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*Episodic memory: from the hippocampal-
entorhinal circuit to a distributed network*

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1. Abstract

Episodic memory is defined as the ability to recollect events with their specific spatiotemporal details. In rodents, the study of episodic-like memory relies on their spontaneous tendency to explore novelty in a familiar environment. Based on this innate behavior, the Novel Object-Place-Context Recognition Task (OPCRT) was developed as a spontaneous recognition paradigm to assess episodic-like memory performance in rodents. Using this task, it has been demonstrated that episodic-like memory critically depends on the hippocampus and the lateral part of the entorhinal cortex (LEC). Recently, neurons exhibiting properties of engram cells for OPC memory have been identified in the LEC. This brain region is among the earliest affected in Alzheimer's disease (AD), a pathology characterized by severe episodic memory deficits. To determine whether episodic-like memory alterations in a transgenic mouse model of AD (APPJ20) arise from impairments in memory encoding or retrieval, we selectively expressed the excitatory chemogenetic receptor hM3Dq in the subpopulation of LEC neurons activated during the learning phase of the OPCRT. Notably, chemogenetic activation of these ensembles restored OPC memory performance in young APPJ20 mice. We also hypothesized that the impairment in novel object recognition (ORT) observed in 6-month-old APPJ20 mice results from the progression of amyloid pathology to the hippocampus and confirmed that memory expression can be rescued by applying the same chemogenetic strategy to the learning-tagged neuronal populations in the dentate gyrus.

We next investigated whether additional brain regions are recruited as part of a distributed network supporting memory retrieval. To this end, we performed a brain-wide mapping of neurons activated during OPC memory recall in wild-type mice, using c-Fos expression as a marker of neuronal activity. We found that OPC memory recall specifically increased c-Fos expression in associative regions involved in memory processing, navigation, and decision-making. The identified regions form a subnetwork characterized by strong anatomical interconnections, consistent with their coordinated involvement in memory processing. Functional connectivity analysis indicated that OPC memory recall is associated with stronger local specialization and more efficient global communication. To further dissect the individual contribution of the selected brain regions to episodic memory, we expressed chemogenetic receptors in their learning-activated neuronal population and manipulated their activity during the recall phase. Across all regions examined, inhibition of learning-tagged neurons consistently impaired memory recall, whereas chemogenetic reactivation of the retrosplenial cortex alone supported successful performance at a time when the memory trace was no longer accessible through natural cues. These results support a distributed organization of episodic-like memory in mice, in which memory recall is associated with increased neural activity across frontal and posterior medial regions.

2. Introduction

2.1 Episodic memory

An essential function of the nervous system is the capacity to acquire, store, and retrieve information from experience to generate adaptive behavior. Learning and memory are the core mechanisms supporting this ability. Learning refers to the biological process through which new information is acquired, whereas memory involves the retention and reconstruction of information over time (Kandel et al., 2014). Memory is a complex and multifaceted function comprising multiple subcomponents, whose underlying neural substrates remain not fully understood. To approach this complex topic, it is helpful to distinguish different memory systems according to their function and neural organization, such as declarative and nondeclarative memory. Declarative memory refers to the conscious recollection of facts and events and relies primarily on the medial temporal lobe and associated cortical regions. In contrast, nondeclarative memory includes forms of learning that occur without conscious awareness, comprising procedural skills, habits, and conditioning, and depends on subcortical structures such as the basal ganglia, cerebellum, and amygdala (Cohen and Squire, 1980; Squire, 2004).

Within declarative memory, episodic memory is defined as the ability to recall events within their spatiotemporal context, a function that enables the use of past experiences to guide future behavior, such as avoiding danger or locating resources (Tulvig, 1983). Although this capacity was originally considered unique to humans (Tulvig and Markowitsch, 1998; Tulvig, 2002), increasing evidence indicates that the core features of episodic memory are conserved across mammals and are also present in several avian species (Clayton et al., 2003; Crystal et al., 2010), highlights its evolutionary importance as a mechanism for successful adaptive behavior. Since the subjective nature of episodic memory remains not accessible in animals, as it depends on verbal components unique to humans, its investigation typically relies on assessing their ability to remember events within their specific contexts (Allen and Fortin, 2013). For instance, Clayton and Dickinson (1998) demonstrated that food-caching scrub jays could selectively search for recently stored, perishable food items before retrieving nonperishable ones. This behavior indicated that the birds retained information about what they had hidden, where, and when, fulfilling the operational criteria for episodic-like memory (Tulving, 1983). Similarly, rats can combine what and where information to reconstruct the sequence of past events (when), an ability that is abolished following hippocampal lesions (Ergorul and Eichenbaum, 2004; Sugar and Moser, 2019). Historically, the “when” component of the episodic-like memory triad has proven particularly challenging to assess, making it difficult to design appropriate behavioral tasks. Consequently, the role of context has been preferentially investigated in nonhuman species. Scene memory has been proposed as an analogue of episodic memory, where background scenes provide the framework for events (Gaffan, 1994).

In rodents, the study of episodic-like memory largely relies on their spontaneous tendency to explore novelty in a familiar environment (Ennaceur and Delacour, 1988). Based on this innate behavior, Eacott and Norman (2004) developed the Novel Object-Place-Context recognition task (OPCRT), a spontaneous recognition paradigm designed to assess

episodic-like memory performance. In this behavioral paradigm, during the learning phase, rats were first exposed to two distinct objects in a specific context defined by visual cues. In the second part of the learning phase, the same pair of objects was presented again, but placed in opposite locations in a different context. During the test phase, two identical copies of one of the objects were presented in the first context, and the time spent exploring each object was measured. The results showed that rats preferentially interacted with the object presented in the novel combination of object identity, position, and context, indicating that they were able to remember which object had been encountered in which location and under which contextual condition. However, this form of memory, which depends on the encoding and retrieval of object-place-context associations, persisted only for a few hours after the learning phase.

While novelty-based paradigms such as the OPCRT have provided valuable information about the associative components of episodic-like memory, they represent only a subset of the available behavioral approaches. In recent years, a variety of alternative paradigms have been developed to probe different aspects of episodic-like memory encoding and recollection (Kunčická et al., 2024). These include foraging-based tasks that assess the integration of what, where, and when information by requiring animals to recall the location and timing of distinct food rewards (Day et al., 2003; Babb and Crystal, 2006). Also, odor sequence or order task tests were used to investigate the animal's ability to remember the temporal organization of events (Fortin et al., 2002; Ergorul and Eichenbaum, 2004). Variants of the classic contextual fear conditioning paradigm have also been developed to investigate episodic-like memory processes. For example, O'Brien and Sutherland (2007) demonstrated that rats can associate an aversive event with both its spatial and temporal context: animals displayed greater freezing in the environment that matched the time of day at which the foot shock had occurred, suggesting the formation of an integrated what-where-when memory. Similarly, Iordanova and colleagues (2008) showed that rats were able to recall the association between a specific auditory cue, the environmental context, and the time of day in which these elements co-occurred. Although these paradigms rely on conditioned responses rather than spontaneous exploration, they suggest that rodents can flexibly encode and retrieve complex event representations involving multiple dimensions of experience.

In contrast, the OPCRT captures spontaneous forms of episodic-like memory that can be established without prior training, offering an important model to investigate the integration of what, where, and contextual information in rodents. Performance in this task is particularly sensitive to damage within the hippocampal-entorhinal network. Lesions of the hippocampus have been shown to selectively impair recognition of integrated object-place-context associations, while leaving intact simpler forms of associative or non-associative recognition such as object, object-place, or object-context memory (Langston and Wood, 2010). Similarly, excitotoxic lesions of the lateral entorhinal cortex (LEC) disrupt the ability to recognize familiar combinations of object, place, and context, despite preserved recognition of individual elements (Vandrey et al., 2020). These findings indicate that both the hippocampus and the LEC are critical for the integration of object, spatial, and contextual

information, supporting the idea that coordinated activity across these regions underlies the formation and retrieval of episodic-like memories in rodents.

This form of memory is particularly vulnerable to the effects of aging and to neurodegenerative disorders such as Alzheimer's disease (AD) (Tromp et al., 2015; Sugar and Moser, 2019). Age-related decline primarily affects the efficiency of encoding and retrieval processes, which depend on the integrity of medial temporal and prefrontal regions (Morcom et al., 2003; Nyberg et al., 2003; Andreson et al., 2005; Head et al., 2009). In AD, these deficits are more pronounced and originate from early pathological alterations in the hippocampus and adjacent cortical areas, leading to the disruption of episodic memory recollection (Baron et al. 2001; Scheltens et al, 2002; Perri et al., 2007; Sexton et al. 2010; El Haj et al., 2015; Tromp et al., 2015).

2.2 The role of the Medial Temporal Lobe system in episodic-like memory

Since the earliest experimental studies on memory and human amnesia, the medial temporal lobe (MTL) has been recognized as a critical component of the neural system underlying episodic memory. The seminal case of patient H.M., who developed profound memory impairment following bilateral resection of the hippocampus and adjacent cortices, provided evidence that these structures are essential for the formation of episodic memories (Scoville and Milner, 1957). Over the following decades, converging findings from neuropsychological, electrophysiological, and neuroimaging studies have progressively refined this view, establishing the MTL system as a key hub that binds together different elements of experience into coherent and retrievable representations. The general organization of the MTL is largely conserved across mammalian species (Manns and Eichenbaum, 2006). It is classically composed of the hippocampal formation, which includes the CA1-CA3 fields, the dentate gyrus (DG), the subicular complex, and of the parahippocampal region, which comprises the entorhinal (EC), perirhinal (PER), and parahippocampal cortices. In the rodent brain, the parahippocampal cortex is typically referred to as the postrhinal cortex (POR), and the parahippocampal region also includes the presubiculum (PRE) and parasubiculum (PAR) (Squire et al., 2004; Furtak et al., 2007). In general, all neocortical association areas send projections to the parahippocampal region, which in turn projects to different subdivisions of the hippocampal formation. Within the hippocampus, information follows a largely unidirectional pathway that begins in the DG, continues through the CA3 and CA1 fields, and terminates in the subiculum (SUB). The output signals arising from CA1 and the SUB are then sent back to the parahippocampal region, which distributes the processed information to neocortical areas (Dickerson and Eichenbaum, 2009).

Among the structures of the parahippocampal region, the EC occupies a central position for the conduction of neocortical information to the hippocampus. This brain region lies along the rhinal sulcus and displays a regular six-layered organization, with sparse neuronal populations in layers I and IV. This laminar architecture is more clearly delineated in the medial entorhinal cortex (MEC) compared to its lateral subdivision (LEC). Focusing on the input connection to the EC subdivisions, the LEC integrates multimodal information from the PER, orbitofrontal (ORB), medial prefrontal (mPFC), and insular cortices, as well as from

olfactory structures including the olfactory bulb and piriform cortex (Burwell et al., 1995; Vertes, 2004; Kerr et al., 2007; Kondo and Witter, 2014; Witter et al., 2017). In contrast, the MEC primarily receives spatial and contextual inputs from the PRE, PARA, and retrosplenial cortex (RSP) (Caballero-Bleda and Witter, 1993; Burwell and Amaral, 1998; Kerr et al., 2007). Additionally, the POR projects to both MEC and LEC, as well as to the PER, thus connecting the different components of the parahippocampal region (Burwell et al., 1995; Nilssen et al., 2019). Both the medial and lateral entorhinal cortices send extensive projections to the DG, CA3-CA1 fields, and subiculum, providing the principal cortical input to the hippocampal formation. The main projection from the EC to the DG originates from reelin-expressing neurons in Layer II. Axons arising from the LEC terminate in the outer molecular layer of the DG, whereas those from the MEC target the middle molecular layer (Witter, 2007). Axons from the LEC mainly target the distal portion of CA1 and the immediately adjacent subicular region. In contrast, MEC projections terminate in the proximal CA1, and in the portion of the subicular region close to the presubiculum. Reciprocal projections from CA1 and the SUB back to Layer V of the EC preserve this topographic arrangement, confirming segregated connectivity loops between each EC subdivision and specific hippocampal domains (Tamamaki and Nojyo, 1995; Nilssen et al., 2019).

Differences between the two entorhinal subdivisions are not limited to their connectivity but also involve distinct cellular and functional specialization. In the MEC, most functionally defined neuron types contribute to spatial coding and navigation, supporting path integration and self-location through the activity of grid cells and other spatially modulated neurons such as head-direction, speed, border, and object-vector cells (Fyhn et al., 2004; Hafting et al., 2005; Solstad et al., 2008; Kropff et al., 2015; Høydal et al., 2018). In contrast, functional characterization of neurons in the LEC is less extensive, yet available evidence indicates that spatial coding is not the primary function. Unlike in the MEC, grid cells are absent, and spatially modulated neurons are relatively scarce (Hargreaves et al., 2005). Instead, a small subset of LEC neurons displays activity related to object location, developing spatially confined firing fields following object exposure and signalling either the current or previous positions of objects (Tsao et al., 2013). The representation of objects likely reflects the prominent input from the PER, which provides only weak projections to the MEC and is thought to convey information involved in object recognition and novelty-familiarity discrimination (Burwell et al., 1998; Brown, 2008). Moreover, LEC neurons are strongly engaged in olfactory processing, receiving projections from the olfactory bulb and piriform cortex and exhibiting activity modulated by olfactory stimuli, consistent with a key role in olfactory associative learning (Otto et al., 1991; Young et al., 1997; Xu and Wilson, 2013). Increased coherence between the LEC and distal CA1 in the slow gamma range (20-40 Hz) during odor-place associations further support its contribution to olfactory memory and associative coding (Colgin and Moser, 2010; Colgin et al. 2016). The presence of these differences between the two subdivisions strongly supports the view that the MEC constructs a universal allocentric map of the environment through path integration mechanisms, whereas the LEC primarily represents spatial information in an egocentric reference frame and, in parallel, contributes to the encoding of temporal information across different

timescales (McNaughton et al. 2006; Moser et al., 2017; Tsao et al., 2018; Wang et al., 2018; Wang et al., 2020; Wang et al., 2024). This anatomical and functional organization suggests that the entorhinal and hippocampal regions form an integrated network that supports the representation of space, time, and experience.

One major advance in understanding memory processing came with the discovery of place cells in the hippocampus, which are hypothesized to constitute a fundamental component of the brain's cognitive map of space (O'Keefe and Dostrovsky, 1971; O'Keefe and Nadel, 1978). According to this view, place cells with neighboring place fields are functionally interconnected, forming ensembles that together represent a memory system for related locations (Sugar and Moser, 2019). In addition to spatial coding, the hippocampus also encodes the temporal dimension of experience, a fundamental feature for constructing episodic memories. The resulting spatiotemporal representations allow discrete events to be mapped onto unique activity patterns within hippocampal ensembles. In this process, the EC conveys sensory information and the associative relationships between these inputs, which can be detected in the LEC before corresponding activity emerges in hippocampal neurons (Leutgeb et al., 2005; Igarashi et al., 2014; Josselyn et al., 2015). These spatial and temporal representations emerge dynamically as the animal explores: spatial coding is driven by the animal's position and continuously refined by environmental cues, whereas temporal coding in the LEC reflects the content and structure of experience (Sugar and Moser, 2019). While the cognitive map hypothesis provided a foundational framework for linking hippocampal activity to memory, growing evidence highlights the EC as critical for organizing the spatial, temporal, and sensory dimensions of experience into integrated memory representations.

2.3 Neocortical areas involved in episodic-like memory

Unlike the MTL, the organization and function of neocortical regions involved in episodic memory differ substantially among mammalian species, particularly in their size, laminar organization, and patterns of connectivity (Manns and Eichenbaum, 2006). However, there is remarkable similarity in the connections between neocortical associative areas and the MTL (Dickerson and Eichenbaum, 2010). Converging evidence from human neuroimaging studies indicates that episodic memory retrieval engages a distributed network of cortical regions interconnected with the MTL. This network centers on the hippocampus and parahippocampal region and extends to the retrosplenial and posterior cingulate cortices, the angular gyrus within the ventral posterior parietal cortex, and the mPFC (Rugg and Vilberg, 2013). Within this network, the RSP and mPFC appear to play critical and distinct roles in episodic memory, as suggested by their distinctive position and pattern of connectivity. The RSP has been considered an "extended hippocampal system" that contributes to the retrieval of contextual information (Aggleton, 2012), while the mPFC likely contributes to contextual processing by integrating retrieved contextual details into higher-order, goal-directed representations (Kveraga et al., 2010).

The RSP in rodents is a large midline structure extending along the anteroposterior axis, and it includes subregions distinguished by the presence or absence of a distinct granular cell layer. Given its anatomical position and extensive pattern of connectivity, the RSP acts

as a major hub for integration among multiple cortical and subcortical systems (Alexander et al., 2023). Its connectivity is remarkably complex but can be summarized into three major pathways (Todd et al., 2020). The first comprises connections with sensory processing regions, including dense reciprocal projections with primary (V1) and higher-order visual cortices, direct inputs from the auditory cortex, and strong interconnections with the claustrum, a structure that receives polymodal sensory input from several primary sensory areas. In addition, its reciprocal connections with the posterior parietal cortex are thought to play a key role in visuo-spatial processing and working memory (Vogt and Miller, 1983; Miller and Vogt, 1984; Wyss and Van Groen, 1992; Todd et al., 2016). The second major pathway includes connections with limbic and memory-related structures. The RSP is strongly interconnected with the anterior and latero-dorsal thalamic nuclei, which are known to contribute to spatial memory and navigation. In addition, the RSP maintains reciprocal connections with the MTL, including the SUB, postsubiculum (POST), and CA1, as well as the POR, PER, and MEC (Mathiasen et al., 2017; Wyss and Van Groen, 1992; Kerr et al., 2007; Burwell and Amaral, 1998). Each of these regions plays a crucial role in spatial navigation and contextual learning, suggesting that the RSP may serve as an interface through which sensory and thalamic information can influence hippocampal-dependent memory processing (Vann and Aggleton, 2005; Vann et al., 2009). The third major pathway involves connections with neuromodulatory and associative systems. The RSP receives sparse but direct dopaminergic input from the ventral tegmental area (VTA) and substantia nigra (SN), suggesting a potential role in processing salient or reinforcing stimuli. It is also innervated by cholinergic projections from the medial septum and the diagonal band, systems known to modulate hippocampal activity. In addition, the RSP maintains reciprocal connections with the anterior cingulate cortex (ACA) and with anterior thalamic nuclei (anterior medial, anterior ventral, and anterior dorsal), structures that are implicated in associative learning and memory (Loughin and Fallon, 1984; Berger et al., 1985; Eckenstein et al., 1988; Shibata and Naito, 2008). Overall, the anatomical position and connectivity of the RSP place it in a unique position to act as an interface between regions involved in sensorimotor and spatial processing and those engaged in mnemonic and associative functions. This integrative role is supported by the presence of different neuronal populations that are tuned to multiple variables, including spatial location, head direction, and contextual cues (Chen et al., 1994; Vedder et al., 2016; Bicanski and Burgess, 2016; Lozano et al., 2017; Alexander and Nitz, 2017; Chinzorig and al., 2020).

In addition to the RSP, the mPFC plays a crucial role in the cortico-hippocampal network that underlies memory. The precise definition and boundaries of medial prefrontal subregions in rodents remain a matter of debate, as different anatomical and functional criteria have been proposed. For the purpose of this discussion, the subdivision proposed by Euston and colleagues (2012) is adopted. Briefly, the rodent mPFC can be subdivided into a dorsal component (dmPFC), which includes the dorsal prelimbic (dPL) and anterior cingulate (ACC) cortices, and a ventral component (vmPFC), comprising the ventral prelimbic (vPL), infralimbic (IL), and dorsal peduncular (DP) cortices. The different mPFC sub-regions are extensively interconnected through both excitatory and inhibitory pathways, supporting rich intra-cortical communication (Anastasiades and Carter, 2021; Howland et

al., 2022). The mPFC receives a wide range of inputs from motor, sensory, limbic and visceral parts of the brain, reflecting its integrative function. The pattern of connections differs markedly along its dorsoventral axis. The dmPFC receives strong projections from sensory, somatosensory and motor regions, consistent with its preferential role in action selection and cognitive control. In contrast, the vmPFC receives dense projections from limbic and visceral areas including the hippocampal formation, amygdala, and posterior insular cortex, but largely lacks input from sensory-motor areas (Hoover and Vertes, 2007; Euston et al., 2012). In addition, the vmPFC presents reciprocal connections with a wide range of neuromodulatory systems, including dorsal raphe, ventral tegmental area (VTA) and locus coeruleus, involved in adaptive responses to rewarding and stressful events (Euston et al., 2012). A similar difference is present regarding the efferent projections. The dmPFC primarily targets premotor and motor areas, as well as the dorsomedial striatum, contributing to goal-directed and flexible behavioral control. While the vmPFC projects preferentially to autonomic and affective centers such as the hypothalamus, periaqueductal gray, lateral habenula, and amygdala, as well as to the ventral striatum and ventral pallidum, supporting visceromotor and motivational functions (Vertes, 2004; Gabbott et al., 2005; Hoover and Vertes, 2007). Regarding the interaction with the MTL, hippocampus and subiculum send particularly dense projections to the mPFC (Cenquizca and Swanson, 2007). Although this pathway is primarily unidirectional, reciprocal interactions can occur indirectly through polysynaptic routes involving the nucleus reuniens of the thalamus or the LEC, thus establishing functional bidirectional communication between the hippocampal formation and prefrontal cortex (Burwell and Amaral, 1998; Vertes et al., 2007). Recently, Jun and colleagues have further highlighted the importance of the reciprocal interactions between the mPFC and the deep layers of LEC in associative learning, suggesting that this circuit encodes and stores experience within a non-spatial, outcome-based cognitive map. Using an odor-outcome associative learning task in which specific cues predicted either reward or punishment, they showed that when the activity of deep layers LEC neurons was optogenetically suppressed, mPFC neurons failed to segregate novel cues into rewarded and punished categories, preventing new learning. Conversely, inhibition of mPFC neurons caused LEC representations to remain isolated for each cue, disrupting both the acquisition and retrieval of learned associations. These findings suggest that deep layers LEC neurons and mPFC neurons cooperatively encode item memories as a map of associated outcome rules (Jun et al., 2024).

2.4 Episodic memory dysfunction in neurodegeneration

Episodic memory is proposed to rely on a multi-dimensional trace distributed across the brain and formed from a single experience and it is particularly vulnerable to disruption under disease conditions (Pause et al., 2013). Episodic memory impairments are reported across a wide range of pathologies, including medial temporal lobe injury, Alzheimer's disease (AD), Huntington's disease (HD), and Parkinson's disease (PD), as well as several psychiatric disorders, including schizophrenia, major depression, and dissociative disorders (Nyberg et al., 1996; Shing et al., 2009; Ballard et al., 2011). Patients with AD exhibit episodic memory deficits from the earliest stages of the disease. These impairments involve both encoding and retrieval processes and are often accompanied by temporal and spatial disorientation

(Peña-Casanova et al., 2012). Substantial evidence suggests that poor episodic memory performance in AD may depend largely on early retrieval deficits, leading to unusually rapid forgetting (Pause et al., 2013). Clinically, patients show marked difficulty learning new word lists and, even when initial learning occurs, they rapidly fail to recall or recognize the items among distractors (Moulin et al., 2004). They also struggle to suppress irrelevant associations, further impairing retrieval (Berger et al., 2015). Interestingly, patients with AD can imagine future events, but these imagined scenarios tend to be closely tied to the limited past memories they can still access (El Haj et al., 2015).

Typically, AD is characterized by extracellular neuritic plaques containing A β , which are widely distributed across the cerebral cortex, and intracellular neurofibrillary tangles composed of tau, which initially appear in the MTL and later spread to isocortical regions of the temporal, parietal, and frontal lobes (Knopman et al., 2021). A β peptides are generated from amyloid precursor protein (APP) through sequential cleavage by β - and γ -secretases. The resulting peptides, most commonly 27-43 amino acids in length, are secreted extracellularly as monomers. Due to its amino acid sequence, particularly in the case of A β 42, the peptide has a strong tendency to aggregate in a concentration-dependent manner (Holtzman et al., 2011; Haass and Willem, 2019). Accumulation of oligomeric A β profoundly impacts synaptic function: it interacts with metabotropic glutamate receptor 5 (mGluR5) and NMDA receptors and may also engage α 7 nicotinic acetylcholine receptors and insulin receptors (Spires-Jones and Hyman, 2014; Benarroch, 2018). These interactions are associated with pathological alterations in dendritic spine morphology and synaptic efficacy (Dorostkar et al., 2015). Tau is a microtubule-associated protein primarily localized in axons, but it is also found in presynaptic and postsynaptic compartments and associated with the nuclear membrane. Six tau isoforms exist due to alternative splicing of exons 2, 3, and 10, producing proteins with either three (3R) or four (4R) microtubule-binding domains. Tau's main role is stabilizing microtubules, but impaired post-translational modifications can increase its propensity to aggregate. Hyperphosphorylated tau accumulates and can be released extracellularly by mechanism dependent on synaptic activity (Giacobini and Gold, 2014; Pooler et al., 2014). Different post-translational modifications influence tau processing, truncation, and aggregation, and can modulate the rate of AD progression (Yamada et al., 2014; Alonso et al., 2018). Emerging evidence indicates that ApoE, TREM2, and microglia contribute to tau-mediated neurodegeneration, though the mechanisms linking microglial activity to tau toxicity remain not fully understood (Lue et al., 2014; Maphis et al., 2015; Shi et al., 2017; Vogels et al., 2019). Tau aggregation is histologically observed in multiple forms, including intracellular neurofibrillary tangles, neuropil threads composed of tau fragments, and dystrophic neurites, which are degenerated axons and dendrites surrounding A β plaques (Karikari et al., 2019). Tau pathology typically originates in the MTL and later spread to associative areas (Delacourte et al., 1999). Medial temporal tauopathy can occur independently of A β , as seen in primary age-related tauopathy, where it may persist without spreading (Braskie et al., 2010). In contrast, AD-type tau pathology spreads beyond the MTL predominantly in the presence of elevated levels of A β (Jack et al., 2019). The sequential accumulation of tau from the MTL to interconnected temporal, parietal, and frontal association cortices underlies the region-specific cognitive deficits observed in mild

cognitive impairment (MCI) and dementia (Ossenkoppele et al., 2016; Schöll et al., 2016). Cognitive impairments observed during AD progression are largely driven by synaptic alterations. A β plaques are associated with substantial losses of both pre- and postsynaptic markers in AD patients and in animal models of plaque deposition, suggesting a direct link between oligomeric A β and synaptic toxicity (Spires-Jones and Hyman, 2014; Rajmohana and Reddy, 2017). Similarly, models of tau overexpression exhibit comparable synaptic loss, which is further exacerbated when A β mutant transgenes are co-expressed (Busche and Hyman, 2020). Beyond synapse loss, AD is also characterized by pronounced synaptic dysfunction, including neuronal hyperactivity and impaired synaptic plasticity (Yamada et al., 2014; Stargardt et al., 2015; Chen et al., 2019; Targa Dias Anastacio et al., 2022).

In general, episodic memory impairments are linked to dysfunctions of the MTL and key regions of the default mode network, including the prefrontal cortex, posterior cingulate/retrosplenial cortex, precuneus, and the inferior parietal lobule (Greicius et al., 2003; Greicius et al., 2004). Among these regions, the LEC appears to be the first cortical region to show pathology and degeneration in AD (Braak and Braak, 1991). In fact, LEC Layer II neurons are particularly vulnerable, with up to 60% cell loss in mild AD and as much as 90% in severe cases (Gómez-Isla et al., 1996). Reelin-expressing Layer II stellate/fan cells in the entorhinal cortex undergo early neurodegeneration in AD patients as well as in AD mouse models (Chin et al., 2007). Supporting this selective vulnerability, it has been observed that LEC Layer II fan cells accumulate intracellular A β at preclinical stages in an APP transgenic rat model, suggesting that these neurons are particularly susceptible to A β -mediated toxicity (Kobro-Flatmoen et al., 2016; Igarashi, 2023). The vulnerability of the LEC to A β accumulation is further supported by early disruptions in synaptic plasticity observed in superficial Layer II neurons of the APPJ20 transgenic mouse model, which overexpresses human APP carrying the Swedish and Indiana familial Alzheimer's disease mutations (Mucke et al., 2000). These LTP impairments coincide with early deficits in OPC memory recall, whereas non-associative object recognition remains intact (Criscuolo et al., 2017). The early stages of Alzheimer's disease have been proposed to involve the propagation of A β pathology along anatomically connected circuits, rather than occurring independently in isolated regions. According to this hypothesis, A β can spread from initially affected areas, such as the LEC, to downstream hippocampal and cortical targets, progressively disrupting network function and memory (Langer et al., 2011; Musiek and Holtzman, 2015; Sengupta et al., 2016). Experimental evidence indicates that microglial-derived extracellular vesicles can mediate this process, as A β -loaded EVs injected into the LEC rapidly spread along the entorhinal-hippocampal circuit and induce hyperexcitability and the alteration of EEG oscillations in cortical and hippocampal regions. Interestingly, EV-injected mice exhibited rapid and sustained deficits in associative OPC memory, followed by impairments in non-associative hippocampal-dependent recognition memory, supporting the notion of progressive, circuit-specific synaptic dysfunction (Falcicchia et al., 2023).

2.5 Engram as the physical trace of memory

A fundamental step in understanding how the brain acquires, stores, and retrieves information is the idea that memories are encoded as lasting physical changes in the brain,

known as engrams. The term engram was first introduced by Richard Semon in 1904 to describe “the enduring, though primarily latent, modifications in the irritable substance produced by a stimulus,” referring to the way a memory trace persists after an experience (Semon, 1904). Semon described the engram as having four defining features. First, it represents a lasting physical change in the brain produced by a specific experience. Second, it has the capacity to be reactivated and expressed through appropriate retrieval cues (ecphory). Third, its content reflects the original experience, determining what can later be recalled. Finally, the engram can remain in a dormant state between encoding and retrieval, existing independently of the processes that create or access it. In this view, the engram itself is not yet a memory but constitutes the physical substrate from which a memory can emerge (Schacter et al., 1978; Josselyn and Tonegawa, 2020). Semon’s ideas remained largely ignored during his lifetime. Years later, in a series of pioneering experiments on spatial memory in rats, Karl Lashley revived and popularized the concept of the engram. In these studies, Lashley systematically removed varying amounts of cortical tissue in an attempt to locate the neural substrate of memory. However, he found that memory impairments were not linked to the specific location of the lesion but rather to the total amount of cortex removed. This led him to conclude that the engram was “elusive,” not confined to a single cortical area but instead widely distributed across the brain (Franz and Lashley, 1917). It is worth noting, however, that Lashley’s experiments had several limitations: the rats underwent extensive training, making their memories relatively resistant to lesions (Winocur et al., 2005), and the observed deficits were likely associated with an unintended damage to the hippocampus caused by large cortical ablations (Josselyn et al., 2015). Another important contribution to the foundation of engram research came from Donald O. Hebb, who proposed that neurons that are active simultaneously during an experience form cell assemblies through the strengthening of their reciprocal connections. According to this principle, often summarized as “cells that fire together, wire together,” memory traces emerge from the coordinated activity and synaptic reinforcement among groups of neurons (Hebb, 1949). Following these seminal ideas, decades of research have investigated how experience-induced changes arise across different levels of the nervous system, from individual synapses to neuronal ensembles and large-scale networks. Collectively, these studies demonstrate that learning and memory retrieval reshape neural structure and function through a variety of mechanisms. These include persistent modifications in DNA structure (Day and Sweatt, 2011), post-translational regulation of key kinases (Stargardt et al., 2015; Sacktor, 2011; Yasuda et al., 2022), the activation of specific gene-expression programs (Silva et al., 1998; Ortega-de San Luis and Ryan, 2022), the induction of immediate-early genes (IEGs) such as *Fos* and *Arc* (Okuno, 2011; Khan et al., 2025; Sullivan et al. 2025), alterations in receptor trafficking (Hayashi, 2022; Rumpel et al., 2005), and lasting changes in synaptic strength and neuronal excitability (Lisman et al., 2018; Magee and Grienberger, 2020).

Determining whether a neural population constitutes an engram has largely depended on four principal experimental strategies: observational studies, loss-of-function, gain-of-function, and mimicry paradigms (Josselyn and Tonegawa, 2020). Each approach relies on the fact that IEGs are expressed in response to neuronal activation, enabling the labeling

and genetic targeting of neurons engaged during memory encoding. Fluorescent reporters (e.g., GFP) or functional effectors such as optogenetic channels and DREADDs can be expressed under the control of IEG promoters like *Fos*, allowing neurons activated within a defined temporal window to be permanently tagged (Denny et al., 2014; Sørensen et al., 2016). Several genetic strategies have been developed to precisely control the timing of neuronal tagging. The tTA-TetO system restricts reporter expression to periods when doxycycline is absent (Tet-Off), because the tetracycline transactivator (tTA) can bind the TetO sequence only in the absence of the drug (Lewandoski, 2001). In this state, activation of the specific IEG promoter controlling tTA expression (e.g., c-Fos) results in tTA-mediated induction of stable reporter expression, restricted to the doxycycline-free window (Reijmers et al., 2007; Liu et al., 2013; Roy et al., 2016). A modified version of this system (Tet-On), replaces tTA with a reverse transactivator (rtTA) that binds the TetO sequence only in the presence of doxycycline, allowing reporter expression to be triggered upon doxycycline administration (Mansuy et al., 1998; Choi et al., 2018). Another strategy, the Targeted Recombination in Active Populations (TRAP) approach, employs a tamoxifen-inducible Cre recombinase (CreER^{T2}). In this system, IEG-driven expression of CreER^{T2} identifies neurons that are active, but recombination occurs only when tamoxifen is administered. As a result, tamoxifen defines a transient tagging window during which CreER^{T2} can enter the nucleus and recombine the reporter construct, permanently labeling the neurons that were active within that specific time frame (DeNardo et al., 2019; Matos et al., 2019; Jung et al., 2023). The most commonly used functional effectors in these genetic systems are optogenetic and chemogenetic tools. Optogenetic tools enable millisecond-precise control of neuronal activity through light-sensitive proteins derived from the channelrhodopsin (ChR) of the green alga *Chlamydomonas*. ChRs can be classified as cation- or anion-conducting channels, referred to as CCRs and ACRs, respectively. Upon light activation, CCRs depolarize the membrane by allowing the influx of various cations, with a strong preference for protons. Sodium permeability varies among CCRs, while divalent cations are generally poorly conducted under physiological conditions. In contrast, ACRs mediate chloride influx, and their effect depends on the chloride reversal potential in the targeted cell or subcellular compartment. By driving the membrane potential toward this reversal potential, ACRs can inhibit action potential firing through shunting inhibition (Emiliani et al., 2022). In contrast to optogenetic channels, which are activated by specific wavelengths of light, chemogenetic receptors, referred to as DREADDs (Designer Receptors Exclusively Activated by Designer Drugs), are based on G protein-coupled receptors (GPCRs). Examples include hM3Dq and hM4Di, derived from the human muscarinic acetylcholine receptor and coupled to Gq and Gi proteins, respectively. These receptors exhibit minimal sensitivity to the endogenous ligand acetylcholine but can be selectively activated by synthetic compounds such as clozapine N-oxide (CNO). Activation of DREADDs consistently modulates neuronal excitability, producing either an increase or decrease in activity depending on whether Gq/Gs or Gi signaling pathways are engaged (Gao et al., 2025). Activation of hM3Dq stimulates PLC- β , which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to its receptors (IP3Rs) on the endoplasmic reticulum membrane, triggering the release of intracellular Ca²⁺ stores. The increase in Ca²⁺, together with DAG, activates protein kinase C (PKC), leading to reduced

KCNQ channel activity and membrane depolarization. Activation of hM4Di inhibits adenylyl cyclase (AC), reducing intracellular cAMP and PKA activity, and suppresses presynaptic neurotransmitter release. In addition, Gi-DREADD activation opens G protein-coupled inwardly rectifying potassium channels (GIRKs), causing hyperpolarization (Armbruster et al., 2007).

In one of the first observational studies, Mayford and colleagues tagged neurons activated during an auditory fear-conditioning paradigm in the basal amygdala. By examining the expression of the IEG *zif268* during memory retrieval, they found that approximately 11% of the neurons activated during training were reactivated during testing. This overlap, significantly above chance, supported the idea that the basal amygdala contains a neuronal population with the characteristics of engram cells for this form of associative memory (Reijmers et al., 2007). Similar results have been obtained across several brain regions for different forms of memory, including the hippocampus (Tayler et al., 2013; Deng et al., 2013; Nakazawa et al., 2016; Karaca et al., 2021; Wilmerding et al., 2023) and various cortical areas (Milczarek et al., 2018; DeNardo et al., 2019; Stegemann et al., 2022; Lee et al., 2022; Brodt et al., 2022). Although these findings are informative for identifying regions that participate in a given behavioral paradigm, such observational evidence alone is insufficient to establish the existence of an engram. In this sense, causal evidence is required. For example, in a foundational loss-of-function experiment, memory allocation was biased by overexpressing CREB in a subset of lateral amygdala neurons, and an inducible construct was used to selectively ablate these neurons after training. Eliminating the CREB-overexpressing neurons produced a specific disruption of the auditory fear memory while leaving the animals' ability to acquire new memories intact (Han et al., 2009). The artificial inactivation of tagged neurons, achieved through both optogenetic and chemogenetic approaches, consistently impaired memory performance across different memory tasks (Denny et al., 2014; Tanaka et al., 2014; Kim et al., 2016; Okuyama et al., 2016; Roy et al., 2017; Autore et al., 2023). For instance, Lacagnina and colleagues demonstrated that optogenetic inhibition of DG neurons active during fear-extinction training increased fear responses after extinction, whereas silencing the neurons active during fear conditioning reduced the spontaneous recovery of fear (Lacagnina et al., 2019). Complementary evidence for sufficiency came from Tonegawa and colleagues, who demonstrated that DG engram cells can drive contextual fear memory using a gain-of-function approach. By expressing channelrhodopsin-2 (ChR2) in neurons active during conditioning and then reactivating these tagged cells using optogenetic stimulation in a neutral context, they were able to induce robust freezing behavior. This showed that activation of the DG engram ensemble alone is sufficient to elicit the behavioral expression of the memory in the absence of the original contextual cues (Liu et al., 2012).

Subsequent studies have extensively examined the sufficiency criterion in various brain regions, demonstrating that artificial reactivation of tagged neuronal ensembles can rescue impaired memory performance or prolong the expression of a memory trace beyond its natural duration (Cowansage et al., 2014; Ryan et al., 2015; Roy et al., 2016; Matos et al., 2019; Tozzi et al., 2024; Santos et al., 2024). An elegant example is provided by Abdou and colleagues, who demonstrated that ensembles encoding two associated memories can

overlap at the cellular level while remaining segregated at the synaptic level. In their retrograde amnesia paradigm, they tagged the neurons in the auditory cortex (AC) and medial geniculate nucleus (MGm) that were activated by the first conditioned tone, and then selectively erased either the memory of this tone or the memory of a second different tone using anisomycin+tBC. Although both tone memories were represented within the same population of lateral amygdala neurons, only the synapses encoding the targeted tone were disrupted. Notably, optogenetic stimulation of the tagged presynaptic terminals corresponding to the first memory reinstated freezing behavior only in the group in which this memory had been preserved, demonstrating that specific synaptic inputs can drive memory recall independently of the shared postsynaptic population. These findings reveal a synapse-specific architecture of overlapping engrams and show that targeted reactivation of the appropriate presynaptic ensemble is sufficient to recover a memory trace (Abdou et al., 2018). This experiment provides indirect evidence for the synaptic organization of engrams, as already suggested by the work of Kaang and colleagues (Choi et al., 2018; Choi and Kaang, 2022; Lee et al., 2023).

Optogenetic activation of engram cells has also been used to create artificial memories (mimicry approach). In a landmark experiment, DG neurons activated during exploration of a neutral context were tagged and later stimulated while mice received footshocks in a different environment. During subsequent testing, animals placed back in the first neutral context exhibited robust freezing behavior, despite having never been shocked there, indicating that the association between the neutral context and the aversive stimulus had been artificially imposed (Ramirez et al., 2013). An additional demonstration of the flexibility of engram manipulation comes from studies showing that optogenetic activation can even alter the valence of a memory. Redondo and colleagues (2014) tagged DG or BLA neurons active during either fear or reward conditioning and showed that reactivating these ensembles triggered the corresponding aversive or appetitive responses. Importantly, when the original engram was reactivated during a second conditioning session of opposite valence, only the DG engram adopted the new emotional value. This work demonstrates that, at least in the DG, memory engrams are sufficiently plastic for their emotional valence to be reassigned with an opposite unconditioned stimulus (Redondo et al., 2014). Together, these decades of studies have revitalized Semon's intuition, substantially expanding the original engram concept by revealing part of the mechanisms of memory storage and retrieval. However, many questions remain, including how engrams corresponding to different experiences interact, how they evolve over time and are maintained at the molecular level, what is the role of silent engrams, and what constitutes the minimal engram unit, whether synaptic or cellular.

2.6 Functions and regulation of IEGs in memory

Neurons face a unique challenge compared with other cell types in the body. Although they are postmitotic and maintain stable cellular identities, they must remain highly responsive to external stimuli while reliably encoding, storing, and processing information to support adaptive behavior. This dual requirement, stability and plasticity, necessitates mechanisms that allow neurons to detect and translate specific patterns of activity into lasting molecular

and cellular changes. Immediate early genes (IEGs) are central to this process. The expression of IEGs is rapidly upregulated within minutes of neuronal activation, allowing neurons to adapt to experiences and changing environmental conditions. This rapidity is a key factor for synaptic plasticity and memory processes. In general, IEGs function either as transcription factors that modulate gene expression or as effector proteins, typically involved in synaptic functions (Khan et al., 2025). The complex signaling cascades that drive the expression of IEGs, including *Fos*, have been widely investigated. In general, the induction of IEGs requires an increase in cytoplasmic calcium, which triggers a cascade of signaling events that ultimately lead to changes in gene expression (Yap and Greenberg, 2018). This increase can be mediated by extracellular calcium entering through N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptors, as well as through voltage-gated calcium channels, and by the release of calcium from intracellular pools (West et al., 2001). The change in gene expression is particularly associated with calcium entry through L-type voltage-sensitive calcium channels (L-VSCCs), which are localized in the cell body and proximal dendrites, in close proximity to the nucleus and to signaling molecules such as calmodulin (Deisseroth et al., 1998; Dolmetsch et al., 2001). The resulting increase in cytoplasmic calcium stimulates a cascade of signaling events, including the activation of the Ras-mitogen-activated protein kinase (MAPK) pathway, calcium/calmodulin-dependent protein kinases (CaMKs), and calcineurin-dependent signaling pathways (Yap and Greenberg, 2018).

In the case of *Fos*, its rapid induction depends on the activation of transcription factors already present in the neuron, specifically the cyclic adenosine monophosphate (cAMP)-responsive element binding protein (CREB), the serum response factor (SRF), and the myocyte enhancer factor 2 (MEF2) (Sheng et al., 1988; Sheng and Greenberg, 1990). In the nucleus, c-Fos forms a heterodimer with c-Jun, creating the activating protein-1 (AP-1) transcription complex. AP-1 is activated by upstream protein kinases, after which it binds to AP-1 sites containing the TRE sequence (5'-TGAG/CTCA-3') in the promoter regions of target genes through its DNA-binding domain, regulating gene expression (Chiu et al., 1988). It is important to note that AP-1 consists of dimers formed by members of the Fos, Jun, ATF, and Maf families. While Fos, ATF, and Maf proteins require heterodimerization, members of the Jun family can also form homodimers. Numerous AP-1 combinations are therefore possible, with partially overlapping functions and gene targets, although the specific roles of many of these dimeric configurations remain unknown (Khan et al., 2025). One of the most studied IEGs is c-Fos, which contains a kinase-binding domain, a transactivation domain, a DNA-binding domain, and a leucine zipper. The leucine zipper is essential for dimerization with Jun family proteins to form the AP-1 complex. In this configuration, AP-1 generally functions as an activator of genes containing TRE sequences (Morgan and Curran, 1989; Curran and Morgan, 1995; Chiu et al., 1998). In contrast, when c-Fos dimerizes with ATF family members, the resulting AP-1 complex can act as a repressor (Lucibello et al., 1989). The kinase-binding domain, which is also present in c-Jun, is critical for AP-1 activation and for its translocation to the nucleus (Smeal et al., 1991). Numerous stimuli, beyond neuronal activity, can lead to the activation of this complex, including growth factors and inflammatory cytokines. These signals activate mitogen-

activated protein kinases (MAPKs), such as ERK, p38, and JNK, through the RAS-Raf-MEK or Rac-MEKK-MKK pathways (Deng and Karin, 1994; Chang and Karin, 2001). Once activated, MAPKs phosphorylate components of the AP-1 complex, leading to its activation (Shaulian and Karin, 2002).

Despite its enormous popularity as an activity marker, the global transcriptomic changes associated with *Fos* expression have been poorly investigated. *Fos* deletion in the dentate gyrus leads to the misregulation of approximately 200 genes (Benito and Barco, 2014), whereas its overexpression in hippocampal neurons results in the upregulation of nearly 300 differentially expressed genes (Benito et al., 2014). However, the cisRED database for conserved regulatory elements indicates that 1703 regions in the mouse genome contain c-Fos interaction motifs (Robertson et al., 2006). It is possible that the exact number and identity of genes regulated by c-Fos depend on the specific chromatin accessibility landscape of each cell type and brain region. Numerous studies have shown that c-Fos expression depends on neuronal activity and is strongly associated with memory processes (Vann et al., 2000; Bertaina-Anglade and Destrade, 2000; Maviel et al., 2004). c-Fos is robustly induced during spatial exploration, spatial novelty, exposure to a novel object, a novel taste, or a novel tone, with marked differences in the brain regions activated depending on the nature of the cue (Keilmann and Herdegen, 1997; Tischmeyer and Grimm, 1999; Okuno, 2011; Aggleton et al., 2012; Nonaka et al., 2014; Chen et al., 2020). Moreover, blocking c-Fos activation in the hippocampus impairs several forms of memory, including water-maze learning, radial-arm maze performance, contextual fear learning and retrieval, and the extinction of conditioned taste aversion (He et al., 2001; Guzowski, 2002; Countryman et al., 2004; Katche et al., 2009). Interestingly, c-Fos knockout mice show selective impairments in hippocampus-dependent spatial and associative learning. These deficits correlate with reduced LTP at CA3-CA1 synapses. LTP can be restored by repeated tetanization, indicating an alteration in LTP induction. This rescue is blocked by a selective NR2B-type NMDA receptor inhibitor, suggesting a specific impairment in this pathway (Fleischmann et al., 2003).

Another IEG that has been widely used to study memory function is *Npas4*, a member of the PAS family characterized by a conserved basic helix-loop-helix motif and a PAS domain. *Npas4* plays a key role in the formation and regulation of inhibitory synapses (Sun and Lin, 2016). Its expression depends on the increase in intracellular calcium that follows the activation of NMDARs and voltage-dependent calcium channels (VDCCs), which in turn activates MAP kinases (p38 and ERK1/2) and CaM kinases (CaMKII and CaMKIV) through distinct signaling pathways. These activated kinases translocate to the nucleus and phosphorylate transcription factors such as ATF2, CREB, and MEF2 at their respective binding sites (e.g., CRE and MEF2) on the *Npas4* promoter, initiating *Npas4* transcription (Fu et al., 2020). Overall, increased NPAS4 activity enhances the number of inhibitory synapses formed onto pyramidal neurons. Reducing *Npas4* expression decreases the density of inhibitory inputs onto hippocampal pyramidal neurons, whereas its overexpression increases them (Lin et al., 2008). The selective deletion of *Npas4* in somatostatin-expressing interneurons lowers the density of excitatory synapses onto these cells. Thus, NPAS4 regulates excitatory-inhibitory balance by controlling the formation of

both inhibitory and excitatory synapses, maintaining circuit homeostasis essential for information processing and memory formation (Spiegel et al., 2014). The distinct synaptic roles of *Fos* and *Npas4* suggest that these genes can be used to tag neuronal ensembles activated by the same cue but serving different functions. Sun and colleagues demonstrated that contextual fear engrams in the DG comprise two genetically and functionally distinct neuronal populations defined by their expression of *Fos* or *Npas4*. The *Fos*-dependent ensemble promotes memory generalization and receives stronger excitatory input from the MEC. In contrast, the *Npas4*-dependent ensemble supports memory discrimination and is preferentially targeted by local CCK-expressing interneurons. Chemogenetic manipulations showed that selectively activating or inhibiting these populations shifts behavioral performance toward generalization or discrimination. Consistent with this, MEC inputs to the DG drive generalization, whereas CCK+ interneurons are specifically required for discrimination (Sun et al., 2020). These findings indicate that functionally distinct neuronal ensembles can coexist within a single memory engram, with each ensemble supporting distinct aspects required for correct memory retrieval.

Another important IEG is *Arc*, a critical mediator of synaptic plasticity and memory. The role of *Arc* is more complex than that of many other IEGs, as it depends on local dendritic transport and translation, interactions with multiple synaptic proteins, and a uniquely specialized protein structure (Sullivan et al., 2025). *Arc* contains retroviral Gag-like elements that originated from the Ty3 retrotransposon family. The retroviral Gag domain encodes a polyprotein involved in the formation of retroviral capsids (Campillos et al., 2006; Cottee et al., 2020). Because of these elements, the *Arc* protein assembles into virus-like particles capable of encapsulating mRNAs and transferring them to neighboring neurons, a function that has been implicated in learning and memory (Ashley et al., 2018). The transcription of *Arc* depends on activity-dependent calcium influx through VDCCs and activation of the MAPK/ERK pathway. After transcription, *Arc* mRNA binds RNA granules and translation regulatory proteins, including fragile X mental retardation protein (FMRP) to form a complex that is rapidly transported to dendrites via a kinesin and microtubule-dependent mechanism (Kanai et al., 2004; Wilkerson et al., 2018). This dendritic transport depends on the sequence of its 3'-UTR (Chen et al., 2023). Once in the dendrites, *Arc* facilitates the internalization of AMPA receptors from the synaptic surface and plays a crucial role in both Hebbian (LTP and LTD) and non-Hebbian (homeostatic) forms of plasticity (Khan et al., 2025). A large body of evidence shows that *Arc* is directly involved in the expression of LTD across hippocampal, cortical, and cerebellar circuits (Waung et al., 2008; McCurry et al., 2010; Smith-Hicks et al., 2010; Wall et al., 2018). The role of *Arc* is particularly important for mGluR-dependent LTD (Park et al., 2008). This effect is mediated by *Arc*'s regulation of AMPAR trafficking, achieved through its interactions with dynamin, endophilin, and the clathrin adaptor protein AP-2 (Chowdhury et al., 2006; Shepherd et al., 2006), as well as by its ability to interfere with the PSD-95-TARP complex (Chen et al., 2022). The role of *Arc* in LTP is more debated. *Arc* knockout mice show intact NMDA-dependent LTP in both CA1 and the dentate gyrus, suggesting that *Arc* is not required for LTP expression (Kyrke-Smith et al., 2021). LTP robustly induces *Arc* transcription, and reducing *Arc* levels blocks BDNF-induced LTP, indicating that *Arc* may be necessary for specific forms or phases of LTP (Messaoudi et al.,

2007). The mechanism by which Arc influences LTP is thought to involve the regulation of actin polymerization and its interaction with the β -isoform of CaMKII. Arc may contribute to heterosynaptic LTD by preferentially targeting weak synapses, where its interaction with CaMKII β promotes AMPAR removal from non-potentiated synapses in a neuron undergoing LTP (Okuno et al., 2012). Such heterosynaptic plasticity may help maintain overall synaptic strength within a controlled dynamic range, preventing the saturation of potentiated synapses and contributing to the homeostatic regulation of synaptic transmission (Sullivan et al., 2025). Another important role of Arc is in homeostatic scaling, the process by which neurons adjust AMPAR levels in response to chronic changes in activity (Shepherd et al., 2006). Arc KO mice fail to exhibit homeostatic scaling in vivo, as shown in visual cortex slices following dark exposure (Gao et al., 2010). Similarly, dysregulation of Arc expression in UBE3A knockout neurons, a model of Angelman syndrome, leads to deficits in the homeostatic scaling of AMPARs (Pastuzyn and Shepherd, 2017). The complexity of Arc's effects on neuronal plasticity reflects its critical role in memory. In fact, Arc is required for the consolidation of several forms of long-term memory, including fear memory, long-term spatial memory, and inhibitory avoidance, while Arc deficiency does not alter short-term memory performance (Guzowski et al., 2000; Plath et al., 2006; Czerniawski et al., 2011; Chia and Otto, 2013; Onoue et al., 2014). Because its expression reliably marks neurons engaged by ongoing experience, Arc has been widely used to tag activated ensembles. Consistent with its role in memory consolidation, optogenetic inhibition of hippocampal neurons expressing Arc during the acquisition of contextual fear memory suppresses subsequent memory retrieval, demonstrating that Arc-expressing ensembles are functionally necessary for the stabilization and later expression of the memory trace (Denny et al., 2014). Finally, a recent work emphasized the distinction between Arc and c-Fos comparing their expression across brain regions and memory states during contextual fear conditioning. By quantifying both proteins after context exposure and fear conditioning at multiple post-encoding and retrieval time points, Bulthuis and colleagues found that Arc and c-Fos exhibit markedly divergent expression patterns. In the DG, Arc and c-Fos tend to label overlapping yet partly distinct neuronal populations, with memory state exerting only a minor influence on their expression. In the BLA and ventral RSP, but not in the ventral ACA, memory state influenced the relative expression of Arc and c-Fos. Although Arc and c-Fos expression patterns were not fully independent, as shown by co-labeling rates consistently above chance in every region, these findings strongly indicate that factors beyond neuronal activity itself determine whether Arc or c-Fos is expressed, suggesting that they cannot be treated as interchangeable markers of memory-related activity (Bulthuis et al., 2025).

2.7 Brain-wide approach for the study of memory

Much of our current understanding of neural mechanisms has come from studies targeting single regions or circuits, an approach that remains essential for identifying the functional role of specific brain structures. However, emerging data from multi-region recordings and activity-mapping techniques indicate that local computations may be part of wider circuits involving several interconnected brain areas (Wheeler et al., 2013; Vetere et al., 2017; Grewe et al., 2017; Musall et al., 2019; Stringer et al., 2019; Salkoff et al., 2019; Gilad and Helmchen, 2020; Terstege et al., 2022; Roy et al., 2022; Shine et al., 2023; Angelaki et al.,

2025). Across sensory, associative, and subcortical structures, populations of neurons participate concurrently in representing stimuli, integrating information, and producing appropriate behavioral outputs. The development of tissue clearing techniques, such as CLARITY (Chung et al., 2013) and iDISCO (Renier et al., 2014), combined with advanced microscopy (Hillman et al., 2019), has enabled high-throughput analyses of intact mouse brains. Similarly, advances in the miniaturization of high-density probes allow simultaneous recordings from multiple brain regions using hundreds of channels (Jun et al., 2017). These technological improvements have greatly expanded the ability to investigate brain-wide neural activity. Recently, a major scientific collaboration generated a comprehensive brain-wide map of neural activity in mice performing a complex decision-making task, using nearly 700 Neuropixels probes across 139 animals (Angelaki et al., 2025). This effort provided an unprecedented view of how distributed neural circuits encode sensory inputs, choices, actions, highlighting that complex processes such as decision-making and reward representation are remarkably widespread across the brain. Also, the brain-wide detection of immediate early genes provides a powerful tool to map how neuronal populations across the entire brain are activated by a given stimulus (Wheeler et al., 2013; Vetere et al., 2017; Franceschini et al., 2023). This strategy has been applied to contexts such as drug administration, sensory stimulation, social and parental behaviors, and fear memory (Wheeler et al., 2013; Renier et al., 2016; Ye et al., 2016; Vetere et al., 2017; DeNardo et al., 2019; Roy et al., 2022; Hu et al., 2024; Aboharb et al., 2025). It is worth noting that, although detecting significant activation in a given brain region does not satisfy the classical criteria for identifying an engram, it can still provide a useful indication of where functionally relevant neuronal populations may reside.

Among immediate early genes, c-Fos has emerged as a popular marker in brain-wide activity-mapping studies. Its broad and reliable induction across diverse brain regions, coupled with its responsiveness to a wide spectrum of experimental stimuli, makes it a preferred marker for large-scale analyses. However, c-Fos also presents several limitations as a marker of neuronal activity. Its expression depends on the molecular and electrophysiological properties of specific brain regions and cell types. For example, the accumulation of Δ FosB in the DG can suppress c-Fos induction, decoupling its expression from neuronal activity under certain conditions (Hope et al., 1994; Lamothe-Molina et al., 2022). In the DG, c-Fos levels may also fail to distinguish subtle differences in memory states (Bulthuis et al., 2025). Furthermore, recent data suggest that a non-negligible subset of c-Fos-positive cells are glial cells (Cruz-Mendoza et al., 2022), and coordinated c-Fos expression in astrocyte ensembles can causally influence performance during contextual fear memory recall (Williamson et al., 2024). Preliminary evidence by Oertner and colleagues suggest that in hippocampal neurons, very low-frequency stimulation (0.1 Hz) induces higher c-Fos expression than high-frequency stimulation (50 Hz). Intermediate frequencies (1-5 Hz) largely fail to induce c-Fos. These findings suggest that c-Fos induction is not simply a cell-autonomous response to spike-induced calcium influx, but rather a network phenomenon: individual neurons detect peaks in extracellular glutamate via mGluR activation, integrating this signal over extended periods (~1 h). These observations suggest that c-Fos-positive neurons may not necessarily have fired at high frequency but rather are

characterized by lasting participation in highly synchronized network activity (Anisimova et al., 2023).

In a seminal study on brain-wide activity mapping, Frankland and colleagues analyzed c-Fos expression across 84 brain regions to identify areas co-active during memory recall. Mice underwent contextual fear conditioning, learning to associate a context with mild footshocks, and memory recall was assessed via freezing behavior, which remained stable at both 1 and 36 days. To assess neuronal population activation across the entire brain, c-Fos protein expression was detected using immunohistochemistry. Comparisons with unshocked control mice revealed that c-Fos expression patterns depended on the interval between training and memory recall: at 1 day, c-Fos activation was mainly observed in hippocampal regions, whereas at 36 days, activation shifted to distributed cortical, thalamic, hypothalamic, and subcortical areas, demonstrating that memory recall induces region-specific neuronal activation that evolves over time (Wheeler et al., 2013). To test for significant differences between selected brain regions, they used task PLS (partial least squares) analysis, a multivariate statistical technique that identifies optimal patterns of activity differentiating experimental conditions (McIntosh, 1999). PLS detects similarities and differences between groups by finding brain regions whose activation varies with the condition. Using singular value decomposition, PLS generates a set of mutually orthogonal latent variable (LV) pairs: one element represents the contrast, reflecting similarities or differences between conditions, while the brain region salience identifies regions whose activation patterns across tasks contribute most strongly to the LV. For brain region salience, reliability was assessed using bootstrap estimation of the standard error. Regions with a bootstrap ratio exceeding a chosen threshold were considered most important for differentiating conditions. An advantage of this approach over univariate methods is that no corrections for multiple comparisons are needed, as brain region saliences are computed across all regions simultaneously in a single mathematical step (McIntosh et al., 1996; Wheeler et al., 2013). To characterize which regions were co-activated following memory recall, they used a graph theory approach to calculate a functional connectivity profile of c-Fos expression. Graph theory is a branch of mathematics used to study relationships within large-scale systems, defining nodes and the edges that connect them. Using this approach, they calculated pairwise correlations of c-Fos expression across all brain regions, with each correlation defining an edge between nodes representing neuroanatomical regions. Measures such as network density and global efficiency provide insight into network-wide information flow, while clustering coefficients and local efficiency assess the extent to which information processing is localized within specialized subpopulations. Global network parameters characterize the overall organization of the system, which is often analyzed to determine whether it exhibits a small-world topology, a feature commonly observed in efficient biological networks (Terstege and Epp, 2023). Interestingly, functional connectivity analysis supported contextual fear memory changes over time, reflecting systems consolidation. Memories are initially encoded in hippocampal-cortical networks, but with increasing retention delay, inter-cortical connectivity strengthens, and the prefrontal cortex becomes more engaged, while hippocampal activity overall declines. Nonetheless, hippocampal connectivity with cortical and thalamic regions remains strong, suggesting a

sustained but more integrated role in remote memory retrieval. As the retention delay increases, functional connections become more numerous and involve a wider set of areas, reflecting a more extensive and integrated network that likely supports the integration of new information. Central hubs, particularly in prefrontal, thalamic, and cortical regions, emerge in networks supporting remote memory, reflecting their critical role in coordinating distributed activity necessary for long-term memory retrieval (Wheeler et al., 2013). However, this study identified brain regions activated during memory retrieval, without directly demonstrating whether these regions contain engram cells. In this context, a more informative brain-wide approach was presented by Roy and colleagues, who designed an experiment to identify engram cell ensembles for contextual fear memory. The authors used Fos-TRAP mice to map neurons activated during contextual fear memory encoding and recent recall, quantifying activity across 247 brain regions. To identify the most promising candidates, they developed an engram index to rank putative engram ensembles for further investigation. After determining which regions contained tagged neurons that were reliably reactivated during recall, they performed targeted optogenetic manipulations, providing causal evidence that several of these regions contain neurons with properties of engram cells. Notably, this approach not only confirmed that previously established regions, such as the hippocampus and BLA, contain engram ensembles for contextual fear memory but also revealed additional areas that had not been implicated before. These newly identified regions include brainstem nuclei (e.g., LDTg), hypothalamic structures (e.g., AHN), thalamic nuclei (e.g., RE, AM, PVT), cortical areas (e.g., VLO), and midbrain regions (e.g., PAG). Moreover, several areas that displayed stronger activation during recall than during encoding (e.g., dSUB, Ol, LH, and AV) likely supported retrieval-related processes (Roy et al., 2022). This work is particularly important because it supports the idea, originally proposed by Richard Semon, that memories are not stored in a single engram cell ensemble but are instead distributed across multiple, interconnected neuronal populations (Semon, 1904). Their results support the presence of multiple engram ensembles distributed across numerous brain regions. Optogenetic activation of a single engram ensemble was sufficient to recruit other anatomically distinct engram populations, suggesting a degree of functional connectivity among engram ensembles distributed throughout the brain.

2.8 Disruption of memory engrams in neurodegeneration

Memory loss is a hallmark of many neurodegenerative disorders, most notably Alzheimer's disease, the leading cause of dementia (Li et al., 2022). Memory impairments in AD have been linked to the development of its two classical pathological hallmarks observed in later stages: extracellular amyloid- β plaques and intracellular tau protein aggregates (Selkoe et al., 2001; John and Reddy, 2022). Decades of research on postmortem Alzheimer's disease brains and transgenic mouse models have shown that the disease involves multiple cellular alterations, including neuronal and synaptic loss, abnormal mitochondrial functions, glial dysfunction, disrupted neurotransmitter signaling, neurofibrillary tangles, and amyloid plaques (Selkoe, 2002; Reddy et al., 2011; Uddin and Lim, 2022; Congdon et al., 2023; Akyuz et al., 2024; Rohden et al., 2025). Several studies have proposed that episodic memory deficits in Alzheimer's disease patients largely reflect impaired encoding of new information (Weintraub et al., 2012). However, because most cognitive assessments rely on

memory retrieval, it remains difficult to distinguish deficits in encoding from impairments in accessing previously stored memories (Roy et al., 2016). Investigating memory deficits in terms of engram formation and stability may help to provide insights into how AD affects the cellular substrates of memory at different stages. For instance, Roy and colleagues showed that 7-month-old APP/PS1 mice exhibit an early impairment in contextual fear memory, characterized by a deficit in long-term memory recall while short-term memory remains intact. Remarkably, optogenetic reactivation of DG neurons tagged during contextual fear conditioning was sufficient to restore freezing behavior in mice that showed retrograde amnesia, indicating that the memory trace was intact but inaccessible. This model also exhibited reduced dendritic spine density in DG engram neurons, a deficit that could be rescued by inducing LTP in the EC-DG pathway (Roy et al., 2016). Similarly, Perusini and colleagues investigated memory dysfunction in APP/PS1 mice crossed with ArcCreERT2 and Chr2-EYFP lines, generating a model that enables tagging and manipulating activity-defined neuronal ensembles in a pathological context. Using this model, they reported impaired contextual fear memory at 6 months, accompanied by reduced reactivation of DG neurons tagged during learning. Notably, optogenetic reactivation of these DG ensembles rescued memory performance, also increasing the reactivation rate in the targeted DG region (Perusini et al., 2017). In another important work, Poll and colleagues investigated how neuronal ensembles representing specific memories fail to be properly reactivated during natural recall. Based on the hypothesis that contextual fear memory ensembles persist but become non-functional, they used an APP/PS1 x FosGFP model to track FosGFP expression in dorsal CA1 neurons during memory acquisition and recall. They revealed two subpopulations of CA1 neurons likely representing stable or variable contextual components of the environment. In APP/PS1 mice, learning triggered a normal increase in c-Fos expression in CA1, and optogenetic activation of this ensemble was sufficient to rescue freezing behavior. Interestingly, analysis of the activity history of individual neurons across trials identified a subpopulation that expressed FosGFP for the first time during memory recall, present only in mice with impaired memory or when exposed to a novel context, likely representing “novelty-like” cells. This finding suggests that APP/PS1 mice may perceive a familiar context as novel, leading these novelty-like ensembles to interfere with the correct memory recall. Indeed, chemogenetic activation of neurons recruited during exposure to a novel context mimicked the interference caused by novelty-like ensembles, reducing freezing in the conditioned context. Silencing this ensemble to suppress novelty-like cell activity during recall restored memory performance. Notably, they showed that engram formation and sparseness remain largely intact in this model, but recall is impaired due to interference from a novelty-like ensemble, highlighting the impact of network-level competitive interactions on memory retrieval (Poll et al., 2020). Together, this evidence suggests that memory impairment in AD involves specific subpopulations of neurons implicated in mnemonic processing that exhibit deficits in reactivation and in structural and functional plasticity, representing potential targets for therapeutic intervention.

3. Aims of the Thesis

Previous work has established a direct role of the LEC in the recall of episodic-like memory in mice. In particular, retrieval of OPC associations is accompanied by an increase in c-Fos expression in the LEC, specific changes in synaptic plasticity, and the recruitment of learning-tagged neuronal populations that exhibit properties of engram cells (Tozzi et al., 2024). In line with these findings, the first aim of this thesis was to further examine the contribution of learning-tagged LEC ensembles to episodic-like memory by investigating their role in the deficits observed in a mouse model of Alzheimer's disease, which is characterized by early synaptic dysfunction and neuronal degeneration (Braak and Braak, 1991; Pause et al., 2013). First, we characterized c-Fos expression in the LEC and other subregions of the MTL at 2 and 6 months of age following the encoding of the OPCRT, in order to assess the activation of their neuronal populations to behavioral stimulation. In addition, we performed chemogenetic manipulations of learning-tagged neuronal populations in the LEC during the OPCRT and in the DG during the ORT to determine their contribution to memory deficits and to test whether reactivation of these ensembles could rescue memory performance. Finally, we examined alterations in dendritic spine morphology in learning-tagged neuronal populations to investigate whether deficits in structural plasticity are affected by chemogenetic intervention.

However, focusing on the entorhinal-hippocampal system alone may not fully capture the complexity of the neural mechanisms underlying episodic-like memory. While these structures are important hubs for memory formation, recent evidence suggests that individual memory traces are supported by distributed networks of engram cells across multiple cortical and subcortical regions (Roy et al., 2022). This idea suggests that episodic-like memory may be better understood as a system-level process, in which critical brain regions operate in a broader network rather than as a function localized to a single structure.

Accordingly, the second aim of this thesis was to investigate episodic-like memory at the brain-wide level, testing the hypothesis that memory retrieval engages a distributed network extending beyond the medial temporal lobe system. To this end, we performed a brain-wide mapping of neuronal activation during the recall phase of the OPCRT to identify neuronal populations involved in this form of memory. We generated a multi-level dataset of c-Fos⁺ cells across the brain and applied anatomical and functional network analyses to characterize the pattern of activation associated with OPC memory. Moreover, we employed chemogenetic manipulations of learning-tagged neuronal populations in the identified brain regions to dissect their contribution to memory retrieval. Finally, we focused on the mPFC and RSP to characterize their activity during episodic memory recall using in vivo electrophysiology.

4. Results

4.1 OPC memory encoding is not detectably altered in young APP mice

Using a transgenic mouse model that overexpress human APP with two mutations linked to familial AD (the Swedish and Indiana mutations, the APPJ20 strain) (Mucke et al., 2000), it has been previously shown that synaptic function and dendritic spine morphology in the EC are altered prior to detectable amyloid plaque accumulation, leading to impairments in episodic-like memory retrieval as assessed by the OPCRT. Subsequently, synaptic dysfunction extends to the primary entorhinal target region, the hippocampus, resulting in deficits in non-associative memory, as measured by the novel object recognition task (ORT) (Criscuolo et al., 2017). However, it remains unclear whether episodic memory impairments in Alzheimer's disease primarily arise from alterations in memory encoding or from deficits in memory retrieval. To address this question, we first examined potential alterations in behavioral performance during the learning phase of the OPCRT in 2-month-old APP mice (Figure 1A). During the learning, APP mice and age-matched wild-type (WT) controls spent a comparable amount of time exploring the objects (Figure 1B and 1C), with no preference observed for either object across the two configurations (Figure 1D and 1E). In addition, no significant differences were detected in the total distance traveled in the arena during either trial, indicating similar levels of exploratory activity and motor behavior (Figure 1F and 1G). These findings suggest that, during OPC memory encoding, young APP mice do not exhibit detectable alterations in behavioral performance compared to WT controls. To determine whether episodic-like memory encoding is associated with comparable activation of MTL regions, we quantified the number of neurons expressing the immediate early gene c-Fos 90 minutes after the learning phase (Figure 1H). No significant differences in c-Fos⁺ cell density were observed between young APP mice and age-matched WT controls in the DG, CA1, CA3, or in either subdivision of the entorhinal cortex (LEC and MEC). Baseline c-Fos expression was also assessed in home-cage (HC) control animals, revealing no significant differences between 2-month-old WT and APP mice (Figure 1I). Consistently, following behavioral stimulation, both groups exhibited comparable levels of c-Fos activation across medial temporal lobe regions (Figure 1J). Together, these findings indicate that, at 2 months of age, APP mice display intact behavioral performance during the learning phase of the OPCRT and a preserved pattern of c-Fos activation within the MTL, suggesting that the encoding of OPC associations might be unaffected at this early stage.

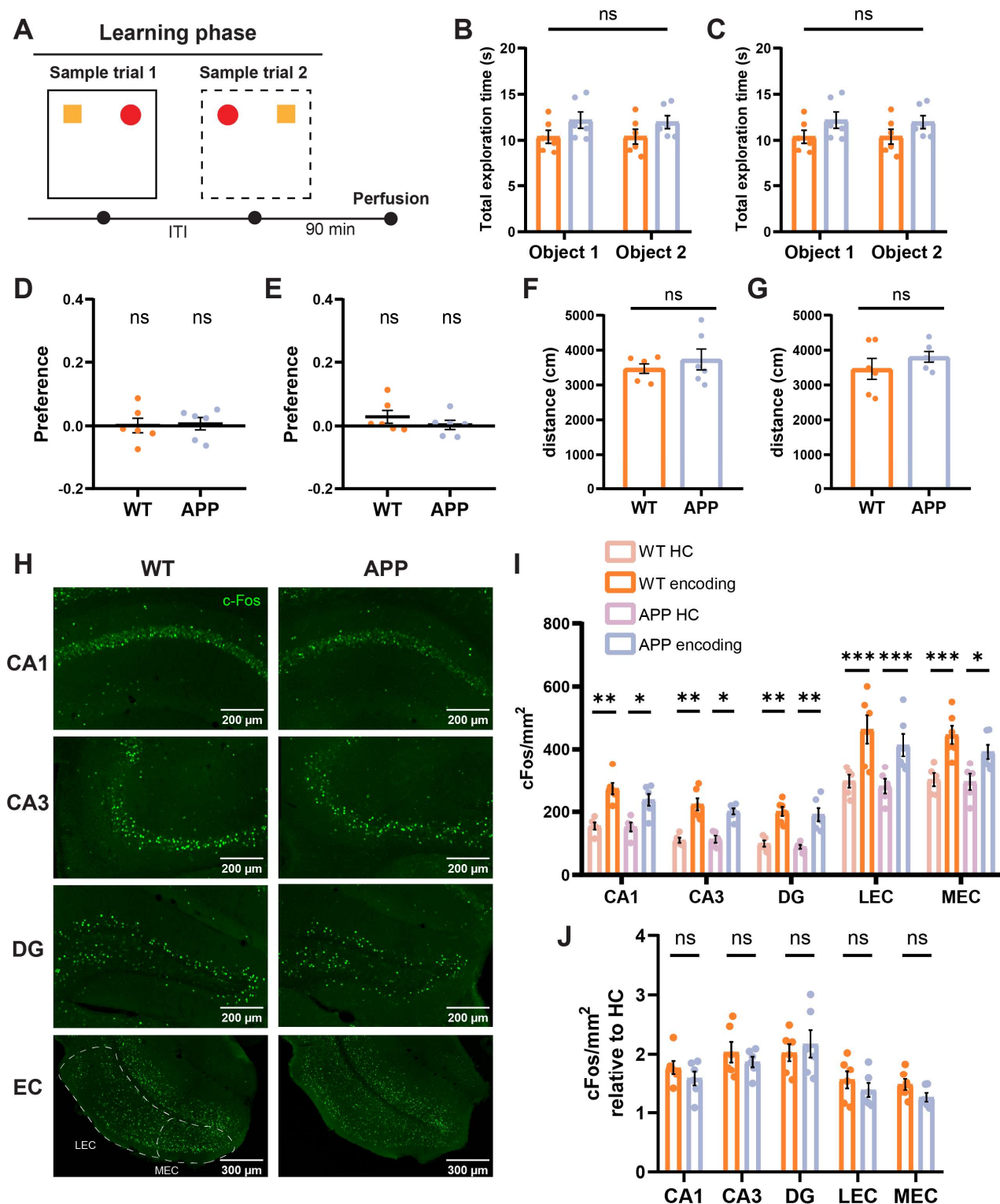


Figure 1. Behavioral analysis and c-Fos quantification showed no differences in OPC memory encoding in 2-month-old APP mice.

(A) Schematic representation of the learning phase of the OPCRT, with animal perfusion that was performed 90 minutes after the end of Sample trial 2. (B) Total exploration time during Sample trial 1 showed no differences between groups (two-way ANOVA with Sidak's multiple comparisons test: WT Object 1 10.39 ± 0.70 $n = 6$ vs APP Object 1 12.19 ± 0.89 $n = 6$, ns $p = 0.2223$; WT Object 2 10.40 ± 0.80 $n = 6$ vs APP Object 2 11.97 ± 0.70 $n = 6$, ns $p = 0.31$). (C) Total exploration time during

Sample trial 2 showed no differences between groups (two-way ANOVA with Sidak's multiple comparisons test: WT Object 1 9.87 ± 0.90 $n = 6$ vs APP Object 1 11.03 ± 1.01 $n = 6$, ns $p = 0.6293$; WT Object 2 7.85 ± 0.67 $n = 6$ vs APP Object 2 11.01 ± 1.09 $n = 6$, ns $p = 0.3583$). (D) During Sample Trial 1, mice showed no preference for either object, with exploration not differing from chance (WT 0.028 ± 0.02 , $n = 6$, ns $p = 0.9575$, $df = 5$, $t = 0.055$; APP 0.003 ± 0.014 , $n = 6$, ns $p = 0.7437$, $df = 5$, $t = 0.3456$; one-sample t test) or between groups (WT vs APP, ns $p = 0.8585$, $df = 10$, $t = 0.1829$, two-tailed unpaired t test). (E) During Sample Trial 2, mice showed no preference for either object, with exploration not differing from chance (WT 0.001 ± 0.02 , $n = 6$, ns $p = 0.2168$, $df = 5$, $t = 1.413$; APP 0.006 ± 0.02 , $n = 6$, ns $p = 0.8293$, $df = 5$, $t = 0.2271$; one-sample t test) or between groups (WT vs APP, ns $p = 0.3302$, $df = 10$, $t = 1.023$, two-tailed unpaired t test). (F) No significant difference in the distance travelled during Sample trial 1 (WT 3470 ± 136.7 vs APP 3732 ± 301.8 , ns $p = 0.4469$, $df = 10$, $t = 0.7917$, unpaired t test). (G) No significant difference in the distance travelled during Sample trial 2 (WT 3460 ± 299.5 vs APP 3805 ± 154.7 , ns $p = 0.3302$, $df = 10$, $t = 1.023$, unpaired t test). (H) Representative images showing c-Fos expression (green) in the regions of interest for both groups, 90 minutes after the end of the learning phase. Scale bars are shown in the figures. (I) A significant difference in c-Fos+ density was observed across all regions of interest between the home-cage (HC) and encoding conditions in both WT and APP groups (two-way ANOVA with Sidak's multiple comparisons test: WT HC CA1 154.6 ± 11.69 $n = 5$ vs WT encoding CA1 273.4 ± 17.97 $n = 6$, $**$ $p = 0.0019$; WT HC CA3 109.9 ± 7.75 $n = 5$ vs WT encoding CA3 223.6 ± 19.12 $n = 6$, $**$ $p = 0.0033$; WT HC DG 99.22 ± 9.82 $n = 5$ vs WT encoding DG 200.9 ± 14.37 $n = 6$, $*$ $p = 0.0110$; WT HC LEC 297 ± 20.68 $n = 5$ vs WT encoding LEC 463.4 ± 44.82 $n = 6$, $***$ $p < 0.001$; WT HC MEC 301.7 ± 20.75 $n = 5$ vs WT encoding MEC 446.1 ± 28.80 $n = 6$, $***$ $p < 0.001$; APP HC CA1 152 ± 14.45 $n = 5$ vs APP encoding CA1 237.8 ± 18.62 $n = 6$, $*$ $p = 0.0476$; APP HC CA3 113.2 ± 11.11 $n = 5$ vs APP encoding CA3 201.1 ± 9.61 $n = 6$, $*$ $p = 0.0396$; APP HC DG 88.83 ± 6.37 $n = 5$ vs APP encoding DG 190.9 ± 20.45 $n = 6$, $*$ $p = 0.0106$; APP HC LEC 281.1 ± 23.48 $n = 5$ vs APP encoding LEC 413.3 ± 35.61 $n = 6$, $***$ $p < 0.001$; APP HC MEC 294.9 ± 25.93 $n = 5$ vs APP encoding MEC 392 ± 22.76 $n = 6$, $*$ $p = 0.0171$). (J) No difference in c-Fos protein expression following behavioral activation between groups across all regions of interest (two-way ANOVA with Sidak's multiple comparisons test: WT encoding CA1 1.768 ± 0.116 $n = 6$ vs APP encoding CA1 1.584 ± 0.124 $n = 6$, ns $p = 0.8877$; WT encoding CA3 2.034 ± 0.144 $n = 6$ vs APP encoding CA3 1.87 ± 0.089 $n = 6$, ns $p = 0.9282$; WT encoding DG 2.025 ± 0.144 $n = 6$ vs APP encoding DG 2.174 ± 0.233 $n = 6$, ns $p = 0.9501$; WT encoding LEC 1.56 ± 0.15 $n = 6$ vs APP encoding LEC 1.383 ± 0.119 $n = 6$, ns $p = 0.9022$; WT encoding MEC 1.478 ± 0.095 $n = 6$ vs APP encoding MEC 1.262 ± 0.073 $n = 6$, ns $p = 0.8016$). c-Fos density was normalized to the home-cage (HC) control condition. Data are presented as mean $\pm$ SEM.

4.2 Chemogenetic reactivation of LEC learning-tagged neurons restores OPC memory retrieval in 2-month-old APP mice

To investigate whether memory deficits in young APP mice arise from impairments in retrieval rather than encoding, we employed a TRAP-based dual-viral strategy to selectively express chemogenetic receptors in a defined temporal window. This approach has been successfully used to tag and manipulate neurons in the LEC, identifying a subpopulation with properties consistent with engram cells for episodic-like memory (Tozzi et al., 2024). Briefly, CreER^{T2} recombinase is expressed under the control of the c-fos promoter and translocates to the nucleus upon administration of 4-hydroxytamoxifen (4OH-TAM) to drive the expression of the excitatory chemogenetic receptor hM3Dq in the subpopulation of LEC neurons activated during the learning phase of the OPCRT. The activity of the learning-

tagged neuronal population is subsequently manipulated using CNO during memory recall (Figure **2A**). At the behavioral level, 2-month-old APP control mice injected with the AAV encoding the mCherry reporter alone failed to retrieve the episodic-like memory trace when tested 12h after the learning phase. In contrast, chemogenetic activation of learning-tagged LEC neurons in APP mice expressing hM3Dq successfully rescued memory recall at this time point. Behavioral performance in the APP hM3Dq group was significantly different compared to APP mCherry controls but not differ from that of WT mCherry control mice (Figure **2B**, **2C** and **2D**). To assess activation of the medial temporal lobe network following episodic-like memory retrieval, c-Fos⁺ neuron density was quantified 90 minutes after the recall phase of the behavioral task. No significant differences were observed among groups in hippocampal subregions, including the DG, CA1, and CA3, nor in the MEC. In contrast, within the LEC, chemogenetic activation was associated with a significant increase in c-Fos⁺ cell density, indicating that the activation of this specific neuronal population was successfully achieved and may be associated with the observed behavioral effects (Figure **2E**). Further immunofluorescence analysis of learning-tagged LEC neurons revealed that the overlap between mCherry and c-Fos signals was significantly reduced in APP mCherry control mice compared to WT mCherry controls, suggesting a selective impairment in the reactivation of this specific neuronal subpopulation rather than a global reduction in LEC activation. Notably, chemogenetic reactivation of the tagged ensembles induced high levels of c-Fos expression, confirming the effectiveness of the chemogenetic stimulation (Figure **2F** and **2G**). To further assess ensemble reactivation, we quantified the proportion of co-localization between c-Fos and mCherry in neuronal populations relative to chance levels. Interestingly, successful natural recall in WT control mice was associated with a significantly higher reactivation rate compared to APP mCherry control mice (Figure **2H**). In the LEC, chemogenetic activation in APP hM3Dq mice resulted in a significant increase in mean fluorescence intensity relative to APP mCherry controls. This effect was specifically driven by a marked increase in fluorescence within the mCherry⁺ neuronal subpopulation targeted by chemogenetic manipulation, whereas the mCherry⁻ population remained unaffected, indicating that the observed change was selective to the learning-tagged ensemble (Figure **2I**). Together, these findings indicate that young APP mice exhibit a specific deficit in the reactivation of LEC neurons recruited during the learning phase of the OPCRT. Importantly, chemogenetic reactivation rescued memory performance, indicating that OPC memory is encoded in APP mice, but not accessible through natural cues alone.

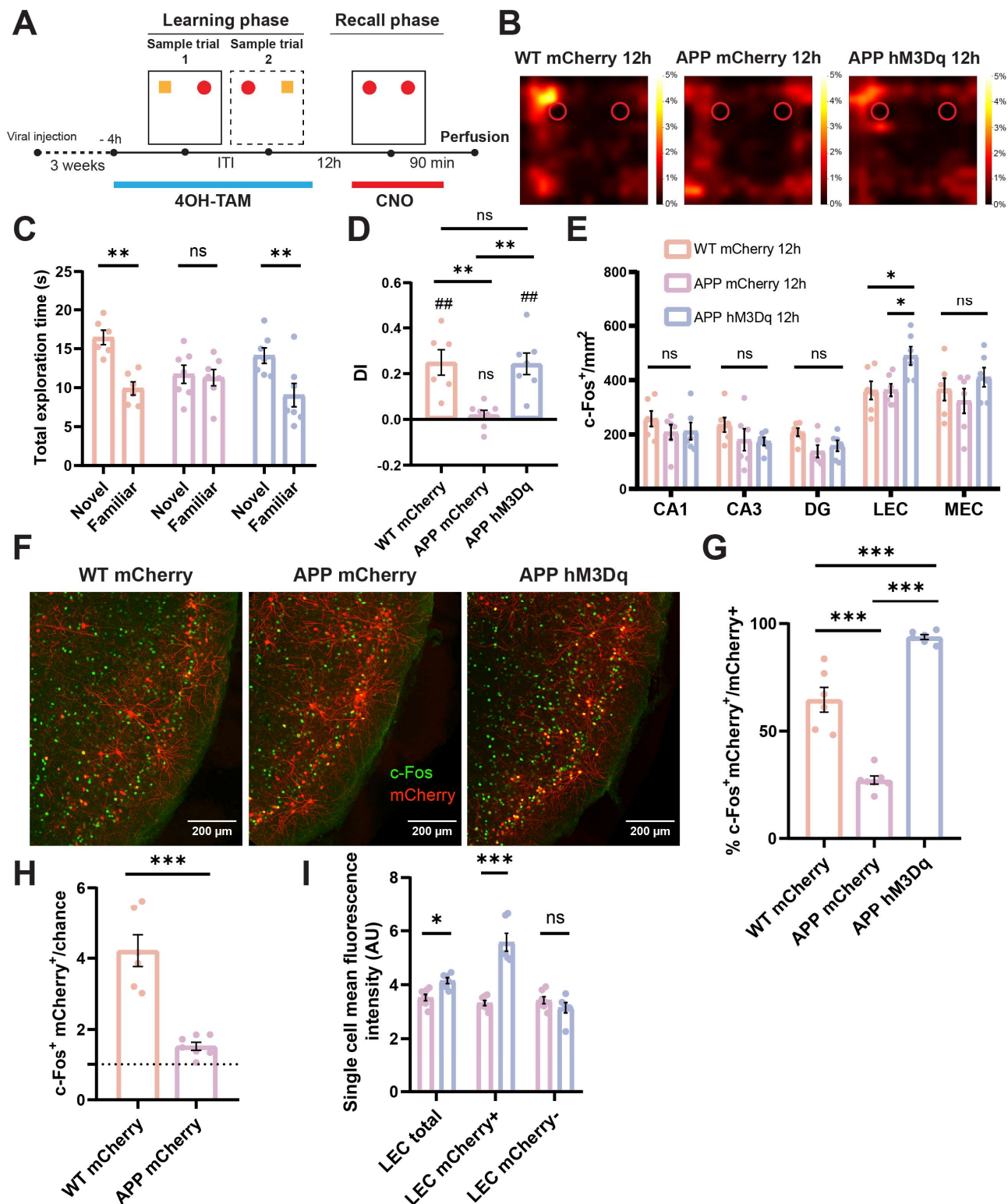


Figure 2. Chemogenetic reactivation of lateral entorhinal cortex learning-tagged neurons restores OPC memory in 2-month-old APP mice.

(A) Schematic representation of the OPCRT: 4OH-TAM treatment was administered 4 hours prior to the learning phase, while CNO injection was given 30 minutes before the recall phase. Animals were perfused 90 minutes after the recall phase. (B) Examples of heatmaps showing the exploratory activity of mice (nose position) during the Recall phase for the three groups. WT control and APP hM3Dq mice exhibited a preference for the novel object, while APP mCherry mice explored both

objects equally. The color bar represents the percentage of total time spent in each position. (C) Analysis of total exploration time revealed a significant exploration of the novel object in both the WT control and APP hM3Dq groups. In contrast, the APP mCherry control group failed to recognize the novel object during the recall phase (two-way ANOVA with Sidak's multiple comparisons test: WT mCherry Novel 16.43 ± 0.93 $n = 6$ vs WT mCherry Familiar 9.9 ± 0.843 $n = 6$, ** $p = 0.0013$; APP mCherry Novel 11.71 ± 1.161 $n = 7$ vs APP mCherry Familiar 11.29 ± 1.05 $n = 7$, ns $p = 0.99$; APP hM3Dq Novel 14.1 ± 0.992 $n = 7$ vs APP hM3Dq Familiar 9.053 ± 1.507 $n = 7$, ** $p = 0.0078$). (D) The discrimination index (DI) analysis showed that the WT control and APP hM3Dq groups exhibited significant exploration of the novel object above chance levels, whereas the APP mCherry control group showed no preference (WT mCherry 0.248 ± 0.055 , $n = 6$, ** $p = 0.0065$, $df = 5$, $t = 4.476$; APP mCherry 0.018 ± 0.02 , $n = 7$, ns $p = 0.3983$, $df = 6$, $t = 0.9091$; APP hM3Dq 0.242 ± 0.046 , $n = 7$, ** $p = 0.002$, $df = 6$, $t = 5.183$; one-sample t test). Additionally, the DI was significantly higher in the WT control and APP hM3Dq groups compared to the APP mCherry control group (one-way ANOVA with Sidak's multiple comparisons test: WT mCherry vs APP mCherry, ** $p = 0.0045$; WT mCherry vs APP hM3Dq, ns $p = 0.9996$; APP mCherry vs APP hM3Dq, ** $p = 0.004$). (E) A significant increase in c-Fos⁺ cell density was detected in the LEC for the APP hM3Dq group compared to the WT and APP mCherry group (two-way ANOVA with Sidak's multiple comparisons test: WT mCherry CA1 258 ± 28.55 $n = 6$ vs APP mCherry CA1 208.2 ± 27.84 $n = 6$, ns $p = 0.4887$; WT mCherry CA1 vs APP hM3Dq CA1 212.3 ± 32.15 $n = 6$, ns $p = 0.5458$; APP mCherry CA1 vs APP hM3Dq CA1, ns $p = 0.9952$; WT mCherry CA3 235.5 ± 26.88 $n = 6$ vs APP mCherry CA3 180.6 ± 40.35 $n = 6$, ns $p = 0.4193$; WT mCherry CA3 vs APP hM3Dq CA3 174.2 ± 14.99 $n = 6$, ns $p = 0.3400$; APP mCherry CA3 vs APP hM3Dq CA3, ns $p = 0.9882$; WT mCherry DG 208.5 ± 14.90 $n = 6$ vs APP mCherry DG 137.9 ± 22.58 $n = 6$, ns $p = 0.2402$; WT mCherry DG vs APP hM3Dq DG 158.5 ± 19.88 $n = 6$, ns $p = 0.4853$; APP mCherry DG vs APP hM3Dq DG, ns $p = 0.8828$; WT mCherry LEC 361.9 ± 33.59 $n = 6$ vs APP mCherry LEC 363.3 ± 23.23 $n = 6$, ns $p = 0.9994$; WT mCherry LEC vs APP hM3Dq LEC 489 ± 34.31 $n = 6$, * $p = 0.0124$; APP mCherry LEC vs APP hM3Dq LEC, * $p = 0.0136$; WT mCherry MEC 365.6 ± 40.89 $n = 6$ vs APP mCherry MEC 323.4 ± 45.13 $n = 6$, ns $p = 0.5960$; WT mCherry MEC vs APP hM3Dq MEC 410.3 ± 35.26 $n = 6$, ns $p = 0.5603$; APP mCherry MEC vs APP hM3Dq MEC, ns $p = 0.1185$). (F) Representative images of mCherry (red) and c-Fos (green) expressing neurons in the LEC of the three groups. Scale bars are shown in the figures. (G) Co-localization analysis of mCherry and c-Fos signals revealed a significant reduction in the APP mCherry group compared to the WT mCherry control. In contrast, a significant increase was observed in the APP hM3Dq group, associated with the chemogenetic activation of learning-tagged LEC neurons (one-way ANOVA with Sidak's multiple comparisons test: WT mCherry 64.57 ± 5.748 $n = 6$ vs APP mCherry 27.17 ± 1.886 $n = 7$, *** $p < 0.001$; WT mCherry 64.57 ± 5.748 $n = 6$ vs APP hM3Dq 93.76 ± 1.151 $n = 7$, *** $p < 0.001$; APP mCherry 27.17 ± 1.886 $n = 7$ vs APP hM3Dq 93.76 ± 1.151 $n = 7$, *** $p < 0.001$). (H) The reactivation of the LEC learning-tagged neurons was measured as the percentage of c-Fos⁺ mCherry⁺ overlap over chance levels. A significant reduction of the reactivation level was observed in the APP mCherry group compared to the WT mCherry group (WT mCherry 4.216 ± 0.447 $n = 6$ vs APP mCherry 1.532 ± 0.112 $n = 7$, *** $p < 0.001$, $df = 11$, $t = 6.262$, unpaired t test). (I) A significant increase in the mean fluorescence intensity of individual cells was found in the total population of c-Fos⁺ LEC neurons of the APP hM3Dq group compared to the APP mCherry control. This difference depends on a strong increase in the mean fluorescence of the mCherry⁺ population but not of the mCherry⁻ (two-way ANOVA with Sidak's multiple comparisons test: APP mCherry LEC total 3.522 ± 0.1214 $n = 7$ vs APP hM3Dq LEC total 4.151 ± 0.11 $n = 7$, * $p = 0.0454$; APP mCherry LEC mCherry⁺ 3.324 ± 0.095 $n = 7$ vs APP hM3Dq LEC mCherry⁺ 5.572 ± 0.33 $n = 7$, *** $p < 0.001$; APP mCherry LEC mCherry⁻ 3.424 ± 0.13 $n = 7$ vs APP hM3Dq LEC mCherry⁻ 3.147 ± 0.191 $n = 7$, ns $p = 0.6091$). Data are presented as mean $\pm$ SEM.

To further assess the functional consequences of chemogenetic manipulation on learning-tagged neurons, we performed whole-cell patch-clamp recordings from LEC neurons expressing hM3Dq. Electrophysiological recordings were consistent with a CNO-induced depolarization of the resting membrane potential and an increase in excitability in hM3Dq-expressing neurons (Figure **3A**, **3B** and **3C**). Moreover, CNO-treated neurons exhibited increased amplitude and frequency of spontaneous synaptic activity (Figure **3D**, **3E**, **3F** and **3G**), along with a step-dependent increase of action potential firing in response to depolarizing current injections (Figure **3H** and **3I**).

To evaluate whether the rescue of episodic-like memory retrieval was specific to LEC ensembles or reflected a more general effect of engram reactivation within the medial temporal lobe, we also targeted learning-tagged neuronal populations in the DG during the OPCRT. Interestingly, chemogenetic reactivation of DG learning-tagged ensembles at both 2 months and 6 months of age failed to rescue behavioral performance in APP mice. This suggests that, in APP mice, reactivation of LEC learning-tagged ensembles is specifically required for the retrieval of OPC memory (Figure **4A**, **4B** and **4C**).

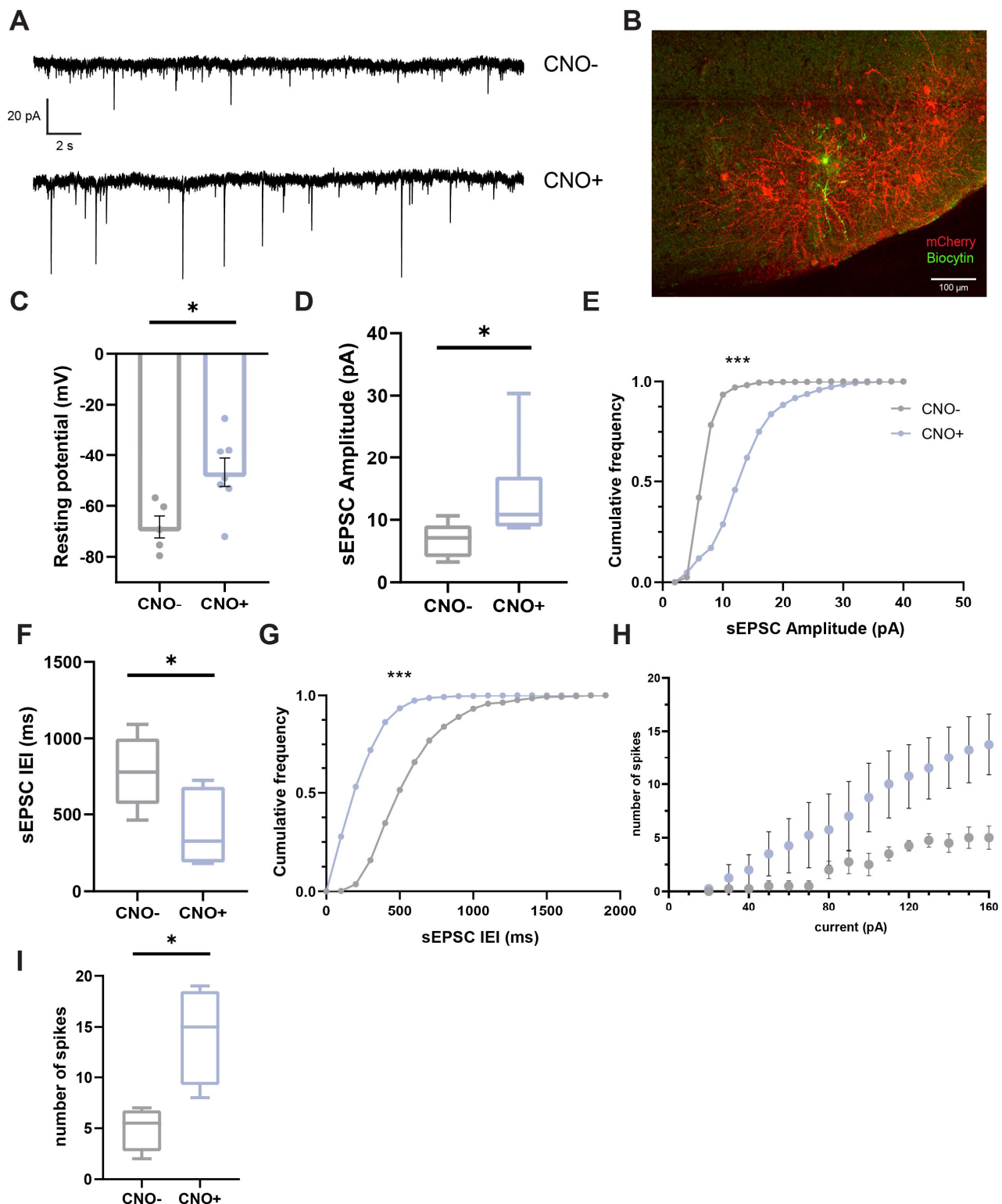


Figure 3. Chemogenetic activation of learning-tagged LEC neurons increases excitability and membrane potential.

(A) Representative traces of spontaneous excitatory postsynaptic currents (sEPSCs) recorded from superficial layer LEC neurons in slices obtained from APP mice following CNO or vehicle treatment. (B) Example of a recorded neuron labeled with mCherry and biocytin (mCherry red, biocytin green). (C) Neurons treated with CNO exhibited a higher resting membrane potential compared to CNO⁻ neurons (unpaired *t* test: CNO⁻ -68.25 ± 4.311 $n = 5$ vs CNO⁺ -46.81 ± 5.580 $n = 6$, * $p = 0.0180$, df

= 10, $t = 2.825$). (D) Neurons treated with CNO exhibited a higher peak amplitude compared to CNO⁻ neurons (Mann-Whitney test: CNO⁻ 6.688 ± 1.262 $n = 5$ vs CNO⁺ 13.71 ± 3.372 $n = 6$, * $p = 0.03$, $U = 3$). (E) Cumulative frequency curves showed that sEPSC peak amplitudes were significantly larger in CNO⁺ neurons than in CNO⁻ neurons (Kolmogorov-Smirnov test: *** $p < 0.001$, $D = 0.6671$). (F) The inter-event interval (IEI) of sEPSCs was significantly shorter in CNO⁺ neurons relative to controls (Mann-Whitney test: CNO⁻ 782.4 ± 106.1 $n = 5$ vs CNO⁺ 401.9 ± 98.06 $n = 6$, * $p = 0.03$, $U = 3$). (G) Cumulative frequency analysis revealed a leftward shift in the distribution of inter-event intervals in CNO-treated neurons (Kolmogorov-Smirnov test: *** $p < 0.001$, $D = 0.5749$). (H) Number of action potentials per current step showed a tendency to increase in CNO⁺ neurons compared to CNO⁻ neurons (two-way repeated-measures ANOVA: interaction $F(14, 84) = 3.945$, $p < 0.001$; current step $F(14, 84) = 23.18$, $p < 0.001$; treatment factor $F(1, 6) = 3.926$, $p = 0.0948$; subject effect $F(6, 84) = 50.93$, $p < 0.001$). (I) The number of action potentials evoked by the maximal current step (160 pA) was significantly higher in CNO⁺ neurons than in controls (Mann-Whitney test: CNO⁻ 5 ± 1.08 $n = 4$ vs CNO⁺ 14.25 ± 2.428 $n = 4$, * $p = 0.0286$, $U = 0$). Each data point represents a single recorded cell; n refers to the total number of patched neurons obtained from 3 APP mice (2 m.o.). Data are presented as mean $\pm$ SEM.

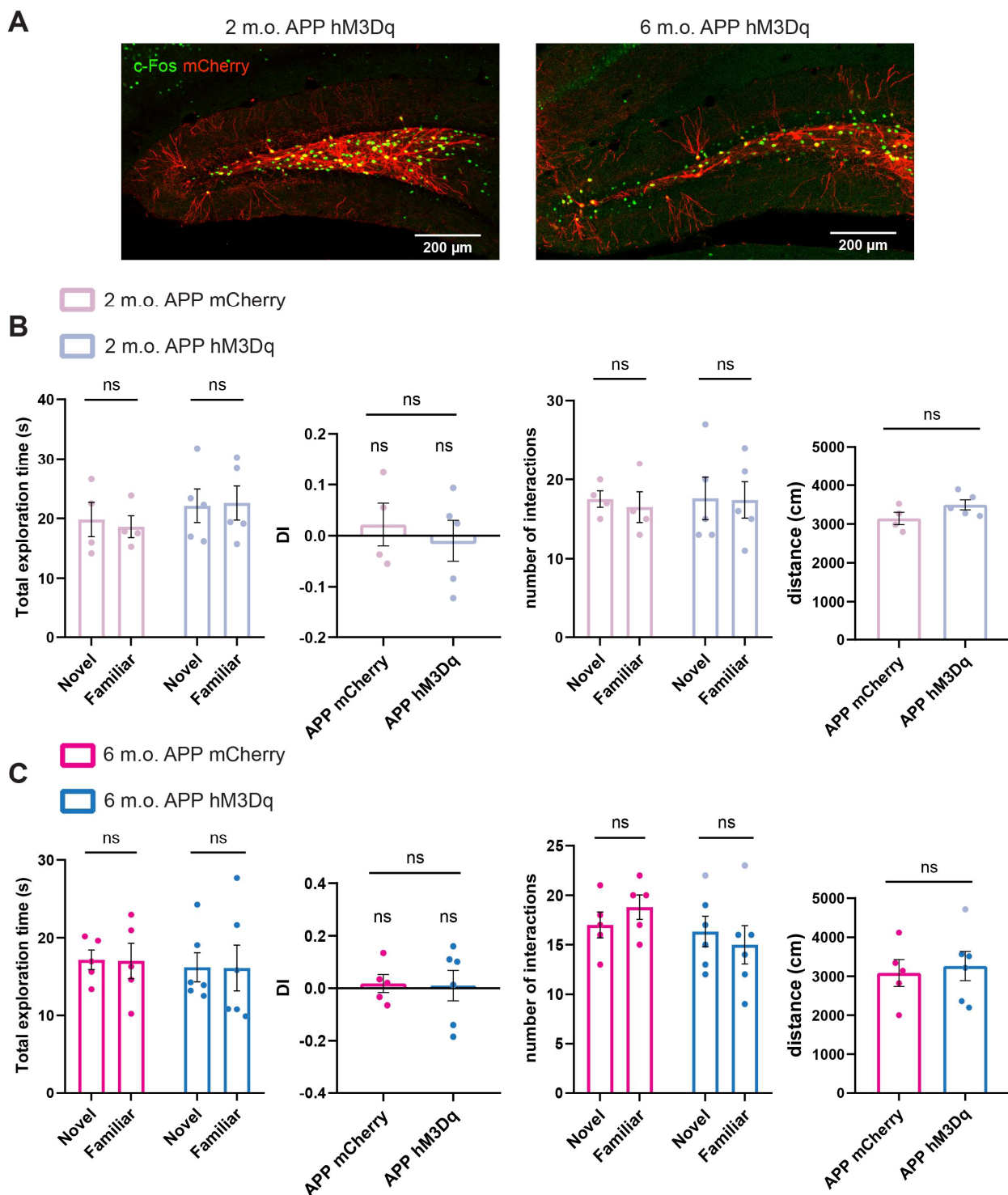


Figure 4. Chemogenetic reactivation of DG learning-tagged ensembles fails to rescue OPC memory in APP mice.

(A) Representative images of mCherry (red) and c-Fos (green) expressing neurons in the DG of 2 m.o. and 6 m.o. APP hM3Dq groups. Scale bars are shown in the figures. (B) Chemogenetic manipulation of DG learning-tagged ensembles in 2 m.o. APP mice failed to rescue memory performance (Time exploration two-way ANOVA with Sidak's multiple comparisons test: APP mCherry Novel 19.80 ± 2.88 $n = 4$ vs APP mCherry Familiar 18.60 ± 1.83 $n = 4$, ns $p = 0.9482$; APP

hM3Dq Novel 22.10 ± 2.80 $n = 5$ vs *APP hM3Dq Familiar* 22.58 ± 2.85 $n = 5$, ns $p = 0.9891$; *DI one-sample t test: APP mCherry* 0.021 ± 0.041 , $n = 4$, ns $p = 0.6383$, $df = 3$, $t = 0.5211$; *APP hM3Dq* -0.01 ± 0.04 , $n = 5$, ns $p = 0.8116$, $df = 4$, $t = 0.2546$; *DI two-tailed unpaired t test: APP mCherry vs APP hM3Dq*, ns $p = 0.6011$, $df = 7$, $t = 0.5474$; *interactions two-way ANOVA with Sidak's multiple comparisons test: APP mCherry Novel* 17.50 ± 1.04 $n = 4$ vs *APP mCherry Familiar* 16.50 ± 1.93 $n = 4$, ns $p = 0.9457$; *APP hM3Dq Novel* 17.60 ± 2.67 $n = 5$ vs *APP hM3Dq Familiar* 17.40 ± 2.29 $n = 5$, ns $p = 0.9972$; *distance two-tailed unpaired t test: APP mCherry* 3142 ± 159.3 , $n = 4$ vs *APP hM3Dq* 3493 ± 129.6 , $n = 5$, ns $p = 0.1272$, $df = 7$, $t = 1.730$). (C) *Chemogenetic manipulation of DG learning-tagged ensembles in 6 m.o. APP mice failed to restore OPC memory performance (Time exploration two-way ANOVA with Sidak's multiple comparisons test: APP mCherry Novel* 17.15 ± 1.26 $n = 5$ vs *APP mCherry Familiar* 17.01 ± 2.27 $n = 5$, ns $p = 0.9989$; *APP hM3Dq Novel* 16.19 ± 1.86 $n = 6$ vs *APP hM3Dq Familiar* 16.11 ± 2.94 $n = 6$, ns $p = 0.9995$; *DI one-sample t test: APP mCherry* 0.018 ± 0.034 , $n = 4$, ns $p = 0.6252$, $df = 4$, $t = 0.5284$; *APP hM3Dq* 0.009 ± 0.058 , $n = 6$, ns $p = 0.8723$, $df = 5$, $t = 0.1692$; *DI two-tailed unpaired t test : APP mCherry vs APP hM3Dq*, ns $p = 0.9112$, $df = 9$, $t = 0.1147$; *interactions two-way ANOVA with Sidak's multiple comparisons test: APP mCherry Novel* 17 ± 1.30 $n = 5$ vs *APP mCherry Familiar* 18.80 ± 1.24 $n = 5$, ns $p = 0.7011$; *APP hM3Dq Novel* 16.33 ± 1.54 $n = 6$ vs *APP hM3Dq Familiar* 15 ± 1.93 $n = 6$, ns $p = 0.79$; *distance two-tailed unpaired t test: APP mCherry* 3083 ± 344.4 , $n = 5$ vs *APP hM3Dq* 3260 ± 374.4 , $n = 6$, ns $p = 0.7396$, $df = 9$, $t = 0.3429$). Data are presented as mean $\pm$ SEM.

4.3 Chemogenetic reactivation of learning-tagged neurons promotes a shift in dendritic spine morphology

Previous work has shown that behavioral deficits in young APP mice are associated with aberrant formation of thin dendritic spines in layer II neurons of the LEC (Criscuolo et al., 2017). To investigate differences between WT and APP mice, as well as the effect of chemogenetic stimulation on dendritic spines, we examined the morphology of apical dendritic spines on learning-tagged pyramidal neurons in Layer II/III. Spine density and morphological features were quantified using three-dimensional reconstructions of selected dendritic segments following chemogenetic stimulation (Figure 5A and 5B). Interestingly, this analysis revealed that spine density was significantly increased in APP mice compared to WT controls (Figure 5C). In addition, APP mCherry control mice exhibited a significant reduction in spine head diameter, spine area, and spine volume relative to WT animals. Notably, chemogenetic activation of learning-tagged neurons in APP mice restored these structural parameters to levels comparable to those observed in WT controls (Figure 5D, 5E and 5F). This indicates that chemogenetic reactivation is associated with a structural remodeling of dendritic spines on learning-tagged LEC pyramidal neurons. To further characterize these morphological changes, dendritic spines were classified into discrete categories as described by Barón-Mendoza and colleagues (2021). Compared to WT controls, APP mCherry mice displayed a significantly higher proportion of thin and long thin spines, whereas mushroom-shaped spines were more prevalent in WT neurons, consistent with an enrichment of immature and potentially dysfunctional spines in APP mice. Following chemogenetic stimulation, we observed a significant reduction in thin spines accompanied by an increase in mushroom-shaped spines (Figure 5G). To further characterize morphological changes associated with chemogenetic activation in APP mice, we applied an unsupervised classification approach to dendritic spine morphology. Sixteen

morphological parameters extracted from three-dimensional spine reconstructions were first subjected to principal component analysis (PCA) to reduce dimensionality. Subsequently, k-means clustering was used to identify distinct groups of spines based on their morphological properties (Figure **5H**). Inspection of the first two principal components did not reveal a clear separation among clusters, consistent with the continuous nature of dendritic spine morphology. Nevertheless, marked differences emerged in the relative distribution of spines across clusters between the APP mCherry and APP hM3Dq groups. In particular, clusters 2 and 4 showed the most pronounced changes, with a substantial reduction in cluster 2 and a concomitant increase in cluster 4 following chemogenetic activation (Figure **5I**). To interpret the unsupervised clusters in terms of established spine categories, we examined their correspondence with spine types identified by supervised classification. This analysis revealed that the clusters corresponded to distinct morphological spine classes. Specifically, cluster 0 predominantly comprised long protrusions such as filopodia, whereas cluster 2 was enriched in thin and long thin spines. Clusters 0, 1, and 3 collectively represented mushroom-shaped spines, consistent with their larger head diameters. In APP hM3Dq mice, the reduction in cluster 2 spines aligned with the observed decrease in thin spines, while the increase in clusters corresponding to mushroom spines reflected a shift toward more mature spine morphologies. Notably, chemogenetic activation also led to an increase in cluster 4 spines, which appear to represent an intermediate morphological state between long thin and mushroom spines. This transitional population is not fully captured by supervised classification schemes, underscoring the added value of the unsupervised approach in identifying intermediate forms that may reflect ongoing structural remodeling (Figure **5J** and **5K**). Collectively, these analyses provide converging evidence for chemogenetic-induced structural remodeling of dendritic spines in learning-tagged LEC neurons. The combined supervised and unsupervised approaches reveal a redistribution of spine subtypes characterized by a reduction in immature thin spines, an increase in mature mushroom spines, and the emergence of a transitional spine population. These findings suggest that the memory rescue induced by chemogenetic stimulation in early-stage APP mice is associated with a facilitation of the transition toward a more mature and stable spine morphology in LEC neurons, as evidenced by structural enlargement.

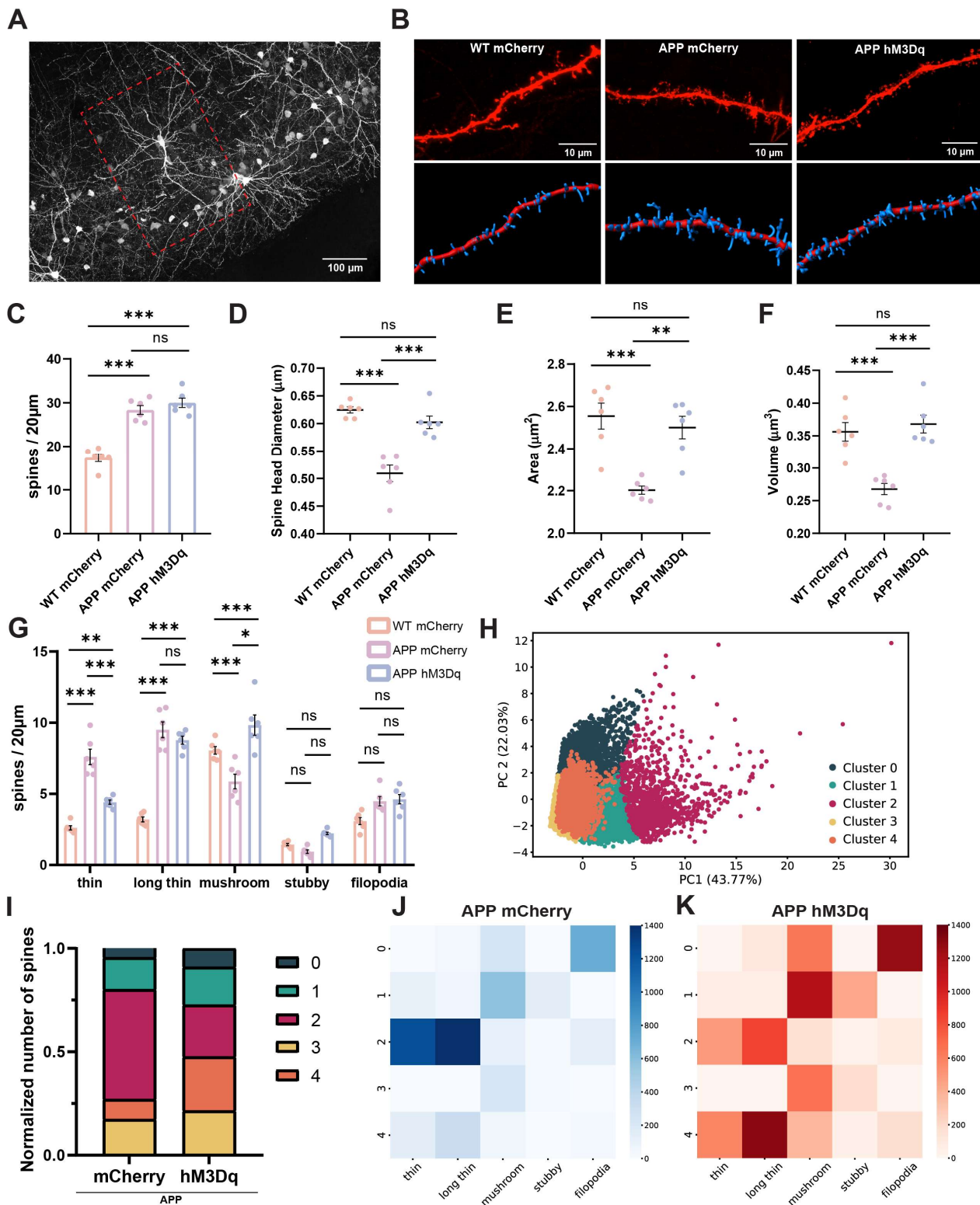


Figure 5. Chemogenetic reactivation of learning-tagged neurons promotes a shift in dendritic spine morphology.

(A) Representative image showing an EC pyramidal neuron in layer II/III from the population of *mCherry*⁺ learning-tagged neurons, captured at 40x magnification. (B) Representative images of selected dendrites at 63x magnification, with corresponding 3D reconstructions shown below. (C) Spine density was significantly lower in WT *mCherry* compared to APP groups, with no difference between APP *mCherry* and hM3Dq (one-way ANOVA with Sidak's multiple comparisons test: WT

mCherry 17.39 ± 0.899 $n = 6$ vs APP mCherry 28.38 ± 1.019 $n = 6$, *** $p < 0.001$; WT mCherry vs APP hM3Dq 29.99 ± 1.066 , *** $p < 0.001$; APP mCherry vs APP hM3Dq, ns $p = 0.61$). (D) A significant reduction in spine head diameter was detected in the APP mCherry group compared to both WT mCherry and APP hM3Dq (one-way ANOVA with Sidak's multiple comparisons test: WT mCherry 0.624 ± 0.005 $n = 6$ vs APP mCherry 0.509 ± 0.015 $n = 6$, *** $p < 0.001$; WT mCherry vs APP hM3Dq 0.602 ± 0.011 , ns $p = 0.4554$; APP mCherry vs APP hM3Dq, *** $p < 0.001$). (E) A significant reduction in spine area was detected in the APP mCherry group compared to both WT mCherry and APP hM3Dq (one-way ANOVA with Sidak's multiple comparisons test: WT mCherry 2.554 ± 0.061 $n = 6$ vs APP mCherry 2.203 ± 0.019 $n = 6$, *** $p < 0.001$; WT mCherry vs APP hM3Dq 2.501 ± 0.053 , ns $p = 0.8254$; APP mCherry vs APP hM3Dq, *** $p < 0.001$). (F) A significant reduction in spine volume was detected in the APP mCherry group compared to both WT mCherry and APP hM3Dq (one-way ANOVA with Sidak's multiple comparisons test: WT mCherry 0.356 ± 0.014 $n = 6$ vs APP mCherry 0.267 ± 0.008 $n = 6$, *** $p < 0.001$; WT mCherry vs APP hM3Dq 0.3681 ± 0.013 , ns $p = 0.8814$; APP mCherry vs APP hM3Dq, *** $p < 0.001$). (G) Using a 5-category classification, it was possible to observe a significant decrease in thin spines and a concomitant increase in mushroom spines in hM3Dq mice. Data are shown as the number of spines per 20 μm (two-way ANOVA with Sidak's multiple comparisons test: APP mCherry thin 7.592 ± 0.549 $n = 7$ vs APP hM3Dq thin 4.407 ± 0.151 $n = 7$, *** $p < 0.001$; APP mCherry long thin 9.497 ± 0.552 $n = 7$ vs APP hM3Dq long thin 8.773 ± 0.277 $n = 7$, ns $p = 0.7134$; APP mCherry mushroom 5.873 ± 0.508 $n = 7$ vs APP hM3Dq mushroom 9.829 ± 0.707 $n = 7$, *** $p < 0.001$; APP mCherry stubby 0.936 ± 0.126 $n = 7$ vs APP hM3Dq stubby 2.224 ± 0.089 $n = 7$, ns $p = 0.1507$; APP mCherry filopodia 4.478 ± 0.318 $n = 7$ vs APP hM3Dq filopodia 4.625 ± 0.337 $n = 7$, ns $p = 0.9997$). (H) PCA-based clustering of the dataset using K-means. The scatter plot represents the first two principal components (PC1 and PC2) of the scaled data, with each point color-coded according to its cluster assignment. The clusters were determined using K-means ($k=5$), and distinct colors are used to represent each cluster. The percentage of variance explained by each principal component is indicated on the axes. On the right, cluster proportions for mCherry and hM3Dq groups. (I) Stacked bar plot displaying the proportion of data points in each cluster following K-means clustering. For both mCherry and hM3Dq groups, the proportions reflect the relative distribution of data points across clusters, normalized to the total number of spines in the dataset. (J-K) Heatmaps showing the absolute abundance of spines across morphological categories (thin, long thin, mushroom, stubby, and filopodia) coupled with clusters identified through the unsupervised approach (PCA and K-means). Panel (J) represents the APP mCherry group, while panel (K) corresponds to the APP hM3Dq group. Color intensity indicates the absolute number of spines within each morphological category for a given cluster. The clusters are shown on the y-axis, and the spine classifications are shown on the x-axis. Spine morphological features are presented in μm . Data are presented as mean $\pm$ SEM.

4.4 Impaired hippocampal c-Fos activation following behavioral stimulation in 6-month-old APP mice

At a later stage of neurodegeneration (i.e., 6 months of age), APP mice exhibited a significant impairment in recognizing the familiar object in the less cognitively demanding novel object recognition task (ORT), consistent with the progression of amyloid pathology to the hippocampus (Criscuolo et al., 2017). To determine whether this deficit in object recognition recall was associated with alterations during memory encoding, we assessed both behavioral performance and hippocampal neuronal activation during the learning phase of the ORT (Figure 6A). Despite the impairment observed during recall, 6-month-old APP mice did not display detectable alterations in behavioral performance during the

learning phase. In fact, both APP and age-matched WT mice showed comparable exploration of the objects, with no preference for either object or position (Figure **6B**, **6C** and **6D**). To examine whether the encoding of non-associative memory engaged hippocampal subregions similarly in the two groups, we quantified the density and relative fluorescence intensity of c-Fos⁺ cells 90 minutes after the learning phase of the task. Baseline c-Fos expression was also assessed in home-cage control animals to evaluate hippocampal response to behavioral stimulation (Figures **6E** and **6F**). Whereas WT mice exhibited a robust increase in c-Fos expression in CA1, CA3, and the DG following object exploration relative to HC conditions, APP mice displayed elevated basal c-Fos levels in the HC control and failed to show further activation in response to the behavioral task. Indeed, basal c-Fos expression in APP mice was significantly higher than in WT controls and comparable to the levels observed in WT mice following encoding. This effect was particularly evident when c-Fos density was normalized to HC conditions, revealing a marked absence of task-induced activation in APP mice (Figure **6G**). These findings collectively indicate that 6-month-old APP mice exhibit an elevated basal level of c-Fos expression in the hippocampus. Notably, this expression level remained unchanged following the encoding of the ORT, suggesting an impaired ability to modulate hippocampal activation in response to behavioral stimuli.

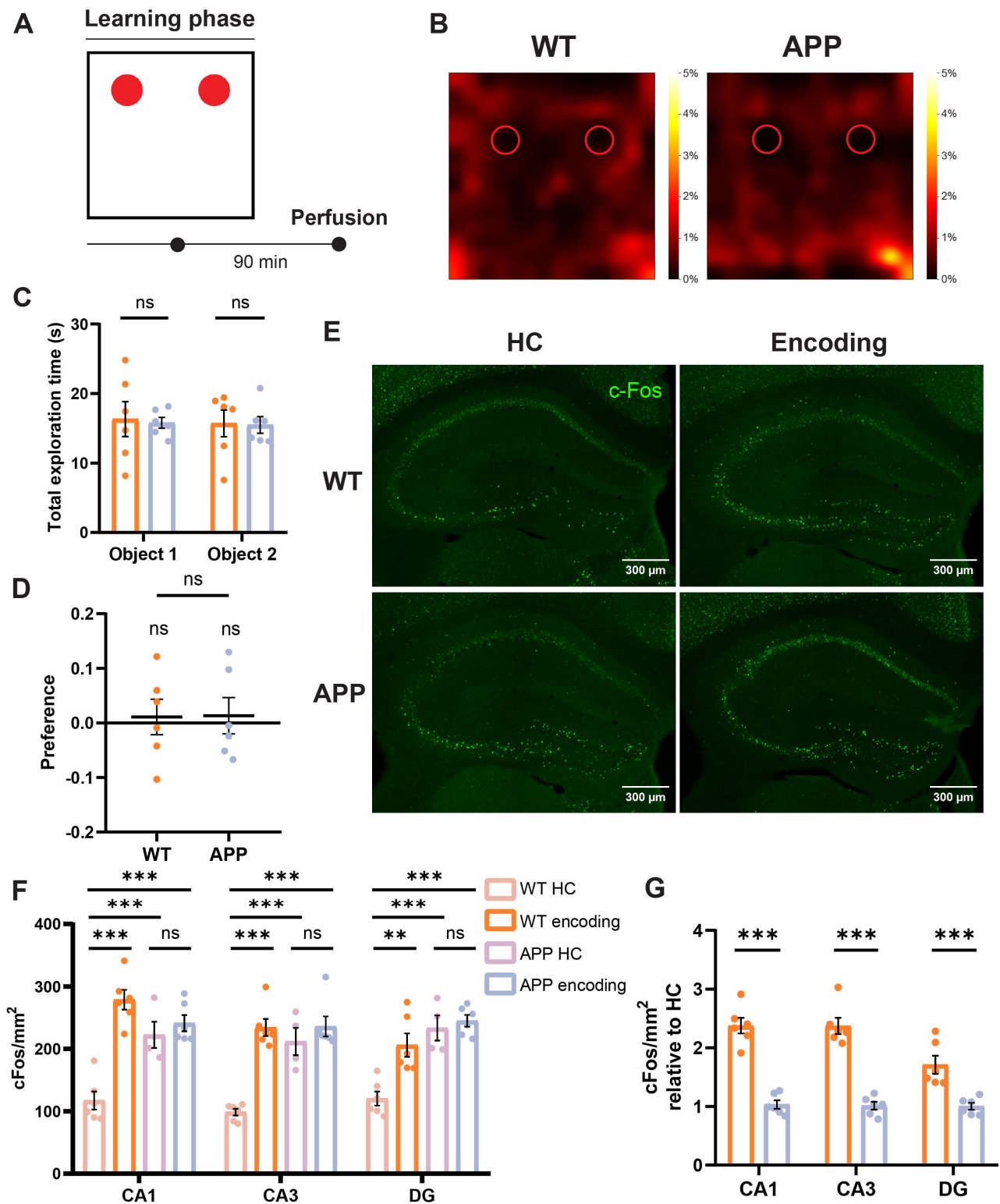


Figure 6. Impaired hippocampal c-Fos activation following behavioral stimulation in 6-month-old APP mice.

(A) Schematic representation of the learning phase of the ORT. Animals were perfused 90 minutes after the end of the task. (B) Examples of heatmaps showing the exploratory activity of mice (nose position) during the task. 6-month-old WT control and APP mice exhibited no preference for either object. (C) Analysis of total exploration time revealed that mice spent a comparable amount of time interacting with both objects (two-way ANOVA with Sidak's multiple comparisons test: WT encoding ns, WT encoding ns).

Object 1 16.32 ± 2.524 $n = 6$ vs APP encoding Object 1 15.8 ± 0.778 $n = 7$, ns $p = 0.9724$; WT encoding Object 2 15.71 ± 1.925 $n = 6$ vs APP encoding Object 2 15.49 ± 1.189 $n = 7$, ns $p = 0.9951$). (D) During Learning phase, mice showed no preference for either object, with exploration not differing from chance (WT encoding 0.01 ± 0.032 , $n = 6$, ns $p = 0.7511$, $df = 5$, $t = 0.3352$; APP encoding 0.013 ± 0.033 , $n = 6$, ns $p = 0.7049$, $df = 5$, $t = 0.4012$; one-sample t test) or between groups (WT encoding vs APP encoding, ns $p = 0.9594$, $df = 10$, $t = 0.0521$, unpaired t test). (E) Representative images showing c-Fos expression (green) in the hippocampus of 6-month-old WT and APP mice in the home cage control condition or following behavioral stimulation during the learning phase of the ORT. Scale bars are included in the figures. (F) Analysis of c-Fos⁺ cell density revealed a specific alteration in c-Fos expression in 6-month-old APP mice. While WT mice exhibited a significant increase in c-Fos density across the hippocampus following behavioral stimulation, APP mice displayed high basal home cage levels of c-Fos protein, which remained unchanged after the task. Moreover, c-Fos levels in APP mice, both in the home cage and after encoding, did not differ from those observed in the WT encoding group (two-way ANOVA with Sidak's multiple comparisons test: WT HC CA1 117.2 ± 14.43 $n = 6$ vs WT encoding CA1 278.7 ± 15.81 $n = 6$, *** $p < 0.001$; WT HC CA1 vs APP HC CA1 206.1 ± 18.65 $n = 4$, *** $p < 0.001$; WT HC CA1 vs APP encoding CA1 241.2 ± 12.90 $n = 6$, *** $p < 0.001$; WT encoding CA1 vs APP encoding CA1, ns $p = 0.2451$; APP HC CA1 vs APP encoding CA1, ns $p > 0.9999$; WT HC CA3 98.75 ± 5.371 $n = 6$ vs WT encoding CA3 234.2 ± 13.71 $n = 6$, *** $p < 0.001$; WT HC CA3 vs APP HC CA3 211.8 ± 21.81 $n = 4$, *** $p < 0.001$; WT HC CA3 vs APP encoding CA3 235.8 ± 15.99 $n = 6$, *** $p < 0.001$; WT encoding CA3 vs APP encoding CA3, ns $p > 0.9999$; APP HC CA3 vs APP encoding CA3, ns $p = 0.9979$; WT HC DG 92.17 ± 11.29 $n = 6$ vs WT encoding DG 206.1 ± 18.65 $n = 6$, ** $p = 0.0015$; WT HC DG vs APP HC DG 233.4 ± 20.02 $n = 4$, *** $p < 0.001$; WT HC DG vs APP encoding DG 245 ± 9.417 $n = 6$, *** $p < 0.001$; WT encoding DG vs APP encoding DG, ns $p = 0.6615$; APP HC DG vs APP encoding DG, ns $p > 0.9999$). (G) Analysis of hippocampal c-Fos⁺ density relative to home cage control (HC) revealed a lack of c-Fos protein increase after the encoding phase in APP mice compared to WT controls. Each encoding group was normalized to the mean c-Fos⁺ density of its respective HC control of the same genotype (two-way ANOVA with Sidak's multiple comparisons test: WT encoding CA1 2.377 ± 0.134 $n = 6$ vs APP encoding CA1 1.033 ± 0.071 $n = 6$, *** $p < 0.001$; WT encoding CA3 2.372 ± 0.138 $n = 6$ vs APP encoding CA3 1.014 ± 0.065 $n = 6$, *** $p < 0.001$; WT encoding DG 1.711 ± 0.154 $n = 6$ vs APP encoding DG 1.004 ± 0.058 $n = 6$, *** $p < 0.001$). Data are presented as mean $\pm$ SEM.

4.5 Chemogenetic reactivation of DG learning-tagged neurons rescues novel object recognition in 6-month-old APP mice

Following the observation that chemogenetic reactivation of learning-tagged neuronal ensembles in the LEC restores memory retrieval in young APP mice, we investigated whether a similar strategy could rescue object recognition memory at a later stage of disease progression. To this end, we applied the same TRAP-based chemogenetic approach to selectively target and reactivate learning-tagged neurons in the DG. Memory performance was assessed 12 hours after the learning phase following CNO administration (Figure 7A). Consistent with the progression of pathology to the hippocampus at this stage, 6-month-old APP mice injected with the mCherry control virus exhibited impaired novel object recognition performance. Notably, chemogenetic reactivation of DG learning-tagged neurons in APP mice restored a robust preference for the novel object, as reflected by both total exploration time and discrimination index values (Figure 7B, 7C and 7D). At the cellular level, analysis of c-Fos expression following the recall phase revealed no significant differences in overall

hippocampal c-Fos density between APP and WT mice. Chemogenetic activation of the DG was associated with a slight increase in c-Fos⁺ cell density ($p = 0.0583$), likely reflecting activation of a sparse subpopulation of tagged neurons (Figure **7E**). Immunofluorescence analysis of DG learning-tagged neurons further revealed a significant reduction in the overlap between mCherry and c-Fos signals in APP mCherry control mice compared to WT controls (Figures **7F** and **7G**), suggesting a selective impairment in the reactivation of this neuronal ensemble. Consistent with this, ensemble reactivation above chance levels was significantly reduced in APP mCherry mice compared to WT controls (Figure **7H**). Moreover, chemogenetic reactivation in APP hM3Dq mice resulted in a marked increase in mean fluorescence intensity selectively within the mCherry⁺ neuronal population, with no effect observed in the mCherry⁻ population (Figure **7I**). These data indicate that 6-month-old APP mice exhibited a disruption in the reactivation of the DG neuronal subpopulation engaged during the learning phase of a non-associative memory. Chemogenetic stimulation of DG learning-tagged neurons in 6-month-old APP mice was sufficient to restore the memory retrieval. Together, this finding suggests that during progressive amyloid- β accumulation, the memory trace becomes inaccessible rather than lost (Guglielmo et al., 2025).

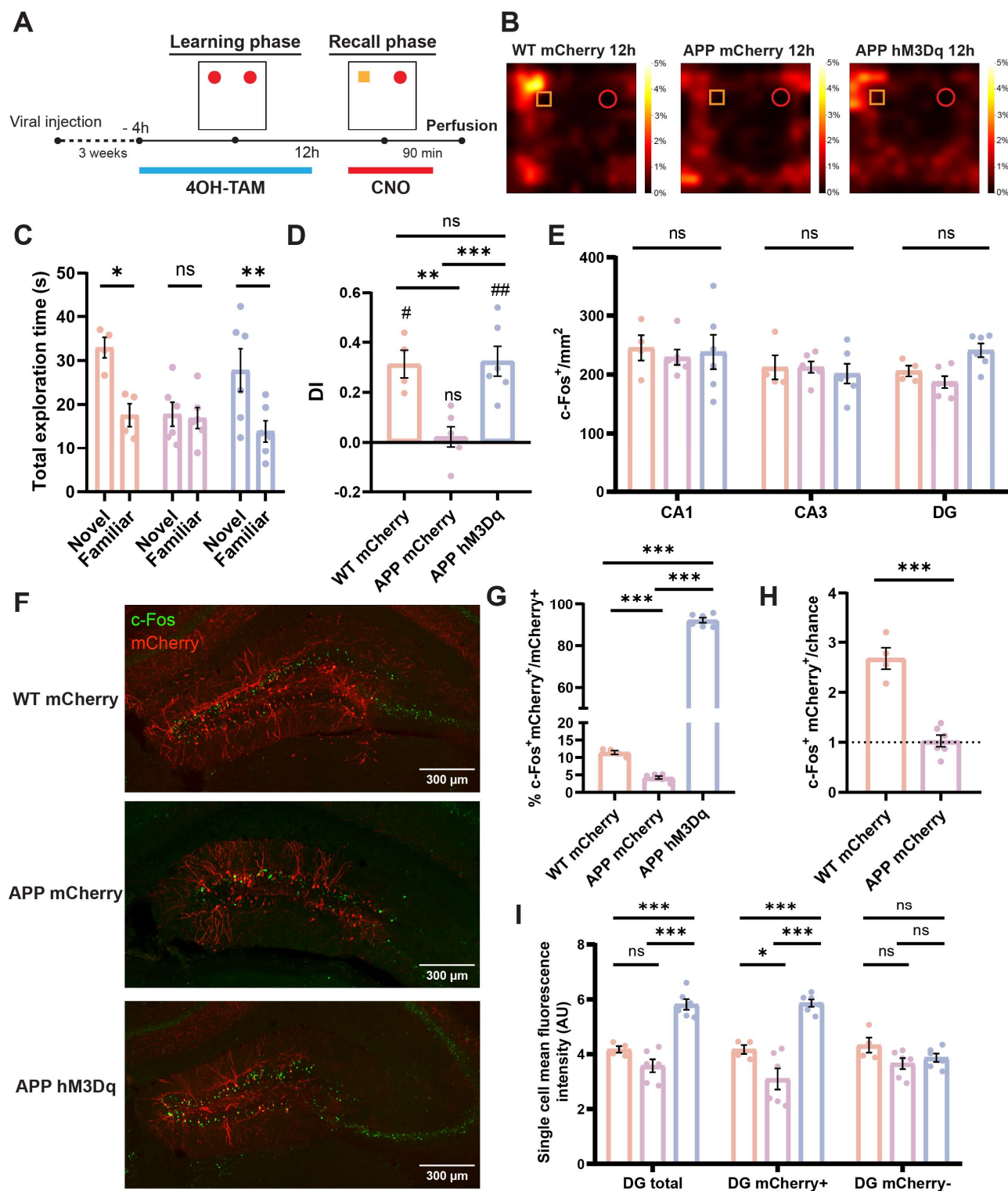


Figure 7. Chemogenetic reactivation of dentate gyrus learning-tagged neurons rescues Novel Object Recognition in 6-month-old APP mice.

(A) Schematic representation of the ORT: 4OH-TAM treatment was administered 4 hours prior to the learning phase, while CNO injection was given 30 minutes before the recall phase. Animals were perfused 90 minutes after the recall phase. (B) Examples of heatmaps showing the exploratory activity of mice (nose position) during the Recall phase for the three groups. WT control and APP hM3Dq mice showed a preference for the novel object, whereas APP mCherry mice explored both

objects equally. The color bar represents the percentage of total time spent in each position. (C) Total exploration time analysis showed significant exploration of the novel object in both the WT control and APP hM3Dq groups. In contrast, the APP mCherry control group did not recognize the novel object during the recall phase (two-way ANOVA with Sidak's multiple comparisons test: WT mCherry Novel 32.92 ± 2.321 $n = 6$ vs WT mCherry Familiar 17.57 ± 2.609 $n = 4$, * $p = 0.0210$; APP mCherry Novel 17.78 ± 2.736 $n = 6$ vs APP mCherry Familiar 16.90 ± 2.376 $n = 6$, ns $p = 0.9958$; APP hM3Dq Novel 27.81 ± 4.875 $n = 6$ vs APP hM3Dq Familiar 13.90 ± 2.442 $n = 6$, ** $p = 0.0096$). (D) The discrimination index (DI) analysis revealed that the WT control and APP hM3Dq groups significantly explored the novel object above chance levels, while the APP mCherry control group showed no preference (WT mCherry 0.3127 ± 0.055 , $n = 4$, ** $p = 0.0110$, $df = 3$, $t = 5.643$; APP mCherry -0.022 ± 0.040 , $n = 6$, ns $p = 0.6051$, $df = 5$, $t = 0.5514$; APP hM3Dq 0.3238 ± 0.059 , $n = 6$, ** $p = 0.0029$, $df = 5$, $t = 5.434$; one-sample t test). Furthermore, the DI was significantly higher in both the WT control and APP hM3Dq groups compared to the APP mCherry control group (one-way ANOVA with Sidak's multiple comparisons test: WT mCherry vs APP mCherry, ** $p = 0.008$; WT mCherry vs APP hM3Dq, ns $p = 0.9987$; APP mCherry vs APP hM3Dq, ** $p = 0.0026$). (E) No significant differences in c-Fos cell density were detected in the regions of interest across the three groups. A trend toward higher values was observed in the dentate gyrus of APP hM3Dq, which may be associated with the chemogenetic activation of subpopulations of learning-tagged DG neurons (two-way ANOVA with Sidak's multiple comparisons test: WT mCherry CA1 245.1 ± 21.51 $n = 4$ vs APP mCherry CA1 229.1 ± 13.10 $n = 6$, ns $p = 0.8050$; WT mCherry CA1 vs APP hM3Dq CA1 238 ± 29.08 $n = 6$, ns $p = 0.9576$; APP mCherry CA1 vs APP hM3Dq CA1, ns $p = 0.9197$; WT mCherry CA3 212.1 ± 20.34 $n = 4$ vs APP mCherry CA3 212.4 ± 9.49 $n = 6$, ns $p > 0.9999$; WT mCherry CA3 vs APP hM3Dq CA3 201.5 ± 16.68 $n = 6$, ns $p = 0.9089$; APP mCherry CA3 vs APP hM3Dq CA3, ns $p = 0.8814$; WT mCherry DG 205.8 ± 9.02 $n = 4$ vs APP mCherry DG 187.2 ± 10.08 $n = 6$, ns $p = 0.7450$; WT mCherry DG vs APP hM3Dq DG 240.9 ± 11.65 $n = 6$, ns $p = 0.3591$; APP mCherry DG vs APP hM3Dq DG, ns $p = 0.0583$). (F) Representative images of mCherry (red) and c-Fos (green) expressing neurons in the DG of the WT mCherry, APP mCherry and APP hM3Dq group. Scale bars are shown in the figures. (G) Co-localization analysis of mCherry and c-Fos signals showed a significant reduction in the APP mCherry group compared to the WT mCherry control (WT mCherry 11.44 ± 0.0548 $n = 4$ vs APP mCherry 4.261 ± 0.4206 , *** $p < 0.001$, $df = 8$, $t = 10.53$, unpaired t test). (H) The reactivation of LEC learning-tagged neurons was assessed as the percentage of c-Fos⁺ mCherry⁺ co-localization relative to chance levels. A significant reduction in reactivation was observed in the APP mCherry group compared to the WT mCherry group (WT mCherry 2.677 ± 0.2147 $n = 4$ vs APP mCherry 1.028 ± 0.1168 , *** $p < 0.001$, $df = 8$, $t = 7.365$, unpaired t test). (I) A significant increase in the mean relative fluorescence intensity was observed in the total population of c-Fos⁺ DG neurons in the APP hM3Dq group compared to the APP mCherry control. This difference was driven by a marked increase in the mean fluorescence of the mCherry⁺ population, with no change in the mCherry⁻ population (two-way ANOVA with Sidak's multiple comparisons test: WT mCherry DG total 4.177 ± 0.118 $n = 4$ vs APP mCherry DG total 3.577 ± 0.2366 $n = 6$, ns $p = 0.5672$; WT mCherry DG total vs APP hM3Dq DG total 5.814 ± 0.1939 $n = 6$, *** $p < 0.001$; APP mCherry DG total vs APP hM3Dq DG total, *** $p < 0.001$; WT mCherry DG mCherry⁺ 4.170 ± 0.165 $n = 4$ vs APP mCherry DG mCherry⁺ 3.099 ± 0.384 $n = 6$, * $p = 0.0307$; WT mCherry DG mCherry⁺ vs APP hM3Dq DG mCherry⁺ 5.862 ± 0.133 $n = 6$, *** $p < 0.001$; APP mCherry DG mCherry⁺ vs APP hM3Dq DG mCherry⁺, *** $p < 0.001$; WT mCherry DG mCherry⁻ 4.328 ± 0.273 $n = 4$ vs APP mCherry DG mCherry⁻ 3.659 ± 0.2024 , ns $p = 0.4209$ $n = 6$; WT mCherry DG mCherry⁻ vs APP hM3Dq DG mCherry⁻ 3.869 ± 0.151 $n = 6$, ns $p = 0.8494$; APP mCherry DG mCherry⁻ vs APP hM3Dq DG mCherry⁻, ns $p = 0.9980$). Data are presented as mean $\pm$ SEM.

4.6 Brain-wide mapping of c-Fos activation during episodic-like memory recall

Starting from the hypothesis that episodic-like memory is encoded and stored across multiple neuronal populations throughout the brain, we examined whether memory recall relies on a distributed network of interacting brain regions. To identify neuronal populations specifically involved in OPC memory recall, we conducted a brain-wide analysis of neuronal activation using c-Fos as a marker of activity. To this end, we induced behavioral activation under different conditions and collected the brains 90 minutes later to capture the peak of c-Fos protein expression. To better characterize the activation patterns associated with memory retrieval, while excluding areas involved in other aspects of exploration, we compared our experimental group subjected to the OPCRT with three control conditions: home-cage control (HC), context-exposed control (CNTX), and a group exposed to the same pair of objects across all contexts (OCT) (Figure **8A**). Following behavioral activation, the collected brains were serially sectioned to obtain a coronal representation of the entire brain and subjected to immunohistochemical staining for c-Fos. The resulting sections were imaged using a confocal microscope and registered onto the Allen Brain Atlas for subsequent quantification and analysis (Figure **8B** and **8C**). This process produced a comprehensive dataset of c-Fos⁺ cells across multiple levels of granularity, from major brain subdivisions to fine subregions, and across different behavioral conditions. Using PCA followed by k-means clustering, we assessed whether the c-Fos dataset could discriminate between the different behavioral groups. The analysis confirmed that the c-Fos activation profiles were sufficient to accurately distinguish all conditions. Inspection of the contributions of individual brain regions revealed that isocortical areas were the dominant contributors across the first three components (Figure **8D**). This suggests that isocortical regions critically shape the activation profiles that differentiate behavioral conditions. We then examined activation patterns across the eleven major brain subdivisions defined by the Allen Brain Atlas (Figure **8E**). As expected, groups subjected to behavioral activation exhibited stronger c-Fos expression compared to HC controls, reflecting the widespread recruitment of neurons during task performance. This effect was particularly pronounced in regions critically involved in sensory processing, memory, and integrative functions, including the isocortex, olfactory areas, hippocampal formation, cortical subplate, pallidum, and hypothalamus (Figure **8F**).

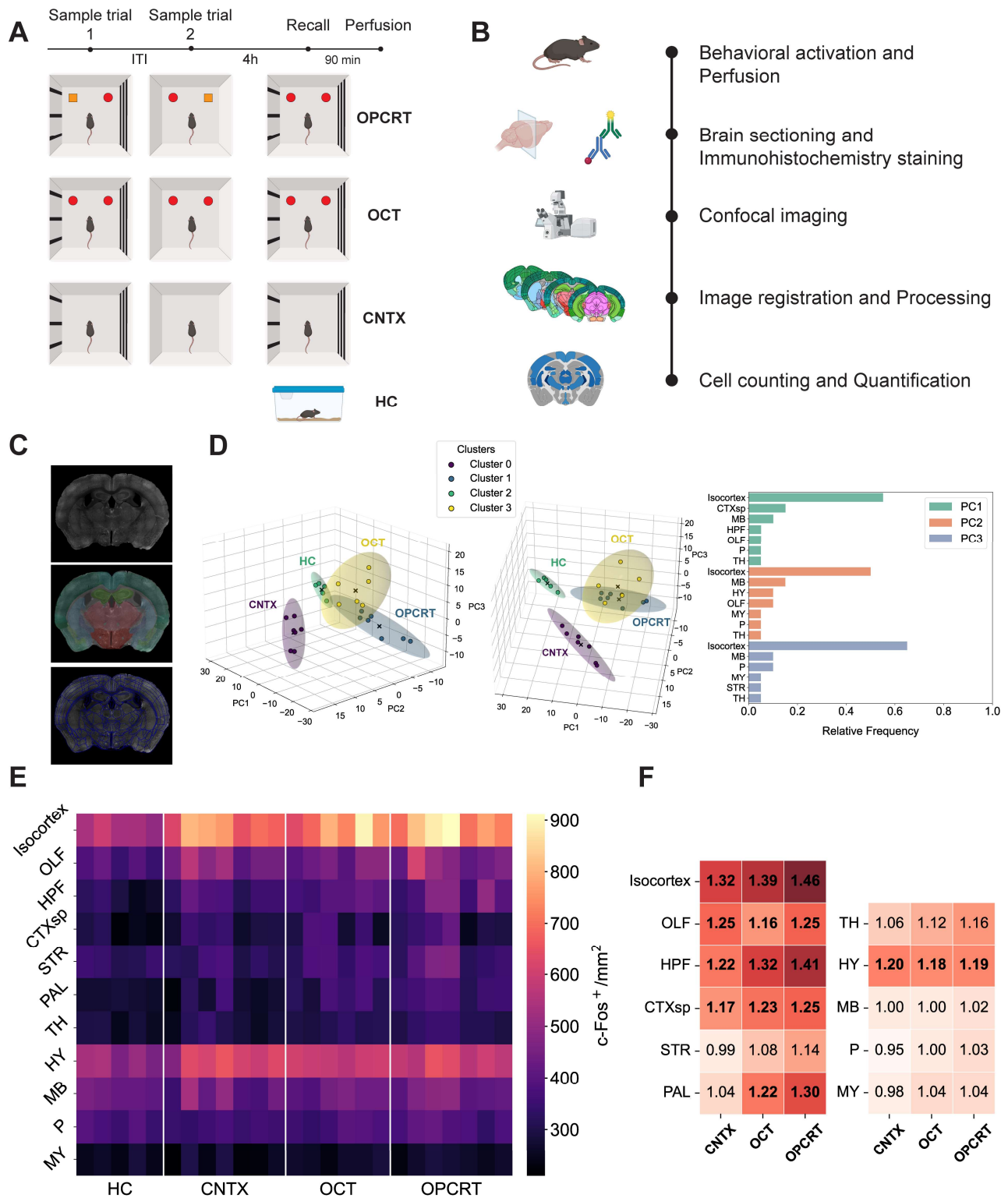


Figure 8. Brain-wide mapping of c-Fos activation during episodic-like memory recall.

(A) Schematic representation of the behavioral paradigm used. Mice were assigned to four experimental groups: home cage (HC, $n=5$), context exposure (CNTX, $n=7$), object-context task (OCT, $n=6$), and object-place-context recognition task (OPCRT, $n=7$). In all conditions, both the sample trials and the recall phase lasted 10 min. (B) Overview of the experimental phases of the brain-wide c-Fos mapping. (C) From top to bottom, representative images of raw acquisition of a whole-mount brain slice stained for c-Fos, its registration to the Allen Brain Atlas, and alignment

refinement. (D) Three-dimensional PCA representation of the c-Fos density dataset based on the first three principal components, with clusters identified by k-means clustering. Each dot represents an individual mouse. Two views of the same projection are shown from different angles. Right, analysis of the contribution of individual brain regions to each principal component showed a predominant involvement of isocortical areas across all three components. (E) Heatmap of c-Fos density across the eleven major brain subdivisions, displayed for individual mice across the four behavioral conditions. (F) Heatmap showing c-Fos fold change for each brain major subdivision in behaviorally activated groups (CNTX, OCT and OPCRT) relative to the HC condition. Fold-change values shown in bold are significantly different from HC, as assessed by two-way ANOVA followed by two-stage FDR post hoc correction ($Q = 0.05$; Isocortex: CNTX 1.323 ± 0.05 $p < 0.001$, OCT 1.389 ± 0.07 $p < 0.001$, OPCRT 1.458 ± 0.061 $p < 0.001$; OLF: CNTX 1.246 ± 0.058 $p < 0.001$, OCT 1.163 ± 0.047 $p = 0.0311$, OPCRT 1.253 ± 0.071 $p < 0.001$; HPF: CNTX 1.220 ± 0.047 $p = 0.0028$, OCT 1.321 ± 0.037 $p < 0.001$, OPCRT 1.406 ± 0.084 $p < 0.001$; CTXsp: CNTX 1.172 ± 0.052 $p = 0.0189$, OCT 1.230 ± 0.086 $p = 0.0025$, OPCRT 1.252 ± 0.080 $p < 0.001$; STR: CNTX 0.990 ± 0.034 $p = 0.8929$, OCT 1.078 ± 0.034 $p = 0.0025$, OPCRT 1.139 ± 0.073 $p < 0.001$; PAL: CNTX 1.038 ± 0.062 $p = 0.5976$, OCT 1.220 ± 0.051 $p = 0.0038$, OPCRT 1.296 ± 0.034 $p < 0.001$; TH: CNTX 1.059 ± 0.048 $p = 0.4197$, OCT 1.117 ± 0.021 $p = 0.1217$, OPCRT 1.163 ± 0.032 $p = 0.0261$; HY: CNTX 1.202 ± 0.046 $p = 0.0058$, OCT 1.184 ± 0.012 $p = 0.0153$, OPCRT 1.191 ± 0.028 $p = 0.0092$; MB: CNTX 0.995 ± 0.059 $p = 0.9503$, OCT 0.997 ± 0.027 $p = 0.9918$, OPCRT 1.022 ± 0.036 $p = 0.6662$; P: CNTX 0.951 ± 0.013 $p = 0.4980$, OCT 0.999 ± 0.028 $p = 0.9918$, OPCRT 1.031 ± 0.030 $p = 0.6662$; MY: CNTX 0.983 ± 0.054 $p = 0.8150$, OCT 1.036 ± 0.028 $p = 0.6361$, OPCRT 1.035 ± 0.029 $p = 0.6288$).

4.7 Recruitment of a fronto-posterior medial network during episodic memory retrieval

To investigate the specific activation of individual brain regions to episodic memory retrieval, we performed a mid-level analysis using 75 aggregated brain regions. To account for the relevance of isocortical areas, these regions were selected to provide an extensive representation of isocortical subregions (Figure 9A). In order to understand the patterns of activation associated with memory retrieval, we compared the c-Fos density of the OPCRT group with that in the control conditions using task partial least squares analysis (task PLS). In the comparison between the OPCRT group and the HC control, task PLS revealed a widespread increase in c-Fos expression across multiple cortical and subcortical regions. Significant activation was observed in primary sensory cortices, including somatosensory and visual areas, as well as in associative cortical regions. Robust engagement was also detected in olfactory structures, hippocampal subfields CA1 and CA3, and the LEC. Within the cortical subplate, strong activation was evident in the claustrum (CLA), basomedial amygdala (BMA), and lateral amygdala (LA). Subcortically, increased activity involved the ventral striatum (STRv), lateral septum (LSX), ventral and caudal pallidum (PALv, PALc), multiple thalamic nuclei, and large parts of the hypothalamus (Figure 9B). A refined number of significantly activated regions was observed when comparing the OPCRT group with the CNTX control. Interestingly, primary sensory cortices, including somatosensory and visual areas, remained significantly activated, likely reflecting the interaction with objects during the task. In addition, frontal cortical regions and the RSP showed higher activation in the OPCRT group. In the hippocampal formation, both the POST and the CA3 subfield displayed significantly stronger activation relative to CNTX, and the CLA emerged as the main subplate

structure with increased activity. Among subcortical regions, the anterior thalamic nuclei (ATN), the reticular nucleus of the thalamus, as well as medial and epithalamic regions showed enhanced activation. In the hypothalamus, significant effects were observed in the periventricular zone (PVZ) and the premammillary region (PM) (Figure **9C**). Finally, when comparing the OPCRT group with the most cognitively demanding control condition (OCT), a strong reduction in the number of significantly activated regions was observed. This result indicates that most of the brain areas recruited in the previous comparisons are likely related to spatial navigation and object interaction rather than to episodic memory retrieval itself. Notably, a small subset of regions remained significantly activated in the OPCRT group, including the mPFC, comprising the anterior cingulate (ACA), prelimbic (PL), and infralimbic (ILA) cortices, along with the RSP, ORB and the POST (Figure **9D**).

These comparisons indicate a progressive restriction of recruited brain regions associated with increasing task complexity. Specifically, areas uniquely activated relative to the OCT control were contained within those activated relative to the CNTX control, which in turn represented a subset of the broader activation observed relative to the HC control. This hierarchical organization suggests that increasing cognitive demands progressively exclude regions primarily related to sensorimotor exploration, retaining only those specifically engaged in OPC memory retrieval. This progression is illustrated by the spatial distribution of c-Fos fold change across coronal brain sections (Figure **10A**), which reveals widespread activation relative to HC that becomes progressively confined when comparisons are restricted to CNTX and OCT controls. This pattern is further confirmed by the nested structure of the Venn diagram showing significantly activated brain regions across behavioral conditions (Figure **10B**). The core component of this structure, comprising the mPFC, RSP, ORB, and POST, identifies brain regions that are robustly and consistently engaged during episodic-like memory recall (Figure **10C**). To explore the anatomical organization of the activated subnetwork, we constructed an anatomical connectome using data from the Allen Brain Connectivity Atlas (Knox et al., 2019). We focused on direct connections among the activated regions, based on the hypothesis that co-activated areas may form a dedicated subnetwork with preferential anatomical connectivity. We observed two highly connected divisions composed of frontal and posterior medial areas (Figure **10D**). Interestingly, this subnetwork exhibited significantly higher mean connectivity compared to equivalent random networks, suggesting that it constitutes a specialized anatomical network activated during memory retrieval (Figure **10E**).

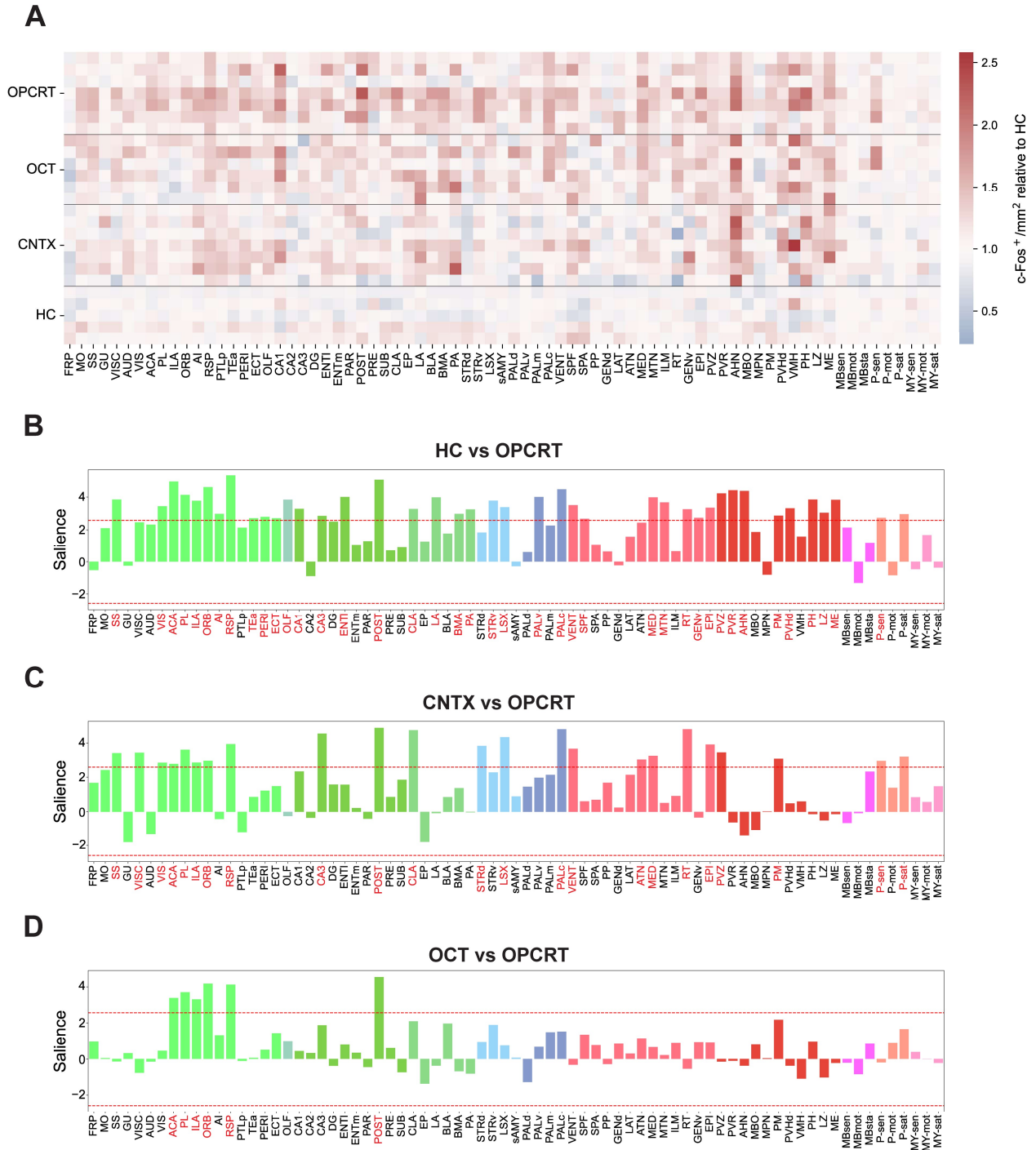


Figure 9. c-Fos activation at the aggregated mesoscale level.

(A) Heatmap showing c-Fos density across 75 aggregated brain regions at the mesoscale level for individual mice across the four behavioral conditions. c-Fos density is shown relative to the HC condition. (B-D) Partial least squares (PLS) analysis of c-Fos expression comparing the OPCRT group with control conditions: HC (B), CNTX (C), and OCT (D). Salience scores, normalized by the standard deviation estimated via bootstrap, identify brain regions that contribute most strongly to condition separation. Red dashed lines indicate the statistical threshold at salience scores of ± 2.58

($p < 0.01$). Brain regions with salience scores exceeding the threshold are shown in red (Task PLS significant salience scores: HC vs OPCRT: VIS 3.418, ACA 4.801, PL 3.967, ILA 3.786, ORB 4.621, AI 2.970, RSP 5.329, TEa 2.696, PERI 2.716, ECT 2.689, OLF 3.866, CA1 3.331, CA3 2.851, ENTI 3.928, POST 5.064, CLA 3.258, LA 3.981, BMA 2.983, PA 3.188, STRv 3.796, LSX 3.449, PALv 4.008, PALc 4.479, VENT 3.559, SPF 2.659, MED 3.828, MTN 3.664, RT 3.254, GENv 2.719, EPI 3.363, PVZ 4.186, PVR 4.424, AHN 4.356, PM 2.845, PVHd 3.277, PH 3.760, LZ 3.008, ME 3.834, P-sen 2.678, P-sat 2.948; CNTX vs OPCRT: SS 3.453, VISC 3.464, VIS 2.866, ACA 2.790, PL 3.696, ILA 2.859, ORB 2.968, RSP 4.006, CA3 4.593, POST 5.003, CLA 4.768, STRd 3.884, LSX 4.367, PALc 4.790, VENT 3.760, ATN 3.047, MED 3.276, RT 4.876, EPI 3.999, PVZ 3.483, PM 3.040, P-sen 3.016, P-sat 3.246; OCT vs OPCRT: ACA 3.334, PL 3.684, ILA 3.328, ORB 4.218, RSP 4.144, POST 4.48).

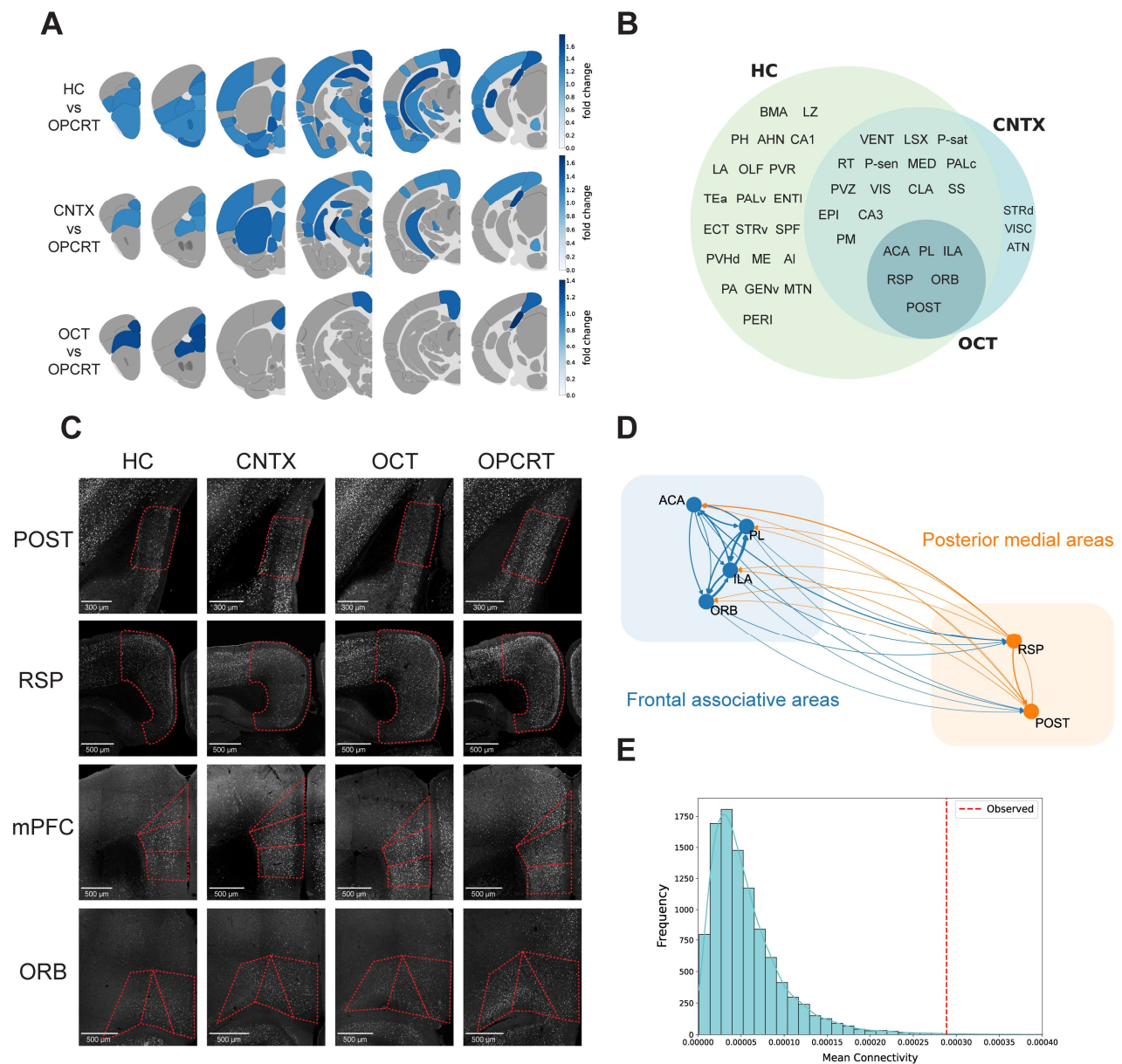


Figure 10. Brain-wide c-Fos mapping reveals a core episodic-like memory network.

(A) Brain-wide heatmaps showing c-Fos density fold-change during OPC memory recall relative to each control condition (HC, CNTX, and OCT). Only significantly activated brain regions are colored

and mapped across coronal brain sections at the aggregated mesoscale level. (B) Venn diagram summarizing significantly activated brain regions identified by task PLS for each comparison with the OPCRT group. (C) Representative c-Fos immunohistochemistry for the four brain regions significantly activated relative to the OCT group (mPFC, RSP, ORB, and POST), shown across all experimental groups. Brain region boundaries are indicated by red dashed outlines; scale bars are shown in the images. (D) Anatomical network composed of areas significantly activated relative to the OCT group, with brain regions shown as nodes and directed anatomical connections represented as arrows. The network reveals two major divisions corresponding to frontal associative and posterior medial components. (E) Distribution of mean connectivity of equivalent random anatomical networks obtained by bootstrapping ($n = 10000$). Mean connectivity represents the average normalized projection density between region pairs. The red dashed line indicates the mean connectivity of the subnetwork composed of brain regions activated relative to the OCT group. The observed value was significantly greater than values obtained from equivalent random networks (observed mean = 0.00029; random mean = 0.00006; one-sided permutation test, $**p = 0.0027$).

4.8 Network analysis indicates a shift toward a more efficient network topology during memory recall

While the quantification of c-Fos expression provides a map of activated brain areas, it does not capture how these regions operate as an integrated system. To address this limitation, patterns of functional connectivity can be estimated by assessing how regional activity covaries across animals following behavioral activation. This approach quantifies relationships between pairs of brain regions, potentially helping identify network properties, such as modular organization and changes in global efficiency, which are not evident from regional activation levels alone. To estimate patterns of functional connectivity, we computed interregional correlation matrices for each experimental group subjected to behavioral activation (CNTX, OCT, and OPCRT) at the aggregated level of anatomical granularity. Pearson correlation matrices were then thresholded by retaining only the strongest statistically significant correlations ($r > 0.80$; $p < 0.05$), from which we generated functional networks in which nodes represent individual brain regions and edges represent suprathreshold functional connections (Figure **11A**, **11B** and **11C**). To further characterize the topological organization of the resulting functional networks, we quantified a set of graph metrics, including node degree, network density, global efficiency, clustering coefficient, betweenness centrality, and small-worldness. Compared to CNTX and OCT networks, the OPCRT network exhibited a significant increase in node degree and overall network density (Figure **12A** and **12B**), indicating a more densely interconnected structure. In parallel, global efficiency was significantly elevated in the OPCRT network (Figure **12C**), suggesting that information transfer across the network can occur through shorter and more efficient paths. At the local scale, the OPCRT network showed an increased clustering coefficient (Figure **12D**), consistent with tighter local connectivity and a more pronounced modular organization. In contrast, betweenness centrality did not increase in the OPCRT network relative to the OCT network, indicating no shift toward greater network centralization (Figure **12E**). Finally, the OPCRT network exhibited a higher small-world index (Figure **12F**), a topological profile that optimally balances local specialization with global integration. Taken together, these alterations in topological parameters indicate that memory retrieval in the OPCRT condition was associated with a more densely connected and efficient network

organization. To further investigate the internal organization of the functional networks identified across conditions, we next focused on the identification of hub regions (Figure **12G**, **12H** and **12I**). In complex brain networks, hubs are nodes that play a critical role in coordinating information flow, either by integrating activity across distinct functional modules or by supporting communication in local subnetworks. To characterize the contribution of each brain region to network organization, nodes were classified based on their within-module connectivity and their participation coefficient, allowing their assignment to the following topological roles: ultra-peripheral (R1), peripheral (R2), non-hub connector (R3), non-hub kinless (R4), provincial hub (R5), connector hub (R6), and kinless hub (R7). Briefly, ultra-peripheral and peripheral nodes (R1-R2) primarily interact within their own module, non-hub connector and non-hub kinless nodes (R3-R4) link multiple modules without showing a critical role, while provincial and connector hub (R5-R6) represent highly connected regions that respectively support within-module processing and inter-module integration. Finally, the kinless hub (R7) represents a global integrator node with links distributed evenly across all modules. Focusing on the OPCRT network (Figure **12I**), the POST, RSP, SUB, and posterior hypothalamic nucleus (PH) exhibited connector hub (R6) characteristics, indicating a central role in mediating communication between distinct network modules. In contrast, several regions, including perirhinal cortex (PERI), auditory cortex (AUD), anterior hypothalamic nucleus (AHN), lateral entorhinal cortex (ENTI), lateral amygdala (LA), entorhinal cortex (ECT), posterior parietal association area (PTLp), and somatosensory cortex (SS), were classified as provincial hubs (R5). These regions displayed high within-module connectivity, consistent with a role in supporting locally specialized processing in individual functional modules rather than inter-module integration. It is also worth noting that the VIS and DG displayed non-hub connector (R3) properties. Although these regions did not reach hub-level connectivity, their elevated participation coefficients indicate that they contribute to linking multiple network modules. The attribution of these topological roles, together with the observation that RSP and POST are significantly activated during episodic-like memory retrieval, emphasizes the contribution of the posterior medial component of the proposed anatomical network, suggesting that the functional network is organized around a set of posterior medial integrative nodes that coordinate interactions between different modules during episodic-like memory recall. To further assess whether each behavioral condition is associated with a distinct pattern of interregional co-activation, we applied a low-dimensional embedding approach using Uniform Manifold Approximation and Projection (UMAP). In this embedding, each brain region was represented by its pattern of correlations with all other regions, and these high-dimensional profiles were projected into a two-dimensional space such that regions with similar correlation patterns were positioned closer together. The resulting UMAP representation revealed a clear segregation of regions according to behavioral conditions, indicating that each condition is characterized by a distinct configuration of functional interactions of c-Fos activity (Figure **12J**).

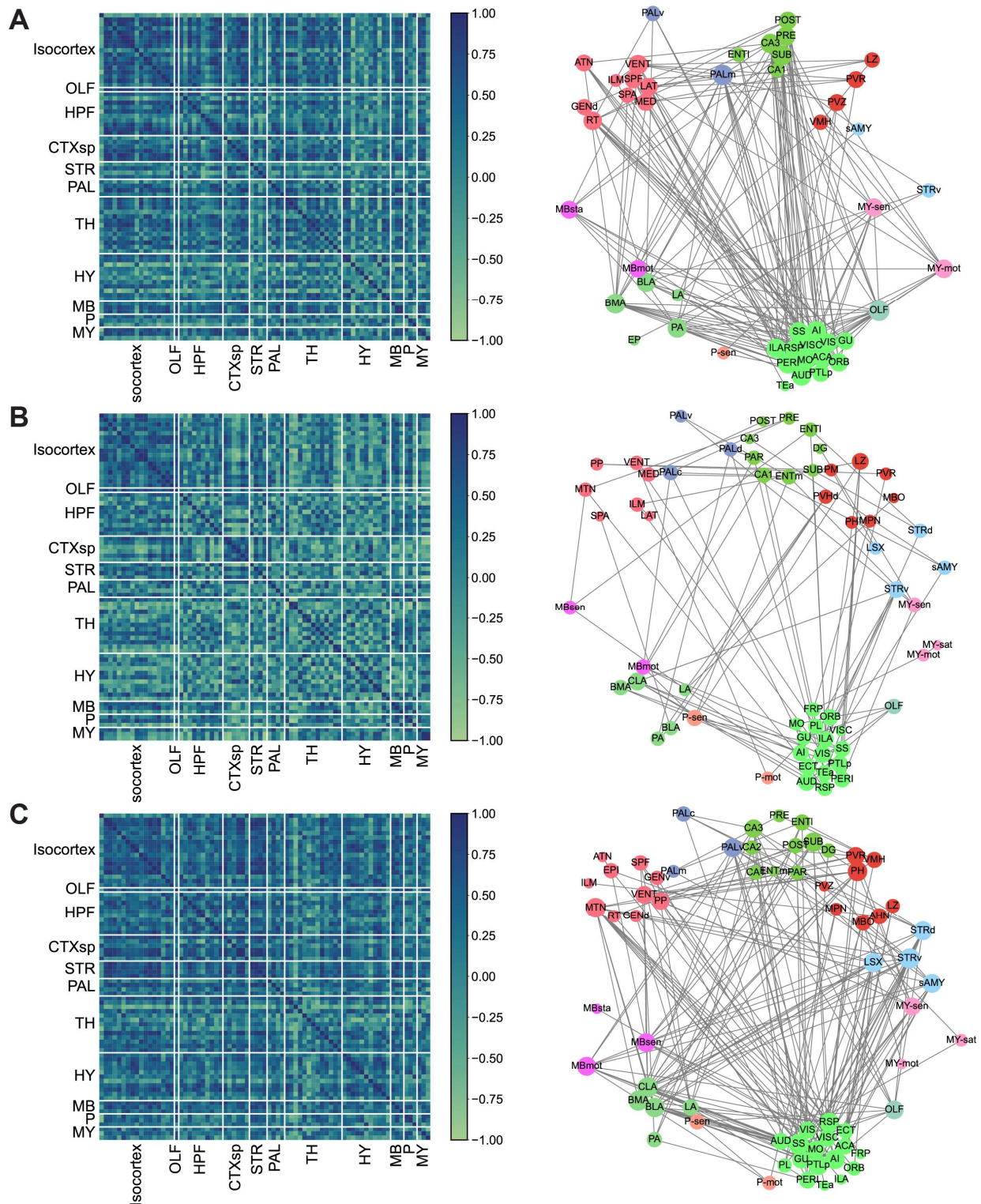


Figure 11. Generation of functional networks from interregional correlation matrices.

(A-C) Functional connectivity analysis for the CNTX (A), OCT (B), and OPCRT (C) conditions. For each condition, the unfiltered interregional Pearson correlation matrix computed across aggregated mesoscale brain regions is shown on the left. Major anatomical subdivisions are indicated along the matrix axes. On the right, the corresponding functional network is shown, obtained by retaining only positive correlations exceeding $r > 0.8$ that were statistically significant ($p < 0.05$). Nodes represent

individual brain regions and are colored by major anatomical subdivision, while edges represent suprathreshold functional connections.

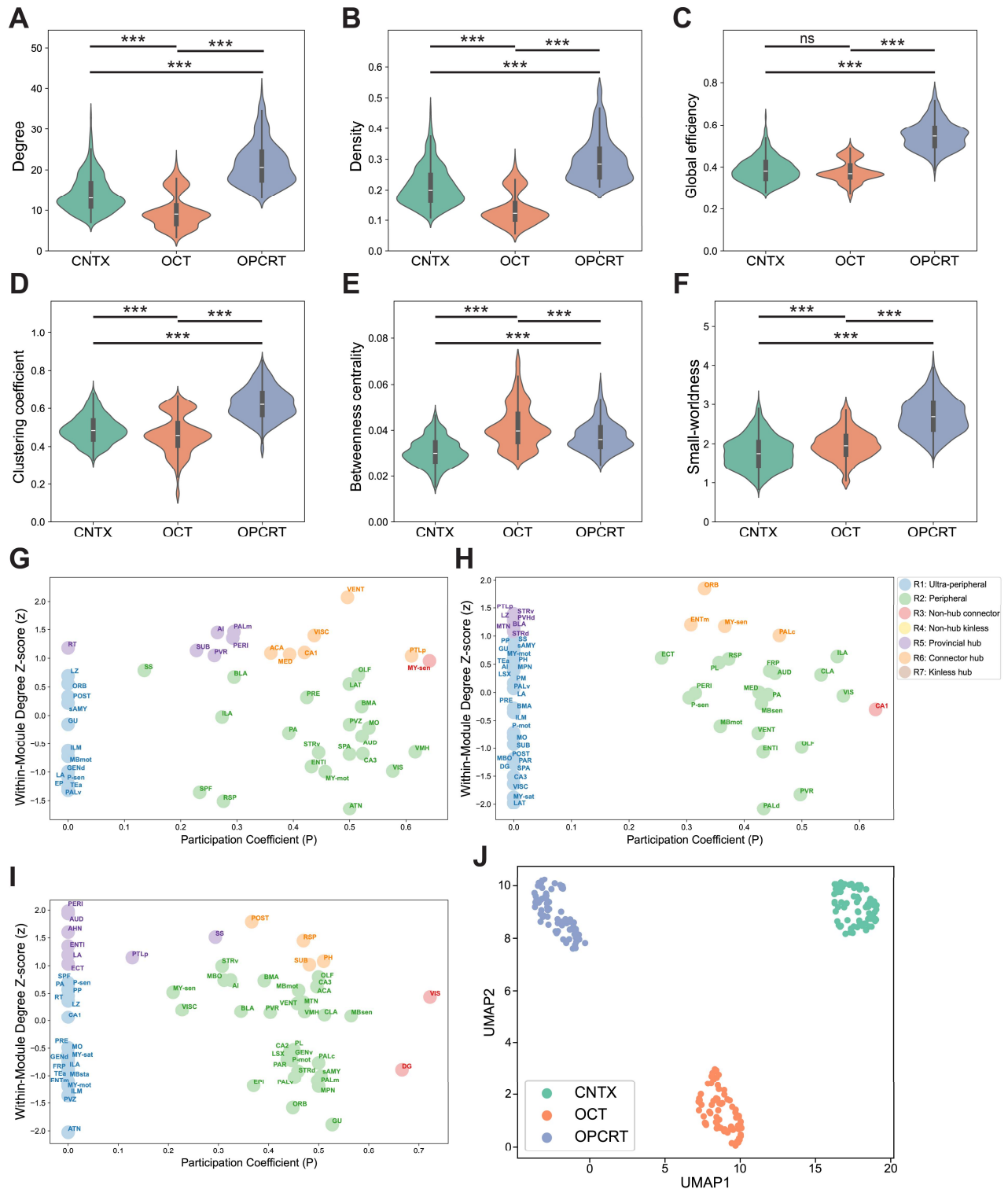


Figure 12. Topological analysis of functional brain networks.

Topological properties of functional networks were estimated using bootstrap resampling across subjects. Functional networks were recomputed over 1000 bootstrap iterations, and graph metrics were calculated for each iteration to obtain distributions of network topology measures. (A) Node

degree was significantly higher in the OPCRT group compared to CNTX and OCT (Kruskal-Wallis test with Dunn's multiple comparisons test: CNTX 14.16 ± 0.143 vs OCT 9.912 ± 0.136 , *** $p < 0.001$; CNTX vs OPCRT 21.50 ± 0.167 , *** $p < 0.001$; OCT vs OPCRT, *** $p < 0.001$). (B) Network density was significantly increased in OPCRT relative to both control conditions (Kruskal-Wallis test with Dunn's multiple comparisons test: CNTX 0.2127 ± 0.002 vs OCT 0.138 ± 0.001 , *** $p < 0.001$; CNTX vs OPCRT 0.299 ± 0.002 , *** $p < 0.001$; OCT vs OPCRT, *** $p < 0.001$). (C) Global efficiency was significantly higher in OPCRT compared to CNTX and OCT (Kruskal-Wallis test with Dunn's multiple comparisons test: CNTX 0.388 ± 0.001 vs OCT 0.379 ± 0.001 , ns $p = 0.0859$; CNTX vs OPCRT 0.550 ± 0.002 , *** $p < 0.001$; OCT vs OPCRT, *** $p < 0.001$). (D) Clustering coefficient was significantly increased in OPCRT relative to both control groups (Kruskal-Wallis test with Dunn's multiple comparisons test: CNTX 0.490 ± 0.002 vs OCT 0.464 ± 0.003 , *** $p < 0.001$; CNTX vs OPCRT 0.627 ± 0.002 , *** $p < 0.001$; OCT vs OPCRT, *** $p < 0.001$). (E) Betweenness centrality in OPCRT was significantly higher than CNTX but lower than OCT (Kruskal-Wallis test with Dunn's multiple comparisons test: CNTX 0.030 ± 0.0001 vs OCT 0.041 ± 0.0003 , *** $p < 0.001$; CNTX vs OPCRT 0.037 ± 0.0002 , *** $p < 0.001$; OCT vs OPCRT, *** $p < 0.001$). (F) Small-worldness was significantly higher in OPCRT compared to CNTX and OCT (Kruskal-Wallis test with Dunn's multiple comparisons test: CNTX 1.777 ± 0.013 vs OCT 1.975 ± 0.011 , *** $p < 0.001$; CNTX vs OPCRT 2.714 ± 0.014 , *** $p < 0.001$; OCT vs OPCRT, *** $p < 0.001$). (G-I) Identification of hub regions and topological roles within functional networks. Nodes were classified based on their within-module degree z-score and participation coefficient, allowing assignment to specific topological roles. Topological role distributions are shown for the CNTX (G), OCT (H), and OPCRT (I) networks. (J) UMAP embedding of interregional functional connectivity profiles. Each point represents a brain region within a given condition, positioned according to its correlation pattern. Brain regions are colored by behavioral conditions, revealing a clear segregation of CNTX, OCT, and OPCRT networks based on distinct patterns of c-Fos co-activation.

4.9 Dissecting the role of learning-tagged neurons during episodic memory retrieval using chemogenetics

Although significant activation of specific neuronal populations suggests their engagement in the network supporting memory retrieval, such evidence alone is insufficient to define these populations as memory engrams. To causally assess the functional contribution of brain regions identified as activated during OPCRT recall, we employed the TRAP-based dual-viral strategy described above to selectively express chemogenetic receptors during memory encoding. The activity of the learning-tagged neuronal population is subsequently manipulated using CNO during memory recall, either at 12h to induce inhibition, when memory expression is still intact, or at 48h to induce activation, when the memory is no longer accessible by natural cues alone (Figure **13A**). Using this strategy, we examined the contribution of selected brain regions to memory recall, focusing on the postsubiculum (POST), retrosplenial cortex (RSP), medial prefrontal cortex (mPFC), and orbitofrontal cortex (ORB) (Figure **13B**).

We first targeted the mPFC (ACA, PL, and ILA) by expressing chemogenetic receptors in learning-tagged neurons using the TRAP system, enabling either inhibition or activation of this subpopulation. Following the recall phase of the OPCRT, we performed c-Fos immunohistochemistry to quantify neuronal activation within the mPFC (Figure **13C**). Control mice tested at 12h exhibited the expected preference for the object in the novel

configuration, whereas control mice tested at 48h showed no preference, indicating that OPC memory is no longer accessible at this time point. Chemogenetic inhibition of learning-tagged mPFC neurons resulted in a significant impairment of memory performance when mice were tested at 12h, suggesting that the activity of this neuronal population is necessary for successful memory retrieval. In contrast, chemogenetic reactivation of the same population at 48h did not facilitate memory recall, indicating that mPFC learning-tagged neurons activation is not sufficient to restore memory expression once natural retrieval has decayed (Figure **13D** and **13E**). These changes in memory performance were not accompanied by alterations in locomotor activity or in the number of object interactions (Figure **13F** and **13G**). At the cellular level, the density of c-Fos⁺ cells across the entire mPFC did not differ following either inhibitory or excitatory chemogenetic manipulations. Interestingly, under control conditions, c-Fos density was significantly higher at 12h compared to 48h, suggesting that recent memory recall was associated with elevated mPFC activation (Figure **13H**). Analysis of ensemble reactivation revealed that chemogenetic inhibition reduced the proportion of reactivated cells, whereas chemogenetic stimulation induced c-Fos expression in nearly all tagged neurons. Notably, successful natural memory recall at 12h was associated with a significantly higher level of ensemble reactivation compared to 48h, when the memory trace is no longer accessible by natural cues (Figure **13I**). However, no significant correlation was observed between behavioral performance and either c-Fos density or the percentage of ensemble reactivation (Figure **13J**).

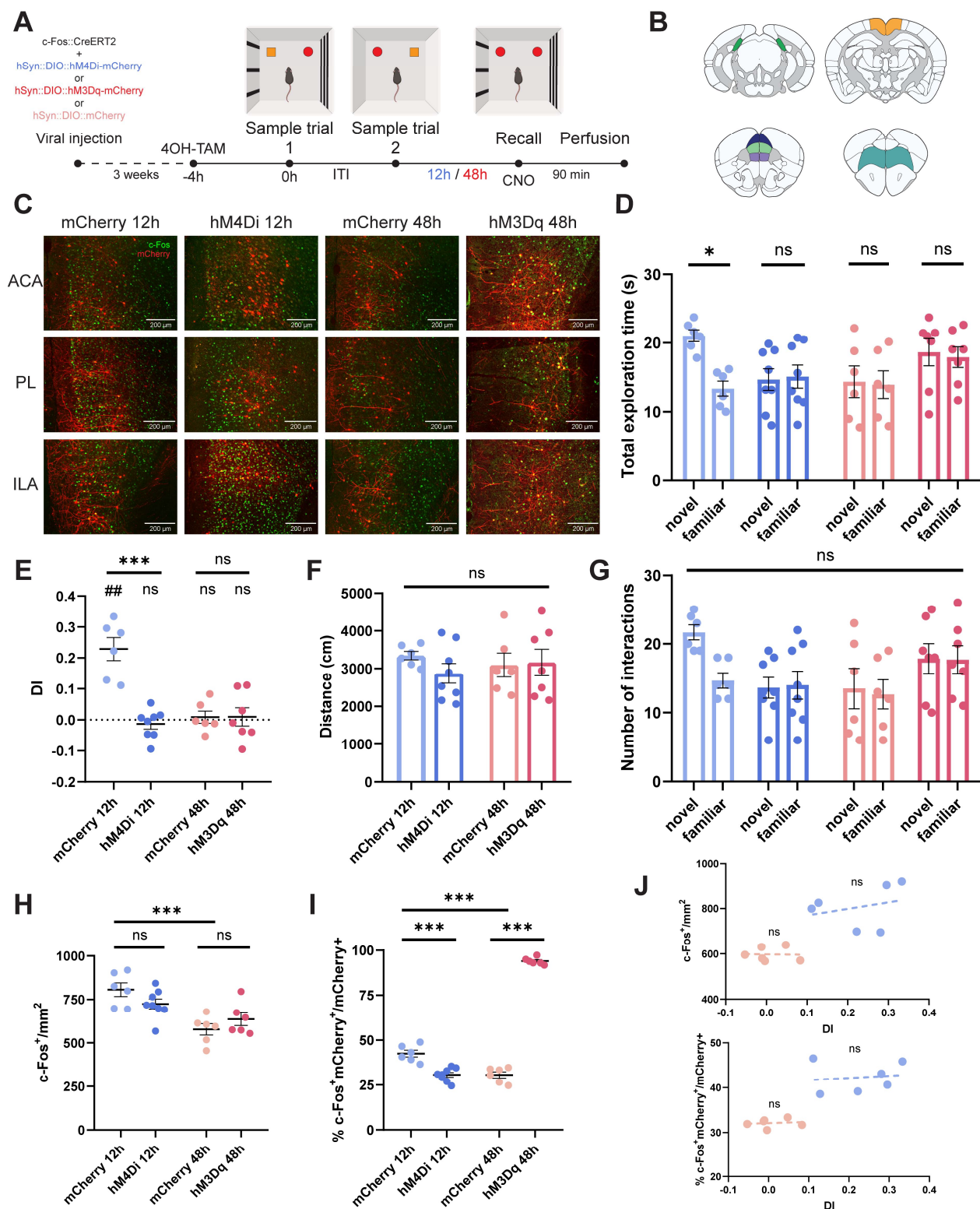


Figure 13. Chemogenetic manipulation of mPFC learning-tagged neuronal populations during OPC memory retrieval.

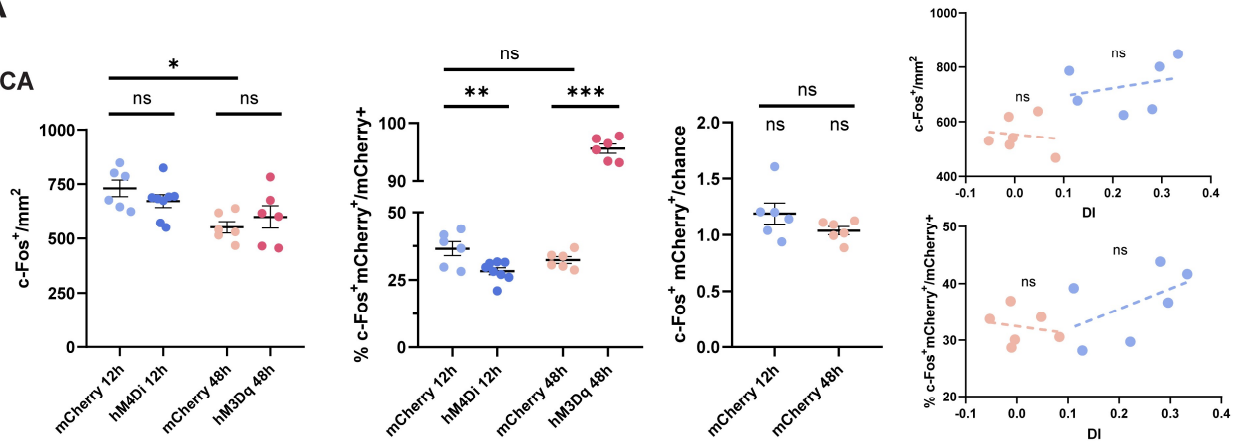
(A) Schematic representation of the TRAP-based chemogenetic manipulation during the OPCRT. The AAV vectors used for neuronal tagging and chemogenetic manipulation are shown on the left. Neuronal populations activated during OPC learning were tagged by 4OH-TAM administration and subsequently manipulated during the recall phase using CNO, either at 12h to induce inhibition or at 48h to induce activation. Mice were perfused 90 min after the recall session. (B) Coronal brain

schematics illustrating the anatomical location of the regions targeted for chemogenetic manipulation, including the postsubiculum (POST), retrosplenial cortex (RSP), medial prefrontal cortex (mPFC), and orbitofrontal cortex (ORB). (C) Representative c-Fos immunohistochemistry of the medial prefrontal cortex (mPFC), shown separately for its three subregions: anterior cingulate cortex (ACA), prelimbic cortex (PL), and infralimbic cortex (ILA). Images are shown for the mCherry 12h, hM4Di 12h, mCherry 48h, and hM3Dq 48h groups. mCherry-expressing neurons are shown in red and c-Fos⁺ cells in green. Scale bars are indicated in the images. (D) Analysis of total object exploration time during the recall phase revealed differential preference for the novel association across experimental groups. A significant increase in exploration of the novel relative to the familiar configuration was observed in the mCherry 12h group, whereas this preference was impaired following chemogenetic inhibition at 12h. No significant difference in exploration time between novel and familiar configuration was detected in either group tested at 48h (two-way ANOVA with Sidak's multiple comparisons test: mCherry 12h Novel 21.04 ± 0.85 $n = 6$ vs mCherry 12h Familiar 13.34 ± 1.08 $n = 6$, * $p = 0.162$; hM4Di 12h Novel 14.66 ± 1.57 $n = 8$ vs hM4Di 12h Familiar 15.08 ± 1.68 $n = 8$, ns $p = 0.995$; mCherry 48h Novel 14.34 ± 2.29 $n = 6$ vs mCherry 48h Familiar 13.92 ± 2.02 $n = 6$, ns $p = 0.9997$; hM3Dq 48h Novel 18.62 ± 1.97 $n = 7$ vs hM3Dq 48h 12h Familiar 17.91 ± 1.50 $n = 7$, ns $p = 0.9969$). (E) Discrimination index (DI) during the recall phase across experimental groups. DI values were significantly greater than chance level only in the mCherry 12h group (mCherry 12h 0.228 ± 0.037 , $n = 6$, ** $p = 0.0017$, $df = 5$, $t = 6.105$; hM4Di 12h -0.013 ± 0.016 , $n = 8$, ns $p = 0.4418$, $df = 7$, $t = 0.8152$; mCherry 48h 0.008 ± 0.019 , $n = 6$, ns $p = 0.6821$, $df = 5$, $t = 0.4344$; hM3Dq 48h 0.009 ± 0.029 , $n = 7$, ns $p = 0.7529$, $df = 6$, $t = 0.3295$; one-sample t test). A significant difference in DI was observed between the mCherry 12h and hM4Di 12h groups, whereas no significant difference was detected between the two groups tested at 48h (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h vs hM4Di 12h, *** $p < 0.001$; mCherry 48h vs hM3Dq 48h, ns $p = 0.9999$). (F-G) No significant differences were observed between experimental groups in total distance traveled (F) (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 3345 ± 112.4 $n = 6$ vs hM4Di 12h 2059 ± 258.1 $n = 8$, ns $p = 0.8142$; mCherry 48h 3094 ± 316.9 $n = 6$ vs hM3Dq 48h 3159 ± 350.2 $n = 7$, ns $p = 0.9999$) or in the number of object interactions (G) (two-way ANOVA with Sidak's multiple comparisons test: mCherry 12h Novel 21.66 ± 1.08 $n = 6$ vs mCherry 12h Familiar 14.66 ± 1.11 $n = 6$, ns $p = 0.0845$; hM4Di 12h Novel 13.62 ± 1.51 $n = 8$ vs hM4Di 12h Familiar 14 ± 2.04 $n = 8$, ns $p = 0.9998$; mCherry 48h Novel 13.5 ± 2.93 $n = 6$ vs mCherry 48h Familiar 12.66 ± 2.12 $n = 6$, ns $p = 0.9976$; hM3Dq 48h Novel 17.85 ± 2.17 $n = 7$ vs hM3Dq 48h 12h Familiar 17.71 ± 2.03 $n = 7$, ns $p = 0.9999$). (H) Quantification of c-Fos⁺ cell density across the entire mPFC. c-Fos density did not differ following inhibitory or excitatory chemogenetic manipulation. Under control conditions, c-Fos density was significantly higher at 12h compared to 48h (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 806.6 ± 39.26 $n = 6$ vs hM4Di 12h 724.4 ± 28.84 $n = 8$, ns $p = 0.3204$; mCherry 48h 578 ± 32.34 $n = 6$ vs hM3Dq 48h 636.4 ± 37.05 $n = 6$, ns $p = 0.6530$; mCherry 12h vs mCherry 48h, *** $p < 0.001$). (I) Ensemble reactivation of learning-tagged neurons in the mPFC. Chemogenetic inhibition significantly reduced the proportion of reactivated neurons, whereas chemogenetic stimulation induced c-Fos expression in nearly all tagged cells. Under control conditions, ensemble reactivation was significantly higher at 12h compared to 48h (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 42.47 ± 1.88 $n = 6$ vs hM4Di 12h 30.67 ± 1.32 $n = 8$, *** $p < 0.001$; mCherry 48h 30.61 ± 1.7 $n = 6$ vs hM3Dq 48h 93.97 ± 0.79 $n = 6$, *** $p < 0.001$; mCherry 12h vs mCherry 48h, *** $p < 0.001$). (J) No significant correlation was observed between DI and c-Fos density (top) ($R^2 = 0.0008$, $p = 0.9556$), or between DI and the percentage of ensemble reactivation (bottom) ($R^2 = 0.0158$, $p = 0.8120$). Data are presented as mean $\pm$ SEM.

To further assess whether these effects differed across mPFC subregions, we next examined c-Fos expression, ensemble reactivation, and their relationship with behavioral performance separately within the ACA, PL, and ILA. Focusing on the ACA, we observed that c-Fos density was significantly higher in the 12h control group compared to the 48h control group. In contrast, the percentage of ensemble reactivation did not differ between these conditions, suggesting that both successful and unsuccessful memory recall engaged a similar proportion of the learning-tagged neurons in this subregion. No significant deviation from chance-level reactivation was observed in either group, indicating that ACA learning-tagged neurons may be recruited to a comparable extent regardless of memory performance. Consistent with this, we did not observe a significant relationship between DI and either c-Fos expression or ensemble reactivation in this subregion (Figure **14A**). In contrast to the ACA, both the PL and ILA exhibited a pattern closely resembling that observed across the entire mPFC. In both subregions, c-Fos⁺ density and the percentage of ensemble reactivation were significantly higher in the 12h control group compared to the 48h group. Ensemble reactivation exceeded chance levels only in the 12h group, while 48h group levels were not different from the chance. Interestingly, within the PL, we observed a strong positive correlation between memory performance and ensemble reactivation in the mCherry 12h group (Figure **14B** and **14C**). Taken together, these findings suggest that distinct mPFC subregions differentially contribute to OPC memory retrieval, with PL and ILA ensembles showing reactivation patterns closely associated with memory accessibility, whereas ACA involvement appears to reflect a more general engagement during recall that is not accompanied by selective reactivation of learning-tagged ensembles.

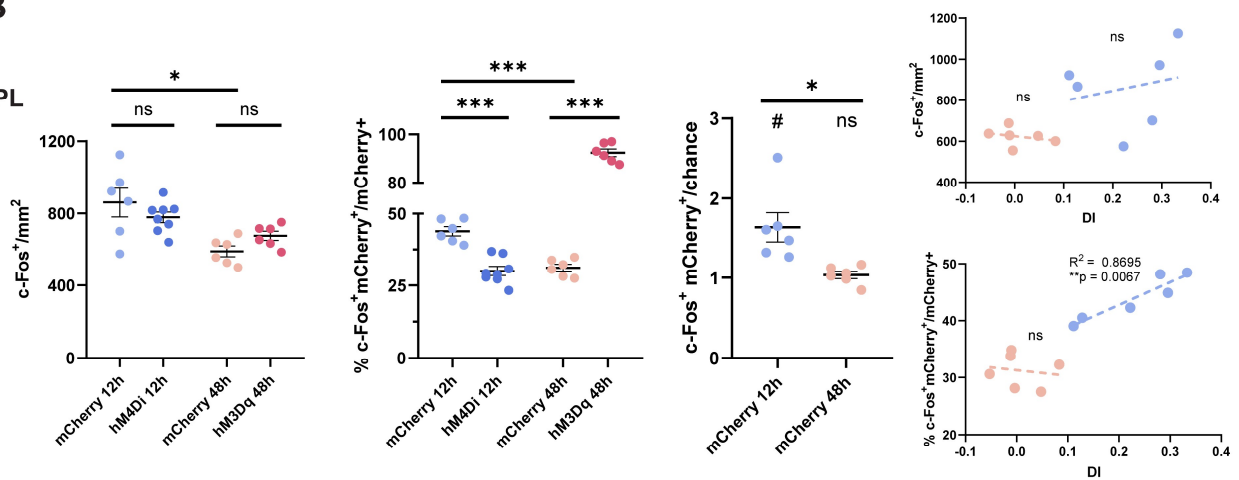
A

ACA



B

PL



C

ILA

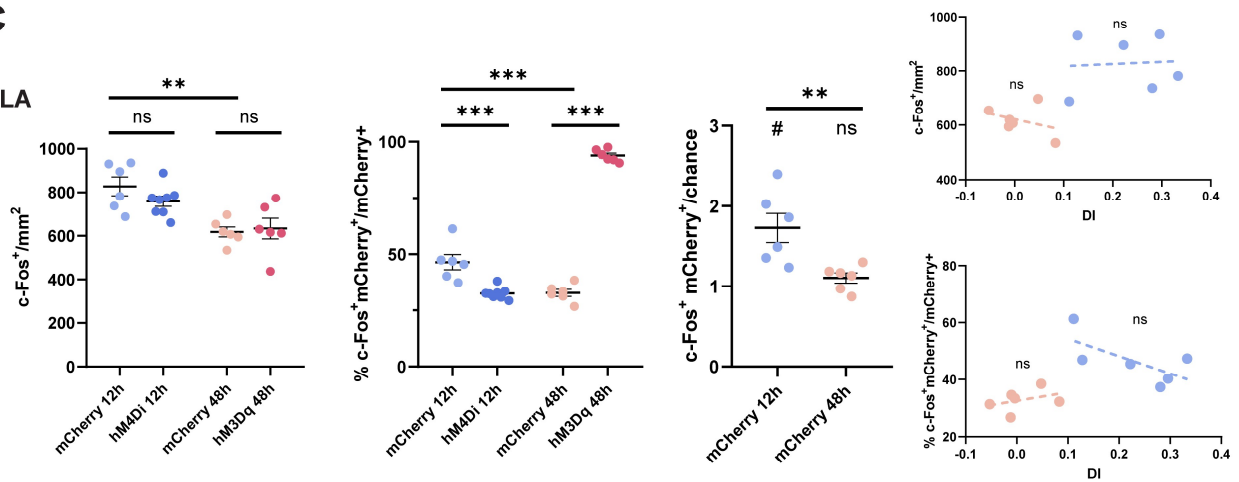


Figure 14. Differential recruitment of mPFC subregions during episodic-like memory recall

(A) Analysis of the ACA. c-Fos⁺ cell density did not differ following inhibitory or excitatory chemogenetic manipulation, but was significantly higher in the mCherry 12h control group compared to the mCherry 48h control group (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 730.7 ± 37.84 $n = 6$ vs hM4Di 12h 672.5 ± 23.65 $n = 8$, ns $p = 0.8363$; mCherry 48h 551.9 ± 26.39 $n = 6$ vs hM3Dq 48h 599.9 ± 51.14 $n = 6$, ns $p = 0.1345$; mCherry 12h vs mCherry 48h, ** $p = 0.0185$). The proportion of ensemble reactivation was significantly reduced following chemogenetic

inhibition and significantly increased following chemogenetic stimulation relative to their respective controls, while no significant difference was observed between the two control groups (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 36.54 ± 2.60 $n = 6$ vs hM4Di 12h 28.28 ± 1.28 $n = 8$, ** $p = 0.0054$; mCherry 48h 32.36 ± 1.26 $n = 6$ vs hM3Dq 48h 95.59 ± 0.79 $n = 6$, *** $p < 0.001$; mCherry 12h vs mCherry 48h, ns $p = 0.3079$). Ensemble reactivation did not differ from chance level in either control group (mCherry 12h 1.189 ± 0.009 , $n = 6$, ns $p = 0.0987$, $df = 5$, $t = 2.025$; mCherry 48h 1.038 ± 0.037 , $n = 6$, ns $p = 0.3591$, $df = 5$, $t = 1.009$; one-sample t test. mCherry 12h vs mCherry 48h, ns $p = 0.1626$, $df = 10$, $t = 1.507$; unpaired t -test). No significant correlation was observed between DI and c-Fos density (top right) (mCherry 12h $R^2 = 0.0811$, $p = 0.5842$; mCherry 48h $R^2 = 0.0178$, $p = 0.8010$), nor between DI and ensemble reactivation for either control group (bottom right) (mCherry 12h $R^2 = 0.2637$, $p = 0.2975$; mCherry 48h $R^2 = 0.039$, $p = 0.7076$).

(B) Analysis of the PL. c-Fos density did not differ following inhibitory or excitatory chemogenetic manipulation, but was significantly higher in the mCherry 12h control group compared to the mCherry 48h control group (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 861.1 ± 80.18 $n = 6$ vs hM4Di 12h 779 ± 30.02 $n = 8$, ns $p = 0.5566$; mCherry 48h 589.5 ± 29.80 $n = 6$ vs hM3Dq 48h 675.9 ± 25.48 $n = 6$, ns $p = 0.5698$; mCherry 12h vs mCherry 48h, ** $p = 0.0025$). Ensemble reactivation was significantly reduced following chemogenetic inhibition and significantly increased following chemogenetic stimulation relative to their respective controls. Ensemble reactivation differed significantly between the two control groups, being higher in mCherry 12h than in mCherry 48h (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 43.88 ± 1.6 $n = 6$ vs hM4Di 12h 30.04 ± 1.28 $n = 8$, *** $p < 0.001$; mCherry 48h 31.20 ± 1.21 $n = 6$ vs hM3Dq 48h 92.41 ± 1.57 $n = 6$, *** $p < 0.001$; mCherry 12h vs mCherry 48h, *** $p < 0.001$). Reactivation in the mCherry 12h group was also significantly higher than both chance level and the mCherry 48h group (mCherry 12h 1.264 ± 0.185 , $n = 6$, * $p = 0.0186$, $df = 5$, $t = 3.430$; mCherry 48h 1.166 ± 0.045 , $n = 6$, ns $p = 0.4912$, $df = 5$, $t = 0.7424$; one-sample t test. mCherry 12h vs mCherry 48h, * $p = 0.0103$, $df = 10$, $t = 3.151$; unpaired t -test). No significant correlation was observed between DI and c-Fos density in either control group (mCherry 12h $R^2 = 0.0530$, $p = 0.6606$; mCherry 48h $R^2 = 0.0828$, $p = 0.3614$); however, a significant positive correlation was observed between ensemble reactivation and DI in the mCherry 12h group (mCherry 12h $R^2 = 0.8695$, $p = 0.0067$; mCherry 48h $R^2 = 0.0252$, $p = 0.7636$).

(C) Analysis of the ILA. c-Fos⁺ cell density did not differ following inhibitory or excitatory chemogenetic manipulation, but was significantly higher in the mCherry 12h control group compared to the mCherry 48h control group (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 827.9 ± 43.45 $n = 6$ vs hM4Di 12h 759.1 ± 24.12 $n = 8$, ns $p = 0.4905$; mCherry 48h 617.6 ± 22.55 $n = 6$ vs hM3Dq 48h 633.5 ± 47.63 $n = 6$, ns $p = 0.9893$; mCherry 12h vs mCherry 48h, ** $p = 0.0024$). The proportion of ensemble reactivation was significantly reduced following chemogenetic inhibition and significantly increased following chemogenetic stimulation relative to their respective controls, with greater reactivation in mCherry 12h compared to mCherry 48h (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 46.48 ± 3.38 $n = 6$ vs hM4Di 12h 32.56 ± 0.91 $n = 8$, *** $p < 0.001$; mCherry 48h 32.78 ± 1.58 $n = 6$ vs hM3Dq 48h 93.92 ± 1.11 $n = 6$, *** $p < 0.001$; mCherry 12h vs mCherry 48h, *** $p < 0.001$). Reactivation exceeded chance levels in the mCherry 12h group but not in the mCherry 48h group, and the two control groups differed significantly (mCherry 12h 1.728 ± 0.181 , $n = 6$, * $p = 0.0103$, $df = 5$, $t = 4.006$; mCherry 48h 1.106 ± 0.063 , $n = 6$, ns $p = 0.1577$, $df = 5$, $t = 1.661$; one-sample t test. mCherry 12h vs mCherry 48h, ** $p = 0.0090$, $df = 10$, $t = 3.231$; unpaired t -test). In contrast to the PL, no significant association was detected between DI and either c-Fos density (mCherry 12h $R^2 = 0.004$, $p = 0.8979$; mCherry 48h $R^2 = 0.1242$, $p = 0.4933$.) or ensemble reactivation (mCherry 12h $R^2 = 0.4589$, $p = 0.1393$; mCherry 48h $R^2 = 0.1414$, $p = 0.4626$.) in this subregion. Data are presented as mean $\pm$ SEM.

We next applied the same TRAP-based chemogenetic approach to assess the contribution of the RSP to episodic memory retrieval (Figure **15A**). Chemogenetic inhibition of learning-tagged RSP neurons resulted in a significant impairment of memory performance at 12h. In addition, chemogenetic reactivation of this neuronal population at 48h significantly facilitated memory recall, a time point at which natural cues alone were insufficient to support memory expression (Figure **15B** and **15C**). As observed for the mPFC, chemogenetic manipulation in the RSP did not affect locomotor activity or object interaction, indicating that the behavioral effects were not driven by nonspecific changes in exploration or motor function (Figure **15D** and **15E**). At the level of neuronal activation, chemogenetic manipulation had no major effect on overall c-Fos expression. However, in the control groups, c-Fos density was significantly higher at 12h compared to 48h, consistent with greater regional activation during successful recall (Figure **15F**). In contrast, the effects of chemogenetic manipulation were pronounced in the learning-tagged neuronal population: inhibition significantly reduced the proportion of reactivated cells, whereas chemogenetic stimulation was associated with a strong increase in ensemble reactivation. In the control condition, a significantly higher proportion of learning-tagged neurons was reactivated during successful memory recall at 12h compared to the later time point (Figure **15G**). Ensemble reactivation exceeded chance levels in the mCherry 12h group but not in the mCherry 48h group (Figure **15H**). While overall c-Fos density did not appear to be directly related to memory performance, a strong positive correlation was observed between DI and the percentage of reactivated neurons in the mCherry 12h group (Figure **15I** and **15J**). Overall, these findings indicate that the RSP is strongly involved in successful episodic memory recall and contains a subpopulation of learning-tagged neurons with properties of engram cells.

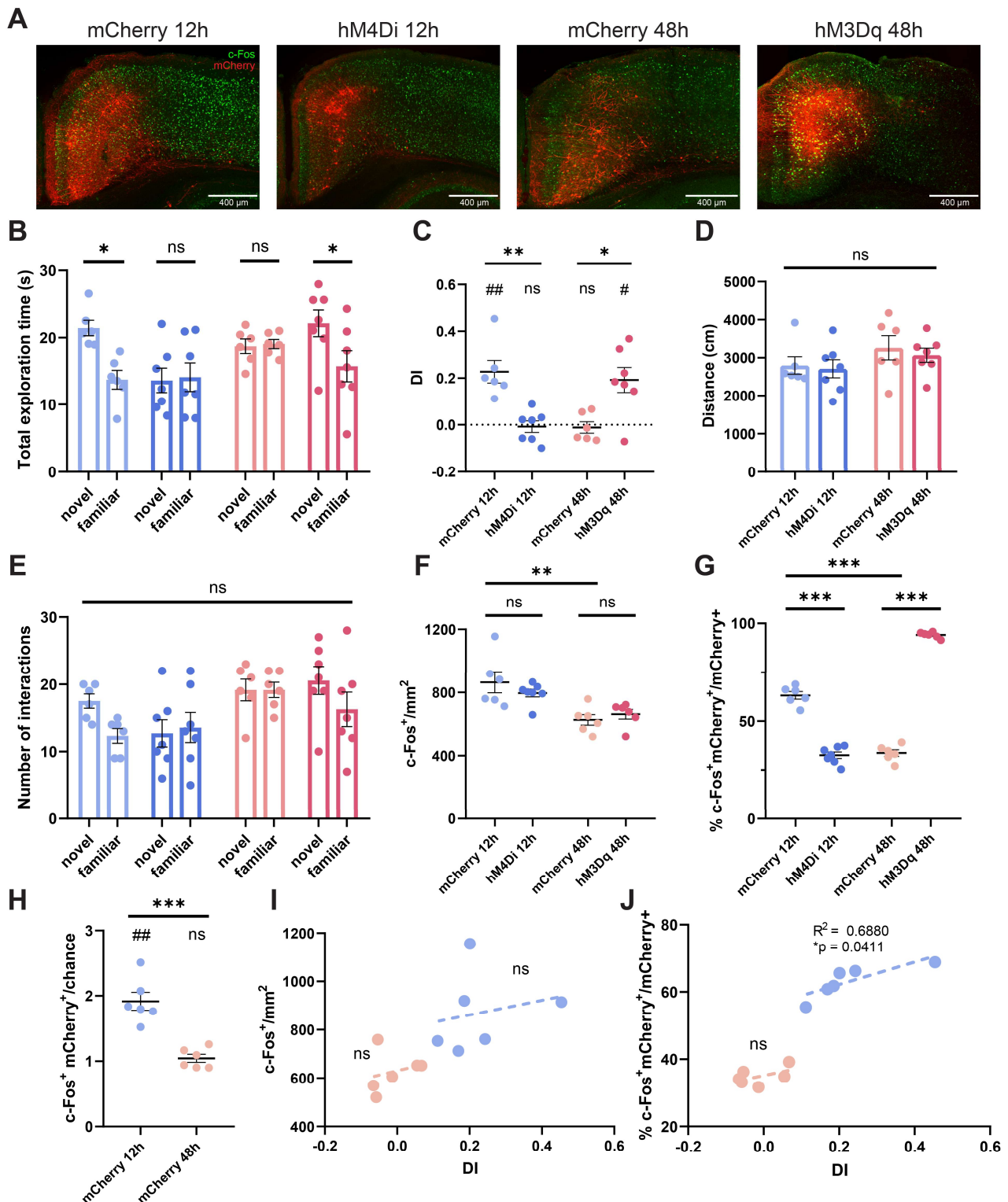


Figure 15. Chemogenetic manipulation of RSP learning-tagged neurons during OPC memory retrieval.

(A) Representative images for the four experimental groups (mCherry 12h, hM4Di 12h, mCherry 48h, and hM3Dq 48h). mCherry⁺ neurons are shown in red and c-Fos⁺ cells in green. Scale bars are indicated in the images. (B) mCherry 12h group showed a significant preference for the object in the novel configuration relative to the familiar one. Chemogenetic inhibition at 12h (hM4Di 12h) impaired novel object exploration, with no difference between novel and familiar exploration times. At 48h, no

preference was observed in the mCherry group, whereas chemogenetic activation (hM3Dq 48h) resulted in significantly greater exploration of the novel object compared to the familiar one (two-way ANOVA with Sidak's multiple comparisons test: mCherry 12h Novel 21.44 ± 1.15 $n = 6$ vs mCherry 12h Familiar 13.72 ± 1.41 $n = 6$, * $p = 0.0176$; hM4Di 12h Novel 13.62 ± 1.83 $n = 8$ vs hM4Di 12h Familiar 14.11 ± 2.13 $n = 7$, ns $p = 0.9993$; mCherry 48h Novel 18.72 ± 1.07 $n = 6$ vs mCherry 48h Familiar 19.05 ± 0.67 $n = 6$, ns $p = 0.9999$; hM3Dq 48h Novel 22.12 ± 1.98 $n = 7$ vs hM3Dq 48h 12h Familiar 15.73 ± 2.31 $n = 7$, * $p = 0.0403$). (C) DI values were significantly greater than 0 in the mCherry 12h and hM3Dq 48h groups, indicating a preference for the object in the novel configuration. In contrast, DI following chemogenetic inhibition at 12h did not differ from chance, indicating a loss of preference for the novel association (mCherry 12h 0.227 ± 0.048 , $n = 6$, ** $p = 0.0054$, $df = 5$, $t = 4.684$; hM4Di 12h -0.007 ± 0.024 , $n = 7$, ns $p = 0.7687$, $df = 6$, $t = 0.3078$; mCherry 48h -0.011 ± 0.024 , $n = 6$, ns $p = 0.6571$, $df = 5$, $t = 0.4716$; hM3Dq 48h 0.1922 ± 0.053 , $n = 7$, * $p = 0.0118$, $df = 6$, $t = 3.572$; one-sample t test). DI also differed significantly between each chemogenetic group and its respective control (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h vs hM4Di 12h, ** $p = 0.003$; mCherry 48h vs hM3Dq 48h, * $p = 0.0111$). (D-E) No differences were detected between experimental groups in locomotor activity (D) (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 2799 ± 229.5 $n = 6$ vs hM4Di 12h 2710 ± 238 $n = 7$, ns $p = 0.9999$; mCherry 48h 3262 ± 319.5 $n = 6$ vs hM3Dq 48h 3068 ± 185.2 $n = 7$, ns $p = 0.9944$) or in the number of interactions with objects (E) (two-way ANOVA with Sidak's multiple comparisons test: mCherry 12h Novel 17.50 ± 1.05 $n = 6$ vs mCherry 12h Familiar 12.33 ± 1.08 $n = 6$, ns $p = 0.2541$; hM4Di 12h Novel 12.71 ± 2.02 $n = 7$ vs hM4Di 12h Familiar 13.57 ± 2.23 $n = 7$, ns $p = 0.9955$; mCherry 48h Novel 19.16 ± 1.62 $n = 6$ vs mCherry 48h Familiar 19.16 ± 1.13 $n = 6$, ns $p = 0.9999$; hM3Dq 48h Novel 20.57 ± 2.09 $n = 7$ vs hM3Dq 48h 12h Familiar 16.28 ± 2.56 $n = 7$, ns $p = 0.3557$). (F) Quantification of c-Fos density in the RSP. c-Fos density did not differ between groups subjected to chemogenetic manipulation and their respective controls, but was significantly higher in the mCherry 12h group compared to the mCherry 48h group (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 864.2 ± 66.93 $n = 6$ vs hM4Di 12h 796 ± 24.85 $n = 7$, ns $p = 0.6375$; mCherry 48h 626.3 ± 33.57 $n = 6$ vs hM3Dq 48h 662.3 ± 30.44 $n = 6$, ns $p = 0.9289$; mCherry 12h vs mCherry 48h, ** $p = 0.0033$). (G) Ensemble reactivation of learning-tagged neurons in the RSP. Chemogenetic inhibition significantly reduced the proportion of reactivated neurons compared to the mCherry 12h control group, whereas chemogenetic stimulation at 48h induced a strong increase in ensemble reactivation. Notably, ensemble reactivation was significantly higher in the mCherry 12h group compared to the mCherry 48h group (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 63.12 ± 1.94 $n = 6$ vs hM4Di 12h 32.56 ± 1.64 $n = 7$, *** $p < 0.001$; mCherry 48h 33.70 ± 1.69 $n = 6$ vs hM3Dq 48h 94.11 ± 0.62 $n = 6$, *** $p < 0.001$; mCherry 12h vs mCherry 48h, *** $p < 0.001$). (H) Analysis of ensemble reactivation relative to chance level in control groups revealed that only the mCherry 12h group showed reactivation significantly above chance and significantly greater than the mCherry 48h group (mCherry 12h 1.916 ± 0.138 , $n = 6$, ** $p = 0.0012$, $df = 5$, $t = 6.614$; mCherry 48h 1.045 ± 0.061 , $n = 6$, ns $p = 0.5039$, $df = 5$, $t = 0.7198$; one-sample t test. mCherry 12h vs mCherry 48h, *** $p < 0.001$, $df = 10$, $t = 5.746$; unpaired t -test). (I) No significant correlation was observed between DI and c-Fos density in the RSP for either control group (mCherry 12h $R^2 = 0.0474$, $p = 0.6786$; mCherry 48h $R^2 = 0.0659$, $p = 0.6232$). (J) A significant positive correlation was observed between ensemble reactivation and DI in the RSP for the mCherry 12h group (mCherry 12h $R^2 = 0.6880$, $p = 0.0411$; mCherry 48h $R^2 = 0.2770$, $p = 0.2834$). Data are presented as mean $\pm$ SEM.

We next characterize the contribution of the ORB using the same approach (Figure **16A**). In control groups, mice tested at 12h displayed the expected significant preference for the

object in the novel configuration. In contrast, chemogenetic inhibition of learning-tagged ORB neurons was associated with a loss of preference, indicating that activity in this population is required for successful memory retrieval. By comparison, chemogenetic reactivation at 48h did not facilitate memory recall, similarly to the results observed in the mPFC (Figure **16B** and **16C**). These behavioral effects were not accompanied by changes in locomotor activity or object exploration (Figure **16D** and **16E**). Quantification of c-Fos expression revealed that chemogenetic manipulation did not significantly alter overall c-Fos density in the ORB. However, considering the control conditions, c-Fos density was significantly higher at 12h compared to 48h, indicating increased ORB engagement during recent memory recall (Figure **16F**). Notably, the proportion of ensemble reactivation did not differ between the mCherry 12h and 48h groups, suggesting that learning-tagged ORB neurons do not exhibit a specific reactivation pattern associated with memory performance (Figure **16G**). Ensemble reactivation exceeded chance levels in both groups, indicating that this behavioral task robustly engages ORB learning-tagged neurons regardless of memory accessibility (Figure **16H**). Consistent with this, no significant relationship was observed between DI and either c-Fos density or ensemble reactivation (Figure **9I** and **9J**).

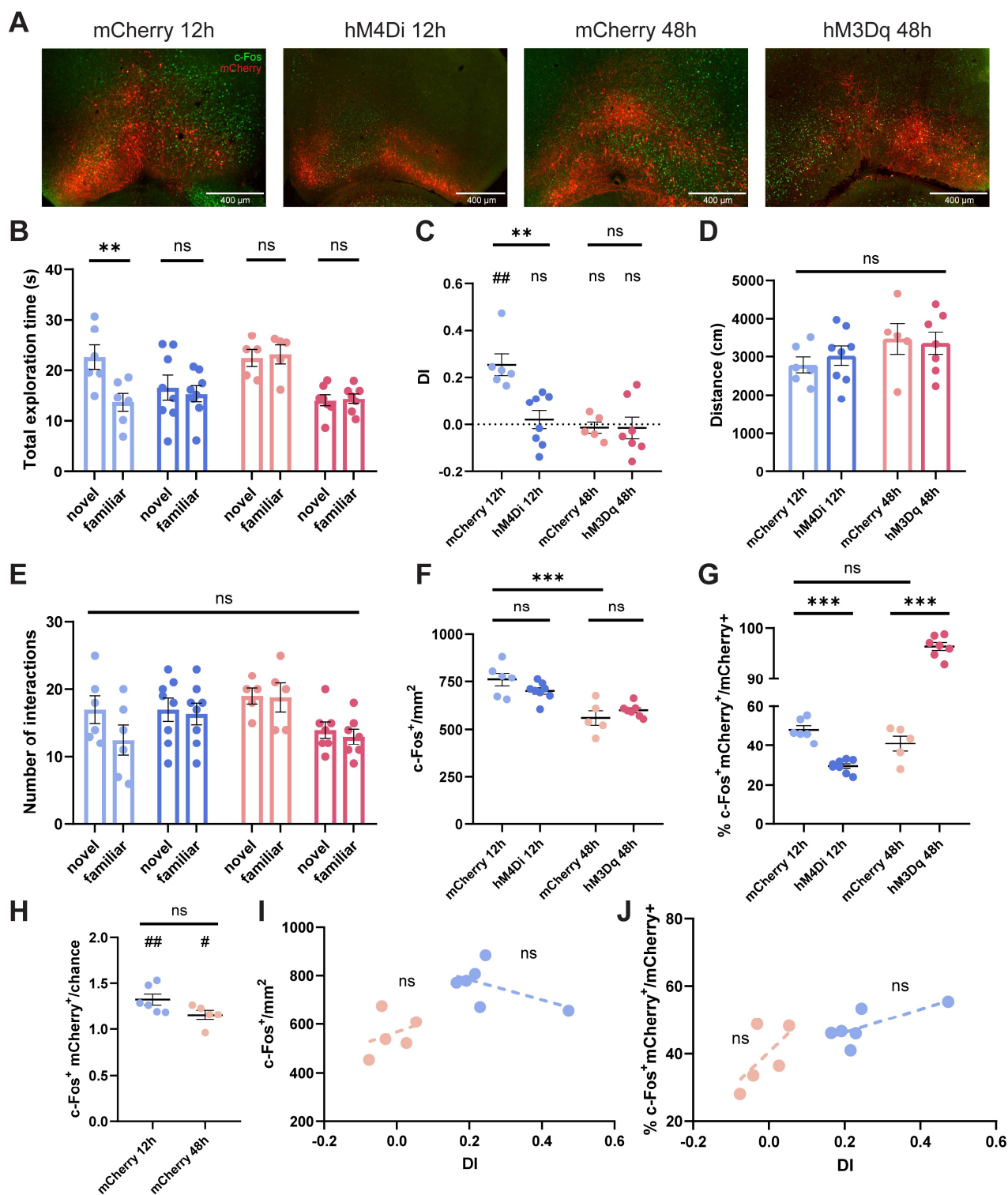


Figure 16. Chemogenetic manipulation of ORB learning-tagged neurons during OPC memory retrieval.

(A) Representative c-Fos immunohistochemistry images for the four experimental groups (mCherry 12h, hM4Di 12h, mCherry 48h, and hM3Dq 48h). mCherry⁺ neurons are shown in red and c-Fos⁺ cells in green. Scale bars are indicated in the images. (B) Analysis of total object exploration time during the recall phase. The mCherry 12h group showed a significant preference for the object in the novel configuration. Chemogenetic inhibition at 12h (hM4Di 12h) resulted in a loss of preference

for the novel association. At 48h, neither the mCherry nor the hM3Dq group showed a significant preference (two-way ANOVA with Sidak's multiple comparisons test: mCherry 12h Novel 22.59 ± 2.40 $n = 6$ vs mCherry 12h Familiar 13.63 ± 1.81 $n = 6$, ** $p = 0.008$; hM4Di 12h Novel 16.62 ± 2.45 $n = 8$ vs hM4Di 12h Familiar 15.36 ± 1.63 $n = 8$, ns $p = 0.9738$; mCherry 48h Novel 22.41 ± 1.66 $n = 5$ vs mCherry 48h Familiar 23.11 ± 1.86 $n = 5$, ns $p = 0.9988$; hM3Dq 48h Novel 14.09 ± 1.16 $n = 7$ vs hM3Dq 48h Familiar 14.40 ± 0.99 $n = 7$, ns $p = 0.9999$). (C) The mCherry 12h group showed a DI significantly greater than chance, indicating a preference for the novel association. Chemogenetic inhibition at 12h reduced DI to chance level, and chemogenetic activation at 48h failed to restore memory performance group (mCherry 12h 0.253 ± 0.045 , $n = 6$, ** $p = 0.0026$, $df = 5$, $t = 5.565$; hM4Di 12h -0.020 ± 0.037 , $n = 8$, ns $p = 0.6061$, $df = 7$, $t = 0.5397$; mCherry 48h -0.013 ± 0.023 , $n = 5$, ns $p = 0.5947$, $df = 4$, $t = 0.5772$; hM3Dq 48h -0.014 ± 0.045 , $n = 7$, ns $p = 0.7542$, $df = 6$, $t = 0.3279$; one-sample t test). DI differed significantly between the mCherry 12h and hM4Di 12h groups, but not between the two groups tested at 48h (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h vs hM4Di 12h, ** $p = 0.002$; mCherry 48h vs hM3Dq 48h, ns $p = 0.9999$). (D-E) No significant differences were observed between experimental groups in total distance traveled (D) (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 2786 ± 206.1 $n = 6$ vs hM4Di 12h 3024 ± 251.5 $n = 8$, ns $p = 0.9923$; mCherry 48h 3466 ± 410.2 $n = 5$ vs hM3Dq 48h 3354 ± 299.1 $n = 7$, ns $p = 0.9999$) or in the number of object interactions (E) (two-way ANOVA with Sidak's multiple comparisons test: mCherry 12h Novel 17 ± 2.03 $n = 6$ vs mCherry 12h Familiar 12.5 ± 2.26 $n = 6$, ns $p = 0.2759$; hM4Di 12h Novel 17 ± 1.71 $n = 8$ vs hM4Di 12h Familiar 16.37 ± 1.59 $n = 8$, ns $p = 0.9974$; mCherry 48h Novel 19 ± 1.18 $n = 5$ vs mCherry 48h Familiar 18.80 ± 1.23 $n = 5$, ns $p = 0.9999$; hM3Dq 48h Novel 14 ± 1.21 $n = 7$ vs hM3Dq 48h Familiar 13 ± 1.13 $n = 7$, ns $p = 0.9876$). (F) c-Fos density did not differ between chemogenetically manipulated groups and their respective controls, but was significantly higher in the mCherry 12h group compared to the mCherry 48h group (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 761.4 ± 34.71 $n = 6$ vs hM4Di 12h 700.4 ± 16.64 $n = 8$, ns $p = 0.3061$; mCherry 48h 560 ± 38.18 $n = 5$ vs hM3Dq 48h 600.2 ± 13.05 $n = 7$, ns $p = 0.7023$; mCherry 12h vs mCherry 48h, *** $p < 0.001$). (G) The proportion of reactivated neurons did not differ between the mCherry 12h and mCherry 48h control groups. Reactivation was significantly reduced in the hM4Di 12h group compared to its mCherry 12h control, whereas chemogenetic stimulation at 48h (hM3Dq 48h) resulted in higher ensemble reactivation (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 48.07 ± 2.18 $n = 6$ vs hM4Di 12h 29.50 ± 1.14 $n = 8$, *** $p < 0.001$; mCherry 48h 41.01 ± 3.94 $n = 5$ vs hM3Dq 48h 96.40 ± 0.78 $n = 7$, *** $p < 0.001$; mCherry 12h vs mCherry 48h, ns $p = 0.1228$). (H) Analysis of ensemble reactivation relative to chance level revealed significant reactivation in both control groups, with no difference between mCherry 12h and mCherry 48h (mCherry 12h 1.187 ± 0.06 , $n = 6$, ** $p = 0.0029$, $df = 5$, $t = 5.398$; mCherry 48h 1.156 ± 0.052 , $n = 5$, * $p = 0.0410$, $df = 4$, $t = 2.974$; one-sample t test. mCherry 12h vs mCherry 48h, ns $p = 0.0696$, $df = 9$, $t = 2.059$; unpaired t -test). (I-J) No significant correlation was observed between DI and c-Fos density (I) (mCherry 12h $R^2 = 0.2897$, $p = 0.2706$; mCherry 48h $R^2 = 0.1004$, $p = 0.6035$), or between DI and ensemble reactivation (J) (mCherry 12h $R^2 = 0.5209$, $p = 0.1054$; mCherry 48h $R^2 = 0.9729$, $p = 0.2740$). Data are presented as mean $\pm$ SEM.

Finally, using the same viral strategy, we targeted the POST to investigate the contribution of learning-tagged neurons in this region to OPC memory retrieval (Figure 17A). As observed in other brain regions, chemogenetic inhibition of learning-tagged POST neurons significantly impaired memory performance, whereas chemogenetic reactivation failed to facilitate memory recall at 48h (Figure 17B and 17C). These behavioral effects were not associated with significant alterations in locomotor activity or object interactions (Figure 17D

and **17E**). Quantification of c-Fos expression revealed that POST activation was significantly higher at 12h compared to 48h under control conditions, while chemogenetic manipulation did not affect overall c-Fos density across the entire region (Figure **17F**). In contrast, strong effects were observed at the level of ensemble reactivation. The proportion of reactivated learning-tagged neurons was significantly higher during recent recall compared to the recall at 48h. Moreover, chemogenetic manipulation strongly modulated ensemble reactivation, with inhibition reducing reactivation in the hM4Di group and stimulation driving near-complete reactivation in the hM3Dq group (Figure **17G**). Although ensemble reactivation was significantly greater at 12h than at 48h, reactivation levels in both groups exceeded chance (Figure **17H**). Finally, no significant relationship was observed between discrimination DI and either c-Fos density or ensemble reactivation (Figure **17I** and **17J**).

Overall, these results indicate that multiple brain regions recruited during OPC memory recall are necessary for successful retrieval, as demonstrated by the consistent impairment observed following chemogenetic inhibition of the selected learning-tagged neuronal populations. However, in the mPFC, ORB, and POST, artificial reactivation of learning-tagged neurons failed to facilitate memory recall at a late time point, suggesting that these regions contribute to the coordination and expression of this memory rather than serving as primary sites of storage. In contrast, chemogenetic reactivation of learning-tagged neurons in the RSP was sufficient to drive successful memory recall, indicating that this region contains neuronal populations with characteristics of engram cells for OPC memory.

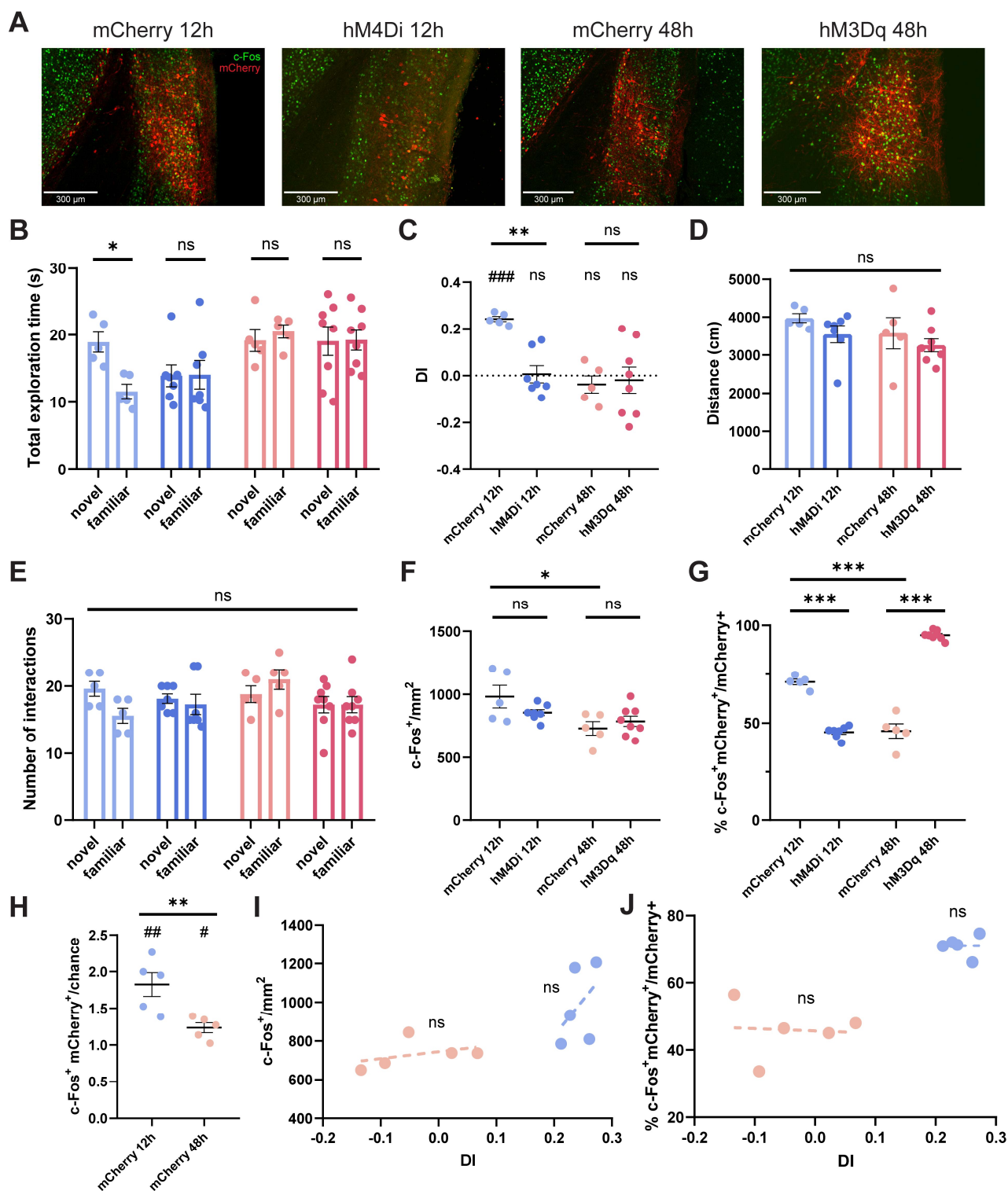


Figure 17. Chemogenetic manipulation of POST learning-tagged neuronal populations during OPC memory retrieval.

(A) Representative c-Fos immunohistochemistry images for the four experimental groups (mCherry 12h, hM4Di 12h, mCherry 48h, and hM3Dq 48h). mCherry⁺ neurons are shown in red and c-Fos⁺ cells in green. Scale bars are indicated in the images. (B) The mCherry 12h group showed a significant preference for the object in the novel configuration. Chemogenetic inhibition at 12h (hM4Di 12h) impaired novel object exploration. At 48h, neither the mCherry nor the hM3Dq group

showed a significant preference (two-way ANOVA with Sidak's multiple comparisons test: mCherry 12h Novel 18.92 ± 1.48 $n = 5$ vs mCherry 12h Familiar 11.60 ± 1.07 $n = 5$, * $p = 0.0498$; hM4Di 12h Novel 13.92 ± 1.61 $n = 7$ vs hM4Di 12h Familiar 14.08 ± 2.12 $n = 7$, ns $p = 0.9999$; mCherry 48h Novel 19.09 ± 2.13 $n = 5$ vs mCherry 48h Familiar 20.52 ± 0.99 $n = 5$, ns $p = 0.9831$; hM3Dq 48h Novel 19.09 ± 2.13 $n = 8$ vs hM3Dq 48h 12h Familiar 19.27 ± 1.54 $n = 8$, ns $p = 0.9999$). C) DI values were significantly greater than 0 only in the mCherry 12h group. Chemogenetic inhibition at 12h reduced DI to chance level, and chemogenetic activation at 48h did not facilitate memory recall (mCherry 12h 0.241 ± 0.011 , $n = 5$, *** $p < 0.001$, $df = 4$, $t = 21.81$; hM4Di 12h 0.006 ± 0.036 , $n = 7$, ns $p = 0.8695$, $df = 6$, $t = 0.1715$; mCherry 48h -0.037 ± 0.036 , $n = 5$, ns $p = 0.3647$, $df = 4$, $t = 1.022$; hM3Dq 48h -0.019 ± 0.055 , $n = 8$, ns $p = 0.7392$, $df = 7$, $t = 0.3465$; one-sample t test). DI differed significantly between the mCherry 12h and hM4Di 12h groups, but not between the two groups tested at 48h (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h vs hM4Di 12h, ** $p = 0.009$; mCherry 48h vs hM3Dq 48h, ns $p = 0.9999$). (D-E) No significant differences were observed between experimental groups in total distance traveled (D) (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 3975 ± 120.2 $n = 5$ vs hM4Di 12h 3546 ± 226.5 $n = 7$, ns $p = 0.7937$; mCherry 48h 3575 ± 412.7 $n = 5$ vs hM3Dq 48h 3254 ± 168 $n = 8$, ns $p = 0.9283$) or in the number of object interactions (E) (two-way ANOVA with Sidak's multiple comparisons test: mCherry 12h Novel 19.6 ± 1.12 $n = 5$ vs mCherry 12h Familiar 15.6 ± 1.12 $n = 5$, ns $p = 0.1746$; hM4Di 12h Novel 18.14 ± 0.70 $n = 7$ vs hM4Di 12h Familiar 17.28 ± 1.49 $n = 7$, ns $p = 0.9760$; mCherry 48h Novel 18.8 ± 1.22 $n = 5$ vs mCherry 48h Familiar 21 ± 1.48 $n = 5$, ns $p = 0.7103$; hM3Dq 48h Novel 17.25 ± 1.22 $n = 8$ vs hM3Dq 48h 12h Familiar 17.28 ± 1.17 $n = 8$, ns $p = 0.9999$). (F) Quantification of c-Fos density in the POST. c-Fos density was significantly higher in the mCherry 12h group compared to the mCherry 48h group, while chemogenetic manipulation did not significantly alter overall c-Fos density relative to respective controls (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 982.4 ± 89.39 $n = 5$ vs hM4Di 12h 855.8 ± 24.01 $n = 7$, ns $p = 0.3459$; mCherry 48h 731.2 ± 54.33 $n = 5$ vs hM3Dq 48h 787.5 ± 41.04 $n = 8$, ns $p = 0.8628$; mCherry 12h vs mCherry 48h, * $p = 0.0239$). (G) Ensemble reactivation of learning-tagged neurons in the POST. The percentage of reactivated neurons was significantly higher in the mCherry 12h group compared to the mCherry 48h group. Chemogenetic inhibition significantly reduced ensemble reactivation relative to the mCherry 12h control, whereas chemogenetic stimulation at 48h (hM3Dq 48h) induced a strong increase in reactivation (one-way ANOVA with Sidak's multiple comparisons test: mCherry 12h 71 ± 1.39 $n = 5$ vs hM4Di 12h 45.35 ± 1.12 $n = 7$, *** $p < 0.001$; mCherry 48h 45.91 ± 3.64 $n = 5$ vs hM3Dq 48h 94.97 ± 0.80 $n = 8$, *** $p < 0.001$; mCherry 12h vs mCherry 48h, *** $p < 0.001$). (H) Analysis of ensemble reactivation relative to chance level revealed that reactivation exceeded chance in both control groups, despite significantly higher reactivation in the mCherry 12h group compared to the mCherry 48h group (mCherry 12h 1.825 ± 0.161 , $n = 5$, ** $p = 0.0069$, $df = 4$, $t = 5.108$; mCherry 48h 1.235 ± 0.068 , $n = 5$, * $p = 0.0264$, $df = 4$, $t = 3.435$; one-sample t test. mCherry 12h vs mCherry 48h, ** $p = 0.0098$, $df = 8$, $t = 3.367$; unpaired t -test). (I-J) No significant correlation was observed between DI and c-Fos density (I) (mCherry 12h $R^2 = 0.2078$, $p = 0.4404$; mCherry 48h $R^2 = 0.1601$, $p = 0.5044$), or between DI and ensemble reactivation (J) (mCherry 12h $R^2 = 0.0001$, $p = 0.9825$; mCherry 48h $R^2 = 0.0004$, $p = 0.9110$). Data are presented as mean $\pm$ SEM.

4.10 Proposed distributed network for episodic-like memory

Considering the activation patterns observed in the brain-wide c-Fos mapping together with the region-specific functional dissection obtained through chemogenetic manipulation, a frontal and posterior medial network emerges as necessary for episodic-like memory recall. In addition, previous evidence demonstrated that episodic-like memory expression

correlated with LEC synaptic plasticity changes, and that this subregion contains neuronal populations that are both necessary and sufficient for OPC memory recall (Tozzi et al., 2024). Taken together, these findings support a distributed network sustaining episodic-like memory in mice, in which the RSP and LEC contain neuronal populations with properties of engram cells that are fundamental for successful memory retrieval (Figure 18A). The remaining brain regions within this network are necessary for the correct behavioral expression, with a prominent involvement of the POST, PL and ILA which showed a significant reactivation of learning-tagged neurons during recent memory recall. Based on the major anatomical connections between these brain regions (Know et al., 2019), the LEC emerges as a key area linking the frontal and posterior medial components of the network. Notably, information from the RSP and POST is likely conveyed to the LEC through strong connections with the MEC; however, this subdivision appears to be less directly involved in OPC memory recall (Tozzi et al., 2024). Direct anatomical connections are also present between the RSP and ACA. Although the ACA showed a less direct involvement in OPC memory, its connectivity suggests a potential role as a relay between the RSP and the frontal associative hub.

A

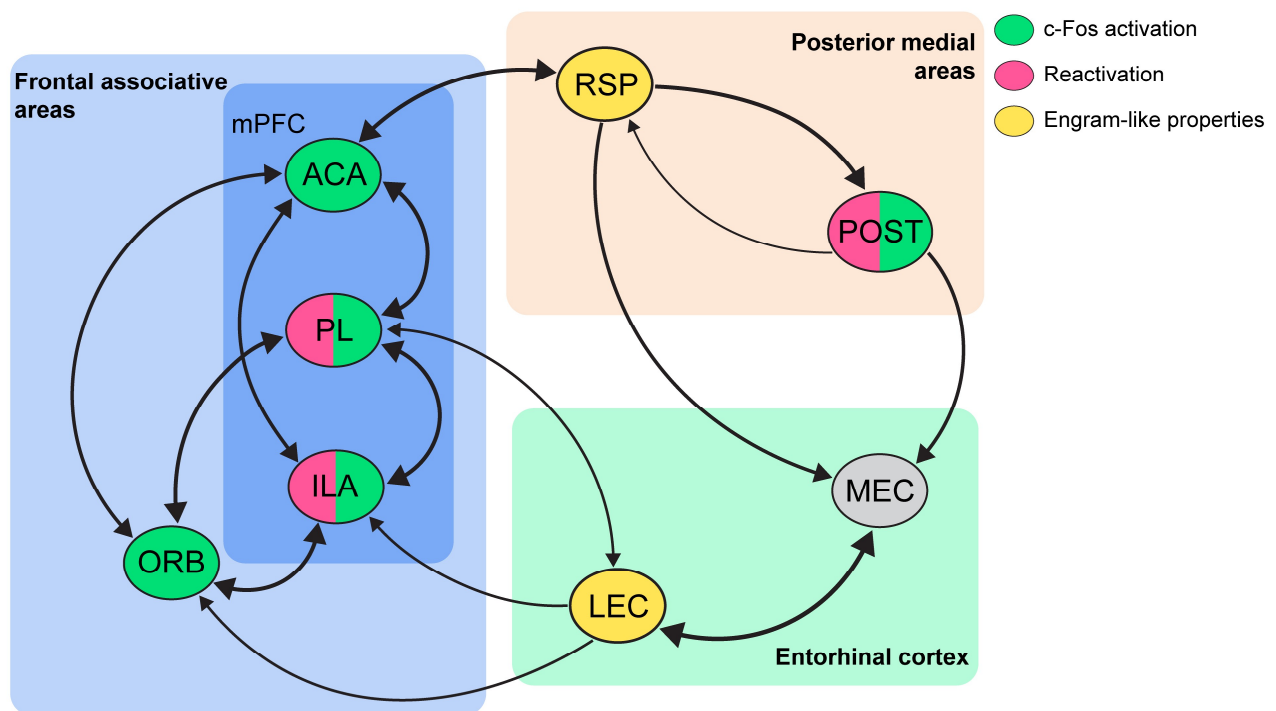


Figure 18. Proposed distributed network for episodic-like memory.

(A) The schematic illustrates a distributed network organized into three major components: frontal associative areas, posterior medial areas, and the entorhinal cortex. Nodes represent brain regions identified as part of the OPC memory network based on the present study and previous work. Green nodes indicate brain regions that showed increased c-Fos activation during OPC memory recall at 12h compared to 48h. Red nodes indicate regions in which learning-tagged neuronal ensembles

exhibited significantly higher reactivation at 12h relative to 48h. Yellow nodes highlight brain regions containing learning-tagged neuronal populations with properties of engram cells for OPC memory, as demonstrated by both necessity and sufficiency for memory recall. The MEC is included based on its anatomical position linking posterior medial areas with the LEC. Edges represent major direct anatomical connections between regions, with edge width proportional to connection density.

4.11 Simultaneous recordings of mPFC and RSP neural activity during memory recall

To further investigate the specific contribution of the mPFC and RSP during episodic-like memory recall, we analyzed changes in local field potential (LFP) signals simultaneously recorded from the two regions during the recall phase of the OPCRT in freely moving mice. LFP activity was recorded using tetrodes and aligned to object exploration epochs; the location of individual tetrodes was confirmed by post-hoc histological analysis (Figure **19A** and **19B**). Spectral analysis of mPFC activity during novel and familiar object exploration revealed a selective increase in low-frequency power during novel object interaction (Figure **19C** and **19D**). Focusing on the theta (5-10 Hz), low-gamma (30-55 Hz), and high-gamma (55-80 Hz) bands, we observed a significant increase in theta power during novel exploration (Figure **19E**). In the RSP, exploration of both novel and familiar objects was associated with a selective increase in theta-band activity; however, no significant differences were observed between the two conditions across the frequency bands considered (Figure **19F**). Interestingly, a modest increase in high-gamma power was observed during novel object exploration, although this effect was not statistically significant (Figure **19G** and **19H**).

We next examined functional coupling between the mPFC and RSP by analyzing LFP coherence during novel and familiar object exploration (Figure **19I**). Coherence analysis provides a measure of frequency-specific synchronization between neural signals recorded from distinct brain regions. Coherence spectra obtained during novel and familiar exploration were largely overlapping, and no statistically significant differences were detected across the frequency range examined (Figure **19J**). Interestingly, for both conditions coherence analysis produced a U-shaped profile, characterized by higher coherence values in the theta and high-gamma bands and lower coherence at intermediate frequencies.

Finally, we assessed the directionality of mPFC-RSP interactions using Granger causality analysis during novel and familiar object exploration. Granger causality analysis assesses whether activity in one time series predicts future activity in another. When expressed in the frequency domain, this approach allows the investigation of directional interactions between brain areas in specific frequency bands. For both conditions, Granger causality was higher in the theta band for the RSP to mPFC direction compared to the mPFC to RSP direction, suggesting that RSP played a leading role in driving mPFC activity during novel exploration (Figure **19K**, **19L**, **19M** and **19N**). By contrast, Granger causality in the low- and high-gamma bands was low and did not differ between directions. Notably, in the RSP to mPFC direction, Granger causality values were higher during familiar object exploration compared to novel object exploration, suggesting that the strength of directional interactions varies with recognition of the OPC association. This effect was specific to the theta frequency band and was not observed in the gamma ranges. Taken together, these results indicate that, during episodic-like memory recall, exploration of the novel object is associated with increased

theta power in the mPFC, whereas no differences in theta or gamma power were detected in the RSP comparing novel and familiar exploration. In the theta band, directional interactions from RSP to mPFC were observed in both conditions and were more pronounced during familiar object exploration, suggesting that contextual input from the RSP partially drives prefrontal activity, particularly during familiarity recognition.

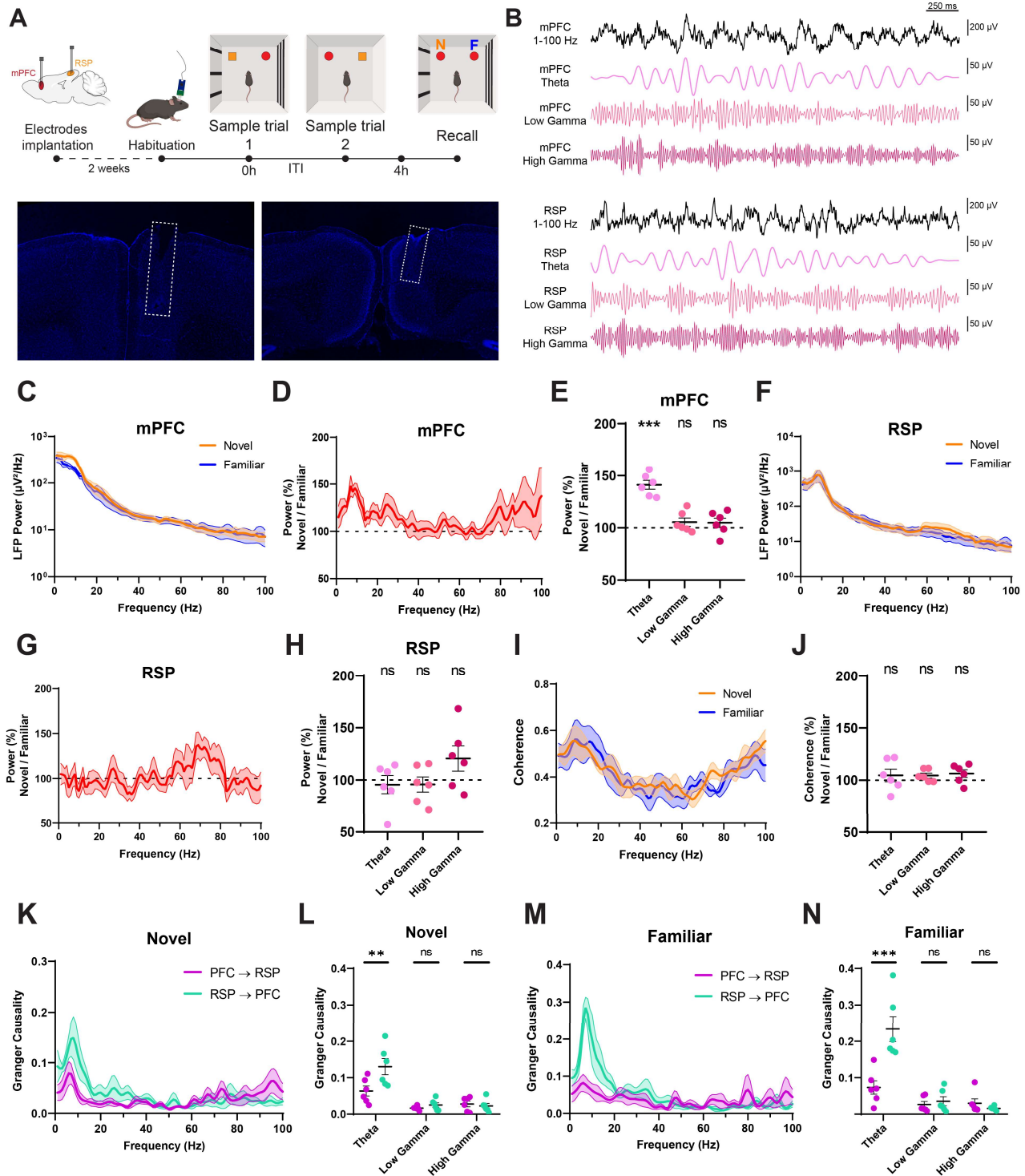


Figure 19. Simultaneous recordings of mPFC and RSP neural activity during memory recall.

(A) *In vivo* simultaneous recordings from the medial prefrontal cortex (mPFC) and retrosplenial cortex (RSP) in freely moving mice during the recall phase of the object-place-context recognition task (OPCRT). Histological confirmation of electrode locations in the mPFC and RSP are shown below. (B) Representative examples of band-pass filtered LFP traces from both regions during object exploration. (C) mPFC power spectral density during novel and familiar object exploration epochs. (D) mPFC power expressed as the percentage of the novel exploration epochs relative to the familiar exploration epochs. (E) Band-averaged mPFC power in the theta (5-10 Hz), low-gamma (30-55 Hz), and high-gamma (55-80 Hz) frequency ranges, expressed as the percentage of the novel exploration epochs relative to the familiar exploration epochs. Theta-band power was significantly higher during novel compared to familiar exploration, whereas no significant differences were observed in the gamma bands (Theta 141.2 ± 4.290 , $n = 6$, *** $p = 0.0002$, $df = 5$, $t = 9.597$; Low Gamma 105.4 ± 3.978 , $n = 6$, ns $p = 0.2317$, $df = 5$, $t = 1.361$; High Gamma 104.9 ± 4.469 , $n = 6$, ns $p = 0.3272$, $df = 5$, $t = 1.086$; one-sample t test). (F) RSP power spectral density during novel and familiar object exploration epochs. (G) mPFC-RSP power expressed as the percentage of the novel exploration epochs relative to the familiar exploration epochs. (H) Band-averaged RSP power during novel and familiar object exploration epochs. No significant differences were observed between conditions across the frequency bands examined (Theta 95.57 ± 8.686 , $n = 6$, ns $p = 0.6321$, $df = 5$, $t = 0.5094$; Low Gamma 95.76 ± 7.286 , $n = 6$, ns $p = 0.5863$, $df = 5$, $t = 0.5812$; High Gamma 120.6 ± 12.09 , $n = 6$, ns $p = 0.1486$, $df = 5$, $t = 1.707$; one-sample t test). (I) Coherence between mPFC and RSP during novel and familiar object exploration epochs. Coherence spectra were computed across the 1-100 Hz range and exhibited comparable profiles between conditions. (J) mPFC-RSP coherence expressed as the percentage of the novel exploration epochs relative to the familiar exploration epochs. No significant differences were observed between conditions across the frequency bands considered (Theta 104.7 ± 5967 , $n = 6$, ns $p = 0.4675$, $df = 5$, $t = 0.7859$; Low Gamma 104.6 ± 2.347 , $n = 6$, ns $p = 0.1098$, $df = 5$, $t = 0.1942$; High Gamma 106.4 ± 3.635 , $n = 6$, ns $p = 0.1366$, $df = 5$, $t = 1.772$; one-sample t test). (J) Granger causality spectra during novel object exploration epochs for both directions of interaction between RSP and mPFC. (L) During novel exploration, Granger causality in the RSP to mPFC direction was significantly higher than in the mPFC to RSP direction (two-way ANOVA with Sidak's multiple comparisons test: PFC to RSP Theta 0.064 ± 0.014 $n = 6$ vs RSP to PFC Theta 0.130 ± 0.022 $n = 6$, ** $p = 0.0011$; PFC to RSP Low Gamma 0.017 ± 0.002 $n = 6$ vs RSP to PFC Low Gamma 0.025 ± 0.006 $n = 6$, ns $p = 0.9400$; PFC to RSP High Gamma 0.028 ± 0.008 $n = 6$ vs RSP to PFC High Gamma 0.022 ± 0.007 $n = 6$, ns $p = 0.9836$). (M) Granger causality spectra during familiar object exploration epochs for both directions of interaction between RSP and mPFC. (N) During familiar exploration, Granger causality in the RSP to mPFC direction was significantly higher than in the mPFC to RSP direction (two-way ANOVA with Sidak's multiple comparisons test: PFC to RSP Theta 0.073 ± 0.018 $n = 6$ vs RSP to PFC Theta 0.234 ± 0.035 $n = 6$, *** $p < 0.001$; PFC to RSP Low Gamma 0.026 ± 0.008 $n = 6$ vs RSP to PFC Low Gamma 0.035 ± 0.013 $n = 6$, ns $p = 0.9795$; PFC to RSP High Gamma 0.030 ± 0.012 $n = 6$ vs RSP to PFC High Gamma 0.016 ± 0.002 $n = 6$, ns $p = 0.9273$). Scale bars are shown in the images. Data are presented as mean $\pm$ SEM.

5. Discussion

Episodic memory refers to the ability to recollect past experiences together with their specific spatial and temporal details (Tulvig, 1983). Rodents exhibit an intrinsic tendency to explore novel stimuli, making them a valuable experimental model for the study of episodic-like memory without the need for explicit training paradigms (Ennaceur and Delacour, 1988). In this context, the Object-Place-Context Recognition Test (OPCRT) has been widely used to investigate the neural substrates of episodic-like memory, revealing the critical contribution of the hippocampus and the lateral entorhinal cortex (LEC) (Eacott and Norman, 2004; Wilson et al., 2013). The unique anatomical and functional organization of the hippocampus enables discrete experiences to be represented by distinct patterns of neuronal activity across hippocampal ensembles. This sparse coding supports pattern separation during memory encoding, allowing similar experiences to be stored as non-overlapping representations, while the coordinated reactivation of hippocampal ensembles supports memory retrieval (Sugar and Moser, 2019). Given its central role in the encoding and storage of relevant experiences, it is not surprising that hippocampal lesions critically impair the ability of rodents to recall object-place-context (OPC) associations (Langston and Wood, 2010). Interestingly, the LEC has also emerged as a critical component of the circuit required for episodic-like memory retrieval (Wilson et al., 2013; Vandrey et al., 2020). Accordingly, the traditional view of the LEC as a subdivision primarily conveying contextual information to the hippocampus has been substantially expanded. Recent evidence indicates that the LEC can directly contribute to the formation of associations and encode detailed features of experiences, including spatial and temporal information relevant for episodic memory (Tsao et al., 2013; Xu and Wilson, 2013; Tsao et al., 2018; Wang et al., 2020; Wang et al., 2024). Moreover, using a TRAP-based strategy to express chemogenetic receptors in learning-activated LEC neurons, we demonstrated that the LEC contains a subpopulation of neurons with characteristics of engram cells for OPC memory (Tozzi et al., 2024). This evidence indicates that LEC neurons may retain information about recent past experiences and raises the question of whether dysfunction of this population could contribute to episodic memory deficits observed in neurodegenerative conditions. In fact, episodic memory impairment emerges as one of the earliest symptoms in the cognitive decline of Alzheimer's disease (AD) (Korczyn and Grinberg, 2024). These deficits have been classically associated with dysfunction of medial temporal lobe circuits, particularly those involving the entorhinal cortex and hippocampus, which are essential for the integration of contextual features of experience (Igarashi, 2023). Our findings provide compelling evidence that memory deficits observed in APP mice primarily reflect impairments in memory retrieval rather than failures of memory storage (Guglielmo et al., 2025), reinforcing the idea that early AD pathology disrupts the mechanisms necessary for accessing previously encoded information, consistent with findings previously reported in the DG (Roy et al., 2016). We initially focused on the LEC in 2-month-old APP mice as a model of early neurodegeneration in the medial temporal lobe circuits. Previous work has demonstrated that synaptic function in the LEC is disrupted at early stages in APP mice, leading to a selective impairment in OPC memory. These deficits have been linked to the accumulation of amyloid- β , which promotes the induction of pro-inflammatory factors and the activation of stress-related protein kinases,

including JNK and p38 MAPK (Criscuolo et al., 2017). We demonstrated that 2-month-old APP mice exhibit normal exploratory behavior during the learning phase of the OPCRT, which is associated with the activation of the immediate early gene *c-fos* in the EC and hippocampus compared to HC control mice. The pattern of c-Fos protein expression did not differ from that observed in age-matched WT controls, suggesting that early A β accumulation may not affect the response to behavioral stimuli. However, this observation alone is insufficient to demonstrate that APP mice successfully encode OPC associations. Rather, it indicates that the neural mechanisms supporting the processing and initial encoding of environmental stimuli remain largely intact at this early stage of pathology.

To further test the hypothesis that episodic memory deficits arise from impaired accessibility of stored memory traces, we combined the TRAP method with a chemogenetic approach. Using a dual-viral strategy, we selectively expressed the excitatory chemogenetic receptor hM3Dq in the subpopulation of LEC neurons activated during the learning phase of the OPCRT. Chemogenetic reactivation of these learning-tagged LEC neurons was sufficient to rescue OPCRT memory performance in 2-month-old APP mice. Immunofluorescence analyses further confirmed that this behavioral rescue was associated with selective reactivation of the LEC neuronal ensemble engaged during learning. In fact, the overlap between mCherry and c-Fos signals was significantly reduced in APP mice compared to WT controls under control conditions, indicating a selective impairment in the endogenous reactivation of learning-tagged neurons. Patch-clamp recordings confirmed that, in APP hM3Dq mice, LEC neurons respond to CNO with a depolarization of the resting membrane potential and an increase in excitability. This suggests that chemogenetic activation likely facilitates reactivation of the learning-tagged ensemble, contributing to the rescue of memory retrieval observed in APP mice. Moreover, chemogenetic reactivation of learning-tagged DG ensembles failed to rescue OPC memory in either 2-month-old or 6-month-old APP mice, supporting the hypothesis that OPC associations are primarily encoded within LEC neuronal ensembles. The DG may instead play a complementary role by integrating spatial and contextual information and relaying it to downstream hippocampal circuits, contributing to the formation of a coherent memory representation. However, in the absence of appropriate reactivation of LEC ensembles, stimulation of DG learning-tagged neurons alone appears insufficient to drive retrieval of the OPC association.

To further investigate the mechanisms underlying memory impairments, we examined dendritic spines, which serve as critical hubs of synaptic plasticity and neuronal function (Holtmaat and Svoboda, 2009; Borczyk et al., 2019). Dendritic spines are highly dynamic structures that undergo activity-dependent remodeling, playing a fundamental role in encoding, storing, and retrieving information (Choi et al., 2018; Lee et al., 2022). Previous studies have demonstrated an increased proportion of thin spines on LEC neurons in APP mice (Criscuolo et al., 2017), a phenotype associated with immature or dysfunctional synapses (Berry and Nedivi, 2017). These structural changes may contribute to impaired synaptic stability and impaired memory retrieval. We found that chemogenetic activation of learning-tagged neurons was associated with structural enlargement of dendritic spines and a shift toward a more mature morphology. This evidence supports the idea that targeted chemogenetic activation of LEC neurons can restore synaptic properties critical for memory

retrieval. Consistent with this idea, Roy and colleagues demonstrated that optogenetic induction of long-term potentiation at perforant path synapses of the DG engram cells restores both spine density and long-term memory (Roy et al., 2016). This suggests that structural modifications at the level of dendritic spines are a key mechanism for rescuing memory function and that enhancing synaptic connectivity within memory-relevant neuronal ensembles can effectively counteract deficits associated with early neurodegeneration.

As AD pathology progresses, hippocampal dysfunction emerges, leading to additional cognitive deficits, including impairments in non-associative recognition memory such as those observed in the novel object recognition task (Criscuolo et al., 2017). We found that in 6-month-old APP mice basal c-Fos expression in the hippocampus was abnormally elevated and unresponsive to behavioral stimulation. Although the total number of tagged neurons did not differ between WT and APP mice, the absence of stimulus-evoked c-Fos induction suggests that a substantial fraction of tagged neurons in APP mice was already active at the time of the experience. This reduction in ensemble sparsity may compromise the specificity of memory-related neuronal populations, increasing interference from non-relevant ensembles and impairing engram-specific retrieval. This suggests a compromised ability to modulate hippocampal c-Fos expression in response to environmental cues, potentially reflecting dysregulated network activity. The elevated basal c-Fos levels in the hippocampus of 6-month-old APP mice may reflect A β -driven disruptions in excitatory-inhibitory balance. It has been shown that 6-month-old APP mice exhibit aberrant excitatory activity, leading to epileptiform discharges, compensatory inhibitory remodeling, and synaptic plasticity deficits, particularly in the DG (Korczyn and Grinberg, 2024). This maladaptive network state is likely to interfere with the precise recruitment and reactivation of learning-tagged neuronal ensembles during recall. In fact, chemogenetic reactivation of learning-tagged DG ensembles was sufficient to restore novel object recognition memory in 6-month-old APP mice. It is important to note that the natural-cues induced reactivation of learning-tagged neuronal subpopulations in the DG is significantly reduced in APP mice compared to WT controls. This suggests that elevated basal c-Fos in the DG may affect the functional recruitment of engram cells, thus disrupting the precise reactivation of memory-specific neural ensembles. In conclusion, our findings contribute to the understanding of the progressive memory impairment in the APP mouse model. We demonstrate that at early-stage deficits are characterized by a selective dysfunction in the LEC, specifically in the reactivation of learning-tagged neurons, which impairs memory retrieval. In contrast, at later stages, behavioral deficits also emerge in less cognitively demanding tasks, indicating a progression of neuronal impairment along the entorhinal-hippocampal network. Chemogenetic reactivation of neuronal ensembles in the LEC and DG was sufficient to restore memory performance, emphasizing the potential of targeting these circuits to mitigate memory deficits in Alzheimer's disease.

However, the neural substrates supporting episodic-like memory may not be confined exclusively to the hippocampus and the LEC. In line with early theoretical proposals by Richard Semon (Semon, 1904), memory traces may be distributed across networks of interconnected neuronal populations, with episodic memory emerging from the coordinated activity of multiple brain regions. Starting from this hypothesis, the second aim of this work

was to identify brain regions specifically involved in episodic memory retrieval in mice using the OPCRT. To this end, we examined patterns of neuronal activation by analyzing the expression of the immediate early gene c-Fos. To disentangle retrieval-specific activation from nonspecific sensory or contextual effects, three control conditions were included: a home-cage control (HC), a context-exposed control (CNTX), and a group exposed to the same pair of objects across all experimental phases (OCT). Specifically, HC control was used to assess baseline neuronal activity, CNTX control to identify patterns of neuronal activation associated with context exposure and spatial navigation, and OCT condition to control also for activation associated with object interaction. Although the OCT condition engages memory retrieval processes, the nature of the retrieved information differs fundamentally from that required for episodic-like memory. In this condition, during the recall phase no novel OPC configuration or sensory mismatch is introduced, and all stimulus features remain identical to those encountered during part of the learning phase. In the absence of a mismatch between expected and experienced stimulus configurations, there is no requirement to retrieve and compare a stored OPC association. Consequently, novelty-driven exploration and prediction-error signals that normally trigger episodic-like retrieval are not engaged (Ranganath and Rainer, 2003; Kumaran and Maguire, 2007). Under these conditions, retrieval of the OPC association, even if stored, is neither required nor behaviorally expressed, and task performance can be supported by recognition of individual stimulus elements without accessing their associative configuration within a specific episode.

To characterize the patterns of neuronal activity specifically associated with episodic-like memory, we mapped c-Fos expression across the entire brain following behavioral testing. This whole-brain analysis revealed that distinct behavioral conditions were associated with reproducible and clearly separable activation patterns. In fact, dimensionality reduction and clustering analyses showed that these c-Fos profiles were sufficient to reliably discriminate all experimental conditions, indicating that each behavioral state engages a distinct pattern of neural activity. Analysis of the regional contributions to the principal components driving condition separation indicated an important involvement of isocortical areas across the first three components. This dominance of isocortical regions suggests that cortical circuits play a major role in shaping the network-level activity that distinguish different behavioral states. The brain-wide mapping approach generated a comprehensive dataset of c-Fos⁺ cells spanning multiple levels of anatomical granularity. When examined at the level of the eleven major brain subdivisions, behavioral conditions were associated with a widespread increase in neuronal activation compared to the HC control. This effect was particularly evident in brain systems implicated in sensory processing, memory formation and retrieval, and higher-order integrative functions, including the isocortex, olfactory areas, hippocampal formation, cortical subplate, pallidum, and hypothalamus. Increased activation in sensory and olfactory regions likely reflects processing of environmental and object-related cues, while recruitment of hippocampal and cortical integrative regions is likely associated with spatial exploration and memory processes related to the task performance.

To further investigate the contribution of specific brain regions to episodic-like memory retrieval, we performed a mid-level analysis by selecting 75 aggregated brain regions. This

level of anatomical granularity represents a trade-off between the need to resolve the contributions of individual regions within major brain subdivisions and the requirement to retain sufficient statistical power for robust comparisons across experimental conditions. Brain regions were selected to ensure extensive representation of the isocortex, in accordance with the dominant contribution of cortical areas observed in the principal component analysis. To characterize patterns of neuronal activation specifically associated with episodic-like memory retrieval, we compared c-Fos density in the OPCRT group with each control condition using task partial least squares (PLS) analysis. PLS is a multivariate statistical approach that identifies optimal patterns of activity differentiating experimental conditions. This method detects differences between groups by identifying brain regions whose activation changes as a function of experimental condition. Through singular value decomposition, task PLS generates a set of mutually orthogonal latent variable (LV) pairs, each consisting of a contrast that reflects similarities or differences between conditions and a corresponding pattern of brain region saliences that identifies regions contributing most strongly to that contrast. Saliences were assessed using bootstrap estimation of standard errors, and regions with bootstrap ratios exceeding a defined threshold were considered to contribute significantly to experimental group separation. In the comparison of the OPCRT group with the HC condition, task PLS analysis revealed a widespread increase in c-Fos expression across cortical and subcortical regions, reflecting the global engagement induced by exploration. In this contrast, robust activation of primary sensory cortices, including somatosensory and visual areas, was observed alongside recruitment of associative cortical regions. This pattern likely reflects the combined contribution of sensory processing, spatial exploration, and object interaction rather than the specific pattern associated with episodic-like memory retrieval. Consistent with this interpretation, additional engagement of olfactory structures, hippocampal subfields, and multiple subcortical regions further supports the notion that this comparison captures broad task-related activation relative to baseline conditions. When OPCRT was compared with the context-exposed control (CNTX), the number of significantly activated regions was reduced. Notably, primary sensory cortices remained significantly engaged, suggesting that object interaction drives a strong activation of these regions. In addition, associative cortical regions, including frontal areas and the RSP, showed increased activation, together with selective recruitment of hippocampal and parahippocampal regions such as CA3 and the POST. This pattern suggests that, beyond sensorimotor processing, OPCRT engages higher-order associative circuits involved in integrating object-related information with contextual features. Finally, comparison with the OCT condition, which controls for both spatial navigation and object interaction, resulted in a marked restriction of the activated network. The substantial reduction in significantly activated regions indicates that most areas identified in the previous contrasts are primarily associated with exploration, sensory processing, and object interaction rather than with memory retrieval. Under these conditions, only a limited subset of regions remained selectively activated during OPCRT, including the mPFC, ORB, RSP and POST. Analysis of activation patterns across the three contrasts reveals a hierarchical organization of recruited brain regions that reflects the increasing cognitive demands of the control conditions. Regions activated relative to OCT represent a subset of those activated relative to CNTX, which in turn are part of the broader set activated relative to HC controls.

This progressive restriction suggests that the increasing cognitive load of the tasks effectively filter out regions primarily involved in sensory processing and object exploration, isolating a core network of regions specifically implicated in OPC memory retrieval.

Between the brain regions found activated, the mPFC is implicated in a broad range of cognitive functions, including attention, decision making, and memory (Preston and Eichenbaum, 2013). Through its extensive glutamatergic projections, the mPFC can exert top-down control over sensorimotor, emotional, and mnemonic systems. Consistent with this anatomical organization, the mPFC has been associated with higher-order processes such as planning, temporal organization of behavior, attentional control, behavioral flexibility, and goal-directed actions, as well as social and emotional regulation (Hoover and Vertes, 2007). Importantly, interactions between the mPFC and the hippocampus have been proposed to play a key role in memory processing, particularly in tasks requiring integration of information across time and context, such as episodic memory (Eichenbaum, 2017). At the cellular level, prefrontal neurons have been shown to encode object-related information, and activity in the ACA correlates with exploratory behavior during NOR task (Smith and Jonides, 1999; Weible et al., 2009). However, lesions of the mPFC do not impair performance in the NOR, suggesting a more complex contribution to memory processing. This apparent discrepancy highlights that the involvement of the mPFC in memory depends on the temporal scale and cognitive demands of the task. To reconcile this multifaceted role, Euston and colleagues (2012) proposed that the mPFC functions as a flexible modulator of memory, becoming particularly engaged when tasks require high specificity. According to this view, the mPFC plays a critical role in learning and representing associations between contexts, locations, events, and their appropriate adaptive responses (Euston et al., 2012; Chao et al., 2020). The relationship with the medial temporal lobe mnemonic system is reflected by the pattern of direct and indirect connections. A prominent direct projection arises from ventral hippocampal CA1 and the subiculum and targets ventral regions of the mPFC. These hippocampal inputs form synaptic contacts with both glutamatergic pyramidal neurons and local GABAergic interneurons within the mPFC. In turn, the mPFC sends direct projections back to the hippocampus, primarily targeting CA1 and CA3 subfields through pathways originating largely from the ACA (Tierney et al., 2004; Rajasethupathy et al., 2015). In addition to direct projections, communication between the prefrontal cortex and the hippocampus is mediated by several indirect, multisynaptic pathways. Key relay structures include the nucleus reuniens (NR) of the medial thalamus, the perirhinal cortex, and the LEC, all of which are reciprocally connected with both the mPFC and the hippocampus. Through these intermediary regions, indirect prefrontal-hippocampal pathways are well positioned to support and modulate functional interactions between the two regions (Hoover and Vertes, 2007). Notably, the mPFC-NR circuit has been proposed to regulate the balance between memory generalization and specificity in contextual fear memory (Xu and Südhof, 2013). Recent work further underscores the importance of reciprocal interactions between the mPFC and the deep layers of the LEC in associative learning. In fact, it has been suggested that this circuit supports the encoding and storage of experience within a non-spatial, outcome-based cognitive map. Disrupting activity in either region impairs the ability to form or retrieve cue-outcome associations, indicating that coordinated mPFC-LEC

interactions are required to organize and access associative memory representations (Jun et al., 2024).

The role of the ORB in episodic memory is comparatively less well understood. Traditionally, the ORB has been primarily associated with decision-making processes that rely on the outcomes of prior experiences (Rushworth et al., 2011; Stalnaker et al., 2015). In this sense, the ORB is thought to encode the predicted and experienced value of behavioral outcomes and to convey this information to other brain regions to guide current and future choices (Padoa-Schioppa and Conen, 2017). However, accumulating evidence suggests that the function of the ORB extends beyond value representation. In particular, the ORB has been proposed to represent the current task state within a cognitive map of task spaces (Wilson et al., 2014; Mizrak et al., 2021). Notably, similar to the hippocampus, the ORB has also been shown to encode information about past, present, and anticipated sensory stimuli, influenced by temporal, spatial, and contextual factors (Schoenbaum et al., 2009; Rudebeck et al., 2014; Zhou et al., 2021). Studies in rodents and monkeys have demonstrated that the identity and expected values of specific outcomes are represented in the ORB, and that disruption of ORB function impairs behavior guided by outcome expectancy. Such deficits extend to associative learning paradigms, including Pavlovian conditioning and extinction, suggesting an important role of the ORB in updating and maintaining outcome-based representations (Thorpe et al., 1983; Schoenbaum and Eichenbaum, 1995; Schoenbaum et al., 1998; McDannald et al., 2005; Takahashi et al., 2009). This function is sustained by direct projections to key limbic and neuromodulatory structures, including the basolateral amygdala, ventral tegmental area, and striatum. In addition, the ORB is anatomically and functionally connected with sensory cortices, suggesting that it may exert top-down modulation of sensory representations in accordance with learned outcome expectations (Schoenbaum et al., 2009; Takahashi et al., 2011; Rudebeck and Murray, 2014). Although the role of the ORB in episodic memory remains comparatively less well defined, its established involvement in representing task states, outcomes, and temporally structured information indicates that it may contribute to episodic-like memory retrieval.

Another region significantly recruited in the present work is the RSP, a medial parietal area anatomically positioned at the interface between neocortical association areas and the medial temporal lobe, receiving convergent inputs from visual, parietal, and prefrontal cortices while maintaining dense reciprocal connections with the hippocampal formation and the EC (Todd et al., 2020). This connectivity places the RSP in an optimal position to integrate spatial, contextual, and mnemonic information. Functionally, the RSP has been implicated in the encoding and retrieval of context-specific memory, spatial navigation, the temporal component of short-term recognition memory, associations between multiple sensory stimuli, prospective thinking as part of the default mode network, and memory extinction (Gabriel, 1993; Corcoran et al., 2013; Robinson et al., 2014; Powell et al., 2017; Villena-Gonzalez et al., 2018; Mitchell et al., 2018; Kaboodvand et al., 2018). Recently, de Landeta and colleagues (2020) demonstrated that anterior RSP is necessary for the formation and the expression of two of the main features of recognition memory: the “what” and the “where” components (de Landeta et al., 2020). The RSP has also been implicated in systems-level memory consolidation. In particular, reactivation of learning-related

neuronal ensembles within the RSP has been shown to promote a shift in memory representations toward a more consolidated state. Artificial reactivation of RSP ensembles associated with contextual fear learning induces systems consolidation, as suggested by the reduced hippocampal dependence, increased generalization across contexts, and enhanced recruitment of neocortical areas during retrieval (de Sousa et al., 2019). Moreover, neuronal activity in the RSP has been shown to exhibit temporally structured firing patterns, including off-response time fields characterized by reliable periods of suppressed activity. While such temporal modulation is present in simpler tasks, it is less prevalent in more demanding paradigms, suggesting that temporal coding in the RSP scales with task mnemonic demands. These findings suggest that temporal coding is a prominent feature of RSP firing patterns, consistent with a role of the RSP in episodic memory (Subramanian and Smith, 2024). In addition, human fMRI studies have reported robust engagement of the RSP during both episodic memory encoding and retrieval (Wiggs et al., 1998; Maguire, 2001; Trinkler et al., 2009).

The last brain region identified in this work for its possible role in episodic-like memory retrieval is the POST. This small brain region occupies an important anatomical position within the hippocampal-parahippocampal network, lying at the interface between the subiculum, the principal output structure of the hippocampus, and the RSP (Nitzan et al., 2020). This location places the POST at the center of the neural systems supporting spatial navigation and orientation. Functionally, neuronal activity in the POST is strongly associated with the animal's head direction, providing a directional reference signal anchored to allocentric space (Duszkiewicz et al., 2025). Importantly, this directional signal is thought to be largely sensory, arising from a brainstem-diencephalic circuit involving the dorsal tegmental nucleus and the lateral mammillary nucleus, where angular head velocity is integrated over time to generate stable directional tuning (Huang et al., 2017). The POST is a transitional region characterized by a six-layered organization, although the boundaries between the deeper layers are relatively indistinct. As in other cortical areas, laminar positioning in the POST reflects projection-specific neuronal populations, corresponding to distinct input and output connections (Herkenham, 1980). However, the POST deviates from the canonical organization of primary sensory cortices. Rather than targeting layer 4, thalamocortical afferents from the anterodorsal thalamic nucleus preferentially innervate layer 3. POST layer 3 also serves as an important output to the cortex, with small pyramidal neurons projecting to higher-order association areas involved in spatial navigation, most prominently the MEC (Nassar et al., 2018). Given its central position, the POST may contribute to episodic-like memory by providing a stable spatial scaffold upon which hippocampal and entorhinal representations can bind object and contextual information. In fact, Bett and colleagues (2013) showed that the POST plays a selective and context-dependent role in spatial memory processing. POST activity is required to maintain stable hippocampal representations in familiar environments, but not for the formation of new place representations. Instead, plasticity in the POST is specifically required for establishing new associations between object locations and familiar spatial landmarks. This dissociation suggests that the POST primarily functions as a relay and integrative node that conveys landmark and directional information to downstream regions, supporting the expression and

updating of spatial reference frames rather than serving as the primary locus of spatial memory storage (Bett et al., 2013).

Despite the widespread use of c-Fos to identify neuronal populations activated by specific stimuli or behavioral tasks, several limitations inherent to its use as a marker of neural activation should be considered. First, c-Fos expression reflects neuronal activation integrated over an extended temporal window, providing an aggregate measure of neural activation across time rather than a precise representation of neuronal dynamics (Eagle et al., 2016; Yap and Greenberg, 2018). As a consequence, it does not inform on the nature or quality of the underlying neural computations. Second, the likelihood of c-Fos induction is not uniform across brain regions, as it depends on region- and cell-type-specific molecular landscape, including differential accessibility and regulation of the *Fos* promoter (Nonaka et al., 2014). As a consequence, comparable levels of neural activity may not translate into equivalent c-Fos expression across structures. Finally, the functional significance of c-Fos induction may differ across brain regions and circuits, such that similar levels of c-Fos expression do not necessarily reflect similar roles in information processing or memory function. This suggests the need for caution when interpreting region-to-region differences in activity based solely on immediate early gene expression.

Beyond changes in regional activation, memory retrieval is expected to engage coordinated interactions across multiple brain systems. We therefore examined the co-variation of c-Fos expression across areas, an approach that has been applied to study functional networks (Wheeler et al., 2013; Vetere et al., 2017). In human neuroimaging, regional activity is typically inferred from electrophysiological signals or from indirect metabolic measures, and functional connectivity is assessed by quantifying the temporal covariance of these signals across regions during task performance. In experimental animal models, by contrast, neural activation is often evaluated using activity-dependent molecular markers such as c-Fos, which provide a single activation value per region and per subject. Although these approaches differ in temporal resolution and interpretability, across-subject covariance analyses provide a means to infer large-scale functional interactions between different neural systems. We estimated patterns of functional connectivity by computing interregional correlations for each experimental condition and used these relationships to generate condition-specific functional networks. At the network level, episodic-like memory retrieval in the OPCRT condition was associated with a marked reorganization of large-scale functional interactions. Relative to CNTX and OCT, the OPCRT network displayed increased connectivity, reflected by higher node degree and network density, together with elevated global efficiency and an increase of the small-world index. This pattern suggests a network state that supports more efficient integration of information across distributed brain regions. At the same time, the increase in clustering coefficient indicates higher local connectivity, consistent with a modular organization. As suggested also by the low-dimensional embedding analysis, each behavioral condition is associated with a distinct pattern of interregional co-activation. Analysis of topological roles suggests that the POST and RSP occupy a central position in mediating communication between distinct functional modules, reinforcing the idea of an involvement of posterior medial structures in the network engaged during episodic-like memory retrieval.

While brain-wide analyses of neuronal activation during OPCRT recall identified a core subset of selectively engaged brain regions, activity alone was insufficient to define the contribution or functional role of individual neuronal populations. By combining TRAP-based activity-dependent tagging with chemogenetic manipulation, we directly tested whether learning-activated neuronal ensembles are necessary and/or sufficient for memory expression. This approach has previously been applied to characterize learning-activated neurons in the LEC, providing causal evidence for the existence of a neuronal subpopulation with properties of engram cells for OPC memory recall. Across all regions examined, chemogenetic inhibition of learning-tagged neuronal populations consistently impaired OPCRT performance, suggesting that these ensembles are not only active during recall but are necessary for successful memory expression. At the behavioral level, this impairment manifested as a loss of preference for the object presented in the novel configuration, indicating a failure to appropriately retrieve or utilize OPC associations. Importantly, although the behavioral outcome was similar across regions, the underlying mechanisms leading to this deficit are likely to be region specific, reflecting distinct contributions to memory retrieval. For instance, inhibition of learning-tagged neurons in the mPFC may disrupt higher-order processes required for the integration and selection of task-relevant mnemonic information, impairing the expression of the appropriate behavioral response. In the ORB, the observed deficit may reflect a failure to assign behavioral significance of competing object configurations, consistent with a role in representing task states or expected outcomes. The disruption of activity of learning-tagged neurons in the POST may impair the integration of spatial information necessary to anchor object representations within a context, preventing discrimination between familiar and novel configurations. In contrast, reactivation of learning-tagged neurons in the RSP was sufficient to restore memory expression when natural cues were no longer effective, thus inhibition of this population is likely to directly interfere with reactivation of the memory trace itself. This suggests that learning-tagged RSP neurons possess functional properties of engram cells.

Analysis of ensemble reactivation revealed a further dissociation across cortical areas. Learning-tagged neuronal populations in the PL, ILA, RSP, and POST showed significantly greater reactivation during successful memory recall compared to later time points at which the memory was no longer accessible. In contrast, learning-tagged neurons in the ACA and ORB did not exhibit a significant difference in reactivation between recent and late recall. This pattern suggests that learning-tagged populations in the ACA and ORB may be recruited in shaping the coordination of distributed network activity or motivational state during recall, rather than supporting the selective reactivation of the memory trace. The PL and the RSP exhibited a significant relationship between the proportion of ensemble reactivation and behavioral performance. In both areas, higher levels of reactivation of learning-tagged neurons were associated with high discrimination performance. This correlation was absent in other regions, including the ILA and POST, despite their showing significant reactivation during natural memory expression. These findings suggest that while reactivation of learning-tagged ensembles in multiple regions is necessary for successful memory retrieval, PL and RSP may play a direct role in driving memory-guided behavior.

Based on the activation patterns observed in the brain-wide c-Fos mapping and the region-specific functional dissection obtained through chemogenetic manipulation, we hypothesized a network composed of a frontal and posterior medial part that is activated during OPC memory recall. Within this network, the RSP appears as a critical node containing neuronal populations with characteristics of engram cells for OPC memory. In addition, previous evidence showed that episodic-like memory is associated with synaptic plasticity changes in the LEC, and that this region contains neuronal populations that are both necessary and sufficient for OPC memory recall (Tozzi et al., 2024). These results support a model in which episodic-like memory in mice is sustained by a distributed network, with the RSP and LEC representing brain regions fundamental for successful memory retrieval. Other regions within this network appear to contribute to memory recall by supporting the correct behavioral expression of the memory rather than directly storing its content. In particular, POST, PL and ILA showed strong c-Fos activation and significant reactivation of learning-tagged neuronal populations during recent memory recall. Interestingly, PL reactivation was strongly correlated with behavioral performance at 12h, similar to that observed in the RSP and previously reported for the LEC (Tozzi et al., 2024). While learning-tagged neurons in the PL may be specifically engaged during successful memory retrieval to coordinate memory expression at the behavioral level, their activation may also reflect the activity of direct anatomical connections between the LEC and PL, particularly projections from LEC layers 5/6 that have been shown to be critical for associative learning (Jun et al., 2024). The LEC receives indirect inputs from posterior medial areas through the MEC and directly projects to multiple frontal associative regions, consistent with a central role within this circuit in mediating interactions between the two major components of the network. Interestingly, visual information reaches the entorhinal cortex directly through projections from the RSP to MEC layer 3, but also via an indirect pathway involving RSP projections to POST layer 3, which in turn projects to MEC layer 3 (Dubanet and Higley, 2024; Duszkievicz et al., 2025). Entorhinal cortex layer 3 is particularly important because it provides direct input to CA1, constituting a parallel information stream to the canonical EC-DG-CA3-CA1 circuit (Guardamagna et al., 2025). Another important circuit supporting associative learning and memory is the strong anatomical connectivity between the RSP and ACA (Todd et al., 2019). Although the ACA showed a more limited involvement in OPC memory, particularly due to the lack of significant reactivation of learning-tagged neurons during recent memory recall, these connections may convey RSP information to other frontal associative regions.

Our chemogenetic experiments further suggest that brain regions strongly activated during OPC memory recall are involved in storing or processing information critical for the recognition of novel associations. However, these approaches provide limited temporal resolution to characterize the dynamics of neural activity in individual regions. To investigate how neural activity changes during exploration of novel and familiar configurations, we conducted electrophysiological recordings in freely moving animals during the recall phase of the OPCRT. We focused on local field potential (LFP) signals, obtained by low-pass filtering the extracellular electrical activity simultaneously recorded from the mPFC and RSP. For this experiment, we selected the RSP and mPFC based on results from our previous

experiments, which identified the RSP as a region involved in storing OPC associations and highlighted the role of the mPFC as necessary for the correct expression of this memory, as well as its importance in coordinating behavioral output during decision-making tasks (Jun et al., 2024). To understand how these brain regions are involved in memory recall, we examined their LFP signals, which reflect the integrated excitatory and inhibitory synaptic activity of local neuronal populations within a radius of few hundred micrometers around the recording electrode (Lindén et al., 2011; Buzsáki et al., 2012). As a result, LFP signals reflect both synchronized synaptic currents and subthreshold integrative processes, providing information about the functional state of the local network. LFPs typically contain a broad spectrum of oscillatory components, which likely reflect the contribution of multiple neural processes (Buzsáki and Draguhn, 2004; Lakatos et al., 2005; Lakatos et al., 2008). This spectral structure allows the separation of distinct functional components of the neural activity. Spectral analysis of LFP power, used to assess the relative contribution of different frequency components, indicated a predominance of low-frequency activity, which was relatively higher in the RSP compared to the mPFC. In particular, we focused our analysis on the theta (5-10 Hz), low-gamma (30-55 Hz), and high-gamma (55-80 Hz) components of the LFP spectrum. Extensive research on the neural basis of episodic memory has highlighted a prominent role for theta and gamma oscillations during memory retrieval (Jensen et al., 2007; Yamamoto et al., 2014; Spellman et al., 2015; Herweg et al., 2020; Griffiths et al., 2021). Theta oscillations have been associated with locomotion, memory-related processes, and spatial navigation. More generally, hippocampal theta activity has been proposed to serve as a mechanism for encoding continuous, task-relevant information and for establishing temporal associations between elements (MacDonald et al., 2011; Herweg et al., 2016; Aronov et al., 2017). This rhythm is thought to play a critical role in integrative processes underlying higher cognitive functions. In fact, neuronal spiking in widespread brain regions, including somatosensory, entorhinal, and prefrontal cortices, has been shown to be phase-locked to hippocampal theta oscillations (Hanada et al., 1999; Sirota et al., 2008; Tamura et al., 2017). Beyond the hippocampus, cortical theta activity has also been linked to memory processes. In humans, increases in theta power have been observed during the recall of associative information, and theta-band activity has been shown to positively correlate with subjective confidence during memory retrieval (Gruber et al., 2008; Addante et al., 2011; Wynn et al., 2019). Gamma oscillations (30-80 Hz) have also been proposed to play a critical role in memory retrieval. Numerous studies have reported a close association between hippocampal gamma activity and neocortical oscillations during both memory encoding and retrieval (Pacheco Estefan et al., 2019; Griffiths et al., 2019; Griffiths and Jensen, 2023). The contribution of gamma oscillations to memory encoding is thought to occur primarily through theta-gamma phase-amplitude coupling. It has been proposed that gamma cycles, representing specific elements of memory, are nested and organized across successive phases of the theta cycle, allowing the formation of specific episodes (Tort et al., 2009; Belluscio et al., 2012; Heusser et al., 2016). Moreover, increases in gamma power during memory retrieval have been suggested to reflect hippocampal pattern completion processes that support the reinstatement of stored information in neocortical circuits (Staresina et al., 2016; Griffiths et al., 2019; Wynn et al., 2024). Consistent with previous findings, power spectral analysis revealed that LFP activity in both

the mPFC and RSP was dominated by theta-frequency components. We also observed that theta power was higher in the RSP than in mPFC, a pattern that may reflect the strong functional coupling of the RSP with hippocampal theta oscillations (de Almeida-Filho et al., 2021). Comparing novel and familiar exploration epochs, we observed that novel object exploration was associated with increased theta power in the mPFC. In contrast, no significant differences in theta power were detected between novel and familiar conditions in the RSP. In the mPFC, gamma power exhibited a similar profile during novel and familiar object exploration. In the RSP, gamma-band activity was also largely comparable between conditions; however, a modest increase in high-gamma power was observed during novel exploration, although this effect was not statistically significant. While this trend should be interpreted with caution, it may reflect increased local network engagement in the RSP during the processing of novel information, consistent with its role in integrating cortical and hippocampal inputs.

Because phase synchronization has been proposed as a mechanism underlying coordination between brain regions during behavior and cognition (Varela et al., 2001; Buzsáki, 2006), we next quantified functional coupling between the mPFC and RSP using coherence and Granger causality analyses. Coherence measures the correlation between neural signals recorded from two brain regions and reflects the degree of phase consistency between them (Fries, 2005; Aoi et al., 2014). Elevated coherence may reflect bidirectional coupling between oscillatory sources or synchronization driven by a common pacemaker, which can align windows of excitability across regions and facilitate information transfer (Bressler and Kelso, 2001; Varela et al., 2001; Palmigiano et al., 2017). Alternatively, coherence may arise from anatomical connectivity, in which spiking activity in a source region generates correlated synaptic activity locally and in downstream target areas with a temporal delay, giving rise to correlated LFP signals (Pesaran et al., 2018; Schneider et al., 2021). Coherence analysis indicated that novel and familiar exploration epochs did not differ in the degree of phase consistency between the mPFC and RSP. In both conditions, coherence spectra exhibited a characteristic U-shaped profile, with higher coherence in the theta and high-gamma bands and reduced coherence at intermediate frequencies. This frequency-dependent pattern suggests that mPFC-RSP interactions are organized preferentially within specific frequency bands that are preserved across behavioral conditions. Coherence remained consistent across novel and familiar exploration epochs, indicating that this measure alone is not sufficient to distinguish the mnemonic content associated with the objects. The absence of condition-dependent changes in coherence may also reflect the similarity of exploratory behavior between novel and familiar conditions, potentially masking more subtle differences related to memory recall or novelty detection. To further characterize inter-regional interactions beyond phase synchronization and to assess the directionality of information flow between mPFC and RSP, we next applied Granger causality analysis. Granger causality is a statistical approach developed to assess information flow between time series and has been widely used to characterize temporal relationships in the activity between brain regions, by testing whether past activity in one region improves the prediction of future activity in another (Seth et al., 2015). Applying this approach to our data revealed that directional interactions between mPFC and RSP were

frequency-specific and predominantly expressed in the theta band. In both novel and familiar exploration conditions, theta-band Granger causality was consistently higher in the RSP to mPFC direction than in the opposite, suggesting an asymmetric pattern of inter-regional coupling. By contrast, Granger causality in the low- and high-gamma frequency ranges was low and did not differ between directions, suggesting that interactions between these regions are not primarily mediated by gamma-band activity. This observation is consistent with the idea that gamma oscillations are largely generated locally within cortical microcircuits and are less dependent on long-range inter-regional interactions (Bartos et al., 2007). Notably, theta-band Granger causality in the RSP to mPFC direction was stronger during familiar object exploration compared to novel exploration. This finding indicates that the strength of directional coupling between RSP and mPFC varies as a function of object configuration. Given the possible role of the RSP in integrating contextual information, this directional coupling may reflect the transmission of previous contextual representations to prefrontal circuits, facilitating the access to previously learned OPC associations.

Together with previous evidence, these findings emphasize the role of the RSP and LEC in episodic-like memory retrieval. However, how these two regions interact during the encoding and recall of OPC associations remains largely unexplored. This interaction could be explored in future studies using simultaneous electrophysiological recordings and targeted manipulations of learning-tagged neuronal populations. In particular, important information about the RSP-LEC interaction may be obtained by assessing the reactivation of learning-tagged neuronal ensembles in one region in response to artificial manipulation of the other, while simultaneous electrophysiological recordings during both encoding and recall would allow the characterization of functional coupling, including correlation and directionality of the interaction between these two regions. In addition, the precise functional contribution of the other brain regions characterized in this study remains to be elucidated: although we demonstrated that their learning-tagged neuronal populations are necessary for memory retrieval, the precise functional contribution of each structure, whether related to sensory integration, contextual representation, or executive control, remains unknown. Finally, while this work focused on the role of entorhinal and hippocampal ensembles in memory impairment in APP mice, the contribution of the RSP to these deficits remains to be determined. In particular, future studies may explore potential synaptic dysfunction in this region, assess whether memory recall reactivates learning-tagged neuronal populations, and determine whether targeted reactivation of RSP ensembles can rescue memory performance.

6. Conclusions

We conducted a comprehensive investigation of the neural representation of episodic-like memory in rodents. Starting from previous evidence highlighting the critical role of the LEC in the processing of OPC memory, we examined the contribution of this brain region to episodic-like memory processing by studying its alteration in a transgenic mouse model of AD. Our results indicate that, at early disease stages, memory encoding processes were largely preserved in APP mice. Despite the presence of early alterations in synaptic plasticity within the LEC, c-Fos expression across the MTL was not globally impaired. Importantly, tagging the learning-activated neuronal ensemble during the encoding of OPC associations revealed a significant reduction in the reactivation of this specific subpopulation during recall. This finding suggests a specific deficit affecting neuronal populations that are directly relevant for memory expression, rather than a widespread disruption of activity. By selectively manipulating the learning-tagged ensemble in the LEC using chemogenetic approaches, we demonstrated that artificial reactivation of this population is sufficient to rescue behavioral performance in the OPCRT. This finding provides direct causal evidence that the impaired reactivation of LEC ensembles underlies the early episodic-like memory deficits observed in APP mice. Consistent with this interpretation, learning-tagged LEC neurons exhibited alterations in dendritic spine morphology, which could be rescued through chemogenetic intervention. At a later stage, we observed an elevated basal level of c-Fos expression in the hippocampus of APP mice. Notably, this expression level did not further increase following the encoding phase of the novel object recognition task, suggesting an impaired ability to modulate hippocampal activation in response to behavioral stimuli. Using the same chemogenetic approach, we also demonstrated that artificial reactivation of learning-tagged neurons in DG was sufficient to restore object recognition memory performance. Collectively, these findings suggest that during progressive amyloid- β accumulation, memory traces become inaccessible rather than lost.

To further characterize how episodic-like memory is represented at the network level, we examined the profile of activation across the entire brain by performing a brain-wide c-Fos mapping experiment following the recall phase of the OPCRT. This analysis revealed that isocortical regions critically contribute to the activation profiles distinguishing episodic-like memory recall from control conditions, highlighting their dominant involvement during memory retrieval. To investigate region-specific activation during memory recall, we performed a mid-level analysis selecting 75 aggregated brain regions. By comparing activation profiles during the OPCRT with those observed in control conditions, we identified a progressive restriction in the set of recruited brain regions as task complexity increased. This analysis further revealed a core set of regions selectively engaged during OPC memory recall, comprising the mPFC, RSP, ORB and POST. Examination of the anatomical relationships among these regions demonstrated that they are significantly interconnected, suggesting the existence of a subnetwork organized around frontal and posterior medial components. Functional connectivity analysis confirmed that OPC memory retrieval is associated with a more densely connected and efficient network organization. This analysis further highlighted the prominent role of the RSP and POST in mediating communication between distinct network modules. Using the same chemogenetic approach described

above, we assessed the functional contribution of the identified brain regions. Inhibition of learning-tagged neuronal ensembles in each of these areas was associated with impaired OPC memory performance, indicating that all components of this subset are necessary for successful memory expression. Analysis of ensemble reactivation revealed that neurons in the PL, ILA, RSP and POST were significantly reactivated during recall, suggesting their selective engagement during recent memory retrieval. Notably, behavioral performance showed a significant correlation with the proportion of ensemble reactivation in the PL and RSP. Importantly, chemogenetic reactivation failed to facilitate memory recall at a later time point for all regions examined, with the exception of the RSP. Learning-tagged neuronal populations in the RSP therefore satisfy both necessity and sufficiency criteria for engram definition. This conclusion is further supported by the observation that the RSP is strongly activated at 12h, when the memory trace is accessible, but not at 48h, when recall is no longer possible, as well as by its prominent role within the functional network identified during OPC memory retrieval. Taken together, these findings support the existence of a distributed network sustaining episodic-like memory in mice, composed of frontal and posterior medial components. Within this network, the LEC and RSP contain neuronal populations exhibiting properties of engram cells that are critical for successful memory retrieval.

7. Materials and methods

7.1 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). 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. For brain-wide c-Fos mapping, experiments were performed in male wild-type C57BL/6J mice (Charles River, Italy) at 3 months of age. For chemogenetic manipulation and electrophysiology experiments, both male and female wild-type C57BL/6J mice at 3 months of age were used. PDGF-APPSw, Ind (line J20) transgenic mice overexpressing an alternatively spliced human APP minigene that encodes hAPP695, hAPP751, and hAPP770 bearing mutations linked to familial AD (V717F, K670N/M671L) have been used (Charles River, Italy). Heterozygous J20-hAPP male mice were bred with C57BL/6J female mice to maintain the hAPP transgene as heterozygous. Throughout this work, the APPJ20 mouse model will be referred to as the APP mouse model for consistency. The resulting litters, which included both male and female heterozygous APP mice along with their non-transgenic C57BL/6J littermates, were used for experiments after genotype confirmation. Experimental procedures were performed on 2-month-old and 6-month-old APP mice. All behavioral procedures were performed 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. The number of animals used in each experiment is provided in the figure legends.

7.2 AAV vectors and stereotaxic injections

The following AAV vectors were used for the chemogenetic manipulation: AAV5-Fos::CreER^{T2} (titer 1.2×10^{13}), AAV5-hSyn::DIO-hM3Dq-mCherry, AAV5-hSyn::DIO-hM4Di-mCherry and AAV5-hSyn::DIO-mCherry (titers: $5.0\text{--}6.0 \times 10^{12}$). For stereotaxic injections, 2-month-old or 5-month-old mice were deeply anesthetized using an intraperitoneal injection of Zoletil 100 (zolazepam and tiletamine, 1:1, 40 mg/kg; Laboratoire Virbac) and Xilor (xylazine 2%, 10 mg/kg; Bio98). Lidocaine (2%) was topically applied to the skull to provide local analgesia. A bilateral craniotomy was performed at the stereotaxic coordinates targeting: LEC (AP -4.0 mm, ML $\pm$ 4.0 mm), DG (AP -2.0 mm, ML $\pm$ 1.0 mm, DV -1.72 mm), mPFC (AP +1.8 mm, ML $\pm$ 0.2 mm, DV -1.55 mm), RSP (AP -2.5 mm, ML $\pm$ 0.2 mm, DV -0.7 mm), ORB (AP +2.6 mm, ML $\pm$ 1.0 mm, DV -1.95 mm), and POST (AP -4.2 mm, ML $\pm$ 2.1 mm, DV -1.9 mm). Dorso-ventral coordinates are reported relative to the brain surface. For all regions, a viral mixture of AAV5-FosCreER^{T2} and Cre-dependent AAV (ratio 1:500, AAV5-FosCreER^{T2} at a final titer of 2.43×10^{10}) was injected. Injections were performed bilaterally using a glass micropipette connected to a microinjection system at a constant flow rate of 0.1 μ L/min. Injection volumes were region-specific: 300 nL were delivered into the mPFC and ORB, whereas 250 nL were injected into the RSP, POST, DG, and LEC. Following each injection, the pipette was left in place for an additional 5 min to allow diffusion

of the viral solution before slow withdrawal. For LEC injections, the glass pipette was lowered from the brain surface at an 11° angle until a slight bending of the pipette indicated contact with the dura. The pipette was then retracted by 0.1 mm, and 250 nL of the viral mixture were injected per hemisphere following the procedure described above. After surgery, animals were returned to their home cages and allowed to recover for 3 weeks before the start of behavioral experiments.

7.3 Behavioral tasks

The test environment was composed of two square boxes (length 40cm, width 40cm, height 40cm) with different visual cues on the walls to provide distinct contexts. The wall and floor of the environment were cleaned with 30% alcohol before each trial. The 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.

Before the behavioral tests, mice were habituated to the experimenter and room by extensive handling for two weeks. For the Novel Object-Place-Context Recognition Task (OPCRT), mice were allowed to explore the first context for 5 minutes before the beginning of the sample phases. Subsequently, mice were presented with two distinct novel objects in Context A during the first sample trial. After this trial, the mice were removed from the box and placed in a holding cage for a 1-minute inter-trial interval (ITI) while the box was cleaned. In the second sample trial, the same pair of objects was presented in Context B, but their positions were reversed relative to the first trial. During the test trial, mice were exposed to a pair of identical objects from the sample trials in the same context and positions as in the first sample trial. Both the sample phases and the test phase were conducted for a duration of 10 minutes each. Animals were tested after a retention interval of 4h, 12h, or 48h, depending on the experimental group, as indicated in the figures.

For the context-exposure group (CNTX), mice were exposed to the same two contexts used in the OPCRT without the presence of objects, following the same context sequence, exposure duration, and retention interval as in the OPCRT.

For the object-context task (OCT), mice were exposed to the same two contexts used in the OPCRT while maintaining the same pair of objects in the same positions across all sample trials, following the same context sequence, exposure duration, and retention interval as in the OPCRT.

The Novel Object Recognition Test (ORT) was performed following the same procedure described for the OPCRT. In this case, mice were exposed to two identical objects during the sample trials, and 12h later, the test trial consisted of one familiar and one novel object. The duration of exploration in each phase was the same as in the OPCRT.

In all the experiments, mice were judged to be exploring an object when it was in proximity with their nose directed towards it. Exploration time was not counted when the mouse's nose was directed away from the object. To ensure reliability, the same separate observer re-scored all the videos in a blind fashion for each task, and these scores consistently differed

by less than 10% from those of the experimenter. For each task, observation scores were converted into discrimination indices (discrimination index = (time at novel – time at familiar) / (time at novel + time at familiar)) to evaluate the extent to which mice explored novel versus familiar objects. The scoring of the videos was performed using Chronotape (Philipsberg et al., 2023), a software designed for precise manual analysis of behavior during experimental trials. The marker output files generated by Chronotape were processed using a custom Python script, which allowed for the quantification of the total exploration time as well as the total number of interactions with both the novel and familiar objects. Behavioral videos were recorded using an AUKEY 1080p full HD webcam and were subsequently analyzed offline. Different body regions of the mouse, namely the nose, two ears, back, middle portion, and tip of the tail, were labeled using the open-source tool DeepLabCut (Nath et al., 2019) for markerless pose estimation. This animal tracking enabled the automated calculation of various motor parameters, facilitating a more detailed analysis of the animal's behavior.

7.4 Pharmacological treatment

4OH-TAM (H6278, Sigma-Aldrich) was prepared for injection by dissolving it in saline. A stock solution of 50 mg/mL 4OH-TAM in DMSO (D8418, Sigma-Aldrich) was initially prepared and stored at -20°C. On the day of the experiment, the final working solution of 2.5 mg/mL 4OH-TAM was achieved in two stages: first, by diluting the stock 1:10 in saline that contained 2% Tween80 (P1754, Sigma-Aldrich), and then adding a volume of saline. This final solution consisted of 2.5 mg/mL 4OH-TAM, 5% DMSO, and 1% Tween80 in saline. Mice were given an intraperitoneal injection of 4OH-TAM at a dose of 25 mg per kg body weight, 4 hours prior to the sample trials.

Clozapine N-oxide hydrochloride (CNO; cat. no. 34233-7, Merck) was dissolved in sterile saline for injection. For the behavioral experiments, mice were administered 3 mg per kg (i.p.) of CNO 30 minutes before each test phase.

7.5 Immunohistochemistry

Mice were deeply anesthetized with urethane (Merck, 20% solution, 0.1 mL/100 g body weight) and perfused intracardially with PBS (pH 7.4), followed by 4% paraformaldehyde (PFA) in PBS (pH 7.4). The brains were carefully removed, post-fixed overnight in 4% PFA (w/v), and transferred to a 30% sucrose solution (w/v) in PBS for dehydration and cryoprotection. Coronal sections, 50 µm thick, were prepared using a Leica freezing microtome, spanning from the main olfactory bulbs to the cerebellum, and the free-floating slices were subsequently processed for immunofluorescence analysis. For the brain-wide c-Fos mapping, one out of every 3 sections was collected for further processing leading to a sampling of one slice every 150 µm. For each animal, slices were assigned a unique ID and pooled in a 24-well plate for free-floating staining. Each well contained 5-6 sections that sampled the brain at an equally spaced position in the anterior-posterior axis. For the other experiments, the appropriate brain slices were manually selected.

All sections were incubated for 2h at room temperature in a blocking solution containing 5% BSA (w/v) and 0.5% Triton X-100 (v/v) in PBS. For brain-wide c-Fos mapping, sections were incubated overnight at 4°C with a monoclonal anti-c-Fos antibody (cat. no. 226 008, Synaptic

Systems) diluted 1:1000 in PBS containing 1% BSA (w/v) and 0.1% Triton X-100 (v/v). Sections were then washed three times for 5 min in PBS and incubated for 2 h at room temperature with an Alexa Fluor 488-conjugated anti-rabbit secondary antibody (cat. no. 711-545-152, Jackson ImmunoResearch) diluted 1:500 in the same solution. For the other experiments, sections were incubated overnight at 4°C with the anti-c-Fos antibody together with a monoclonal anti-mCherry antibody (cat. no. M11217, ThermoFisher Scientific), both diluted 1:1000 in PBS containing 1% BSA and 0.1% Triton X-100. Corresponding Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 568-conjugated anti-rat secondary antibodies (cat. nos. 711-545-152, Jackson ImmunoResearch; A11077, ThermoFisher Scientific) were applied for 2 h at room temperature at a dilution of 1:500. Finally, sections were washed three times in PBS, mounted onto glass slides, air-dried, and coverslipped using Fluoromount™ aqueous mounting medium (cat. no. F4680, Merck).

7.6 Image acquisition

Fluorescence images were acquired using a Leica Stellaris 8 confocal microscope equipped with 10x/0.3 NA dry and 20x/0.75 NA dry objectives. For brain-wide c-Fos mapping, whole coronal sections were imaged using the 10x objective at a scan speed of 600 Hz and a resolution of 2048 x 2048 pixels, acquiring the optical plane exhibiting the maximal c-Fos signal. For the other experiments, tiled acquisitions spanning 4-6 z-planes were performed using the 10x or 20x objectives at a scan speed of 600 Hz and a resolution of 1024 x 1024 pixels.

For dendritic spine imaging, a Zeiss LSM 800 confocal laser scanning microscope equipped with an AiryScan detector, and a 63x/1.40 NA oil immersion objective was used. Images were acquired at 16-bit depth using AiryScan imaging, a super-resolution microscopy technique. Z-stacks were captured with a step size of 0.15 µm, spanning the entire volume of the selected dendritic region. Raw images were processed using AiryScan image reconstruction in ZEN software.

7.7 Image processing and registration

Image registration to the Allen Brain Atlas CCFv3-2017 was performed as previously described (Lupori et al., 2023). For each brain, images were ordered along the posterior–anterior axis according to their unique slice ID. All images were visually inspected and, when necessary, mirrored to ensure consistent left-right hemisphere orientation across sections. To restrict the analysis of c-Fos⁺ cell density to image regions containing brain tissue, binary masks were generated for each image using an Ilastik-trained machine learning model. All masks were manually inspected and, when required, corrected using a custom MATLAB script. Alignment to the Allen Brain Atlas was performed in three steps. First, experimental images were matched to the corresponding atlas planes using QuickNII v2.2. Alignment was then refined in VisuAlign v0.9 by manually applying local transformations based on anatomical landmarks and slice-specific distortions. Finally, custom MATLAB functions (Lupori et al., 2023) were used to map each image pixel to its corresponding voxel in the reference atlas. Brain-wide heatmaps of c-Fos density fold-change were generated in Python using the BraiAnalyse library (Chiaruttini et al., 2025).

7.8 Cell counting

For brain-wide c-Fos mapping, c-Fos⁺ cells were detected using a custom Python pipeline based on StarDist (Schmidt et al., 2018), a deep learning-based method for cell and nuclear segmentation. The raw segmentation output was subsequently refined by applying semi-automated thresholds on fluorescence intensity and object area to exclude background signal and segmentation artifacts. Threshold values were manually adjusted on a per-image basis when required to account for variations in staining quality and background fluorescence. All adjustments were visually inspected to ensure accurate identification of c-Fos⁺ while minimizing technical artifacts.

For the other experiments, regions of interest (ROIs) were manually outlined in ImageJ based on anatomical boundaries defined using the mouse brain atlas (Paxinos and Franklin, *The Mouse Brain in Stereotaxic Coordinates*, 2004). Detection of c-Fos⁺ cells within these ROIs was performed using the same custom Python pipeline described for brain-wide analysis. c-Fos⁺ cell density (c-Fos⁺/mm²) was calculated and averaged across 4-5 sections per animal. The same procedure was applied to quantify DAPI⁺ cells. For the mCherry⁺ cell detection, cell counting was manually performed in ImageJ and the mCherry⁺ cell density (mCherry⁺/mm²) was averaged over 4-5 sections per animal. The number of c-Fos⁺/mCherry⁺ cells per section was manually counted in ImageJ. Z-planes were examined to ensure accurate co-localization of the c-Fos and mCherry signals within the same cell. The percentage of reactivation within the tagged neuronal population was calculated as $((\text{c-Fos}^+ \text{ mCherry}^+)/(\text{mCherry}^+)) \times 100$. To assess the level of reactivation, the proportion of overlap with DAPI⁺ was calculated as $(\text{c-Fos}^+ \text{ mCherry}^+)/\text{DAPI}^+$. The expected chance level of overlap was determined as $(\text{c-Fos}^+/\text{DAPI}^+) \times (\text{mCherry}^+/\text{DAPI}^+)$, and this calculation was performed for each slice. Overlap/chance was then computed by dividing the overlap/DAPI value by the chance value for each slice. Overlap/chance was averaged across all slices for each mouse, generating a single average value used for statistical analysis.

For single-cell mean fluorescence intensity analysis, the mask generated during the c-Fos⁺ cell detection segmentation was imported into ImageJ. This mask was then used to select the population of c-Fos⁺ cells. ImageJ was used to extract and average the mean intensity for each c-Fos⁺ cell. The average intensity per animal was calculated from 4-5 slices.

7.9 Spine analysis

For spine analysis, 8 pyramidal neurons were selected per mouse from layer II/III of the lateral entorhinal cortex. From each neuron, two 20 µm segments of second-order apical dendrites were randomly chosen for detailed spine morphology analysis using the Filament Tracer module in Imaris software (version 7.4.2). In the Filament Tracer's Surpass mode, the Autopath algorithm was used to trace dendrites and spines. Reconstruction was conducted using a semi-automatic approach, with dendrite diameters constrained to 1-5 µm and a minimum spine diameter of 0.4 µm. Automated thresholds were initially applied to identify spine seed points and enable surface rendering; however, threshold values were manually adjusted for each dendrite segment to ensure accuracy, adding true spines or removing artifacts as necessary. The morphological properties of dendrites and spines were

exported in an .xls table using a custom-made Xtension script in MATLAB. A data refinement procedure was conducted to enhance the reliability of the dataset by removing spines generated during automated reconstruction that either lacked a spine neck or were protrusions incorrectly identified as spines.

Spine morphology was then analyzed according to their width, length and length/width ratio (LWR), with the criteria from Barón-Mendoza et al. (2021) modified as follows:

- Filopodia: length > 2 μm , LWR > 1 spine head max diameter $\leq$ 0.6 μm
- Long thin: 1 μm < length $\leq$ 2 μm , spine head max diameter $\leq$ 0.6 μm
- Thin: length $\leq$ 1 μm , spine head max diameter $\leq$ 0.6 μm
- Stubby: LWR $\leq$ 1
- Mushroom: spine head max diameter $\geq$ 0.6 μm

Spine area and volume were computed following the approach described in Imaris software. Specifically, the surface area (or volume) of the hemisphere at the spine attachment point was subtracted from the sum of the surface area (or volume) of the spine frustum and the terminal hemisphere. Data processing and spine classification were performed in Python using a custom-made script.

7.10 Whole-cell patch-clamp recordings

Whole-cell patch-clamp recordings were performed as previously described (Tozzi et al., 2024) on visually identified EC neurons. Briefly, for acute LEC slices preparation, the head was submerged in ice-cold, oxygenated cutting solution containing (in mM): Sucrose 248, HEPES 20, glucose 10, MgCl₂ 10, KCl 2, CaCl₂ 0.5, Na⁺ ascorbate 5, Na⁺ pyruvate 3, pH adjusted to 7.4 with NaOH, where the brain was quickly extracted from the skull. The brain was immersed in the same ice-cold, oxygenated cutting solution, and 250 μm -thick brain sections were cut using a vibratome (Leica VT1200S, Weitzlar, Germany), then transferred to a recovery chamber containing oxygenated artificial cerebrospinal fluid (aCSF) composed of (in mM): NaCl 119, HEPES 10, glucose 10, NaHCO₃ 6.2, KCl 2.5, CaCl₂ 2, MgCl₂ 1.2 NaH₂PO₄ 1, Na⁺ ascorbate 0.4, pH adjusted to 7.4 with NaOH, in which they were incubated at 32 °C for 30 min. After moving the chamber to room temperature and allowing an additional 60 min, slices were transferred to the recording chamber, where recordings were performed under continuous perfusion with oxygenated aCSF at 32 °C. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded in patch-clamp mode. Borosilicate glass micropipettes were fabricated from 1B150F-4 capillaries (WPI, Sarasota, FL, USA) using a PC-100 vertical puller (Narishige International, London, UK), and had a 4-6 M Ω resistance when filled with an internal solution containing (in mM): K⁺-gluconate 145, HEPES 10, phosphocreatine 5, Mg²⁺-ATP 2.5, MgCl₂ 2, Na⁺-GTP 0.25, EGTA 0.1, plus biocytin (HB5035, Hello Bio, Dunshaughlin, Ireland) 0.2% W/V, pH adjusted to 7.35 with KOH. Neurons of the superficial layers of the LEC were approached under DIC illumination, using a 63x immersion objective. After establishing a gigaseal, the patch was broken by applying negative pressure to achieve a whole-cell configuration. A series resistance lower than 15

MΩ was considered acceptable and monitored constantly throughout the entire recording. At least 3 min were allowed for complete cytosol dialysis, then sEPSCs were recorded while holding the neuron at -70 mV, using a MultiClamp 700A amplifier and a Digidata 1550B card, controlled via Clampex 10 software (Molecular Devices, San Jose, CA, USA). Signal analysis was performed with the Clampfit 10 software (Molecular Devices) by employing the template-based search and identification function, with a template match threshold of 4.

7.11 In vivo electrophysiology

Custom electrode assemblies were prepared by inserting Formvar-insulated nichrome wires (50.8 μm diameter, A-M systems, Cat. #762000) into the channel holes of a gold-plated electronic interface board (EIB-16, Neuralynx), forming two electrode bundles of eight electrodes each, which were secured with gold pins (Neuralynx). Ground/reference channels were connected to a silver wire soldered to a stainless-steel screw. For chronic implantation, 3-month-old C57BL/6J mice were deeply anesthetized with isoflurane (3% for induction, 1.5% for maintenance) and positioned in a stereotaxic frame. The scalp was disinfected with 70% ethanol followed by betadine solution before incision. Craniotomies were performed stereotaxically above the mPFC and RSP using the same coordinates described for viral injections, and an additional hole was drilled above the cerebellum for ground/reference placement. Electrode bundles were slowly lowered into the target regions to the appropriate dorsoventral coordinates. The stainless-steel screw connected to the reference/ground channel was implanted over the cerebellum. The EIB and ground screw were secured to the skull using dental cement (Super-Bond C&B) and further reinforced with acrylic dental cement (Paladur). Following surgery, mice were allowed to recover from anesthesia at 37°C before they returned to their home cages.

Recordings were performed 15 days after the surgery using an Open Ephys acquisition board and the Open Ephys GUI data acquisition software. Briefly, signals from the EIB-16 were amplified, filtered, and digitized using a 16-channel Intan RHD headstage and acquired via the digital input of the acquisition board. To synchronize electrophysiological recordings with behavioral data, an LED was positioned within the camera's field of view in a location not visible to the animal, while behavioral activity was recorded at 30 FPS. The LED was controlled by an Arduino microcontroller, which simultaneously triggered LED illumination and sent a synchronization pulse to the analog input of the Open Ephys acquisition board. A custom Python script was used offline to align video frames containing LED activation with the corresponding analog synchronization signal, enabling precise temporal alignment between electrophysiological and behavioral data. Electrophysiological signals were recorded at a sampling rate of 30 kHz. Animals were habituated to experimenter handling and to the recording apparatus for five days before the start of behavioral procedures.

For LFP analyses, raw traces were visually inspected to identify and exclude channels with excessive noise or unstable signals. Remaining channels were downsampled to 1 kHz and band-pass filtered between 1 and 100 Hz. Object-exploration epochs were manually annotated from video recordings, aligned to the exploration midpoint, and divided into 3-s time windows; corresponding LFP segments were then extracted from the filtered traces for subsequent analyses. LFP power spectral density was estimated using a multitaper method

(DPSS tapers) in the 1-100 Hz frequency range. Inter-regional functional coupling was quantified using coherence and Granger causality analyses. Coherence between prefrontal and retrosplenial LFP signals was computed for each exploration epoch across frequencies. Directional interactions were assessed using spectral Granger causality, estimated for each exploration epoch and computed separately for each direction. For band-specific analyses, the theta band was defined as 5-10 Hz, low-gamma as 30-55 Hz, and high-gamma as 55-80 Hz. Absolute LFP power, coherence, and Granger causality values were averaged across these frequency bands for each exploration epoch and then averaged within condition (novel or familiar object exploration). All electrophysiological analyses were performed in Python using the SpikeInterface, MNE, and SciPy (scipy.signal) libraries.

7.12 Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA), and detailed statistical information for each experiment is provided in the corresponding figure legends. Data normality was assessed using the Shapiro-Wilk test. Depending on the experimental design, comparisons were performed using one-way or two-way ANOVA or two-tailed unpaired t-tests, with Tukey's and Sidak's post hoc tests applied after one-way and two-way ANOVA, respectively. For behavioral experiments, these analyses were used to evaluate differences in discrimination index and exploration parameters. One-sample t-tests were performed to determine whether discrimination index differed significantly from chance (hypothesized mean = 0) and whether ensemble reactivation differed from chance level (hypothesized mean = 1). For dendritic spine analysis, principal component analysis (PCA) was performed in Python using the scikit-learn library. A dataset comprising 16 morphological features was extracted from the 3D reconstruction of dendritic spines. Data were normalized using StandardScaler, and PCA was subsequently applied to reduce dimensionality. K-means clustering was performed, with the optimal number of clusters determined using the elbow method. For brain-wide c-Fos mapping, dimensionality reduction and clustering analyses were conducted in Python using the scikit-learn and UMAP libraries. PCA and Uniform Manifold Approximation and Projection (UMAP) were used for dimensionality reduction and low-dimensional embedding, followed by K-means clustering. Task partial least squares (task-PLS) analysis was performed in Python using a custom script implementing the method described in Franceschini et al. (2023). For functional network analysis, interregional correlation matrices were computed in Python, and network graphs and topological metrics were obtained using the NetworkX library.

8. References

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