Monosynaptic connection from the subiculum to medial mammillary nucleus neurons projecting to the anterior thalamus and Gudden’s ventral tegmental nucleus

Ryoko Umaba a, 1, Takuma Kitanishi b, c, *, Kenji Mizuseki b, *

a Department of Neurosurgery, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan
b Department of Physiology, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan
c PRESTO, Japan Science and Technology Agency (JST), Kawaguchi, Saitama 332-0012, Japan

A R T I C L E   I N F O

Article history:
Received 22 October 2020
Received in revised form 29 December 2020
Accepted 14 January 2021
Available online 18 January 2021

Keywords:
Anterograde transsynaptic tracing
Papez circuit
Hippocampal formation
Hypothalamus
Thalamus
Hippocampus
Memory

A B S T R A C T

As a major hippocampal output structure, the subiculum projects to diverse cortical and subcortical areas, and its projection to the medial mammillary nucleus (MM) has been implicated in memory. Major efferent targets of the MM are the anteroventral and anteromedialthalamic nuclei and Gudden’s ventral tegmental nucleus. These projections may play a key role in distributing subicular information. However, it remains unknown whether neurons in the MM that receive monosynaptic input from the subiculum project to these target regions. We addressed this issue with anterograde transsynaptic tracing mediated using adeno-associated virus serotype 1 (AAV1). Injection of AAV1-Cre and a Cre-dependent AAV encoding enhanced yellow fluorescent protein (EYFP) into the rat dorsal subiculum and MM, respectively, labeled the soma of the MM and axons in the anteroventral/antem medialthalamic nuclei and Gudden’s ventral tegmental nucleus with EYFP. The EYFP-positive neurons in the MM were immunoreactive for glutamate and leu-enkephalin and received perisomatic GABAergic inputs. These results revealed monosynaptic projections from the subiculum to MM neurons projecting to the anteroventral/anterm edialthalamic nuclei and Gudden’s ventral tegmental nucleus. This monosynaptic connection may support a fast and robust signal flow through the hippocampal–mammillothalamic and hippocampal–mammillotegmental pathways.

© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Although the hippocampus is essential for spatial and episodic memory (Scoville and Milner, 1957; O’Keefe and Dostrovsky, 1971; Buzsáki and Moser, 2013), the entire memory process including consolidation and recall of memory requires precise communication between the hippocampus and extra-hippocampal areas (Squire, 1986; Mcclelland et al., 1995; Buzsáki, 1996; Wiltgen et al., 2004; Tse et al., 2007; Preston and Eichenbaum, 2013; Ciocchi et al., 2015; Kitamura et al., 2017; Roy et al., 2017). Despite this essential role in memory processes, the distribution of information from the hippocampus to its downstream targets, particularly to its subcortical targets, has received considerably lesser investigative attention than the role of the hippocampus itself.

The subiculum (SUB) is a major output structure of the hippocampal formation (Matsumoto et al., 2019). The hippocampal CA1 area and the entorhinal cortex innervate the SUB, while SUB neurons provide excitatory projection to diverse cortical/subcortical areas, including the nucleus accumbens, retrosplenial cortex, entorhinal cortex, anterior thalamic nuclei (ATN), and medial mammillary nucleus (MM) (Allen and Hopkins, 1989; Witter et al., 1990; Honda and Ishizuka, 2015; Roy et al., 2017; Cembrowski et al., 2018; Kinnavane et al., 2018; Matsumoto et al., 2019; Kitanishi et al., 2020). The SUB and MM constitute parts of a multiregion loop called the Papez circuit, which has been implicated in memory processing; originating in the hippocampus, it extends through the SUB, MM, ATN, and cingulate cortex before returning to parahippocampal areas (Papez, 1937; Bubb et al., 2017). Dam-
age to components of the Papez circuit impairs memory. Atrophy of the MM is closely associated with an amnestic disorder called Korsakov syndrome (Vann, 2010). In animal models, lesions in the MM, ATN, or the mammillothalamic tract connecting the two structures impair memory (Vann, 2010). In addition to projecting to the ATN, the MM shares reciprocal connections with Gudden’s ventral tegmental nucleus (VTg) (Hayakawa and Zyo, 1990; Wirtshafter and Stratford, 1993). The association between lesions in the VTg and impaired memory implicates the interaction between the MM and VTg in memory processing (Vann, 2009, 2013).

The aforementioned anatomical and behavioral evidence highlights the potential role of the MM in distributing information sourced from the SUB to the ATN and VTg. In the MM, there are at least two types of projection neurons selectively targeting distinct nuclei in the ATN, anteroverentral thalamic nucleus (AV), and anteromedial thalamic nucleus (AM) (Hayakawa and Zyo, 1989; Shibata, 1992). Moreover, recent single-cell RNA sequencing found five transcriptionally-distinct neuronal populations in the MM, two of which were likely to overlap the AV-projecting and AM-projecting populations (Mickelsen et al., 2020). While SUB–MM and MM–ATN/VTg projections have been extensively studied and well established (Takeuchi et al., 1985; Allen and Hopkins, 1989; Roy et al., 2017; Bienkowski et al., 2018; Cembrowski et al., 2018; Kinnavane et al., 2018; Ding et al., 2020), the type of MM projection neurons receiving monosynaptic input from SUB neurons has not yet been identified. To identify this, we used antero- grade transsynaptic tracing with adeno-associated virus serotype 1 (AAV1) (Zingg et al., 2017, 2020). While AAV-mediated transgene expression is generally confined to neurons that are primarily infected by the AAV, high titer AAV1 exhibits anterograde transsynaptic transport that depends on neurotransmitter release machinery. Hence, by injecting AAV1-Cre and the AAV with a Cre-dependent expression cassette into brain regions containing presynaptic and postsynaptic neurons, respectively, the postsynaptic neurons receiving monosynaptic input from the presynaptic region are labeled (Zingg et al., 2017, 2020). Moreover, the downstream targets of the labeled postsynaptic neurons can be identified by tracing their axons (Zingg et al., 2017, 2020). Using this method to investigate the neuroanatomy of rats, we found that dorsal SUB neurons synapse onto MM neurons projecting to the AV/AM and VTg.

2. Materials and methods

All procedures related to animal care and use were approved by the Institutional Animal Care and Use Committee of Osaka City University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

2.1. Adeno-associated virus (AAV)

AAV serotype 1 expressing Cre recombinase under the control of human Synapsin (hSyn) promoter, AAV1-Cre, was obtained from Penn Vector Core [Lot No. CS1087, AV-1-PV2676; 4.37 × 10^{13} genome copies (GC)/ml]. AAV serotype 5 expressing mCherry under the control of hSyn promoter, AAV5-mCherry, was obtained from UNC Vector Core [Lot No. AV5034c, 4.8 × 10^{12} GC/ml]. AAV chimeric serotype 1/2 expressing enhanced yellow fluorescent protein (EYFP) under the control of elongation factor 1α (EF1α) promoter after recombination with Cre, AAV-DIO-EYFP (9.2 × 10^{11} GC/ml), was produced by co-transfecting plasmids into 293 T cells (RCB2202, Riken BRC) and was purified with heparin columns (HiTrap, GE Healthcare) as previously described (Kitanishi et al., 2015; Kitanishi and Matsu, 2017). Its titer was measured using qPCR (StepOnePlus, Applied Biosystems). The transfected plasmids were as follows: pAAV-EF1α-DIO-EYFP (No. 20296, Addgene), pXR1 (National Gene Vector Biorepository), pAAV-RC (AAV Helper-Free System, Stratagene), and pHelper (AAV Helper-Free System).

2.2. Surgery

Stereotaxic injections were performed with male Long-Evans rats (n = 5, 2, and 4 for anterograde transsynaptic tracing, control experiment 1, and control experiment 2, respectively; weight, 307–341 g; age on the day of surgery, 8–9 weeks; SLC, Japan) under anesthesia (5% and 2% isoflurane for induction and maintenance, respectively, with 0.5 L/min oxygen and 0.5 L/min air). AAV1-Cre diluted with phosphate-buffered saline (PBS) (1:3; v/v) (200 nL, 5,000 nL/h) was injected into the left dorsal SUB (anterior-posterior from bregma [AP], –6.1 mm; mediolateral from the midline [ML], 3.3 mm; dorsoventral from the cortical surface [DV], 2.8 mm) (Paxinos and Watson, 2006). Seven days later, undiluted AAV-DIO-EYFP was injected into the MM. To spare the superior sagittal sinus running along the midline, this injection was performed through a craniotomy hole made above the right hemisphere (AP, –4.2 mm; ML, 1.5 mm), from which a glass pipette was inserted at an angle of 10 degrees in the coronal plane with the tip pointing toward the medial direction. To reliably deliver the AAV-DIO-EYFP to the MM, the injection was performed at three depths (DV, 8.9, 9.1, and 9.3 mm; 400 nL/site; 5,000 nL/h) (Paxinos and Watson, 2006). Although this multi-depth injection could introduce the AAV-DIO-EYFP not only in the MM but also in the adjacent areas, such as the supramammillary nucleus (SuM), we observed few EYFP-positive somata in the SuM (Figs. 1 and 2) since the SuM lacks afferent inputs from the SUB (Pan and McNaughton, 2004). The aforementioned two-step injection of the AAV1-Cre and AAV-DIO-EYFP was performed to minimize any risk of contamination between the two AAVs during surgery (Zingg et al., 2017). For control experiment 1, PBS (200 nL) instead of AAV1-Cre was injected into the SUB. For control experiment 2, AAV1-Cre (200 nL) was applied to the cortical surface above the dorsal SUB (AP, –6.1 mm; ML, 3.3 mm; DV, 0 mm) instead of the SUB. For both control experiments, the AAV-DIO-EYFP was injected into the MM as described above. In the control experiment 2, the AAV-DIO-EYFP was supplemented with a one-tenth volume of AAV5-mCherry, the expression of which was used to confirm the injection site in the post-hoc histologic analysis. We administered analgesics (meloxicam, 1 mg/kg body weight, subcutaneous) and antibiotics (cefazolin, 50 mg/kg body weight, intramuscular) after the surgery.

2.3. Histology

Fourteen days following the second surgery, rats were transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were stored in the same fixative overnight at 4 °C. They were then sectioned with a vibratome (VT1200S, Leica) at a thickness of 50 μm parallel to the coronal plane. The sections were incubated sequentially with 5% bovine serum albumin (BSA) / 0.3 % Triton X-100 in PBS for 30 min at room temperature, primary antibodies in 5% BSA/PBS overnight at 4 °C, and corresponding secondary antibodies conjugated with Alexa Fluor dyes in 5% BSA / PBS overnight at 4 °C. The sections were counterstained with DAPI (0.5 μg/mL, D1306, Thermo Fisher). The sections were washed with PBS between the incubations. The following primary antibodies were used: Chicken anti-GFP (1:2,000, ab13970, Abcam), mouse anti-Cre recombinase (1:2,000, MAB3120, Millipore), rabbit anti-glutamate (1:2,500, G6642, Sigma-Aldrich), mouse anti-GAD67 (1:1,000, MAB5406, Millipore), and rabbit anti-leu-enkephalin (1:500, AB5024, Millipore). The following secondary antibodies were used at a dilution of 1:50.
of 1:800; goat anti-chicken IgY conjugated with Alexa Fluor 488 (A-11039, Thermo Fisher), goat anti-mouse IgG with Alexa Fluor 594 (A-11032, Thermo Fisher), goat anti-mouse IgG with Alexa Fluor 647 (A-21236, Thermo Fisher), and goat anti-rabbit IgG with Alexa Fluor 594 (A-11037, Thermo Fisher). The sections were mounted on coverslips with an antifade mountant (P36961, Thermo Fisher). Tiled fluorescent images were obtained with a confocal microscope (LSM700, Zeiss) equipped with 20× (numerical aperture = 0.8) and 40× (numerical aperture = 0.95) objectives. Cre-positive and EYFP-positive soma were detected from the fluorescent images using an automated software (Image-based Tool for Counting Nuclei, Center for Bio-image Informatics at University of California, Santa Barbara), followed by the manual correction of erroneous detections (Kitanishi and Matsuo, 2017). EYFP-positive axons in the ATN and VtG were semi-automatically traced with Neurolucida software (Rodriguez et al., 2008; Kitanishi et al., 2009) and SNT toolbox (Arshadi et al., 2020).

3. Results

To specifically label MM neurons that receive monosynaptic input from the SUB neurons, we injected AAV1-Cre and AAV-DIO-EYFP into the left dorsal SUB and MM, respectively (Fig. 1a). Cre recombinase expression was largely confined to the dorsal SUB (Figs. 1b and 2). A relatively high titer AAV1-Cre is necessary for its anterograde transsynaptic spread (Zingg et al., 2017); however, highly expressed Cre can damage mammalian cells by recognizing genomic DNA sequences that resemble loxP sites (Loonstra et al., 2001; Semprini et al., 2007; Rezai Amin et al., 2019). Thus, we diluted AAV1-Cre to minimize cell damage while maintaining the transsynaptic spread. Nonetheless, Cre was often undetected in the cells surrounding the injection sites (~200 μm from the injection sites; Figs. 1b and 2), which might be due to the adverse effects of Cre. Numerous EYFP-positive somata were observed in the MM but only a few in the adjacent lateral mammillary nucleus (LM) or SuM (Figs. 1c, d and 2). EYFP-positive neurons in the MM often expressed Cre (Fig. 1d). This result supports the idea that AAV1-Cre in SUB neurons showed trans-neuronal spread to the MM neurons, which in turn induced Cre-dependent EYFP expression in the MM neurons. In cases of Cre expression into the presubicular (PrS), more EYFP-positive somata were observed in the LM (cases ru0047 and ru0050, Fig. 2), which is consistent with the known PrS layer 4–LM projection (Vann and Aggleton, 2004; Simonnet and Fricker, 2018). Considering the anatomical division of the MM into the medial (pars medialis and pars medius) and lateral (pars lateralis) components situated medially and laterally to the principal mammillary tracts (pm), respectively (Dillingham et al., 2015), the EYFP-positive cells were found to be dense in the pars medialis / pars medius and left pars lateralis ipsilateral to the AAV1-Cre injection site and sparse in the right pars lateralis (Figs. 1c and 2). EYFP-positive axons were observed in the bilateral pm (Fig. 1c, e) connecting MM to the ATN and Vtg. The bilateral AV and AM contained EYFP-positive axons, while the anterodorsal thalamic nucleus (AD) contained no or very few EYFP-positive axons (Figs. 1f, g and 2). In one case with EYFP-positive somata predominantly in the pars medialis / pars medius (case ru0049, Fig. 2), EYFP-positive axons were primarily observed in the AM but sparsely in the AV. This is in agreement with the known topography of MM–ATN connections where lateral and medial parts of the MM preferentially project to AV and AM, respectively (Hayakawa and Zyo, 1989; Shibata, 1992; Mückelsen et al., 2020). EYFP-positive axons were also observed in the bilateral Vtg (Figs. 1h and 2). The axonal labeling in the AV, AM, and VtG was denser in the left hemisphere than in the right (Figs. 1f–h and 2).
To confirm the specificity of the observed EYFP expression, we performed two control experiments. In the first control experiment, we injected PBS – instead of AAV1-Cre – and AAV-DIO-EYFP into the SUB and MM, respectively (Fig. 3a). As Cre and EYFP expression was not observed in the SUB and MM, respectively (Fig. 3b, c), the AAV-DIO-EYFP injection revealed no leaky EYFP expression independent of Cre recombinase. In the second control experiment, we applied AAV1-Cre to the cortical surface dorsal to the SUB and injected AAV-DIO-EYFP into the MM (Fig. 3d). While Cre recombinase was occasionally expressed in the superficial cerebral cortex (Fig. 3e), no EYFP expression was detected in the MM (Fig. 3f). These results suggest that EYFP labeling in the MM (as shown in Fig. 1) requires the injection of AAV1-Cre into the SUB and not the cerebrospinal fluid or nearby areas, such as the cerebral cortex, that do not share monosynaptic connections with the MM (Vann and Aggleton, 2004). These findings collectively demonstrate that dorsal SUB neurons synapse onto MM neurons projecting to the AV/AM and VTg.

We subsequently characterized EYFP-positive cells in the MM (Fig. 1) using immunohistochemistry (Fig. 4). Most EYFP-positive cells were found to be immunoreactive for glutamate (Fig. 4a, 4b) and leu-enkephalin (Fig. 4c, d). The somata of most EYFP-positive cells were surrounded by glutamic acid decarboxylase 67 (GAD67)-positive terminals (Fig. 4e, f), suggesting that these cells receive perisomatic GABergic inputs. Interneurons are thought to be absent in the rodent MM (Veazey et al., 1982; Mugnaini and Oertel, 1985; Allen and Hopkins, 1989; Gonzalo-Ruiz et al., 1996). Consistently, none of the EYFP-positive somata were immunoreactive for GAD67 in the MM (Fig. 4e, f). Thus, our findings indicate that GAD67-positive terminals may originate from a source outside the MM, such as the VTg (Gonzalo-Ruiz et al., 1993; Wirtshafter and Stratford, 1993). These immunohistochemical features were consistent between the pars medialis/medianus and pars lateralis of the MM (Fig. 4a–f).

4. Discussion

Using AAV1-mediated anterograde transsynaptic tracing, we found that dorsal SUB neurons send monosynaptic efferents to MM neurons that project to the AM, AV, and/or VTg. We also
Fig. 3. Control experiments of anterograde transsynaptic tracing.
(a) Schematic of the first control experiment. We injected phosphate-buffered saline (PBS) and AAV-DIO-EYFP into the subiculum (SUB) and medial mammillary nucleus (MM), respectively.
(b,c) Coronal sections showing no Cre (red) expression in the SUB (b) and no EYFP (green) expression in the MM (c). Dotted lines represent borders of the SUB, parahippocampal areas, CA1 pyramidal cell layer, and the superficial layer of the presubiculum (b) and MM (c). D, dorsal; V, ventral; L, lateral; M, medial.
(d) Schematic of the second control experiment. We applied AAV1-Cre to the cortical surface and injected AAV-DIO-EYFP into the MM.
(e,f) Coronal sections showing Cre (red) expression in the cerebral cortex (CC) dorsal to the SUB (e) and no EYFP (green) expression in the MM (f). Dotted lines represent borders of the SUB, parahippocampal areas, CA1 pyramidal cell layer, and the superficial layer of the presubiculum (e) and MM (f). LV, lateral ventricle.

Fig. 4. Immunohistochemical characterization of the medial mammillary nucleus (MM) neurons receiving subiculum (SUB) output.
(a–f) Coronal MM sections showing EYFP (green)-positive cells and cellular markers (purple), such as glutamate (a, b), leu-enkephalin (c, d), and GAD67 (e, f) in the pars medialis/medianus (a, c, e) and left pars lateralis (b, d, f). Columns show merged, EYFP, and marker images (from left to right). (a–d) Arrowheads, double-positive cells for EYFP and marker. (e, f) Arrowheads, EYFP-positive cells whose soma is surrounded by GAD67-positive terminals. EYFP, enhanced yellow fluorescent protein; GAD, glutamic acid decarboxylase.
showed that the MM neurons receiving SUB input are immunoreactive for glutamate and leu-enkephalin and receive perisomatic GABAergic inputs. Comprehensive characterization using anatomical, functional, and molecular approaches has shown that the AAV1-Cre selectively spreads to postsynaptic neurons with minimal non-specific labeling (approximately 1–4% of innervated cells) (Zingg et al., 2017, 2020). A known limitation of AAV1-mediated anterograde transsynaptic tracing is its unsuitability for identifying monosynaptic connections when a AAV1-Cre injected region and a Cre-dependent reporter virus injected region have reciprocal connections: if the Cre-injected and reporter-injected areas have reciprocal connections, the labeling of postsynaptic neurons can be compromised by a possible retrograde infection (i.e., infection from axons) of AAV1 (Zingg et al., 2017). As the SUB unidirectionally projects to the MM (Bubb et al., 2017), the observed EYFP expression cannot be accounted for by the retrograde infection. Therefore, most, though not all, EYFP-positive cells in the MM should constitute the postsynaptic neurons of SUB projection neurons.

The ATN is comprised of AM, AV, and AD (Jankowski et al., 2013). We observed axonal EYFP labeling in the AM and AV, but only scarcely in the AD. This is consistent with the parallel hippocampal–mammillothalamic circuit model (Vann and Aggleton, 2004): the MM receives inputs from the SUB and projects to the AM and AV, while the LM receives inputs from the pre-subiculum / parasubiculum and projects to the AD. The MM has at least two types of projection neurons; the medial and lateral parts of MM neurons selectively innervate the AM and AV, respectively (Hayakawa and Zyo, 1989; Shibata, 1992; Mickelsen et al., 2020). Most MM projection neurons share collateral projections to the VtG (Takeuchi et al., 1985; Valverde et al., 2000). Thus, our observations of EYFP-positive somata in both the medial and lateral MM and EYFP-positive axons in the AM, AV, and VtG suggest that SUB neurons synapse onto both the MM neuronal populations that project to the AM and to the AV. In addition to this topography along the MM–ATN projection, accumulating evidence suggests that the entire SUB–MM–ATN network has matched topography: the proximal SUB projects to the AM and medial parts of the MM, while the distal SUB innervates the AV and lateral parts of the MM (Christiansen et al., 2016; Bienkowski et al., 2018; Pederick et al., 2020). Thus, the AM neurons may receive the monosynaptic input from the proximal SUB projection neurons and disynaptic input from the proximal SUB via projection neurons in the medial MM, while the AV neurons may receive the monosynaptic input from the distal SUB projection neurons and disynaptic input from the distal SUB via projection neurons in the lateral MM. The monosynaptic connection found in the present study seems to be consistent with this possibility, as the AAV1-Cre injection into the proximal SUB resulted in the EYFP-labeled cells preferentially in the medial MM and EYFP-labeled axons preferentially in the AM (case ru0049). How these projections (that is, proximal SUB–medial MM–AM and distal SUB–lateral MM–AV) are associated with the diverse SUB and MM neuronal populations in terms of electrophysiological, transcriptional, and neurochemical properties (Alonso and Linas, 1992; Ishihara and Fukuda, 2016; Bienkowski et al., 2018; Ding et al., 2020; Ishihara et al., 2020; Kitanishi et al., 2020; Mickelsen et al., 2020) and how their functions differ warrant further investigations.

Although we injected AAV1-Cre unilaterally into the left SUB, EYFP labeling was observed bilaterally in the MM, AV, AM, and VtG. This is consistent with previously established evidence of bilateral projections from the SUB to the MM (Allen and Hopkins, 1989; Witter et al., 1990). Considering that the SUB and its monosynaptic upstream projection from the hippocampal CA1 area lack commissural connections (Andersen et al., 2007) and that the MM projects unilaterally to its downstream targets (Takeuchi et al., 1985; Hayakawa and Zyo, 1989; Shibata, 1992), the subicular projection to the bilateral MM may play a key role in integrating bilateral hippocampal outputs.

The MM contains projection neurons that are immunoreactive for glutamate and leu-enkephalin (Fujii et al., 1987; Gonzalo-Ruiz et al., 1998). Consistent with this finding, we observed EYFP-positive MM cells that were immunoreactive for leu-enkephalin and glutamate, though whether the EYFP-positive cells contain both or either of them, remains unaddressed. It also remains unknown to what degree the glutamate- and leu-enkephalin-containing neuronal populations overlap in the MM (Gonzalo-Ruiz et al., 1998). In contrast to the excitatory projection from the MM to VtG, the VtG sends dense GABAergic projections terminating at the somatodendritic compartments of MM neurons (Mugnaini and Oertel, 1985; Gonzalo-Ruiz et al., 1993; Wirsthafter and Stratford, 1993). As the rodent MM is thought to contain no GABAergic interneurons (Vezey et al., 1982; Mugnaini and Oertel, 1985; Allen and Hopkins, 1989; Gonzalo-Ruiz et al., 1996; Mickelsen et al., 2020), the observed perisomatic GABAergic terminals around the EYFP-positive cells might originate from the VtG neurons. Note that, in humans, ~2% of MM neurons express GAD (Dixon et al., 2004; Bernstein et al., 2007), raising the possibility that the existence of GABAergic interneurons in the MM is species-dependent.

The ATN receives SUB output in two ways: directly from the SUB and indirectly through the MM (Jankowski et al., 2013). The monosynaptic connection from the SUB to the MM projection neurons suggests that this indirect pathway can quickly transmit signals and modulate the impact of the direct pathway on the ATN with minimal temporal delay. Perisomatic GABAergic inputs that presumably project from the VtG to MM projection neurons could potentially control the signal flow through the indirect pathway. Considering the recurrent excitatory and inhibitory connections between the MM and VtG (Hayakawa and Zyo, 1990; Wirsthafter and Stratford, 1993), this loop may control the rhythmic signal flow – e.g., at theta frequency – through the indirect pathway (Bland et al., 1995; Bassant and Poidoussou-Jazat, 2001; Sharp and Koester, 2008).

Hence, this study proposes that a monosynaptic connection from the SUB to the MM projection neurons supports fast and robust information flow through the hippocampal–mammillothalamic and hippocampal–mammillotegmental circuits. The information flow through both these aforementioned circuits warrants further investigation to reveal their exact roles in memory processing.

Author contributions

T.K. and K.M. conceived the project. R.U. and T.K. performed the experiments. T.K. and K.M. wrote the manuscript with inputs from all authors.

Funding

This work was supported by JSPS KAKENHI (20H03356, 18H05137, and 16H04656) (K.M.), (20K06878, 19H04937, 17H05977, and 17H05575) (T.K.), JST PRESTO (JPMJPR1882) (T.K.), Toray Science Foundation (K.M.), Takeda Science Foundation (K.M. and T.K.), The Uehara Memorial Foundation (K.M. and T.K.), The Naito Foundation (K.M. and T.K.), The Nakajima Foundation (T.K.), SEI Group CSR Foundation (T.K.), and Osaka City University Strategic Research Grant for young researchers (T.K.).

Data availability

The data supporting this study are available from the corresponding authors upon reasonable request.
Declaration of Competing Interest
The authors declare no competing interests.

Acknowledgments
We thank Osaka City University Research Support Platform for assisting with the confocal microscopy.

References
Allen, G., Hopkins, D.A., 1989. Mammillary body in the rat: topography and synaptology of projections from the subcommissural prefrontal cortex, and midbrain tegmentum. J. Comp. Neurol. 286, 311–336.
Alonso, A., Llinas, R.R., 1992. Electrophysiology of the mammillary complex in vitro. II. Medial mammillary neurons. J. Neurophysiol. 68, 1321–1331.
Andersen, P., Morris, R., Amaral, D., Bliss, T., O’Keefe, J., 2007. The Hippocampus Book. Oxford University Press, Oxford, New York.
Arshadi, C., Günther, U., Eddison, M., Harrington, K.L.S., Ferreira, T.A., 2020. SNT: a unifying toolbox for quantification of neuronal anatomy. bioRxiv, http://dx.doi.org/10.1101.2020.07.13.179325.
Bassant, M.H., Posnennou-Jazat, F., 2001. Ventral tegmental nucleus of Gudden: a pontine hippocampal theta generator? Hippocampus 11, 809–813.
Bernstein, H.C., Krause, S., Kröll, D., Dobrowolny, H., Wolter, M., Stauf, R., Rantl, K., Käpollner, J.P., Jirikowski, G.F., Bogerts, B., 2007. Strongly reduced number of parvalbumin-immunoreactive projection neurons in the mammillary bodies in schizophrenia: further evidence for limbic neuropathy. Ann. N. Y. Acad. Sci. 1120, 120–127.
Bienkowski, M.S., Bowman, I., Song, M.Y., Gou, L., Ard, T., Cotter, K., Zhu, M., Benavidez, N.L., Yamashita, S., Abu-Jaber, J., Azam, S., Lo, D., Foster, N.N., Hirtny, H., Dong, H.W., 2018. Integration of gene expression and brain-wide connectivity reveals the multiscale organization of mouse hippocampal networks. Nat. Neurosci. 21, 1628–1643.
Bland, B.H., Konopacki, J., Kirk, I.J., Oddie, S.D., Dickson, C.T., 1995. Discharge patterns of hippocampal theta-related cells in the caudal diencephalon of the urethane-anesthetized rat. J. Neurophysiol. 74, 322–333.
Bubb, E.J., Kinnavane, L., Aggleton, J.P., 2017. Hippocampal—diencephalic—cingulate networks for memory and emotion: an anatomical guide. Brain Res. Adv. 1, 1–20.
Buzsáki, G., 1996. The hippocampo-neocortical dialogue. Cereb. Cortex 6, 81–92.
Buzsáki, G., Moser, E.I., 2013. Memory, navigation and theta rhythm in the hippocampal-entorhinal system. Nat. Neurosci. 16, 130–138.
Cembroroski, M.S., Phillips, M.G., Diliso, S.F., Shields, R.C., Winnubst, J., Chandrashekar, J., Bas, E., Spruston, N., 2018. Dissociable structural and functional hippocampal outputs via distinct subicular cell classes. Cell 173 (1280–1292), e12181.
Christiansen, K., Dillingham, C.M., Wright, N.F., Saunders, R.C., Vann, S.D., Aggleton, J.P., 2016. Complementary subicular pathways to the anterior thalamic nuclei and mammillary bodies in the rat and macaque monkey brain. Eur. J. Neurosci.
Cioccì, S., Passecker, J., Malagon-Vina, H., Mikus, N., Klausberger, T., 2015. Selective information routing by ventral hippocampal CA1 projection neurons. Science 349, 560–563.
Dillingham, C.M., Fritzazi, A., Nelson, A.J., Vann, S.D., 2015. How do mammillary body inputs contribute to anterior thalamic function? Neurosci. Biobehav. Rev. 54, 108–119.
Ding, S.L., Yao, Z., Hirokawa, K.E., Nguyen, T.N., Graybucket, L.T., Fong, O., Bohn, P., Ngo, K., Smith, K.A., Koch, C., Phillips, J.W., Lein, E.S., Harris, J.A., Tasic, B., Zeng, H., 2020. Distinct transcriptomic cell types and neural circuits of the subiculum and prosubiculum along the dorsal-ventral Axis. Cell Rep. 31, 107648.
Dixon, C., Garrick, T., Whiteman, L., Sarris, M., Sthamparanathan, S., Harper, C.G., 2004. Characterization of gabanergic neurons within the human medial mammillary nucleus. Neuroscience 127, 365–372.
Fuji, S., Senba, E., Kiyama, H., Ueda, Y., Tohyama, M., 1987. Mammillothalamic ekephalinergic pathway in the rat: an immunocytochemical analysis. Brain Res. 401, 1–8.
Gonzalo-Ruiz, A., Sanz-Anquela, J.M., Spencer, R.F., 1993. Immunohistochemical localization of GABA in the mammillary complex of the rat. Neuroscience 54, 143–156.
Gonzalo-Ruiz, A., Sanz, J.M., Lieberman, A.R., 1996. Immunohistochemical studies of localization and co-localization of glutamate, aspartate and GABA in the anterior thalamic nuclei, retrosplenial granular cortex, thalamic reticular nucleus and mammillary nuclei of the rat. J. Chem. Neuroanat. 12, 77–84.
Gonzalo-Ruiz, A., Mortel, L., Sanz, J.M., 1998. Glutamate/aspartate and leu-enkephalin immunoreactivity in mammillothalamic projection neurons of the rat. Brain Res. Bull. 47, 565–574.
Hayakawa, T., Zyo, K., 1989. Retrograde double-labeling study of the mammillothala-
mic and the mammillomammillotegmental projections in the rat. J. Comp. Neurol. 284, 466–475.
Hayakawa, T., Zyo, K., 1990. Ultrastructure of the mammillothalamotegmental projections to the ventral tegmental nucleus of Gudden in the rat. J. Comp. Neurol. 293, 466–475.
Valverde, F., Garcia, C., Lopez-Mascaraque, L., De Carlos, J.A., 2000. Development of the mammillothalamic tract in normal and Pax-6 mutant mice. J. Comp. Neurol. 419, 485–504.

Vann, S.D., 2000. Gudden’s ventral tegmental nucleus is vital for memory: re-evaluating diencephalic inputs for amnesia. J. Comp. Neurol. 419, 485–504.

Vann, S.D., 2009. Gudden’s ventral tegmental nucleus is vital for memory: re-evaluating diencephalic inputs for amnesia. J. Comp. Neurol. 419, 485–504.

Vann, S.D., 2010. Re-evaluating the role of the mammillary bodies in memory. Neuropsychoologia 48, 2316–2327.

Vann, S.D., 2013. Re-evaluating the role of the mammillary bodies in memory. Neuropsychologia 48, 2316–2327.

Vann, S.D., 2013. Dismantling the Papez circuit for memory in rats. Elife 2, e00736.

Veazey, R.B., Amaral, D.G., Cowan, W.M., 1982. The morphology and connections of the posterior hypothalamus in the cynomolgus monkey (Macaca fascicularis). I. Cytoarchitectonic organization. J. Comp. Neurol. 207, 114–134.

Wiltgen, B.J., Brown, R.A., Talton, L.E., Silva, A.J., 2004. New circuits for old memories: the role of the neocortex in consolidation. Neuron 44, 101–108.

Wirtshafter, D., Stratford, T.R., 1993. Evidence for GABAergic projections from the tegmental nuclei of Gudden to the mammillary body in the rat. Brain Res. 630, 188–194.

Witter, M.P., Ostendorf, R.H., Groenewegen, H.J., 1990. Heterogeneity in the dorsal subiculum of the rat. Distinct neuronal zones project to different cortical and subcortical targets. Eur. J. Neurosci. 2, 718–725.

Zingg, B., Chou, X.L., Zhang, Z.G., Mesik, L., Liang, F., Tao, H.W., Zhang, L.I., 2017. AAV-mediated anterograde transsynaptic tagging: mapping corticocollicular input-defined neural pathways for defense behaviors. Neuron 93, 33–47.

Zingg, B., Peng, R., Huang, J., Tao, H.W., Zhang, L.I., 2020. Synaptic specificity and application of anterograde transsynaptic AAV for probing neural circuitry. J. Neurosci. 40, 3250–3267.