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## Complementary roles of differential medial entorhinal cortex inputs to the hippocampus for the formation and integration of temporal and contextual memory

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### Abstract

In humans and rodents, the entorhinal cortical (EC) -hippocampal (HPC) circuit is crucial for the formation and recall of memory, preserving both spatial and temporal information about the occurrence of past events. Both modeling and experimental studies have revealed circuits within this network that play crucial roles in encoding space and context. However, our understanding about the time-related aspects of memory are just beginning to be understood. In this review, we first describe updates regarding recent anatomical discoveries for the EC-HPC network, as several important neural circuits critical for memory formation have been discovered by newly developed neural tracing technologies. Second, we examine the complementary roles of multiple medial entorhinal cortical inputs, including newly discovered circuits, into the hippocampus for the temporal and spatial aspects of memory. Finally, we will discuss how temporal and contextual memory information is integrated in hippocampal CA1 cells. We provide new insights into the neural circuit mechanisms for anatomical and functional segregation and integration of the temporal and spatial aspects of memory encoding in the EC-HPC networks.

### Keywords

Time; Space; Cell type specific; Trace fear conditioning; Contextual fear conditioning

### Introduction

The entorhinal cortical (EC)-hippocampal (HPC) network is necessary for formation of memory across mammalian species, playing a critical role in the spatial and temporal aspects of memory (Scoville & Milner, 1957; Mahut *et al.*, 1982; Kim & Fanselow, 1992; Phillips & LeDoux, 1992; Frankland *et al.*, 1998; Eichenbaum, 2000; Tulving, 2002;

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WDM, NY, and TK wrote the manuscript. WDM, NY, and TK designed the figures. NY created the figures. WDM, NY, and TK edited and revised the manuscript. WDM, NY, and TK approved the final manuscript.

Sutherland *et al.*, 2008; Kitamura *et al.*, 2012; Pilkiw *et al.*, 2017). Memory formation is typically comprised of “what”, or representation of objects (Mahut, 1971; Eacott & Norman, 2004; Knierim, 2004; Deshmukh & Knierim, 2013; Laurent *et al.*, 2016; Connor & Knierim, 2017; Zimmermann & Eschen, 2017; Schurgin & Flombaum, 2018), “where” for spatial orientation and navigation, as well as context (O’Keefe & Dostrovsky, 1971; Morris *et al.*, 1982; Nakazawa *et al.*, 2004; Moser *et al.*, 2008; Smith & Bulkin, 2014), and “when”, which is fulfilled by the timing of event sequences, and the linkage of those events across temporal gaps (McEchron *et al.*, 2003; MacDonald *et al.*, 2011; Eichenbaum, 2014; Kitamura *et al.*, 2014; Kitamura *et al.*, 2015a; Eichenbaum, 2017; Kitamura, 2017). Importantly, each of these aspects have their own distinct mechanisms for encoding and retrieval. The central focus in this review is how differential neural circuits in the EC-HPC network encode different types of memory and how these memories are integrated in the HPC. In order for each of these memory modalities to be processed and integrated, an array of local cellular microcircuits in the EC-HPC networks must interact with each other both within and across brain regions, ultimately ending in successful memory encoding. Therefore, we first examine recent advances in anatomical knowledge of the EC-HPC network, as several important neural circuits crucial for memory formation have been discovered using the anatomical gene expression atlas project, viral-mediated neural tracing, and cell type-specific optogenetic circuit stimulation (Wickersham *et al.*, 2007; Thompson *et al.*, 2008; Varga *et al.*, 2010; Wickersham *et al.*, 2010; Kitamura *et al.*, 2014; Kohara *et al.*, 2014; Ray *et al.*, 2014; Kitamura *et al.*, 2015b).

In experimental animal studies, the EC-HPC network is traditionally known for its critical role in the processing of spatial information and there has been significant progress toward understanding the neural circuit mechanisms underlying the spatial/contextual memory (O’Keefe & Dostrovsky, 1971; Morris *et al.*, 1982; Wilson & McNaughton, 1993; Eichenbaum *et al.*, 1999; Moser *et al.*, 2008; Hasselmo, 2011; Buzsaki, 2013; Ekstrom & Ranganath, 2017). However, neural circuit mechanisms and their process in the EC-HPC network on temporal memory are just beginning to be understood (Buzsaki, 2013; Eichenbaum, 2014; 2017; Ekstrom & Ranganath, 2017). As our second topic, we examine the structure and function of neural circuits in the EC-HPC network involved in temporal memory by focusing on temporal association learning, which allows animals to associate two temporally segregated events (Wolff, 1966; Solomon *et al.*, 1986; Moyer *et al.*, 1990; Wallenstein *et al.*, 1998; McEchron *et al.*, 1999). As a complementary function to temporal association learning, we also examine the neural circuit activity essential for contextual memory in the EC-HPC network as a component of spatial memory (Marr, 1971; O’Reilly & McClelland, 1994; Treves & Rolls, 1994; Leutgeb *et al.*, 2007; McHugh *et al.*, 2007).

We review here the differential neural circuits in the EC-HPC system that contribute to temporal and spatial memory which can be integrated to form a more robust event memory (Fortin *et al.*, 2002; MacDonald *et al.*, 2011; Naya & Suzuki, 2011; MacDonald *et al.*, 2013; Kitamura *et al.*, 2014; Sakon *et al.*, 2014; Allen *et al.*, 2016; Aronov *et al.*, 2017; Eichenbaum, 2017). We lastly discuss possible neural mechanisms describing how temporal and contextual memory are integrated in hippocampal CA1 pyramidal cells in order to elucidate the circuit functionality underlying these processes as they pertain to the EC-HPC network.

## Multiple entorhinal cortical inputs to hippocampus

Previous studies with physical or chemical lesions and anterograde/retrograde tracing have revealed the extensive reciprocal synaptic connections between EC and HPC; superficial layers of EC (II/III) project to HPC and HPC projects back to the deep layer of EC (V/VI) (Amaral & Witter, 1989; Amaral & Lavenex, 2007; Kitamura, 2017). Specifically, the superficial layer of entorhinal cortex provides two major excitatory inputs into hippocampus (Fig 1), the direct (EC layer III → HPC CA1), and the indirect (tri-synaptic) pathway (EC layer II → HPC dentate gyrus (DG), DG → CA3 and CA2, CA3 and CA2 → HPC CA1) (Amaral & Witter, 1989; Amaral & Lavenex, 2007; Kitamura, 2017; Kitamura *et al.*, 2017). Excitatory stellate cells in EC layer II (Reelin<sup>+</sup> cells) project to DG, CA3 and CA2 (Tamamaki & Nojyo, 1993; Kohara *et al.*, 2014), while Excitatory pyramidal cells in EC layer III (ECIII cells) directly project to CA1 (Amaral & Witter, 1989; Amaral & Lavenex, 2007; Kohara *et al.*, 2014) (Fig. 1). Though previous findings made using broadly-acting electrical stimulation of fiber bundles suggested direct input from ECIII to CA2 (Chevalayre & Siegelbaum, 2010), Kohara et al determined that such a direct connection does not exist from their more finely tuned experiments (Kohara *et al.*, 2014). The experimenters expressed ChR2 specifically in medial ECIII (MECIII) neurons and subsequently performed whole-cell patch clamp on the downstream CA2 neurons. They then optogenetically stimulated MECIII axons with brief pulses of blue light. Following these stimulatory pulses, a lack of mono-synaptic response activity was found in the CA2 pyramidal cells (Kohara *et al.*, 2014), suggesting that rather than receiving input from MECIII, CA2 receives inputs primarily from the tri-synaptic pathway (Fig. 1). The excitatory population of cells in ECII is comprised of two morphological subpopulations; stellate and pyramidal cells (Alonso & Klink, 1993; Klink & Alonso, 1997) (Fig. 1). Stellate cells in ECII express Reelin (we refer to these as Reelin<sup>+</sup> cells), while pyramidal cells in ECII express both CalbindinD-28K (CalB) and Wolfram syndrome 1 (Wfs1) (we refer to these as CalB<sup>+</sup> cells) (Varga *et al.*, 2010; Kitamura *et al.*, 2014; Ray *et al.*, 2014). CalB<sup>+</sup> cell populations are not homogeneously distributed, but rather CalB<sup>+</sup> cells form clusters arranged in a hexagonal formation along the border of ECII and ECI, surrounded by Reelin<sup>+</sup> cells (Kitamura *et al.*, 2014; Ray *et al.*, 2014). These clustered CalB<sup>+</sup> cells have been referred to as “island cells”, and Reelin<sup>+</sup> cells as “ocean cells” (Kitamura *et al.*, 2014). In addition to morphological and broader cytoarchitectural differences in the distribution of these populations, they also possess differential physiological properties, molecular markers, and most importantly, project to different areas of the EC-HPC circuit (Alonso & Klink, 1993; Tamamaki & Nojyo, 1993; Klink & Alonso, 1997; Varga *et al.*, 2010; Kitamura *et al.*, 2014; Ray *et al.*, 2014). While it has been well known that ECII stellate cells directly project to DG, CA3, and CA2, the projection patterns of ECII pyramidal cell remained unknown. In 2014, using a cell type-specific optogenetic approach, Kitamura et al. described a previously undiscovered direct projection originating from the CalB<sup>+</sup> pyramidal cells of ECII to the GABAergic interneurons of stratum lacunosum (SL) in CA1 (SL-INs), while MECIII neurons project directly to distal pyramidal cell dendrites in stratum moleculare (SM), in close proximity to SL, (Fig. 1) (Kitamura *et al.*, 2014). Optogenetic stimulation of the terminals of MECII ChR2-expressing CalB<sup>+</sup> neurons coupled with whole cell patch-clamp recordings in vitro demonstrated monosynaptic glutamatergic inputs onto the SL-INs. Moreover, simultaneous

recordings from SL-INs and CA1 pyramidal cells along with optogenetic stimulation of axons from MECIII cells revealed a novel gating circuit system in CA1 driven by the CalB<sup>+</sup> cells of MECII (Kitamura *et al.*, 2014). Entorhinal projections from CalB<sup>+</sup> cells in ECII to hippocampal CA1 have since been confirmed by other groups (Shu *et al.*, 2016; Surmeli *et al.*, 2016; Yang *et al.*, 2016; Zutshi *et al.*, 2018; Ohara *et al.*, 2019). In addition to excitatory neurons, GABAergic inhibitory interneurons in EC also project to the HPC. Melzer et al showed long-range projections of entorhinal interneurons onto HPC interneurons (Melzer *et al.*, 2012). Similarly, Basu et al. found long-range projecting GABAergic interneurons in EC, which mediate gating mechanisms in CA1 (Basu *et al.*, 2016). While it would be important to re-confirm these projections by using traditional tracing methods, newly developed viral tracing methods and cell type-specific optogenetic stimulation have significantly advanced our understanding of neural circuits, even in the heavily studied EC-HPC network.

## Pyramidal cells in hippocampal CA1 are crucial for temporal association learning

During the memory formation, temporally discontinuous events are linked by a process known as temporal association learning (Wolff, 1966; Solomon *et al.*, 1986; Moyer *et al.*, 1990; Wallenstein *et al.*, 1998; McEchron *et al.*, 1999). In experimental animal models, temporal association memory paradigms have been made possible with the application of Pavlovian conditioning (Pavlov, 1927; Solomon *et al.*, 1986; Maren, 2001). Delay fear conditioning, in which a neutral conditioned stimulus (CS), often in the form of a tone, is paired with a shock or other aversive unconditioned stimulus (US) in such a way that the two events occur sequentially with a small overlap in timing and then terminate simultaneously, can be used to elicit a conditioned freezing response in trained animals (Weiss & Disterhoft, 2011; Tipps *et al.*, 2014). Trace fear conditioning (TFC) introduces a temporal gap, in which the CS ends before delivery of the US begins. The time between the end of the CS and beginning of the US is called the “trace period” or “trace interval” (Solomon *et al.*, 1986; Moyer *et al.*, 1990; McEchron *et al.*, 1999). These temporally based behavioral paradigms allow for assessment of differential contributions of different brain structures to temporal association learning. The hippocampus has been shown to be indispensable for TFC, but delay fear conditioning does not appear to be hippocampus dependent (Solomon *et al.*, 1986; Moyer *et al.*, 1990; Weiss *et al.*, 1999; Tseng *et al.*, 2004). This suggests that the phenomenon of temporal association learning that bridges the gap between events across time is heavily dependent on the functional processes happening within the hippocampus (Meck *et al.*, 1984; Solomon *et al.*, 1986; Moyer *et al.*, 1990; Lyford *et al.*, 1993; Jackson *et al.*, 1998; McEchron *et al.*, 1999; Takehara *et al.*, 2002; Takehara *et al.*, 2003). In a CA1 specific N-methyl-D-aspartate (NMDA) receptor knockout mouse model, there is an observed deficit in TFC but not delay fear conditioning (Tsien *et al.*, 1996; Huerta *et al.*, 2000; Fukaya *et al.*, 2003). Subregion specific lesions within CA1 were also found to cause similar disruption of TFC. Lesions to CA3, by contrast, did not (Kesner, 2000; Kesner *et al.*, 2005). These findings suggest that the synaptic plasticity of CA1 pyramidal cells is necessary for TFC to bridge the temporal gap.

## Input from pyramidal cells in MECIII into hippocampal CA1 drives temporal association learning

Lesions to the EC have also been shown to impair temporal association memory, specifically trace-eyeblink conditioning, but again not delay eyeblink conditioning (Ryou *et al.*, 2001). Similar impairments were seen in TFC following EC lesions (Esclassan *et al.*, 2009). Additionally, infusions of muscimol into the LEC specifically resulted in impaired recall during trace eyeblink testing (Morrissey *et al.*, 2012). These findings provide evidence that in addition to CA1, the function of the EC is also necessary for temporal association memory. The use of various physical and chemical lesions, and genetic alterations to target portions of the EC-HPC network in order to determine their general contribution to a temporal association memory has been critical in uncovering the role of the hippocampus in this function, however the techniques are limited in their ability to determine the contributions of specific cell-types and local circuit activities. Two main excitatory inputs to the hippocampus arise from the EC; the tri-synaptic pathway and the direct pathway as described above (Fig. 1) (Kitamura *et al.*, 2010; Kitamura & Inokuchi, 2014; Kohara *et al.*, 2014; Kitamura *et al.*, 2015a). In order to determine the importance of the inputs from ECIII to the CA1 pyramidal cells, a critical experiment was performed by Suh *et al.* utilizing a specifically designed transgenic mouse line expressing the Tetanus-toxin light chain (TeTX) in the dorsal MECIII. The expression of TeTX caused a blockade of signal from MECIII to CA1 pyramidal cells (Suh *et al.*, 2011). The mutant mice showed a deficit in TFC but not delay fear conditioning. Utilizing a similar TeTX line to assess CA3 input to pyramidal cells, no deficit in TFC was shown (Nakashiba *et al.*, 2008; Suh *et al.*, 2011). The CA3 may still have a limited role in temporal association learning, as multiple lesion studies have shown disruptions to spatial working memory (Handelmann & Olton, 1981; Jarrard, 1983; Kesner, 2000), however, optogenetic silencing of MECII inputs from Reelin<sup>+</sup> cells to the tri-synaptic pathway did not result in reduced TFC (Kitamura *et al.*, 2015b), indicating that the monosynaptic inputs from MECIII to CA1 have a direct role in temporal association learning.

To better understand the circuit dynamics underlying temporal association memory, the use of optogenetics became necessary. With optogenetic approaches, either inhibition or activation of specific cell populations or terminals with precise timing on the order of milliseconds is possible (Boyden *et al.*, 2005; Tye *et al.*, 2011; Deisseroth, 2015), allowing for a more focused approach to understanding the individual contributions of components within the EC-HPC circuit. Importantly, this approach allows for inhibition of cellular populations at different points in a behavioral testing paradigm. Kitamura *et al.* in 2014 showed that optogenetic inhibition of MECIII input into HPC during TFC results in deficient TFC test performance (Kitamura *et al.*, 2014). Oppositely, optogenetic activation of MECIII inputs to HPC by 4Hz light stimulation enhances TFC (Kitamura *et al.*, 2014). This reveals that MECIII signal to CA1 during the gap between events is required for temporal association, and opens up a new line of questioning into what the nature of the signal itself is that allows the linkage of events across time. A possible, but still unproven, hypothesis is that persistent spiking activity of these neurons generates a consistent, pacemaker like signal that may support a framework for temporal processing downstream in the hippocampus

(Frank & Brown, 2003; Major & Tank, 2004), by creating a consistent, repetitive input from MECIII to the hippocampus acting as a backdrop against which events can be ordered. This sort of activity is observed in other cortical regions, and even within the MEC itself for other functions (Kubota & Niki, 1971; Egorov *et al.*, 2002). Persistent firing activity has been identified in MECIII cells *in vitro* (Yoshida *et al.*, 2008), but not *in vivo*. Although the phenomenon has yet to be seen, the observation *in vitro*, which is dependent on glutamatergic activation of mGluR1 and Muscarinic 5-HT receptors (Egorov *et al.*, 2002; Yoshida *et al.*, 2008), is suggestive of possible persistent firing activity *in vivo*. Blocking cholinergic inputs to EC chemically or by deafferentation causes reduced TFC performance (Esclassan *et al.*, 2009). Furthermore, strong inhibition of TFC is observed when antagonists for both mGluR1 and muscarinic 5-HT receptors are injected together into the MEC (Suh *et al.*, 2011). Additionally, increased phasic multiunit activity bursts during the delay period in a delayed-non-matching-to-place task have been observed in wild type mice, and these bursts were weaker in MECIII-TeTX mice (Yamamoto *et al.*, 2014). When all these lines of evidence are considered, it presents the possibility that the burst activity in the MECIII may be related to the observed bursting *in vitro*, and that this activity could be a mechanism for the association of event-related information across gaps in time. This theoretical backdrop of activity might then serve as a modifiable element to enable temporal encoding.

### **Input from CalB<sup>+</sup> pyramidal cells in MECII cells into hippocampal CA1 regulates temporal association learning**

Temporal association learning provides a critical means for cognitive adaptation to both dangerous and beneficial events (McEchron & Disterhoft, 1997; Kitamura, 2017). It stands to reason that such a critical process must be tightly regulated. If the association is too weak, a memory may not form. Conversely, overly strong association making may result in inappropriate linkages or the inability to distinguish useful from nonsense associations (Kitamura, 2017). A neural mechanism to regulate the formation of temporal association memories was discovered by Kitamura *et al* in the form of a novel circuit in the EC-HPC network, as described above (Fig. 2). CalB<sup>+</sup> cells of ECII project to a population of interneurons within SL of CA1 (Kitamura *et al.*, 2014). These inputs from the CalB<sup>+</sup> cell clusters run parallel to the ECIII projections to the distal dendrite segments of CA1 pyramidal cells, forming a laminar structure (Kitamura *et al.*, 2014). The ECII CalB<sup>+</sup> activation of SL-interneurons near the inputs from ECIII provides the substrate for a feed-forward inhibitory circuit that regulates MECIII inputs into hippocampal CA1 (Kitamura *et al.*, 2014) (Fig 2).

Differential contributions of both these circuits to temporal association learning were assessed using optogenetic tools for activation by Channelrhodopsin2 (ChR2) or inactivation by Archaelrhodopsin (ArchT, eArch3.0) of specific cell types during TFC. (Han *et al.*, 2011; Mattis *et al.*, 2011; Kitamura *et al.*, 2014; Okuyama *et al.*, 2016). Inhibition of the MECIII input into HPC CA1 during TFC (tone + trace + shock), as was expected, caused reduced the TFC performance (Suh *et al.*, 2011). Importantly, optogenetic activation of the CalB<sup>+</sup> cells input into HPC CA1 during TFC inhibited TFC learning. Neither condition, however, had an effect on contextual fear conditioning (Mingote *et al.*, 2015), indicating

that both circuit components deal primarily with the transmission of temporal information and regulation of temporal association learning. Additionally, Inactivation of the CalB<sup>+</sup> cells caused enhanced performance in TFC, showing a bidirectionality in the regulation of MECIII inputs by the CalB<sup>+</sup> cells control of SL-interneurons, supporting the view of a feedforward inhibitory mechanism for the regulation of temporal association learning (Kitamura *et al.*, 2014). The relative strengths of these two inputs is believed to be critical in determining the strength and longevity of these temporal associations.

## Sequential time cell activity in CA1 pyramidal cells may link events across the trace period

If the MEC is the upstream driver of temporal association memory formation, the CA1 pyramidal cells, as the recipients, are likely to in some way act to encode this memory according to Hebbian principles (Hebb, 1949). This is supported by observations of temporal memory deficits with genetic knockout of NMDARs in the hippocampal CA1 pyramidal cells (Huerta *et al.*, 2000). The hippocampus is well known for its role in memory of spatial and temporal information (Scoville & Milner, 1957; Waxler & Rosvold, 1970; Ekstrom & Ranganath, 2017). Hippocampal pyramidal cells have been observed to be responsive to specific places, acting as “place cells”, demonstrating that the pattern of hippocampal pyramidal cell activity can directly encode spatial features (O’Keefe & Dostrovsky, 1971). Interestingly, hippocampal CA1 pyramidal cells exhibit temporal organization of their neural firing (McEchron & Disterhoft, 1997; McEchron *et al.*, 2003; Pastalkova *et al.*, 2008; MacDonald *et al.*, 2011). The temporal organization of this activity spans the entirety of the trace period, with individual neurons firing at specific moments within that period. For example, one cell may consistently fire at 1–2 sec from the start of the trace period, while another fires consistently at 5–6 sec. This activity, known as “internally generated cell assembly sequences” or “time cell sequences”, allows the hippocampus to form a representation of the temporal gap between events (Pastalkova *et al.*, 2008; MacDonald *et al.*, 2013).

It has been demonstrated that rodents run on a treadmill also showed distinct temporal tuning during repetitive runs (Kraus *et al.*, 2013; Salz *et al.*, 2016; Mau *et al.*, 2018). Mau *et al.* utilized *in vivo* calcium imaging to identify time cell sequences across differential timescales (Mau *et al.*, 2018). The activity of time cells has typically been measured in the scale of seconds (Kraus *et al.*, 2013; Modi *et al.*, 2014), however, Mau *et al.* discovered differential mechanisms that allow the representation of time in the range of seconds, minutes, and days simultaneously utilizing repetitive runs on a treadmill based task. Second-scale activity is encoded by the repetitive activity of time cells at particular point in a gap phase period, as previously examined. Importantly, it was possible to decode the temporal sequences of the cellular activity to predict the temporal location with a 10s running period. Successful reconstructions of minute-scale representations are achieved by observing the activity of any individual time cell relative to the order of the trials presented. Shifts in the populations of time cells which were active across multiple days could also be decoded to indicate which day a particular trial occurred on (Mau *et al.*, 2018).

Even though time cell activity can be observed within the delay period of timing-related behavioral tasks despite the presence of body movement in the subject animal, a more desirable condition for analysis of time cell activity would be for animals to be immobile during the delay or timing period (Kraus *et al.*, 2013; Marks *et al.*, 2019). Utilizing tetrode-implanted, head fixed rats MacDonald *et al.* performed a delayed matching to sample task for odor cues. Time-sensitive cells in CA1 that fired during the delay period between matching sets were observed, and individual cells fired consistently within the delay period at a specific temporal bin across the theta cycle (MacDonald *et al.*, 2013). Each odor pair memory presented with a unique set of neurons firing in the delay period, with some neurons being active across multiple odor pairs. Those cells that fired across multiple memories either fired at the same point in the delay across memories or showed differential temporal tuning by odor pair memory. Hippocampal time cell activity has been also observed in head fixed animals trained in the trace eyeblink paradigm with two-photon calcium imaging. Within the delay period of the trace eyeblink task, individual neurons were observed firing sequentially at specific points within the delay period (Modi *et al.*, 2014). More recently, Marks *et al.* developed new behavioral paradigms, in which mice must maintain a nose poke during auditory cue presentation for up to ten seconds, and further need to discriminate the duration of cue presentation, in order to get a reward (Marks *et al.*, 2019). These tasks have been designed for freely moving animals, and thus are compatible with activity measurement/manipulation methods free of body or head restraint. Furthermore, by combining these new tasks with head-mounted microscope imaging (Ziv *et al.*, 2013; Sun *et al.*, 2015; Okuyama *et al.*, 2016; Kitamura *et al.*, 2017; Roy *et al.*, 2017), Marks *et al.* successfully recorded time cell activity in the hippocampus while animals were voluntarily holding still during the nose poking period (Marks *et al.*, 2019).

The origin of time cell activity in the hippocampal CA1 is not fully understood. Questions remain as to whether sequences are generated locally in the hippocampus and biased/modulated by EC and other brain regions, or if time cell sequences are transmitted from MEC directly. Time cell activity is abolished by inactivation of the medial septum, which generates the hippocampal theta (Lee *et al.*, 1994; Wang *et al.*, 2015). At the same time, optogenetic inactivation of MEC also causes loss of time sequence coherence (Robinson *et al.*, 2017). In light of the previous findings on the role of theta and MEC inputs in memory formation, we speculate that the CA1 time cell sequences are generated by concerted action of both EC and medial septum in order to associate temporally discontinuous events and remember specific timing in memory formation (Fig. 2).

## **Inputs from Reelin<sup>+</sup> stellate cells in MECII into hippocampus are crucial for contextual memory**

The ECII-DG-CA3 pathway has historically been considered to be crucial for the generation of discriminatory representations of spaces and contexts bearing strong similarity to each other, with processing occurring at each step along the tri-synaptic pathway. The paradigms used in this context separation were understood to occur along this pathway based in part on observations of the large amount of DG granule cells, the sparse activity of those same cells, and the low redundancy observed in DG-CA3 synaptic connections (Marr, 1971; O'Reilly



& McClelland, 1994; Treves & Rolls, 1994; Leutgeb *et al.*, 2007; Bakker *et al.*, 2008). These theories are supported with experimental data showing the physiological responses of CA3 pyramidal cells during alternating exposure to pairs of closely related contexts (Leutgeb, 2004; Leutgeb *et al.*, 2007; McHugh *et al.*, 2007; Wintzer *et al.*, 2014), and data showing the behavioral phenotype of mice without functional NMDAR expression in the dentate granule cells (McHugh *et al.*, 2007). More recent studies utilizing mice with impaired adult neurogenesis (Altman & Das, 1965; Schlessinger *et al.*, 1975; Seki & Arai, 1993; Eriksson *et al.*, 1998) and/or blockade of DG-CA3 inputs, however, showed that the input from the mossy fibers from DG to CA3 were not necessary for the discrimination of similar contexts (Nakashiba *et al.*, 2012). Instead, a relatively small contingent of the larger granule cell population, comprised of newly generated granule cells (Kitamura *et al.*, 2009; Arruda-Carvalho *et al.*, 2011; Gage & Temple, 2013; Kitamura & Inokuchi, 2014; Alam *et al.*, 2018; Terranova *et al.*, 2019), was found to be responsible for the contextual discrimination (Clelland *et al.*, 2009; Scobie *et al.*, 2009; Creer *et al.*, 2010; Sahay *et al.*, 2011; Nakashiba *et al.*, 2012). Mutant mice lacking neural transmission from CA3 outputs due to expression of TeTX in CA3 pyramidal cells also showed deficits in contextual fear conditioning. These studies have all been directed towards understanding the roles of the various HPC subfields and their activity patterns as the driving force underlying contextual memory. However, these studies have not touched on upstream circuits, like those in ECII, and their potential contributions to contextual memory and context discrimination. The possibility exists, however, that EC cells may demonstrate a heterogeneous pattern of activity such that differential contexts drive differential patterns of neuronal activity in the region. This type of activity may be capable of driving the discrimination of contextual cues downstream in the HPC, and in that way contributing to contextual memory encoding. Kitamura *et al.* in 2014 applied a novel approach to differentiate two populations of MECII excitatory neurons, and examine their unique functionalities (Kitamura *et al.*, 2014). Their study focused on the respective roles of Reelin<sup>+</sup> and CalB<sup>+</sup> neurons of MECII in the process of contextual discrimination by applying *in vivo* Ca<sup>2+</sup> imaging to observe the activity patterns of Reelin<sup>+</sup> and CalB<sup>+</sup> cells while freely moving mice were exposed to two different contexts with an alternating presentation. Cell type-specific *in vivo* Ca<sup>2+</sup> imaging revealed that Reelin<sup>+</sup> cells rapidly form a distinct representation for novel contexts, while CalB<sup>+</sup> cells in MEC did not have any context-specific activity (Kitamura *et al.*, 2015b). Consistent with these findings, other groups using single unit tetrode neural recording found similar context-specific neural firing in the MEC (Pérez-Escobar *et al.*, 2016; Diehl *et al.*, 2017). What neural inputs generate such context-specific neural activity of Reelin<sup>+</sup> cells in MECII? One possibility is that context-specific activity is generated by a diverse set of external inputs from outside of the EC-HPC networks including postrhinal cortex, visual cortex, medial septum, para-subiculum, and pre-subiculum (Canto *et al.*, 2008). Another possibility is that the back projection from hippocampal CA1 into ECV/VI could contribute to the context-specific activity of Reelin<sup>+</sup> cells, however,, Kitamura *et al.* showed pharmacological inhibition of CA1 activity did not have a strong effect on the context-specific activity of Reelin<sup>+</sup> cells in MECII (Kitamura *et al.*, 2015b), indicating that MECII rapidly processes contextual information and sends it to the hippocampal DG, CA3, and CA2 neurons (Fig 3).

To investigate the role of MEC inputs into the HPC on the formation and recall of contextual memory, Kitamura et al, further examined optogenetic manipulation of each type of HPC-projecting MEC excitatory neuron during contextual fear conditioning (CFC), which is an association between context and aversive foot shock (Pavlov, 1927; Kim & Fanselow, 1992; Phillips & LeDoux, 1992; Frankland *et al.*, 1998; Amaral & Lavenex, 2007; Sutherland *et al.*, 2008; Maren *et al.*, 2013; Kitamura *et al.*, 2015b; Kitamura *et al.*, 2017). They showed that optogenetic cell body inhibition of Reelin<sup>+</sup> cells during CFC disrupted the formation of CFC memory, while optogenetic inhibition of CalB<sup>+</sup> cells or MECIII cells during CFC did not affect CFC memory (Kitamura *et al.*, 2014; Kitamura *et al.*, 2015b). Optogenetic inhibition of Reelin<sup>+</sup> cells during testing of CFC also disrupted the recall of CFC memory, indicating that neural activity of Reelin<sup>+</sup> cells is essential for both formation and recall of CFC memory. While Reelin<sup>+</sup> cells in MECII have been also found as a critical functional component for spatial navigation memory (Kanter *et al.*, 2017; Qin *et al.*, 2018; Tennant *et al.*, 2018), importantly, Reelin<sup>+</sup> cells in MECII did not have a critical role in trace fear conditioning (Kitamura *et al.*, 2014), which requires two MEC direct inputs (MECIII cells and CalB<sup>+</sup> cells) into CA1, indicating that the multiple MEC inputs into the HPC have complementary roles for temporal and contextual memory formation respectively (Fig. 2–3).

### **Inputs from Reelin<sup>+</sup> stellate cells in MECII into hippocampus are crucial for the generation and reactivation of hippocampal memory engram cells for a specific context**

Approaches for targeting specific cell populations, in this case active cells as a functional subset rather than an anatomical or biochemical classification, have yielded crucial information about the encoding and recall of contextual memories in the form of engram cells (Guzowski *et al.*, 1999; Han *et al.*, 2007; Reijmers *et al.*, 2007; Han *et al.*, 2009). Richard Semon first developed the physical theory of human memory in the early 19<sup>th</sup> century (Semon, 1921). To define a hypothetical physical substrate of memory, he developed the term “engram”. The currently accepted idea of what defines a true memory engram can be described by a set of three criteria (Josselyn, 2010; Tonegawa *et al.*, 2015; Tonegawa *et al.*, 2018). An engram i) is a substantive and long-term alteration to a neural network either in its physical structure or chemical activity/composition, ii) results from induction of activity in a subpopulation of neurons by event stimulus, and iii) can be reactivated by a stimulus that was a part of the original set of stimuli to initiate recall of the memory. Activity-dependent cell labeling, made possible by using immediate early gene promoters can be paired with optogenetics to drive expression of an opsin protein in specific neurons activated during an event, has resulted in the first observations of an “engram cell” (Reijmers *et al.*, 2007; Liu *et al.*, 2012). Labeling of DG cells active during CFC with ChR2 was performed by Liu et al in 2012, utilizing a doxycycline dependent ChR2 expression paradigm. They observed that a 20Hz pulse stimulation with blue light along implanted optic fibers terminating in DG was able to induce memory recall in the absence of the original cue in a context specific manner (Liu *et al.*, 2012). All three of the criteria for a true engram are met by this activity in the dentate gyrus granule cells (Josselyn, 2010; Tonegawa *et al.*, 2015; Tonegawa *et al.*, 2018). These studies highlight the idea that a specific subset of neurons

activated during a particular event can be artificially reactivated with optogenetic stimulation is sufficient for recall of a memory, and furthermore provide evidence for **engrams** in the dentate gyrus. Subsequent studies also showed that memory engram cells can be also formed in CA3 and CA1 for contextual memory (Ramirez *et al.*, 2013a; Ramirez *et al.*, 2013b; Denny *et al.*, 2014; Ohkawa *et al.*, 2015; Ryan *et al.*, 2015; Guo, 2018; Tanaka *et al.*, 2018; Ghandour *et al.*, 2019; Oishi *et al.*, 2019). Furthermore, Choi et al recently found that engram cells preferentially form synapses with other engram cells in the HPC (Choi *et al.*, 2018). Therefore, engram cells are described as cell networks which physically store the information pertinent to a memory, and activation of a local engram network is necessary and sufficient to recall memory (Tonegawa *et al.*, 2015; Denny *et al.*, 2017).

Using targeted approaches can allow for a more in-depth analysis of how MEC inputs into the HPC contribute to the formation of engram networks for encoding contextual memory. Kitamura et al examined the effects of optogenetic inhibition of Reelin<sup>+</sup> or CalB<sup>+</sup> cell activity on the generation of contextual memory engrams in HPC sub-regions (Kitamura *et al.*, 2015b). They found that optogenetic inhibition of Reelin<sup>+</sup> cells inhibited the number of c-Fos<sup>+</sup> cells activated by novel context exposure in both hippocampal DG and CA3, while optogenetic inhibition of CalB<sup>+</sup> cells in MEC did not, indicating that MECII input into hippocampal DG and CA3 is crucial for the generation for contextual memory engrams. They also tested the reactivation of the memory engrams upon re-exposure to the same context. Memory engram cells in CA3 were reactivated when an animal re-explored the same context after its initial presentation (Nakashiba *et al.*, 2009; Denny *et al.*, 2014; Kitamura *et al.*, 2015b; Lacagnina *et al.*, 2019). Kitamura et al demonstrated that the MECII Reelin<sup>+</sup> input into the HPC is crucial for the reactivation of CA3 engram cells (Kitamura *et al.*, 2015b), which is essential for the recall of contextual memory. In summary, contextual memory for specific contexts are stored in a small population (5–10%) of neurons activated during contextual learning as memory engrams which are necessary and sufficient for the recall of contextual memory. Reelin<sup>+</sup> cells in MECII drive context-specific activation to downstream hippocampal circuits to generate and reactivate hippocampal memory engrams cells to form and recall contextual memory, respectively (Kitamura *et al.*, 2015b) (Fig. 3).

## Integration mechanisms of temporal and contextual memory in hippocampal CA1 pyramidal cells

While previous sections examined how differential entorhinal inputs contribute to temporal or contextual memory, how these memories are integrated to in successful memory encoding remains an open question. Eichenbaum theorized that the integration process would involve a “mixed selectivity” of neurons within a population, meaning that elements are independently selected from encoded sequences rather than having a unified memory engram. In this model, A memory would be constructed by the activation of differentially coded data sets within the same population (Eichenbaum, 2017). We believe that this integration would be conducted within the population of CA1 pyramidal cells. Multiple studies demonstrate that time-responsive and place or movement responsive cell populations can overlap in CA1 (MacDonald *et al.*, 2011; Kraus *et al.*, 2015; Mau *et al.*, 2018), however, some data exists that suggests that this may be an integrated response; for example, a

context-dependent time cell, or a place-dependent speed cell. This is likely due not to a complete unification of data, but rather a stratification in selection of a particular ensemble during encoding and recall, with the overlap occurring between population subsets serving as a functional linkage. Evidence of such activity dependent ensemble selection has been observed in the form of object-relative spatial coding (Connor & Knierim, 2017).

MacDonald et al in 2011 demonstrated that while some neurons are responsive to time regardless of location, a subpopulation of time cells were directly influenced by an animals' location (MacDonald *et al.*, 2011). This is supported by a more recent study showing that the CA1 network fluctuates between spatially and temporally permissive states, usually occupying a state permissive to both (Haimerl *et al.*, 2019). Close examination of active time cells between different odor presentations in MacDonald et als' 2013 examination of time cell activity in rats shows that some time cells maintain a specific temporal profile across two different odors, while one slightly shifts its activation time across differing presentations (MacDonald *et al.*, 2013). Furthermore, analysis of the time cell activity in Sabariego et als' 2019 study suggests that there is a contextual element overlapping the temporal processing (Sabariego *et al.*, 2019). These data stand in support of variable, overlapping activity in CA1 pyramidal cells. In addition, mathematical modeling suggests the possibility of the already differential activity of place cells in a history or path dependent manner behaving differentially across time, indicating that the processing boundary between specific places or contexts and temporal activity may occur on a longer scale of time (minutes to hours vs seconds) (Howard *et al.*, 2014). Gradual changing of time cell sequences across hours and days has been observed, which was found to be encoded by a general shift in the population of pyramidal cells acting as time cells from one day to the next (Mau *et al.*, 2018). CA2 place cells have been observed to have a greater level of instability across time than place cells in CA1 (Mankin *et al.*, 2015; Hainmueller & Bartos, 2018). within hours, it may be possible for CA2 to generate a gradual shift of time cell activity concerted with MECIII and MECII CalB<sup>+</sup> inputs. Could this process be occurring in CA1 as well? CA1 serving as the site of integration for these data streams is probable, considering the tight regulation of inputs by a vast array of local interneuron interactions (Ali *et al.*, 1998; Klausberger & Somogyi, 2008; Cutsuridis *et al.*, 2010; Stark *et al.*, 2014; Amilhon *et al.*, 2015; Del Pino *et al.*, 2017). While the CA1 interneuron network functions as a large network, anatomically, a few specific subpopulations are more obvious contributors to the integration processes due to their known associations with dendritic sub-compartments of the CA1 pyramidal cells. Firstly, Somatostatin positive cells of the stratum oriens, the oriens-lacunosum moleculare cells (OLMs), project to stratum lacunosum moleculare and inhibit the distal terminals of CA1 pyramidal cells (Fig 1B) (Leao *et al.*, 2012; Orban-Kis *et al.*, 2015) which receive temporal information from the entorhinal cortex layer III (Fig 1) (Kitamura *et al.*, 2014). The Parvalbumin (PV) positive cells of the stratum radiatum, which include bistratified cells and PV<sup>+</sup> basket cells (Ali *et al.*, 1998; Klausberger & Somogyi, 2008), preferentially innervate the more proximal apical dendrites of CA1 pyramidal cells in the stratum radiatum (Sik *et al.*, 1997; Ali *et al.*, 1998; Muller & Remy, 2014; Pelkey *et al.*, 2017) which receive contextual information via CA3 (Fig. 1) (Kitamura *et al.*, 2015b). While the anatomical evidence alone is compelling, the OLMs and at least one subset of PV<sup>+</sup> interneurons have been shown to interact with each other to form a feedback control/

input gating loop that differentially interacts with the ECIII and CA3 inputs in a theta dependent manner (Buhl *et al.*, 1996; Sik *et al.*, 1997; Klausberger *et al.*, 2004; Leao *et al.*, 2012; Muller & Remy, 2014). Our interest in the hippocampal representation of memory is the differential processing of different information types within the hippocampus, and the integration strategies used to link the different memory components. For example, while contextual memory components discussed in this review were examined in terms of engrammatic storage, no true time engram has yet been identified. It would be reasonable to conclude that a continuous variable like time would need to be encoded by a more dynamic process, as it is not a discrete event or object that can be recalled like a snapshot, but rather acts more as a linkage between snapshots. The time engram may be widely distributed to allow its integration into various memory engram structures. This is not far off the theory proposed by Yntema & Trask in 1963, and further refined by Wolff in 1966, that a sort of temporal stamp is somehow associated with a particular object or event to enable sequential orientation of remembered units (Yntema & Trask, 1963; Wolff, 1966). It has been demonstrated that dendritic plateau potentials in CA1 pyramidal cells originating from ECIII can act to predispose a pyramidal cell to burst firing activity resulting from concurrent CA3 input activity (Bittner *et al.*, 2015; Grienberger *et al.*, 2017). Bittner *et al.* demonstrated that an individual pyramidal cell is not tuned to a given location prior to place field formation, but rather is recruited by these concurrent inputs, which they suggest is driven in part by local plasticity in CA1 and differential grid cell firing patterns from ECIII (Bittner *et al.*, 2015), which in addition to GABAergic interneuron regulation (Cutsuridis & Hasselmo, 2012; Grienberger *et al.*, 2017), causes a long term alteration in the local microcircuit environment to support integrative activity for these two signals in a given spatial environment. Calcium imaging in the dorsal hippocampus reveals that stable place fields can persist on the scale of days to weeks (Rubin *et al.*, 2015; Kinsky *et al.*, 2018), however periodic place independent representations and spatial remapping events occur which were discovered to contain functional timestamps as assessed by the rate of change in overlap between representations against the stable rate of place field remapping. These decoded time sequences were able to predict sequential order of events, and were able to link events in different locations across various timescales (Rubin *et al.*, 2015). Moreover, time fields have been observed to shift across the scale of days, such that over a given period of time the identity of individual time cells will change, however, some overlap across timescales will remain, potentially allowing the linkage of events across time as individual cells have active representations in multiple timescales (Mau *et al.*, 2018). In conjunction with observations of differing engram stability across hippocampal subregions (Mankin *et al.*, 2015; Hainmueller & Bartos, 2018), these findings may support the idea that the imposition of sequential timing information onto memory structures involves an interplay between the rate of change of relatively static vs. dynamic engrams, allowing the linkage of events across time into a set of associated recallable units. This makes sense in light of the findings from Susumu Takahashi's 2013 study, which showed that place fields can represent the same place across multiple tasks by changing the frequency of their firing, or rate remapping, rather than a complete spatial remapping. The observed rate remapping was tied to a hierarchical representation of different contexts (Takahashi, 2013). It is not a far leap to consider similar processes may be proceeding with other forms of information, and that a hierarchical approach to rate remapping across related events

within existing engrams is a more broadly active component of the hippocampal integration strategy. Indeed, the mechanisms behind the integration of individual spatial components are still severely understudied, in part due to the complexities of the processing of these individual components themselves.

Here, we speculate contextual activity may influence which cells become active time cells during an event, and that those contextual memory engram cells can work as an index to retrieve context-dependent time cell sequences (Fig. 4). For example, if memory engram cells for context A in HPC CA3 are activated, Context A dependent time cell sequences are generated in hippocampal CA1 cells. If memory engram cells for context B in HPC CA3 are activated, Context B dependent time cell sequences are generated in hippocampal CA1 cells. This differential temporal encoding dependent on contextual representations could be a mechanism for the linkage of temporal information with engrammatic storage of event information.

## Conclusions

In this review, we have addressed the current understanding of the circuit mechanisms behind temporal association learning and contextual memory in the EC-HPC networks. Crucial inputs from MEC to CA1 were observed using cell-type specific TeTX or optogenetic approaches. MECIII inputs to the distal dendrites of CA1 were shown to be crucial for temporal association, while MECII CalB<sup>+</sup> cell inputs to SL interneurons in CA1 engage in feed-forward regulation of the temporal signal from MECIII. Reelin<sup>+</sup> cells in MECII, by contrast did not carry temporal information, but were involved in contextual memory formation via the hippocampal tri-synaptic pathway (Nakashiba *et al.*, 2008; Suh *et al.*, 2011; Kitamura *et al.*, 2014; Kitamura *et al.*, 2015b). In this way, the MEC presents the hippocampus with two distinct information streams; context via the tri-synaptic synapses, and time, primarily via the temporoammonic pathway and regulated by a novel feedforward circuit originating in MECII island clusters. These two modalities and others (e.g. smell, object) may be integrated at the CA1 subfield, with both sequential neural activity and engrammatic neural activity being combined into a singular, but multi-faceted representation during memory formation.

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## List of Abbreviations

<b>CA1</b>	Cornu Ammonis 1
<b>CA2</b>	Cornu Ammonis 2
<b>CA3</b>	Cornu Ammonis 3

<b>CalB</b>	Calbindin
<b>CFC</b>	Contextual fear conditioning
<b>DG</b>	Dentate gyrus
<b>EC</b>	Entorhinal cortex
<b>HPC</b>	Hippocampus
<b>Wfs1</b>	Wolfram Syndrome 1
<b>LEC</b>	Lateral entorhinal cortex
<b>MEC</b>	Medial entorhinal cortex
<b>OLM</b>	Oriens – Lacunosum Moleculare projecting cell
<b>PV</b>	Parvalbumin
<b>SST</b>	Somatostatin
<b>TeTX</b>	Tetanus toxin light chain
<b>TFC</b>	trace fear conditioning

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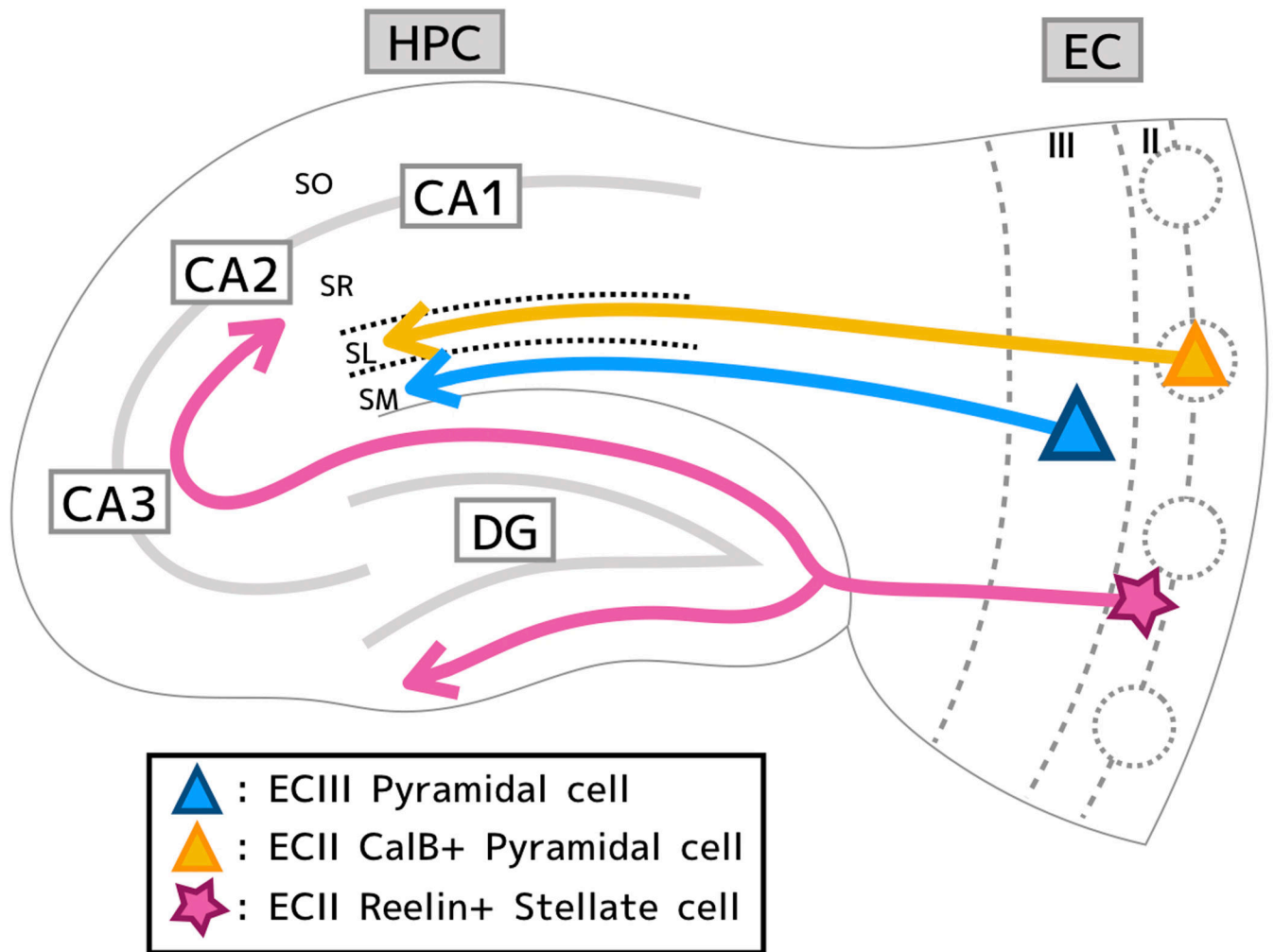
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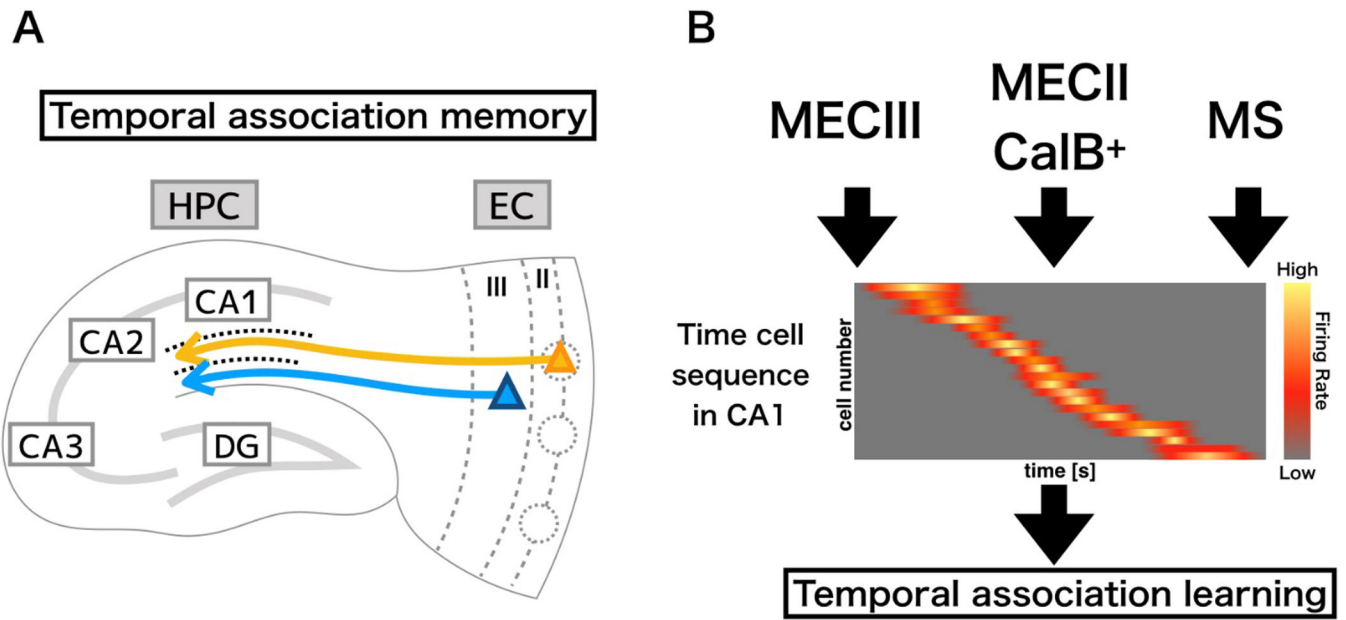
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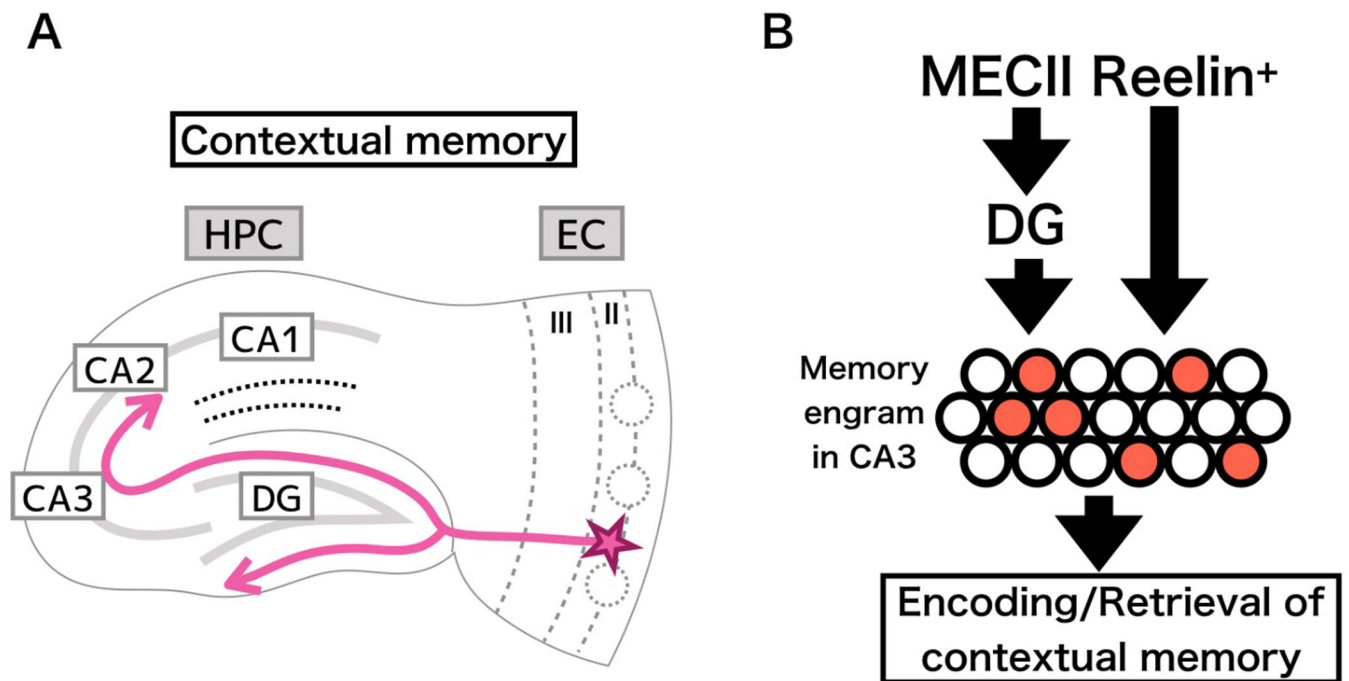




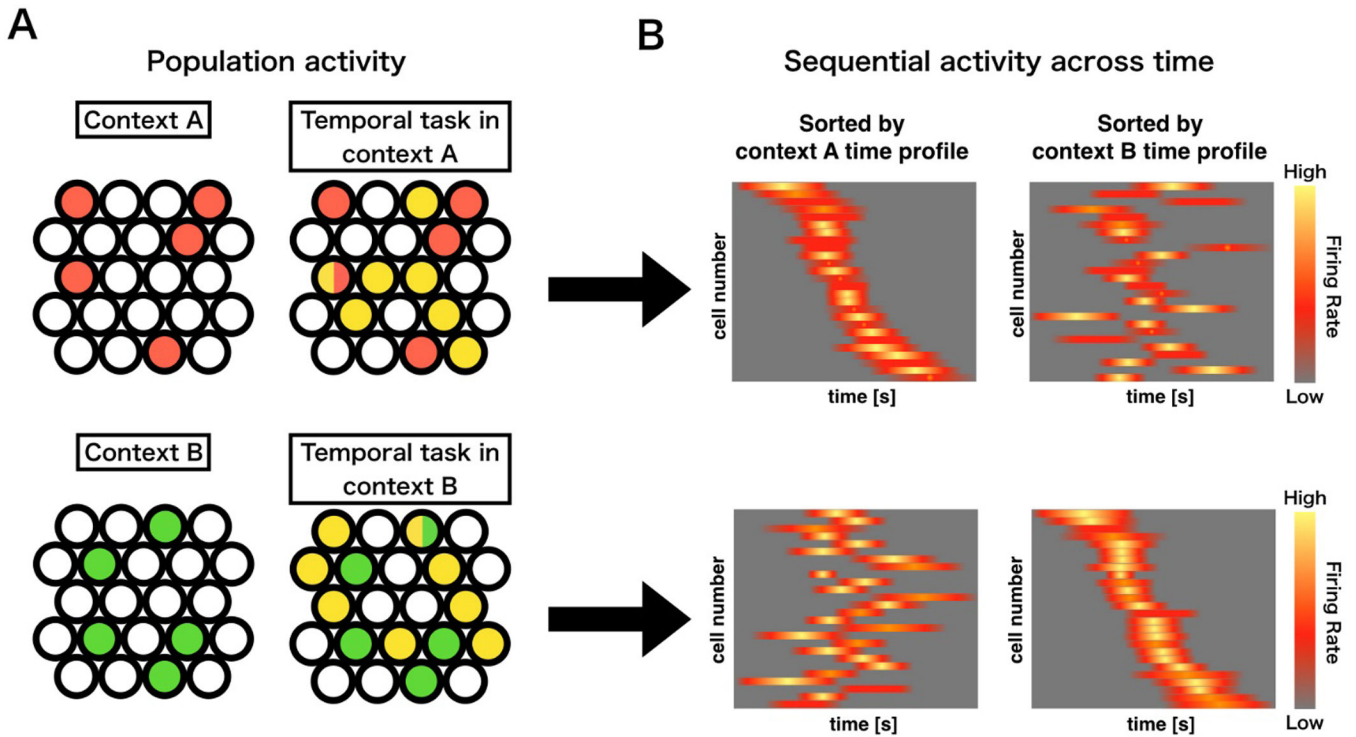
**Figure 1,**  
**Diagram** of the EC-HPC circuit depicting the layout of three major inputs from EC to HPC. I) The direct pathway from ECIII pyramidal cells (blue) to stratum moleculare (SM). II) The indirect (trisynaptic) pathway from ECII Reelin<sup>+</sup> stellate cells (pink) projects to the granule cells of the DG. Mossy fibers project to CA3 pyramidal which then project to CA1. Mossy fibers from DG also project to CA2. III) A regulatory pathway projecting from ECII CalB<sup>+</sup> pyramidal cells (orange) to stratum lacunosum (SL) interneurons which regulate ECIII input onto hippocampal CA1 pyramidal cells.



**Figure 2.** Circuitry involved in temporal association memory. A) The pyramidal cells of MECIII project directly to distal dendrites of CA1 pyramidal cells, while pyramidal cell clusters in ECIII project to interneurons in SL. B) this interplay between excitatory ECIII and inhibitory action driven ECII CalB<sup>+</sup> inputs in CA1 tightly regulates temporal encoding, along with input from the medial septum (MS) resulting in a temporal sequence, and a refined temporal association memory. The temporal sequence can be seen in the sequential activation of individual neurons at a specific point in time within a larger time interval, with the total population covering the entirety of the interval period.



**Figure 3.** Circuitry involved in contextual memory. A) Reelin<sup>+</sup> stellate cells in ECII act on CA1 by means of the traditional tri-synaptic pathway. B) parallel inputs along the tri-synaptic pathway and collateral direct inputs to CA1 enables the encoding of a contextual representation in the form of an engram, a specific subset of cells activated by a particular stimulus (indicated by red filled circles) that can be reactivated to initiate memory recall.



**Figure 4.**

A hypothetical process for context-time integration in CA1. Panel A depicts the summation of population activity within two different hypothetical contexts; A (upper), and B (lower). Differential exposure to contexts will result in formation of a particular memory engram specific to that context (red or green). Active timing or temporal task performance within a specific context will result in a time cell assembly unique to that particular context (Yellow) and having some overlap with the contextual engram cells (mixed color). Panel B depicts the sequential activity across time of the time cell populations specific to the differential contexts in panel A. The resultant time cell sequences will be indistinguishable in a different context, allowing for a specific linkage between contextual memory units and temporal information