

GPR6 Protects Medial Entorhinal Cortex in 5×FAD Mice from Early Pathology in Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is characterized by progressive memory decline, with the medial entorhinal cortex (MEC) representing an early vulnerable hub for spatial cognition. Here, using 10× Visium spatial transcriptomics, we identified *Gpr6* as a MEC-enriched and layer III-biased gene whose expression was reduced in 5×FAD mice and in the entorhinal cortex of human AD patients. Bilateral MEC-targeted *Gpr6* overexpression in young 5×FAD mice ameliorated early spatial memory deficits. In vivo two-photon calcium imaging further revealed MEC layer II/III network hyperactivity in 5×FAD mice, together with disrupted spatial and directional coding. *Gpr6* overexpression attenuated MEC neuronal hyperactivity, improved spatial coding, and enhanced the stability of spatial representations across contexts. These effects were observed in both *Gpr6*-overexpressing and neighboring non-overexpressing neurons, consistent with both cell-associated and local circuit-level effects. In parallel, MEC-targeted *Gpr6* overexpression reduced local A β plaque burden, altered microglial morphology, and increased dendritic spine density. Together, these findings identify GPR6 as an AD-vulnerable molecular regulator in the MEC and suggest a potential entry point for understanding and modulating early entorhinal circuit dysfunction in AD.

Introduction

Spatial cognitive deficits and impaired navigation are among the earliest cognitive manifestations of Alzheimer's disease (AD), often appearing before overt dementia and reflecting dysfunction within neural systems that support cognitive maps [1, 2]. The medial entorhinal cortex (MEC) and hippocampus form a core circuit for allocentric spatial representation, path integration, and memory-guided navigation [3–6]. Within this circuit, MEC neurons encode complementary spatial and self-motion variables through functionally specialized populations, including grid cells, head-direction cells, speed cells, border cells, and non-grid spatially tuned neurons [4, 7–10]. These neuronal populations convey information about position, direction, movement, and environmental boundaries, thereby supporting stable yet flexible spatial representations and self-motion-based navigation [5, 11–13]. This integration of heading, speed, and spatial metric signals is particularly important for path integration, and disruption of grid-cell activity impairs path integration in mice [14–16]. Disruption of MEC spatial coding is therefore likely to contribute to the navigation and spatial memory deficits that characterize early AD.

The entorhinal cortex is one of the earliest cortical regions affected in AD. Neuropathological studies have shown pronounced neuronal loss and pathological alterations in the entorhinal cortex even at very mild disease stages [17, 18]. This early vulnerability is consistent with the clinical observations that spatial cognitive deficits and impaired navigation can emerge before overt dementia [1, 2]. In humans, cognitively normal APOE ϵ 4 carriers show reduced grid-cell-like representations during virtual navigation, suggesting that entorhinal spatial-coding abnormalities may precede clinical symptoms in genetically at-risk individuals [19]. Although tau pathology classically begins in the transentorhinal and entorhinal regions before spreading to hippocampal and neocortical areas, amyloid- β deposition and APP/A β -related dysfunction also involve medial temporal and neocortical networks during disease

progression[20–23]. In experimental models, APP/A β expression in entorhinal cortex neurons can drive transsynaptic dysfunction within the entorhinal–hippocampal circuit[24]. Thus, early entorhinal dysfunction may directly impair spatial information flow and memory-guided navigation.

At the circuit level, AD mouse models provide direct evidence that MEC–hippocampal spatial coding is disrupted during disease progression. APP knock-in mice show impaired grid-cell activity, altered hippocampal remapping, and reduced integration of self-motion cues[25–27]. Recent work in aged AppNL-G-F knock-in mice further showed reduced MEC spatial information, unstable spatial representations, impaired position and speed decoding, and mild hyperactivity driven in part by putative interneurons [28]. Tau-based models also exhibit grid-cell dysfunction accompanied by spatial memory deficits[29]. Together, these findings indicate that AD-related cognitive impairment involves dysfunction of spatial circuits, not merely neuronal loss or end-stage degeneration. Importantly, this vulnerability is increasingly understood at finer anatomical and cellular resolution: layer II entorhinal neurons are selectively affected during aging and early AD progression[18, 29–31], and in vivo studies suggest that AD-related network dysfunction can depend on cortical layer and interneuron subtype[28, 32]. This layer- and cell-type-specific view raises the possibility that molecular programs enriched in defined MEC populations may contribute to early circuit instability in AD.

Despite these advances, the intrinsic molecular mechanisms that maintain MEC circuit stability remain incompletely understood. Recent single-cell, cell-type-specific, and spatial transcriptomic studies have shown that different brain regions and cell types exhibit distinct gene expression changes in AD, indicating that disease vulnerability is not uniform but instead depends on region- and cell-type-specific molecular programs[30, 31, 33–37]. However, the molecular markers and endogenous regulators that support MEC circuit function during early AD-related dysfunction remain poorly defined. Because the MEC is a key node in the entorhinal–hippocampal network, molecules enriched in MEC neuronal populations may be positioned to influence spatial information flow, representation stability, and early circuit dysfunction in AD.

Among molecular candidates that may link regional gene-expression programs to circuit function, cell-surface signaling receptors are of particular interest because they can couple extracellular or neuromodulatory signals to intracellular pathways controlling neuronal excitability, synaptic function, and network dynamics. G protein-coupled receptors (GPCRs) represent a major class of such signaling molecules, yet many orphan GPCRs remain poorly characterized despite their broad expression in the central nervous system[38, 39]. Several orphan GPCRs have been implicated in cognition and AD-related pathology. For example, loss of GPR3 reduces amyloid plaque burden and improves memory in AD mouse models [40], whereas GPR12 regulates short-term memory through effects on thalamocortical network synchrony[41]. These findings suggest that orphan GPCRs may influence neural circuit processes relevant to cognition and neurodegenerative disease, but whether they contribute to MEC circuit function remains unclear.

In our spatial transcriptomic screen for MEC-enriched molecular candidates, *Gpr6* emerged as a gene with enriched expression in MEC neuronal populations. GPR6 is a class A orphan GPCR closely related to GPR3 and GPR12[38]. Previous studies have examined GPR6 primarily in striatal circuits, where it regulates intracellular signaling and motor-related functions [42–45]. GPR6 also shows high constitutive activity and has been linked to Gs-dependent cAMP signaling and downstream PKA–CREB pathways [46, 47], although its endogenous ligand and physiological functions remain incompletely characterized [39, 48]. Additional evidence suggests potential roles in neuronal development and neuroprotection, including promotion of neurite outgrowth and involvement in pathways that mitigate amyloid- β -induced toxicity [47, 49]. However, whether GPR6 is expressed in entorhinal spatial circuits or contributes to AD-related MEC dysfunction has not been established.

In this study, we investigated whether GPR6 represents a candidate molecular regulator of MEC circuit stability under AD-related conditions. Using spatial transcriptomics, molecular validation, behavioral analyses, and in vivo two-photon calcium imaging, we examined the expression, disease-associated regulation, and functional consequences of *Gpr6* in the MEC. We further tested whether MEC-targeted *Gpr6* overexpression could modulate neuronal activity, spatial and directional coding stability, local AD-related pathology, and spatial memory impairment in 5 $\times$ FAD mice.

Results

Spatial transcriptomics identifies *Gpr6* as a layer III-enriched MEC neuronal gene

To identify molecular candidates associated with MEC circuit function, we first generated 10 $\times$ Visium spatial transcriptomic data from adult mouse brain sections. Among genes showing regional enrichment, *Gpr6*, an orphan G protein-coupled receptor, exhibited prominent expression in the medial entorhinal cortex (MEC) (Figure 1A). Quantification of spatial transcriptomic spots showed that *Gpr6*-positive spots were more abundant in the MEC than in cortical and hippocampal regions. Spot-level *Gpr6* expression was also higher in the MEC than in the cortex, but was comparable to that in the hippocampus and lower than that in the striatum, where GPR6 has been studied in striatal signaling and Parkinsonian circuitry [43–45] (Figure 1B). We next validated this regional pattern by in situ hybridization, which confirmed enriched *Gpr6* signal in the MEC (Figure 1C). Laminar quantification further showed that *Gpr6*-positive cells were preferentially distributed in layer III rather than uniformly across MEC layers, and co-labeling with NeuN indicated that *Gpr6* expression was largely neuronal (Figure 1D).

To define the cellular identity of *Gpr6*-expressing cells more precisely, we analyzed public mouse MEC single-nucleus transcriptomic data, with all public datasets summarized in Table S1. Unsupervised clustering separated neuronal and non-neuronal populations (Figure 1E). *Gpr6* expression was enriched in a subset of excitatory neuronal populations, with *Gpr6*-positive cells distributed predominantly among layer III excitatory clusters (Figure 1F). Together, spatial transcriptomics, in situ hybridization, and single-nucleus analyses indicate that *Gpr6* is an MEC-enriched neuronal gene preferentially expressed in layer III.

We next asked whether this expression pattern was conserved in humans. Sequence analysis showed that GPR6 was highly conserved across mouse, rat, and human (Figure S1A). In public human entorhinal cortex spatial transcriptomic data, GPR6 showed a laminar expression pattern with enrichment in superficial entorhinal layers (Figure 1K), consistent with the superficial-layer bias observed in mouse MEC. We then asked whether this MEC-enriched expression pattern was a general feature of closely related orphan receptors. Despite sequence similarity among GPR6, GPR3, and GPR12 (Figure S1B), their entorhinal expression patterns were distinct. In mouse spatial transcriptomic and single-nucleus datasets, *Gpr3* and *Gpr12* were detectable in entorhinal regions but showed weaker or less selective MEC enrichment than *Gpr6* (Figures S1C–S1F). A similar distinction was observed in public human entorhinal cortex spatial transcriptomic data, where GPR3 and GPR12 did not recapitulate the superficial entorhinal enrichment pattern observed for GPR6 (Figures S1G and S1I).

MEC-enriched *Gpr6* is reduced under AD-related conditions

Having identified *Gpr6* as a conserved, superficial-layer-enriched entorhinal gene, we next asked whether its expression was altered under AD-related conditions. Analysis of public mouse MEC single-nucleus transcriptomic data showed reduced *Gpr6* expression in AD-model samples at 2 and 8 months, particularly within *Gpr6*-expressing excitatory neuronal clusters (Figure 1G). In our independent MEC tissue analysis, *Gpr6* RNA expression was reduced in 5-month-old 5×FAD mice and remained decreased at 10 months, whereas no significant genotype difference was detected at 2 or 14 months (Figure 1H and Figure S2A). Across the mouse lifespan, *Gpr6* expression showed an age-associated decline in WT mice, with additional reductions in 5×FAD mice (Figure 1I). At the protein level, western blotting detected reduced MEC GPR6 protein in 5-month-old 5×FAD mice (Figure 1J and Figures S2B). However, no significant difference was detected at 10 months (Figure S2C). These analyses suggest that *Gpr6* is reduced in the MEC under AD-related conditions, with the most robust protein-level difference detected during an early stage of amyloid pathology.

We next examined whether this disease-associated reduction extended to human AD datasets. Human bulk transcriptomic datasets showed reduced GPR6 expression in the AD entorhinal cortex, whereas comparable reductions were not observed in the temporal cortex, frontal cortex, or hippocampus (Figure 1L). Human bulk transcriptomic analyses further showed that AD-associated expression changes of GPR3 and GPR12 did not mirror the entorhinal-selective reduction observed for GPR6 (Figures S1H and S1J). Together with the mouse data, these findings indicate that *Gpr6* downregulation is observed across mouse AD-related conditions and human AD entorhinal cortex, supporting its association with AD-related entorhinal vulnerability.

Because *Gpr6* was preferentially expressed in the MEC layer III, we further asked whether the laminar distribution of endogenous *Gpr6*-expressing cells was related to local A β plaque deposition. *Gpr6*-iCre; tdTomato mice confirmed labeling of endogenous *Gpr6*-expressing cells (Figure S2D). In 5×FAD; *Gpr6*-iCre mice, A β plaque burden varied across MEC layers and was relatively lower in layer III (Figures S2E and S2F). However, distance analyses showed no preferential proximity or exclusion of *Gpr6*-labeled

cells relative to A β plaques compared with NeuN-positive neurons (Figure S2G). Thus, the observed Gpr6 expression pattern is not simply explained by local plaque proximity.

MEC Gpr6 overexpression ameliorates early spatial-navigation deficits in 5 $\times$ FAD mice

Given that Gpr6 reduction was most evident during an early stage of amyloid pathology, we next tested whether MEC-targeted Gpr6 overexpression could modify spatial-memory deficits in 5 $\times$ FAD mice. Young 5 $\times$ FAD mice received bilateral MEC injections of either a Gpr6-overexpression AAV or a control virus, followed by behavioral testing across the 5–9-month age window (Figure 2A). Histological analysis confirmed viral expression in the MEC and local A β pathology within the targeted region (Figure 2B).

We first assessed spatial-memory-related behavior using the Barnes maze (Figure 2C). Compared with control-virus-injected 5 $\times$ FAD mice, Gpr6-overexpressing mice showed a shorter latency to locate the target hole, whereas movement speed was comparable between groups. In the Y-maze spontaneous alternation task, Gpr6-overexpressing mice showed increased alternation performance, accompanied by a small but significant difference in mean speed (Figure 2D). By contrast, no significant differences were detected in the Morris water-maze probe test, novel object recognition, or novel location recognition assays (Figures S3A, S3C, and S3D). Elevated plus maze performance was also comparable between groups, arguing against a major contribution of anxiety-related behavior to the observed behavioral effects (Figure S3B).

We next examined path-integration-related navigation using the path-integration water maze, a task designed to place greater demand on self-motion-based spatial updating and MEC spatial circuitry[4, 13, 14] (Figure 2E). Gpr6-overexpressing mice showed swim trajectories more closely aligned with the platform direction and improved performance across training days, as reflected by increased cosine similarity to the target direction, while mean swim speed was comparable between groups. Together, these findings indicate that MEC-targeted Gpr6 overexpression improves spatial-memory performance and enhances path-integration-related navigation in young 5 $\times$ FAD mice.

MEC Gpr6 overexpression attenuates AD-related neuronal hyperactivity

To determine how Gpr6 overexpression affects MEC circuit function during spatial behavior, we performed in vivo two-photon calcium imaging of MEC layer II/III neurons after viral injection and microprism implantation (Figures 3A and 3B). We first analyzed calcium-event activity across all recorded MEC neurons and classified cells into low-, intermediate-, and high-activity bins according to calcium-event frequency: 0–2, 2–10, and >10 transients/min, respectively. Compared with WT mice, control 5 $\times$ FAD mice showed a shift toward higher calcium-event frequencies, with an increased proportion of high-activity neurons. MEC-targeted Gpr6 overexpression shifted this distribution away from the high-activity range and reduced the proportion of high-activity neurons (Figure 3C).

Quantification further showed that mean calcium-event frequency was elevated in control 5 $\times$ FAD mice and decreased after Gpr6 overexpression, indicating attenuation of AD-related MEC hyperactivity (Figure

3D). In contrast, the coefficient of variation of calcium-event frequency increased after Gpr6 overexpression, suggesting that the reduction in average event frequency did not reflect a simple global suppression of neuronal activity. These activity differences were unlikely to be explained by differences in imaging quality or behavior during recording, because spatial coverage, calcium signal-to-noise ratio, and running speed were comparable across groups (Figure S5D).

We next examined how this altered activity profile related to spatial coding. Control 5×FAD mice showed reduced spatial information and a relative increase in other unclassified cells among recorded MEC neurons, whereas Gpr6 overexpression increased spatial information and reduced the proportion of unclassified cells (Figure 3D). Activity-bin analysis further showed that spatial cells and unclassified cells were differentially distributed across activity ranges, with a higher proportion of other cells observed in the high-activity range. Within the intermediate activity range, spatial information was reduced in control 5×FAD mice and increased toward WT levels after Gpr6 overexpression (Figure 3E). Regression analyses further showed that mean calcium-event frequency and event-frequency variability improved the prediction of spatial information beyond the experimental group alone (Table S2). Together, these analyses suggest that AD-related MEC hyperactivity is associated with impaired spatial coding, whereas Gpr6 overexpression shifted the activity distribution away from excessively high event rates and toward a more balanced activity range.

Gpr6 overexpression improves MEC spatial and directional coding in 5×FAD mice

MEC-dependent navigation is supported by multiple spatially tuned neuronal populations, which may be differentially affected by AD-related circuit dysfunction. We therefore examined whether Gpr6 overexpression altered the coding properties of defined MEC cell classes. In non-grid spatial cells, control-virus-injected 5×FAD mice showed reduced spatial information and enlarged spatial fields compared with WT mice. Gpr6 overexpression increased spatial information and reduced field size, indicating improved spatial selectivity within this population (Figure 3F). A similar improvement was observed in head-direction cells. Control 5×FAD mice showed reduced head-direction information and lower mean vector length, whereas Gpr6 overexpression increased both measures, suggesting more reliable directional tuning (Figure 3G).

By contrast, border- and grid-cell measures showed limited improvement. Border-cell analyses showed no significant increase in border score after Gpr6 overexpression (Figure S5E). For grid cells, grid scores among cells that met the grid-cell classification criterion did not differ significantly across groups. When grid scores were calculated across all recorded MEC neurons, group differences were detected, suggesting that grid-score distributions differed at the population level but did not translate into a clear increase among classified grid cells (Figure S5F). Consistently, the observed proportions of classified grid and border cells remained relatively low after Gpr6 overexpression. Thus, MEC-targeted Gpr6 overexpression preferentially improved non-grid spatial and head-direction coding, with weaker effects on grid and border representations.

Gpr6 overexpression stabilizes MEC spatial and directional representations across contexts

Stable spatial cognition requires reliable representations across repeated experiences and appropriate separation between distinct contexts. We therefore examined MEC coding stability across three open-field sessions: Context A, Context B, and repeated Context A' (Figures 4A and 4B). This design allowed us to compare coding stability during repeated exposure to the same environment with coding separation across different environments.

Across all recorded neurons, group-level map-correlation patterns indicated weaker distinction between repeated and distinct contexts in control 5×FAD mice, whereas this distinction was more evident after Gpr6 overexpression (Figure 4C). Quantification further showed group differences in both repeated-context and distinct-context map correlations. The corresponding Δ map correlation, used to summarize the difference between repeated- and distinct-context correlations, was reduced in control 5×FAD mice and increased after Gpr6 overexpression (Figure 4D). Context-pair comparisons further showed that control 5×FAD mice exhibited an abnormal reversal of context-dependent map correlations, with A–B correlations exceeding A–A' correlations (Figures S6B and S6C). Control analyses showed that these effects were not explained by differences in movement coverage, calcium signal-to-noise ratio, or movement speed across sessions (Figure S6A).

Given that Gpr6 overexpression improved non-grid spatial and head-direction coding in the analyses above, we next asked whether these two affected cell populations also showed improved representational stability across contexts. We first focused on non-grid spatial cells. Control 5×FAD mice showed weaker separation between repeated and distinct contextual representations, as reflected by reduced A–A' map correlations and a lower Δ map correlation. Gpr6 overexpression increased A–A' map correlations and elevated the Δ map correlation (Figure 4E and Figure S7A). Joint-distribution analyses further illustrated this effect at the single-cell level, showing a clearer separation between A–A' and A–B map correlations after Gpr6 overexpression (Figure 4E).

We then examined whether head-direction cells showed a similar improvement in cross-context stability. Aligned head-direction tuning heatmaps illustrated the organization of preferred directions across Context A, B, and A', and preferred-direction difference distributions showed greater directional drift in control 5×FAD mice, particularly between repeated Context A and A'. Gpr6 overexpression reduced this repeated-context drift. To summarize context-dependent directional stability, we calculated the difference in preferred-direction drift between distinct and repeated contexts, defined as $\Delta PD(A-B) - \Delta PD(A-A')$. This value was increased after Gpr6 overexpression, mainly reflecting reduced directional drift between repeated Context A and A', and indicating improved anchoring of head-direction tuning across repeated exposures to the same environment (Figure 4F). Context-pair analyses further showed that the difference between repeated-context and distinct-context directional drift was reduced in control 5×FAD mice and increased after Gpr6 overexpression (Figure S8). Tuning-curve correlation analyses further supported improved cross-session directional consistency after Gpr6 overexpression. However, ADOe mice showed relatively high correlations for both A–A' and A–B comparisons, indicating that this improvement did not fully match the WT pattern (Figures S7C–S7F).

Together, these results indicate that Gpr6 overexpression improves the context-dependent stability of MEC spatial representations and strengthens the anchoring of head-direction tuning across repeated experiences, although the directional correlation pattern did not fully match the WT pattern.

MEC Gpr6 overexpression is associated with local pathological and synaptic molecular changes

We next examined whether MEC-targeted Gpr6 overexpression was associated with local pathological, structural, and molecular changes in 5×FAD mice. In 9-month-old 5×FAD mice, A β immunostaining revealed reduced plaque burden in the MEC of Gpr6-overexpressing mice compared with control 5×FAD, including decreases in small and intermediate plaque numbers, total plaque number, and A β -positive area fraction (Figure 5A). Microglial analysis further showed that this local reduction in A β burden was not accompanied by a broad reduction in microglial abundance. The total number of IBA1-positive microglia per ROI did not differ significantly between groups (Figure 5B). However, high-magnification imaging and skeleton-based morphological analysis revealed altered microglial structure, including changes in endpoint number, maximum branch length, and soma size (Figures 5C and 5D). These findings indicate that Gpr6 overexpression was associated with remodeling of local microglial morphology rather than a simple decrease in microglial number.

We also examined dendritic spine density as a structural readout of neuronal integrity in the MEC. Gpr6-overexpressing mice showed higher total spine density than control 5×FAD (Figure 5E). Subtype-specific density analysis indicated that this increase was mainly reflected in filopodia and thin spines, whereas mushroom and stubby spine densities were not significantly changed (Figure S10A). Thus, the spine phenotype was primarily associated with increased overall spine density and dynamic spine classes (filopodia and thin) rather than a selective increase in mature spine classes. To determine whether these pathological effects were broadly distributed outside the targeted region, we examined A β and IBA1 staining in the hippocampus and cortex. These analyses did not reveal comparable reductions in total A β plaque burden, A β -positive area, or microglial number, indicating that the pathological effects of MEC-targeted Gpr6 overexpression were most evident in the targeted region (Figures S10B–S10F).

Finally, we examined molecular changes associated with Gpr6 overexpression. Bulk RNA-seq analysis of MEC tissue identified differentially expressed genes between the control and Gpr6-overexpression groups (Figure 5F). Gene Ontology analysis highlighted pathways related to antioxidant and anti-peroxidation processes, together with neuronal and synaptic functions (Figure 5G). These transcriptomic changes were consistent with the local reduction in A β burden, microglial morphological remodeling, and increased dendritic spine density observed after MEC-targeted Gpr6 overexpression. Together, these results indicate that MEC-targeted Gpr6 overexpression improves MEC circuit function and spatial-navigation behavior in 5×FAD mice, accompanied by local pathological and structural changes.

Discussion

In this study, we identified GPR6 as a superficial MEC-enriched molecular feature that is reduced under AD-related conditions and functionally linked to entorhinal circuit instability. This conclusion is supported

by convergent spatial transcriptomic, single-nucleus transcriptomic, and human expression analyses, suggesting that GPR6 enrichment reflects a reproducible molecular property of superficial MEC and entorhinal populations rather than a dataset-specific observation. Functionally, MEC-targeted Gpr6 overexpression in young 5xFAD mice improved selected spatial-memory and path-integration-related behaviors, attenuated excessive MEC calcium-event activity, enhanced non-grid spatial and head-direction coding, and improved representational stability across repeated experience. These circuit-level effects were accompanied by reduced local A β burden, microglial morphological remodeling, increased dendritic spine density, and transcriptomic changes related to antioxidant and synaptic processes. Together, these findings suggest that GPR6 may help maintain local MEC circuit and synaptic states that support reliable spatial coding in an AD-vulnerable entorhinal network.

The laminar enrichment of Gpr6 provides a molecular entry point into MEC organization, but it should not be interpreted as evidence that layer III neurons are universally more vulnerable or more important than other entorhinal populations. Classical neuropathological studies have emphasized early pathological changes in layer II entorhinal neurons during aging and AD [17, 18, 31, 35]. Our data reveal a related but distinct superficial MEC-associated pattern: Gpr6 is biased toward layer III in the mouse MEC and shows superficial entorhinal enrichment in human datasets, where layer II and layer III cannot always be cleanly separated. Thus, Gpr6 is better viewed as a molecular feature of a superficial MEC population that may participate in circuit processes disrupted early in AD, rather than as a general marker of entorhinal vulnerability. Given the role of the MEC in entorhinal–hippocampal spatial information processing [6, 50–52], functional changes in this Gpr6-enriched superficial population may provide a local MEC circuit basis for the improved spatial-navigation and spatial-updating behaviors observed here. Whether these effects also involve downstream hippocampal or broader entorhinal–hippocampal circuits remains to be determined.

The temporal and regional pattern of GPR6 reduction further supports its relevance to early entorhinal dysfunction. In 5xFAD mice, Gpr6 RNA and GPR6 protein were reduced at 5 months, an early symptomatic stage marked by established amyloid pathology and emerging circuit dysfunction, whereas this reduction was less evident at later ages examined. Human datasets also showed reduced GPR6 expression in the AD entorhinal cortex, but not comparably in the hippocampus, frontal cortex, or temporal cortex. This pattern argues against GPR6 downregulation being merely a nonspecific consequence of global AD pathology or advanced degeneration. Instead, reduced GPR6 may reflect an early entorhinal stress state associated with local circuit dysfunction. This interpretation is consistent with previous work implicating GPR6 in C1q-associated neuronal protection against A β toxicity [49]. Comparison with the related orphan receptors GPR3 and GPR12 further suggests that the MEC-focused expression and AD-related entorhinal reduction observed here are most closely associated with GPR6 within this receptor family [38, 40, 41].

The selectivity of the behavioral effects is informative for interpreting the functional relevance of Gpr6 overexpression. Spatial-navigation and path-integration impairments are increasingly recognized as early AD-related phenotypes in humans, including individuals with MCI, prodromal AD, or elevated AD risk [19,

53, 54]. In this context, the improvement in Barnes maze performance, Y-maze spontaneous alternation, and path-integration water-maze performance suggests that MEC-targeted Gpr6 overexpression preferentially benefits behaviors related to spatial updating and entorhinal–hippocampal processing. This interpretation is particularly relevant for path integration, which depends on heading and speed information, both represented in MEC circuits [7, 8, 55, 56]. By contrast, Morris water-maze probe performance, novel object recognition, and novel location recognition were not significantly changed. Thus, the behavioral data are best interpreted as evidence for improved MEC-related spatial updating and navigation rather than generalized cognitive enhancement.

The calcium-imaging results provide a circuit-level framework for these behavioral effects. AD-related hyperactivity has been reported across multiple models and brain regions [57–59], including entorhinal or MEC circuits [28, 60, 61], and such hyperactivity may interfere with the formation of reliable spatial representations. In the present study, Gpr6 overexpression shifted MEC activity away from an excessively high calcium-event-rate state. However, this effect should not be interpreted as simple neuronal suppression. If Gpr6 overexpression merely reduced activity globally, spatial coding might be expected to deteriorate. Instead, spatial information improved, and calcium-event activity became more heterogeneous. These observations suggest that Gpr6 overexpression moved the MEC network toward an activity regime more compatible with spatially informative coding.

A plausible interpretation is that GPR6 reshapes the local MEC activity state rather than directly suppressing neuronal activity. GPR6 is known to exhibit constitutive activity and to engage Gs/cAMP signaling [46, 47], but the reduction in excessive MEC calcium-event activity observed here is not readily explained by this canonical pathway alone. Instead, our data suggest that Gpr6 overexpression may act through AD-context-dependent network, cellular, or microenvironmental mechanisms, affecting synaptic output, excitatory–inhibitory interactions, cellular resilience, or the local tissue environment. This interpretation is compatible with the multifactorial origin of AD-related hyperactivity, including soluble A β , impaired inhibition, astrocytic calcium dysregulation, and intracellular calcium-store abnormalities [59, 60, 62–64]. This interpretation is particularly relevant to the MEC, where spatial coding depends on a balanced local network state shaped in part by recurrent and feedback inhibitory interactions [65, 66]

The mCherry-defined population analysis further supports this local circuit-level interpretation. Coding changes were detected not only in Gpr6-overexpressing neurons but also in neighboring non-overexpressing neurons within the same imaging fields. Although several measures tended to show stronger effects in Gpr6-overexpressing neurons, the differences between mCherry-positive and mCherry-negative populations were not statistically significant. Together, these findings support a local circuit-level effect of Gpr6 overexpression within the MEC, extending beyond the transduced neuronal population. The accompanying increase in dendritic spine density and transcriptomic enrichment of antioxidant and synaptic processes further suggests that Gpr6 overexpression may improve a local synaptic and cellular resilience state that supports MEC coding.

The uneven effects across MEC coding features also fit this activity-state interpretation. Gpr6 overexpression preferentially improved non-grid spatial selectivity and head-direction tuning, whereas grid- and border-cell measures showed limited improvement. This selectivity is biologically plausible because different MEC coding features depend on different circuit requirements. Grid-cell periodicity requires coordinated heading, speed, and recurrent network signals [7, 8, 55, 56, 65–67], whereas border-related representations are shaped by environmental boundaries and boundary-responsive signals distributed across the MEC, subiculum, and pre-/Para subiculum [9, 11, 12]. Thus, MEC-targeted Gpr6 overexpression may improve local activity balance and selected spatial or directional coding features without fully restoring the distributed circuit interactions required for robust grid periodicity or boundary anchoring.

The representational-stability findings extend this interpretation from single-session coding to the maintenance of MEC representations across experience. Previous AD-related MEC studies have emphasized hyperactivity and impaired spatial or grid-cell coding under familiar–novel transitions, cue-changing conditions, or self-motion-integration demands [25, 27, 28]. Our A–B–A' paradigm addressed a related but distinct question: whether MEC representations can return to a stable state when animals re-encounter the same context after exposure to a different environment. In this setting, Gpr6 overexpression improved repeated-context stability of MEC spatial and directional representations, but the pattern remained more generalized than WT-like. Thus, Gpr6 overexpression stabilizes MEC coding but does not fully restore the dynamic, context-dependent flexibility of MEC circuits.

The local pathological and structural findings place these circuit effects within a broader MEC tissue context, while leaving causality unresolved. Gpr6 overexpression was associated with reduced local A β burden, microglial morphological remodeling, increased dendritic spine density, and transcriptomic changes related to antioxidant and synaptic processes. These changes may be intertwined with circuit stabilization, because synaptic activity can regulate interstitial A β levels in vivo [68], and A β -dependent neuronal hyperactivation can form a pathological feedback loop that further destabilizes circuit function [69]. Alternatively, local amyloid reduction and microglial remodeling may contribute to a tissue environment that supports more stable neuronal activity and spatial coding. The altered microglial morphology and increase in dynamic spine classes may therefore reflect remodeling of the local plaque-, synapse-, and plasticity-related microenvironment [70–73]. Together, these findings link Gpr6-dependent circuit effects to local pathological and structural remodeling, without establishing their temporal or causal order.

Several considerations temper the interpretation of these findings. First, the study relies primarily on the 5 $\times$ FAD model, which captures amyloid-associated pathology but does not fully reproduce tau progression, neuronal loss, or the clinical heterogeneity of human AD. Second, viral overexpression tests the functional sufficiency of increasing Gpr6 in the MEC, but it does not necessarily mimic physiological restoration of endogenous GPR6. Third, the temporal and causal relationship between circuit stabilization and tissue-level remodeling remains unresolved. Finally, the conclusions are centered on the

MEC, where Gpr6 is physiologically enriched; whether Gpr6 modulation in other regions would produce similar or distinct effects remains unknown.

In summary, we identify GPR6 as a superficial MEC-enriched molecular feature that is reduced under AD-related conditions and functionally linked to entorhinal circuit stability. MEC-targeted Gpr6 overexpression improved selected spatial-navigation behaviors and MEC circuit function in 5×FAD mice, accompanied by local pathological, structural, and transcriptomic changes. These findings suggest that GPR6 helps maintain the activity and synaptic state required for reliable MEC spatial coding, and that its reduction may contribute to early entorhinal circuit dysfunction in AD.

Declarations

Resource availability

Data availability

All data necessary to assess the conclusions in this manuscript are provided in the main text and supplemental information. Additional information is available from the corresponding author upon reasonable request.

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Author contributions

Methodology, S.U., S.C., Z.W., X.Z., X.F., and Y.L.; software, X.Z. and Z.W.; formal analysis, Z.W., X.Z., and X.F.; investigation, S.U., S.C., Z.W., X.Z., and Y.L.; data curation, Z.W., X.Z., and X.F.; writing—original draft, Z.W. and C.M.; writing—review and editing, all authors; visualization, Z.W., X.Z., and X.F.; supervision, C.M.; project administration, C.M.; funding acquisition, C.M.

Declaration of interests

The authors declare no competing interests.

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Methods

Animals

All animal procedures were performed in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of Peking University. JAX C57BL/6J mice were used as the wild-type background strain. The 5×FAD line was originally obtained from The Jackson Laboratory (JAX stock no. 008730) and maintained on a C57BL/6J background by crossing hemizygous 5×FAD males with wild-type C57BL/6J females. Transgenic 5×FAD mice and wild-type littermates were used for molecular, behavioral, histological, and in vivo calcium-imaging experiments. Offspring were genotyped by PCR before experimental assignment. Transgenic 5×FAD mice and wild-type littermates were used for molecular, behavioral, histological, and in vivo calcium-imaging experiments.

Custom-generated Gpr6-iCre mice were used for endogenous Gpr6-cell labeling experiments. For reporter validation, Gpr6-iCre mice were crossed with Ai14 Cre reporter mice, B6. Cg-Gt (ROSA)26Sor^{tm14}(CAG-tdTomato) Hze/J (JAX stock no. 007914), to generate Gpr6-iCre; Ai14 mice. Gpr6-iCre mice were also used together with Cre-dependent reporter labeling for plaque-proximity analyses in 5×FAD mice.

Mice were housed under a 12-h light/dark cycle in a temperature- and humidity-controlled facility, with food and water available ad libitum unless otherwise specified for behavioral testing. Both male and female mice were included, and animals were assigned to experimental groups in a balanced manner when possible.

For endogenous Gpr6 expression analyses, WT and 5×FAD mice were examined at 2, 5, 10, and 14 months of age, as indicated in the corresponding figure legends. For overexpression experiments, young 5×FAD mice received bilateral MEC injections of either a control AAV or a Gpr6-overexpression AAV. Mice were subsequently used for behavioral testing, in vivo calcium imaging, and post hoc histological or

molecular analyses. Sample sizes, animal numbers, and statistical tests for each experiment are provided in the figure legends.

Transcriptomic datasets

Mouse 10x Genomics Visium spatial transcriptomic data were generated in-house from mouse brain sections. Processed spatial gene-expression matrices and corresponding tissue images were reanalyzed in this study. Loupe Browser 8 was used to visualize Visium spatial transcriptomic data, inspect tissue morphology, and assist in anatomical annotation of spatial spots. Regional expression patterns of Gpr6, Gpr3, and Gpr12 were examined across anatomically defined regions, including the MEC, cortex, hippocampus, and striatum.

Public transcriptomic datasets were analyzed to examine the regional, cellular, and disease-associated expression patterns of Gpr6 and the related orphan receptors Gpr3 and Gpr12. The public datasets included human entorhinal cortex spatial transcriptomic data, human bulk transcriptomic datasets, and mouse MEC single-nucleus RNA-seq data. Gene-expression visualization, regional comparison, cluster-level analysis, and statistical testing reported in this study were performed by the authors.

For human entorhinal cortex spatial transcriptomic analysis, 10x Genomics Visium data were obtained from GEO accession GSE307990, associated with Huuki-Myers et al.[74]. This dataset contains spatially resolved transcriptomic profiles from postmortem human entorhinal cortex tissue. Spatial gene-expression matrices and spot-level metadata were used to visualize GPR6, GPR3, and GPR12 expression across entorhinal cortex spatial spots. Laminar information was assigned according to the dataset metadata and associated spot-level annotations when available.

For human bulk transcriptomic analysis, expression matrices and sample metadata were obtained from the public GEO datasets integrated in the study by Xu et al.[75]. The frontal cortex analysis included GSE12685, GSE36980, GSE48350, GSE5281, GSE53890, GSE66333, and GSE15222. The temporal cortex analysis included GSE29652, GSE36980, GSE37263, and GSE5281. The hippocampus analysis included GSE28146, GSE29378, GSE36980, GSE48350, and GSE5281. The entorhinal cortex analysis included GSE26927, GSE26972, GSE48350, and GSE5281. These datasets were analyzed to compare GPR6, GPR3, and GPR12 expression between control and AD samples across brain regions.

For mouse MEC single-nucleus RNA-seq analysis, count matrices and metadata were obtained from GEO accession GSE296091, associated with Bartas et al.[76]. The data were analyzed to examine Gpr6 expression across MEC cell clusters. Cluster-level expression, the percentage of Gpr6-positive cells, and disease-associated changes in clusters with high baseline Gpr6 expression were quantified.

Dataset accession numbers, species, brain regions, data modalities, and figure-panel usage are summarized in Table S1.

RNA extraction and quantitative PCR

Mice were anesthetized with isoflurane, and MEC tissue was rapidly dissected on ice. Total RNA was extracted using either TRIzol reagent or the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturers' protocols. RNA concentration and purity were assessed using a NanoDrop spectrophotometer. cDNA was synthesized from equal amounts of total RNA using HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech Co., Ltd., Nanjing, China).

Quantitative PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China) on a LightCycler 96 real-time PCR system. Relative gene-expression levels were normalized to Gapdh and calculated using the $\Delta\Delta C_t$ method. Primer sequences are provided in Table.

RNAscope in situ hybridization

RNAscope in situ hybridization was performed using the RNAscope Multiplex Fluorescent Reagent Kit V2 (323100, Advanced Cell Diagnostics, Inc., Newark, CA, USA) according to the manufacturer's protocol. Fresh brain tissue was embedded in OCT compound (4583, Sakura, Tokyo, Japan), frozen on dry ice, and sectioned at 15 μ m thickness using a cryostat. Sections were mounted onto glass slides, fixed, dehydrated, and treated with hydrogen peroxide and protease solution before probe hybridization.

A Gpr6-specific RNAscope probe was hybridized to tissue sections, followed by signal amplification and fluorescent labeling. Sections were counterstained with DAPI and mounted with antifade medium. Images were acquired using a Nikon AXR laser scanning confocal microscope (Nikon, Tokyo, Japan). MEC laminar boundaries were determined based on cytoarchitecture, DAPI staining, NeuN staining, and anatomical landmarks. Gpr6-positive cells were quantified across MEC layers. To assess neuronal enrichment, Gpr6 signal was quantified in NeuN-positive and NeuN-negative cells. Image analysis was performed using HALO software (Indica Labs Inc., Albuquerque, NM, USA).

For validation of Gpr6-iCre; Ai14 reporter labeling, Gpr6 RNAscope signal was compared with tdTomato reporter fluorescence, and the overlap between reporter-positive cells and Gpr6-positive cells was quantified from confocal images.

Western blotting

MEC tissue was collected from WT and 5x*FAD* mice at the indicated ages and homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors. Lysates were centrifuged to remove debris, and protein concentrations were measured using a BCA protein assay. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked using X-Blocking Low-Background Rapid Blocking Solution (Xblot-004, X-BLOT Life Science Ltd.) according to the manufacturer's instructions and incubated overnight at 4°C with primary antibodies against GPR6 (ab85005, Abcam; 1:500) and ATP1A1/Sodium Potassium ATPase (T55159, Abmart; 1:30,000).

After washing, membranes were incubated with HRP-conjugated secondary antibodies at room temperature. Chemiluminescent signals were detected using a Touch Imager (e-BLOT Life Science Co.,

Ltd., Shanghai, China). Band intensities were quantified using Fiji, and GPR6 levels were normalized to ATP1A1.

Sequence conservation analysis

Protein sequences of GPR3, GPR6, and GPR12, as well as GPR6 sequences from mouse, rat, and human, were obtained from UniProt. Multiple sequence alignment was performed using MEGA. Pairwise sequence identity and similarity were calculated and displayed as heatmaps

Viral vectors and stereotaxic injection

For MEC-targeted Gpr6 overexpression, mice were injected with an AAV2/9 vector encoding mouse Gpr6. Control mice received an AAV2/9 control vector expressing mCherry. Both the Gpr6-overexpression and mCherry control viruses were packaged in-house. The Gpr6-overexpression virus was used without dilution, whereas the mCherry control virus was diluted 1:10 before injection because of its higher viral titer. For calcium imaging experiments, GCaMP6f was expressed in MEC neurons using rAAV-hSyn-GCaMP6f-WPRE-hGH pA (PT-0147, AAV2/9; BrainVTA, Wuhan, China).

Mice were anesthetized with isoflurane and secured in a stereotaxic frame. Anesthesia was induced at approximately 3% isoflurane and maintained at 1–2% during surgery. Body temperature was maintained using a heating pad. For MEC injection, small craniotomies were made above the target sites. Virus was delivered using a NanoFil syringe or a glass micropipette connected to a microsyringe pump. The MEC was targeted bilaterally using coordinates relative to the transverse sinus and midline. Injection sites were positioned approximately 0.3 mm anterior to the transverse sinus, 3.3–3.6 mm lateral to the midline, and 1.6–1.8 mm below the brain surface. Virus was infused at approximately 0.08 $\mu\text{L}/\text{min}$, with a total volume of 500 nL per injection site and 1000 nL per hemisphere. The injection needle was left in place for 10 min after infusion before being slowly withdrawn to reduce backflow.

Microprism implantation for MEC imaging

For chronic two-photon imaging of MEC neurons, a microprism was implanted above the right MEC. After anesthesia and stereotaxic positioning, a craniotomy was made over the right posterior cortical region above the MEC. Tissue above the imaging target was carefully removed or displaced to provide optical access. The implantation site was positioned approximately 0.5 mm anterior to the transverse sinus, 2.9 mm lateral to the midline, and 1.5–1.6 mm below the brain surface. The microprism was inserted through the craniotomy and tilted approximately 8–10° posteriorly during implantation. The microprism and a custom head plate were secured to the skull using dental cement. Mice were allowed to recover before habituation and imaging. During postoperative recovery, mice received ceftriaxone sodium for 1 week. Prism location, viral expression, and imaging fields were verified by post hoc histology.

Behavioral testing

Behavioral assays were conducted after viral expression and postoperative recovery. Mice were habituated to the testing room before each experiment, and behavioral apparatuses were cleaned between trials. Movement trajectories and behavioral performance were recorded using a video-tracking system.

Barnes maze

Spatial reference memory was assessed using the Barnes maze, adapted from a previously described protocol[77]. The maze consisted of a circular platform containing multiple equally spaced holes, one of which led to an escape box. The escape-hole position remained fixed throughout training. Distal visual cues were placed around the testing room, and mild aversive stimuli, including bright light, 90-dB white noise, and a fan blowing across the maze, were used to motivate escape behavior. The testing area was surrounded by curtains to minimize uncontrolled external cues.

During habituation, each mouse was first placed in the escape box for 2 min and was then allowed to explore the maze for 5 min. If the mouse failed to enter the escape box during habituation, it was gently guided to the escape hole. During acquisition training, mice were trained for 5 days with four trials per day. At the beginning of each trial, the mouse was placed in a start chamber for 10 s and then released onto the maze. The starting location was varied across trials relative to the fixed escape-hole position. Each trial ended when the mouse entered the escape box or after 180 s. If a mouse failed to locate the escape hole within 180 s, it was gently guided to the escape hole and assigned an escape latency of 180 s. Mice were allowed to remain in the escape box for 30 s after each trial. The maze was rotated between trials when needed to reduce the use of olfactory cues. Escape latency, target-location accuracy, movement trajectory, and movement speed were quantified. A 60-s probe trial was performed to assess target-hole searching behavior.

Y-maze spontaneous alternation

Spatial working memory was assessed using spontaneous alternation in the Y-maze. Each mouse was placed in the center of a three-arm maze and allowed to explore freely for 10 min. Arm entries were recorded, and spontaneous alternation was calculated as the percentage of successive entries into all three arms without repetition.

Path-integration water maze

Path-integration-related navigation was assessed using an L-shaped water-maze task in a circular pool 120 cm in diameter. A 60° swimming corridor was placed in the pool, and the platform was positioned at the end of the corridor. Behavioral training was conducted in complete darkness without external visual cues. Swimming trajectories were recorded using a camera equipped with infrared illumination.

Training was performed for 5 days. Each training session consisted of four sample trials and four test trials, performed in an alternating sequence. At the beginning of each trial, mice remained in the start box for 10 s before being released. During sample trials, mice swam through the corridor to reach the

platform and remained on the platform for at least 10 s before being removed from the pool and dried in a warming chamber. Test trials were performed immediately after removal of the corridor. During each test trial, mice were released from the start box and allowed to swim freely for 90 s to locate the platform. If a mouse failed to find the platform within 90 s, it was gently guided to the platform and allowed to remain there for 10 s before being returned to the warming chamber. The interval between each sample trial and test trial was 15–20 min. Swimming trajectories were used to calculate angular deviation relative to the platform direction. Directional performance was further summarized using polar plots and cosine similarity to the platform direction.

Morris water maze test

Spatial memory was further assessed using the Morris water maze. Mice were trained for 5 days with five trials per day to locate a hidden platform. On day 6, a 90-s probe test was performed with the platform removed. Target-zone searching behavior, swim trajectory, and movement speed were quantified.

Novel object recognition and novel location recognition

Novel object recognition and novel location recognition were used to assess recognition memory and spatial location memory. Before each session, mice were allowed to acclimate briefly to the testing room. On day 0, mice were habituated to the empty arena for 10 min. On day 1, mice were exposed to two identical objects for 10 min. After a 6-h interval, one object was replaced with a novel object for the novel object recognition test or moved to a new location for the novel location recognition test, and mice were allowed to explore for 10 min. Exploration time directed toward each object was quantified to calculate recognition or location preference.

Elevated plus maze

Anxiety-related behavior was assessed using the elevated plus maze, as shown in the supplementary figures. Mice were placed in the center of the maze and allowed to explore the open and closed arms. Open-arm and closed-arm exploration were quantified.

In vivo two-photon calcium imaging

For in vivo calcium-imaging experiments, 5xFAD mice received viral injections into the MEC at approximately 5 months of age and were imaged at approximately 8 months of age. After recovery from microprism implantation, mice underwent contextual habituation and training before calcium imaging. To promote exploration during training and imaging, mice were placed under mild food restriction starting several days before contextual training and maintained at approximately 85–90% of their individual baseline body weight, with water available ad libitum. Body weight was monitored throughout the training and imaging period. Each training or imaging session lasted 40 min. Mice were first exposed to the same white context for two consecutive days to establish familiarity with the environment. A distinct black context was then introduced. During the following 10 days, mice were trained in an A–B–A'

sequence every other day for a total of five training sessions. Context A and repeated Context A' were the same white environment, whereas Context B was a distinct black environment.

Calcium imaging preprocessing and behavioral tracking

Calcium imaging was performed in freely moving mice using a miniature two-photon microscope (LFMTPM614 V2.0.1, Transcend Vivoscope Biotech Co., Ltd., China). Before imaging, the protective cover of the GRIN/prism optical window was removed, optical coupling gel was applied to the lens surface, and the microscope was attached to the baseplate and secured with screws. GCaMP6f fluorescence was excited at 920 nm, with laser power adjusted between 30 and 80 mW at the sample. Image acquisition was performed using GINKGO-MTPM software (Transcend Vivoscope Biotech Co., Ltd., China). Images were acquired at 600×512 pixels with a spatial resolution of approximately 0.89 $\mu\text{m}/\text{pixel}$ and a frame rate of 9–9.5 Hz using a femtosecond fiber laser (TVS-FL-01, Transcend Vivoscope Biotech Co., Ltd., China). Animal behavior was simultaneously recorded using an overhead camera at 25 Hz. Behavioral videos and calcium imaging data were synchronized and acquired using GINKGO-MTPM software. Imaging fields, viral expression, and prism placement were verified by post hoc histological analysis.

Calcium imaging movies were processed using Suite2p[78]. Motion correction, ROI detection, and extraction of fluorescence traces were performed in Suite2p, followed by manual inspection of ROIs when necessary. Fluorescence traces were converted to $\Delta F/F$, and calcium events were inferred from $\Delta F/F$ traces using a deconvolution-based procedure[79]. All downstream analyses of neuronal activity, spatial tuning, and directional tuning were performed using inferred calcium events.

Behavioral videos were analyzed using DeepLabCut[80]. The mouse head center was used to define position, and head direction was calculated from the vector between the head center and the nose. Behavioral trajectories were aligned to calcium-imaging timestamps before spatial and directional coding analyses.

Functional classification of MEC neurons

MEC neurons were classified into grid cells, border cells, spatially tuned non-grid cells, and head-direction cells based on calcium-event-derived spatial and directional tuning properties.

Grid cells

Grid-cell properties were evaluated from the spatial autocorrelogram of each smoothed event-rate map. Grid scores were calculated by comparing rotational correlations at 60° and 120° with those at 30°, 90°, and 150°, as described in previous studies [4, 55]. A neuron was classified as a grid cell if its grid score exceeded the 95th percentile of its shuffled distribution and its within-session spatial map stability was greater than 0.3. Within-session spatial map stability was calculated as the Pearson correlation between event-rate maps generated from the first and second halves of the session.

Border cells

Border-cell tuning was quantified using a border score based on the relationship between calcium-event fields and environmental boundaries[9]. Event fields used for border-score calculation were detected from smoothed event-rate maps using a peak-based thresholding procedure. The border score reflected the extent to which a cell's event field was aligned with the walls of the arena. A neuron was classified as a border cell if its border score exceeded the 95th percentile of its shuffled distribution and its within-session spatial map stability was greater than 0.3.

Non-grid spatial cells

Non-grid spatial cells were defined as neurons with significant spatial information and stable spatial maps that did not meet the criteria for grid or border cells[10]. Spatial information was considered significant if it exceeded the 95th percentile of the shuffled distribution, and spatial map stability was required to be greater than 0.3. For field-size analysis, spatial fields of non-grid cells were identified as contiguous regions in the smoothed event-rate map surrounding a local peak. Field size was calculated as the area occupied by the identified spatial field.

Head-direction cells

Head-direction tuning was assessed from calcium-event-based directional tuning curves[7]. Calcium events were binned according to the animal's head direction, and the resulting tuning curves were smoothed before calculation of the mean vector length. A neuron was classified as a head-direction cell if its mean vector length exceeded the 95th percentile of its shuffled distribution and its within-session angular stability was greater than 0.3. Within-session angular stability was calculated as the Pearson correlation between head-direction tuning curves generated from the first and second halves of the session. Preferred direction, head-direction information, peak event rate, and tuning width were calculated from the smoothed tuning curve.

mCherry-defined neuronal population analysis

To distinguish virally labeled neurons from neighboring non-labeled neurons, mCherry fluorescence images were acquired separately from the GCaMP6f calcium-imaging sessions. Before each freely moving imaging session, and again after completion of the three context-imaging sessions, mice were briefly head-fixed to acquire mCherry fluorescence images. For each acquisition, 2000 frames were collected over approximately 3.5–3.7 min.

mCherry fluorescence images were processed using Suite2p to identify mCherry-positive cellular regions. Cell identity across mCherry fluorescence images and GCaMP6f imaging sessions was matched using CellReg [81] followed by manual correction when necessary. mCherry-defined ROIs were then aligned with GCaMP6f-derived neuronal ROIs to classify each recorded neuron as mCherry-positive or mCherry-negative.

Immunohistochemistry

Mice were deeply anesthetized and transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA). Brains were dissected, post-fixed in 4% PFA, cryoprotected in 30% sucrose, embedded in OCT compound, and sectioned at 30 μ m thickness using a cryostat. Brain sections were washed in PBS, permeabilized, blocked with blocking buffer, and incubated with primary antibodies overnight at 4°C. After washing, sections were incubated with species-appropriate Alexa Fluor-conjugated secondary antibodies, counterstained with DAPI, and mounted with antifade mounting medium. Fluorescent images were acquired using confocal microscopy or slide-scanning microscopy, depending on the experiment.

Primary antibodies included anti-A β (mouse, BioLegend, 803001), anti-IBA1 (rabbit, FUJIFILM Wako, 019-19741), anti-NeuN (rabbit monoclonal, Abcam, ab177487), anti-GFP (chicken IgY polyclonal, Invitrogen, A10262), and anti-mCherry/RFP (rabbit, Rockland, 600-401-379). DAPI was obtained from Sigma-Aldrich (D9542). Secondary antibodies included Alexa Fluor 488 goat anti-rabbit (Invitrogen, A11034), Alexa Fluor 488 goat anti-chicken (Invitrogen, A11039), Alexa Fluor 568 goat anti-rabbit (Invitrogen, A11036) and Alexa Fluor 647 donkey anti-mouse (Invitrogen, A31571), depending on the primary antibody combination used in each staining experiment.

Quantification of A β plaques and microglia

After immunostaining, brain sections were imaged using an Olympus VS120-S6-W slide scanner or a SpinSR10 confocal microscope (Evident/Olympus), depending on the experiment. Anatomical regions of interest, including the MEC, hippocampus, and cortex, were defined according to anatomical landmarks. All image preprocessing, thresholding, and ROI selection were performed in Fiji. Quantification was then performed using Fiji measurements and custom MATLAB scripts.

For A β analysis, A β -positive plaques were segmented in Fiji using intensity-based thresholding followed by size filtering to remove background signal and small nonspecific particles. The same thresholding and filtering criteria were applied across groups within each experiment. Plaque number, plaque size, and A β -positive area were quantified using Fiji and MATLAB scripts. A β -positive area fraction was calculated as the total A β -positive area divided by the total area of the corresponding anatomical region.

For microglial analysis, IBA1-positive cells were segmented from fluorescence images in Fiji. Microglial number was quantified within each anatomical region of interest. For microglial morphology analysis, high-magnification images were acquired using a 100 $\times$ objective on a SpinSR10 confocal microscope (Evident/Olympus). Intact individual microglia were manually selected as ROIs before skeletonization. Cells that were incomplete, located at image borders, or strongly overlapping with neighboring cells were excluded from morphology analysis. Skeletonized microglial ROIs were then analyzed to quantify endpoint number, maximum branch length, and soma size.

For plaque-proximity analysis, A β plaques, NeuN-positive neurons, and endogenous Gpr6-labeled cells were segmented from fluorescence images. Cell centroids and plaque boundaries were extracted, and the distance from each NeuN-positive neuron or Gpr6-labeled cell to nearby A β plaques was calculated. Mean plaque distance and minimum plaque distance were quantified to compare the spatial relationship of endogenous Gpr6-labeled cells and the broader neuronal population with A β plaques.

Golgi staining and dendritic spine analysis

Golgi staining was performed using the FD Rapid GolgiStain Kit (PK401A, FD NeuroTechnologies) according to the manufacturer's instructions. After staining, brain sections containing the MEC were prepared and mounted for bright-field imaging. Neurons located in the superficial MEC were imaged using a 100 $\times$ objective on a SpinSR10 microscope (Evident/Olympus). Neurons with complete impregnation, clear dendritic morphology, and minimal overlap with neighboring cells were selected for analysis.

Dendritic segments were selected from comparable neuronal compartments across animals. Segment length was measured, and dendritic spines were manually identified and classified according to morphological criteria as mushroom, stubby, thin, or filopodia spines. Total spine density was calculated as the total number of spines divided by dendritic length. Subtype-specific spine densities were calculated separately for each spine category. Analyses were performed blind to group identity when possible.

Bulk RNA sequencing and enrichment analysis

MEC tissue was dissected from control-virus and Gpr6-overexpression mice and processed for total RNA extraction. RNA-seq libraries were constructed and sequenced by GeekGene using the Illumina paired-end 150-bp platform.

RNA-seq data were processed on the High Performance Computing Platform of Peking University. Raw reads were quality-controlled, trimmed, and aligned to the mouse GRCm39 reference genome. Gene-level count matrices were generated from the aligned reads and used for downstream analysis.

Differential gene-expression analysis was performed in R using DESeq2, with log2 fold-change shrinkage performed using apegglm. Genes with mean expression greater than 50, nominal $P < 0.05$, and absolute log2 fold change > 0.25 were considered differentially expressed. Upregulated and downregulated genes were analyzed separately. Gene Ontology enrichment analysis was performed using clusterProfiler and org.Mm.eg.db after conversion of gene symbols to Entrez IDs. Enriched biological processes related to antioxidant responses, anti-peroxidation, neuronal function, and synaptic regulation were visualized.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10, R, MATLAB R2024/R2025, and Python. $P < 0.05$ was considered statistically significant.

Figures

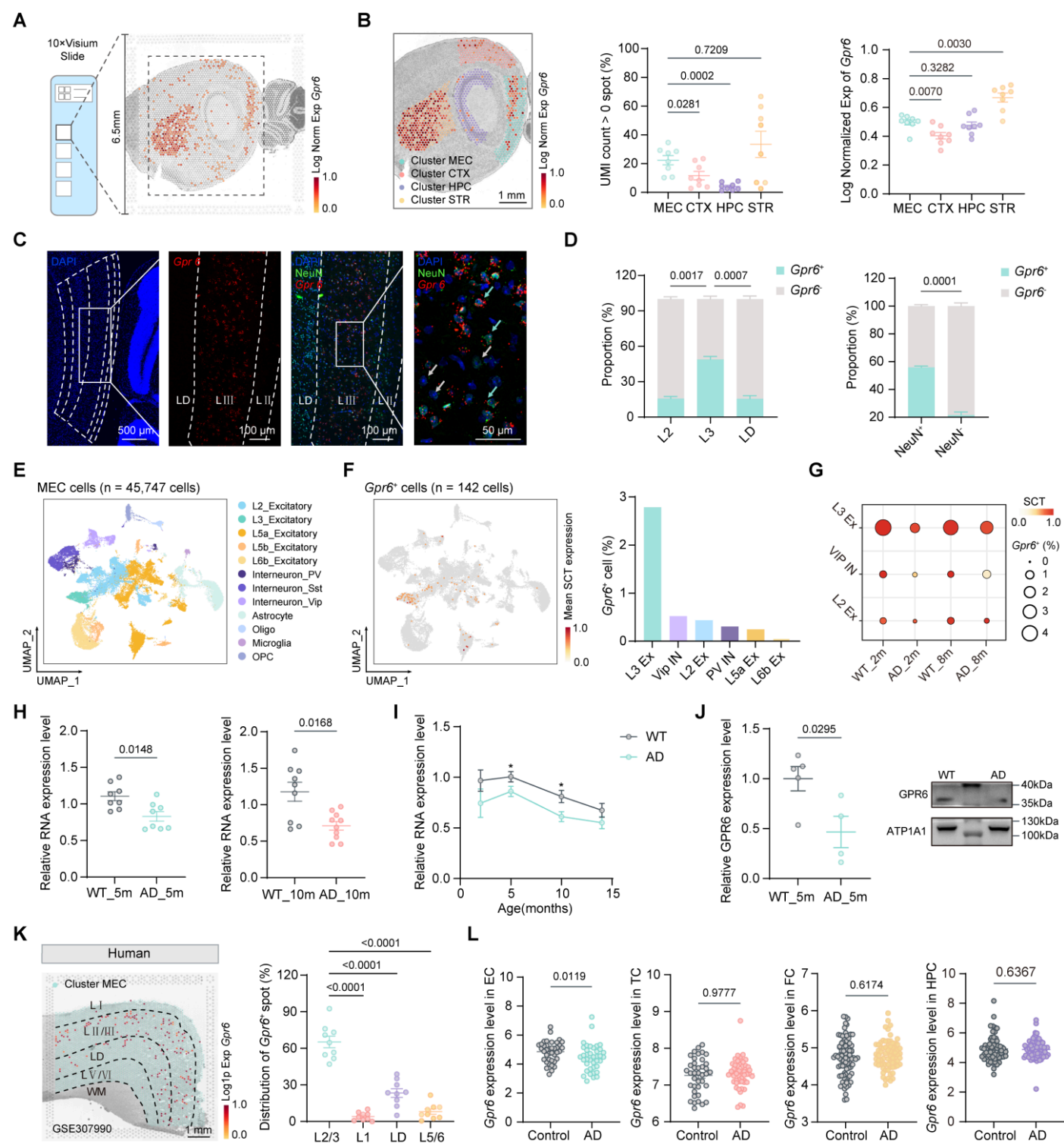


Figure 1

Gpr6 is enriched in the MEC and reduced in AD-related conditions.

(A) Mouse whole-brain 10×Visium spatial transcriptomic map showing *Gpr6* expression.

(B) Regional annotation of MEC, CTX, HPC, and STR spots, with quantification of the proportion of Gpr6-positive spots and log-normalized Gpr6 expression across regions. Gpr6-positive spots were defined as spots with UMI count > 0, and the proportion was calculated relative to the total number of spots in each region (n = 8 sections, N = 2 mice; Mann–Whitney U test; P values are shown in the panel).

(C) Representative in situ hybridization images showing Gpr6 expression in the MEC. White dashed lines indicate laminar boundaries estimated from cell-density differences. Enlarged images show Gpr6-positive and Gpr6-negative cells. Green arrows, Gpr6-positive cells; gray arrows, Gpr6-negative cells.

(D) Quantification of Gpr6-positive and Gpr6-negative cells across MEC layers and in NeuN-positive and NeuN-negative cells (n = 10 slices, N = 3 mice; P values are shown in the panel).

(E) UMAP visualization of mouse MEC single-nucleus RNA-seq (GSE296091) data showing annotated neuronal and non-neuronal clusters (n = 45,747 cells, N = 8 mice).

(F) Feature plot and cluster distribution of Gpr6-positive cells in mouse MEC single-nucleus RNA-seq data (n = 142 Gpr6-positive cells, N = 8 mice).

(G) Dot plot showing Gpr6 expression in major Gpr6-expressing MEC clusters across WT and AD-model samples at 2 and 8 months. Dot color indicates SCTransform-normalized expression; dot size indicates the percentage of Gpr6-positive cells.

(H) Gpr6 RNA expression in the MEC of WT and 5×FAD mice at 5 and 10 months (5 months: WT N = 8, 5×FAD N = 8, P = 0.0148; 10 months: WT N = 9, 5×FAD N = 10, P = 0.0168; Mann–Whitney U test).

(I) Age-dependent trajectory of Gpr6 RNA expression in WT and 5×FAD mouse MEC. Asterisks indicate significant group differences.

(J) GPR6 protein levels and representative western blots from 5-month-old WT and 5×FAD mouse MEC samples (WT N = 5, 5×FAD N = 4; P = 0.0295; Mann–Whitney U test). ATP1A1 was used as the loading control.

(K) Human EC 10× Visium data (GSE307990) showing GPR6 expression and the laminar distribution of GPR6-positive spots. Black dashed lines indicate approximate laminar boundaries adapted from the published annotation of the source dataset (P values are shown in the panel).

(L) Human bulk transcriptomic datasets comparing GPR6 expression between control and AD samples in EC, TC, FC, and HPC (EC: control N = 39, AD N = 39, P = 0.0119; TC: control N = 39, AD N = 52, P = 0.9777; FC: control N = 88, AD N = 73, P = 0.6174; HPC: control N = 66, AD N = 74, P = 0.6367; Mann–Whitney U test).

= 0.7212; ADCon: n = 35 daily values, N = 7; ADOe: n = 45 daily values, N = 9; Mann–Whitney U test). Asterisks indicate significant group differences at the indicated training days.

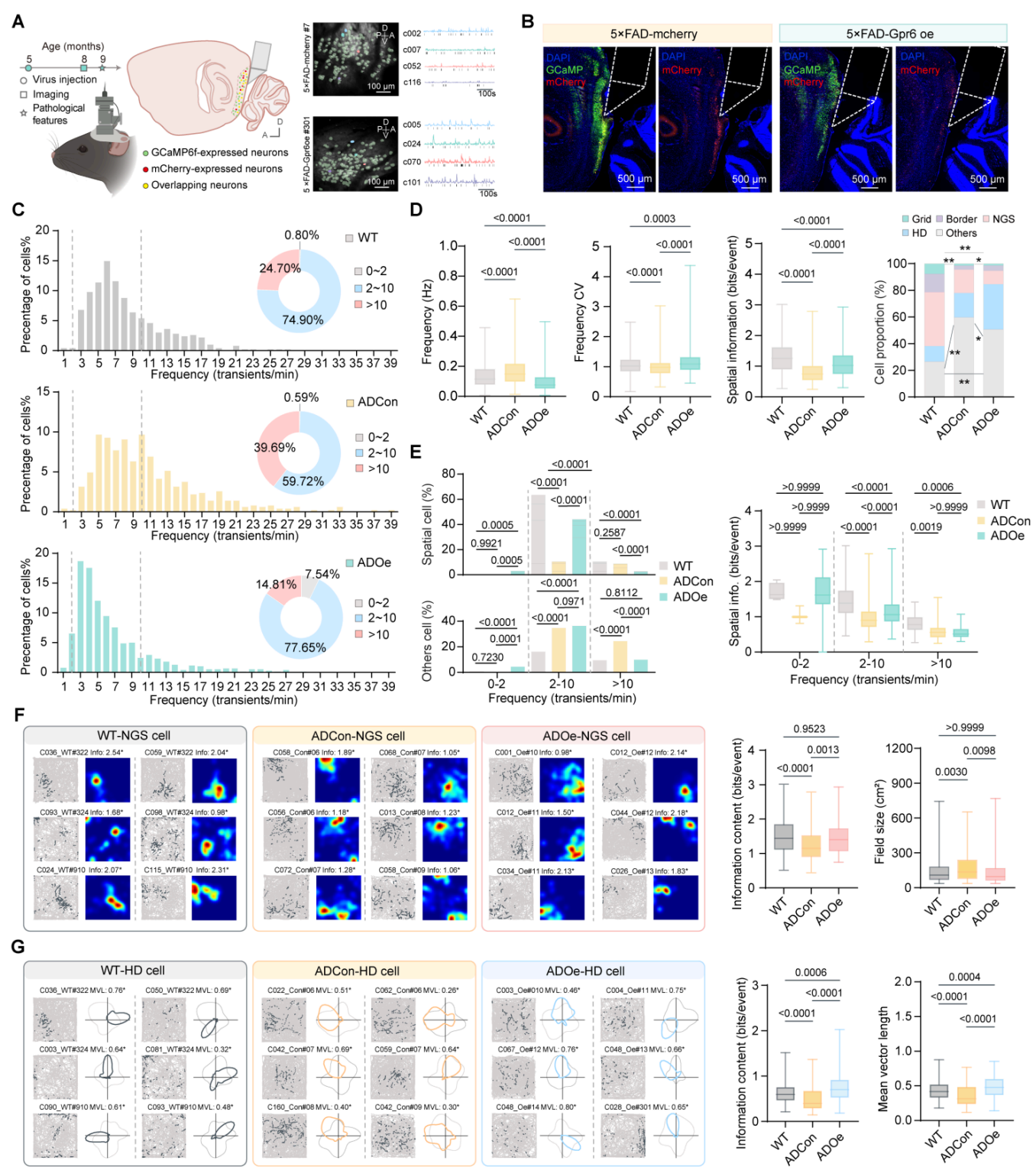


Figure 3

Gpr6 overexpression reduces MEC hyperactivity and improves spatial and directional coding in 5x FAD mice.

- (A) Schematic of viral injection, microprism implantation, and in vivo two-photon calcium imaging in the MEC. Representative imaging ROIs and calcium traces are shown.
- (B) Representative brain sections showing prism location and viral expression. GCaMP6f-expressing neurons are shown in green, mCherry-expressing neurons in red, and overlapping neurons in yellow. White dashed outlines indicate the approximate shape and position of the implanted microprism inferred from the implantation track.
- (C) Distribution of calcium-event frequency and proportions of low-, intermediate-, and high-activity neurons, defined as 0–2, 2–10, and >10 transients/min, respectively (WT: n = 502 cells, N = 3; ADCon: n = 508, N = 5; ADOe: n = 783, N = 6).
- (D) Mean calcium-event frequency, event-frequency CV, spatial information, and functional coding identity across groups. Functional identities included grid, border, NGS, HD, and other undefined cells (same n/N as in C). Pairwise adjusted P values are listed in the order WT vs ADCon, WT vs ADOe, and ADCon vs ADOe. Mean calcium-event frequency: P < 0.0001, P < 0.0001, and P = 0.0003. Event-frequency CV: P < 0.0001, P < 0.0001, and P < 0.0001. Spatial information: P < 0.0001, P < 0.0001, and P < 0.0001. Cell-type composition was analyzed by χ^2 test, with adjusted pairwise comparisons shown in the panel.
- (E) Activity-bin analysis of functional identity and spatial information. Recorded MEC neurons were stratified into low-, intermediate-, and high-activity bins as defined in C. Left, proportions of spatial cells and other cells across activity bins are shown separately in the upper and lower plots; spatial cells include grid, border, NGS, and HD cells. Right, spatial information of all recorded neurons within each activity bin. Pairwise adjusted P values are shown in the panel.
- (F) Representative NGS cells and spatial maps. Quantification of information content and field size is shown on the right. P values are listed in the same order as in D. Information content: P < 0.0001, P = 0.9523, and P = 0.0013. Field size: P = 0.0030, P > 0.9999, and P = 0.0098 (WT: n = 308 cells, N = 3; ADCon: n = 114, N = 5; ADOe: n = 125, N = 6).
- (G) Representative HD cells and head-direction tuning curves. Quantification of HD information content and mean vector length is shown on the right. P values are listed in the same order as in D. HD information content: P < 0.0001, P = 0.0006, and P < 0.0001. Mean vector length: P < 0.0001, P = 0.0004, and P < 0.0001 (WT: n = 202 cells, N = 3; ADCon: n = 124, N = 5; ADOe: n = 375, N = 6).

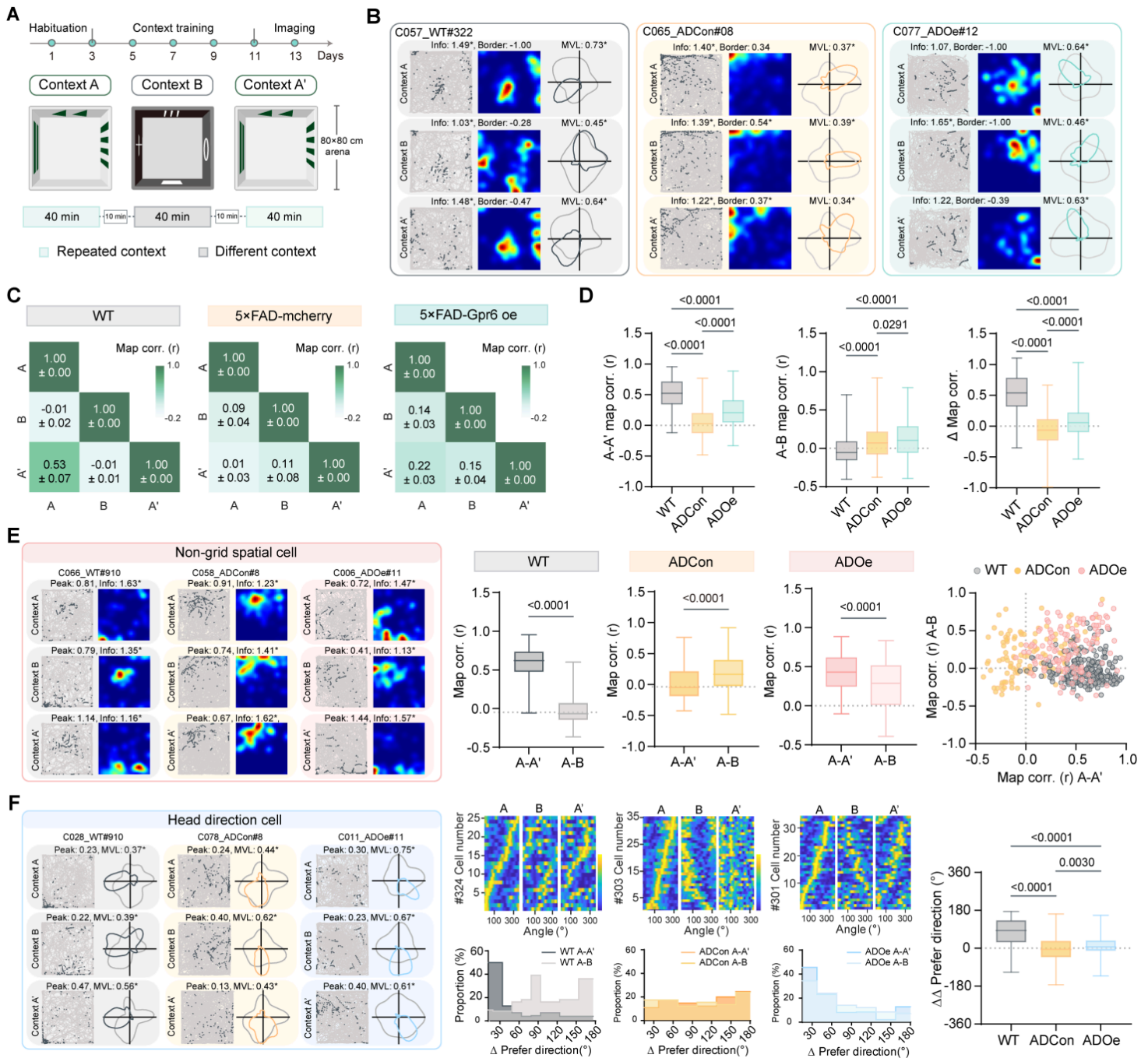


Figure 4

Gpr6 overexpression enhances the contextual stability of MEC spatial and directional coding in 5x^{FAD} mice.

(A) A three-session contextual recording paradigm. Mice underwent context training before imaging and were then sequentially recorded in Context A, Context B, and repeated Context A'.

(B) Representative MEC neurons recorded across Context A, Context B, and Context A'. Spike-location plots, spatial maps, and HD tuning curves are shown.

(C) Heatmaps of mean spatial map Pearson correlations across sessions (WT: N = 3; ADCon: N = 5; ADOe: N = 6).

(D) Spatial map correlations across all recorded MEC neurons for A–A', A–B, and Δ map correlation, calculated as $\text{corr}(A-A') - \text{corr}(A-B)$ (WT: n = 502 cells, N = 3; ADCon: n = 508, N = 5; ADOe: n = 783, N = 6; Kruskal–Wallis test with multiple-comparison correction; P values are shown in the panel).

(E) Context-dependent map correlations of NGS cells. Representative cells, within-group A–A' versus A–B comparisons, and joint distributions are shown (WT: n = 217 cells, N = 3; ADCon: n = 114, N = 5; ADOe: n = 136, N = 6; WT, $P < 0.0001$; ADCon, $P < 0.0001$; ADOe, $P < 0.0001$; Wilcoxon matched-pairs test).

(F) Context-dependent preferred-direction analyses of HD cells. Representative cells, aligned tuning heatmaps, preferred-direction differences, and preferred-direction stability, calculated as $\Delta\text{PD}(A-B) - \Delta\text{PD}(A-A')$, are shown (WT: n = 202 cells, N = 3; ADCon: n = 156, N = 5; ADOe: n = 197, N = 6; Kruskal–Wallis test with multiple-comparison correction). Pairwise adjusted P values for preferred-direction stability are listed in the order WT vs ADCon, WT vs ADOe, and ADCon vs ADOe: $P < 0.0001$, $P < 0.0001$, and $P = 0.0030$.

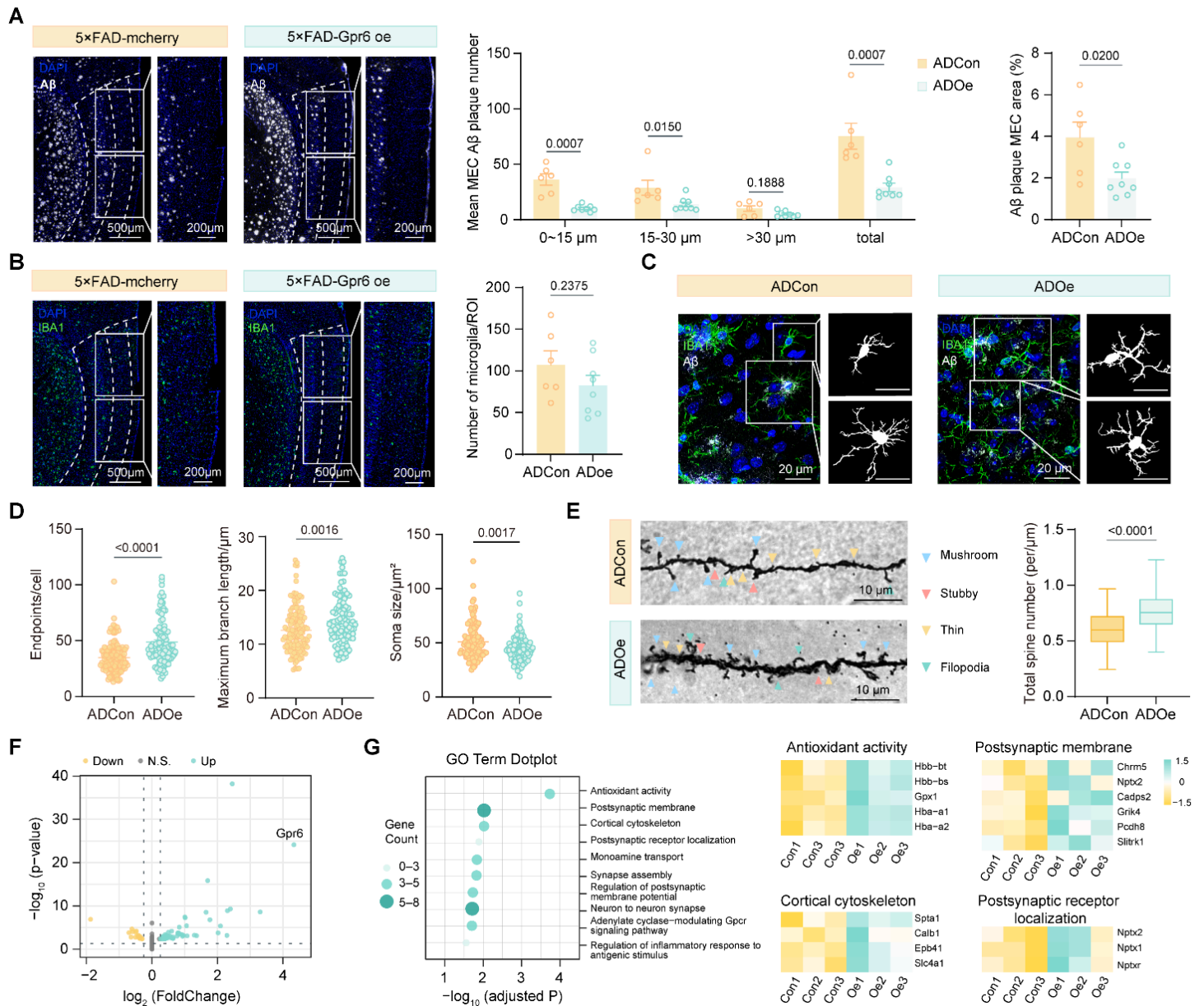


Figure 5

Gpr6 overexpression reduces the local Aβ burden and preserves neuronal structural integrity in the MEC.

(A) Representative Aβ immunostaining images in the MEC of ADCon and ADOe mice. Aβ is shown in white and DAPI is shown in blue. White dashed lines indicate approximate MEC boundaries and coarse laminar subdivisions. Quantification of Aβ plaque number by diameter, total plaque number, and Aβ-positive area is shown on the right (ADCon: N = 6; ADOe: N = 8; Mann–Whitney U test; P values are shown in the panel).

(B) Representative IBA1 immunostaining images in the MEC. IBA1 is shown in green and DAPI is shown in blue. Quantification of microglial number per ROI is shown on the right (P = 0.2375; ADCon: N = 6; ADOe: N = 8; Mann–Whitney U test).

(C) High-magnification images and skeletonized reconstructions of MEC microglia. IBA1 is shown in green, A β in white, and DAPI in blue.

(D) Microglial morphology, including endpoint number, maximum branch length, and soma size (endpoints: $P < 0.0001$; branch length: $P = 0.0016$; soma size: $P = 0.0017$; ADCon: $n = 134$ cells, $N = 5$; ADOe: $n = 110$, $N = 5$; Mann–Whitney U test).

(E) Representative dendritic spine subtypes and quantification of total spine density ($P < 0.0001$; ADCon: $n = 188$ dendrites, $N = 4$; ADOe: $n = 184$, $N = 4$; Mann–Whitney U test).

(F) Volcano plot of bulk RNA-seq differential expression analysis comparing ADCon and ADOe MEC samples ($N = 3$ per group).

(G) GO enrichment analysis and heatmaps of representative genes from selected enriched categories

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTables.docx](#)
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