Title: Transection of the ventral hippocampal commissure impairs spatial but not contextual memory in split-brain mice

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Author Contributions: JJ conceptualized the study. JJ and CP designed the experiments. JJ and YT performed the experiments. JJ analyzed the data. JJ and CP wrote the manuscript.

Abstract

The hippocampus is essential for spatial and contextual memory; however, little is known about the functional role of inter-hemispheric communication between hippocampi. We found that split-brain mice, with a severed hippocampal commissure, were impaired in spatial learning and memory. Unexpectedly, split-brain mice were not impaired in hippocampus-dependent contextual fear memory. Thus, interhemispheric communication is not required for hippocampus-dependent memory *per se*, but instead may be necessary for binding composite memories of events to spatial coordinates.

Decades of experiments have described interhemispheric information exchange and its role in cognition via examination of the corpus callosum^{1,2}. However, very little is known about the function of the ventral hippocampal commissure (VHC), an interhemispheric pathway that directly connects the left and right hippocampi in rodents³. The mouse hippocampus is lateralized in NMDA receptor subunit densities^{4,5}, microanatomy⁵, and synaptic plasticity^{6,7}. Further, functional hemispheric specialization of the hippocampus has been reported in rodents⁷⁻¹⁰ and humans¹¹⁻¹³. It is not known whether integration of hippocampal lateralized processing occurs and whether such integration is required for hippocampal function.

To address this, we severed interhemispheric pathways in C57BL6J mice (Fig. 1a,b). SHAM surgeries consisted of a rostral to caudal incision in the cortex immediately left or right of the superior sagittal sinus, dorsal to the corpus callosum, leaving inter-hemisphere fibers intact. In CC mice, corpus callosal fibers (CC) were sectioned in addition to overlying cortex. In VHC+CC mice, the VHC and overlying corpus callosum and cortex were sectioned (Fig. 1b, and Supplementary Methods). We then compared hippocampal-dependent short- and long-term spatial memory and contextual fear memory across treatments. We performed a short-term memory version of the spatial Y-Maze task (Fig. 2a)⁷. During encoding, mice were allowed to explore two of the three-arms of a Y-Maze, with access to one arm blocked off, for two minutes. After a 1-minute intertrial interval, all arms were accessible and we measured time spent exploring the familiar arm versus the novel arm during a two-minute retrieval trial. SHAM and CC mice exhibited a preference for the novel arm over the familiar arm during the retrieval session, whereas VHC+CC mice showed no preference (Fig. 2b) suggesting they did not remember which arm they had previously experienced.

Mice were then trained on a spatial version of the MWM in which the escape platform was hidden below the surface of the water and could be found using distal spatial cues (Fig. 2c). Spatial acquisition was assessed by measuring the average escape latency on each day over the five days of training. VHC+CC mice showed impaired acquisition of the platform location compared to SHAM and CC mice, on day 4, with escape latencies longer than those of SHAM or CC mice (Fig. 2d). Twenty-four hours after the end of acquisition training, a 60-second probe trial was conducted in which the escape platform was removed and time spent searching in the target quadrant versus time spent searching in others was measured. VHC+CC were impaired in memory for platform location. SHAM and CC mice searched selectively, exhibiting a preference for the target quadrant over others. VHC+CC mice did not appear to distinguish between the target quadrant and others (Fig. 2e). To rule out potential effects of split-brain surgery on vision, locomotion, motivation, or non-hippocampus-dependent procedural learning, mice were trained on a visible version of the MWM in which mice could see the platform throughout the duration of each trial. There was no effect of surgical treatment on escape latencies throughout training (Supplementary Fig. 1). As an additional test of non-specific surgical effects, we compared shortest latencies at the end of training to determine whether fastest trials differed across groups. There were no differences across treatments (data not shown). Collectively, these data indicate impaired spatial learning and memory following loss of interhippocampal communication.

To determine whether surgical treatment affected contextual fear memory, we used a one-shock contextual fear conditioning protocol. This paradigm is extremely sensitive to hippocampal manipulations, as lesions of the dorsal hippocampus and even ablation of adult hippocampal neurogenesis produce anterograde amnesia of contextual fear memory when only a single training trial is given (Fig. 3a)¹⁴. We conducted retrieval sessions 1 and 24 hours after conditioning in which the animal was returned to the context in which they had been shocked and we measured time spent freezing, a naturally occurring fear response indicating strength of memory for the context. There appeared to be no impairment of either short- or long-term contextual fear memory in VHC+CC, CC, or SHAM mice during retrieval sessions occurring one (Fig. 3b) and 24 hours (Fig. 3c) after conditioning. Although there was a trend towards an effect

of surgical treatment during the long-term contextual fear memory test, this did not indicate decreased memory in split-brain mice, but rather a possible enhancement of fear memory in CC mice (Fig. 3c). Unlike in the Y-Maze and spatial MWM paradigms, the contextual fear memory task does not require retrieval of a particular location within an environment. Therefore, impairments following VHC+CC surgery may be limited to hippocampus-dependent tasks that require retrieval of memories at particular locations within the behavioral environment.

Earlier work suggested that split-brain mice may have higher levels of anxiety, which may have contributed to impaired performance on a Barnes Maze spatial memory test⁹. To determine whether split-brain surgery affected anxiety, we exposed mice to a single session of exploration of an elevated plus maze. Reduced exploration of the open arms of the maze indicates increased anxiety. There was no difference across groups in exploration of open arms (Supplementary Fig. 2), indicating no difference in levels of anxiety across treatment groups.

In summary, transection of the VHC, but not the corpus callosum alone, impaired spatial learning and memory and had no effect on hippocampus-dependent contextual fear memory. Because the contextual fear conditioning protocol we used reveals slight manipulations of hippocampal circuitry¹⁴⁻¹⁷; we believe it is unlikely that cellular remodeling following VHC transection contributed to the impairments seen in our spatial memory experiments. Further, although our VHC+CC surgery necessarily damaged overlying structures, such as the fimbria, fornix, and septum (Fig. 1a), contextual fear memory has been shown to be impaired following lesions to the fimbria and fornix, and conditioned contextual fear expression is suppressed during inhibition of the septum¹⁸. Taken together, our data indicate that loss of interhippocampal communication does not abolish the ability to acquire, store, and retrieve new hippocampal memories, but does impair learning and memory involved in spatial recognition and spatial navigation.

A number of influential theories have been proposed to characterize the function of the hippocampus, and yet it has been difficult to reconcile the dual roles of the hippocampus in memory and in spatial navigation¹⁹. Consideration of hemispheric lateralization may be a solution to this problem. Consistently, the left hippocampus has been found to be dominant in memory acquisition in mice⁷, rats⁸, and humans¹², and the right hemisphere is dominant in memory-guided spatial navigation in rats⁸ and humans^{11,12,20}. One model of bilateral hippocampal function suggests that the left CA3 acquires and stores new memories via its capacity for synaptic plasticity⁵⁻⁷ and that the right CA3 rapidly provides spatial representations via stable neural networks that were preconfigured during development¹⁰. Rapid emergence of bilateral CA1 cognitive maps in new environments would be contributed by right CA3. These maps could then be modified via spatial learning that engages left CA3, allowing the binding of acquired memories and learned associations to these spatial representations¹⁰. Our findings of impaired spatial memory, but intact contextual fear memory, following VHC transection provide behavioral evidence for this model, unifying seemingly disparate left and right functions. Accounting for interhemispheric communication and lateralization is critical to understand hippocampal function and, more broadly, how the brain carries out mnemonic and spatial cognition.

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Fig. 1 Transection of interhemispheric pathways. **a**, Anatomical illustration of area targeted by split-brain surgery. Dotted line indicates path of surgical knife. ACAV, ventral anterior cingulate cortex; cing, cingulum bundle; cc, corpus callosum; CTX, cortex; df, dorsal fornix; fi, fimbria; IG, induseum griseum; LSc, TRS, and SF are all nuclei of the septum; SFO, subfornical organ; V3, third ventricle; vhc, ventral hippocampal commissure; VL, lateral ventricle. Image credit: Allen Institute, © 2004 Allen Institute for Brain Science. Allen Mouse Brain Atlas, available from <http://mouse.brain-map.org/> **b**, Experimental design and surgical treatment groups. Myelin is stained with luxol fast blue and counterstained with cresyl violet. SHAM surgery consisted of lowering the surgical knife into the cortex, superficial to the cc, leaving the cc and vhc intact. CC surgery consisted of transection of the cc only (white arrow). VHC+CC surgery consisted of transection of both the vhc (black arrow) and overlying cc (white arrow). Scale bar = 1 mm.

Fig. 2 VHC+CC transection impairs spatial memory. **a**, Y-Maze short-term spatial memory paradigm. Mice were allowed to explore a three-arm Y-Maze during an encoding trial with one arm blocked off (green). Following a 1-minute intertrial, mice were placed back into the apparatus with all arms open. S indicates the start arm; F indicates the familiar arm that the mouse had access to during the encoding trial; N indicates the novel arm that was blocked off during encoding. SHAM: N = 9; CC: N = 9; VHC+CC: N = 10. **b**, Time spent in the novel and familiar arms during the retrieval trial. SHAM and CC mice spent significantly more time exploring the novel arm than the familiar arm (SHAM: $t(8) = 3.502$, $p = 0.008$; CC: $t(8) = 4.641$, $p = 0.002$). VHC+CC mice exhibited no such preference ($t(9) = 0.205$; $p = 0.842$). **c**, MWM spatial learning and memory paradigm. Mice were trained to locate a hidden escape platform by its relation to extra-maze spatial cues. After 5 days of training, mice were given a single probe trial with the escape platform removed. SHAM: N = 8; CC: N = 9; VHC+CC: N = 11. **d**, Average escape latencies on

each day of training. We found a main effect of training day ($F(4,25) = 8.304$, $p < 0.0001$) and of treatment group ($F(2,25) = 4.268$, $p = 0.025$) on escape latency during acquisition, with no interaction between these factors ($F(8,25) = 0.513$, $p = 0.845$). Post hoc analyses showed that VHC+CC mice were significantly slower on training day 4 compared to the other groups (VHC+CC vs. SHAM: $p = 0.026$; VHC+CC vs. CC: $p = 0.036$). CC mice did not differ from SHAM mice at any point during acquisition ($p > 0.79$ for each day). **e**, Time spent searching in the target quadrant (T) compared to the average of the times spent in the other quadrants (O) for each treatment group on the probe trial. SHAM and CC mice searched selectively, exhibiting a preference for the target quadrant over others (SHAM: $t(7) = 3.924$, $p = 0.003$; CC: $t(8) = 2.480$, $p = 0.038$). VHC+CC mice did not appear to distinguish between the target quadrant and others ($t(10) = 1.320$; $p = 0.216$). Histograms indicate group means; lines indicate individual mice. * $P < 0.05$, ** $P < 0.01$; NS indicates no significance.

Fig. 3 VHC+CC transection spares hippocampus-dependent contextual fear memory. a, Hippocampus-dependent contextual fear memory paradigm. Mice were placed in a behavioral chamber where they received a single mild foot-shock during a conditioning session. Mice were re-exposed to the chamber one and 24 hours later to assess short- and long-term contextual fear memory. SHAM: N = 9; CC: N = 9; VHC+CC: N = 11. **b,** Time spent freezing during context re-exposure one hour after conditioning. There was no effect of surgical treatment on freezing ($F(2,26) = 0.69$; $p = 0.511$). **c,** Time spent freezing during context re-exposure 24 hours after conditioning. There was no effect of surgical treatment on freezing ($F(2,26) = 2.66$; $p = 0.089$). Error bars represent SEM; dots indicate individual mice; NS indicates no significance.

Supplemental Figures

Supplementary Figure 1. MWM acquisition with a visible platform. **a**, We found a main effect of training day on escape latency ($F(4,26) = 98.1$, $p < 0.0001$), no effect of treatment ($F(2,26) = 0.024$, $p = 0.976$), and no interaction between these factors ($F(8,26) = 0.766$, $p = 0.6332$). **b**, Minimum escape latency for each mouse on the final day of training revealed no effect of treatment ($F(2,26) = 0.632$, $p = 0.539$). SHAM: $N = 11$; CC: $N = 9$; VHC+CC: $N = 9$.

Supplementary Figure 2. Exploration of the open arms on an elevated plus maze apparatus. Surgical treatment did not appear to alter levels of anxiety as there were no differences among groups in time spent in open arms ($F(2,25) = 0.867$, $p = 0.433$). SHAM: $N = 10$; CC: $N = 9$; VHC+CC: $N = 9$.

Methods

Animals. We used adult male and female C57/BL6J mice (Jackson Labs) that have been bred in-house for 2-5 generations. All mice were adults aged 2.5-7 months at the time of surgery. Mice were housed on a 12 h light/dark cycle, with all behavioral sessions occurring during the light phase. All procedures were approved by the Queens College Institutional Animal Care and Use Committee (protocol # 177).

Experimental Design. The ventral hippocampal commissure contains axons connecting the left and right hippocampi³. The term ventral hippocampal commissure distinguishes this structure from the dorsal hippocampal commissure, which connects extra-hippocampal cortical areas in the left and right hemisphere and should not be confused with the ventral/dorsal distinction of the hippocampus itself²¹. Fibers in the VHC originate and terminate throughout the entire dorsal-ventral extent of the hippocampus. We performed “complete” or “partial” split-brain surgeries. A complete split-brain surgery consisted of transection of both the VHC and the overlying corpus callosum (CC), as the VHC cannot be accessed without transecting the corpus callosum. Partial split-brain surgery consisted of transection of only the corpus callosum located over the VHC to control for possible contributions of the corpus callosum to the behavioral assays, and hippocampus-dependent memory more specifically²². Sham surgeries consisted of sectioning cortex overlying the corpus callosum. We refer to mice receiving complete split-brain surgery as VHC+CC ($n = 11$; 4 females, 7 males), mice receiving partial split-brain surgery as CC ($n = 9$; 5 females, 4 males), and sham-operated mice as SHAM ($n = 9$; 4 females, 5 males). There were no sex differences in any behavioral measure, therefore males and females were combined for all analyses. Sample sizes were corrected following histological confirmation of surgery and varied by behavioral test, see individual sections in Results.

Surgery. In order to sever interhemispheric pathways, we modified a method developed by Schalomon & Wahlsten²³. We used an L-shaped, sharpened piece of tungsten wire (0.25 mm in diameter) as a knife. The body temperature of the mice was monitored and maintained with heating pad. Mice were anesthetized with ketamine/xylazine (intraperitoneal, 90-120 mg/kg and 5-10 mg/kg, respectively) and further anesthetized for 1 minute in an isoflurane chamber (4.5% isoflurane). Mice were placed in a stereotaxic apparatus, receiving a constant flow of 1.5% isoflurane and oxygen (1.5 L/minute) and were given an injection of bupivacaine (1.25-2 mg/kg) under the scalp for local analgesia. An opening was made by drilling two adjacent 1 mm-wide holes into the skull to access the brain. To avoid the superior sagittal sinus, openings in the skull were made 0.5 mm off the midline and the side of surgery for each animal was randomly chosen (± 0.5 ML, -0.8 AP from bregma for the first hole, ± 0.5 ML, -1.6 AP from bregma for the second hole). To sever both the VHC and CC, the short, sharpened end of the L-knife was placed on the surface of the brain along the medial side of the hole and was then slowly lowered 3.4 mm. Once lowered, the knife was translated anteriorly so that the knife moved posterior to anterior to “hook” the VHC and CC fibers. The knife was then raised 3.4 mm until the short arm reached the underside of the skull. The knife was then translated back and raised out of the hole. To sever the CC only, we performed the same procedure as VHC+CC transection, however the knife was only lowered 2.2 mm. For sham surgeries, we used the same procedure, but the knife was lowered 1.0 mm. Mice were administered buprenorphine following surgery (0.1 mg/kg, subcutaneous). Transected and sham mice were indistinguishable by blind observation of their behavior in the homecage.

Behavior. Behavioral testing began approximately four weeks after surgery. Mice were habituated to handling for one day by the experimenter. On testing days, mice were transported to a designated behavior room and were allowed to acclimate for a minimum of 20 minutes before the start of the task. All behavior was scored by an experimenter blind to surgical condition and sex using the Stopwatch+ program.

Short-term spatial memory was measured using the Y-Maze, known to be equally sensitive to inactivation of either the left or the right hippocampus⁷. The Y-maze apparatus was constructed of clear acrylic and had three arms (height: 20 cm; length: 30 cm; width: 8 cm) 120 degrees apart. The room contained many spatial cues including light fixtures and furniture. In addition, a painting and a movie poster were placed on the walls in line with the axes of the familiar and novel arms, while the experimenter stood along the axis of the start arm during each trial. Each arm was marked with a black line at the entrance to determine whether the mouse was in the arm or not. Mice were considered to be in an arm if all four paws were across the entrance line. The paradigm consisted of a 2-minute encoding trial during which one arm was blocked off, followed by a 1-minute intertrial interval, then a 2-minute retrieval session. The start arm remained the same in both the encoding and retrieval trial, while the exposed arm during the encoding trial was considered the familiar arm and the blocked arm was considered the novel arm. At the start of the encoding trial, mice were placed facing outward in the start arm and were allowed to explore the start and familiar arms for two minutes, beginning when the mouse left the start arm. Mice were removed from the Y-Maze and placed back into their home cage for one minute. While mice were in the home cage, the block was removed to expose the novel arm. To remove any potential confounds from odor cues, the apparatus was wiped with 70% ethanol, rotated 120 degrees, and then wiped with a dry paper towel before the retrieval trial. After the intertrial interval, the mice were again placed facing out in the start arm and were allowed to explore the entire maze for two minutes. At the end of the retrieval session, mice were placed back in their home cages and the maze wiped and dried before the next animal was run. Y-Maze spatial memory was scored as the time spent in the novel and familiar arms during the retrieval paradigm. One HC+CC mouse was removed from analysis for failure to leave the start arm during the retrieval trial and therefore had scores of zero for both the novel and familiar arms.

Long-term spatial learning and memory was measured using the Morris Water Maze (MWM), following the protocol of Vorhees & Williams²⁴. The pool was 110 cm in diameter and was filled with opaque water colored with non-toxic white paint maintained at a temperature of 25.3°C + 0.5°C. The escape platform was white and was submerged approximately 0.5 cm under the surface of the water. The platform remained in the same location throughout training. Salient room cues were visible from the surface of the pool and included colored and patterned posters, lighting, and furniture. Training consisted of four trials per day for five days with the starting location varying on each trial. Intertrial intervals were 30 seconds, during which the mice remained on the platform before starting the next trial. Mice that did not reach the platform within 60 seconds were placed onto the platform. Twenty-four hours after training, a 60-second probe trial was given during which the escape platform was removed. To measure spatial learning, latencies to the escape platform were recorded for each training trial and were averaged across trials for each mouse on each of the 5 training days. For the probe trials, spatial memory was scored by time spent in the target quadrant versus the average of the times spent in non-target quadrants, computed by summing the time spent in non-target quadrants divided by three²⁵⁻²⁷. One SHAM mouse was removed from analysis for exhibiting signs of hypothermia after a training session.

Hippocampus-independent learning was assessed using a version of the MWM in which the escape platform was visible, as described by Vorhees & Williams²⁴. Mice were tested in the same pool as in the spatial paradigm. However, the platform was above water level and a red disk was placed on top to contrast the platform with the white pool and water. Training consisted of three trials per day over five days. Mice were placed in the water facing the wall on the opposite side of the pool from the target platform. After finding the platform, mice were left for 15 seconds before being moved to the home cage for the intertrial interval and the platform was moved to a new spatial location. The intertrial interval had no set time and ended when the platform was moved and the water settled (approximately 30 seconds). If mice did not find the

platform within 60 seconds, they were placed onto it by the experimenter and remained for 30 seconds.

Short- and long-term contextual fear memory was tested using a one-shock conditioning paradigm that is particularly sensitive to hippocampal manipulations¹⁴. Conditioning took place in a fear conditioning chamber housed in a sound-attenuating cubicle (Med Associates). Before each session, the chamber was wiped down with 70% ethanol and dried. On the first day, mice were placed in the chamber and allowed to explore freely for 3 minutes, then given a mild foot-shock (2 s, 0.75 mA), and removed 15 seconds later (total conditioning session time = 3 minutes, 17 seconds). Two 3-minute retrieval sessions occurred 1 and 24 hours after conditioning, a protocol previously used to dissociate molecular contributions to short- and long-term contextual fear memory²⁸. Fear expression was scored as time spent freezing during each minute of the three-minute retrieval trial (absence of all movement, except breathing).

Anxiety was measured using the elevated plus maze, a paradigm sensitive to both dorsal and ventral hippocampal manipulations^{21,29}. The elevated plus apparatus consisted of four arms (30.5 cm long, 6.4 cm wide), two of which were enclosed on three sides with walls (20.3 cm high). Mice were placed in the center of the apparatus and were allowed to explore freely for 5 minutes and were then returned to their home cage. The apparatus was wiped with 70% ethanol and dried both before and after each trial. Anxiety was scored as time with all four paws on an open arm. One male HC+CC mice was removed from analysis as it fell off the apparatus during the session.

Tissue Processing and Surgical Verification. Mice were euthanized with 0.3 mL of euthasol. Brains were extracted and post-fixed in 4% paraformaldehyde. Brains were then cryoprotected in 30% sucrose before cryosectioning at 60 μ m. To assess transection to the corpus callosum and hippocampal commissure, we stained sections with luxol blue and cresyl violet. Slides were dried overnight at 37°C. Histology began with a de-fat step in which sections were serially dehydrated in ethanol and then placed in xylene (twice for 5 minutes each). Sections were then rehydrated and submersed in 70% ethanol for one hour at room temperature. Sections were then incubated in a 0.1% luxol blue solution in 95% ethanol overnight at 56°C. Myelin was differentiated via rinses in deionized water, followed by 0.05% lithium carbonated in deionized water, followed by 70% ethanol (2 minutes each; differentiation was repeated as necessary). Sections were then stained in 0.1% cresyl violet in deionized water, serially dehydrated, cleared in xylene and coverslipped using Krytoxal.

Statistical Analysis. To assess short-term memory during the Y-Maze retrieval trial, we planned comparisons to determine whether each treatment group exhibited a preference for the novel arm over the familiar arm, but not whether treatment groups differed in exploration times. Therefore, we compared time spent in the novel arm to time spent in the familiar arm within each group using Student's paired t-tests. To assess cued and spatial learning on the MWM, we performed two-way mixed model ANOVAs (treatment x training day, with training day as the repeating factor) on escape latency, followed by post hoc tests (Tukey's HSD). To assess spatial memory on the 60-second MWM probe trial we used Student's paired t-tests within each treatment group, comparing time spent in the target to time spent in an average of the other quadrants²⁵⁻²⁷. We used a one-way ANOVA to determine whether minimum escape latencies differed across groups on the final day of visible MWM training. To assess short- and long-term contextual fear memory, we performed a two-way mixed model ANOVA (treatment x minute, with minute as the repeating factor) on time spent freezing during each of the two retrieval trials. To assess anxiety during the elevated plus maze test, we performed a one-way ANOVA on time spent in the open arms across all groups.

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