**LRP4 is required for the olfactory association task 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 potentiation (LTP).

**Results:** In this study, we found that *Lrp4* was highly expressed in layer II of the piriform cortex. Both body weight and brain weight decreased in *Lrp4ECD/ECD* mice without TMD (Transmembrane domain) and ICD (intracellular domain) of LRP4. However, in the piriform cortical neurons of *Lrp4ECD/ECD* mice, the spine density increased, and the frequency of both mEPSC (miniature excitatory postsynaptic current) and sEPSC (spontaneous excitatory postsynaptic current) was enhanced. Intriguingly, finding food in the buried food-seeking test was prolonged in both *Lrp4ECD/ECD* mice and *Lrp4 cKO* (conditional knockout of Lrp4 in the piriform cortex) mice.

**Conclusions:** This study indicated that the full length of LRP4 in the piriform cortex was necessary for maintaining synaptic plasticity and the integrity of olfactory function.

**Keywords:** LRP4, Piriform cortex, Olfactory function, Golgi staining, Spine density

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**Introduction**

LRP4 plays an essential role in synaptic plasticity and excitatory transmission in the 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 Aβ 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]. Recent research shows that LRP4 plays a significant role, including hippocampal synaptic plasticity, excitatory synaptic transmission, fear regulation, and LTP [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 NMJ maintenance [5, 8–10, 13]. Biochemical studies confirm that LRP4 is a crucial protein in the complex with Agrin and MuSK, and importantly, ECD of LRP4 is the direct interaction site among them [5, 10, 12–14]. Though *Lrp4* null is 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].

This research found that Lrp4 was supremely expressed in layer II of the piriform cortex, besides the hippocampus in the CNS in the previous report [1]. To explore whether the full length of LRP4 in the piriform cortex involves the sense of smell, we investigated Lrp4EC/EC mice and Lrp4 cKO mice. The body and brain weight of Lrp4EC/EC mice decreased. Intriguingly, finding food in the buried food-seeking test was prolonged in both types of mice, implying the olfactory function was impaired. In the piriform cortical neurons of Lrp4EC/EC mice, the dendritic spine density increased, and the frequency of both sEPSC and mEPSC was enhanced. These data indicated that the full length of LRP4 in the piriform cortex was necessary to maintain synaptic plasticity and the integrity of olfactory function.

Materials and methods

Animals
Lrp4LacZ/+ 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]. Lrp4EC/EC mice (JAX stock #013157) were described before, which introduced a stop codon just upstream of TMD of Lrp4 [6, 7, 18]. CAG-Cas9 mice (C57BL/6-Gt(ROSA)26Sor tm1(CAG-Cas9)Smoledo, NM-KI-00120, Shanghai Model Organisms Center) was a gift from Dr. Dongmin Yin (East China Normal University). Mice were housed in a 12-h light/dark cycle room, 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), anti-GAPDH (Mouse monoclonal, ab8245, Abcam, 1:2000) or 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 2 h and then washed three 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′-GTGTTGCGAACCCTTGACAGTC-3′ and 5′-TACGGTCTGACCATCCATTCC-3′), and Gapdh (5′-CATCAGGGCCCACTACTGCTG-3′ and 5′-ATGCGAGTCAGCTTCCGGTTC-3′). qPCR was carried out by the Step One Plus Real-Time PCR system (Applied Biosystems) using the mix. mRNA expression levels were normalized to the reference gene Gapdh using the ΔCT method [21, 22].

Open-field test
In behavioral tests, the activity levels of the mice were evaluated at P50. The open field (40 × 40 × 20 cm) measured the mice’s moving distance over 10 min. A video camera recorded the data, 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 days, trained for 2 days, and tested continuously 3 days, with ad libitum access to enough water. Food was randomly placed in the box and was buried under padding for 0.5 cm in testing trials. The mice seizing the food with their front paws and biting 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-galactopyranoside), 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 with PBS. After rinsing for 10 min, adding dye solution, put the slices at 37 °C for 8 h. After the reaction, brain slices were washed three 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. 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) for 2 h. Brain slices were washed with PBS, 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 staining solution for 5 min. The slices were put into glass tanks containing 50%, 75%, and 90% ethanol in sequence, each for 4 min. Then the slices were transferred into 100% ethanol two times. Then the slides were put into xylene for 5 min. At last, the slides were sealed with Hydro mount (National Diagnostics). Images were captured by an inverted fluorescence microscope (Olympus FSX100).

Virus packaging and stereotactic injection (Lrp4 cKO mice)
The AAV-PHP capsids engineering was performed as previously described [23, 24]. pAAV-gRNA-CMV-mCherry contains the rAAV genome of interest, pUCmini-iCAP-PHP encodes the viral capsid and replication proteins, and pHelper encodes adenoviral proteins necessary for replication. Using the triple-transfection (pAAV-gRNA-CMV-mCherry; pUCmini-iCAP-PHP; pHelper = 5:2:1) with polyethylenimine (PEI), a single-stranded rAAV genome is packaged into an AAV-PHP capsid in HEK293 cells. In brief, Lrp4 gRNA (GTACCTGTATCCCGCCCAGTG) was inserted into pAAV-gRNA-CMV-mCherry to produce AAV-Lrp4 gRNA, and the control virus AAV-vector containing pAAV-gRNA-CMV-mCherry vector. Five days after triple-transfection, the AAV (AAV-Lrp4 gRNA or AAV-vector) was performed with harvest, purification, and titration test. CAG-Cas9 mice (6 weeks) were then anesthetized with 1% pentobarbital sodium (100 mg/kg, i.p.). Select the middle position of the brain, cut off the mouse’s topcoat, disinfect with alcohol, cut the scalp longitudinally, and separate the skin with hemostatic forceps, after the peristem was removed, the three-dimensional coordinates (X = ± 3.75; Y, bregma = −1.2; Z, depth = −5.48) were read by brain locator, and 0.2 μl AAV suspension liquid was injected into the piriform cortex. After 21 days, the mice were sacrificed, and their brains were harvested quickly and stored at −20 °C for immunofluorescent staining.

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. Then, the slices were rinsed with PBS at room temperature. 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 three times 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 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 NeuroTechnologies, PK-401, USA). Staining solution D and solution E were mixed with ultrapure water in a ratio of 1:1:2. Dying at room temperature for 10 min. Slides with the slices were washed in ultrapure water twice, then put into the plate hole containing 50%, 75%, 90%, and 100% ethanol in sequence, each for 4 min. After dehydration 3 times, 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 [25, 26]. Brain sections of 300 μm 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
mediated currents and action potentials. For mEPSC recording, 20 µM BMI and 1 µM TTX were added into the perfusion ACSF to block GABA receptor.

0.3 mM Na-GTP and 10 mM phosphocreatine, pH 7.35). Pyramidal neurons in the piriform cortex were visualized with infrared optics using an upright fixed microscope equipped with a 40× 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 sEPSC recording, pyramidal neurons were held at −70 mV in the present of 20 µM bicuculline methiodide (BMI), with the pipette solution (125 mM K-glucorhionate, 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). For mEPSC recording, 20 µM BMI and 1 µM TTX were added into the perfusion ASCF 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 S1B, 5E, 6 J), and t-test (Other figures) analyzed the normality distributed data. All tests were two-sided. * p < 0.05, ** p < 0.01, *** p < 0.001.

Results
Expression of Lrp4 in the piriform cortex
Lrp4LacZ/+ mice were utilized to locate the Lrp4 expression region by X-gal staining because X-gal is the substrate for β-gal. And 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 LRP4 protein relative expression in the brain regions, we conducted western blotting experiments (Fig. 1B). LRP4 relative level in the hippocampus is high, and LRP4 relative level is not different between the piriform cortex and in the cerebral cortex (Fig. 1C). 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. 1D). Lrp4 was at a low and stable level in adolescence and became highly expressed in adulthood in the piriform cortex, suggesting that Lrp4 expression was related to the development.

High expression of Lrp4 in layer II of the adult piriform cortex
Taking advantage of Lrp4LacZ/+ mice, we used immunohistochemical co-staining to identify the location of LRP4. The result indicated that LRP4 was mainly expressed in layer II 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). In LacZ positive cell population, the percent of GFAP positive (GFAP+ LacZ−) cells was higher than the percent of GFAP negative (GFAP− LacZ+) cells (Fig. 2C).

Normal structure of the piriform cortex in Lrp4ECD/ECD mice
ECD of LRP4 could maintain critical signaling thresholds for development [17]. Therefore, Lrp4ECD/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 Lrp4ECD/ECD mice were lighter than the control mice (Additional file 1: Fig S1A–D). Lrp4ECD/ECD mice showed typical tight-knit morphology (Additional file 1: Fig S1E).

It was unclear whether the morphology of the piriform cortex in Lrp4ECD/ECD mice changed. Immunofluorescent staining was carried out to observe the piriform cortex of the mice. There was no remarkable difference in the thickness of Lrp4ECD/ECD mice compared with the control mice (Fig. 2D, E), and the piriform cortical neuron density was similar in the two types of mice (Fig. 2F). We speculated that the ECD of LR4 maintained the typical structure of the piriform cortex.

Increase of neuronal mature spine density in Lrp4ECD/ECD mice
To explore whether the morphology of piriform cortical neurons in Lrp4ECD/ECD mice changed or not, we used Golgi staining to observe the neuronal dendritic spines in the Lrp4ECD/ECD mice, compared with littermate control mice (Fig. 3). There were two different neurons in the second layer of the piriform cortex (Fig. 3B). One type was semilunar (SL) cell lacking basal dendrites, and the other was superficial pyramidal (SP) cell with both apical dendrites and basal dendrites. The mature spines (mushroom type) and total spine density on SP neurons were increased in the piriform cortex of Lrp4ECD/ECD mice (Fig. 3C), which implied a potential increase in functional synaptic transmission. Except for

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 40× 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 sEPSC recording, pyramidal neurons were held at −70 mV in the present of 20 µM bicuculline methiodide (BMI), with the pipette solution (125 mM K-glucorhionate, 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). For mEPSC recording, 20 µM BMI and 1 µM TTX were added into the perfusion ASCF to block GABA receptor-mediated currents and action potentials.

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a little rising of the thin type of spine in SL neurons, other types of spine density in Lrp4ECD/ECD mice exhibited similar to the control mice (Fig. 3D).

Enhanced EPSC of the piriform cortical neurons in Lrp4ECD/ECD mice

In whole-cell patch-clamp configuration, piriform cortex pyramidal neurons were recorded in mice (Fig. 4). Compared with the control mice, Lrp4ECD/ECD mice exhibit a high frequency of sEPSC (Fig. 4B) and mEPSC (Fig. 4D) in the piriform cortical neurons. No change was observed in the amplitude of mEPSC (Fig. 4C) and sEPSC (Fig. 4E). The results suggested hyperfunction of glutamatergic transmission in Lrp4ECD/ECD mice, consistent with increased spine density (Fig. 3).

Impaired olfactory function in both Lrp4ECD/ECD mice and Lrp4 cKO mice

Both spine density and electrophysiology of piriform cortical neurons were changed in Lrp4ECD/ECD mice. In order to explore the function of LRP4 in the olfactory pathway, a buried food-seeking test (Fig. 5) was performed. Firstly,
in the open-field test, we found that the locomotive ability of Lrp4<sup>ECD/ECD</sup> mice was not affected (Fig. 5A), the total travel distance (Fig. 5B) and average speed (Fig. 5C) were not changed. Then, the buried food-seeking test was conducted. The mice were food-deprived for 2 d before training 3 d, and testing was started (Fig. 5D). Mice were free to access enough water all the time. Lrp4<sup>ECD/ECD</sup> mice spent more time finding the buried pellet chow in the test trials than control mice (Fig. 5E, F), suggesting that olfactory function may be impaired.

Besides, Lrp4<sup>cKO</sup> mice, via injecting AAV-Lrp4<sup>gRNA</sup> into the piriform cortex of CAG-Cas9 mice, were engaged in evaluating the olfactory function (Fig. 6). Immunofluorescence staining results showed that Layer II cells infected with AVV appeared red in the piriform cortex region (Fig. 6E, F). The layer II cells of the piriform cortex had higher virus infection compared with layer I and layer III (Fig. 6G). Lrp4 was effectively knockedout in the piriform cortex of Lrp4<sup>cKO</sup> mice (Fig. 6H, I). Moreover, the time was prolonged for searching buried food in Lrp4<sup>cKO</sup> mice (Fig. 6J, K).

In conclusion, we reported that Lrp4 was highly expressed in layer II of the piriform cortex, and piriform cortical neurons in Lrp4<sup>ECD/ECD</sup> mice exhibited...
an increase in mature spine density and enhanced both sEPSC and mEPSC. Moreover, impairment of olfactory function was found in both Lrp4<sup>ECD/ECD</sup> mice and Lrp4 cKO mice, suggesting the non-negligible role of the full length of LRP4 in the piriform cortex. These results implied that the full length of LRP4 in the piriform cortex was necessary to maintain synaptic plasticity and the integrity of the olfactory signal transmission.

**Discussion**

The piriform cortex, a densely packed-cell-body layer, exhibits highly structural plasticity, such as dendritic remodeling, spine genesis and synaptic reorganization [30], and the synaptic plasticity in the piriform cortex encodes olfactory information, associative memory and sensory processing [27–30]. As a higher olfactory center and the largest area of the olfactory cortex, the piriform cortex receives direct input from the olfactory bulb and is connected with all of the entorhinal cortical domains [31, 32]. Similar to the hippocampus being an evolutionarily conserved paleocortex, the piriform cortex is also a phylogenetically ancient structure [30, 33].

Astrocytes, the most abundant cell type in the brain [34, 35], have a star-like morphology. The cell soma radiates many branches, and the primary branches gradually divide into finer and finer processes to form a dense network [34], which is more complex than immunostaining. In our immunostaining result, GFAP positive cells did not have the astrocyte characteristic morphology. The dentate gyrus is a particular region of the dense granular cells, and astrocytes cannot be discriminated in the condensed neuron layer. Herein, the piriform cortex cannot efficiently stain the astrocyte.

Layer II of the piriform cortex is developed prenatally and is regarded as devoid of postnatally proliferative capacity before. However, a subpopulation of immature neurons in layer II express DCX (doublecortin) and PSA-NCAM (polysialylated neural cell adhesion molecule) [30, 36–38]. Therefore, the piriform cortex possesses slight adult neurogenesis [38–40]. So, PSA-NCAM...
positive neurons may also express GFAP, and the GFAP-positive cells in the piriform cortex may not be astrocytes [41]. Zhang et al. found that Lrp4 is indispensable in adult neurogenesis [52], which might bring the complicated effect of LRP4 in the piriform cortex.

The olfactory sensory neurons locate in the olfactory mucosa, bulb, and olfactory cortex [42, 43]. The olfactory cortex integrates the olfactory signals, forms olfactory memories, and integrates specific olfactory signals with sensory information, such as color, taste, shape, and...
spatial location [44]. Complex odor signal analysis and integration rely on higher-level central structures, such as the piriform cortex [45]. The olfactory signal analysis in the piriform cortex relates to specific olfactory memory and involves olfactory sensitization and passivation [46]. Although the recognition mechanism of odor molecules has been deeply understood in the past ten years [47], it is still unclear how to complete the integration and modulation of olfactory signals in the high cortex. Here, LRP4 was highly expressed in the piriform cortex, and \( Lrp4^{ECD/ECD} \) mice exhibited high spine density and high frequency of sEPSC and mEPSC in the piriform cortical neurons, which indicated LRP4 might 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 than in the control mice. The test results suggested that \( Lrp4^{ECD/ECD} \) mice may have olfactory dysregulation. Moreover, similar results were seen in \( Lrp4 \) cKO mice. Strikingly, \( Lrp4^{ECD/ECD} \) mice showed reduced body weight and brain weight, suggesting that LRP4 affected the development. The previous report also found that \( Lrp4^{ECD/ECD} \) mice showed typical tight-knit morphology [6]. This kind of limb clamping 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.

Kariminejad et al. reported one case in which 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 [48]. 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 [49]. These findings suggest that the ECD of LRP4 plays a vital role in limb development, kidney development, and brain development. The results imply that the full length of LRP4 was non-negligible in the development.

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**Fig. 5** 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 days before the training, training 2 days, and testing 3 days; 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 ± SEM. n.s., no significant; *P < 0.05, **P < 0.01)
Wnt signaling regulates brain development and synapse maturation [50]. LRP4 has an antagonistic function on LRPs-mediated Wnt/β-catenin activation. Ramos-Fernández et al. reported that Wnt-7a stimulates dendritic spine formation in the hippocampus via inhibiting GSK-3β (glycogen synthase kinase-3β), triggering TCF/LEF-dependent gene transcription and promoting PSD-95 expression [51]. Our results showed that the dendritic spine density in the piriform cortex of Lrp4<sup>ECD/ECD</sup> mice significantly increased. Therefore, we hypothesized that the ECD of LRP4 might promote dendritic spine formation. SP dendritic mature spine in the piriform cortex increased significantly, related to neuronal physiological functions via participating in different neural circuits.

Excitatory synaptic transmission of the piriform cortical neurons in Lrp4<sup>ECD/ECD</sup> mice was enhanced. Pohlkamp et al. examined synaptic function in the Lrp4<sup>ECD/ECD</sup> mice by recording LTP in CA3-CA1 Schaeffer collaterals [7]. CA3-CA1 projections are a classic model for measuring and understanding synaptic plasticity. There is a substantial deficit in late-phase LTP in Lrp4<sup>ECD/ECD</sup> mice [7], implying the loss of the ICD and TMD may severely impair the LRP4 function. Sun et al. found that conditional knockout of Lrp4 in astrocytes
suppresses glutamatergic transmission in the CNS [1]. The frequency of sEPSC and mEPSC in hippocampal CA1 pyramidal neurons was reduced, and synaptic plasticity was also impaired in Lrp4 conditioned knockout mice [1, 6]. sEPSC and mEPSC frequency in piriform cortical neurons were enhanced in Lrp4ECD/ECD mice. Though the pyramidal cell type recorded for electrophysiology was not clear, the enhancement of EPSCs was consistent with increasing the piriform cortex’s dendritic spine density. The inconsistencies were that ECD of LRP4 remained in piriform cortical neurons in this research, but in previous literature, Lrp4 was conditioned knockout in astrocytes [1, 6]. Moreover, LRP4 is indispensable in adult neurogenesis [52]. We speculated that excitatory neurotransmitters might be impaired in the Lrp4 cKO mice leading to an abnormal behavior similar to Lrp4ECD/ECD mice, but the mechanism might be complicated.

**Conclusions**

In conclusion, our results showed that Lrp4 was highly expressed in layer II of the piriform cortex. In Lrp4ECD/ECD mice, piriform cortical neuronal dendritic mature spine density increased, and both sEPSC and mEPSC were enhanced. Moreover, the olfactory function was impaired in Lrp4ECD/ECD mice and Lrp4 cKO mice. These results suggest that the full length of LRP4 in the piriform cortex was necessary to maintain synaptic plasticity and the olfactory signal transmission pathway. The molecular regulating mechanism needs further exploration.

**Abbreviations**

AD: Alzheimer’s disease; β-gal: β-Galactosidase; cKO: Conditional knockout; CNS: Central nervous system; DDX: Doublecortin; ECD: Extracellular domain; ICD: Intracellular domain; LRP4: Low-density lipoprotein receptor-related protein 4; mEPSC: miniature excitatory postsynaptic current; NMJ: Neuromuscular junction; PBS: Phosphate-buffered sodium; PEI: Polyethylenimine; 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-galactopyranoside.

**Supplementary Information**

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**Author contributions**

MY and SW initiated and designed the study. MY, MX, and HZ performed western blotting, behavior test, immunostaining, and Golgi staining. YW performed the virus instruction and injection. 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 read and approved the final manuscript.

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**Availability of data and materials**

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

**Declarations**

**Ethics approval and consent to participate**

All experiments involving animals were conducted according to the ‘guide-lines 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.

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**References**

1. Sun XD, Li L, Liu F, Huang ZH, Bean JC, Jiao HF, et al. Lrp4 in astrocytes modulates glutamatergic transmission. Nat Neurosci. 2016;19(8):1010–8.
2. Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, et al. Genome-wide atlas of gene expression in the adult mouse brain. Nature. 2007;445(7124):168–76.
3. Tian QB, Suzuki T, Yamauchi T, Sakagami H, Yoshimura Y, Miyazawa S, 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, Chen W, Tan Z, Zhang L, Dong Z, Cui W, et al. A role of low-density lipoprotein receptor-related protein 4 (LRP4) in astrocytic alpha clearance. J Neurosci. 2020;40(28):5347–61.

**Additional file 1:** Fig. S1. Lower body and brain weight of Lrp4ECD/ECD mice. A Representative images of one-month-old Lrp4ECD/ECD mice compared with the control mice. Lrp4ECD/ECD mice were smaller than control mice. B Lrp4ECD/ECD mice’s body weight was significantly lower, compared to the control mice (control mice, n = 16, Lrp4ECD/ECD, n = 12). C Representative brain images of Lrp4ECD/ECD mice and the control mice. D Lrp4ECD/ECD adult mice’s brain weight was lower, compared with the control mice (control mice, n = 12; Lrp4ECD/ECD, n = 12). E Lrp4ECD/ECD mice showed typical tight-knit morphology. (Values were means ± SEM. *P < 0.05, ** P < 0.01).
5. Gomez AM, Burden SJ. The extracellular region of Lrp4 is sufficient to mediate neuromuscular synapse formation. Dev Dyn. 2011;240(12):2626–33.

6. Gomez AM, Froemke RC, Burden SJ. Synaptic plasticity and cognitive function are disrupted in the absence of Lrp4. Elife. 2014;3:e04287.

7. Pothkamp T, Durakoglugil M, Lane-Donovan C, Xian X, Johnson EB, Hammer RE, et al. Lrp4 domains differentially regulate limb/brain development and synaptic plasticity. PLoS ONE. 2015;10(2): e0116701.

8. Shen C, Xiongand WC, Mei L. LRP4 in neuromuscular junction and bone development and diseases. Bone. 2015;60:101–8.

9. Weatherbee SD, Anderson KV, Niswanter LA. LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. Development. 2006;133(24):4993–5000.

10. Zhang B, Luo S, Wang Q, Suzuki T, Xiongand WC, Mei L. LRP4 serves as a coreceptor of agrin. Neuron. 2008;60(1):285–97.

11. Kim N, Burden SJ. Musk controls where motor axons grow and form synapses. Nat Neurosci. 2008;11(1):19–27.

12. Wu H, Lu Y, Shen C, Patel N, Gan L, Xiong WC, et al. LRP4 is a coreceptor for agrin and forms a complex with Musk. Cell. 2008;135(2):334–42.

13. Wu H, Xiongand WC, Mei L. To build a synapse: signaling pathways in neuromuscular junction assembly. Development. 2010;137(7):1017–33.

14. Barik A, Lu Y, Sathyamurthy A, Bowman A, Shen C, Li L, et al. LRP4 is critical for neuromuscular junction maintenance. J Neurosci. 2014;34(42):13892–905.

15. Kim N, Stiegler AL, Cameron TO, Hallock PT, Gomez AM, Huang JH, et al. Lrp4 is a receptor for agrin and forms a complex with Musk. Cell. 2008;135(2):334–42.

16. Tanahashi H, Tian QB, Hara Y, Sakagami H, Endo S, Suzuki T. Polyhydramnios in Lrp4 knockout mouse with bilateral kidney agenesis: defects in the pathways of amniotic fluid clearance. Sci Rep. 2016;6:20241.

17. Dietrich MF, van der Weyden L, Prosser HM, Bradley A, Herzand J, Adams DJ. Ectodomains of the LDL receptor-related proteins LRP1b and LRP4 have anchorage independent functions in vivo. PLoS ONE. 2010;5(4): e9960.

18. Johnson EB, Hammerand RE, Herz J. Abnormal development of the apical ectodermal ridge and polysyndactyly in Megf7-deficient mice. Hum Mol Genet. 2005;14(22):3523–38.

19. Yan M, Guo A, Chen P, Jing H, Ren D, Zhong Y, et al. LRP4 regulates expression of ErbB4 in astrocyte enhance dendrite arborization of the neuron. Mol Physiol Biophys. 2015;34(1):1–12.

20. Loureiro M, Achargui R, Flakowski J, Van Zessen R, Stefanelli T, Pascoli V, et al. Social transmission of food safety depends on synaptic plasticity in the prefrontal cortex. Science. 2019;364(6444):991–5.

21. Vadodaria KC, Yanpallewar SJ, Vaishnavi M, Toshniwal D, Liles LC, Rommelfanger KS, et al. Noradrenergic regulation of plasticity marker expression in the adult rodent piriform cortex. Neurosci Lett. 2017;644:76–82.

22. Wang D, Liu P, Mao X, Zhou C, Tao T, Xu J, et al. Task-demand-dependent neural representation of odor information in the olfactory bulb and posterior piriform cortex. J Neurosci. 2019;39(50):10022–18.

23. Strauch C, Manahan-Vaughn D. Orchestration of hippocampal information encoding by the piriform cortex. Cereb Cortex. 2020;30(1):135–47.

24. Katori K, Manabe H, Nakashima A, Duru F, Sasaki T, Ikeya Y, et al. Sharp wave-associated activity patterns of cortical neurons in the mouse piriform cortex. Eur J Neurosci. 2018;48(10):3246–54.

25. Freeman MR. Specification and morphogenesis of astrocytes. Science. 2010;330(6005):774–8.

26. Pelny M, Pelka M. Astrocyte reactivity and reactive astroglisis: costs and benefits. Physiol Rev. 2014;94(4):1077–98.

27. Gomez-Climent MA, Castillo-Gomez E, Varea E, Guirado R, Blasco-Ibanez JM, Crespo C, 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.

28. Rubio A, Bellés M, Belenguer G, Vidueira S, Farinas I, Nacher J. Characterization and isolation of immature neurons of the adult mouse piriform cortex. Dev Neurobiol. 2016;76(7):748–63.

29. Rothenheinrich P, Bellés M, Benedetti B, König R, Dannehl D, Kreutzer C, et al. Cellular plasticity in the adult murine piriform cortex: continuous maturation of dormant precursors into excitatory neurons. Cereb Cortex. 2018;28(7):2610–21.

30. Chen J, Kwon CH, Lin L, Liand Y, Parada LF. Inducible site-specific recombination in neural stem/progenitor cells. Genesis. 2009;47(2):122–31.

31. Wang D, Liu P, Mao X, Zhou Z, Cao T, Xu J, et al. Task-demand-dependent neural representation of odor information in the olfactory bulb and posterior piriform cortex. J Neurosci. 2017;37(11):4694–9.

32. Mori K, Sakano H. How is the olfactory map formed and interpreted in the mammalian brain? Annu Rev Neurosci. 2011;34:467–99.

33. Wang F, Nemes A, Mendelschon M, Axel R. Odorant receptors govern the formation of a precise topographic map. Cell. 1998;93(1):47–60.

34. Freeman MR. Specification and morphogenesis of astrocytes. Science. 2010;330(6005):774–8.

35. Gottfried JA, Zelano C. The value of identity: olfactory notes on orbitofrontal cortex function. Nat Rev Neurosci. 2007;8(11):840–51.

36. Nait-Oumesmar B, Picard-Riera N, Kernion C, Decker L, Seilhean D, Haglinger GJ, et al. Activation of the subventricular zone in multiple sclerosis: evidence for early glial progenitors. Proc Natl Acad Sci USA. 2007;104(11):4694–9.

37. Leinwand SG, Chalasani SH. Olfactory bulb processes for sensation to perception. Curr Opin Genet Dev. 2011;21(6):806–11.

38. Kariminejad A, Stollfuss B, Li Y, Bogenshauer N, Boss K, Hennekam RC, et al. Severe Cenani-Lenz syndrome caused by loss of LRP4 function. Am J Med Genet A. 2013;161A(6):1475–9.

39. Afzal M, Zaman Q, Kornak U, Mundlos S, Malik S, Flottmann R. Novel splice mutations in LRP4 causes severe type of Cenani-Lenz syndactyly syndrome with oro-facial and skeletal symptoms. Eur J Med Genet. 2017;60(8):421–5.

40. Li Y, Pawlik B, Elcioglu N, Aglan M, Kayserili H, Yigit G, 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.

41. Ramos-Fernández E, Tapia-Rejas C, Ramírez-Vara VT, Inestrosa NC. Wnt-7a stimulates dendritic spine morphogenesis and PSD-95 expression through canonical signaling. Mol Neurobiol. 2018;56(3):1870–82.

42. Zhang H, Sathyamurthy A, Liu F, Li L, Zhang L, Dong Z, et al. Agrin-Lrp4-Ror2 signaling regulates adult hippocampal neurogenesis in mice. Elife. 2019;8:e45303.