promoter and Cre-loxP recombination. Tg3-TcTX and Tg3-GFP; TcTX and GFP transgenic lines, respectively, under control of the tetracycline (Tet) operator. (C and D) Double immunofluorescence staining of coronal sections from a Tg1xTg3-GFP control mouse (C) and chronically de-repressed CA3-GFP mouse (D) with antibodies specific for GFP (green) and for a cell nuclei marker, DAPI (blue). (E) Triple immunofluorescence staining of a hippocampal sagittal section from a chronically de-repressed CA3-GFP mouse with antibodies specific for GFP (green), DAPI (blue) and Netrin-G1 (red, a marker for TA and lateral perforant axons) (34). Outer one-third of DG dendrites stain yellow because they are positive for both Netrin-G1 (red) and GFP (green). (F to H) DAPI and GFP double staining of a hippocampal section from a chronically repressed CA3-GFP mouse (F),
followed by two weeks of Dox withdrawal (G), followed by two weeks of Dox re-administration (H). (I to L) Immunofluorescence staining with VAMP2 antibodies of a hippocampal section from control mice that have been on Dox diet (I). VAMP2 staining of a section from a CA3-TeTX littermate raised on Dox diet (J) and after four weeks of Dox withdrawal (K). CA3-TeTX mouse having undergone three weeks of Dox withdrawal followed by seven weeks of Dox re-administration (L). (M) Locations of various hippocampal strata.

**Fig. 2.** Input-output relationships of SC and TA inputs to CA1 in CA3-TeTX (TG) and control littermates (CT). (A) Repressed (Dox-on) mice. (B) De-repressed (Dox on-off) mice after 4 weeks of Dox withdrawal. (C) De-repressed (Dox-on-off: 3+1w) and re-repressed (Dox-on-off-on: 3+6w) mice. Sample traces are representative of recorded mean maximal fEPSP slopes. Note the absence of population spikes in TG traces of (B) and (C). SC scale bar: 4 mV/2 ms; TA scale bar: 0.4 mV/4 ms. All statistics are given in SOM.

**Fig. 3.** Morris water maze and contextual fear conditioning. (A to D) Performance in MWM of CA3-TeTX (TG) and double transgenic (Tg1xTg3-TeTX) control littermates (CT) having undergone 4 weeks of Dox withdrawal. (A) Averaged latencies. (B) Probe trials by relative quadrant occupancy time. (C) Numbers of platform crossings. Quadrant designations: TA, target; OP, opposite; L/R, left/right to target. (D) Heat maps of average search time during probe trials. (E and F) CFC in a novel context of de-repressed mice (4 weeks off Dox). (E) Kinetics of averaged freezing. (F) Freezing averaged over the 3 minute test session. (G and H) CFC in a novel context of re-repressed mice (3 weeks off Dox followed by 6 weeks of Dox re-administration). (G) Kinetics of freezing. (H) Freezing averaged over the first 3 minute test session. (I and J) CFC in de-repressed mice (4 weeks off Dox) after 3-day familiarization (10 min/day) to the conditioning chamber. (I) Kinetics of freezing. (J) Freezing averaged over the first 3 minute session. (K) PECFC of mice having undergone Dox diet schedules indicated on top. Kinetics of averaged freezing (left). Freezing averaged over the 5 minute test session (right). CS; pre-exposure, US; footshock. All statistics are given in SOM.

**Fig. 4.** CA1 place cells. (A) Representative examples of CA1 firing rate maps in novel (day 1) and familiar (day 3) environments from CA3-TeTX (TG) and their double transgenic control littermates (CT) having undergone 4 weeks Dox withdrawal, as mice completed 10 laps on a linear track. Colors scaled to peak firing rates (in Hz) indicated at the top right of each map (blue, minimum; red, maximum). (B) Size of CA1 place fields, determined by percentage of space where cells fire on the track for each day. (C) Average firing rate of all place cells plotted for each day. (D) Average spatial information (see SOM) for each day. (E) Spatial information for individual cells in novel (day 1) and familiar (day 3) environments. All statistics are given in SOM. In (B) to (D), stars above filled circles indicate highly significant (**) or significant (*) differences between TG and CT. Double stars at the brackets indicate highly significant differences between TG day 1 and TG day 2.