LRP4 mediated synaptic plasticity in the piriform cortex

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Abstract

Background

Low-density lipoprotein receptor-related protein 4(LRP4) plays a critical role in the central nervous system (CNS), including hippocampal synaptic plasticity, maintenance of excitatory synaptic transmission, fear regulation, as well as long-term enhancement.

Results

In this study, we found that *Lrp4* was highly expressed in the piriform cortex and located in the second layer of the piriform cortex. When the transmembrane domain (TMD) and the intracellular domain (ICD) were missing, the *Lrp4*^{ECD/ECD} mice appeared to be smaller, and the brain's weight decreased, compared with the control mice. Simultaneously, finding food was prolonged for *Lrp4*^{ECD/ECD} mice in the buried food-seeking test. In the piriform cortex of *Lrp4*^{ECD/ECD} mice, the spine density of layer II increased, and the frequency of both miniature excitatory postsynaptic current (mEPSC) and spontaneous excitatory postsynaptic current (sEPSC) enhanced.

Conclusions

This study indicated that LRP4 mediated synaptic plasticity in the piriform cortex. Moreover, it also suggested that TMD and ICD of LRP4 are nonnegligible for the LRP4 function in the piriform cortex.

Introduction

LRP4 plays an essential role in forming and maintaining synapses and synaptic plasticity, and excitatory transmission in CNS, and it is expressed in the hippocampus, olfactory bulb, cerebellum, and neocortex[1–3]. Recently, Zhang *et al.* found that genetic deletion of *Lrp4* increased A β plaques formation in Alzheimer's disease (AD) mice and exacerbated the deficits in neurotransmission, cognition, and synchrony between the hippocampus and prefrontal cortex[4]. Astrocytic LRP4 played a crucial role in AD pathology and cognitive function. Sun *et al.* found that astrocytic LRP4 regulates ATP release[1]. Glutamate release of the hippocampal neurons was impaired because of ATP release enhancement in *Lrp4* knockout astrocytes[1]. These research results show that LRP4 plays a significant role, including hippocampal synaptic plasticity, excitatory synaptic transmission, fear regulation, and long-term enhancement[5–7].

LRP4 is a member of the low-density lipoprotein receptor (LDLR) family. As a single transmembrane protein, LRP4 contains a short ICD and a large extracellular domain (ECD) possessing eight LDLA repeats, six EGF repeats, and four β -propeller domains[8–13]. Being a receptor of agrin, LRP4 is critical for MuSK activation, AChR clustering in the neuromuscular junction (NMJ) formation, and maintenance[5, 8–10,

13]. Biochemical studies confirm that LRP4 is a crucial protein in the complex of Agrin and MuSK, and the LRP4 ECD domain is the direct interaction site among them[5, 10, 12–14]. Though *Lrp4* null mutations are perinatally lethal[9, 15, 16], ECD of LRP4 may function as a scavenger for signal modulators or signaling ligands in the extracellular space, consequently maintaining critical signaling thresholds for development[17].

In our study, *Lrp4*^{LacZ/+} mice were utilized to locate the *Lrp4* expressing region. Intriguingly, except the hippocampus in the CNS as previous, *Lrp4* was also supremely expressed in the piriform cortex, which played a critical role in transmitting olfactory signals. To explore whether LRP4 involves the sense of smell, *Lrp4*^{ECD/ECD} mice were investigated. Finding food was prolonged in the buried food-seeking test, implying the olfactory function was impaired in *Lrp4*^{ECD/ECD} mice. Besides, we also found that the body and brain weight of *Lrp4*^{ECD/ECD} mice were lighter than the control mice. A further study utilizing *Lrp4*^{ECD/ECD} mice indicated that piriform cortical neuronal dendritic spine density increased. Compared with the control mice, *Lrp4*^{ECD/ECD} mice exhibited a higher frequency of sEPSC and mEPSC in the piriform cortical neurons. These data indicate that the TMD and ICD of LRP4 are nonnegligible for the LRP4 mediating synaptic plasticity in the piriform cortex.

Materials And Methods

Animals

Lrp4^{LacZ/+} mice were described before; in brief words, β -galactosidase (β -gal) protein cassette, including stop code and a polyadenylation termination signal, was inserted into the downstream of *Lrp4* promoter[1]. *Lrp4*^{ECD/ECD} mice (JAX stock #013157) were described before, which introduced a stop codon just upstream of *Lrp4* TMD[6, 7, 18]. Mice were housed in a room 12-h light/dark cycle, 23-25°C, with ad libitum access to rodent chow diet and water. Experiments involving animals were conducted according to the “guidelines for the care and use of experimental animals” issued by Nanchang University, following the directive 2010/63/EU to protect animals used for scientific purposes. For *in vivo* experiment, surgery was executed with sodium pentobarbital anesthesia (50 mg/kg, ip injection), and all efforts were made to minimize suffering [19]. Male mice were utilized for the experiments, and after terminal experiments, the mice were euthanized by carbon dioxide inhalation.

Western blotting

Western blotting was performed as described previously[20] with minor modifications. In brief, total proteins were extracted by RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM pH 8.0 Tris), supplementary with phenyl methane sulfonyl fluoride (PMSF) and proteinase inhibitor mix before using. After electrophoresis, samples were transferred to the PVDF membrane (Millipore, USA) with transfer buffer (25 mM Tris, 192 mM Glycine, 20%(v/v) Methanol). The membrane was blocked by blocking buffer(5%(m/v) Skim-milk, 20 mM Tris, 150 mM NaCl, 0.1%(v/v) Tween20) for 2 h and was washed 3 times with washing buffer (20 mM Tris, 150 mM NaCl, 0.1%(v/v) Tween20). Anti-LRP4 (Rabbit-

anti-mouse, Lab produced, 1:1000) and anti- α -tubulin (mouse monoclonal, SC-23948, Santa Cruz Biotechnology, 1:1000) primary antibody was added and incubated at 4°C overnight. The HRP-labeled secondary antibody (Goat anti-Mouse IgG 31431, Goat anti-Rabbit IgG, 31466, Thermo Fisher Scientific, 1:2000) was added to incubate at room temperature for 2h and then washed 3 times. LuminataTM Crescendo Western HRP Substrate was added. Immunoreacted bands were captured by an enhanced chemiluminescence system (BIO-RAD, USA).

Quantitative real-time PCR (qPCR)

Total RNA was isolated from mice brain tissues according to the manufacturer's instructions of TRIzol Reagent (Invitrogen), and complementary DNA (cDNA) was synthesized following the manufacturer's protocol of High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368814). The qPCR primer sets as below: *Lrp4* (5'-GTGTGGCAGAACCTTGACAGTC-3' and 5'-TACGGTCTGAGCCATCCATTCC-3'), and *Gapdh* (5'-CATCACTGCCACCCAGAAGACTG-3' and 5'-ATGCCAGTGAGCTTC CCGTTCAG-3'). qPCR was carried out by the StepOnePlus Real-Time PCR system (Applied Biosystems) using the mix. Expression levels of mRNA were normalized to the reference gene *Gapdh* using the Δ CT method.

Open-field test

In behavioral tests, the activity levels of the mice were evaluated at P50. The open field (40 × 40 × 20cm) was used to measure the mice's moving distance over 10 min. The data were recorded using a video camera, and the data were analyzed using the behavior analysis software ANY-maze (Stoelting Co., Wood Dale, IL, USA).

Buried food-seeking test

Mice were food-deprived for 2 d, trained for 2 d, and tested continuously 3 d, with ad libitum access to enough water all the time. Food was randomly placed in the box and was buried under padding for 0.5cm in testing trials. The mice seizing the food with their front paws and biting with mouth were regarded as finding the food. The time was recorded when the mice were placed in the container and found the food.

X-gal staining

X-gal(5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside), the inert chromogenic substrate for β -gal, hydrolyzes X-gal into colorless galactose and 4-chloro-3-brom-indigo, forming an intense blue precipitate. Mice brains were fixed for 8-10 h in 2%(m/v) paraformaldehyde (PFA) at room temperature and then were transferred into 30% sucrose solution at 4°C. The brain slices were added PBS (phosphate-buffered sodium, pH 7.4) in a wet box, washing the slices at room temperature 3 times with PBS. After rinsing for 10 min, adding dye solution, putting the slices at 37°C for 8 h. After the reaction, brain slices were washed 3 times with PBS.

Immunohistochemistry co-staining with X-gal

X-gal-stained brain slices were immersed in blocking solution (10%(v/v) donkey serum, 1%(m/v) calf serum albumin, 0.5%(v/v) Triton X-100 in PBS) for 2 h. Then the slices were rinsed with PBS at room temperature 3 times. Incubating the brain slices with the primary antibody (Rabbit anti-GFAP antibody, Z0334, Dako, 1:1000) at 4°C overnight. The slices were incubated with a secondary antibody (Alexa Fluor® 488 Goat anti-rabbit, A32731TR, Thermo Fisher Scientific, 1:1000) at room temperature for 2 h. Brain slices were washed with PBS 3 times, and then the images were captured by a microscope (Olympus FSX100).

Nuclear fast red counterstaining

Put the X-gal-stained or co-stained brain slices into a vitreous tank containing nuclear fast red staining solution for 5 min. Slides with the slices were put into glass tanks containing 50%, 75%, and 90% ethanol in sequence, each for 4 min. The slides were transferred into 100% ethanol 2 times. Then the slides were put into xylene for 5 min. At last, the slides were sealed with mounted in Hydro mount (National Diagnostics). Images were captured by an inverted fluorescence microscope (Olympus FSX100).

Immunofluorescent staining

The brain slices were rinsed with PBS at room temperature and were immersed with antibody blocking solution (0.5%(v/v) Triton X-100 10%(v/v) donkey serum, 1%(m/v) calf serum albumin, in PBS) at room temperature for 2 h. And then, the slices were rinsed with PBS at room temperature 3 times. The slices were incubated with primary antibody anti-NeuN (mouse monoclonal, MAB377, Merck Millipore, 1:1000) at 4°C overnight. The slices were washed with PBS at room temperature 3 times, each time for 10 min. The secondary antibody (Alexa Fluor® 568 Goat anti-Mouse A-11019, Thermo Fisher Scientific, 1:1000) was added, and then the slices were incubated at room temperature for 2 h in the dark. After washing 3 times with PBS, samples were mounted in a Hydro mount (National Diagnostics).

Golgi staining

Golgi staining was performed following the FD Rapid Golgi Stain™ Kit (FD Neuro Technologies, PK-401, USA). Staining solution D and solution E were mixed with ultra-pure water in a ratio of 1:1:2. Dying at room temperature for 10 min. Slides with the slices were washed in ultra-pure water twice, then put into the plate hole containing 50%, 75%, 90%, and 100% ethanol in sequence, each for 4 min. Dehydration for 3 times, then the slides were put into xylene for 1 h and mounted in Hydro mount (National Diagnostics). Images were captured by an Olympus fluorescence microscope (FSX100), and dendritic spines were counted with image J.

Electrophysiological recording

The electrophysiological recording was performed as previously described[21, 22]. Brain sections of 300µm thickness were cut with a vibratome (Leica, VT1000S) in oxygenated (95% O₂, 5% CO₂) sectioning buffer (120 mM Choline-Cl, 2.5 mM KCl, 0.5 mM CaCl₂, 7 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃,

and 25 mM glucose) at 4°C. Slices were then placed into the oxygenated artificial cerebrospinal fluid (ACSF) (124 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl₂, 2 mM MgSO₄, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 10 mM glucose) at 34°C for 30 min and recovery at room temperature (25 ± 1°C) for more than 1 h before recording. Slices were transferred to a recording chamber under perfusion ACSF (2 ml/min, 32-34°C). Pyramidal neurons in the piriform cortex were visualized with infrared optics using an upright fixed microscope equipped with a 40x water-immersion lens (FN1, Nikon) and CCD monochrome video camera (IR-1000, DAGE-MTI). Patch pipettes (resistance of 3–5 MΩ) were prepared by a horizontal pipette puller (P-1000; Sutter Instruments). For spontaneous excitatory postsynaptic current (sEPSC) recording, pyramidal neurons were held at -70 mV in the presence of 20 μM bicuculline methiodide (BMI), with the pipette solution (125 mM K-gluconate, 5 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 1 mM MgCl₂, 4 mM Mg-ATP, 0.3 mM Na-GTP and 10 mM phosphocreatine, pH 7.35, 290 mOsm). For miniature excitatory postsynaptic current (mEPSC) recording, 20 μM BMI and 1 μM TTX were added into the perfusion ACSF to block GABA receptor-mediated currents and action potentials.

Statistical analysis

Data were statistically analyzed with GraphPad Prism 6.0 software systems, and the values were expressed as means ± standard error (means ± SEM). One-way ANOVA (Fig 1C), two-way ANOVA (Fig 3B), and t-test analyzed the normality distributed data. All tests were two-sided. * p < 0.05, ** p < 0.01.

Results

Lrp4 was highly expressed in the piriform cortex

Lrp4^{LacZ/+} mice were utilized to locate the *Lrp4* expression region by X-gal staining because X-gal is the substrate for β-gal. X-gal staining results showed that *Lrp4* was expressed in many brain regions, such as the piriform cortex, hippocampus, and cerebral cortex (Fig. 1A). To verify the expression of *Lrp4* in the brain regions, as mentioned above, we conducted western blotting experiments. The results also showed that *Lrp4* was highly expressed in the piriform cortex, hippocampus, and cerebral cortex (Fig. 1B). To detect the expression profile of *Lrp4* in the piriform cortex, we also used quantitative fluorescence PCR to quantify the expression of *Lrp4* in the piriform cortex in postnatal wild-type mice (Fig. 1C). *Lrp4* was at a low and stable level in adolescence and began to be highly expressed in adulthood in the piriform cortex, suggesting that *Lrp4* expression was related to the development and may involve the olfactory pathway.

GFAP-positive cells in layer I of the adult piriform cortex have high expression of Lrp4

Taking advantage of *Lrp4*^{LacZ/+} mice, we used immunohistochemical co-staining to identify the location of LRP4. The results indicated that *Lrp4* was mainly expressed in the layer I of the piriform cortex (Fig. 2A). The co-staining assay results suggested that X-gal co-stained with anti-GFAP (astrocyte and neuron stem cell marker) and GFAP-negative cells (Fig. 2B).

Lrp4^{ECD/ECD} mice have a regular structure of the piriform cortex

ECD of LRP4 could maintain critical signaling thresholds for development[17]. Therefore, *Lrp4*^{ECD/ECD} mice could develop much better than *Lrp4* null mice because the latter mice are dead at birth[9, 15, 16]. The body and brain weight of *Lrp4*^{ECD/ECD} mice were lighter than the control mice (Fig. 3A-3D). *Lrp4*^{ECD/ECD} mice showed typical tight-knit morphology (Fig. 3E).

It was unclear whether the morphology of the piriform cortex in *Lrp4*^{ECD/ECD} mice changed. Firstly, immunofluorescent staining was carried out to observe the piriform cortex region of the mice. There was no remarkable difference in the thickness of *Lrp4*^{ECD/ECD} mice compared with the control mice (Fig. 2C, 2D), and the piriform cortical neuron density was similar in the two types of mice (Fig. 2E). We speculated that LRP4 ECD maintained the typical structure of the piriform cortex.

Morphology of neurons in *Lrp4*^{ECD/ECD} mice brain changed

To explore whether the morphology of piriform cortical neurons in *Lrp4*^{ECD/ECD} mice change or not, we used Golgi staining to observe the neuronal dendritic spines in the *Lrp4*^{ECD/ECD} mice, compared with littermate control mice (Fig. 4). There are two different types of neurons in the second layer of the piriform cortex (Fig. 4B). One type is semilunar (SL) cell lacking basal dendrites, and the other one is superficial pyramidal (SP) cell with both apical dendrites and basal dendrites. In the piriform cortex, *Lrp4*^{ECD/ECD} mice showed an increase in the mature spine (mushroom type) and total spine density on SP neurons than the control mice (Fig. 4C), which implies a potential increase in functional synaptic transmission. Except for a bit of rising of the thin type of spine, *Lrp4*^{ECD/ECD} mice exhibited similar spine density on SL neurons than the control mice (Fig. 4D). The results suggested that the TMD and ICD of LRP4 were nonnegligible for the spine maturation and maintaining of neurons in the piriform cortex.

Enhanced excitatory postsynaptic transmission of the piriform cortical neurons in *Lrp4*^{ECD/ECD} mice

In whole-cell patch-clamp configuration, piriform cortex pyramidal neurons were recorded in mice. Compared with the control mice, *Lrp4*^{ECD/ECD} mice exhibit a high frequency of sEPSC (Fig. 5B) and mEPSC (Fig. 5D) of piriform cortical neurons. No change was observed in the amplitude of mEPSC and sEPSC (Fig. 5C, 5E). The results suggested hyperfunction of glutamatergic transmission in *Lrp4*^{ECD/ECD} mice, consistent with increased spine density (Fig. 4).

Impaired olfactory function in *Lrp4*^{ECD/ECD} mice

Both spine density and electrophysiology of piriform cortical neurons were changed in *Lrp4*^{ECD/ECD} mice. In order to explore the function of LRP4 in the olfactory pathway, a buried food-seeking test (Fig. 6) was performed in the two types of mice. Firstly, in the open-field test, we found that the locomotive ability of *Lrp4*^{ECD/ECD} mice was not affected (Fig. 6A), without change in total travel distance (Fig. 6B) and average speed (Fig. 6C). Then, the buried food-seeking test was conducted. The mice were food-deprived for 2 d before training 3 d, and testing was started following training (Fig. 6D). Mice were free to access enough

water all the time. *Lrp4*^{ECD/ECD} mice spent more time finding the buried pellet chow in the test trials than control mice (Fig. 6E, 6F), suggesting that olfactory function may be impaired.

In conclusion, we reported that *Lrp4* was highly expressed in the piriform cortex, and *Lrp4*^{ECD/ECD} mice exhibited enhanced spine density in the piriform cortex, hyperfunction of the excitatory postsynaptic current in the piriform cortical neurons, and impaired olfactory function. These results implied that LRP4 might maintain the olfactory signal transmission pathway in the piriform cortex. Although LRP4 ECD seems to play a prominent role in piriform cortical development, both the TMD and the ICD of LRP4 may also be nonnegligible to the piriform cortical development and function.

Discussion

The piriform cortex with synaptic plasticity involves the encoding of olfactory information, associative memory, and sensory processing[23–25]. The piriform cortex containing the densely packed cell bodies exhibits highly structural plasticity, such as dendritic remodeling, spine genesis, and synaptic reorganization[26]. As a higher olfactory center receiving direct input from the olfactory bulb, the piriform cortex comprises the largest area of the olfactory cortex, which is vital for olfactory function processing its connection with all of the entorhinal cortical domains[27, 28]. Like the hippocampus, the piriform cortex with a three-layered cortical structure belongs to an evolutionally conserved paleocortex, phylogenetically ancient structure[26, 29].

Layer II of the piriform cortex is developed prenatally and is devoid of postnatally proliferative capacity. However, in layer II, it is often found that a subpopulation of immature neurons is characterized by the expression of doublecortin (DCX) and polysialylated neural cell adhesion molecule (PSA-NCAM)[26, 30–32]. Therefore, it is believed that the piriform cortex has a slight potential for adult neurogenesis[32–34]. Therefore, all GFAP-positive cells in the piriform cortex may not be astrocytes because PSA-NCAM positive neurons may also express GFAP[35].

The olfactory system's essential components include sensory neurons located on the olfactory mucosa, olfactory bulb, and olfactory cortex[36]. The most critical part of the olfactory cortex is the piriform cortex[37]. The olfactory cortex mainly integrates the olfactory signals, forms olfactory memories, and integrates specific olfactory signals with sensory information, such as color, taste, shape, and spatial location[38]. Much complex odor signal analysis and integration rely on higher-level central structures, such as the piriform cortex[39]. The olfactory signal analysis in the piriform cortex may be related to specific olfactory memory and may involve olfactory sensitization and passivation[40]. It was deeply understood the recognition mechanism of odor molecules in the past ten years, the projection of olfactory mucosa to the olfactory bulb, and olfactory bulbs' spatial orientation to specific olfactory signals[41]. However, it is still unclear how to complete the integration and modulation of olfactory signals by the high cortex. Here we demonstrated that *Lrp4* was highly expressed in the olfactory pathway, especially in the piriform cortex. In this area, we found that *Lrp4* was also highly expressed in piriform cortical GFAP-

positive cells except for GFAP-negative cells, and LRP4 may regulate the transmission of olfactory signals.

In the buried food-seeking test, results showed that the time to find food was significantly longer in *Lrp4*^{ECD/ECD} mice comparing with the control mice. The test results suggested that *Lrp4*^{ECD/ECD} mice may have olfactory dysregulation. At the same time, *Lrp4*^{ECD/ECD} mice also showed reduced body weight and brain weight. We also found that *Lrp4*^{ECD/ECD} mice showed typical tight-knit morphology in the previous report[6]. This kind of limbs clinging to the tail after the tail's suspension also appeared in the neurological disease model mice, suggesting that the brain's neurological function in the *Lrp4*^{ECD/ECD} mice may be impaired.

Ariana Kariminejad *et al.* had reported one case that a patient identified the novel homozygous mutation c.289G > T in *Lrp4* exon 3. This nucleotide exchange leads to a premature stop codon at amino acid 97 (p.E97X) at the beginning of the large extracellular domain. The patient had mixed-type hearing loss, vertebral anomalies, and renal hypoplasia[42]. Another study reported a novel splice variant in *Lrp4* (c.316t1G > A), and the missense variant adds 29 non-native amino acids with premature stop-codon, which causes the *Lrp4* encoding to terminate prematurely. The patient had short feet, frontal bossing, and other symptoms[43]. However, these findings suggest that the ECD of LRP4 plays a vital role in limb development, kidney development, and brain development; they may also imply that both the TMD and ICD of LRP4 may be nonnegligible in the development.

Wnt signaling regulates brain development and synapse maturation[44]. LRP4 has an antagonistic function on LRP6-mediated Wnt/ β -catenin activation. Eva Ramos-Fernández *et al.* reported that Wnt-7a stimulates dendritic spine formation in the hippocampus through glycogen synthase kinase-3 β (GSK-3 β) inhibition, triggering β -catenin/T cell factor/lymphoid enhancer factor (TCF/LEF)-dependent gene transcription and promoting postsynaptic density-95 (PSD-95) protein expression, infecting the synapse plasticity[45]. Our results showed that the dendritic spine density in the piriform cortex of *Lrp4*^{ECD/ECD} mice significantly increased. Furthermore, we hypothesized that the ECD of LRP4 might promote dendritic spine formation. SP dendritic spines in the piriform cortex increased significantly, while the density of dendritic spines of SL cells did not change. The neuronal dendritic spines are related to neuronal physiological functions, and they are likely to participate in different neural circuits. On the other hand, neuronal dendritic spine density also affects the same afferent arrival of neurons. Two types of particular neurons in the piriform cortex may be involved in different neural circuits.

This study found enhanced excitatory synaptic transmission of the piriform cortical neurons in *Lrp4*^{ECD/ECD} mice. Pohlkamp *et al.* examined synaptic function in the *Lrp4*^{ECD/ECD} mice by recording theta-burst long-term potentiation (LTP) in CA3-CA1 Schaeffer collaterals[7]. CA3-CA1 projections are a classic model for measuring and understanding synaptic plasticity. There was a substantial deficit in late-phase LTP in *Lrp4*^{ECD/ECD} mice compared with the control mice[7], and the loss of the ICD and TMD may severely impair the LRP4 function. Sun *et al.* found that *Lrp4* knockout astrocytes suppress glutamatergic transmission in the CNS[1]. The frequency of sEPSC and mEPSC in hippocampal CA1 pyramidal neurons

was reduced, and synaptic plasticity was impaired in *Lrp4* knockout mice[1, 6]. In our research, sEPSC and mEPSC frequency in piriform cortical neurons were enhanced in *Lrp4*^{ECD/ECD} mice. Although consistent with the increase of the piriform cortex's dendritic spine density, it was different from other reports[1, 6]. There are two possible reasons for the inconsistencies. One is that different brain areas were investigated, and the other reason is that the mechanism involving in the dysfunction may be caused by *Lrp4* expression in GFAP-negative cells.

Conclusions

In conclusion, our results showed that *Lrp4* was highly expressed in the piriform cortex, especially in layer Ia, and LRP4 localized in both GFAP-positive and GFAP-negative cells. In *Lrp4*^{ECD/ECD} mice, piriform cortical neuronal dendritic spines density increased, and the olfactory function was impaired. The results suggest that the TMD and ICD of LRP4 are nonnegligible for the LRP4 function in the piriform cortex while maintaining the olfactory signal transmission pathway. The molecular regulating mechanism needs further exploration.

Abbreviations

AD: Alzheimer's disease; β -gal: β -galactosidase; CNS: Central nervous system; DCX: Doublecortin; ECD: Extracellular domain; ICD: Intracellular domain; LRP4: Low-density lipoprotein receptor-related protein 4(LRP4); mEPSC: miniature excitatory postsynaptic current; NMJ: Neuromuscular junction; PBS: Phosphate-buffered sodium; PFA: Paraformaldehyde; PSA-NCAM: Polysialylated neural cell adhesion molecule; sEPSC: spontaneous excitatory postsynaptic current; TMD: Transmembrane domain; X-gal: 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside.

Declarations

Acknowledgments

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Author Contributions

MY and SW initiated and designed the study. MY, HJ, MX, and HZ performed western blotting, behavior test, immunostaining, and Golgi staining. DL, JC, ZL, and PC performed the electrophysiological recordings and analysis. DR, EF, XL, and SZ analyzed data. MY and SW wrote the manuscript with input from all coauthors. All authors have read and agreed to the published version of the manuscript.

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Availability of Data and Materials

The datasets used or analyzed in our study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

All experiments involving animals were conducted according to the “guidelines for the care and use of experimental animals” issued by Nanchang University. The Committee on the Ethics of Animal Experiments of the University of Nanchang approved the protocol.

Consent for Publication

Not applicable

Competing Interests

The authors have no conflicts of interest to declare.

References

1. Sun, X.D., L. Li, F. Liu, Z.H. Huang, J.C. Bean, H.F. Jiao, et al. Lrp4 in astrocytes modulates glutamatergic transmission. *Nat Neurosci.* 2016; 19(8):1010-8.
2. Lein, E.S., M.J. Hawrylycz, N. Ao, M. Ayres, A. Bensinger, A. Bernard, et al. Genome-wide atlas of gene expression in the adult mouse brain. *Nature.* 2007; 445(7124):168-76.
3. Tian, Q.B., T. Suzuki, T. Yamauchi, H. Sakagami, Y. Yoshimura, S. Miyazawa, et al. Interaction of LDL receptor-related protein 4 (LRP4) with postsynaptic scaffold proteins via its C-terminal PDZ domain-binding motif, and its regulation by Ca/calmodulin-dependent protein kinase II. *Eur J Neurosci.* 2006; 23(11):2864-76.
4. Zhang, H., W. Chen, Z. Tan, L. Zhang, Z. Dong, W. Cui, et al. A Role of Low-Density Lipoprotein Receptor-Related Protein 4 (LRP4) in Astrocytic Abeta Clearance. *J Neurosci.* 2020; 40(28):5347-5361.
5. Gomez, A.M. and S.J. Burden. The extracellular region of Lrp4 is sufficient to mediate neuromuscular synapse formation. *Dev Dyn.* 2011; 240(12):2626-33.
6. Gomez, A.M., R.C. Froemke and S.J. Burden. Synaptic plasticity and cognitive function are disrupted in the absence of Lrp4. *Elife.* 2014; 3:e04287.
7. Pohlkamp, T., M. Durakoglugil, C. Lane-Donovan, X. Xian, E.B. Johnson, R.E. Hammer, et al. Lrp4 domains differentially regulate limb/brain development and synaptic plasticity. *PLoS One.* 2015; 10(2):e0116701.

8. Shen, C., W.C. Xiong and L. Mei. LRP4 in neuromuscular junction and bone development and diseases. *Bone*. 2015; 80:101-108.
9. Weatherbee, S.D., K.V. Anderson and L.A. Niswander. LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. *Development*. 2006; 133(24):4993-5000.
10. Zhang, B., S. Luo, Q. Wang, T. Suzuki, W.C. Xiong and L. Mei. LRP4 serves as a coreceptor of agrin. *Neuron*. 2008; 60(2):285-97.
11. Kim, N. and S.J. Burden. MuSK controls where motor axons grow and form synapses. *Nat Neurosci*. 2008; 11(1):19-27.
12. Wu, H., Y. Lu, C. Shen, N. Patel, L. Gan, W.C. Xiong, et al. Distinct roles of muscle and motoneuron LRP4 in neuromuscular junction formation. *Neuron*. 2012; 75(1):94-107.
13. Kim, N., A.L. Stiegler, T.O. Cameron, P.T. Hallock, A.M. Gomez, J.H. Huang, et al. Lrp4 is a receptor for Agrin and forms a complex with MuSK. *Cell*. 2008; 135(2):334-42.
14. Wu, H., W.C. Xiong and L. Mei. To build a synapse: signaling pathways in neuromuscular junction assembly. *Development*. 2010; 137(7):1017-33.
15. Barik, A., Y. Lu, A. Sathyamurthy, A. Bowman, C. Shen, L. Li, et al. LRP4 is critical for neuromuscular junction maintenance. *J Neurosci*. 2014; 34(42):13892-905.
16. Tanahashi, H., Q.B. Tian, Y. Hara, H. Sakagami, S. Endo and T. Suzuki. Polyhydramnios in Lrp4 knockout mice with bilateral kidney agenesis: Defects in the pathways of amniotic fluid clearance. *Sci Rep*. 2016; 6:20241.
17. Dietrich, M.F., L. van der Weyden, H.M. Prosser, A. Bradley, J. Herz and D.J. Adams. Ectodomains of the LDL receptor-related proteins LRP1b and LRP4 have anchorage independent functions in vivo. *PLoS One*. 2010; 5(4):e9960.
18. Johnson, E.B., R.E. Hammer and J. Herz. Abnormal development of the apical ectodermal ridge and polysyndactyly in Megf7-deficient mice. *Hum Mol Genet*. 2005; 14(22):3523-38.
19. Yan, M., A. Guo, P. Chen, H. Jing, D. Ren, Y. Zhong, et al. LRP4 LDLalpha repeats of astrocyte enhance dendrite arborization of the neuron. *Mol Brain*. 2020; 13(1):166.
20. Yu, Z., D. Lin, Y. Zhong, B. Luo, S. Liu, E. Fei, et al. Transmembrane protein 108 involves in adult neurogenesis in the hippocampal dentate gyrus. *Cell Biosci*. 2019; 9:9.
21. Chen, W.B., J. Chen, Z.Y. Liu, B. Luo, T. Zhou and E.K. Fei. Metformin Enhances Excitatory Synaptic Transmission onto Hippocampal CA1 Pyramidal Neurons. *Brain Sci*. 2020; 10(10).
22. Zhao, K., C. Shen, L. Li, H. Wu, G. Xing, Z. Dong, et al. Sarcoglycan Alpha Mitigates Neuromuscular Junction Decline in Aged Mice by Stabilizing LRP4. *J Neurosci*. 2018; 38(41):8860-8873.
23. Hori, N., T. Kadota and N. Akaike. Functional changes in piriform cortex pyramidal neurons in the chronic methamphetamine-treated rat. *Gen Physiol Biophys*. 2015; 34(1):5-12.
24. Strauch, C. and D. Manahan-Vaughan. In the Piriform Cortex, the Primary Impetus for Information Encoding through Synaptic Plasticity Is Provided by Descending Rather than Ascending Olfactory Inputs. *Cereb Cortex*. 2018; 28(2):764-776.

25. Loureiro, M., R. Achargui, J. Flakowski, R. Van Zessen, T. Stefanelli, V. Pascoli, et al. Social transmission of food safety depends on synaptic plasticity in the prefrontal cortex. *Science*. 2019; 364(6444):991-995.
26. Vadodaria, K.C., S.U. Yanpallewar, M. Vadhvani, D. Toshniwal, L.C. Liles, K.S. Rommelfanger, et al. Noradrenergic regulation of plasticity marker expression in the adult rodent piriform cortex. *Neurosci Lett*. 2017; 644:76-82.
27. Wang, D., P. Liu, X. Mao, Z. Zhou, T. Cao, J. Xu, et al. Task-Demand-Dependent Neural Representation of Odor Information in the Olfactory Bulb and Posterior Piriform Cortex. *J Neurosci*. 2019; 39(50):10002-10018.
28. Strauch, C. and D. Manahan-Vaughan. Orchestration of Hippocampal Information Encoding by the Piriform Cortex. *Cereb Cortex*. 2020; 30(1):135-147.
29. Katori, K., H. Manabe, A. Nakashima, E. Dunfu, T. Sasaki, Y. Ikegaya, et al. Sharp wave-associated activity patterns of cortical neurons in the mouse piriform cortex. *Eur J Neurosci*. 2018; 48(10):3246-3254.
30. Gomez-Climent, M.A., E. Castillo-Gomez, E. Varea, R. Guirado, J.M. Blasco-Ibanez, C. Crespo, et al. A population of prenatally generated cells in the rat paleocortex maintains an immature neuronal phenotype into adulthood. *Cereb Cortex*. 2008; 18(10):2229-40.
31. Rubio, A., M. Belles, G. Belenguer, S. Vidueira, I. Farinas and J. Nacher. Characterization and isolation of immature neurons of the adult mouse piriform cortex. *Dev Neurobiol*. 2016; 76(7):748-63.
32. Rotheneichner, P., M. Belles, B. Benedetti, R. Konig, D. Dannehl, C. Kreutzer, et al. Cellular Plasticity in the Adult Murine Piriform Cortex: Continuous Maturation of Dormant Precursors Into Excitatory Neurons. *Cereb Cortex*. 2018; 28(7):2610-2621.
33. Chen, J., C.H. Kwon, L. Lin, Y. Li and L.F. Parada. Inducible site-specific recombination in neural stem/progenitor cells. *Genesis*. 2009; 47(2):122-31.
34. Sakurai, M., H. Suzuki, N. Tomita, Y. Sunden, A. Shimada, H. Miyata, et al. Enhanced neurogenesis and possible synaptic reorganization in the piriform cortex of adult rat following kainic acid-induced status epilepticus. *Neuropathology*. 2018; 38(2):135-143.
35. Nait-Oumesmar, B., N. Picard-Riera, C. Kerninon, L. Decker, D. Seilhean, G.U. Hoglinger, et al. Activation of the subventricular zone in multiple sclerosis: evidence for early glial progenitors. *Proc Natl Acad Sci U S A*. 2007; 104(11):4694-9.
36. Mori, K. and H. Sakano. How is the olfactory map formed and interpreted in the mammalian brain? *Annu Rev Neurosci*. 2011; 34:467-99.
37. Wang, F., A. Nemes, M. Mendelsohn and R. Axel. Odorant receptors govern the formation of a precise topographic map. *Cell*. 1998; 93(1):47-60.
38. Boyd, A.M., J.F. Sturgill, C. Poo and J.S. Isaacson. Cortical feedback control of olfactory bulb circuits. *Neuron*. 2012; 76(6):1161-74.
39. Bekkers, J.M. and N. Suzuki. Neurons and circuits for odor processing in the piriform cortex. *Trends Neurosci*. 2013; 36(7):429-38.

40. Gottfried, J.A. and C. Zelano. The value of identity: olfactory notes on orbitofrontal cortex function. *Ann N Y Acad Sci.* 2011; 1239:138-48.
41. Leinwand, S.G. and S.H. Chalasani. Olfactory networks: from sensation to perception. *Curr Opin Genet Dev.* 2011; 21(6):806-11.
42. Kariminejad, A., B. Stollfuss, Y. Li, N. Bogershausen, K. Boss, R.C. Hennekam, et al. Severe Cenani-Lenz syndrome caused by loss of LRP4 function. *Am J Med Genet A.* 2013; 161A(6):1475-9.
43. Afzal, M., Q. Zaman, U. Kornak, S. Mundlos, S. Malik and R. Flottmann. Novel splice mutation in LRP4 causes severe type of Cenani-Lenz syndactyly syndrome with oro-facial and skeletal symptoms. *Eur J Med Genet.* 2017; 60(8):421-425.
44. Li, Y., B. Pawlik, N. Elcioglu, M. Aglan, H. Kayserili, G. Yigit, et al. LRP4 mutations alter Wnt/beta-catenin signaling and cause limb and kidney malformations in Cenani-Lenz syndrome. *Am J Hum Genet.* 2010; 86(5):696-706.
45. Ramos-Fernández, E., C. Tapia-Rojas, V.T. Ramírez and N.C. Inestrosa. Wnt-7a Stimulates Dendritic Spine Morphogenesis and PSD-95 Expression Through Canonical Signaling. *Molecular Neurobiology.* 2018; 56(3):1870-1882.

Figures

Figure 1

Highly expression of *Lrp4* in piriform cortex (A) X-gal staining of *Lrp4*^{+/Lac} mice brain slice. PIR, piriform cortex; CTX, cerebral cortex; HIP, hippocampus; TH, thalamus; *Lrp4*^{+/Lac} mice, n = 5. (B) *Lrp4* expression in wild-type adult mice brain was confirmed by Western blotting (Wild type mice, n = 6). (C) mRNA expression of *Lrp4* in piriform cortex in postnatal wild type mice; *Lrp4* expression values were calculated relative to *Gapdh* by using the $2^{-\Delta CT}$ methods, and the P18 group was set to a value of 1. (Wild type mice per group, n ≥ 5; RNA pool was made for qPCR in each group; Values were means ± SEM, one-way ANOVA with multiple comparisons, compared other group with P18 group. n.s. no significant, * P < 0.05, *** P < 0.001)

Figure 2

Lrp4 was expressed in GFAP-positive cells in the layer I of the piriform cortex (A) *Lrp4*^{+/Lac} mice brain slice was co-stained with X-gal and anti-GFAP. GFAP positive cells were mainly distributed in the second superficial area of the piriform cortex, and X-gal staining positive cells were primarily located in layer I of the piriform cortex; nuclei were counterstained fast red. (B) Layer I of the piriform cortex. X-gal co-stained with GFAP-positive cells (red arrow) and GFAP-negative cells (white arrow; *Lrp4*^{+/Lac} mice, n = 5). (C) Representative images of neuron staining (anti-NeuN) of the two types of mice brain. (D) The thickness of the piriform cortex in layers I, II, and III did not change in *Lrp4*^{ECD/ECD} mice. (E) The neuronal density was not different in the three layers (mice per type, n = 7; scale bar = 50 μm; values were means ± SEM).

Figure 3

Lower body and brain weight of *Lrp4^{ECD/ECD}* mice (A) Representative images of one-month-old *Lrp4^{ECD/ECD}* mice comparing with the control mice. *Lrp4^{ECD/ECD}* mice were smaller than control mice. (B) *Lrp4^{ECD/ECD}* mice's body weight was significantly lower, compared to the control mice (control mice, $n = 16$; *Lrp4^{ECD/ECD}* mice, $n = 12$). (C) Representative brain images of *Lrp4^{ECD/ECD}* mice and the control mice. (D) *Lrp4^{ECD/ECD}* adult mice's brain weight was lower, compared with the control mice

(control mice, n = 12; Lrp4ECD/ECD mice, n = 12). (E) Lrp4ECD/ECD mice showed typical tight-knit morphology. (Values were means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$)

Figure 4

Increased spine density of superficial pyramidal neuron in Lrp4ECD/ECD mice (A) Representative Golgi staining of piriform cortical neurons in mice. (B) The representative spine on superficial pyramidal neuron (SP, arrowhead in panel A) and semilunar neuron (SL, arrow in panel A) in mice. (C) Quantification of spine density on SP neurons. Mature and total spine density of SP neurons in Lrp4ECD/ECD mice were more than that in the control mice. (D) Quantification of spine density on SL neurons. Lrp4ECD/ECD mice exhibited a similar spine density of SL neurons than the control mice, except for a little increase in the thin type of spine. (Control mice, n = 6; Lrp4ECD/ECD mice, n = 6. Values were means $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$)

Figure 5

Enhanced excitatory synaptic transmission of the piriform cortical neurons in *Lrp4*^{ECD/ECD} mice (A) Representative recording trace of sEPSC and mEPSC in the piriform cortex. (B) Frequency of spontaneous excitatory postsynaptic current (sEPSC) of the piriform cortical neurons in *Lrp4*^{ECD/ECD} mice increased. (C) The amplitude of the piriform cortical neuronal sEPSC in *Lrp4*^{ECD/ECD} mice was similar to that in the control mice (control mice, n = 4, neurons, n = 18; *Lrp4*^{ECD/ECD} mice, n = 3, neurons, n = 15). (D)

Frequency of miniature excitatory postsynaptic current (mEPSC) of the piriform cortical neurons in *Lrp4*^{ECD/ECD} mice elevated. (E) The amplitude of the piriform cortical neuronal mEPSC in *Lrp4*^{ECD/ECD} mice was no different from the control mice (control mice, n = 5, neurons, n = 16; *Lrp4*^{ECD/ECD} mice, n = 6, neurons, n = 18). (Values were means $\pm$ SEM. n.s., no significant, ** P < 0.01)

Figure 6

The impaired olfactory function of *Lrp4*^{ECD/ECD} mice (A) Representative trace of mice in the open-field test. (B-C) There was no difference between *Lrp4*^{ECD/ECD} mice and the control mice in the total travel distance(B) and average speed(C) (control mice, n = 10; *Lrp4*^{ECD/ECD} mice, n = 10). (D) Schematic diagram of buried food-seeking test. Food deprived for 2 d before training, training 2 d, and testing 3 d; mice were free to access enough water all the time; food was visible in training trials and buried in testing trials. (E) *Lrp4*^{ECD/ECD} mice spent more time finding the buried pellet chow than control mice. (F) The latency to find pellet chow in the testing days. (Control mice, n = 6; *Lrp4*^{ECD/ECD} mice, n = 5. Values were means $\pm$ SEM. * P < 0.05, ** P < 0.01)