Title

Intrinsic Projections of Layer Vb Neurons to Layers Va, III, and II in the Lateral and Medial Entorhinal Cortex of the Rat

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Intrinsic Projections of Layer Vb Neurons to Layers Va, III, and II in the Lateral and Medial Entorhinal Cortex of the Rat

Graphical Abstract

Highlights
- Layer V (LV) circuitry in lateral and medial entorhinal cortex is similar
- LV comprises two sublayers, Va and Vb, with Vb neurons projecting locally
- LVb neurons contact telencephalic projecting neurons in LVa
- LVb neurons also contact hippocampus-projecting neurons in LII and LIII

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In Brief
Ohara et al. demonstrate the intrinsic connectivity of layer Vb neurons of both the medial and lateral entorhinal cortex. Layer Vb neurons are key elements of two circuits in the hippocampus-memory system: a hippocampal-output circuit and a feedback loop to the hippocampus.
Intrinsic Projections of Layer Vb Neurons to Layers Va, III, and II in the Lateral and Medial Entorhinal Cortex of the Rat

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SUMMARY

Layer V of the entorhinal cortex (EC) receives input from the hippocampus and originates main entorhinal outputs. The deep-sublayer Vb, immunopositive for the transcription factor Ctip2, is thought to be the main recipient of hippocampal projections, whereas the superficial-sublayer LVa, immunonegative for Ctip2, originates the main outputs of EC. This disrupts the proposed role of EC as mediating hippocampal-cortical interactions. With the use of specific (trans)synaptic tracing approaches, we report that, in medial entorhinal cortex, layer Vb neurons innervate neurons in layers Va, II, and III. A similar circuitry exists in the lateral entorhinal cortex. We conclude that EC-layer Vb neurons mediate two circuits in the hippocampus-memory system: (1) a hippocampal output circuit to telencephalic areas by projecting to layer Va and (2) a feedback projection, sending information back to the EC-hippocampal loop via neurons in layers II and III.

INTRODUCTION

The entorhinal cortex (EC) constitutes the major gateway between the hippocampus and the neocortex and, together with the hippocampus, plays a critical role in memory and spatial navigation. Previous anatomical studies have shown that connectivity patterns of the superficial layers (layers I–III) and the deep layers (layers V and VI) of EC are strikingly different (Cappaert et al., 2015). The superficial EC neurons are the main though not exclusive recipients of cortical inputs, either directly or through adjacent cortices, and provide inputs to all subfields of the hippocampus via the perforant pathway. On the other hand, deep layer V (LV) neurons receive a substantial part of the hippocampal output via projections arising in field CA1 and the subiculum. This hippocampal output circuit via LV is considered to play an important role in transferring transiently stored information in the hippocampus to downstream neocortical networks for long-term memory formation (Buzsáki, 1996; Eichenbaum et al., 2012; Knierim, 2015). Entorhinal LV neurons also project to the superficial layers (Dolorfo and Amaral, 1998; Köhler, 1986, 1988; 1996; van Haeften et al., 2003), and it has been shown that the hippocampal information may re-enter the entorhinal-hippocampal loop (Iijima et al., 1996; Klosterman et al., 2003). This re-entrant activity (reverberation) is one of the mechanisms proposed for temporal storage of information in a neuronal network (Edelman, 1989; Iijima et al., 1996; Klosterman et al., 2003). Alternatively, these deep to superficial inputs would allow superficial neurons to compare incoming entorhinal information with hippocampally processed information (Buzsáki, 1996). This circuitry is assumed to be present in both the medial (MEC) and lateral subdivision (LEC) of the entorhinal cortex.

LV in rodents is commonly subdivided into two sublayers, layers Va (LVa) and Vb (LVb). The superficial LVa, adjacent to layer IV (lamina dissecans), comprises mainly large pyramidal neurons that are unequally distributed along the extent of both MEC and LEC. Cells in LVb appear smaller, more uniform in soma size and are more densely packed than their counterparts in LVa (Insausti et al., 1997). Recent studies in the mouse showed that these two sublayers in MEC can also be differentiated with respect to the expression patterns of transcription factors and their main connectivity (Ramsden et al., 2015; Sürmeli et al., 2015). Whereas LVa neurons express E twenty-six (ETS) variant 1 (Etv1), LVb neurons express chicken ovalbumin upstream promoter transcription factor (COPU-TF) interacting protein 2 (Ctip2). Regarding the connectivity, the latter authors showed that hippocampal afferents from CA1 terminate preferentially in LVb of MEC, whereas the efferent projections to telencephalic domains preferentially originate in LVa. This thus puts an additional synapse between neurons in LVb and LVa to close the postulated output circuit from the hippocampus to the neocortex. Neurons in LVb are known to originate long-range and local intrinsic projections (Dolorfo and Amaral, 1998; van Haeften et al., 2003; Witter et al., 1989). Therefore, Sürmeli et al. (2015) hypothesized that neurons in LVb might...
contact neurons in LVa. However, proof of such a synaptic connection from neurons in LVb to neurons in LVa is currently lacking.

We further do not know whether a similar connectional differentiation between LVa and LVb exists in LEC. This might be hypothesized in view of convincing data that the population of pyramidal neurons in LV of LEC and MEC are morphologically and electrophysiologically indistinguishable and that also in LEC, LV originates the main entorhinal efferents to telencephalic areas (Canto et al., 2008; Hamam et al., 2000, 2002; Insausti et al., 1997).

In this study, we therefore aimed to identify the projection targets of LVb neurons in MEC and LEC. We opted to carry out these analyses in the rat, because this rodent species is still commonly used as an experimental animal in neuroscience. We first examined whether LV in LEC and MEC can be subdivided into LVa and LVb based on differential protein expression. To this end, we used immunolabeling to assess the distribution of Ctip2 and Purkinje cell protein 4 (PCP4) in EC and confirmed that LV in both LEC and MEC can be divided into Ctip2/PCP4-positive LVb and Ctip2/PCP4-negative LVa. With the use of anterograde tracing, we established in the rat that transsynaptic retrograde tracing approach with rabies virus, we show that LVb neurons of LEC and MEC likely target both telencephalic-projecting LVa neurons and the hippocampus-projecting neurons in LII and LIII. We thus conclude that LVb neurons are the key elements of two main circuits in the hippocampus-memory system: a hippocampal output circuit to telencephalic areas by projecting to neurons in LVa and a feedback loop by projecting to neurons in LII and LIII.

RESULTS

In Both LEC and MEC, LVa and LVb Differ with Respect to Molecular Identity and Projections

To examine whether the EC LV of the rat can be further divided into two sublayers, we examined the distribution of Ctip2- and PCP4-positive neurons in both MEC and LEC (Figures 1A and 1B). In line with the previous mouse study, Ctip2- and PCP4-positive neurons distributed densely in MEC LVb (Kitamura et al., 2017; Sürmeli et al., 2015). Ctip2-positive neurons were also observed in MEC LII, and PCP4-positive neurons were observed in MEC LIII, which is in line with a previous study (Figure 1A; Tang et al., 2015).
In LEC, Ctip2- and PCP4-positive neurons were preferentially and densely present in the deeper portion of LV (Figure 1B), similar to MEC. In both MEC and LEC, the Ctip2- and PCP4-immunopositive neurons in LVb mainly had a small cell soma, and the overall distribution and density of these labeled neurons was similar in both EC divisions. As for the other layers in LEC, Ctip2-positive neurons were seen in superficial portion of LII (LIIa) as well. In contrast to MEC, PCP4-labeled neurons were not prominent in LII. Our data thus indicate that not only in MEC but also in LEC in the rat, LV can be divided into two sublayers by a layer-specific gene expression pattern, similar to what was reported for the mouse MEC (Sürmeli et al., 2015).

In the latter study, it was further reported that LVa and LVb in mouse MEC differ with respect to their main efferent projections such that telencephalic projections originate mainly from LVa neurons, but not from LVb neurons. To test whether this is true in the rat and whether LV in LEC shows a similar organization, we conducted a series of retrograde tracing experiments with a focus on main telencephalic targets of projections from MEC and LEC (Insausti et al., 1997; Agster and Burwell, 2009). Retrogradely transported chemical tracers were injected into either the basolateral amygdala (BLA) (n = 2), nucleus accumbens (NAC) (n = 2), prelimbic cortex (PrL) (n = 2), or the retrosplenial cortex (RSC) (n = 2). Injections into the BLA, NAC, and PrL resulted in numerous labeled neurons in LVa of both LEC and MEC. In case of RSC injections, labeled neurons were observed in MEC LVa, but not in LEC. In all cases, retrogradely labeled neurons were rarely observed in LVb of either LEC or MEC (Figures 1C–1F). These results thus show that main telencephalic projections originate preferentially from LVa neurons, but not from LVb neurons, in both LEC and MEC. These results in the rat are thus in line with and extend the observations in mouse MEC (Sürmeli et al., 2015).

This is not due to a failure of transport of the tracers as many retrogradely labeled neurons were observed in the deep layers of presubiculum (PrS) and parasubiculum (PaS), in line with previous studies (Sürmeli et al., 2015; Vertes et al., 2015). Our results thus indicate that, in the rat, in contrast to the mouse but in line with previous rat studies (Kerr et al., 2007), neurons in LVb of both LEC and MEC do not project to the anterior thalamus.

LVa and LVb Differ with Respect to Inputs

Layer V of EC is considered as the main recipient of hippocampal projections originating in CA1 and subiculum (Kloosterman et al., 2003b; Köhler, 1985a; van Haeften et al., 2003). Additional inputs arise from the medial septal complex (MS) and medial prefrontal cortex (Alonso and Köhler, 1984; Czajkowski et al., 2013; Fuchs et al., 2016; Hasselmo, 2013; Jones and Witther, 2007; Sugar et al., 2011). In none of these studies, a separation between LVa and LVb has been made, and it is only in a recent paper in mice that it is reported that CA1 project almost exclusively to LVb of MEC (Sürmeli et al., 2015). We therefore set out to investigate whether the CA1 projection in rats is equally selective in rat MEC and LEC and whether cortical and MS projections show preferential distributions to either one of the sublayers. Injecting the anterograde viral tracer, Tet-off lentivirus, into CA1 (n = 2), we confirmed and extended the previous observations in mice that axons originating in dorsal CA1 preferentially target LVb not only in MEC but also in LEC (Figure 2). We also confirmed a previous report that CA1 sends a weak projection to superficial layers of both entorhinal subdivisions (Cenquizca and Swanson, 2007; Kloosterman et al., 2003b). Injections into MS (n = 4) resulted in a densely labeled plexus in LII (Alonso and Köhler, 1984; Fuchs et al., 2016) as well as in LVa, whereas innervation of LVb was weak (Figure S2). In contrast, projections that arise from the ventral medial prefrontal cortex distribute in...
LVb of both LEC and MEC, with a higher innervation density in LEC (n = 3; Figure S3). Projections from the retrosplenial cortex distribute selectively to MEC but, like the medial prefrontal projections, preferentially terminate in LVb (n = 4; Figure S4).

LVb Neurons Originate Intrinsic Projections Targeting Projection Neurons in LVa

For the mouse MEC, Sürmeli et al., (2015) hypothesized that LVb neurons might be the main origin of the well-known local projections within the entorhinal cortex and thus would innervate neurons in LVb. However, no experimental evidence for this was provided. To assess whether this suggestion is actually correct, we traced the origin of local projections in the entorhinal cortex. After large retrograde tracer injections into the superficial layers of EC at the border between LEC and MEC (n = 3), numerous labeled neurons were observed more anteriorly in LVb of LEC (Figures 3A and 3B). This supports that neurons in LVb are the main source of the long-range deep originating EC intrinsic connections reported previously (Dolorfo and Amaral, 1998). Small retrograde injections in the superficial layers (layers I–III) of MEC resulted in labeled LVb neurons, directly deep to the injection (Figures 3C and 3D), in line with a previously described column-like short-range projection arising from LV (van Haeften et al., 2003). To further examine the target layer of these local projections of LVb, we injected the anterograde tracer (PHA-L) into LVb of EC (Figures 3E–3G; n = 2). Such injections resulted in labeled axons that traversed LVa, the lamina dissecans, and the superficial layers, eventually reaching layer I. These results indicate that LVb neurons may innervate neurons in LVa, LIII, and LII.

To substantiate that LVb neurons indeed innervate neurons in LVa that project to telencephalic structures and neurons in LII and LIII projecting to the hippocampus, we conducted transsynaptic tracing experiments with rabies virus (RV). We used a glycoprotein-deleted RV vector (ΔG-RV), which, due to the lack of the gene encoding the glycoprotein, will only label the two sets of direct projecting neurons in LII, LIII, and LVa. In contrast, a CVS strain of RV (CVS-RV) can propagate transsynaptically and thus will additionally label neurons that make synaptic contacts with the 1st order infected projection neurons identified using ΔG-RV. We predicted that neurons in LVb are among this transsynaptically labeled population. We first assessed whether LVb neurons project to principal neurons in LVa (Figure 4) by injecting either one of the two rabies strains into NAc or RSC (Figures 4A and 4E). In animals with a ΔG-RV injection into NAc (n = 7), we observed many retrogradely labeled neurons in LVa but only few labeled neurons in other layers, including LVb (Figure 4B). A comparable pattern of labeling was observed following injections with CVS-RV injection with a short survival time (36 hr; data not shown). In contrast, sixty hours after a CVS-RV injection into NAc (n = 4), many labeled neurons were observed not only in LVa but also in LVb of both LEC and MEC (Figure 4C; Table S1). These labeled neurons in LVb were Ctip2 positive (Figure S5). The number of labeled LVb neurons in case of the CVS-RV experiments was significantly higher than in case of the ΔG-RV-injected samples (p < 0.01; Mann-Whitney U test; Figure 4D). This indicates that most of LVb neurons were transsynaptically labeled. Because LVb neurons do not project to NAc directly, as concluded based on the above described “classic” tracing experiments, these results strongly indicate that LVb neurons make mono-synaptic contacts with the NAc-projecting neurons in LVa. Injections of RV into RSC (n = 4 for ΔG-RV injection; n = 4 for CVS-RV injection) also resulted in labeled neurons in various regions of EC, including LVb (Figure 4F).
Injection) resulted in labeled neurons distributed predominantly in MEC, in line with the previous experiments and the literature (Burwell and Amaral, 1998). Similar to NAc injection samples, many labeled neurons were observed in MEC LVa in both ΔG-RV- and CVS-RV-injected samples (Figures 4F and 4G; Table S2). In contrast, the number of labeled LVb neurons increased significantly in case of CVS-RV injections compared with ΔG-RV cases (p < 0.05; Mann-Whitney U test; Figure 4H). These results thus indicate that MEC neurons in LVb are synaptically connected with the RSC-projecting MEC LVa neurons.

**LVb Neurons Originate Intrinsic Projections Targeting Hippocampal-Projecting Neurons in LII and LIII**

We next assessed whether LVb neurons target principal neurons in the superficial LII and LIII that project to the hippocampus. We injected the same pair of RV into the dorsal hippocampus involving both dentate gyrus (DG) and CA1 (Figure 5A). Injections of ΔG-RV into the hippocampus (n = 6) resulted in retrograde labeling in layer II and III of both LEC and MEC (Figure 5B). A very low number of labeled neurons were also present in layers Va, Vb, and VI, in line with previous reports about sparse hippocampal projections from layer V and VI neurons (Deller et al., 1996; Gloveli et al., 2001; Köhler, 1985b; Table S3). In contrast, in cases with CVS-RV injection (n = 4), in addition to labeling in the superficial layers, many labeled neurons were observed in LVb of both LEC and MEC (Figure 5C). The number of LVb-labeled cells, normalized over the total number of LII and LIII-labeled cells in each experimental animal, was significantly higher in CVS-RV-injected samples than in the ΔG-RV-injected samples in both LEC (p < 0.01; Mann-Whitney U test) and MEC (p < 0.01; Mann-Whitney U test; Figure 5D).

Similar to what we observed following ΔG-RV injections, we observed labeled neurons in LVa of both LEC and MEC following CVS-RV injections (Figure 5C; Table S3). The labeling of LVa neurons can thus reflect either direct projections or can be the result of transsynaptic labeling through their local projections to the superficial layers (Canto and Witter, 2012a, 2012b), although we cannot exclude that some LVa neurons were labeled transsynaptically through other possible extrahippocampal targets, such as the thalamic nucleus of reuniens (Herkenham, 1978) or the septum (Alonso and Köhler, 1984).

Because we have shown that LVb neurons do not project to brain targets outside the entorhinal cortex, our data support the conclusion that the LVb neurons were transsynaptically labeled by way of the hippocampal-projecting neurons located in superficial layers II and III.

**DISCUSSION**

It is well-established that neurons in entorhinal LV are the recipients of hippocampal output originating in CA1 and subiculum (Cenquizca and Swanson, 2007; Kloosterman et al., 2003b; Köhler, 1985a). Recently, an interesting detail was added in mouse MEC, that in particular, entorhinal LVb neurons are the main recipients of the hippocampal projections but that they essentially lack projections to telencephalic structures (Sürmeli et al., 2015). In our study, we confirmed in the rat that LVb neurons do not project to telencephalic regions, in accordance with previously
published data in the mouse, but we could not replicate that MEC LVb neurons project to the anterior thalamus (Sürmeli et al., 2015). Our major finding is that LVb neurons in both MEC and LEC mediate two circuits in the hippocampus-memory system: a hippocampal output circuit to telencephalic areas by projecting to LVa and a feedback loop by projecting back to the EC-hippocampal loop via neurons in LII and LIII (Figure 6). Our findings thus position LVb neurons as key elements of these two networks of the entorhinal cortex.

Our experimental data, based on transsynaptic tracing, prove the postulate that LVb neurons originate intrinsic connections within MEC (Sürmeli et al., 2015) correct by showing that indeed LVb cells send axons toward layers Va, III, and II. We further show that this holds true not only in MEC but also in LEC. We thus conclude that the hippocampal-cortical output circuit and the hippocampal re-entry circuit are not simple disynaptic pathways but more complicated trisynaptic pathways, including a third synapse involving LVb neurons.

We argue that the transsynaptic labeling is due to the transsynaptic spread of RV via EC neurons in layers II, III, and Va. The labeling of LVb neurons might be the result of indirect multisynaptic labeling through interneurons that target principal neurons in LVa, LII, and LIII. We deem this unlikely in view of the limited survival time (Iwata et al., 2011; Kelly and Strick, 2003; Miyachi et al., 2005, 2006) and, in case of LII and LIII, the fact that the majority of postsynaptic targets of local projections from LV to superficial layers are spiny principal neurons (van Haeften et al., 2003).

Previous studies have shown LV projections to superficial LII and LIII without differentiating between a potential preferred origin in LVb over LVa (van Haeften et al., 2003; Czajkowski et al., 2013; Dolorfo and Amaral, 1998). Both anatomical and electrophysiological studies indicated that these projections mainly originate from excitatory neurons (Gloveli et al., 1997; van Haeften et al., 2003) and that the net effect of activation of LV neurons is the generation of excitatory responses in layers II and III principal neurons (Iijima et al., 1996; Kloosterman et al., 2003a). It is thus likely that the labeled LVb neurons, which we observed in this study, are excitatory neurons. Thus, the information that is processed through the hippocampus will be sent back to the hippocampus through this excitatory entorhinal-hippocampal loop. Although the function of this re-entrant activity (reverberation) has not been examined directly, it is thought that this is one of the mechanisms underlying temporal storage of information in neuronal networks. However, it must be noted that, irrespective of the above-mentioned net excitatory effects, 44% of the excitatory deep-to-superficial projections make synapses on non-spiny dendritic shafts, indicative for interneurons as postsynaptic partners (van Haeften et al., 2003). It remains to be established how these excitatory and feedforward inhibitory inputs cooperatively influence the re-entry circuit and whether the deep-to-superficial inputs differ depending on whether they target DG/CA3/2-projecting LII neurons or CA1/sub-projecting LIII neurons (Iijima et al., 1996).

Our findings indicate that the hippocampal-cortical output circuit, like the hippocampal re-entry circuit, is not simple disynaptic pathways but more complicated trisynaptic pathways, mediated by neurons in LVb. What we do not know yet is whether the same neuron in LVb is involved in both pathways or acts as a...
selective component of one of the two. Irrespective of this, the
canonical cortical projection systems target the entorhinal cortex. One of
the most studied inputs in this respect originates in MS, known to
be critically involved in synchronization between hippocampal
and parahippocampal structures in the theta frequency band
(Lopes da Silva et al., 1990). Although much focus has been on the
role of this complex with respect to theta generation in LII
of MEC (Deshmukh et al., 2010; Jeeawaje et al., 2008; Tahvildari
and Alonso, 2005), we show that projections also target deeper
layers of both LEC and MEC, showing a striking preference for
LVa, corroborating previously published findings (Gonzalez-
Sulser et al., 2014). Interestingly, projections from the claustrum
also preferentially terminate more heavily in LVa and VI than in
LVb (Eid et al., 1996; Kitanishi and Matsuo, 2017). The preferred
input to LVa may control the gating of the information flow from
the hippocampus to the neocortex. This notion is supported by
reports that the projection from MEC LVa to medial prefrontal
cortex is crucial for remote memory of contextual fear conditioning
(Kitamura et al., 2017) and that this process depends on the
claustrum (Kitanishi and Matsuo, 2017). The latter authors re-
ported that inactivating the input from the claustrum to MEC
LVa impaired the long-term memory retrieval of a contextual fear
memory. In contrast, cortical inputs arising from the medial
prefrontal cortex and the retrosplenial cortex show a clear prefer-
ence for LVb of LEC and/or MEC. These inputs thus likely influ-
ence both hippocampal-EC LVb-mediated projections in a similar way, because preliminary data indicate that, in case of
MEC, inputs from RSC and subiculum converge on neurons in
LVb (Simonsen et al., 2012, FENS, abstract).

In this study, we conclude that LVb neurons of LEC and MEC
constitute local circuit elements, involved in both the hippocam-
pal re-entry circuit via LII and LIII and the hippocampal-output
circuit via LVa. The fact that both LEC and MEC share this
unique feature of having a sublayer of LV neurons dedicated to
short- and long-range intrinsic connections to both of the main
entorhinal projection systems is exceptional for cortex. In the
neocortex, neurons in deep layer V (Vb) give rise to descending
projections to brainstem structures and striatum, whereas pro-
jections from more superficial neurons (Va) seem to selectively
originate inter-telencephalic and local projections, with a sub-
class projecting also to the striatum (Gerfen et al., 2016; Kim
et al., 2015; Shipp, 2007). Whether the specific connectivity of
LVb in EC is related to the overall unique organization of EC, lack-
ing strong descending projections, and originates not only the
canonical cortical projection systems from layers V, III, and II
but also the massive hippocampal projections from LII and LIII
(Witter et al., 2017) remains to be elucidated.

EXPERIMENTAL PROCEDURES

Surgical Procedures
Young adult male Wistar rats weighing 200–250 g were used in this study. All
experiments were approved by the Center for Laboratory Animal Research,
we used a survival time of the CVS-RV-injected rats of 2.5 days in this study. After the appropriate survival periods, the animals were deeply anaesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused and fixed with 10% sucrose in 0.1 M phosphate buffer (PB) (pH 7.4) followed by 4% freshly prepared paraformaldehyde in 0.1M PB. The brains were removed from the skulls, postfixed in the same fresh fixative for 4 hr at 4°C, and then cryoprotected for at least 48 hr at 4°C in PB containing 30% sucrose. The brains were coronally, horizontally, or sagittally sectioned at 40 μm on a freezing microtome.

The Citp2- and PCP4-positive neurons, anterograde labeling, and the RV-infected neurons were visualized by immunostaining as described below. All brain sections were soaked in PBS containing 5% goat serum and 0.1% Triton X-100 (blocking solution) for an hour at room temperature. Sections were then incubated overnight at 4°C with primary antibodies diluted in the same blocking solution. Sections were subsequently washed three times with PBS containing 0.1% Triton X-100 (PBT) and incubated with secondary antibodies diluted in PBT for 4 hr at room temperature. The sections were counterstained with NeuroTrace 500/525 green fluorescent Nissl stain (1:250; Thermo Fisher Scientific) or Hoechst 33342 (1:1,000; Dojindo), washed three times with PBS, mounted onto gelatin-coated glass slides, air-dried, soaked in xylene, and coverslipped with mounting medium (Entellan new; Merck Millipore). The following antibodies were used in this study: anti-Citp2 rat immunoglobulin G (IgG) (1:250; Abcam; ab18465); anti-PCP4 rabbit IgG (1:250; Sigma; HPA005792); anti-GFP rabbit IgG (1:400; Life Technologies; A11212); anti-GFP mouse IgG (1:400; Invitrogen; A-11210); anti-PHA-L rabbit IgG (1:800; Vector Laboratories); anti-DsRed rabbit IgG (1:400; Clontech Laboratories; 632496); and monospecific rabbit anti-N anticoreum (1:25,000; supplied by Dr. Satoshi Inoue, National Institute of Infectious Diseases, Japan; Inoue et al., 2003) as primary antibodies and Alexa-Fluor-488-conjugated anti-rat; goat IgG (1:400; Jackson ImmunoResearch); Cy3-conjugated anti-rabbit goat IgG (1:400; Jackson ImmunoResearch); Alexa-488-conjugated anti-mouse goat IgG (1:400; Jackson ImmunoResearch); and Cy3-conjugated anti-rabbit goat IgG (1:400; Jackson ImmunoResearch) as secondary antibodies. Sections were examined using an Axiovert 200 M microscope (Carl Zeiss) and imaged either in the rat entorhinal cortex. II. The medial entorhinal cortex. Hippocampus 22, 1256–1276.

The numbers of RV-infected neurons in LII, LIL, and LV of LEC and MEC were counted in every section from one series, which were obtained with NAc and RSC injection, the number of labeled LvB neurons was normalized to that of labeled LII and LIII neurons. All numerical data are expressed as mean values ± the SEM. The statistical significance between direct and transsynaptic inputs was evaluated by using Mann-Whitney U test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.06.014.

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AUTHOR CONTRIBUTIONS

S.O. and M.P.W. conceived the study design. M.O., S.O., O.W.S., and R.Y. collected and analyzed the experimental data. All quantifications were carried out by M.O. All authors contributed to the discussions that resulted in the current paper, which was written by S.O., M.O., and M.P.W. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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