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Specific contribution of the Lateral Entorhinal Cortex to episodic-like memory recall

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Abstract

Episodic memory, the extraordinary capacity to recall specific events and experiences in a particular context, relies on the entorhinal cortex (EC) as a crucial bridge connecting the hippocampus and other sensory processing regions in the brain.

Traditionally, it was believed that the lateral entorhinal cortex (LEC) and its counterpart, the medial entorhinal cortex (MEC), performed distinct roles in relaying information to the hippocampus. The MEC was specialized in processing spatial information, while the LEC was thought to handle contextual details. However, recent evidence challenges this simplistic division, proposing that the LEC may have a more direct role in integrating diverse information to form a coherent experience even before reaching the hippocampus.

In this study, we investigated the entorhinal cortex's involvement in episodic-like memory processing in mice, utilizing the object-place-context-recognition task (OPCRT), a well-established behavioral paradigm for studying episodic-like memory in rodents. To identify a link between entorhinal cortex activation and memory processing, we employed the protein encoded by the immediate early gene (IEG) c-fos as a marker of neuronal activity and observed its level in the EC after executing the OPCRT. Our results showed that mice engaged in the OPCRT task exhibited a higher number of c-fos-positive cells in the LEC compared to control mice confined to their home cages. Remarkably, the increase in c-fos staining was specific to the LEC and not observed in other cortical regions like the MEC or V1, strongly supporting the notion of the LEC's specific involvement in episodic-like memory processing.

Furthermore, we observed changes in synaptic plasticity specific to the LEC circuitry. Using field excitatory post-synaptic potential (fEPSP) recordings in brain slices containing the EC, we found that mice exposed to OPCRT displayed a significant reduction in long-term potentiation (LTP) in the superficial layers of the LEC 12 hours after memory acquisition. Notably, the decrease in LTP was limited to the OPCRT group and absent in control animals, and was associated with an increase in basal synaptic transmission. Interestingly, these changes were reversible and returned to baseline 48 hours after task execution when mice could no longer recall the memory, suggesting that heightened synaptic transmission may play a crucial role in episodic memory recall.

Finally, we conducted chemogenetic manipulations of LEC learning-tagged neurons, i.e. those activated during the learning phase of the paradigm. The results demonstrated that the inhibition of LEC neurons impaired the mice's performance in the memory task, while their activation facilitated memory recall, providing conclusive evidence of the existence of an episodic-like memory engram in the lateral entorhinal cortex.

Together, these findings offer compelling evidence of the central role of the LEC in episodic-like memory processing and its integration into the broader episodic memory network.

Introduction

Introduction to Episodic Memory

Episodic memory constitutes a crucial facet of long-term memory, facilitating individuals in the retrieval of personal experiences from their past. It encompasses the remarkable capacity to recall intricate details of events, including the spatial and temporal context in which they unfolded. As one of the constituents of explicit memory, alongside semantic memory that encompasses the acquisition of facts and concepts, episodic memory plays a pivotal role in shaping our understanding of the world. These diverse memory mechanisms synergistically collaborate to facilitate individuals in learning, adapting, and effectively navigating their surroundings by leveraging the wealth of past experiences. The inception of the concept of episodic memory can be attributed to the pioneering work of Endel Tulving in the early 1970s (Tulving 1972, 1993). Tulving's original formulation of episodic memory operationalized it as a memory system uniquely capable of encoding and storing individualized personal experiences, encompassing vital aspects such as the specific details of what transpired, the spatial context in which it occurred, and the temporal dimension defining when it took place. As the understanding of episodic memory evolved, Tulving further expanded his definition to incorporate autonoetic awareness—a remarkable cognitive ability enabling individuals to mentally traverse the corridors of time and relive past personal events (Tulving 1985). Unlike other memory modalities, episodic memory stands apart by its inherent connection to the past, accompanied by a vivid sense of recollection. In contrast, other forms of knowledge predominantly manifest as factual representations devoid of the experiential dimension. The identification of episodic memory in humans can be effectively achieved using the remembering/knowing paradigm. This paradigm helps determine whether an individual's ability to recall a specific object, for example, depends on their capacity to mentally relive the past event in which they encountered the object (remembering, or episodic memory). Alternatively, it ascertains whether the person is merely aware of having encountered the object before without recalling how or when (knowing). However, demonstrating episodic memory in non-human animals, particularly in the absence of language, poses challenges due to the lack of consensus on non-linguistic behavioral indicators of conscious experience in these animals (Griffiths, Dickinson, and Clayton 1999). Nevertheless, content-based behavioral approaches rooted in Tulving's original definition of episodic memory as a system that "stores information about temporally dated episodes and temporal-spatial relations between them" have allowed researchers to dissociate the content of episodic memory from its subjective experience. These approaches have led to the description of a form of episodic-like memory (Clayton and Dickinson 1998) in non-human animals, closely resembling human episodic memory. Clayton and Dickinson's seminal experiment involving scrub jays marked a significant milestone in the study of animal cognition by providing the initial evidence of animals' ability to recollect specific past experiences (Clayton and Dickinson 1998). In this experiment, scrub jays were allowed to cache and retrieve two distinct types of food, differing in palatability and perishability. The results demonstrated that these animals could remember both the type of food they had hidden and its specific location. Additionally, they exhibited the capability to keep track of the temporal dimension by recalling how long ago they had cached different types of

perishable foods that decayed at varying rates. This finding suggested that certain animals can form integrated episodic-like memories, encompassing the integration of three distinct elements into a unified memory. Subsequently, numerous other animal species have been shown to acquire and retrieve episodic-like memories. Nonetheless, despite endeavors to establish similarities between animal memory and human episodic memory, unequivocally demonstrating the presence of episodic memory in non-human animals remains a challenging task.

The novel object-place-context recognition paradigm to assess episodic-like memory in animals

Research conducted on animals has played a crucial role in identifying objective behavioral criteria for different features of episodic-like memory, enabling their experimental operationalization and assessment in both animals and humans (Clayton and Dickinson 1998; Ekrem Dere, Huston, and De Souza Silva 2005b, 2005a; Dere et al. 2006). One essential characteristic of episodic-like memory is its automatic and one-trial learning nature, distinguishing it from memory processes that require explicit training or learning rules. This definition of episodic-like memory aligns well with spontaneous and innate forms of memory, such as human episodic memory, which can be formed without the need for prior training sessions. To evaluate episodic-like memory in rats without pre-training, Eacott and colleagues (Eacott, Easton, and Zinkivskay 2005; Eacott and Norman 2004; Easton, Zinkivskay, and Eacott 2009) developed a modified version of the spontaneous recognition paradigm known as the Novel Object-Place-Context recognition test (OPCRT). In this behavioral paradigm, rats were initially exposed to two different objects within a specific context defined by visual cues. Subsequently, the animals were presented with a different context featuring alternative visual cues, wherein the locations of the two objects were swapped. Finally, during the test phase, two identical copies of one of the two objects were presented either in the first or second context, and the animals were assessed for their ability to recognize the novel object-place-context configuration. The findings revealed that the animals demonstrated the capability to remember which object was seen in which location and on which occasion for periods lasting up to 1 hour. Moreover, this ability was found to rely on episodic-like processes rather than mere familiarity mechanisms since all components of the task (objects, locations, and contexts) were equally familiar to the animals.

The OPCRT paradigm has been widely utilized in the study of episodic memory, leading to important findings. For instance, it has provided insights into the involvement of the entorhinal cortex in processing episodic-like memories in mice (D. I. G. Wilson et al. 2013; Vandrey et al. 2020). One distinctive aspect of this task is its utilization of context as an "occasion specifier" to differentiate between different occasions. This may seem inconsistent with Tulving's original description of episodic memory as a memory of "temporally dated events" and "temporal-spatial relations" between events. However, research has revealed that humans have a limited ability to recall the temporal order of events and instead rely on the specific occasion on which an event occurred to retrieve temporal information about a past event. Similarly, animals are capable of remembering the spatial arrangements of objects on specific occasions indicated by distinct visual cues.

Hence, it has been proposed that episodic memory can be viewed as the ability to remember what happened on a specific occasion rather than at a precise time.

However, it is intriguing to note that an alternative version of the OPCRT exists, enabling the investigation of temporal aspects of episodic-like memory. In this paradigm, developed by Kart-Teke et al. (Kart-Teke et al. 2006), rodents are allowed to explore an environment containing four copies of an object situated at various locations during the initial sample trial. In the subsequent sample trial, the animals are exposed to the same environment with four copies of different objects placed in different locations. Finally, during the test phase, the animals are presented with two copies of each object, with one copy placed in a familiar location and the other relocated to a different position. If the animals can remember not only the "what" and "where" information about that specific episode but also the "when" component, they should exhibit a preference for exploring the item that was least recently seen and had been moved to a different location.

Neural basis of episodic and episodic-like memory

In both humans and non-human animals, the formation of new episodic memories relies on a complex network of interconnected brain regions, prominently including the hippocampus and the surrounding parahippocampal areas (Aggleton 2014; Nadel and Moscovitch 1997; Nadel and Peterson 2013; Ranganath and Ritchey 2012; Rugg and Vilberg 2013). This anatomical and functional organization of the brain areas involved in episodic memory is highly conserved across mammalian species (Dickerson and Eichenbaum 2010). According to Eichenbaum (Eichenbaum 2000), memory processing entails the transmission of information from neocortical regions to various subdivisions of the para-hippocampal region, such as the perirhinal cortex, postrhinal cortex, and entorhinal cortex. From these regions, the information is further relayed to different subdivisions of the hippocampus, traversing the dentate gyrus, CA3, CA1, and the subiculum, before being directed back to the para-hippocampal regions and subsequently to the cerebral cortex. The parahippocampal areas have been demonstrated to play a critical role in episodic memory, and selective lesions in these areas in humans have been associated with deficits in this specific memory domain (Annese et al. 2014; Scoville and Milner 1957; Zola-Morgan and Squire 1990). The well-known case of Henry Molaison (HM) serves as an illustrative example, as bilateral temporal lobe resection led to profound and isolated memory deficits, underscoring the importance of the lesioned areas in memory processing (Scoville and Milner 1957). Moreover, studies have revealed increased activity in the hippocampus and parahippocampal regions during the encoding and retrieval of episodic memories in humans (Burgess, Maguire, and O'Keefe 2002; Hayes et al. 2004; Nadel, Campbell, and Ryan 2007; Squire et al. 1992). The impairment of integrating "what-where" and "when" information about episodes, resembling the amnestic syndrome observed in humans with hippocampal damage (Ergorul and Eichenbaum 2004; Fortin, Wright, and Eichenbaum 2004), has been demonstrated through rodent experiments. This evidence strengthens the crucial role of the hippocampus and the surrounding parahippocampal areas in the formation of episodic memories in both humans and non-human animals. Studies in rodents have further shown that hippocampal lesions result in deficits in episodic-like memory tasks involving the integration of object-place-context information (Eacott and Norman 2004; Langston and Wood 2010) or object-place-

temporal information (DeVito and Eichenbaum 2010; Fellini and Morellini 2013). On the other hand, hippocampal lesions typically do not affect general novel object recognition memory (Winters et al. 2004; Ainge et al. 2006), likely due to intact familiarity recognition but disrupted recollection, nor do they typically impair memory for object configurations in contexts (Norman and Eacott 2005; Langston and Wood 2010; D. I. G. Wilson et al. 2013; D. I. Wilson et al. 2013). Therefore, the hippocampus appears to play a critical role in tasks that require rodents to remember spatial information (Mumby et al. 2002; Eacott and Norman 2004).

In addition to the hippocampus, the entorhinal cortex, a region located within the medial temporal lobe, has emerged as a crucial player in the processing of episodic memory. The entorhinal cortex and the hippocampus are intricately connected, forming a functional network that plays a vital role in the formation and retrieval of episodic memories. Recent research has provided insights into the specific involvement of the lateral entorhinal cortex (LEC) in encoding contextual information, which is essential for episodic memory. The LEC receives input from neocortical regions and sends projections to the hippocampus through the perforant pathway. Numerous studies have demonstrated that the LEC is responsible for integrating and associating various sensory inputs, thereby providing a spatial framework for the creation of episodic memories (Burwell 2000; Hargreaves et al. 2005).

In rodent studies, the LEC has been shown to play a fundamental role in encoding associations between objects, places, and contexts. Notably, Wilson et al. (D. I. G. Wilson et al. 2013) demonstrated that the LEC is necessary for recognizing objects within a specific context, highlighting its involvement in object-context associations. Moreover, the same group showed that excitotoxic lesions of the LEC were able to selectively impair the recognition of familiar combinations of objects in place, place in context, or object in place and context, while leaving the recognition of objects and places independently unaffected (D. I. Wilson et al. 2013). These findings suggest that the LEC plays a crucial role in integrating different pieces of information related to an experience. Moreover, emerging evidence indicates that the LEC may contribute to the encoding of episodic memories by processing an individual's first-person perspective in space and time, known as egocentric coding (Wang et al. 2018), a characteristic feature of human episodic memory. Interestingly, the involvement of the entorhinal cortex in episodic memory has also been confirmed by recent studies in humans. These studies examined memory-related changes in the spatial tuning of entorhinal cortex neurons in neurosurgical patients performing virtual reality object-location memory tasks. Remarkably, the researchers identified "memory-trace cells" that exhibited activity specifically tuned to the retrieved location of a particular object participants were cued to remember. The activity of these cells selectively differentiated between memories from a single environment (Qasim et al. 2019). These findings strikingly resemble the discovery of object trace cells in the rat entorhinal cortex by Tsao et al. (Tsao, Moser, and Moser 2013), strengthening the hypothesis that the episodic memory system may operate similarly in animals and humans. Furthermore, recent studies have demonstrated that lesions in the LEC impair odor-context associative memory in rodents, indicating that the entorhinal cortex, particularly the LEC, plays a role in associative properties that extend beyond objects, places, and contexts, encompassing various features that constitute episodic memories (Persson et al. 2022).

Apart from the hippocampus and entorhinal cortex, the prefrontal cortex (PFC) has gained recognition for its vital role in processing episodic memory, particularly in regulating the "top-down" flow of information (Dobbins et al. 2002; Preston and Eichenbaum 2013). The PFC collaborates with the hippocampus to organize memories within appropriate contexts and enhance the retrieval of contextually relevant information by suppressing competing, contextually inappropriate memories. While individuals with prefrontal damage typically do not exhibit severe episodic memory impairment, they often encounter difficulties in remembering information when faced with interference or distractions (Shimamura et al. 1995). Animal studies have further corroborated this notion by demonstrating that PFC damage hampers animals' ability to recall items encountered in specific contexts and their capacity to inhibit irrelevant memories (Barker and Warburton 2020). The PFC and hippocampus maintain robust connections through direct and indirect pathways, with the LEC postulated to play a pivotal role in processing object and event representations. These findings underscore the significance of the interconnected prefrontal cortex, entorhinal cortex, and hippocampus as key brain regions involved in the formation and retrieval of episodic memories.

Cellular basis of episodic and episodic-like memory

During learning, experiences induce lasting changes in the anatomical connections and physiological processes of the brain. These enduring alterations, whether physical or chemical, form the basis for the formation of new memory associations, known as an "engram" or a "memory trace."

The notion that memories are preserved as persistent changes in the brain can be traced back to the times of Plato and Aristotle. However, it was not until the 20th century that the scientific formulation of this theory emerged. Richard Semon, an evolutionary zoologist, coined the term "engram" to elucidate the neural foundation of memory storage and retrieval. Semon's definition of an engram as "the enduring though primarily latent modifications in the irritable substance produced by a stimulus" (Schacter, Eich, and Tulving 1978; Schacter 1982) has laid the groundwork for understanding how experiences are stored within our brains.

Although Semon's ideas were largely overlooked during his lifetime, the concept of the engram is now widely accepted as the substrate for episodic memories. Today, at the cellular level, the engram is recognized as a population of neuronal cells characterized by at least three key features: (i) activation during an experience, (ii) undergoing structural and functional modifications, and (iii) reactivation when recalling that specific experience (Tonegawa et al. 2015).

One of the earliest significant contributions to the understanding of memory engrams was Donald O. Hebb's proposal that "neurons that fire together, wire together" (Hebb 1949). This concept was later experimentally confirmed through the identification of long-lasting activity-dependent changes in synaptic strength between co-active neurons (Bliss and Lomo 1973; Collingridge, Kehl, and McLennan 1983; Malenka and Bear 2004). These findings provided initial insights into the potential mechanisms underlying enduring changes within a hypothetical cellular engram.

However, a major breakthrough in accessing and studying engrams came with the discovery that the proto-onco-gene c-fos could serve as an indicator of plasticity-inducing activity in neurons in vivo (Morgan et al. 1987) and as a genetic proxy for neural activity (Radulovic, Kammermeier, and Spiess 1998). The use of immediate-early genes (IEGs), such as cfos, Arc, and Zif/268, enabled researchers to genetically target and manipulate putative engram ensembles.

Leveraging these cutting-edge technologies, scientists have made significant progress in identifying and characterizing various properties of engrams. For instance, Josselyn, Silva, and colleagues discovered that engram recruitment, also known as "allocation," appears to be a competitive process in which neurons that are relatively more excitable than their neighbors during an experience are preferentially selected compared to less active neurons (Han et al. 2009, 2007, 2008; Zhou et al. 2009; Hsiang et al. 2014; Yiu et al. 2014; Josselyn and Frankland 2018; Josselyn 2010; Silva et al. 2009; Matos et al. 2019). Importantly, this increased excitability can persist for several hours after the event, suggesting that it may be the mechanism by which overlapping engram ensembles encode multiple experiences occurring within that specific time window.

The ability to target and study *in vivo* engram cells has allowed researchers to investigate the nature of the "enduring changes" proposed by Semon. Regarding to contextual fear conditioning, studies have shown that the spines of CA1 engram cells receiving input from CA3 engram cells increase in number and size, as demonstrated by Choi et al. (Choi et al. 2018). This enhanced connectivity between engram cells occludes long-term potentiation (LTP), confirming that a previous LTP-like phenomenon occurs during learning.

To establish a causal link between these synaptic processes and memory, Nabavi et al. (Nabavi et al. 2014) conducted experiments in which rats were conditioned to associate a foot shock with a particular sound using optogenetic stimulation of the auditory inputs to the amygdala. They demonstrated that the delivery of long-term depression (LTD)-inducing stimuli to the auditory input inactivated the memory, while subsequent delivery of LTP-inducing stimuli reactivated it. These findings support the idea that synaptic plasticity and increased connectivity within engram cells contribute to the long-lasting changes induced by learning, as originally proposed by Semon.

In summary, the ability to manipulate and study engram cells *in vivo* has provided evidence for enduring changes in synaptic connectivity and plasticity that underlie memory formation. The mentioned studies highlight the role of synaptic modifications, such as spine changes and the induction of LTP and LTD, in the consolidation and reactivation of engram cells associated with specific memories.

However, memories are not static entities and can undergo dynamic changes over time. Experimental evidence supports the phenomenon of memory loss, where memories that were once accessible can become "lost" after a certain period. For example, in the social discrimination task performed by rodents, the engram ensemble associated with a familiar mouse in the ventral CA1 of the hippocampus became silent when the animals could no longer recall the memory. This silence meant that the engram was not activated by natural recall cues. Remarkably, artificially reactivating the same ensemble using optogenetic tools

could reinstate the "lost" social discrimination memory, indicating that the engram still existed in the brain but was temporarily inaccessible (Okuyama et al. 2016; Kogan, Frankland, and Silva 2000).

Similar findings have been observed in other behavioral paradigms and brain regions, suggesting that the phenomenon of engram silence and memory loss is not specific to a particular type of memory or brain region. The exact mechanism underlying engram silence is still unknown, but one popular theory suggests that it may involve competition between old and new memory traces in controlling behavior (Lacagnina et al. 2019; Khalaf et al. 2018).

Furthermore, there is growing evidence that engrams supporting specific episodic memories are widely distributed throughout the brain. While early studies focused on the hippocampus as a critical structure for episodic memory engrams, recent research has revealed that episodic-like memory engrams can be detected across the brain. For example, Roy and colleagues (Roy et al. 2022) have shown that engram ensembles for contextual fear conditioning are distributed throughout the brain, with the lateral entorhinal cortex (LEC) identified as a potential engram region for this type of memory. This finding strengthens the hypothesis that the LEC may be involved in the storage of episodic memories.

Ongoing research on entorhinal cortex engrams holds significant importance, particularly in the context of neurodegenerative diseases like Alzheimer's disease (AD). Determining whether the entorhinal cortex contributes to the engram network for episodic memories can offer valuable insights for our understanding of memory-related processes and potential implications for AD. Indeed, the entorhinal cortex is one of the earliest regions to degenerate in AD (Van Hoesen, Hyman, and Damasio 1991; Braak and Braak 1991; Gómez-Isla et al. 1996; Hyman et al. 1984; Kordower et al. 2001; Olsen et al. 2017), and episodic memory impairment is among the initial symptoms of neurodegeneration.

Strategies to identify episodic memory engrams

Currently, various methods are available to investigate episodic engrams at the cellular level. One such method involves immunostaining against the protein encoded by Immediate Early Genes (IEGs) to capture the cellular activation associated with specific experiences or behaviors (Morgan et al. 1987; Sagar, Sharp, and Curran 1988; Ramírez-Amaya et al. 2005; Hartzell et al. 2013). IEGs are a group of genes rapidly activated and transiently expressed in response to external stimuli, including neuronal activity, growth factors, neurotransmitters, and hormones. What makes them particularly suitable for mapping brain activity is their ability to be activated without requiring prior protein synthesis or other molecular events. Their rapid appearance after stimulation and low basal expression make them ideal candidates for studying functional activity in the brain. Notable examples of IEGs include cfos, c-jun, and zif268.

While no single IEG can perfectly represent neuronal activity, the use of IEG-dependent labeling, such as cfos, Arc, and other IEGs, has provided valuable insights into engram physiology. By employing this technique, researchers have been able to identify cells that are activated in response to multiple stimuli occurring at different times (Guzowski et al.

1999), visualize active neurons in fixed or live tissue using transgenic animals or animals injected with specific AAVs (Barth, Gerkin, and Dean 2004; Smeyne et al. 1992; Wang et al. 2006), and manipulate the activity of cell populations expressing IEGs (Garner et al. 2012; Koya et al. 2009; Liu et al. 2012; Reijmers et al. 2007).

An illustrative example of how IEG-dependent labeling can be employed to investigate memory engrams involves combining the genetic tagging of cells activated during an experience with IEG immunohistochemistry of cells activated during subsequent memory tests. This approach allows researchers to examine whether there is a greater-than-chance overlap between these two cell populations within a specific brain region following a particular behavior. If the observed overlap exceeds what would be expected by chance, it may indicate significant reactivation of the same cell population during memory recall and provide evidence of memory engrams.

In a notable study by Reijmers and colleagues (Reijmers et al. 2007), IEGs were utilized to explore the activation of a neuronal population in the basal amygdala during auditory fear conditioning. During the experiment, animals were exposed to a harmless tone (the conditioned stimulus) paired with an aversive footshock (the unconditioned stimulus) in a specific context. Subsequently, the researchers assessed whether the animals displayed significant memory of the training experience by observing freezing behavior in the presence of either the tone or the conditioning environment, but without the aversive stimulus. Notably, the authors genetically tagged the neurons that were active during the training session using a fluorescent reporter. They then employed Zif268 immunohistochemistry to label the neuronal population activated during memory recall. Interestingly, they discovered a greater-than-chance overlap between these two populations, suggesting the presence of a potential memory engram that was activated during the initial experience and reactivated during subsequent memory recall of the same event.

Furthermore, in a recent study conducted by Roy and colleagues, a similar technique was employed to construct a comprehensive brain-wide map of the engram complex associated with contextual fear conditioning. The researchers achieved this by tagging neuronal ensembles expressing cfos during the training phase and identifying those that exhibited reactivation during memory recall at a higher rate than expected by chance. Their findings provided evidence that specific memories appear to be stored in functionally connected cell ensembles distributed widely across the brain, including the entorhinal cortex (Roy et al. 2022).

However, to conclusively establish the existence of a memory engram, it is crucial to demonstrate that the activation of a specific group of neurons is both necessary and sufficient for memory recall. To achieve this, numerous loss-of-function studies have sought to identify and disrupt engram cells prior to a memory test. Building upon current scientific understanding of engram allocation, Josselyn and colleagues (Han et al. 2009) conducted the pioneering cellular-level study in which they investigated memory loss through loss-of-function manipulations. In their study, they injected mice with a neurotropic virus that expressed CREB (Ca++/cyclic AMP-responsive element-binding protein), a transcription factor known to increase neuronal excitability (Dong et al. 2006;

Marie et al. 2005; Han et al. 2006; Zhou et al. 2009; Benito and Barco 2010) and spine density (Marie et al. 2005; Sargin et al. 2013), thereby enhancing the likelihood of a cell becoming part of an engram. They hypothesized that the neurons infected with the CREB vector would likely be incorporated into the memory engram. Subsequently, they employed another viral construct that expressed both CREB and an inducible construct capable of inducing cell-autonomous ablation, specifically targeting and eliminating the allocated neurons after the training experience (Han et al. 2009). The researchers made a noteworthy observation: the ablation of neurons overexpressing CREB completely disrupted the fear memory in mice, while the ablation of a comparable number of non-CREB-overexpressing cells did not yield the same effect (Han et al. 2009). This finding provided compelling evidence that the activation of the identified CREB-overexpressing neurons was essential for the formation and recall of the fear memory, indicating their involvement in the memory engram.

Furthermore, optogenetic and chemogenetic techniques have been instrumental in shedding light on memory engrams. These methods allow for the temporary deactivation of putative engram ensembles during memory tests, demonstrating that the activation of these specific cell populations is necessary for successful memory recall (Denny et al. 2014). Conversely, gain-of-function studies have been designed to simulate the retrieval process by directly reactivating engram cells using optogenetic or chemogenetic techniques in the absence of the conditioned stimulus. Remarkably, these studies have shown that the artificial stimulation of engram cells can effectively replace the conditioned stimulus, eliciting memory retrieval even in the absence of any external sensory cue (Redondo et al. 2014).

Intriguingly, these techniques have not only advanced our understanding of natural memory processes but have also enabled the creation of artificial memories. For example, Tonegawa and colleagues (Ramirez et al. 2013) labeled the dentate gyrus (DG) neurons that were activated during the exploration of context A and subsequently reactivated those neurons while mice received foot shocks in a different context, context B. During the memory test, the mice placed in context A exhibited significant freezing behavior, even though they had never experienced the foot shocks in that context. This finding indicated the formation of an artificial memory resulting from the association between the footshock and context A. The same mice froze in context B, indicating natural retrieval, but did not exhibit freezing behavior in a new context, context C. Another study employed a similar approach and demonstrated the implantation of a memory in the complete absence of natural stimuli. The researchers optogenetically stimulated a specific olfactory glomerulus to represent the conditioned stimulus and paired this stimulation with the optogenetic activation of either appetitive or aversive neural pathways. As a result, the mice displayed attraction or aversion towards the actual odor capable of activating the same olfactory glomerulus (Vetere et al. 2019).

These findings highlight the power of optogenetic and chemogenetic techniques in deciphering the mechanisms underlying memory formation, recall, and even the creation of artificial memories. By selectively manipulating and activating engram cells, researchers have made significant strides in unraveling the intricate processes that contribute to the encoding and retrieval of memories.

The Entorhinal Cortex

The entorhinal cortex (EC, Broadmann area 28) is a brain region situated in the medial portion of the temporal lobe. It derives its name from being partially enclosed by the rhinal (olfactory) sulcus, which is a feature more pronounced in non-primate mammals. Currently, the EC is recognized as a crucial intermediary between hippocampal formation and various cortical areas. It forms part of the parahippocampal gyrus, which also includes Brodmann areas 27, 35, and the uncus. The EC serves as a transitional zone between the allocortex and the neocortex, comprising six distinct layers. Based on its cellular architecture, connectivity, and function, the entorhinal cortex can be divided into a medial (MEC) and a lateral (LEC) section. The MEC appears to be primarily involved in spatial processing and navigation (Fyhn et al. 2004; Hafting et al. 2005), while the LEC is implicated in conveying olfactory information (Eichenbaum, Yonelinas, and Ranganath 2007; Habets, Lopes da Silva, and Quartel 1980; Habets, Lopes Da Silva, and Mollevanger 1980) and in processing local landmarks of the environment (Hargreaves et al. 2005; Deshmukh and Knierim 2011; Kuruvilla and Ainge 2017). The MEC and LEC subdivisions of the entorhinal cortex receive inputs from different brain regions associated with various aspects of cognitive processing. The MEC receives inputs from the postrhinal cortex (POR) (Naber et al. 1997), the pre-subiculum (PreS), the parasubiculum (PaS) (Caballero-Bleda and Witter 1993), the retrosplenial cortex (RSC) (Simonsen, Czajkowski, and Witter 2022) and in part also from the perirhinal cortex (PER), all areas primarily involved in spatial processing. These inputs terminate mainly in the superficial layers of the MEC. In contrast, the LEC receives inputs from regions more focused on processing non-spatial features of the environment, such as the PER (Naber, Witter, and Lopez da Silva 1999; Pinto, Fuentes, and Paré 2006), the olfactory cortex (Haberly and Price 1978; Kerr et al. 2007), the insular cortex (Mathiasen, Hansen, and Witter 2015), and the orbitofrontal cortex (Hoover and Vertes 2007, 2011; Kondo and Witter 2014). These inputs also terminate in the superficial layers of the LEC. Importantly, both the MEC and LEC project back to the same regions through neurons located in their deep layers.

The organization of projections from the entorhinal cortex suggests the existence of two parallel streams of information converging onto the hippocampus. According to this hypothesis, one stream conveys spatial information about an experience via the MEC, while the other stream provides non-spatial information through the LEC (Naber et al. 1997; Witter et al. 2000; Knierim, Lee, and Hargreaves 2006; Eichenbaum et al. 2012). Indeed, the segregation of information processing by the MEC and LEC is largely preserved throughout the EC-hippocampal circuit. For example, the projections from the superficial layers of the entorhinal cortex to the hippocampus form the perforant pathway. This pathway consists of excitatory neurons in layer 2 (L2) projecting to the DG and CA3 regions of the hippocampus, and neurons in layer 3 (L3) projecting to CA1 and the subiculum (Steward 1976; Köhler 1986, 1988; Naber, Lopes da Silva, and Witter 2001; Kerr et al. 2007; Strien, Cappaert, and Witter 2009). However, it has been shown that MEC L2 neurons project to the middle molecular layer of the DG and the deep region of CA3, while LEC L2 neurons project to the outer molecular layer of the DG and the superficial region of CA3 (see Van Strien et al. 2009). Furthermore, L3 projections from the MEC reach the proximal CA1 and the distal subiculum, while LEC L3 neurons innervate the distal CA1 and the proximal

subiculum. The hippocampal formation also provides sparse projections to the superficial layers of the EC from the CA1 subregion and the subiculum, with distal CA1 and proximal subiculum projecting to the LEC, and proximal CA1 and distal subiculum projecting to the MEC (Tamamaki and Nojyo 1995; Naber, Lopes da Silva, and Witter 2001; Kloosterman, Haeften, and Lopes da Silva 2004).

Besides the external connections, the MEC and LEC also appear to be extensively connected. Indeed, MEC L2, L3, L5 and L6 project to the L2 and L3 of the LEC (Köhler 1986, 1988; Dolorfo and Amaral 1998). In return, LEC L2 and L5 project to L2 and L3 of the MEC, and L3 and L6 project to the L5 and L6 of the MEC (Köhler 1986, 1988; Dolorfo and Amaral 1998). The connectivity between the two regions of the entorhinal cortex might support the integration of different types of information independently from the hippocampus.

The LEC and MEC can also be differentiated based on their cellular composition. The EC has classically been considered an intermediate structure between the six-layered neocortex and the three-layered archicortex (Groen 2001). Originally, the EC was described as a region characterized by four cellular layers and two relatively cell-free layers. However, recent studies have revealed that the cellular layer 5 is instead composed of two distinct sublayers, a more superficial layer (L5a) expressing the transcription factor Etv1, and a deeper layer expressing the transcription factor Ctip2 (Sürmeli et al. 2015; Ramsden et al. 2015). In the superficial layers of the MEC, the largest population of excitatory cells is composed of the stellate cells, whose name originates from the star-like arrangement of their dendrites around the soma. The second population of excitatory neurons is then represented by pyramidal cells (Canto and Witter 2012b). Stellate cells express the glycoprotein reelin, whereas pyramidal cells express the calcium-binding protein calbindin D-28k (Kitamura et al. 2014). In addition, the superficial layers of the MEC seem to be rich in parvalbumin-expressing interneurons (Wouterlood et al. 1995; Fujimaru and Kosaka 1996; Leitner et al. 2016).

In the LEC, the largest population of excitatory neurons is instead represented by the fan cells, named for the branching arrangement of their dendrites horizontally through layer 2 and vertically towards the pia. Similarly to the MEC, the second largest population of excitatory neurons is composed of pyramidal cells , moreover, the LEC contains a subgroup of multiform cells, whose name originates from their different morphologies (Tahvildari and Alonso 2005; Canto and Witter 2012a; Leitner et al. 2016). LEC fan cells have been shown to express reelin while pyramidal cells are positive for calbindin (Fujimaru and Kosaka 1996; Leitner et al. 2016). However, intermediate cell types expressing both reelin and calbindin can also be observed in both subregions (Witter et al. 2017). What makes this distinction even more interesting is the observation that reelin and calbindin-positive cells project to completely different regions of the hippocampus. Indeed, in both EC subregions, reelin-positive neurons project to the DG and CA3, whereas calbindin-positive neurons project to CA1 and other cortical structures (Kitamura et al. 2014; Sürmeli et al. 2015; Leitner et al. 2016), this may indicate different functional roles. Finally, in contrast to the MEC, the superficial layers of the LEC seem to have a lower number of parvalbumin-positive interneurons (Wouterlood et al. 1995; Fujimaru and Kosaka 1996; Leitner et al. 2016).

Regarding the deep layers, the entorhinal cortex layer 5 appears to be principally composed of large populations of pyramidal neurons, which are further subdivided into different subtypes (Canto and Witter 2012b, 2012a). Interestingly, layer 5 can be subdivided into two sub-layers called L5a and L5BL5b, distinguished by the expression of the transcription factors Etv1 and ctip2, respectively (Ramsden et al. 2015; Sürmeli et al. 2015; Witter et al. 2017). The L5a contains mostly pyramidal neurons, whereas L5b contains a mixed population of pyramidal and multipolar neurons (Hamam et al. 2000; Canto and Witter 2012b, 2012a). Regarding external connections, L5b has been shown to receive input from dorsal CA1 and subiculum, whereas L5a receives input from the ventral CA1. In addition, cortical regions such as the medial prefrontal cortex, the anterior cingulate cortex, the retrosplenial cortex, and the medial secondary visual cortex also project to L5 but the specific sublayer is still not known (Czajkowski et al. 2013; Olsen et al. 2017; Canto, Wouterlood, and Witter 2008; Jones and Witter 2007).

In MEC and LEC neurons in L5a represent the primary telencephalic output, but projections to the retrosplenial cortex, which is likely to be necessary for spatial processing, appear to be more prominent from MEC. This is consistent with the notion that the MEC plays a preferential role in spatial processing. Intriguingly, functional connectivity from L5b to L5a appears more prominent in LEC compared to MEC (Ohara et al. 2021) and this might suggest that the LEC alone may represent the primary route by which memory-related signals reach the neocortex and subcortical telencephalic structures from the hippocampus.

The Lateral Entorhinal Cortex in episodic memory

Episodic memory encompasses various facets of an event, including its specific details, temporal context, and spatial location. Conventionally, the hippocampus has been regarded as the primary site for integrating spatial and non-spatial aspects of an experience. However, recent research has shed light on the pivotal role of the Lateral Entorhinal Cortex (LEC) in this process.

While the Medial Entorhinal Cortex (MEC) has been extensively investigated for its spatial representation capabilities, with distinct cell types encoding movement, speed, and environmental spatial features, the LEC diverges in its functional involvement in spatial navigation. The MEC provides crucial spatial information that contributes to our understanding of how we navigate and orient ourselves in the surrounding environment (Hafting et al. 2005; Barry et al. 2006; Savelli, Yoganarasimha, and Knierim 2008; Solstad et al. 2008; Lever et al. 2009). On the other hand, the LEC receives sensory information related to the characteristics of objects, odors, or other local features of the environment within an experience and integrates this sensory information with temporal and contextual cues to generate a comprehensive episodic memory representation.

Research has revealed that the LEC is crucial for associative memory, recognizing associations between objects, places, and contexts (D. I. Wilson et al. 2013). The LEC's role in associative memory highlights its importance in forming connections between different elements of an experience, allowing us to remember a cohesive episode. It is worth noting that different memory functions might be segregated within different LEC networks. For

instance, Vandrey et al. (2020) demonstrated that selective suppression of synaptic transmission in the LEC's superficial layers, specifically the fan cells, impaired recognition of familiar object-place-context associations but not object-context associations (Vandrey et al. 2020). This suggests that the encoding of object-place-context and object-context information might be segregated within this circuitry, further emphasizing the specialized functions of different LEC networks.

In addition to its role in forming associations, the LEC fan cells to DG circuit appears to be particularly important for pattern separation, a fundamental process for episodic memory. Increased connectivity between fan cells and DG has been shown to improve an animal's ability to separate similar contexts and enhance cognitive flexibility (Yun et al. 2023), highlighting the role of this specific sub-population of superficial LEC neurons in distinguishing and preserving distinct memories, ensuring that similar experiences are not conflated or confused.

The discovery of object trace cells in the LEC was a significant milestone in understanding the LEC's involvement in episodic memory. These cells, identified by Tsao et al. (2013), exhibit spatial tuning to locations where objects were previously loicated, indicating their role in encoding such information within a memory trace (Tsao, Moser, and Moser 2013). This finding not only suggests the LEC's function in forming associations but also reveals its capacity to encode detailed spatial information in memory.

Furthermore, the LEC is involved in encoding the temporal aspects of experiences. Research has demonstrated that LEC neurons possess the intrinsic ability to modulate their firing rates in a ramp-like fashion from the beginning of an event, enabling the encoding of the temporal context within an experience (Tsao et al. 2018; Bright et al. 2020; Bellmund, Deuker, and Doeller 2019; Montchal, Reagh, and Yassa 2019). This temporal encoding adds another layer of complexity to the LEC's role in episodic memory, highlighting its crucial involvement in capturing and maintaining the temporal framework of events in memory.

Notably, LEC neurons exhibit stable firing responses to familiar and structured experiences involving repeated stimuli or movements within a known environment. This stability in firing responses may be attributed to consistent sensory inputs across both novel and structured experiences or the continuous excitatory inputs into specific neuronal ensembles in the hippocampus and neocortex during familiar, repetitive encounters. One possible hypothesis is that these stable firing responses in the LEC facilitate the reinstatement of neural activity patterns associated with specific experiences. In support of this hypothesis, research has shown that pharmacological inactivation of the LEC disrupts the consistency of neural firing patterns in the medial prefrontal cortex (mPFC) across repeated experiences, leading to a decrease in the proportion of mPFC neurons that maintain stable firing selectivity for stimulus associations (Pilkiw, Jarovi, and Takehara-Nishiuchi 2022). This suggests that the LEC may play a crucial role in reinstating cortical patterns linked to specific events and, consequently, in the recall of episodic memories.

Moreover, the relevance of the entorhinal cortex in episodic memory processing is underscored by its vulnerability to Alzheimer's disease. The entorhinal cortex, particularly layer 2, is significantly affected in Alzheimer's disease (Gómez-Isla et al. 1996; Stranahan

and Mattson 2010; Khan et al. 2014; Kobro-Flatmoen, Nagelhus, and Witter 2016). Interestingly, there seems to be a close association between reelin, expressed by fan cells in layer II, and amyloid beta, a hallmark protein implicated in Alzheimer's disease. Recent studies have shown that reducing reelin levels in the LEC leads to a concomitant reduction in amyloid beta peptide, suggesting a potential link between LEC dysfunction and the development of early Alzheimer's disease (Kobro-Flatmoen et al. 2023). In our own lab, we demonstrated that APPJ20 mice, a mouse model of familial Alzheimer's disease, showed initial signs of episodic-like memory impairment along with specific electrophysiological alterations in the LECsuperficial layers. Notably, we observed that these functional deficits were largely mediated by the activation of the receptor for advanced glycation end products (RAGE) (Origlia et al. 2008) by the amyloid-beta peptide and that counteracting amyloid beta activity on the microglial receptor RAGE was sufficient to rescue these behavioral deficits (Criscuolo et al. 2017), further supporting the intimate connection between LEC dysfunction and episodic memory deficits observed in early Alzheimer's disease.

In summary, the Lateral Entorhinal Cortex (LEC) plays a crucial role in episodic memory by integrating sensory information, temporal and contextual cues, and forming associations between objects, places, and contexts. Its involvement extends beyond simple association formation and includes encoding spatial, temporal, and detailed information in memory. The LEC specialized circuits, such as the fan cells to DG circuit, contribute to pattern separation and cognitive flexibility. Additionally, the LEC firing responses and reinstatement of neural activity patterns are crucial for recall. Understanding the intricate functions of the LEC enhances our comprehension of episodic memory processes and sheds light on its vulnerability during the development of neurodegeneration as in Alzheimer's disease..

Synaptic plasticity in the Entorhinal Cortex

To better understand the contribution of the entorhinal cortex to memory processing, an essential area of investigation involves studying the synaptic plasticity mechanisms within its intrinsic circuitry. Like many other brain regions, these mechanisms can be examined through changes in the efficiency of glutamatergic synaptic transmission using established experimental paradigms such as long-term potentiation (LTP) and depression (LTD). These paradigms provide valuable insights into the dynamic nature of synaptic connections within the entorhinal cortex and their potential role in shaping memory-related processes.

Several forms of plasticity have been identified as capable of producing a persistent increase in synaptic transmission. The most extensively studied LTP form involves NMDA receptor-dependent synaptic plasticity, which occurs when calcium influx through NMDA receptors in the postsynaptic neuron triggers potentiation of postsynaptic transmission, via a series of intracellular second messengers such as CaMKII and changes in the number of AMPA receptors expressed on the postsynaptic membrane. Nonetheless, LTP can also occur through non-NMDA dependent mechanisms such as the activation of voltage-dependent calcium channels or metabotropic glutamatergic receptors that can lead to increased calcium influx and postsynaptic increases in AMPA receptors (Gu et al. 1996; Grover and Teyler 1995, 1990). Furthermore, presynaptic LTP has been observed, for

example, at the level of the mossy fiber input to CA3 pyramidal neurons (Nicoll and Malenka 1995; Zalutsky and Nicoll 1990).

In the entorhinal cortex, the first evidence of LTP came from a chronic in vivo recording study in adult rats. The study demonstrated that brief episodes of high-frequency stimulation (HFS) delivered to the amygdala induced LTP in early and late components of field potentials recorded in the EC (Köhler 1986), and that LTP induction in this pathway obeyed Hebbian learning rules (Kourrich and Chapman 2003; Larson and Lynch 1986). However, different forms of LTP have been observed in the EC. HFS stimulation of EC layer I induces a post-tetanic potentiation in EC layer II that can last for more than 30 min (Yun, Mook-Jung, and Jung 2002; Alonso, Curtis, and Llinás 1990; Ma, Alonso, and Dickson 2008) and is NMDA-dependent (Ma, Alonso, and Dickson 2008), while LTP observed in the ascending columnar connections from layer III to layer II neurons relies on both postsynaptic and presynaptic mechanisms (Ma, Alonso, and Dickson 2008), but does not depend on NMDA receptors. Moreover, the LTP induced by high-frequency stimulation in the deep-to-superficial layer connections has been shown to depend on NMDA receptor activation, indicating that different pathways in the EC rely on different mechanisms to regulate synaptic weight.

A similar situation has been observed regarding LTD. Different forms of long-term depression (LTD) have also been observed in the entorhinal cortex (EC), with LFS-inducing synapse-specific, NMDA-dependent LTD in the superficial layers and a presynaptic, NMDA-independent, short-term form of LTD in the deep cortical layers. These differences in plasticity mechanisms may reflect functional segregation between different circuits within the EC. However, the exact role of entorhinal long-term synaptic plasticity in episodic memory processing is still unclear.

Interestingly, studies have shown that neurons in EC layer II are particularly vulnerable to aging and Alzheimer's disease (AD). In a mouse model of AD, an early synaptic impairment in the EC superficial layers was observed, which was associated with disrupted memory for object-place-context associations, but spared object recognition memory. The reduction in LTP induced by high-frequency stimulation in acute brain slices was paralleled by impaired episodic-like memory in the OPCRT behavioral paradigm, suggesting that glutamatergic synaptic plasticity in the superficial layers of the EC might play a critical role in episodic-like memory processing. No memory impairment was observed in the novel object recognition test, indicating that LTP impairment in the superficial EC horizontal connections specifically affected associative, episodic-like memory and did not impair the animal's general ability to process single elements of the task (Criscuolo et al. 2017). Supporting these findings, a study by Cui and colleagues (citation) demonstrated that the conditional knockout of the NMDA NR1 subunit in the forebrain, resulting in non-functional NMDA receptors, led to impaired LTP in the superficial layers of the entorhinal cortex, along with severe deficits in recalling remote fear memories acquired 9 months earlier (Cui et al. 2004). These results further strengthen the relationship between synaptic plasticity within the entorhinal cortex, and episodic-like memory.

Results

c-Fos expression is increased in the EC following OPCRT and context exploration

To investigate whether the processing of an episodic-like memory engages the EC and triggers the expression of plasticity-related genes, the number of cells that are positive for the immediate early-gene (IEG) encoded c-Fos protein were assessed 2 hours after the execution of the behavioral task.

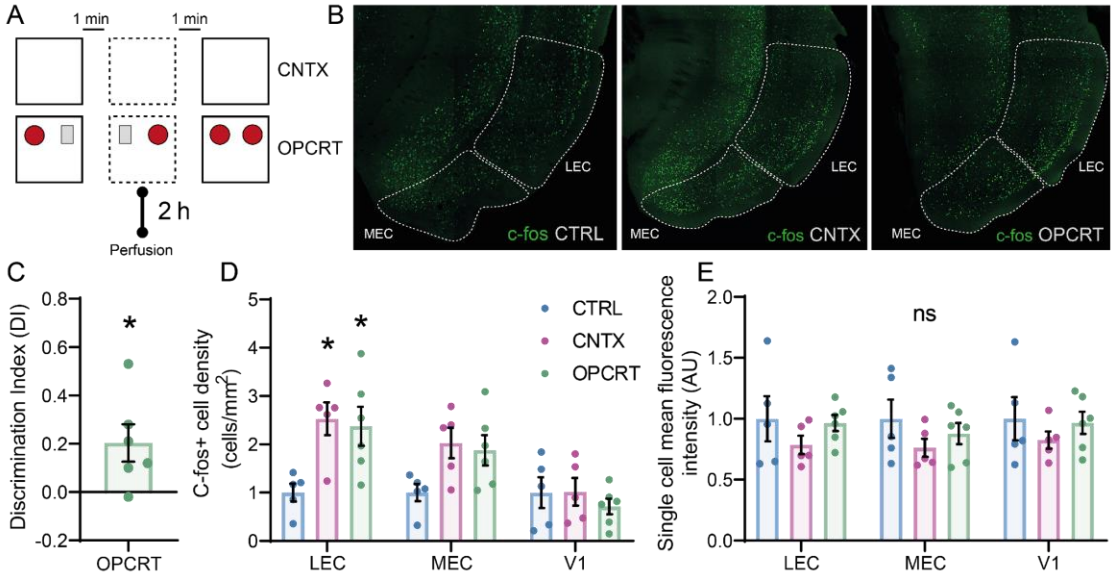

Figure 1. C-fos cell density is increased in the LEC following OPCRT and context exploration. (A) *Schematic representation of context exposure without objects (CNTX group) and the OPCRT test, with animal perfusion taking place 2 hours after the behavioral task.* ***(B)*** *Representative images displaying c-Fos expression (green) in the medial and lateral entorhinal cortex across various experimental groups.* ***(C)*** *The discrimination index (DI) of mice subjected to OPCRT was significantly higher than chance (0.20 ± 0.08, n = 6, p = 0.047, df = 5, t = 2.62, one-sample t-test). *p <0.05.* ***(D)*** *A significant difference in c-Fos+ cell density was observed in the lateral entorhinal cortex (LEC; Two-way ANOVA RM, p(interaction) = 0.011, df = 4, F = 0.24; CTRL 1 ± 0.18 c-fos+ cells/mm2 relative to CTRL n = 5 mice vs CNTX 2.53 ± 0.34 c-fos+ cells/mm2 relative to CTRL n = 5 mice p = 0.017; CTRL vs OPCRT 2.37 ± 0.40 c-fos+ cells/mm2 relative to CTRL n = 6 mice, p = 0.04 Tukey's multiple comparisons test), while no difference was observed either in the medial entorhinal cortex (MEC; CTRL 1 ± 0.18 c-fos+ cells/mm2 relative to CTRL n = 5 mice vs CNTX 2.02 ± 0.32 c-fos+ cells/mm2 relative to CTRL n = 5 mice, p = 0.06; CTRL vs OPCRT 1.88 ± 0.31 c-fos+ cells/mm2 relative to CTRL n = 6, p = 0.09 Tukey's multiple comparisons test) or in the primary visual cortex (V1; CTRL 1 ± 0.32 c-fos+ cells/mm2 relative to CTRL n = 5 mice vs CNTX 1.01 ± 0.32 c-fos+ cells/mm2 relative to CTRL n = 5 mice, p = 0.99; CTRL vs OPCRT 0.71 ± 0.16 c-fos+ cells/mm2 relative to CTRL n = 6 mice, p = 0.71 Tukey's multiple comparisons test). * p <0.05.* ***(E)*** *No significant difference in w the c-Fos single cell mean fluorescence intensity was observed between the experimental groups (p(interaction) = 0.91 Two-way ANOVA RM).*

The levels of c-Fos in 3-month-old male mice subjected to the OPCRT paradigm (OPCRT group) were compared with the levels in mice exposed to different contexts without object presentation (CNTX group) and animals remained in their home cage (CTRL group, Fig1 A). The CNTX group enabled to examine whether any potential engagement of the EC was exclusively linked to the presence of objects or could also be induced by the exploration of novel contexts alone.

The results showed that the mice in the OPCRT group exhibited a significant preference for the novel object-place-context configuration compared to the familiar one, indicating the presence of episodic-like memory (Fig1 C). The processing of this memory was associated with a significant increase in c-Fos cell density in the LEC subdivision compared to CTRL mice, suggesting that this region was strongly recruited during the task. However, a similar increase in c-Fos cell density was also observed in the CNTX group, indicating that the presence of objects is not necessary to engage this circuitry (Fig 1 B, D).

Interestingly, no significant increase in c-Fos cell density was observed in the same conditions either in the MEC or in another cortical area, the primary visual cortex (V1) (Fig 1 B, D). Nevertheless, it is worth noting that, while no specific trend was observed in V1, the MEC displayed a tendency towards increased c-Fos levels both in the CNTX and OPCRT groups, indicating that this region might be slightly affected by episodic-like memory processing, as well.

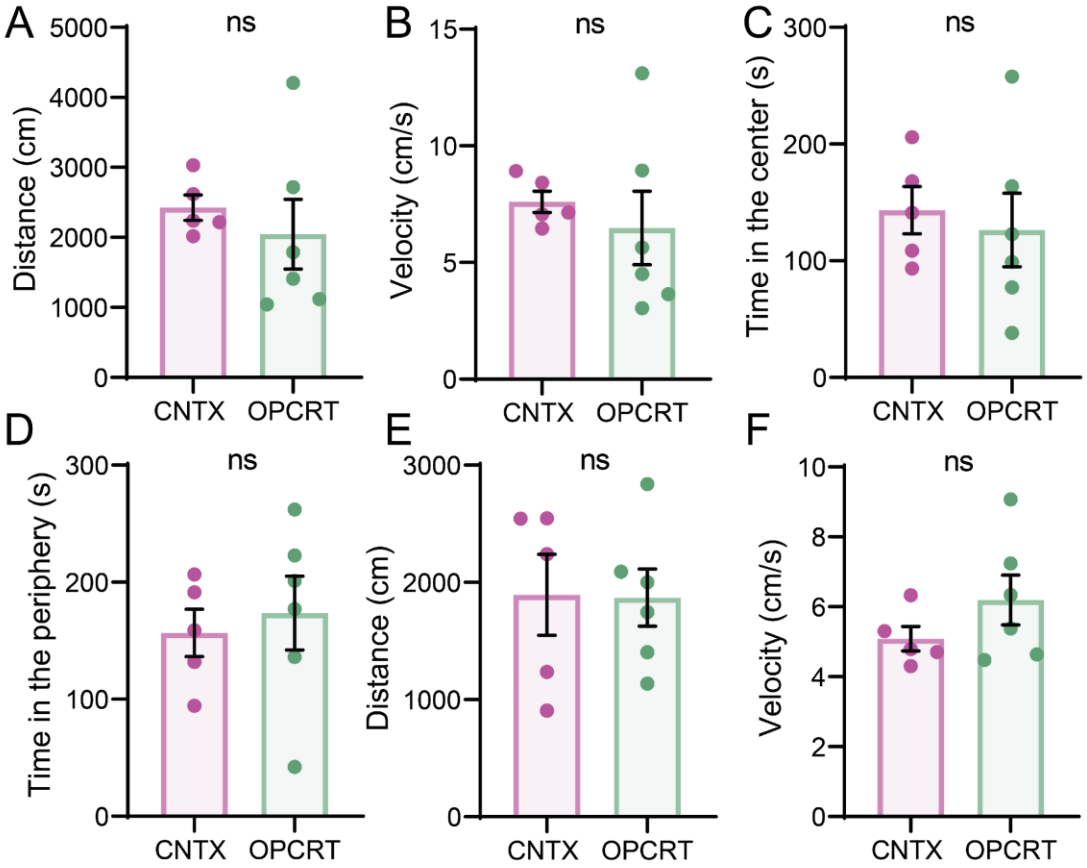

Figure 2. Mouse locomotor activity during the open field test and the OPCRT test trial. ***(A)*** *The average velocity of locomotion during the open field test was not significantly different between CNTX and OPCRT mice (7.6 ± 0.46 cm/s, n = 5 CNTX vs 6.48 ± 1.6 cm/s, n = 6 OPCRT, p = 0.46, df = 9, t = 0.63 two-tailed unpaired t-test).* ***(B)*** *The total distance traveled during the open field test was similar between the CNTX and OPCRT groups (2425 ± 180.6 cm, n = 5 CNTX vs 2047 ± 498.2 cm, n = 6 OPCRT, p = 0.53, df = 9, t = 0.66 two-tailed unpaired t-test).* ***(C)*** *The time spent in the center of the arena during the open field was comparable between CNTX and OPCRT mice (143.4 ± 20.27 s, n = 5 CNTX vs 126.4 ± 31.46, n = 6 OPCRT, p = 0.68, df = 9, t = 0.43 two-tailed unpaired t-test).* ***(D)*** *The time spent in the periphery of the arena during the open field was similar (156.6 ± 20.27, n = 5 CNTX vs 173.6 ± 31.46, n = 6 OPCRT, p = 0.68, df = 9, t = 0.68 two-tailed unpaired t-test).* ***(E)*** *The total distance traveled during the test trial was very similar between the two groups (1894 ± 344.9 cm, n = 5 CNTX vs 1870 ± 243.3 cm, n = 6 OPCRT, p = 0.95, df = 9, t = 0.06 two-tailed unpaired t-test).* ***(F)*** *The average velocity of locomotion during the test trial was similar between the two groups (5.08 ± 0.35 cm/s, n = 5 CNTX vs 6.19 ± 0.72 cm/s, n = 6 OPCRT, p = 0.23, df = 9, t = 1.30 two-tailed unpaired t-test).*

In order to investigate whether episodic-like memory could affect not only the number of c-Fos expressing cells but also the relative amount of c-Fos protein produced by individual EC cells, the mean fluorescence intensity of single cells per animal was examined in the same experimental groups. However, no significant difference in c-Fos fluorescence intensity were found in the areas of interest (Fig 1 E). These results suggest that the recruitment of a large group of LEC neurons, at least in terms of c-Fos staining, is induced by the processing of episodic-like memory, but this phenomenon does not lead to changes in the amount of c-Fos protein produced by each individual cell.

To ensure that the observed effects were not due to behavioral differences in exploratory behavior between the OPCRT and CNTX groups, an open field test was performed before the experiments and compared the time spent in the center *vs.* periphery of the arena, the average velocity, and the traveled distance (Fig 2 A-D). Additionally, exploratory behavior during the test trial did not significantly changed between groups (Fig 2 E-F).

Time course of object-place-context recognition memory

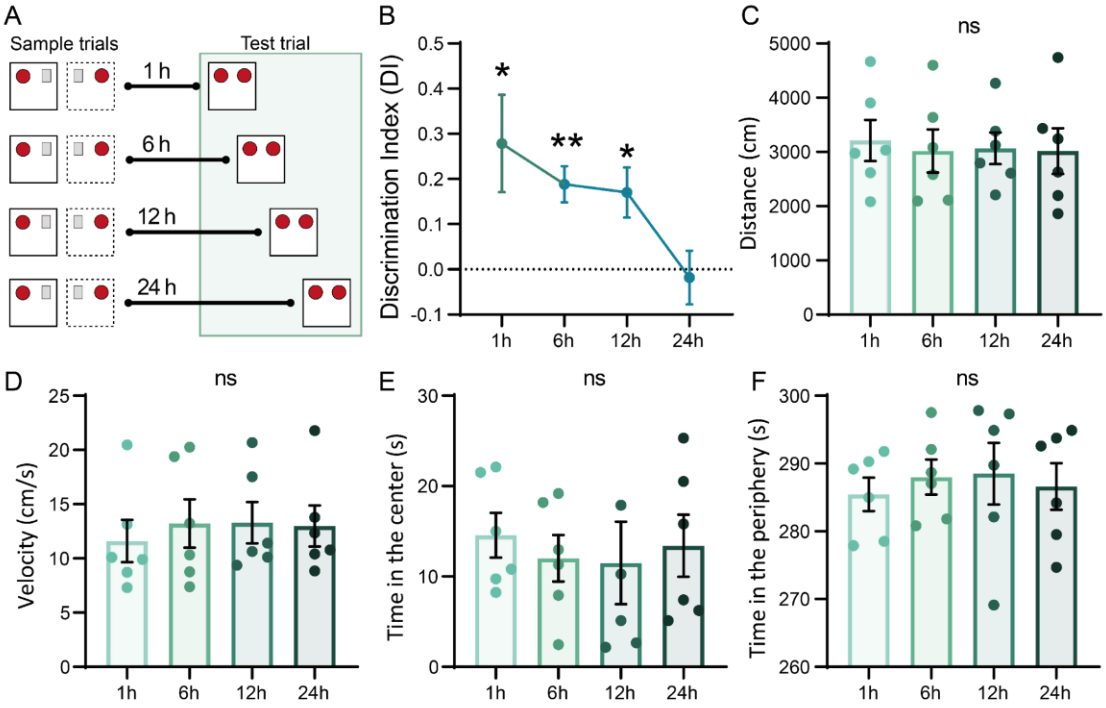

Figure 3. Time course of object-place-context recognition memory. (A) *Schematic representation of the delayed OPCRT test, with the test trial conducted at different time intervals (1h, 6h, 12h, or 24h) following the sample trial presentation.* ***(B)*** *The discrimination index (DI) for the novel OPC association exhibits a significant decline with increasing time intervals from the sample trials presentation (p = 0.037, Kruskal-Wallis test). Mice were able to recall the previously acquired episodic-like memory at either 1h (0.28 ± 0.11, n = 6, p = 0.049, df = 5, t = 2.58), 6h (0.19 ± 0.04, n = 6, p = 0.0053, df = 5, t = 4.71) or 12h (0.17 ± 0.06, n = 6, p = 0.03, df = 5, t = 3.06; one-sample t-test), while no memory was observed at 24h (-0.018 ± 0.06, n = 6, p = 0.77, df = 5, t = 0.31. one-sample t-test).* ***(C)*** *No significant difference in the total distance traveled during the open field test was observed between experimental groups (n = 6, p = 0.97, F = 0.06 one-way ANOVA).* ***(D)*** *The animal velocity during the open field test did not differ significantly among groups (n = 6, p = 0.92, F = 0.16 one-way ANOVA).* ***(E)*** *The time spent in the center of the arena by the animals was similar among the different experimental groups (n = 6, p = 0.92, f = 0.17 one-way ANOVA).* ***(F*** *The animals spent a comparable amount of time in the arena's periphery (n = 6, p = 0.92, f = 0.17 one-way ANOVA). *p <0.05, **p <0.01.*

In order to characterize the time course of object-place-context recognition memory, a delayed OPCRT was performed during which the test trial presentation was delayed by 1, 6, 12, or 24 hours after the sample trials (Fig 3 A). By analyzing changes in the discrimination index over these time points, the temporal expression of episodic-like memory could be estimated, allowing for the identification of the time window during which the memory was behaviorally expressed. Results showed a significant preference for the novel OPC association within the first 12 hours following the sample trials, indicating effective recall

of the previously acquired memory. However, after 24 hours, novelty discrimination significantly declined (Fig 3 B).

It is worth noting that the observed differences in memory expression were not attributed to differences in anxiety levels or exploratory behavior as there was no significant difference in the traveled distance (Fig 3 C), the average velocity (Fig 3 D), and the time spent in the center/periphery of the arena (Fig 3 E-F) among the groups.

OPCRT induces long-term reversible changes in LEC synaptic transmission

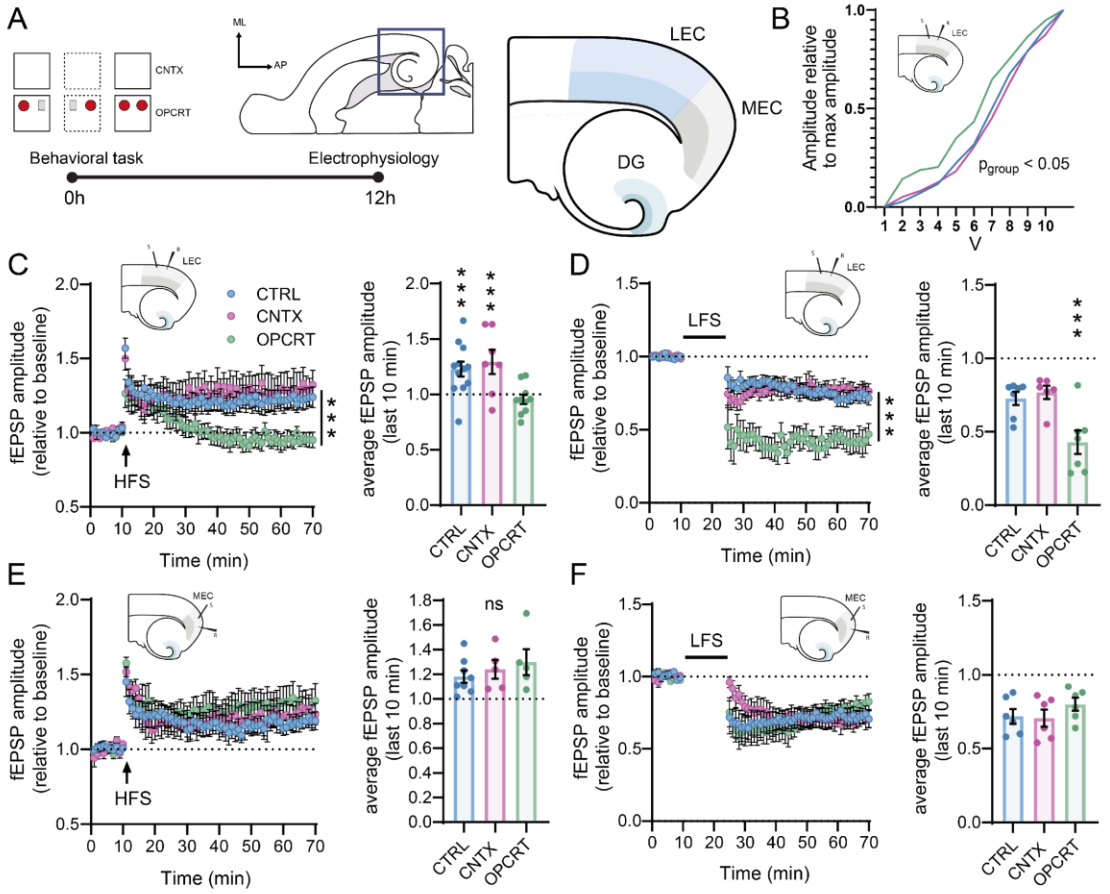

Figure 4. OPCRT-induced long-term changes in LEC synaptic plasticity. (A) *Timeline of the experimental design.* ***(B)*** *The input/output (I/O) relationship in slices obtained from OPCRT mice was significantly rightward-shifted compared to slices obtained from either CNTX or CTRL mice (p(group effect) = 0.037, df = 2, F = 3.62 two-way ANOVA RM).* ***(C)*** *(left) time course of field excitatory post-synaptic potentials (fEPSPs) during the LTP protocol. (right) the average fEPSPs on the last 10 min of recordings. Slices obtained from either CNTX or CTRL mice showed high-frequency stimulation (HFS)-induced long-term potentiation (LTP) 12h after the execution of the behavioral tasks (p(interaction) = 0.007, p(time) = 0.001m p(group) = 0.0066 two-way ANOVA RM; 129 ± 11% of baseline, n = 7, p = 0.005 vs baseline for CNTX; 123 ± 7% of baseline, n = 12, p = 0.0043 vs baseline for CTRL, Sidak's multiple comparisons test), while no significant LTP was observed in OPCRT slices (95 ± 4% of baseline, n = 10, p = 0.89, two-way ANOVA RM and Sidak's multiple comparisons test). OPCRT*

slices were significantly different when compared to CTRL (95 ± 4% of baseline, n = 10 OPCRT vs 123 ± 7% of baseline, n = 12 CTRL, p = 0.0005) or CNTX slices (95 ± 4% of baseline, n = 10 OPCRT vs 129 ± 11% of baseline, n = 7 CNTX, p = 0.0002, two way ANOVA RM and Sidak's multiple comparisons test). ***(D)*** *Same as in (C) but for the LTD protocol. Significant levels of low-frequency stimulation (LFS)-induced LTD were observed in all the groups in the LEC 12h after the execution of the behavioral tasks (73 ± 4% of baseline, n = 7, p = 0.0007 vs baseline for CTRL; 77 ± 5% of baseline, n = 6, p = 0.0063 vs baseline for CNTX; 43 ± 8% of baseline, n = 7, p <0.0001 vs baseline for OPCRT; two-way ANOVA RM and Sidak's multiple comparisons test). However, in OPCRT slices, LTD in the LEC was significantly enhanced compared either to CTRL (43 ± 8% of baseline, n = 7 OPCRT vs 73 ± 4% of baseline, n = 7 CTRL, p <0.001) or CNTX (43 ± 8% of baseline, n = 7 OPCRT vs 77 ± 5% of baseline, n = 6 CNTX, p <0.001, two way ANOVA RM and Sidak's multiple comparisons test).* ***(E)*** *Same as in (C) but for the medial entorhinal cortex (MEC). In the MEC, a significant LTP was observed in all the groups following HFS (118 ± 5% of baseline, n = 8, p = 0.034 vs baseline for CTRL; 124 ± 8% of baseline, n = 5, p = 0.026 vs baseline for CNTX; 130 ± 11% of baseline, n = 5, p = 0.0057 vs baseline for OPCRT; two-way ANOVA RM and Sidak's multiple comparisons tests), with no significant difference between the experimental groups observed 12h following the execution of the behavioral tasks in the superficial layers (p(group effect) = 0.52, df = 2, F = 0.69 two-way ANOVA RM).* ***(F)*** *Same as in (E) but for the LTD protocol. A significant LTD was observed in the MEC in all the groups following LFS (72 ± 5% of baseline, n = 6, p = 0.0002 vs baseline for CTRL; 71 ± 6% of baseline, n = 6, p = 0.0001 vs baseline for CNTX; 80 ± 4% of baseline, n = 6, p = 0.004 vs baseline for OPCRT). However, no significant difference between groups was observed (p(group effect) = 0.39, df = 2, F = 0.99 two-way ANOVA RM). ***p <0.001.*

Memory acquisition has been shown to trigger persistent changes in synaptic structure and function, indicating the involvement of synaptic plasticity in memory storage. To investigate whether processing of episodic-like memory can affect synaptic plasticity in the EC, field excitatory post-synaptic potentials (fEPSPs) were recorded in EC slices obtained from mice subjected to OPCRT and sacrificed 12 hours later. Specifically, it was assessed whether any alterations in long-lasting forms of plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), were present at a time point when memory was still behaviorally expressed, thus exploring the possible association between long-lasting plasticity and memory expression (Fig 4 A).

Indeed, a significant leftward shift in the input/output (I/O) curve was observed in the LEC in the OPCRT group compared to the CNTX and CTRL groups, which could reflect an increased excitatory transmission in the horizontal connections of the LEC following memory acquisition (Fig 4 B). When high-frequency stimulation (HFS) was used to induce LTP and a significant reduction in LTP was observed in slices from OPCRT mice compared to CNTX or CTRL groups, suggesting that the processing of an episodic memory had already potentiated synaptic transmission in the LEC superficial layers (Fig 4 C). This effect was OPCRT-specific since context exploration was not sufficient to induce such plasticity effects. Slices from OPCRT mice also showed enhanced low-frequency stimulation (LFS)-induced LTD compared to CNTX and CTRL groups, indicating that the processing of a new episodic-like memory might specifically induce a shift in the threshold for the induction of synaptic plasticity and strongly affects LEC synaptic transmission (Fig 4 D).

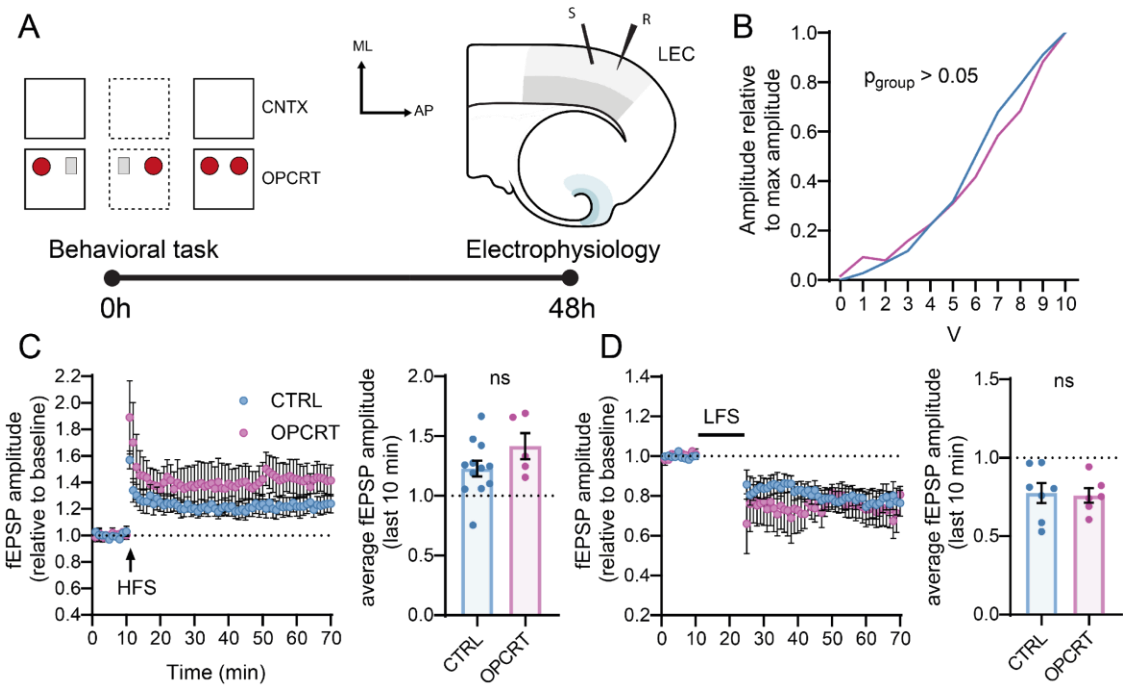

Figure 5. Reversible effects of memory processing on LEC synaptic plasticity. (A) *Timeline of the experimental design.* ***(B)*** *No effect of OPCRT on basal synaptic transmission was observed 48h after the execution of the test (p(group effect) = 0.73, df = 1, F = 0.12 two-way ANOVA RM).* ***(C)*** *(left) the timecourse of field excitatory post-synaptic potentials (fEPSPs) during the LTP protocol. On the right, a histogram showing the average fEPSPs on the last 10 min of recordings. LEC slices obtained from CTRL (123 ± 7% of baseline, n = 12, p = 0.0087 vs baseline) or OPCRT mice (142 ± 1% of baseline, n = 5, p = 0.0026 vs baseline; two-way ANOVA RM and Sidak's multiple comparisons test) showed comparable levels of HFS-induced LTP 48h following the execution of the test (123 ± 7% of baseline, n = 12 CTRL vs 142 ± 11% of baseline, n = 5 OPCRT, p = 0.08; two-way ANOVA RM and Sidak's multiple comparisons test).* ***(D)*** *Same as in (C) but for the LTD protocol. No difference in the LFS-induced LTD was observed between CTRL (78 ± 6% of baseline, n = 7 CTRL, p = 0.003 vs baseline) and OPCRT slices (76 ± 5% of baseline, n = 6 OPCRT, p = 0.004 vs baseline) 48h following the execution of the behavioral tasks (Two-way ANOVA RM, p(group) = 0.85, df = 1, F = 0.04).*

Interestingly, no significant change either in HFS- or LFS-induced synaptic plasticity was observed in the superficial layers of the MEC 12 hours following the execution of the task, suggesting that OPCRT did not affect synaptic plasticity in MEC circuitry or that the changes were not detectable using an extracellular single-electrode electrophysiological technique (Fig 4 E, F).

Long-term plasticity has been hypothesized to be at the basis of learning and memory and to represent a physiological phenomenon to store information for long periods of time. Based on these results, it could be speculated that, if the plasticity effects observed following OPCRT played a role in retaining information about a previous episode, they should return to baseline when the memory was not behaviorally expressed anymore. To test this, the electrophysiological experiments were repeated 48 hours following the

execution of the task (Fig 5 A). At this time point, LEC slices showed normal synaptic transmission when compared to controls (Fig 5 B) and displayed levels of HFS-induced LTP and LFS-induced LTD comparable to that of CTRL (Fig 5 C, D), indicating that the episodic-like memory-induced changes in LEC synaptic transmission were indeed reversible.

The activation of LEC learning-tagged neurons is necessary for episodic-like memory recall

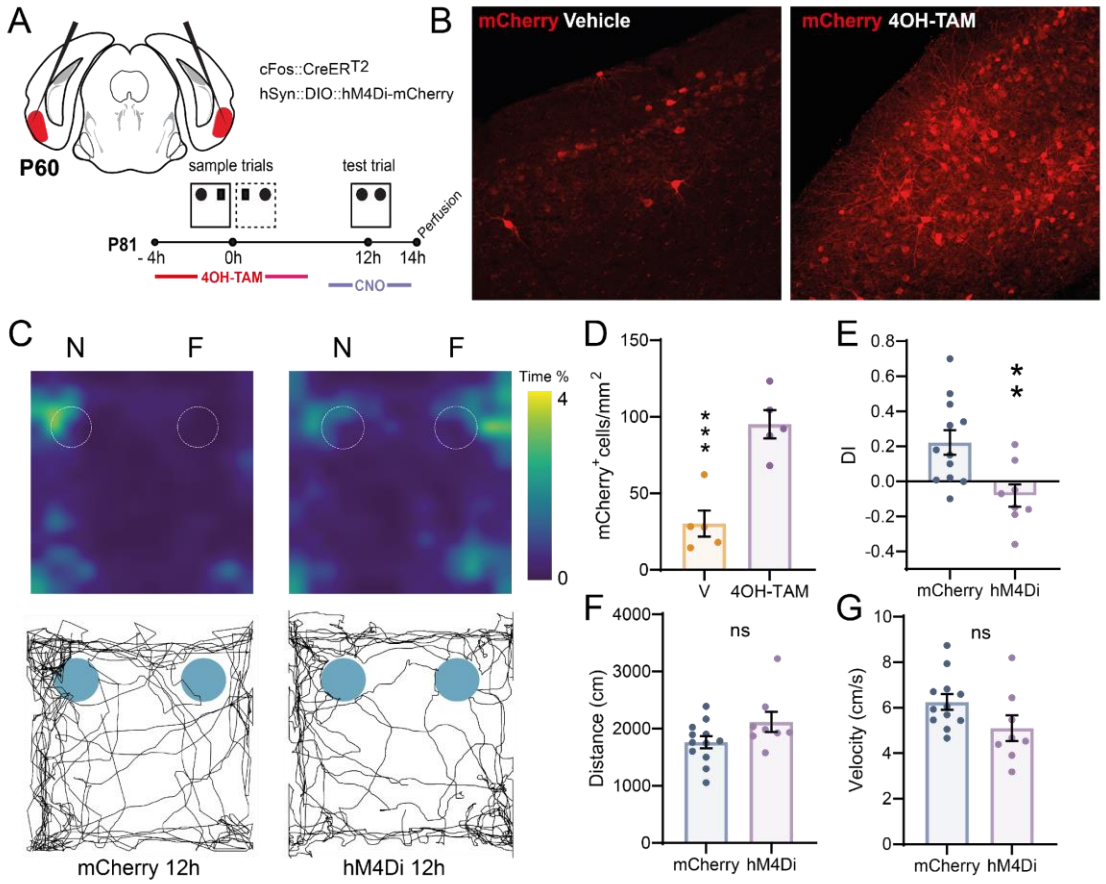

Figure 6. Chemogenetic inhibition of LEC learning-tagged neurons. (A) *Experimental timeline showing the injection of two AAV5 viral constructs, cFos::CreERT2 and hSyn::DIO::hMDDi-mCherry, at p60 and the OPCRT memory paradigm at p81. 4 hours before the execution of the sample trials mice received an i.p. injection of 4OH-TAM to induce the recombination and expression of the inhibitory chemogenetic receptor; 30 min before the execution of the test trial, they received an i.p. injection of CNO to induce the activation of the hM4Di receptor.* ***(B)*** *Representative images showing the expression of hM4Di-mCherry either in mice injected with the vehicle or mice injected with 4OH-TAM.* ***(C)*** *Heatmaps and tracking plots of the exploratory activity of mice (position of the nose) during the test trial of the OPCRT paradigm.* ***(D)*** *The density of hM4Di-expressing neurons was significantly higher in mice injected with 4OH-TAM(25mg/kg) compared to mice injected with the vehicle (95.15 ± 9 cells/mm2, n = 5 4OH-TAM vs 30.16 ± 8 cells/mm2 vehicle, p = 0.0008, df = 5, t = 5.2 two-tailed unpaired t-test).* ***(E)*** *The discrimination index (DI) of mCherry mice 12h following the presentation of the sample trials was significantly higher than chance (0.22 ± 0.07, n = 12, p =*

0.0087, df = 11, t = 3 one-sample t-test). In contrast, the DI of hM4Di mice did not differ from chance levels (-0.08 ± 0.06, n = 8, p = 0.24). Moreover, the two groups were significantly different from each other (mCherry 12h vs hM4Di, p = 0.0073, df = 18, t = 3 two-tailed unpaired t-test). ***(F)*** *No significant difference in the distance traveled during the test trial between groups (1761 ± 106 cm, n = 12 mCherry vs 2113 ± 175 cm, n = 8 hM4Di, p = 0.08, df = 18, t = 1.8 two-tailed unpaired t-test).* ***(G)*** *No significant difference in average velocity between groups during the test trial (6.26 ± 0.34 cm/s, n = 12 mCherry vs 5.1 ± 0.56 cm/s, n = 8 hM4Di, p = 0.08, two-tailed unpaired t-test). ** p <0.01, *** p <0.001.*

Based on previous results, it could be hypothesized that the LEC neurons may retain information from past experiences, which could be accessed during memory recall. To validate this hypothesis, a double virus system, previously developed (Matos et al. 2019) was used, based on the Targeted Recombination in Active Populations (TRAP) technology (Guenthner et al. 2013). This method allowed to target the LEC neurons recruited during the learning phase of the paradigm and manipulate their activity during memory recall. Specifically, an Adeno-Associated Virus (AAV) coding for the inducible Cre recombinase was injected under the control of the c-Fos promoter (AAV-Fos::CreER^{T2}) and Cre-dependent AAV containing the coding sequence of the inhibitory chemogenetic receptor hM4Di in an inverse open reading frame flanked by Cre recognition sites, bilaterally in the LEC of 2-month old male C57BL6 mice. Then, by administering 4OH-TAM i.p. 4h before the presentation of the OPCRT sample trials, d the expression of the chemogenetic receptor was induced in the learning-tagged neurons specifically in the LEC (Fig 6 A).

Initially, the ability of 4OH-TAM administration to induce the expression of the chemogenetic receptor was verified by analyzing the density of mCherry-expressing cells in mice that were injected with 4OH-TAM compared to those injected with the vehicle. Results indicated that mice injected with 4OH-TAM showed significantly higher mCherry expression levels 16h post-treatment compared to those that received the vehicle (Fig 6 B, D).

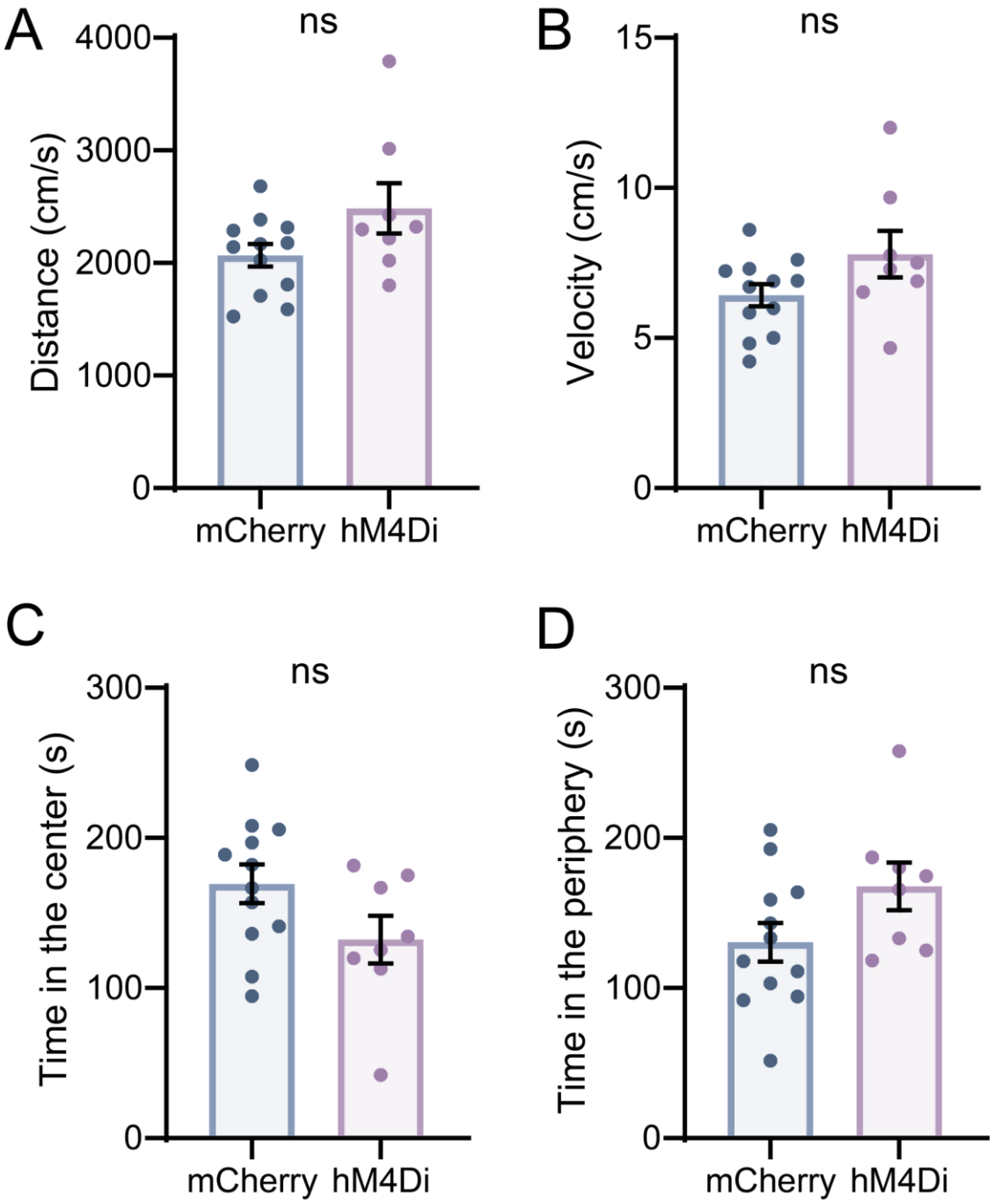

Figure 7. Exploratory activity during the open field test. ***(A)*** *The total distance traveled during the open field test was not different between mCherry and hM4Di mice (2068 ± 101 cm, n = 12 mCherry vs 2486 ± 223.7 cm, n = 8 hM4Di, p = 0.07, df = 18, t = 1.91 two-tailed unpaired t-test).* ***(B)*** *The average velocity during the open field test was not different between mCherry and hM4Di mice (6.43 ± 0.37 cm/s, n = 12 mCherry vs 7.79 ± 0.77, n = 8 hM4Di, p = 0.10, df = 18, t = 1.76 two-tailed unpaired t-test).* ***(C)*** *The time spent in the center of the arena during the open field test was similar between the mCherry and hM4Di groups (169.4 ± 12.87 s, n = 12 mCherry vs 132.3 ± 15.91, n = 8 hM4Di, p = 0.09, df = 18, t = 1.82 two-tailed unpaired*

t-test). ***(D)*** *The time spent in the periphery of the arena during the open field test was similar between the mCherry and hM4Di groups (130.6 ± 12.86 s, n = 12 mCherry vs 167.8 ± 15.91, n = 8 hM4Di, p = 0.09, df = 18, t = 1.82 two-tailed unpaired t-test).*

Next, it was investigated whether the activation of learning-tagged LEC neurons was crucial for achieving a successful memory recall. Notably, pharmacological inhibition of LEC neurons significantly impaired mice's ability to discriminate novel OPC associations in the hM4Di group 12 hours after the sample trials, whereas mice expressing the mCherry reporter alone displayed normal episodic-like memory (Fig 6 E, C).

To exclude the possibility that the observed behavioral effect was caused by differences in exploratory activity or anxiety levels, the average velocity and total distance traveled during the test trial by both hM4Di and mCherry mice were measured but no significant difference was found (Fig 6 C, F, G). As an additional control, an open field test was performed immediately before the execution of the OPCRT and demonstrated that both groups had comparable anxiety levels (Fig 7 A-D), supporting the hypothesis that the disruption in memory recall was the result of the inhibition of a specific neuronal ensemble in the LEC.

The activation of LEC learning-tagged neurons is not required for the expression of non-associative memory

An alternative interpretation of the chemogenetic results is that the reactivation of these neurons during memory recall could be essential for facilitating communication between different brain regions, or be involved in different mechanisms such as novelty detection, rather than be directly related to memory storage.

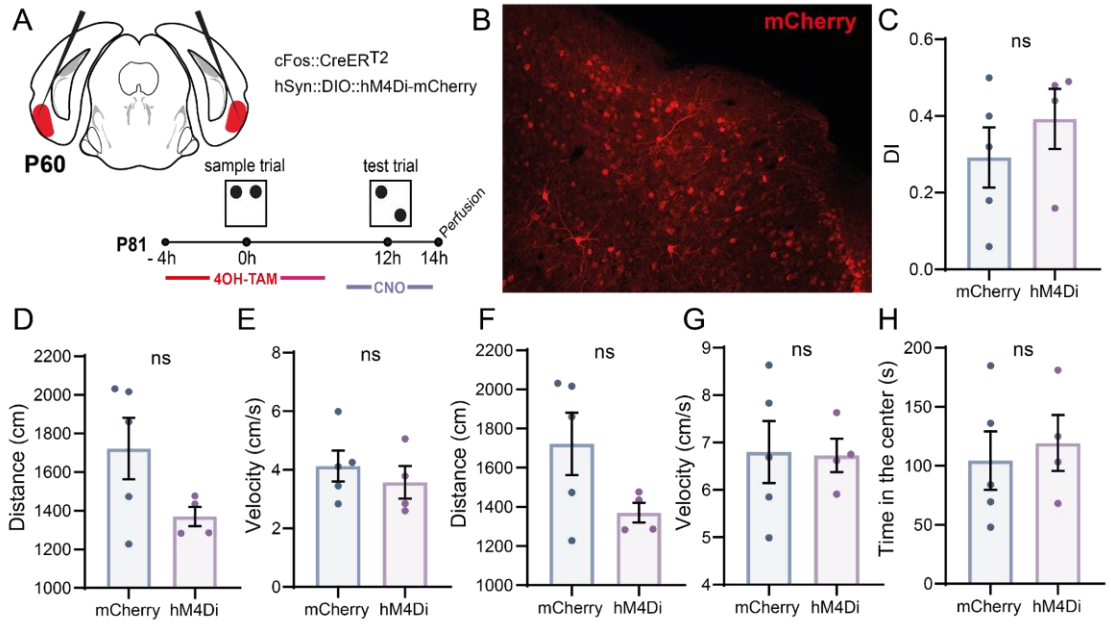

Figure 8. Inhibition of LEC learning-tagged neurons does not affect non-associative memory recall. (A) *Experimental timeline showing the injection of two AAV5 viral*

constructs, cFos::CreER^{T2} and hSyn::DIO::hMDDi-mCherry, at p60 and the novel location test (OLT) memory paradigm at p81. 4 hours before the execution of the sample trials mice received an i.p. injection of 4OH-TAM to induce the recombination and expression of the inhibitory chemogenetic receptor; 30 min before the execution of the test trial, they received an i.p. injection of CNO to induce the activation of the hM4Di receptor. ***(B)*** *Representative image showing the expression of hM4Di-mCherry in the LEC of mice after 4OH-TAM administration.* ***(C)*** *Mice in the mCherry (0.29 ± 0.08, n = 5 vs chance level, p = 0.02, df = 4, t = 3.74 one-sample t-test) and hM4Di (0.39 ± 0.08, n = 4 vs chance level, p = 0.015, df = 3, t = 5 one-sample t-test) showed significant memory for the familiar location. No difference was observed between the two experimental groups (0.29 ± 0.08, n = 5 mCherry vs 0.39 ± 0.08, n = 4 hM4Di, p = 0.40, df = 7, t = 0.90 two-tailed unpaired t-test).* ***(D)*** *Mice in the mCherry and hM4Di groups did not differ in the distance traveled during the test trial (1722 ± 159 cm, n = 5 mCherry vs 1371 ± 50 cm, n = 4 hM4Di, p = 0.10, df = 7, t = 1.89 two-tailed unpaired t-test).* ***(E)*** *No difference in the animal velocity during the test trial (4.13 ± 0.53 cm/s, n = 5 mCherry vs 3.58 ± 0.58 cm/s, n = 4 hM4Di, p = 0.50, df = 7, t = 0.72 two-tailed unpaired t-test).* ***(F)*** *Mice showed similar distance traveled during the open field test (2151 ± 208 cm, n = 5 mCherry vs 2125 ± 110 cm, n = 4 hM4Di, p = 0.92, df = 7, t = 0.10 two-tailed unpaired t-test).* ***(G)*** *No difference was observed in the animal velocity during the open field test (6.80 ± 0.66 cm/s, n = 5 mCherry vs 6.73 ± 0.35 cm/s, n = 4 hM4Di, p = 0.93, df = 7, t = 0.09 two-tailed unpaired t-test).* ***(H)*** *Mice showed no significant difference in the time spent in the center of the arena during the open field test (104.4 ± 24.8 s, n = 5 mCherry vs 119.3 ± 23.7 s, n = 4 hM4Di, p = 0.68, df = 7, t = 0.43 two-tailed unpaired t-test).*

In order to determine whether the impairment of episodic-like memory recall caused by the inhibition of LEC neurons was due to impaired detection of novelty in individual components of the task, a follow-up experiment was conducted using a non-associative task called the novel location test (NLT, Fig 8 A, B). This task did not require the learning of an association between stimuli, but rather, the novelty was represented by a different location within a familiar context. Interestingly, chemogenetic inhibition of learning-tagged LEC neurons did not affect the mice's ability to recognize the novel location compared to the familiar one, and no significant difference was observed between the hM4Di and mCherry groups (Fig 8 C).

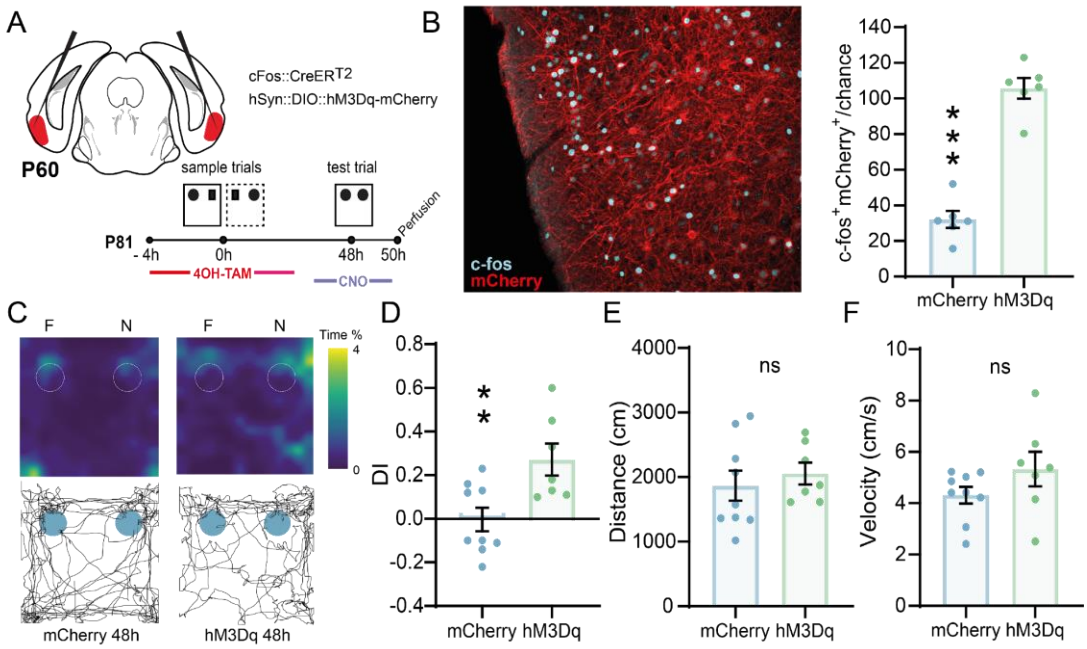

Figure 9. The activation of LEC neurons facilitates episodic-like memory recall. (A) *Experimental timeline showing the injection of two AAV5 viral constructs, cFos::CreER^{T2} and hSyn::DIO::hMDDi-mCherry, at p60 and the OPCRT memory paradigm at p81. 4 hours before the execution of the sample trials mice received an i.p. injection of 4OH-TAM to induce the recombination and expression of the inhibitory chemogenetic receptor; 30 min before the execution of the test trial, they received an i.p. injection of CNO to induce the activation of the hM3Dq receptor.* ***(B)*** *The overlap of mCherry and c-Fos expressing neurons over chance levels was significantly higher in the hM3Dq group compared to the mCherry one (31.98 ± 4.8 c-Fos+mCherry+/chance levels, n = 6 mCherry vs 105.7 ± 5.8 c-Fos+mCherry+/chance levels, n = 6 hM3Dq, p <0.001, df = 10, t = 9.9 two-tailed unpaired t-test).* ***(C)*** *The figures present heatmaps and tracking plots that depict the exploratory activity of mice during the test trial of the OPCRT paradigm. The tracking plots illustrate the trajectory of the mouse's nose.* ***(D)*** *Mice expressing the control reporter mCherry did not remember the previously acquired OPC association 48h following learning (-0.003 ± 0.05, n = 9 vs chance level, p = 0.95, df = 8, t = 0.06), while hM3Dq mice showed a strong preference for the novel association (0.27 ± 0.07, n = 7 vs chance level, p = 0.01, df = 6, t = 3.7 one-sample t-test). Indeed, the DI of mCherry mice was significantly lower than the DI of hM3Dq animals (-0.003 ± 0.05, n = 9 mCherry vs 0.27 ± 0.07, n = 7, p = 0.008, df = 14, t = 3.1 two-tailed unpaired t-test).* ***(E)*** *The total distance traveled during the test trial was not different between the mCherry and hM3Dq groups (1866 ± 232.1 cm, n = 9 mCherry vs 2054 ± 169.2 cm, n = 7 hM3Dq, p = 0.54, df = 14, t = 0.62 two-tailed unpaired t-test).* ***(F)*** *The average velocity was comparable among groups (4.32 ± 0.32 cm/s, n = 9 mCherry vs 5.33 ± 0.68 cm/s, n = 7 hM3Dq, p = 0.17, df = 14, t = 1.5 two-tailed unpaired t-test). ** p <0.01, *** p <0.001.*

The exploratory behavior during the test trial was similar between the experimental groups (Fig 8 D, E) and mice showed comparable anxiety levels during an open field test

(Fig 8 F-H), indicating that the observed differences in episodic-like memory recall were not due to different exploration of the environment or anxiety issues.

Together these results suggest that the activation of learning-tagged LEC neurons is specifically required for the recall of associative episodic-like memory and not for non-associative behavioral tasks, indeed not affecting mice's ability to discriminate novelty or remember the individual non-associative components of the behavior, such as the memory for spatial location.

The activation of LEC learning-tagged neurons facilitates episodic-like memory recall

To investigate whether activation of the LEC learning-tagged neurons was also sufficient to facilitate memory recall, excitatory chemogenetic manipulations of the neurons were conducted using the excitatory chemogenetic receptor hM3Dq receptor in place of the hM4Di receptor.

Prior to sample trials, mice were injected with 4OH-TAM to tag the LEC neurons expressing c-Fos during learning, and then these same neurons were artificially reactivated 48 hours later during the test trial by administering CNO (Fig 9 A). To verify the effectiveness of hM3Dq receptors in activating LEC neurons, we conducted immunofluorescence against c-Fos 2 hours following the manipulation of neurons on a subset of experimental animals. The results showed a significant increase in the overlap of mCherry+c-Fos+ in hM3Dq mice compared to mCherry mice, indicating efficient activation of LEC-tagged neurons (Fig 9 B). However, it is noteworthy that in the mCherry control group, the overlap was also higher than chance, which implies that even in the absence of chemogenetic manipulation, a subset of the original neuronal ensemble was still reactivated when the animal encountered the familiar episode again.

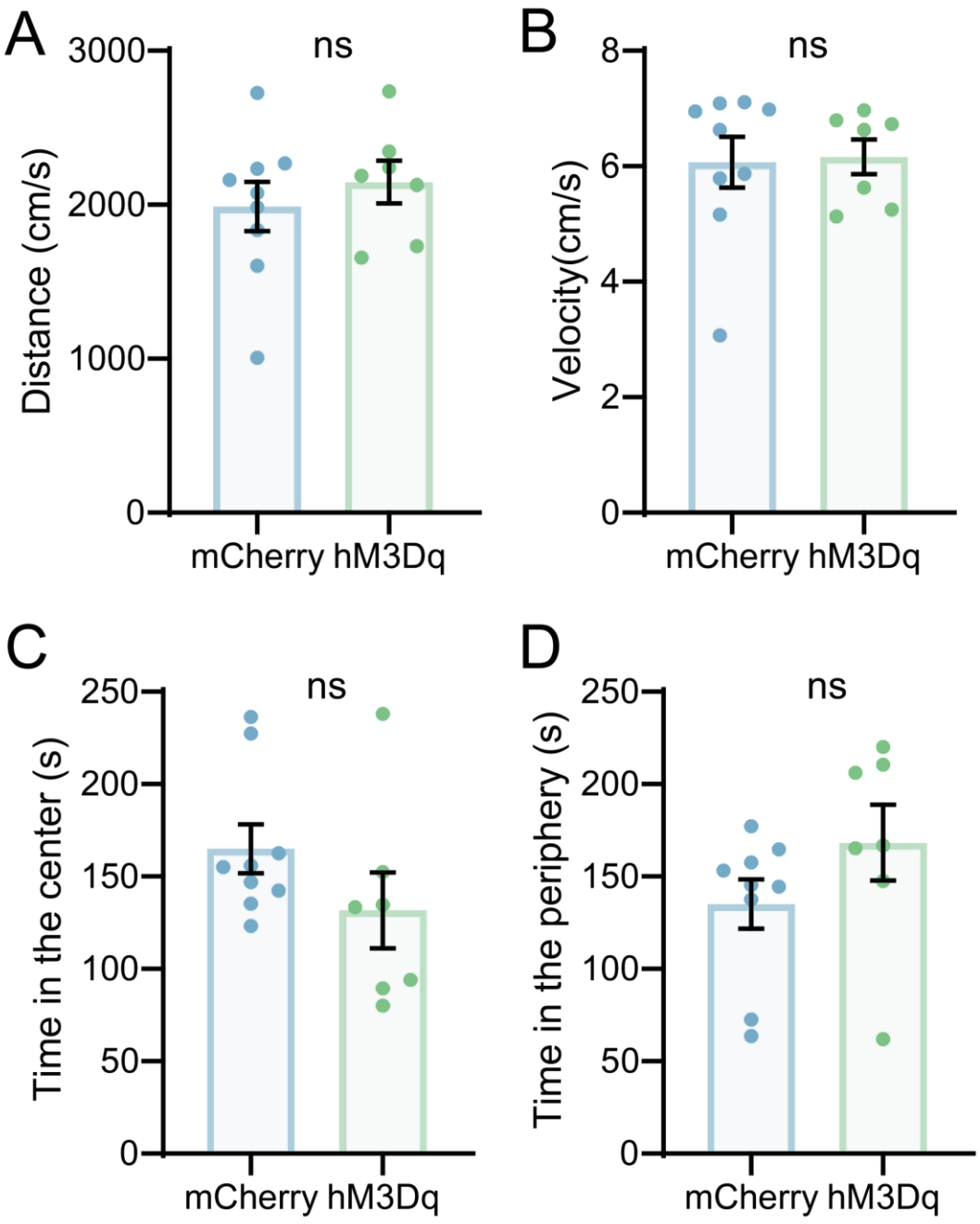

Figure 10. Locomotor activity during the open field test. (A) *The total distance traveled during the open field was not significantly different among the experimental groups (1988 ± 160.5 cm, n = 9 mCherry vs 2147 ± 139 cm, n = 7 hM3Dq, p = 0.48, df = 14, t = 0.072 two-tailed unpaired t-test).* ***(B)*** *The average velocity during the open field test was not significantly different among the experimental groups (6.07 ± 0.44 cm/s, n = 9 mCherry vs 6.16 ± 0.3, n = 7 hM3Dq, p = 0.88, df = 14, t = 0.16 two-tailed unpaired t-test).* ***(C)*** *The time spent in the center of the arena during the open field test was not significantly different among the experimental groups (165 ± 13.2 s, n = 9 mCherry vs 131.7 ± 20.5 s, n = 7 hM3Dq, p = 0.18, df = 14, t = 1.42 two-tailed unpaired t-test).* ***(D)*** *The time spent in the periphery of the arena during the open*

field test was not significantly different among groups (135.1 ± 13.3 s, n = 9 mCherry vs 168.3 ± 20.5 s, n = 7 hM3Dq, p = 0.18, df = 14, t = 1.42 two-tailed unpaired t-test).

At the behavioral level, control mice injected with mCherry did not exhibit episodic-like memory 48 hours after the execution of sample trials, consistent with the behavioral results observed in the time course experiment suggesting the decline of memory recall within 24 hours from the learning phase. However, at the same time point, hM3Dq mice showed a strong preference for the novel OPC association (Fig 9 C, D), and the differences observed in control mice were not due to a different exploratory behavior because mCherry and hM3Dq mice showed comparable velocity and traveled distance during the test trial (Fig 9 C, E, F). Furthermore, mice displayed similar anxiety levels, as well (Fig 10 A-D). Therefore, this evidence indicates that the stimulation of LEC learning-tagged neurons alone is not only necessary but also sufficient to facilitate memory recall, thus strengthening the hypothesis that key information about previous episodes might be retained in LEC neuronal ensembles and that their reactivation might be necessary to access the stored information.

LEC learning-tagged neurons are reactivated by natural recall cues

If the activation of a specific neuronal ensemble is necessary and sufficient for memory recall, as for the definition of a neuronal engram, the same neuronal ensemble should be more likely to be reactivated when the animal expresses the memory behaviorally compared to those situations where the animal fails to retrieve the previous experience.

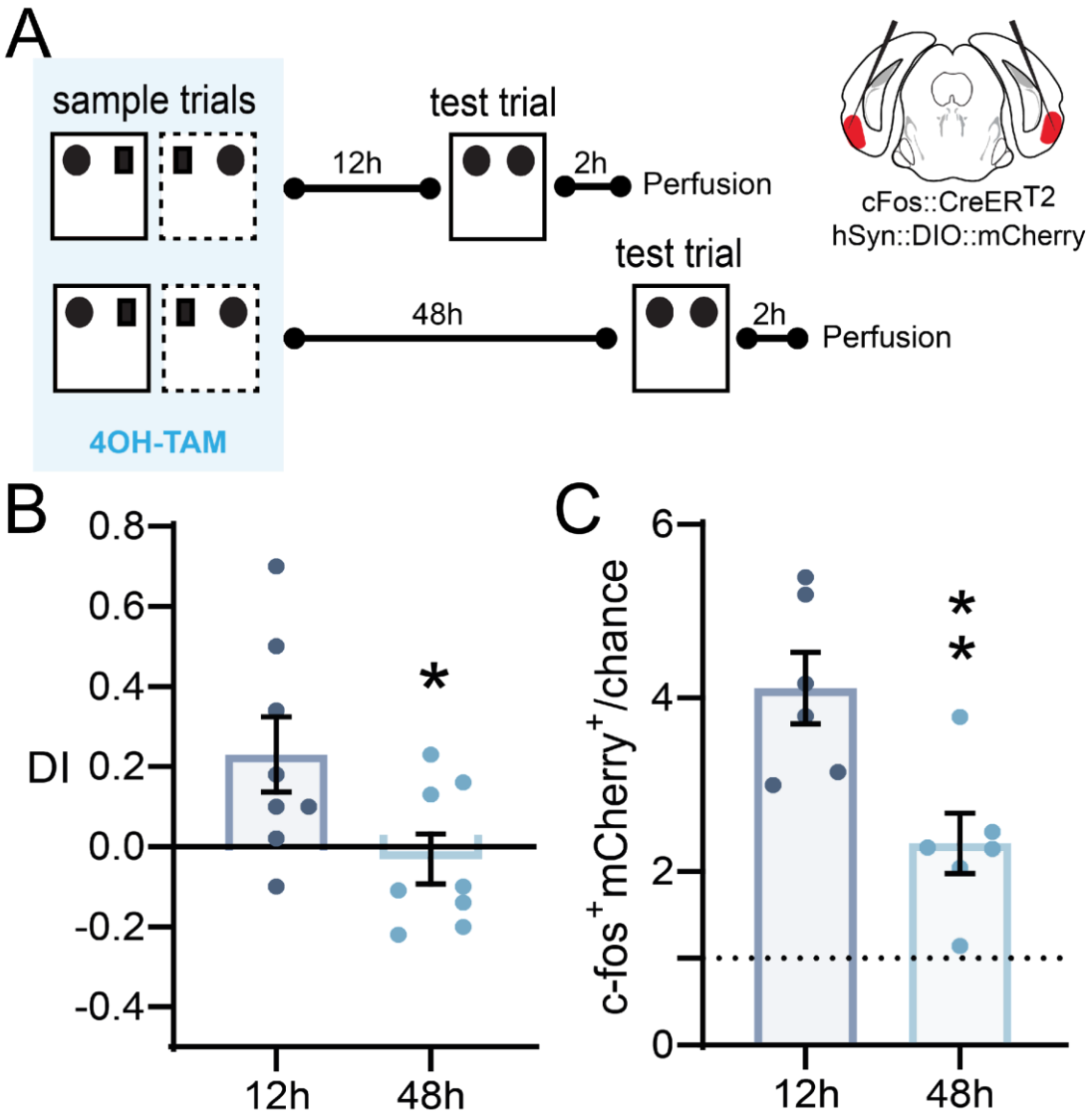

Figure 11. Reactivation of learning-tagged neurons by natural recall cues. (A) *Timeline of the experimental design.* ***(B)*** *Mice expressing the reporter mCherry were able to recall the previously acquired episodic-like memory when the test trial was presented 12h following learning (0.23 ± 0.10, n = 8, p = 0.04, df = 7, t = 2.4 one-sample t-test) but not when the test trial was performed 48h after learning (-0.03 ± 0.06, n = 8, p = 0.63, df = 7, t = 0.5 one-sample t-test). The discrimination indices of the two groups were significantly different (mCherry 12h vs mCherry 48h, p = 0.04, df = 14, t = 2-3 two-tailed unpaired t-test).* ***(C)*** *The reactivation of the LEC learning-tagged neurons, measured as the percetage of cfos+mCherry+ overlap over chance levels, was higher in mCherry mice exposed to the test trial at 12h with respect to mice exposed to the test trial at 48h (4.11 ± 0.41, n = 6 mCherry 12h vs 2.33 ± 0.35, n = 6 mCherry 48h, p = 0.008, df = 10, t = 3.3 two-tailed unpaired t-test). However, both at 12h and 48h, the reactivation was significantly different from chance levels (4.11 ± 0.41, n = 6 mCherry 12h vs chance, p = 0.0006; 2.33 ± 0.35, n = 6 mCherry 48h, p = 0.013 one-sample t-test).*

To investigate whether this was true for LEC neurons, we measured the overlap between mCherry and c-Fos expression in control mice injected with Cre-dependent mCherry reporter and subjected to memory recall either 12h (mCherry 12h group) or 48h (mCherry 48h group) after the sample trials presentation (Fig 11 A). Interestingly, we found that mCherry 12h mice significantly discriminated OPC associations, while mCherry 48h mice failed to do so, indicating that the former group achieved successful recall, whereas the latter group did not (Fig 11 B).

Interestingly, the immunofluorescence analysis revealed that the overlap between mCherry and c-Fos was significantly higher in the mCherry 12h group compared to the mCherry 48h, supporting the hypothesis that the reactivation of LEC neurons induced by natural recall cues may represent the physiological way through which successful recall is achieved (Fig 11 C).

Collectively, these findings corroborate the association between LEC neuronal ensemble activation and episodic-like memory recall, thus reinforcing the proposal that essential information regarding past experiences is encoded within this network and is utilized to accomplish effective memory recall.

Discussion

The entorhinal cortex (EC) serves as a crucial link between the hippocampus and multisensory cortical areas. It consists of two subdivisions, the lateral entorhinal cortex (LEC) and medial entorhinal cortex (MEC), which have been traditionally associated with conveying contextual and spatial information, respectively, to the hippocampus for the integration of memories (Fyhn et al. 2004; Hafting et al. 2005; Hargreaves et al. 2005). However, emerging evidence suggests that this conceptualization of parallel information streams in the EC may be oversimplified. Indeed, the EC itself may play a direct role in integrating different types of information into a cohesive experience even before they reach the hippocampus. Several experiments have emphasized the involvement of the LEC in episodic-like memory tasks such as object-place-context recognition (D. I. G. Wilson et al. 2013; D. I. Wilson et al. 2013; Criscuolo et al. 2017) and odor-context associations (Persson et al. 2022), where different pieces of information need to be combined. Nonetheless, there is still no conclusive evidence regarding the existence of entorhinal engrams, leaving this question partially unanswered.

The LEC is recruited during OPCRT and context exploration in mice

The aim of this study was to investigate the role of the entorhinal cortex (EC), particularly its lateral subdivision (LEC), in the OPCRT memory test, which is commonly used to study episodic-like memory in rodents (Vandrey et al. 2020; Eacott and Norman 2004; Eacott, Easton, and Zinkivskay 2005; Easton, Zinkivskay, and Eacott 2009). To assess the selective involvement of the entorhinal cortex in the OPCRT task, the product of the immediate early gene (IEG) c-fos was utilized as an activity-dependent marker. The c-fos marker has been extensively used in previous research to identify cell populations that are recruited during

specific tasks and may undergo synaptic plasticity as a result of the experience (Guzowski et al. 1999; Barth, Gerkin, and Dean 2004; Smeyne et al. 1992; Wang et al. 2006).

In this study, mice subjected to the OPCRT task exhibited a higher number of c-fos-expressing cells in the LEC compared to mice that remained in their home cages. Notably, the difference between the two experimental groups was primarily observed in the number of c-fos-expressing cells rather than the individual cells' level of c-fos protein fluorescence intensity. However, it should be noted that the relationship between fluorescence intensity and protein quantity in immunofluorescence staining is complex, making it difficult to rule out the possibility of differences in protein expression at the single-cell level, which may have been beyond the resolution of the technique used.

Importantly, the increase in c-fos cell density was specific to the LEC, as no significant increase in c-fos expression was observed in either the medial entorhinal cortex (MEC) or the primary visual cortex (V1), which served as a control region. These findings highlight the selective recruitment of the LEC during the OPCRT task. The c-fos IEG has been widely employed to identify neuronal engrams, which are neuronal ensembles that become activated and undergo enduring changes in response to a specific experience or memory formation process, effectively serving as a memory trace. Therefore, one possible interpretation of these results is that the selective increase in c-fos within a LEC neuronal ensemble after the OPCRT task may indicate the presence of an episodic-like memory neuronal engram that encodes information about the experience over time.

Alternatively, the selective recruitment of the LEC observed in this study may reflect its role in processing local cues within the environment during the ongoing experience, without directly implicating it in memory processing. Previous research by Kuruvilla et al. (Kuruvilla and Ainge 2017) demonstrated that the LEC is responsible for processing local features, whereas the MEC is involved in processing global features of the environment. In the current study, the behavioral apparatus allowed animals to experience local cues such as visual patterns on the arena walls, objects, boundaries, and corners of the arena. However, global cues were absent because the arena was isolated from the surrounding room using a divider placed on top of the arena. Therefore, the specific recruitment of the LEC, rather than the MEC, may be attributed to the presence of local cues and the absence of global cues in the experimental setup.

According to the findings, when animals were exposed solely to different contexts without objects, thereby preventing the formation of object-place-context associations, the observed pattern of c-fos fluorescent staining in the LEC was remarkably similar to that seen in the OPCRT group. This similarity confirms the crucial role of processing contextual features during the ongoing experience in the recruitment of the LEC, even in the absence of object-place-context associations.

However, it is important to note that the processing of contextual information during the ongoing experience and the memory storage hypothesis are not mutually exclusive. It is plausible that the same LEC neurons recruited by the local features of the environment during the experience may also be involved in acquiring and storing that information for longer periods. One key characteristic of episodic-like memory is the automatic association

of an event's memory with the context in which it occurs. Remarkably, LEC neurons have been shown to be crucial in contextualizing items, such as odors or objects, and are significantly modulated by specific combinations of items, places, and contexts during the ongoing experience (D. I. G. Wilson et al. 2013; D. I. Wilson et al. 2013; Vandrey et al. 2020; Burwell 2000; Hargreaves et al. 2005; Persson et al. 2022). This associative capability has also been observed in the hippocampus, a region where the existence of episodic-like memory engrams has been extensively demonstrated. Thus, the LEC emerges as an ideal candidate region not only for the processing of episodes but also for the immediate encoding and storage of new episodic memories. However, in order to conclusively demonstrate this dual role, direct manipulation of the identified neuronal ensembles becomes crucial.

In summary, these results provide evidence for the significant recruitment of the LEC, but not the MEC, during the OPCRT memory test and support the involvement of the LEC in episodic-like memory processing, without excluding the possibility that the selective recruitment may be attributed to the processing of local cues in the environment during the ongoing experience.

The processing of a new episodic-like memory induces reversible long-term changes in LEC synaptic plasticity

The mechanisms underlying the persistent storage and retrieval of memories at the neurobiological level are still not fully understood. However, it is widely believed that strengthened synaptic connectivity between neurons involved in memory formation plays a crucial role in facilitating the reactivation of a group of neurons, known as engram cells, during memory recall (McKernan and Shinnick-Gallagher 1997; Rogan, Stäubli, and LeDoux 1997; Tye et al. 2008; Nabavi et al. 2014). To explore whether the processing of an episodic-like memory could induce long-lasting changes in the entorhinal cortex (EC) circuitry, field excitatory post-synaptic potential (fEPSP) recordings were performed in slices containing the EC obtained from mice subjected to the object-place-context-response-task (OPCRT) paradigm.

The results revealed a significant reduction in long-term potentiation (LTP) in the superficial layers of the lateral entorhinal cortex (LEC) 12 hours after the execution of the OPCRT task. This decrease in LTP was observed only in the OPCRT group and not in the control animals that remained in their home cages (CTRL) or were exposed to the context-only condition (CNTX), where the contextual sequence was experienced without objects. Additionally, the OPCRT group showed an increase in basal synaptic transmission compared to the control and CNTX groups. These findings suggest that the processing of an episodic-like memory may potentiate excitatory synaptic transmission within the LEC circuitry, resulting in long-lasting modifications.

In contrast, the same experimental condition (OPCRT) facilitated long-term depression (LTD), which represents the weakening of synaptic connections, in the LEC compared to the control and CNTX groups at the 12-hour time point. Taken together, these results suggest that the acquisition of a new episodic-like memory can change the treshold for synaptic modification in the LEC circuit, at least within the superficial layers. This leads to

an occlusion-like phenomenon, where LTP is diminished, while LTD is enhanced. Importantly, these effects appear to be specific to the LEC, as there were no significant changes in synaptic plasticity or basal transmission observed in the medial entorhinal cortex (MEC) at the same time point.

These findings resemble previous studies conducted in the lateral amygdala (Hong et al. 2011), which demonstrated that the acquisition of fear conditioning resulted in reversible occlusion of LTP and enhanced LTD in specific neuronal pathways. Similar observations were also made in the cortico-amygdala pathway following the same memory task (Tsvetkov et al. 2002). Moreover, the extinction of fear memory was found to reverse these changes, restoring LTP and occluding LTD, indicating that both memory acquisition and extinction can reversibly modify the upper and lower limits of a fixed modification range (Hong et al. 2011).

To further investigate the phenomenon observed in the lateral entorhinal cortex (LEC), electrophysiological recordings were conducted in brain slices obtained 48 hours after the execution of the memory paradigm (OPCRT), a time point when the animals were no longer able to successfully express the memory for familiar object-place-context associations. Surprisingly, at this time point, long-term potentiation (LTP) induced by electrical stimulation was effectively induced in LEC slices, and no significant changes were observed in basal synaptic transmission or long-term depression (LTD) expression compared to control slices. These findings indicate that the synaptic plasticity changes induced by the acquisition of an episodic-like memory in the LEC are reversible and follow a time course similar to that of memory expression.

One possible explanation for these results is that enhanced synaptic transmission within the LEC circuitry may be crucial for the reactivation of the engram ensemble in the presence of natural recall cues, allowing for successful memory recall. However, when these plasticity changes return to baseline, the natural recall cues may not be as effective in reactivating the memory trace, resulting in the loss of episodic-like memory expression at the behavioral level. It is plausible to hypothesize that long-lasting forms of plasticity serve as a mechanism for recruiting the engram, considering that this episodic-like memory has a relatively short duration of a maximum of 12 hours.

It is worth noting that the observed changes in synaptic efficacy might not be detectable through single-electrode field recordings if the augmentation of synaptic plasticity occurs only between engram neurons, which are believed to constitute a sparse population. Although c-fos staining in LEC slices immediately after OPCRT showed that c-fos positive neurons represent only a small fraction of the total cell population, it is important to highlight that a significant reduction in LTP was not observed in all recorded LEC slices during the electrophysiological experiments. Instead, the probability of observing a reduction in LTP was significantly increased in OPCRT slices compared to control or context-only (CNTX) slices, resulting in an overall decrease in total LTP in the OPCRT group. Therefore, the reduction in LTP should not be interpreted as evidence of complete saturation of excitatory synaptic transmission in the LEC after OPCRT but rather as evidence of increased functional connections among a considerable number of neurons compared to other control conditions.

Additionally, the experimental protocol used in this study resembled an occlusion-like protocol rather than a complete occlusion experiment. Therefore, even slices that seemingly exhibited complete occlusion of LTP could still be capable of increasing their synaptic transmission in response to additional high-frequency stimulation (HFS). Moreover, the setting parameter for the measurement of c-fos through immunostaining techniques could potentially lead to the loss of sub-threshold changes in other cellular populations. These subthreshold changes could instead be detected at the electrophysiological level and have an impact on the network. Furthermore, emerging evidence suggests that c-fos is not the only immediate early gene expressed by engrams during the acquisition and recall of new memories. Different engram populations may exist, characterized by the expression of different immediate early genes and potentially serving different roles in memory processing. Therefore, attributing the observed long-term changes in synaptic plasticity solely to the c-fos expressing population would be simplistic, as they likely result from complex interactions between different neuronal engram populations characterized by different immediate early gene expressions.

In summary, the findings of the study indicate that memory acquisition induces changes in synaptic plasticity, including a decrease in LTP and an increase in LTD, in the lateral entorhinal cortex (LEC). These changes are specific to the LEC and can be reversed, as shown in subsequent experiments. The results suggest that heightened synaptic transmission within the LEC circuitry may play a crucial role in memory reactivation during recall, as the time course of these long-lasting plasticity changes closely aligns with that of memory expression. However, it is important to note that the presence of such plasticity changes following memory tasks does not directly establish their role in memory storage and recall, as they could potentially serve other functions. Therefore, further comprehensive investigation is necessary to definitively establish the involvement of the LEC in memory recall.

The activation of LEC learning-tagged neurons is necessary and sufficient for the recall of episodic-like memories but not for non-associative memories

To establish a neuronal ensemble as an engram, it is crucial to demonstrate that activating that specific ensemble is both necessary and sufficient for memory recall (Ramirez et al. 2013). If these neurons indeed store information through their activity and connections, inactivating them should render the information inaccessible, while artificially stimulating them should evoke the associated memory even without natural cues. The findings of this study confirm this hypothesis. Temporary inactivation of the LEC neuronal population involved in learning an episodic-like memory impaired memory recall during the test phase. In contrast, control animals exhibited discrimination between novel and familiar associations.

Moreover, when the same neuronal ensemble was artificially reactivated at a time when control animals no longer displayed behavioral expression of the memory, complete recall of the previously acquired memory was achieved. This evidence confirms that critical information about past experiences is stored within the LEC network and is necessary and sufficient for memory retrieval. The same neuronal ensemble is also naturally reactivated in response to recall cues. Comparing the reactivation of learning-tagged neurons during

the test trial between mice capable of remembering associations and those that were not, a significant increase in ensemble reactivation was observed in the former group. Notably, reactivation levels exceeded chance levels, indicating that a substantial portion of the engram was reactivated even when the memory was not behaviorally expressed. However, this level of reactivation may not be sufficient for successful memory recall.

These results provide direct and clear evidence of entorhinal engrams for episodic memory and challenge the traditional view of the EC as solely relaying information between the hippocampus and neocortex. The conventional perspective suggests that the EC transmits non-spatial and spatial information to the hippocampus, where it is integrated and stored as a unified memory. However, the evidence that the integration of information about events already occurs within the LEC goes beyond this theory. The findings support this notion, as inhibiting LEC learning-tagged neurons impaired recall of an episodic-like memory without affecting the recognition of individual components. This suggests that the integration of sensory information related to new experiences occurs in the LEC before reaching the hippocampus and that the memory impairment is not due to a general inability to recognize the novelty of single components.

The LEC is an ideal structure for storing episodic-like memories as it encodes all essential components within its circuitry. It is known to be modulated by objects, and odors, and can integrate the "what" and "where" aspects of an experience. Recent studies have also revealed its ability to encode episodic time (Tsao et al. 2018), with a distinct temporal signal that encodes time across different scales and environmental contexts.

Significantly, the encoding of episodic time in the LEC displays distinct characteristics depending on the nature of the experience. During novel and open-ended experiences, around 20% of LEC neurons exhibit a gradual change in firing rate over time, enabling them to distinguish between successive visits to the same location. Similar findings have been observed in primates (Bright et al. 2020). In contrast, during familiar and structured experiences, LEC neuronal activity becomes stable and no longer fluctuates across repeated laps. These findings are noteworthy as they confirm the LEC's capability to encode the temporal aspect of an experience. Consequently, the LEC encompasses all three components of episodic-like memory, rendering it an ideal candidate for encoding and storing such memories (Tsao et al. 2018).

Furthermore, it is essential to acknowledge that chemogenetic and optogenetic techniques have limitations in replicating the natural patterns of neural activity during experiences and recall, as the inactivation and activation they induce are artificial. This raises intriguing questions about how artificial activation can mimic the natural process of memory recall. While these technologies have proven effective in various scenarios, considering our findings from the perspective of encoding episodic time adds an additional dimension to our understanding of the mechanisms underlying memory recall.

In familiar experiences, Tsao et al. (Tsao et al. 2018) have presented evidence suggesting that the LEC exhibits stable neural activity, potentially resulting from continuous excitatory inputs to specific groups of neurons in the hippocampus or neocortex. This mechanism is believed to assist in reinstating neural activity patterns associated with a particular

experience. From this viewpoint, it is conceivable that chemogenetic activation of learning-tagged neuronal ensembles may act as a stabilizer for LEC neuronal activity. By inducing stable and coherent firing of the neurons for a specific duration, this activation could evoke a sense of familiarity associated with the previously encountered combination of items, even if the exact activity patterns from the original experience are not precisely replicated in the engram ensemble.

Conversely, inhibiting the same neuronal ensemble through chemogenetic methods could disrupt the stable neuronal activity that would typically be present when the memory is behaviorally expressed in control animals. Considering the relatively brief time frame for the expression of the receptor on the cell membrane, it is plausible to consider that the number of receptors expressed at the membrane level during the memory test could vary significantly among individual cells. This variability may not reach the required levels to completely suppress synaptic transmission in certain neurons, leading to unstable activity patterns in the learning-tagged neurons that deviate from the expected patterns. Consequently, the familiar experience associated with that specific neuronal ensemble could be perceived as new due to these altered and unstable activity patterns, impairing object-place-context discrimination.

It is important to note that using the human synapsin promoter for viral expression in our study did not allow differentiation between the various cell types within the LEC that contribute to the engram ensemble. Further investigations are necessary to characterize these cell types and the intrinsic circuits within the LEC involved in this phenomenon. However, existing evidence suggests that a specific subpopulation of superficial layer cells in the LEC, known as fan cells, plays a crucial role in the acquisition of episodic-like memory (Vandrey et al. 2020). Therefore, it is reasonable to speculate that fan cells should be part of this engram ensemble, although scientific evidence is still needed. Nevertheless, the precise composition of the engram remains unknown. Exploring how different functional populations of LEC cells converge and coordinate to define an episode would be an intriguing direction for future research.

Conclusions

In conclusion, this work sheds light on the significance of the lateral entorhinal cortex (LEC) in the processing of episodic-like memories and suggests its potential involvement as a component of the episodic engram network. The selective recruitment of the lateral entorhinal cortex (LEC) during the object-place-context recognition test (OPCRT) indicates its involvement in the acquisition phase of memory and its role in processing local cues within the environment during an ongoing experience.

The LEC's involvement in the initial acquisition of episodic-like memories within the OPCRT paradigm indicates its contribution to integrating various sensory cues, contextual details, and, potentially, even temporal information. This integration process is fundamental for creating coherent and meaningful memory representations. The activation of the LEC during the OPCRT supports the idea that it is specifically engaged in the early stages of memory encoding, where the binding of different elements of an experience occurs.

In addition, this study provides compelling evidence that the acquisition of episodic-like memories triggers reversible long-term changes in synaptic plasticity specifically within the LEC circuitry. Indeed, a significant decrease in long-term potentiation (LTP) and an increase in long-term depression (LTD) was observed in the LEC 12 hours after memory acquisition, a time point when the animals demonstrated the ability to discriminate between novel and familiar object-place-context associations.

The specificity of these synaptic plasticity changes to the LEC, as opposed to the MEC, the other subdivision of the entorhinal cortex, highlights the unique role of the LEC in memory expression suggesting that the LEC is capable of undergoing dynamic adaptations in response to memory-related information. This underscores the plasticity of the LEC network and suggests its ability to flexibly encode and store new memories, even after a single learning experience. Importantly, the reversible nature of these synaptic changes further strengthens their association with memory expression, as they can be reversed over time.

Finally, the activation of LEC learning-tagged neurons was shown to be necessary and sufficient for the recall of episodic-like memories. When the specific neuronal ensemble that was engaged during memory learning was inactivated, it resulted in impaired memory recall. This suggests that the activation of these specific entorhinal neuronal ensembles is essential for accessing and retrieving the stored memory information. The findings further support the notion that critical details of past experiences are encoded and stored within the LEC network.

Interestingly, the study also demonstrated that artificial reactivation of the same ensemble of engram cells could evoke the associated memory, even in the absence of natural recall cues. This suggests that the activation of specific engram cells within the LEC is not only necessary but also sufficient for memory retrieval. It highlights the direct link between the activation of these engram cells and the recall of episodic-like memories.

In conclusion, while the findings presented in this study provide valuable insights into the involvement of the LEC in episodic-like memory processing and recall, there is still much to learn about the complex mechanisms and functions of this brain region. Further research is necessary to delve deeper into the specific processes and interactions within the LEC that contribute to memory storage and retrieval. It would be beneficial to explore the roles of other immediate early genes and engram populations in order to obtain a more comprehensive understanding of memory formation and recall processes. Investigating additional molecular and cellular markers associated with memory encoding and retrieval could shed light on the broader network dynamics and mechanisms at play.

Nonetheless, the current findings significantly contribute to our understanding of the role of the LEC in episodic-like memory and synaptic plasticity. They provide important evidence that supports the involvement of the LEC in memory retrieval and deepen our knowledge of the intricate processes underlying episodic-like memory formation and retrieval.

Materials and methods

Animals

All experimental procedures involving animals followed the guidelines defined by the European legislation (Directive 2010/63/EU), and the Italian Legislation (LD no. 26/2014). The Organism Responsible for Animal Welfare (OPBA) of the National Research Council of Italy (CNR) Institute of Neuroscience in Pisa and the Italian Ministry of Health approved the study protocol (authorization n. 16/2022-PR).

Male wild-type C57BL/6J mice were housed in conventional cages (365 x 207 x 140 mm, 2-3 animals per cage) with nesting material on a 12-h light/dark cycle with food and water available ad libitum. Behavioral experiments were performed on 3 months aged male mice during the light phase and mice were randomly assigned to experimental groups. To control for order and cage effects, each cage contained a mixture of mice from the experimental and control groups.

AAV vectors and stereotaxic injections

AAV-Fos::CreER^{T2} (titer: 1.2×10^{13}) and Cre-dependent AAVs AAV-hSyn::DIO-hM3Dq-mCherry, AAV-hSyn::DIO-hM4Di-mCherry and AAV-hSyn::DIO-mCherry (titers: $5.0 - 6.0 \times 10^{12}$) were packaged as serotype 5 virus. The AAV-Fos::CreERT2 vector was obtained from (Matos et al. 2019), while the Cre-dependent AAVs AAV-hSyn::DIO-hM3Dq-mCherry, AAV-hSyn::DIO-hM4Di-mCherry, and AAV-hSyn::DIO-mCherry vectors were purchased from the UZH viral vector facility (Zurich, Switzerland).

For stereotaxic injections, 2-month-old male C57BL/6J mice were deeply anesthetized using an intraperitoneal injection of Zoletil 100 (zolazepam and tiletamine, 1:1, 40 mg/kg; Laboratoire Virbac) and Xilor (xilazine 2%, 10 mg/kg; Bio98). After the tail pinch reflex disappearance, mice were positioned in the stereotaxic apparatus. Lidocaine (2%) was topically applied to the skull to provide local analgesia. The scalp was shaved and a midline incision was made. A bilateral craniotomy was performed at the stereotaxic coordinates targeting the LEC (AP -4.0 mm, ML ± 4.0 mm from Bregma, measured on the skull surface). A glass pipette was lowered from the brain's surface at an 11° angle until a slight bend in the pipette indicated contact with the dura as described by (Vandrey et al. 2020). The pipette was retracted 0.1 mm and 125 nl of a virus mixture of AAV-Fos::CreERT2 and Cre-dependent AAV (ratio 1:500, AAV-Fos::CreERT2 at a final titer of 2.4×10^{10}) was injected. Then, the pipette was retracted again by 0.1 mm and an additional 125 nL of the virus mixture was injected, for a total of 250 nL. This approach minimized the likelihood of the spread of the virus into adjacent cortical structures. For all injections, the pipette was slowly retracted after a stationary period of five minutes. Then, the scalp was sutured and the mouse was brought back to its cage for recovery. Animals remained in their home cage for 3 weeks until the start of behavioral experiments.

Behavioral apparatus

The test environment was composed of two square boxes (length 40 cm, width 40 cm, height 40 cm) with different visual cues on the walls to provide distinct contexts. Context A had gray walls and context B had four different visual cues on the four walls: black and white vertical stripes, black triangles on a white background, black and white horizontal stripes, and black circles on a white background. The wall and floor of the environment were cleaned with 30% alcohol before each trial. Objects were household items of approximately the same size as the mouse and varying in color, shape, and texture. To avoid odor cues, new identical copies of each object were used for each trial, and objects were cleaned with 30% alcohol after each trial.

Behavioral tasks

For the behavioral tests, 3-month-old C57BL/6J mice were habituated to the experimenter by extensive handling for one week before the experiments. The performance of specific tasks that included the novel location test (NLT) and the novel object-place-context recognition test (OPCRT) was always preceded by the open field test to control locomotion and anxious behavior in the same arena used for behavioral testing.

During the behavioral tasks, object exploration was monitored via an overhead camera. For all sample and test trials, mice were allowed to explore the environment freely for 3 min. If the animals did not meet the minimum exploration time of 20 s for both objects, the scoring was continued past 3 min for a maximum of 10 min until total exploration reached 20 sec (Lueptow 2017). Between the two sample trials, mice were removed to a holding cage for approximately 1 minute while the next environment was configured for the subsequent trial. The test trial was instead performed either 1 h, 6 h, 12 h, or 48 h after the sample trials presentation for the OPCRT, and 12 h for the NLT. For each task, the novel object at the test, the context, and the quadrants where the novel object or configuration occurred were counterbalanced across animals and experimental conditions.

Open field

Before the execution of the memory tasks, mice were allowed to explore for 5 minutes the same context in which they were given the same trial of the subsequent behavioral test. A custom-made Python pipeline was used to automatically compute the total ambulatory distance as well as the amount of time spent in the outer zones versus inner zones (24 cm x 24 cm).

Novel location test

During the sample trials, mice were exposed to two copies of an object in a given context. Then, the mice were removed from the box and brought back into their home cages for 12h before the presentation of the test trial. In the test trial, one of the two objects was moved to a novel location (novel location) while the other was kept in the same location as the sample trial (familiar location) and the context remained the same.

Novel Object-Place-Context Recognition Test

In the OPCRT, in the first sample trial mice were presented with two different novel objects in context A or B. After the first sample trial, mice were removed from the box and placed in a holding cage for a 1-minute inter-trial interval (i.t.i.) while the box was cleaned. In the second sample trial, mice were presented with the same objects in opposite locations in different contexts. In the test trial, mice were presented with two copies of one of the objects within the same context used in the first sample trial. At the test, one copy of the object is in a novel location for the test context (novel OPC configuration), whereas the other copy is in a familiar location for the test context (familiar OPC configuration).

Contexts exposure

As a control in electrophysiology experiments, mice of the CNTX group were allowed to explore a sequence of A-B-A or B-A-B empty contexts for 5 minutes each. Between each context exploration, mice were removed from the box and placed in a holding cage for 1-minute i.t.i., while the box was cleaned.

Animal tracking

Behavioral videos were recorded using an AUKEY webcam 1080p full HD camera and were then analyzed offline. Different mouse body regions, namely the nose, the two ears, the back of the animal, the middle portion, and the tip of the tail labeled using the open-source tool DeepLabCut (Mathis et al. 2018) for markerless pose estimation. For this purpose, 460 frames extracted from 23 videos were manually labeled and used to train the DeepLabCut ResNet50 network for 103000 iterations, obtaining a high-fidelity tracking of the selected body parts. The animal tracking allowed to automate of the calculation of the animal speed, the total traveled distances, and the time spent in different regions of the arena (center/periphery) in the open field test, the novel location test, and in the novel object-place-context recognition test. Moreover, the tracking results were also used to automatically calculate the discrimination index of the animals during the memory test by combining pose estimation with a machine learning algorithm.

Behavioral scoring

In order to obtain a reproducible and efficient scoring of the behavioral tests, a random forest classifier was trained to discriminate between epochs of object exploration and epochs of no exploration by using the distances of the tracked body parts from the two objects. This approach allowed us to automatically detect the time spent by the animal exploring either the novel or the familiar object and calculate the corresponding discrimination index. To train the classifier, 156414 frames from 16 videos were manually labeled as “exploration” or “no exploration”, the results of the classifier were compared with the traditional scoring using a stopwatch and the frame-by-frame manual scoring as the ground truth.

To determine the relative exploration of novel and familiar configurations, the time spent exploring the familiar and novel objects was converted into a discrimination index (DI) according to the formula:

DI=TNovel−Tfamiliar / TNovel+Tfamiliar

4-hydroxytamoxifen treatment

4OH-TAM (H6278, Sigma-Aldrich) was injected into an aqueous solution. A 50 mg/ml stock of 4OH-TAM in DMSO (D8418, Sigma-Aldrich) was realized and maintained at -20°C. On the day of the experiment, a final solution of 4OH-TAM 2.5 mg/ml was obtained in two steps: first, diluting the stock solution 1:10 in saline containing 2% Tween80 (P1754, Sigma-Aldrich) and then adding a volume of saline. The final solution contained 2.5 mg/ml of 4OH-TAM, 5% DMSO, and 1% Tween80 in saline. Mice received 4OH-TAM (25 mg per Kg, i.p.) 4h before the sample trials (Matos et al. 2019). To reduce the stress from i.p. injections, mice were anesthetized shortly using isofluorane (3%) and were injected while unconscious.

Chemogenetic intervention

Clozapine N-oxide hydrochloride (CNO; cat. no. 34233-7, Merck) was dissolved in sterile saline. For behavioral experiments, mice received 3 mg per kg (i.p.) CNO 30 min before each test session.

Immunohistochemistry

Mice were deeply anesthetized using urethane (Merck, 20% solution, 0.1 ml/100 g body weight) and perfused with an intracardiac infusion with PBS pH 7.4, followed by 4% paraformaldehyde (PFA) in PBS pH 7.4. Brains were removed, post-fixed for 24h in 4% PFA (w/vol) solution, and then immersed in 30% sucrose (w/vol) in PBS. Brains were then sliced into 50 µm coronal sections using a freezing microtome (Leica) and free-floating sections were processed for immunofluorescence.

The cortical sections were incubated for 2h in a blocking solution at 22-24°C containing 5% BSA (w/vol) and 0.5% Triton X-100 (vol/vol) in PBS, and incubated overnight at 4°C with anti-cfos monoclonal antibody (cat. no. 226 008, Synaptic Systems) diluted 1:1000 and anti-mCherry monoclonal antibody (cat. no. M11217, ThermoFisher Scientific) diluted 1:1000 in PBS with 1% BSA (w/vol) and 0.1% Triton X-100 (vol/vol). Sections were then washed with PBS and incubated for 2h at 22-24°C with Alexa Fluor 488-conjugated secondary antibody (cat. no. 711-545-152, Jackson Immunoresearch) and Alexa Fluor 568-conjugated secondary antibody (cat. no. A11077, ThermoFisher Scientific) added at a dilution of 1:300 in the same solution as the primary antibody. Sections were then washed 3 times with PBS and mounted on slides, then they were air-dried and coverslipped with FluoromountTM aqueous mounting medium (cat. no. F4680, Merck).

Imaging was performed on an Axio Imager Z2 microscope (Carl Zeiss) and multichannel images were produced with ApoTome 2 using an EC Plan-NEOFLUAR 10x/ 0.3 objective.

cFos and mCherry quantification

The cFos+ cell detection was performed using a modified version of the puncta-detecting algorithm described in (Cicconet and Hochbaum 2019) followed by manual refinement of

the detection results. Several parameters in the pipeline were optimized to process the experimental brain slices.

To detect individual cFos+ nuclei via Laplacian of Gaussian filtering of the image, the local maxima that corresponded to cfos puncta were found and distinguished from the local maxima in the background. For the analysis, the software parameters were set to: background threshold: 10, sigma: 3.5, and circularity threshold:0.3. The detection results were then supervised by an operator and eventually modified to correct for inaccuracies.

The density of cFos+ cells (cFos+/mm^2) was averaged over 6-10 sections per animal. For the mCherry+ cell detection, cell counting was manually performed by an operator blind to the experimental conditions, when possible, and the mCherry+ cell density (mCherry+/mm^2) was averaged over 4-6 sections per animal. The LEC and MEC regions of interest (ROI) were manually outlined based on the mouse brain atlas (Paxinos and Franklin's The Mouse Brain in Stereotaxic Coordinates).

To analyze the overlap between cFos and mCherry-expressing cells, the percentage of overlap was calculated as the number of cFos+, mCherry+ double-positive cells divided by the average number of dapi+ cells.

The chance level of overlap was calculated as the product between the number of cFos+ positive and mCherry+ cells, divided by the average number of dapi+ cells. The average number of dapi+ cells was obtained by averaging the number of dapi+ nuclei in 6-9 LEC sections obtained from 7 animals.

In-vitro electrophysiology

Electrophysiology was performed as in Origlia et al., (Origlia et al. 2008). Mice were anesthetized with urethane i.p. injections (20% sol (w/vol), 0.1 ml/100 g of body weight) and decapitated. Horizontal EC-hippocampal slices (400 µm of thickness) were produced using a vibratome (Leica VT1200S). All steps were performed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 119 NaCl, 2 CaCl2, 1.2 MgSO4, 1 NaH2PO4, 6.2 NaHCO3, 10 HEPES, 11 glucose. Slices were then transferred to a chamber and perfused at a 2-3 ml/min rate. Field excitatory post-synaptic potentials (fEPSPs) were evoked by a concentric bipolar stimulating electrode and recorded in the II/III layers of EC.

Basal recordings were carried out using stimulus intensity evoking a response whose amplitude was 50-60% of the maximal amplitude. After 10 min of stable baseline, long-term potentiation (LTP) or long-term depression (LTD) was induced using either high-frequency stimulation (HFS, three trains of 100 pulses at 100 Hz, 10 s interval) or low-frequency stimulation (LFS, 900 paired pulses at 1 Hz, the interval between paired pulses was 30 ms).

After HFS or LFS, fEPSPs were monitored every 20 s for at least 60 min or 40 min respectively. The magnitude of LTP or LTD was calculated as the average of the relative amplitudes (compared to baseline) of fEPSPs recorded in the last 10 min. Values were expressed as percentage changes relative to the baseline.

Statistical analysis

All data are reported as mean ± SEM. For electrophysiological experiments statistical comparisons between experimental groups were performed by applying a two-way repeated-measures ANOVA with pair wise multiple comparison procedure (Holm-Sidak method). For behavioral experiments, a one-way ANOVA or an unpaired t-test were applied to determine differences in average discrimination indices and exploration rates between groups. One-sample t-test was used to determine whether the average discrimination index for each group was different from chance (hypothesized mean = 0). A one-way ANOVA was applied for the evaluation of differences in c-fos cell density or single-cell mean fluorescence intensity. Kruskal-Wallis was used to determine differences in the discrimination indices among different time points in the analysis of the time course of episodic-like memory.

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