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Collateral Projections Innervate the Mammillary Bodies and Retrosplenial Cortex: A New Category of Hippocampal Cells

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Collateral projections innervate the mammillary bodies and retrosplenial cortex: A new category of hippocampal cells

Abbreviated title: Collateral subiculum projections to limbic sites

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Abstract

To understand the hippocampus it is necessary to understand the subiculum. Unlike other hippocampal subfields, the subiculum projects to almost all distal hippocampal targets, highlighting its critical importance for external networks. The present studies, in male rats and mice, reveal a new category of dorsal subiculum neurons that innervate both the mammillary bodies and the retrosplenial cortex. These bifurcating neurons comprise almost half of the hippocampal cells that project to retrosplenial cortex. The termination of these numerous collateral projections was visualized within the medial mammillary nucleus and the granular retrosplenial cortex (area 29). These collateral projections included subiculum efferents that cross to the contralateral mammillary bodies. Within the granular retrosplenial cortex, the collateral projections form a particularly dense plexus in deep layer II and layer III. This retrosplenial termination site co-localized with markers for VGluT2 and neurotensin. While efferents from the hippocampal CA fields standardly collateralize, subiculum projections often have only one target site. Consequently, the many collateral projections involving the retrosplenial cortex and the mammillary bodies present a relatively unusual pattern for the subiculum, which presumably relates to how both targets have complementary roles in spatial processing. Furthermore, along with the anterior thalamic nuclei, the mammillary bodies and retrosplenial cortex are key members of a memory circuit, which is usually described as both starting and finishing in the hippocampus. The present findings reveal how the hippocampus simultaneously engages different parts of this circuit, so forcing an important revision of this network.

Significance Statement

The hippocampus has both cortical and subcortical connections that are critical for spatial learning in rodents and episodic memory in humans. Chief among these connections are the dense hippocampal inputs to the retrosplenial cortex and mammillary bodies, both of which originate in the subiculum. The present experiments reveal that in rodents approximately half of these retrosplenial projections have collaterals that also innervate the mammillary bodies. Consequently, these two areas share common hippocampal information, despite playing different roles in cognition. These same collateral projections contradict longstanding ideas

about extended, serial hippocampal networks for memory. As these networks are affected from the earliest stages of Alzheimer's disease, when memory disorders first appear, there is added significance in understanding their precise connectivity.

Introduction

Within the hippocampus (dentate gyrus, CA fields, and subiculum), the subiculum has a unique status. Unlike any other subfield, the subiculum projects to almost all external sites innervated by the hippocampus (O'Mara, 2005). In addition, some key hippocampal projections arise almost exclusively from the subiculum. Examples include the dense hippocampal efferents to the mammillary bodies, anterior thalamic nuclei, and retrosplenial cortex (areas 29, 30), which together form an extended limbic network (Rolls, 2015; Bubb et al., 2017). These limbic interconnections have been regarded as vital for emotion (Papez, 1937; MacLean, 1949; Dalgleish, 2004) and, more recently, for spatial memory in rodents and episodic memory in humans (Aggleton et al., 2010; Carlesimo et al., 2011; Ritchey et al., 2015). These same hippocampal connections are also directly implicated in the memory loss that characterizes the earliest stages of Alzheimer's disease (Tan et al., 2013; Aggleton et al., 2016). Consequently, understanding the nature of these hippocampal connections remains a priority.

A feature of the projections from the various hippocampal CA fields is that they standardly collaterize to innervate multiple sites (Swanson et al., 1981; Donovan & Wyss, 1983). In contrast, projections from the subiculum are typically segregated by their columnar and laminar site of origin (Witter et al., 1990; Ishizuka, 2001; Witter, 2006; Christiansen et al., 2016). A consequence is that many subiculum neurons only innervate one target site (Swanson et al., 1981; Donovan & Wyss, 1983; Namura et al., 1994; Naber & Witter, 1998; Wright et al., 2010, 2013). There are, however, reasons to suppose that the hippocampal projections to the retrosplenial cortex and mammillary bodies might prove different, as populations of subiculum neurons that project to these two sites seem to be present in overlapping regions of the subiculum in both rats and monkeys (Van Groen & Wyss, 2003; Kobayashi & Amaral, 2007; Christiansen et al., 2016). For these reasons, the present study began by determining whether the source of these hippocampal projections was indeed from the same region

of subiculum, before testing if these two sets of hippocampal efferents remain segregated or whether they provide collateral outputs to both targets. Resolving these issues is valuable as it has been presumed that the retrosplenial cortex and mammillary bodies are concerned with different aspects of hippocampal information processing (Byrne et al., 2007; Dillingham et al., 2015a). One potential basis for this difference would be if they derive information from separate hippocampal outputs.

The initial experiments, therefore, used multiple fluorescent tracers to determine whether the subiculum projections to the mammillary bodies and retrosplenial cortex arise from the same or different cell populations. One of the axonal tracers used in the present study, unconjugated cholera toxin B subunit (CTB), is transported in both anterograde and retrograde directions. A consequence is that 'collateral-collateral' transport can occur (Chen & Aston-Jones, 1998). This form of transport occurs when a tracer is conveyed retrogradely in one collateral to reach the cell soma, where it is then conveyed anterogradely along other collaterals. This property not only makes it possible to specify the location of the particular collateral terminals under investigation, i.e., in either the mammillary bodies or retrosplenial cortex, but it also becomes possible to look for other collateral projections involving these same terminal sites. In follow-up experiments, surgical disconnections helped to test for whether collateral-collateral tracer transport from the hippocampus had, indeed, occurred. Those findings then led to more precise neurochemical characterizations of these shared limbic pathways.

Methods

The principal experiments were performed on 34 adult, male Lister Hooded rats weighing 270-320g (Envigo, Bichester, UK). Additional experiments involved two adult, male C57BL/6 mice weighing 32 and 35g (bred at Cardiff University). Pairs of anatomical tracers were used in combination to allow double fluorescent labelling in the same animal. The fluorescent retrograde tracers Fast Blue (FB; Polysciences Inc, Warrington, PA, USA), FluoroGold (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Cholera Toxin Subunit B-Alexa Fluor-488 (CTB-488) and Cholera Toxin Subunit B-Alexa Fluor-594 (CTB-594; Invitrogen, Waltham, Massachusetts, USA). Additionally, unconjugated Cholera Toxin Subunit B (CTB; List Biological

Laboratories Inc., Campbell, CA, Product # 103B) was used as it is transported along axons in both anterograde and retrograde directions. This tracer was visualized by immunofluorescence. The tracer pairings were as follows: FB + FG, n = 6; CTB-488/CTB-594 + FB, n = 4; CTB in mammillary bodies (MB) + FB in retrosplenial cortex (RSP), n = 5; FB in MB + CTB in RSP, n = 2. Single tracer studies using only CTB were also conducted: CTB in RSP, n = 3; CTB in MB only, n = 4. A final, additional set of two adult male Lister Hooded rats received injections of the anterograde tracer, 3 kD biotinylated dextran amine (BDA; Life Technologies Ltd, Paisley, UK) in the dorsal hippocampus to provide additional information about the termination sites of possible collateral connections. All experiments were in accordance with UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, and approved by local ethical committees at Cardiff University.

Surgical methods - rats

All rats were anesthetized throughout surgery with isofluorane (5% for induction, 2% thereafter). Rats were placed in a stereotaxic frame (Kopf, Tujunga, CA, USA), with the mouth-bar set at +5.0mm. For analgesic purposes, Lidocaine was administered topically (0.1ml of 20mg/ml solution; B. Braun, Melsungen, Germany) and meloxicam was given subcutaneously (0.06ml of 5mg/ml solution, Boehringer Ingelheim Ltd, Berkshire, UK). Under aseptic conditions, small openings were made in the skull and dura to allow access for a 0.5µl Hamilton syringe for pressure injections (25ga, Hamilton, Bonaduz Switzerland).

Single tracer injections (per hemisphere) were made in the mammillary bodies. The coordinates centered on anterior-posterior (AP) -1.9, medial-lateral (ML) +/- 0.5, and dorsal-ventral (DV) -10.4 from bregma, but varied slightly to encompass different subregions. For the retrosplenial cortex, six injections ensured coverage along the full AP plane of this large cortical area. The six coordinates, relative to bregma, with depth relative to top of cortex, were: AP -1.8, ML ±0.5, DV -1.0; AP -2.8, ML ±0.5, DV -1.0; AP -4.0, ML ±0.5, DV -1.0; AP -5.8, ML ±0.5, DV -2.5; AP-5.8, ML ±0.9, DV -1.4; AP-6.6, ML ±0.9, DV -1.8). Animals received either bilateral or unilateral injections in the same structure.

Unconjugated-CTB, CTB488 and CTB594 were made up as a 1% solution in sterile 0.1M phosphate buffered saline (PBS; pH 7.4), Fast Blue was made up as a 3% solution in sterile PBS (pH 7.4), while FluoroGold was made up as a 4% solution in sterile, distilled water. Following pressure injections of 0.06-0.1µl into each site, the syringe was left in place for at least five minutes to help reduce any back flow of the tracer. For the retrosplenial cortex there was no concern about tracers travelling back up the syringe tract, however, some evidence of the tracers could be detected from the syringe tracks immediately above the mammillary body injections.

For the anterograde tracer studies, BDA was made up as a 10% solution in sterile, distilled water (pH 7.4) and injections were made at three sites along the anterior-posterior axis of the dorsal subiculum. The injection coordinates relative to bregma were: AP -4.4, ML ± 2.9, DV -5.8; AP -5.0, ML ± 3.8, DV -6.7; AP -5.3, ML ± 4.9, DV -8.3. Injection volumes were 0.06 - 0.08 µL. The pressure injections were made over 10 minutes with the syringe left in place for at least five minutes to help reduce back flow of the tracer.

After completion of the tracer injections, the scalp was sutured and animals received a 5 ml subcutaneous injection of 5% glucose in 0.9% saline (Baxter Healthcare Ltd, Norfolk, UK). Clindamycin hydrochloride antibiotic powder (Fort Dodge Animal Health Ltd, Southampton, UK) was applied over the closed, sutured scalp. Animals recovered in a thermostatically controlled container before returning to individual housing with *ad lib* food and water.

Surgical methods – mice

The mice were anesthetized throughout surgery with isofluorane (5% for induction, 2% thereafter). Mice were placed in a stereotaxic frame using a flat skull orientation. Lidocaine was administered topically (0.1ml of 2mg/ml solution) and meloxicam was given subcutaneously (0.06ml of 0.5mg/ml solution). Under aseptic conditions, small openings were made in the skull and dura to allow access for a 5µl Hamilton syringe (33ga) connected to a UMP3 microsyringe pump injector (World Precision Instruments, Hertfordshire, UK) with a flow rate of 0.02µl per minute.

A single tracer injection (CTB, 0.05µl) was made in the mammillary bodies with coordinates AP -2.1, ML +0.2, DV -5.5 from bregma. For the retrosplenial cortex, two ipsilateral Fast Blue injections (both 0.1µl) ensured spread along the cortex. The coordinates, relative to bregma were: AP -1.5, ML ±0.2, DV -0.8; AP -2.4, ML ±0.2, DV -1.0. Post-surgical care was the same as for rats, except that the mice received a 0.5 ml subcutaneous injection of 5% glucose in 0.9% saline.

Testing the collateral – collateral transport of CTB: Fornix lesions

Surgical disconnections were used to test whether CTB injected into the mammillary bodies could first be transported retrogradely in the fornix to the hippocampus (subiculum), but then be transported anterogradely in the same subiculum neuron to the retrosplenial cortex ('collateral-collateral transport'). For this reason, in some rats lesions were made in the fornix, followed by CTB tracer injection into the mammillary bodies. Although it was possible to conduct the complementary experiment, i.e., injecting CTB into retrosplenial cortex after fornix lesions, this procedure was not carried out as there are light, direct projections from retrosplenial cortex to the mammillary bodies (Van Groen & Wyss, 2003).

Bilateral radiofrequency lesions were targeted at the postcommissural descending fornix (n = 4). This region of the fornix was the preferred target as it is the subdivision of the fornix taken by neurons projecting from the subiculum to the mammillary bodies (Swanson & Cowan, 1977). The lesions were made using a thermocouple radiofrequency electrode (0.3 mm active tip length, 0.25 mm diameter; Diros Technology Inc., Ontario, Canada). The electrode was lowered vertically and the tip temperature was then raised to 70-74°C for 45 seconds using an OWL Universal RF System URF-3AP lesion maker (Diros Technology Inc. Ontario, Canada). The stereotaxic coordinates from bregma were: AP -0.2, LM ±1.2, DV -8.4, with the mouth-bar set at + 5.0 mm.

Post-operative processing

Following a postoperative period of seven days, the rats were deeply anesthetized with sodium pentobarbital (Euthatal, Merial, Harlow, UK). They were then perfused intracardially with 0.1M PBS at room temperature followed by 4% paraformaldehyde

in 0.1M PBS at ~4°C. Brains were removed and post-fixed in the dark for 4 hours in paraformaldehyde and then transferred to 25% sucrose solution in 0.1M PBS for 24 hours in the dark before sectioning into 40µm coronal sections with a freezing microtome (Leica 1400). A 1-in-4 series of sections was mounted directly onto gelatine-subbed slides and then allowed to dry in the dark at room temperature. This series was stained with cresyl violet to help localize the injection sites. For the surgical cases involving Fast Blue, FluoroGold, CTB488 or CTB594, a second 1-in-4 series was mounted directly onto gelatine-subbed slides, allowed to dry, dehydrated in increasing concentrations of alcohol, then cover-slipped using DPX (Sigma Aldrich, Gillingham, UK).

For the cases involving CTB, the second tissue series was immunohistochemically stained for that tracer. The sections were incubated in a solution of rabbit-anti-cholera toxin primary antibody (1:10,000; Sigma Aldrich, Gillingham, UK, Product # C3062, batch 104M4768V; RRID: AB_258833) and 1% normal goat serum in 0.1M PBS for 24 hours at room temperature. Following washing, the sections were incubated with DyLight 594 – Goat-anti-Rabbit (1 in 200; Vector Laboratories, Peterborough, UK, Product # DI-1594; RRID: AB_2336413) for 24 hours at 4ºC. Sections were then mounted onto gelatine-subbed slides, allowed to dry, dehydrated in increasing concentrations of alcohol and cover-slipped with DPX.

For the cases involving BDA, the second tissue series was incubated in the Vectastain ABC solution (Vector Labs, Peterborough, UK) for 2 hours, then washed in PBST twice for 10min each, followed by a further three washes in 0.1M PBS. Sections were then reacted with diaminobenzidine (DAB; Vector Labs, Peterborough, UK) and intensified with nickel, after which they were mounted, dried, and coverslipped, as described above.

Sections were viewed using a Leica DM5000B microscope for both transmitted white light (for sections stained with cresyl violet) and fluorescence microscopy (for sections with a fluorophore). An attached Leica DFC350FX digital camera and LAS AF image acquisition software (Leica) were used to capture high resolution images.

Experimental design and statistical analysis

Fast Blue in conjunction with FluoroGold was used for initial qualitative analyses of the two pathways. For quantitative analyses, Fast Blue injections were paired with CTB injections into the mammillary bodies or retrosplenial cortex. The combination of Fast Blue and CTB was chosen for quantification as these tracers have distinctive emission wavelengths (420nm and 618nm respectively) and fill neuronal cell bodies in different ways (Köbbert et al., 2000). Cell counts were only taken from those animals in which the respective injections were correctly located.

Double-labelled subicular neurons were counted using the object-based co-localization methods of 'Just Another Co-localization Plugin', a plugin to the public domain, ImageJ software (Bolte & Cordelières, 2006). This software allowed for the initial identification of subicular neurons that project to each region separately. The plugin then determined the fluorescence intensity centers of the CTB-positive subcellular structures and identified the locations at which they coincide with Fast Blue. The system was tested using images that were taken on the same microscope, under the same conditions as the images to be analyzed. These test images had either two overlapping (different fluorophores targeting the same protein) or non-overlapping distributions of fluorescent staining. The co-localization analysis was carried out in four regions of interest across the proximal-distal axis of the dorsal subiculum (see Christiansen et al., 2016). An average of ten dorsal subiculum sections from -5.16 to -6.60 mm posterior to bregma (Paxinos & Watson, 2005) were analyzed for each case. Cell counts were taken from the dorsal subiculum as this is the source of the hippocampal projections to retrosplenial cortex (Van Groen & Wyss, 2003).

Post-operative processing: Additional immunofluorescent targets

These analyses examined the sites of collateral-collateral transport termination. Selected targets followed inspection of the Allen Brain Atlas (http://www.brain-map.org). Accordingly, antibodies for Calbindin D28k (1 in 10,000; Swant, Marly, Switzerland, Product # 300; RRID: AB_10000347), Calretinin (1 in 5,000; Swant, Marly, Switzerland, Product # 6B3; RRID: AB_10000320), Cholecystokinin 8 (1 in 500; Abcam, Cambridge, UK, Product # ab37274; RRID: AB_726010), GAD67 (1 in 1000; Merck Millipore, Hertfordshire, UK, Product # MAB5406; RRID: AB_2278725), Parvalbumin (1 in 15,000; Sigma-Aldrich, Gillingham, UK, Product #

P3088; RRID: AB_477329), Neurotensin (1 in 100; Product # SAB4200703, Sigma-Aldrich Gillingham, UK), VGluT1 (1 in 300; Product # ab193595, Abcam, Cambridge, UK), and VGluT2 (1 in 300; Product # ab7915, Abcam, Cambridge, UK) were included. The secondary antibody, DyLight 488 – Horse-anti-mouse (1 in 200; Vector Laboratories, Peterborough, UK, Product # DI-2488; RRID: AB_2307439) was used for visualization. Processing followed standard protocols (see Dillingham et al., 2015b). All antibodies were tested before use to help confirm regional specificity by reference back to the Allen Brain Atlas. Immunohistochemical analyses were conducted on series of tissue from a subset of the surgical cases described above; CTB in MB + FB in RSP, n = 4; FB in MB + CTB in RSP, n = 1; CTB in MB only, n = 4.

For the examples of the higher magnification (40x) images of VGluT2 and NT, Manders' coefficient of colocalization was estimated, again using 'Just Another Co-localization Plugin' (Bolte & Cordelières, 2006). The M_1 quantifies the proportion of the green signal coincident with a signal in the red channel over its total intensity. This measure can fall between zero (no overlap) and one (complete colocalization).

Anatomical nomenclature

Anatomical names and borders follow Swanson (1992), except for the divisions within the retrosplenial cortex and postsubiculum, which use the terminology of Van Groen and Wyss (2003). The latter authors divide retrosplenial cortex into a dorsal, dysgranular subregion (Rdg, area 30) and two ventral, granular subregions (Rga, Rgb, area 29). [Note, other authors further subdivide area 29, e.g., Jones and Witter (2007).] Here, the rat subiculum is divided into two layers, i.e., a superficial molecular layer and a deeper, thick layer of pyramidal cells (Kloosterman et al., 2003). The term 'intermediate subiculum' refers to that subiculum region at the caudal extent of the hippocampal flexure where the dorsal subiculum and ventral subiculum converge (Bast et al., 2006). In accordance with Witter and Wouterlood (2002), the subiculum is included within the hippocampus, while the presubiculum, parasubiculum (and postsubiculum) form parts of the parahippocampal region.

Results

In an initial series (n = 3), injections of Fast Blue and FluoroGold helped to confirm the presence of overlapping populations of dorsal subiculum neurons that project to the two target regions (Figure 1D). Within these overlapping populations of pyramidal cells (blue to retrosplenial cortex, yellow to mammillary bodies), some cream colored cells were observed (Figure 1D). These additional neurons are presumed to send axons to both the mammillary bodies and retrosplenial cortex. A similar pattern of results was obtained with the reverse tracer-target configuration (n = 3). This pattern was further corroborated using Cholera Toxin Subunit B conjugated to Alexa Fluors (CTB488 and CTB594), in combination with either Fast Blue or FluoroGold (n = 4).

To quantify this population of collateralizing projections more precisely, Fast Blue and CTB were separately injected into the two target sites (Figure 1B,C). Of the acceptable injections, five involved CTB in the mammillary bodies and Fast Blue in retrosplenial cortex, while two rats received the reverse placement of tracers. Double-labelling was observed in pyramidal cells in the middle of layer II of the septal and intermediate (dorsal) subiculum (Figure 1A). The number of labelled neurons was estimated in four regions of interest along the proximal-distal axis of the subiculum (R1-4; Figure 2). Double-labelled neurons were most prevalent in the mid proximal-distal plane (R2 and R3) of the dorsal hippocampus (Figure 1A, 2). The cell counts from these seven cases indicated that an overall mean of 46% (range 41.8% to 64.3%) of the subiculum pyramidal neurons that project to the retrosplenial cortex also collateralize to innervate the mammillary bodies (Figure 2; Extended Data Figure 2-1). (This percentage is an underestimate as complete mammillary body tracer uptake would be needed for a full count.) No apparent morphological characteristics could be discerned to distinguish single from double-labelled cells.

After being transported retrogradely to the subiculum, CTB can travel anterogradely in the same neuron (Chen & Aston-Jones, 1998), labelling its collateral terminal fields (Figure 3A,B). Consequently, four more rats received a CTB injection in the mammillary bodies, while three received CTB in the retrosplenial cortex. The mammillary body CTB injections not only retrogradely labelled numerous cells in the

subiculum of both hemispheres, but also produced a dense band of bilateral terminal label throughout deep layer II and layer III of granular retrosplenial cortex (Figure 3A). This terminal label in areas 29a and 29b stopped abruptly at the border with dysgranular retrosplenial cortex (area 30). This pattern of terminal labelling matches that produced when an anterograde tracer such as BDA is injected into the dorsal subiculum (Figure 3E-G), thus, is consistent with the direct projections from subiculum to retrosplenial cortex. Meanwhile, CTB injections in retrosplenial cortex led to ipsilateral, dorsal subiculum label, accompanied by (bilateral) terminal label in the medial mammillary nucleus, most evident in dorsal pars lateralis (Figure 3B).

In those cases with CTB injections in the mammillary bodies it was possible to look for anterograde label in other sites that do not receive direct mammillary inputs, as such label might reflect additional collateral connections. (The same procedure was not applied to those cases with CTB injections in retrosplenial cortex as, unlike the mammillary bodies, this cortical region innervates many different sites, so making interpretation more difficult.) As expected, dense anterograde label was observed in the anterior thalamic nuclei due to the very large projection via the mammillothalamic tract (Figure 3C). Other sites containing terminal label included the prelimbic cortex, infralimbic cortex, the septum (medial and lateral), and the medial and lateral regions of entorhinal cortex (Figure 3D). This entorhinal label was concentrated in the deep layers, predominantly in layer V.

Testing the collateral-collateral transport of CTB: Fornix lesions

In those cases with the most complete section of the postcommissural descending fornix (compare Figure 4A with 4B), the quantity of retrograde subiculum label was markedly attenuated after CTB injections in the mammillary bodies (Figure 4C,D). In these cases (n = 2), the anterograde label in area 29 was no longer visible (Figure 4E). This result, the elimination of terminal label in retrosplenial cortex, indicated that the anterograde label had originated via the subiculum inputs to the mammillary bodies. To confirm that this absence of tracer signal in the subiculum and retrosplenial cortex was not due to the tracer failing to be taken up by the mammillary bodies following fornix lesions, Gudden's ventral tegmental nucleus was examined as this nucleus projects to the mammillary bodies, but not via the fornix (Allen & Hopkins, 1989). Comparable numbers of neurons labelled with CTB were observed in Gudden's

nucleus, whether the fornix had been cut or spared (Figure 4F,G), confirming tracer uptake in both conditions.

Cross-hemispheric collateral projections

The pattern of double and single labelling in the subiculum following tracer injections into one hemisphere indicated that the projections to the retrosplenial cortex remained ipsilateral to the subiculum while the collaterals to the mammillary bodies could arise from either the ipsilateral or contralateral subiculum.

Cross-species comparisons

To determine whether these bifurcating subicular neurons are present in other rodents, the same anatomical methods were applied to adult mice (C57BL/6 strain). The tracer CTB was injected into the mammillary bodies (Figure 5A) and Fast Blue injected into the retrosplenial cortex (Figure 5B) generating a population of double-labelled neurons in the dorsal subiculum (Figure 5C). Quantification of those subiculum neurons that project to retrosplenial cortex and also project to the mammillary bodies yielded remarkably similar results to those found in the rat (Extended Data Figure 5-1). The co-localization analysis indicated that an overall mean of 41% of those subiculum neurons that project to retrosplenial cortex also collateralize to innervate the mammillary bodies (range across cases 39.8% - 46.5%). Furthermore, CTB tracer injections in the mammillary bodies again resulted in dense terminal label, restricted to area 29 (Figure 5D). This label was concentrated in deep layer II and layer III (Figure 5D), consistent with collateral-collateral transport via the subiculum and the results seen in the rat.

Neurochemistry of subiculum efferents

The ability to visualize the collateral projections within retrosplenial cortex made it possible to determine if these subiculum efferents co-localize with specific neurochemicals. Using tissue from rats with CTB injections in the mammillary bodies, immunofluorescence revealed how the area 29 terminations specifically co-localized with signals for VGluT2 and neurotensin (Figure 6A,B). This co-localization was very precise as both VGluT2 and neurotensin matched the CTB distribution in deep layer II and III, but appeared absent from the rest of area 29. The

co-localization in Figure 6 was estimated using Manders' Coefficient; for VGluT2 signal overlap with the CTB signal was $M_1 = 0.72$, while for neurotensin the overlap with CTB was M1 = 0.96. Signals for neurotensin and VGluT2 were also present in dorsal pars lateralis of the medial mammillary bodies, i.e., those regions receiving collateral innervations. The CTB-positive area 29 terminations did not co-localize with VGluT1, GAD67, calretinin, parvalbumin (PV), calbindin, or cholecystokinin (Extended Data Figure 6-1).

As has been described previously (Varoqui et al., 2002), we found a paucity of VGluT1 label in deep layer II and layer III. GAD67 is a GABA-synthesizing enzyme and so was employed as a crude marker for GABAergic neurons to be followed up by other interneuron markers. GAD67 and CTB-positive terminals showed an almost complementary pattern of staining with GAD67 present in superficial layer II and the deeper cortical layers but not deep layer II and III (Extended Data Figure 6-1). The pattern of PV labelling was, unsurprisingly, very similar to that of GAD67. Although non-overlapping, there was a close association with CTB terminals in area 29 and PV-positive staining as PV cell bodies were found to sit among the CTB-positive terminals in deep layer II and adjacent to PV-positive terminals in superficial layer II (Extended Data Figure 6-1); this pattern of PV staining matches previous descriptions (Salaj et al., 2015). Also consistent with previous reports (Salaj et al., 2015), calretinin had low but detectable levels of staining of both cells bodies and neuropil in retrosplenial cortex but there was a conspicuous absence of label in layers II and III, and so no overlap with CTB. The final interneuron markers to be tested, calbindin and cholecystokinin, had very low levels of expression in retrosplenial cortex. Taken together, these results show that these CTB-labelled projections are excitatory rather than inhibitory.

Discussion

The present study revealed collateral subiculum projections that simultaneously link the hippocampus with two sites, the mammillary bodies and the retrosplenial cortex (Figures 1, 2). These shared projections arise from the dorsal subiculum, comprising almost half of the hippocampal projections to retrosplenial cortex in both rats and mice. For some of these collateral projections, the input from the subiculum to the

mammillary bodies crosses to the opposite hemisphere (Figure 7A). Meanwhile, the retrograde then anterograde movement of CTB, the latter via collateral-collateral transport, showed how the termination sites of these collateral projections are restricted to the medial mammillary nucleus and retrosplenial area 29 (layers deep II and III) (Figure 3). Consequently, these two sites receive shared hippocampal information, despite the different contributions they make to learning and memory (Byrne et al., 2007; Vann et al., 2009; Dillingham et al., 2015a, Roy et al., 2017). This finding of a new category of subiculum neurons may relate to recent electrophysiological descriptions of multiple subpopulations of spatial cells within this same hippocampal region (Brotons-Mas, et al., 2017).

At the outset, it is important to confirm whether the CTB injections did, indeed, result in collateral-collateral transport, as such label best specifies the terminal sites of hippocampal collaterals within the retrosplenial cortex and mammillary bodies. The clearest evidence relates to the anterograde label observed in retrosplenial cortex following CTB injections into the mammillary bodies. First, there are no direct projections from the mammillary bodies to retrosplenial cortex (Van Groen & Wyss, 2003) and although transneuronal tracing has been observed using a biotin conjugate of CTB (Lai et al., 2015), unconjugated CTB is not thought to be trans-synaptically transported under the conditions used in the present study (Bilsland & Schiavo, 2009). While one potential trans-synaptic route would have been via the anterior thalamic nuclei, this would have principally produced anterograde label in layers I and V of retrosplenial cortex (Van Groen & Wyss, 2003). Instead, the observed label was restricted to layers II and III. Second, the distribution of the retrosplenial terminal label precisely matched that of the direct projections from the subiculum to retrosplenial cortex (Figure 3F, see also Van Groen & Wyss, 2003). Perhaps, most compelling, was the finding that surgical disconnection of the hippocampal projections to the mammillary bodies blocked the presence of this terminal label in retrosplenial cortex.

Evidence of transport of CTB from the retrosplenial cortex to the subiculum, and then to the medial mammillary bodies, was also observed, but this potential collateral-collateral label is more difficult to interpret. The difficulty arises because there is a very light, direct projection from granular retrosplenial cortex to the mammillary

bodies (Van Groen & Wyss, 1990, 2003; see also retrograde labelled neurons in Figure 3A). The apparent co-localization of the CTB label in the medial mammillary nucleus with neurotensin is consistent with this being collateral-collateral transport, but not proof. Likewise, the finding that the CTB label was concentrated in the dorsal medial mammillary nucleus is more consistent with a projection from the septal (dorsal) subiculum (Shibata, 1989; Kishi et al., 2000), especially as the sparse, direct retrosplenial inputs from Rga are scattered across the mammillary bodies (Van Groen & Wyss, 1990).

The collateral-collateral transport of CTB made it possible to look for other projections to the mammillary bodies that might collateralize, e.g., from the subiculum. The mammillary bodies lend themselves to this analysis as they only have a restricted set of efferent targets. Aside from the anterior thalamic nuclei, which receive especially dense, direct projections from the mammillary bodies, other sites containing terminal label included the medial and lateral regions of entorhinal cortex, as well as the infralimbic and prelimbic cortices. Of these sites, the entorhinal label is the most likely to reflect collateral-collateral connections via the subiculum as the other sites receive direct mammillary body inputs (Hoover & Vertes, 2007). Furthermore, subiculum neurons that innervate both the mammillary bodies and entorhinal cortex have already been described (Donovan & Wyss, 1983; Roy et al., 2017). As the subiculum inputs to entorhinal cortex terminate in the deep layers (Sorensen & Shipley, 1979), this distribution is consistent with the present entorhinal terminal label reflecting collateral projections. It was, therefore, striking that the density of this terminal label in entorhinal cortex appeared far less than that seen in retrosplenial cortex (Figure 3A,D), even when accounting for the more diffuse termination zone. Meanwhile, the value of appreciating hippocampal collateral projections has been highlighted by recent studies with mice. Roy et al. (2017) demonstrated the importance of subiculum neurons that collateralize to both the entorhinal cortex and mammillary bodies for fear memory retrieval (subiculum to entorhinal cortex) and for coincident fear states associated with fear memory retrieval (subiculum to mammillary bodies). They suggest that in their contextual fear conditioning paradigm the dorsal subiculum to mammillary body projections regulate memory-retrieval-induced stress hormone responses, although it should be pointed out that the mammillary bodies have been implicated in many forms of spatial

558 memory that do not involve an overtly stressful component (Vann & Aggleton, 2004;
559 Vann & Nelson, 2015).
560
561 It should be added that the postsubiculum and regions of the medial prefrontal cortex
562 also project to both mammillary bodies and retrosplenial cortex. Examination of these
563 areas in our paired tracer studies revealed single labelled neurons but not double-
564 labelled neurons. Thus, neurons in these regions are unlikely to contain neurons that
565 collateralize to mammillary bodies and retrosplenial cortex.
566
567 The collateral-collateral transport of CTB also demonstrated the striking overlap
568 between the collateral projections to area 29 and the presence of neurotensin and
569 VGluT2, but not VGluT1. With known neurotensin projections from the subiculum
570 to both the retrosplenial cortex and the mammillary bodies (Roberts et al., 1984;
571 Kiyama et al., 1986), it now appears very likely that many of these same connections
572 collaterize. Meanwhile, VGluT1 and VGluT2, which reflect different subclasses of
573 glutamate terminal (Fremeau et al., 2004), occupy complementary areas within
574 granular retrosplenial cortex (Varoqui et al., 2002). Their respective laminar
575 locations within retrosplenial cortex are notable as they differ appreciably from that
576 found across other cortical areas (Varoqui et al., 2002). Our tissue also indicates that
577 the collateral subiculum projections to the mammillary bodies are again VGluT2 and
578 neurotensin-positive (see also Ziegler et al., 2002). Neurotensin can act as a
579 neuromodulator to several neurotransmitter systems, including the glutamatergic
580 system. A microdialysis study in freely moving rats demonstrated that neurotensin
581 enhances cortical glutamate release, particularly by modulating the functional activity
582 of cortical NMDA receptors (Ferraro et al., 2011). Thus, perhaps amplifying the
583 excitatory signals from the hippocampus to these regions. While the analysis of these
584 terminals permitted precise visualization of these subiculum-limbic efferents, it was
585 not, however, possible to determine if the collateral projections have properties that
586 differ from those connections that only reach one target.
587
588 The present findings challenge notions about subiculum organization. Previous
589 studies have shown that many subiculum connections are segregated by their
590 columnar and laminar origin (Witter et al., 1990; Ishizuka, 2001; Witter, 2006; Wright

et al., 2010, 2013; Christiansen et al., 2016), consequently subiculum neurons often innervate only one target. This property provides a marked contrast with the adjacent hippocampal CA fields (Swanson et al., 1981; Naber & Witter, 1998). The present findings now, however, show that the hippocampal (subiculum) inputs to the mammillary bodies may provide a special case as some of these inputs have collaterals to the retrosplenial cortices (present study) while, as others have already noted, there are also subiculum projections to the mammillary bodies with collaterals to the entorhinal cortex (Donovan & Wyss, 1983). In this way, subiculum neurons that collaterize link the hippocampus simultaneously with other sites that make different contributions to cognition (Vann et al., 2009; Todd & Bucci, 2015; Roy et al., 2017).

With respect to spatial processing, the mammillary bodies are closely linked with learning allocentric-based locations and providing head direction information, while the retrosplenial cortex is closely linked to landmark usage and changing reference frames (Vann & Aggleton, 2004; Byrne et al., 2007; Auger et al., 2012; Dillingham et al., 2015a; Vann & Nelson, 2015). Retrosplenial cortex also contains cells coding for spatial context (Mao et al., 2017), as well as head direction cells linked to landmarks (Jacob et al., 2017). The mechanisms behind these complementary spatial functions become more tractable in light of the discovery of shared hippocampal projections to both sites. These same complementary features also highlight the key position of the anterior thalamic nuclei, which receive dense inputs from both the mammillary bodies and retrosplenial cortex, as well as the hippocampus. Consistent with this strategic location and the partial duplication of hippocampal inputs to the mammillary bodies and retrosplenial cortex, lesion studies in rats have shown that the anterior thalamic nuclei are more critical for hippocampal-sensitive spatial tasks than either the mammillary bodies or retrosplenial cortex (Aggleton et al., 1991, 1995; Neave et al., 1994). In addition, these thalamic nuclei show additional electrophysiological properties relating to spatial information (Tsanov et al., 2011; Jankowski et al., 2015) than either the mammillary bodies or retrosplenial cortex. These findings are consistent with the convergent involvement of the anterior thalamic nuclei in multiple aspects of spatial learning, which is partly fed by the collateral subiculum projections to the mammillary bodies and retrosplenial cortex.

The mammillary bodies, anterior thalamic nuclei, and retrosplenial cortex are key steps along a hippocampal return circuit ('Papez circuit') historically presumed to be vital for emotion (Dalgleish, 2004; see Figure 7B). These same sequential connections also provide the core of an extended hippocampal-limbic circuit, critical for episodic memory (Aggleton & Brown, 2006; Carlesimo et al., 2011; Rolls, 2015). The finding of a bifurcating pathway that allows the hippocampus to influence the diencephalon (mammillary bodies) and cingulate gyrus (retrosplenial cortex) either individually or in parallel (Figure 7B), presents a different perspective. Indeed, in conjunction with other neuroanatomical studies (Jones & Witter, 2007; Kobayashi & Amaral, 2007), there is need to markedly revise this hippocampal-limbic circuit. Three parallel hippocampal-anterior thalamic routes emerge in this new account (Figure 7B). First, a 'ventral' subcortical route, via the fornix to the mammillary bodies and anterior thalamic nuclei, i.e., the original Papez circuit. Second, a 'dorsal' cortical route, containing multiple two-way interconnections between the subiculum, retrosplenial cortex, and anterior thalamus (Bubb et al., 2017). Third, the new collateral pathway that unites both the 'ventral' and 'dorsal' routes. These findings create novel hippocampal networks for information processing in the thalamus, cingulate cortices, and beyond. These anatomical insights are timely as growing evidence links episodic memory loss in Mild Cognitive Impairment and early Alzheimer's disease with the breakdown of this same extended hippocampal network (Tan et al., 2013; Aggleton et al., 2016).

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Figure Legends

Figure 1. Subicular neurons collateralize to innervate the retrosplenial cortex and mammillary bodies. **A.** Coronal photomicrographs of dorsal subiculum in a rat following Fast Blue (FB) injections in retrosplenial cortex (RSP) and Cholera Toxin B (CTB) in the mammillary bodies (MB) with pink double-labelled cells in the overlay panel indicating neurons that collateralize to both regions. Proximal-distal regions (R1-4) were divisions used for subsequent quantification. **B.** Coronal section showing FB injection into retrosplenial cortex. **C.** Coronal section showing CTB injection into mammillary bodies. **D**. Coronal dorsal subiculum section after injections of Fast Blue into the retrosplenial cortex and FluoroGold into the mammillary bodies. The open arrow head points to a single-labelled neuron projecting to MB, the closed arrow head to single-labelled neuron projecting to RSP, the open diamonds indicate double-labelled neurons. Abbreviations: CA1, hippocampal field CA1; LMB, lateral mammillary nucleus; MMB, medial mammillary nucleus; Rga, Rgb, granular retrosplenial cortex, subdivisions a and b, respectively (collectively, area 29); Rdg, dysgranular retrosplenial cortex (area 30). Scale bars = 500μm.

Figure 2. Quantification of extent and location of collateralizing neurons in dorsal subiculum. Histogram illustrates the percentage of subiculum neurons projecting to retrosplenial cortex that co-label with mammillary body tracer. For this analysis, dorsal subiculum was divided by proximal-distal (R1-4) and anterior-posterior (AP) locations (cell counts are presented in Extended Data Figure 2-1). Photomicrographs depict dorsal subiculum (right hemisphere) at five AP levels (numbers indicate distance from bregma in mm), the borders are color-coded to match the corresponding bars in the histogram. The photomicrographs show pink double-labelled cells that innervate both sites, red neurons projecting to MB, and blue neurons projecting to RSP. Additional, higher magnification panels show labelling in more detail; FB (blue) fills the cytoplasm while retrogradely transported CTB (red) remains in vesicles and so appears granular. The open arrow head marks a single-labelled neuron projecting to MB, the closed arrow head marks a single-labelled neuron projecting to RSP, the open diamonds indicate double-labelled neurons. Scale bar = 500μm unless otherwise specified.

Extended Data Figure 2-1. Numbers of Cholera Toxin Subunit B (CTB) and Fast Blue (FB) positive cells within different proximal – distal positions (R1-R4) of the dorsal and intermediate subiculum of the rat, including the number of double-labelled cells. The case numbers and hemisphere of cell counts (R or L) are shown, along with the percentage of subicular cells projecting to the retrosplenial cortex (RSP) that are double labelled. Other abbreviations: MB, mammillary bodies.

Figure 3 Characterization of collateral-collateral transport. **A1.** Photomicrograph of collateral-collateral transport following a Cholera Toxin B (CTB) injection into the mammillary bodies. The section shows CTB terminal label in layers II and III of granular retrosplenial cortex (area 29). The Nissl stained overlay (**A2.**) confirms the abrupt border with dysgranular cortex (area 30). **B.** Coronal section showing terminal label in dorsal pars lateralis (MMBl) and pars medianus (MMBmed) of the medial mammillary nucleus following a retrosplenial CTB injection. Note, pia artefact has been removed. **C**. Coronal section showing dense terminal label in the anterior thalamic nuclei. **D.** Pattern of both retrograde and light terminal label in the entorhinal cortex after a CTB injection into the mammillary bodies. Boxes, **D2** and **D3** correspond to higher magnification images of medial and lateral entorhinal cortex respectively. **E.** Photomicrograph of dorsal subiculum following injection of an anterograde tracer (BDA). **F.** Coronal section of retrosplenial cortex showing pattern of BDA anterograde transport from dorsal subiculum. **G.** Coronal section from same level of retrosplenial cortex as depicted in F., illustrating pattern of CTB terminal label following CTB injection in mammillary bodies. Abbreviations: AD, anterodorsal thalamic nucleus; AM, anteromedial thalamic nucleus; AV anteroventral thalamic nucleus; BDA, biotinylated dextran amine; LMB, lateral mammillary nucleus; MB, mammillary bodies; MMBl, medial mammillary body, pars lateralis; MMBm, medial mammillary body, pars medialis. Scale bars = 500µm unless otherwise specified.

Figure 4. Absence of collateral-collateral transport to retrosplenial cortex following a Cholera Toxin B (CTB) injection into the mammillary bodies combined with lesion involving the postcommissural descending fornix. **A., B.** Nissl stained sections, 1.56 mm behind bregma (according to Paxinos and Watson, 2005), showing postcommissural fornix lesion (**A.**) and intact case (**B.**) respectively. **C.** Coronal photomicrograph showing the very limited retrograde label in proximal dorsal subiculum after a postcommissural fornix lesion. **D**. Typical appearance of retrograde label in the dorsal subiculum in an intact case (CTB in mammillary bodies). **E**. Lack of terminal label in the retrosplenial cortex after postcommissural fornix lesion. The inset provides a comparison with an intact case. **F., G.** Retrogradely labelled neurons in Gudden's ventral tegmental nucleus when the postcommissural descending fornix is lesioned (**F.**) or intact (**G.**) Note, while the label in D appears more restricted, it is denser. Abbreviations: 3V, 3rd ventricle; opt, optic nerve. Scale bars = 500µm.

Figure 5. Cross-species comparisons. **A.** Coronal section showing Cholera Toxin B (CTB) injection into mouse mammillary bodies. **B.** Coronal section showing Fast Blue (FB) injection into mouse retrosplenial cortex. **C.** Coronal photomicrograph of dorsal subiculum. The numerous double-labelled (pink) cells innervate both sites. The open arrow head marks a single-labelled neuron projecting to MB, the closed arrow head marks a single-labelled neuron projecting to RSP, the open diamonds indicate double-labelled neurons. Inset depicts higher magnification of indicated region. The open arrow head points to a single-labelled neuron projecting to MB, the

closed arrow head to a single-labelled neuron projecting to RSP, the open diamonds indicate double-labelled neurons. Associated cell counts are presented in Extended Data Figure 5-1. **D1**. Red terminal label in the granular retrosplenial cortex (area 29) from collateral-collateral transport, alongside scattered retrogradely labelled cells in retrosplenial cortex and the indusium griseum (IG). **D2.** A Nissl stained overlay of section B1 shows the border between area 29 and area 30. The label is concentrated in deep layer II and layer III of area 29. Abbreviations: LMB, lateral mammillary bodies; MMB, medial mammillary bodies; PM, premammillary nucleus. Scale bar = 500µm unless otherwise specified.

Extended Data Figure 5-1. Numbers of Cholera Toxin Subunit B (CTB) and Fast Blue (FB) positive cells within of the dorsal and intermediate subiculum of the mouse, including the number of double-labelled cells. The case numbers and hemisphere of cell counts (R or L) are shown, along with the percentage of subicular cells projecting to the retrosplenial cortex (RSP) that are double labelled. Other abbreviations: MB, mammillary bodies.

Figure 6. Neurochemical characterization of collateral-collateral terminals. **A1.** Combined immunohistochemical signal for VGluT2 matching the distribution of Cholera Toxin B (CTB) terminal label localized in superficial area 29. **A2** shows at greater magnification the separate CTB and VGluT2 label, with the overlay showing co-localization within layers II and III of area 29. **B1**. Combined immunohistochemical signal for neurotensin (NT) matching the distribution of Cholera Toxin B (CTB) terminal label localized in superficial area 29. **B2** shows at greater magnification the separate CTB and NT label, with the overlay showing co-localization within layers II and III of area 29. Scale bar = 500µm unless otherwise specified. Note, pia artefact has been removed. Neurochemicals that did not co-localize with the CTB positive terminals are shown in Extended Data Figure 6-1.

Extended Data Figure 6-1. Series of coronal immunofluorescence images at the level of the retrosplenial cortex in an animal with a Cholera Toxin B (CTB) injection in the mammillary bodies. Left column: Green immunofluorescent label associated with antibodies for VGluT1, GAD67, parvalbumin (PV), calretinin (CR), calbindin (CB), and cholecystokinin (CCK). Middle column: CTB terminal label in the retrosplenial cortex (area 29, layers II and III) highlighting the collateralizing subiculum projections that were present in the same section as depicted in the left column. Right column: The section overlay shows how the distribution of these neurochemicals do not match the termination sites of the collateral projections from the subiculum to area 29. Scale bar = 500µm.

Figure 7. Schematic depictions of described hippocampal network connectivity. **A.** Ipsilateral and crossed collaterals from the subiculum reach the mammillary bodies (MB) and retrosplenial cortex (RSP, area 29). Note, the subiculum projections to area 29 remain ipsilateral while collaterals to MB can remain ipsilateral or cross

hemispheres. **B.** Updated hippocampal-limbic network ('Papez' circuit') showing the ventral (subcortical), dorsal (cingulate), and new 'collateral' routes. Other abbreviations: ATN, anterior thalamic nuclei; MTT, mammillothalamic tract.