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Master Thesis

自閉症相關基因-*Dlgap2* 突變鼠表現異常嗅覺性徵

Autism-Related Gene *Dlgap2* Mutant Mice Display Abnormal Olfactory

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謝辭

從大學到研究所，近七年的時間我成長許多。最後能拿到碩士文憑，要感謝口委高淑芬老師、符文美老師、陳嘉祥老師、王培育老師、我的指導老師李立仁老師及許多人的鼓勵及協助。

報考一個新的研究所多少會有點焦慮，但父母及姊姊在我求學上一路支持，讓我無後顧之憂大膽地嘗試自己想做的事。碩士班研究題目的實驗老鼠，是根據高淑芬醫師、陳嘉祥醫師的臨床研究延伸發想的，如果沒有這兩位老師過去在病人基因上的研究，我也無緣接觸到這麼有趣的題目。曉梅學姊製作出基因突變鼠，並加上動物中心的幸珈花了大量精神照顧這隻老鼠，讓我能順利地進行實驗。研究過程，非常感謝符文美老師實驗室提供資源協助研究，以及邵涵的基因分型。研究夥伴兼腦心所同學兼宿舍鄰居，江謝立峰，我們常常一起討論實驗想法、設計及流程，也把評論分析大大小小事當成訓練腦力及創新的工具。

我的指導老師，李立仁老師，李老師實驗室氣氛融洽，腦心所成立之初沒有專任老師，感謝李老師願意收留一個外所的學生。老師個性活潑，常常找同學們聊天談心，如果沒有李老師的提攜，利用軟硬兼施的方法，不論在求學或是人生上我也不會學習到那麼多。實驗室中的各個成員，思婷、慧宇、亦鈞、克儉，還有已經離開實驗室的廖俊傑學長、亮文、御彰、鋒翌、李泱及王亭都給予相當多的支援及協助。

一開始踏入神經科學領域，完全是一個霧裡看花的菜鳥，但多虧了梁庚辰老師的提攜及教導，還有梁老師實驗中的張世達學長、游文愷學長、劉姿蘭學姊、陳可欣學姊、楊幼屏學姊、田欣華學姊的技術及想法傳授，又或是與思妤及小狼一同討論心理學及腦神經科學，都漸漸讓我更加孰悉神經科學領域。

腦心所的成立，多虧了謝豐舟老師、胡海國老師、嚴震東老師、梁庚辰老師及跨學院熱心的老師們的努力，我才有榮幸能成為腦心所的一員。成立之初，如果沒有幹事士齡的幫忙，許多行政程序完全無法進行，我們也無法順利修課及畢業。第一屆一同打拼的同學們，聲暉、智邦、泓綸、曉鋒、紹維、勤霖、佳達及婉容，是我們一起把所給成立的。第二屆的好學弟妹們，謝成、廷瑋、林醫師、楊醫師、淑韵、及第三屆即將加入的新血及新進的老師們，程淮榮老師、王培育老師、吳恩賜老師及黃憲松老師，都讓腦心所更加熱鬧，領域更加多元專業。

從大一到碩二，台大薰風國樂社是我大學生涯最豐富的地方。就算到了碩士班，偶爾還是要偷閒玩音樂並和大家聊聊天，皓中及博賢兩位國小同學、b95 就認識的大家、還有一屆屆加入的新血陪伴，都幫助我轉換心境並產生更多的想法。宿舍的鄰居，杜律師、蔡醫師還有不知道名字但遇見會打招呼的朋友們，相會時的閒聊也成為一天辛苦的享受。

離開校園，未來的路誰也不知道。但過去假若沒有他們的協助，現在的我也無緣享受畢業帶來的欣喜。

郁唐

中文摘要

嗅覺是腦中一個相當古老的系統，其在哺乳類中有建立社會行為的作用。然而，至今嗅覺與社會行為的分子機制仍不清楚。臨床研究發現，一個突觸後蛋白-DLGAP2 與泛自閉症(autism spectrum disorder, ASD)病人的社會表現損害有關。目前已知 *Dlgap2* 在囓齒類的嗅覺系統有高度表現，故 *Dlgap2* 在嗅覺及社會行為的角色需被闡明。

利用 Cre/lox 系統，*Dlgap2* 基因的第六外顯子被剔除，*Dlgap2* 剔除(knockout, KO)鼠而後製造出來。嗅覺系統的結構及功能則利用生物化學、型態及行為觀察檢定。

雖然 *Dlgap2* 剔除鼠的嗅覺偵測及嗅覺區分能力和野生型 (wildtype, WT)無異，但是 *Dlgap2* 剔除鼠卻對社會味道表現高度反應。此外與野生型鼠比較，一個 AMPA 受器-GluR1 在 *Dlgap2* 剔除鼠的嗅球表現量增加。型態方面，*Dlgap2* 剔除鼠在梨狀皮質(piriform cortex)中的表層錐狀細胞(superficial pyramidal cell)的樹突(dendrite)總長度和節點數上升，而樹突突起(dendritic spine)密度卻下降。

嗅球的突觸蛋白改變及嗅覺皮質的樹突結構改變可能造成 *Dlgap2* 剔除鼠對於社會訊息的偵測或解釋異常，並可說明社會味道所引發的高反應性。

關鍵字: 嗅球、突觸蛋白、嗅覺、梨狀皮質、樹突結構

Abstract

The olfactory system, a relatively primitive system in the brain, plays an important role in the establishment of social behaviors in mammals. However, the molecular mechanisms regarding olfaction and social behavior are still largely unclear. Clinical research has suggested an association between DLGAP2, encoding a postsynaptic protein, and impaired social performance in patients of autism spectrum disorder (ASD). It is clear that *Dlgap2* is highly expressed in the olfactory system in rodents, the roles of *Dlgap2* in olfaction and social behavior can thus be elucidated.

Using the cre/lox system, exon 6 of *Dlgap2* gene was deleted and *Dlgap2* knockout (KO) mice were subsequently generated. The structure and function of the olfactory system were examined with biochemical, morphological and behavioral means.

In *Dlgap2* KO mice, olfactory detection and discrimination were comparable to those in wildtype (WT) littermates. However, they demonstrated hyperactivity towards social odors. Compared with WT mice, the expression of GulR1, a key component of AMPA receptor, was increased in the olfactory bulb of *Dlgap*2 KO mice. In the morphological aspect, the dendritic length was longer and number of segments was increased in superficial pyramidal cells of the piriform cortex in *Dlgap2*

KO mice while the spine density was reduced.

Changes of synaptic proteins in the olfactory bulb and altered dendritic structures in the olfactory cortex might contribute to the disturbance of detection or interpretation of social signals which might account for social odor-induced hyperactivity in *Dlgap2* KO mice.

Keywords:

olfactory bulb, synaptic protein, olfaction, piriform cortex, dendritic structure

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I. Introduction

I-1 Olfactory system and social behaviors

Social behavior is critical for reproduction and survival of many species. In mammals, it is believed that most of mammals use odor cues to establish social behaviors such as mating, parenting, affiliations and prey-predator relationships (Wedekind and Penn, 2000, Brewer et al., 2006). In human brain imaging studies, social stimuli-associated signals are evident in olfaction-related regions such as insular cortex, orbitofrontal cortex and amygdala, indicating a close relationship between olfaction and sociality-related information processes (Singer et al., 2004, Beer et al., 2006, Bickart et al., 2011). However, the neural and molecular mechanism linking olfactory system and social behaviors are still not clear.

In mammal olfactory system, there are two distinct pathway, the main olfactory system (MOS, Fig. A) and the accessory olfactory system (AOS) have been identified. The MOS is the principal olfactory system, known for detecting volatile chemical substance to perceive scent, so that animals can search for food or avoid danger; the AOS is mainly for pheromone detection, contributing to behaviors like mating, parenting, affiliations and prey-predator relationships (Luo et al., 2003). However, recent studies have shown that the two olfactory systems are not mutually exclusive

and the impacts of manipulations on the MOS system are far beyond olfactory function. In the main olfactory bulb (MOB), urine responsive neurons are evident (Lin et al., 2005). Anosmic mice have deficits in olfaction are less aggressive (Ropartz, 1981). Furthermore, in mice lacking *Cnga2*, which is encoding a subunit of cyclic nucleotide-gated channel expressed exclusively in the MOS, the mating and fighting behaviors are decreased (Mandiyan et al., 2005). These evidences suggested that the MOS is important for social behaviors (Choi and Anderson, 2005, Spehr et al., 2006, Sanchez-Andrade and Kendrick, 2009, Keller and Levy, 2012) and implied that social deficits might be resulted from the impairment of the MOS.

I-2 The pathway and function of the main olfactory system

I-2-1 Pathway

The structures and connections of MOS is illustrated as Fig. A. The olfactory sensory neurons (OSNs) are located in the olfactory epithelium (OE). When odor chemicals

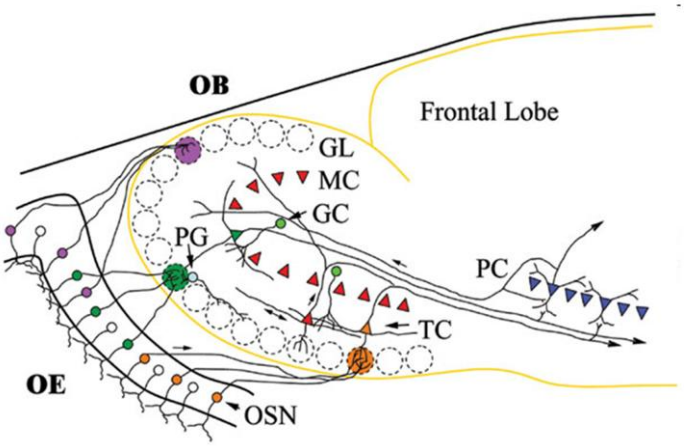

Figure A. The main olfactory system (MOS). OE: olfactory epithelium; OSN: olfactory sensory neurons; PG: periglomerular cells; TC: tufted cells; GC: granule cells; mitral cells; GL: glomeruli; PC: pyramidal cells. (Adopted from Willhite et al., 2006)

bind to OSNs, signals pass to glomerulus in the MOB, where the axons of sensory neurons forming synapse mainly with the dendrite of mitral cells (Murthy, 2011) (Fig. A). The basal dendrites of mitral cells interact with granule cells produce lateral inhibition (Kennedy and Ehlers, 2011). Mitral cells then project to various brain areas like anterior olfactory cortex, amygdala, entorhinal cortex and piriform cortex (Baum and Kelliher, 2009). The olfactory related brain areas connect to each other complicatedly and play roles in a variety of functions like olfactory sensation, perception, memory or emotion.

Axons of tufted/mitral cells are merged into olfactory tract and projects to a number of brain regions. One of the most important areas for olfactory perception and behavioral formation is the piriform cortex. The piriform cortex is a three layer paleocortex and most of the cells are located in Layer II, where the two principal cells called superficial pyramidal cells and semilunar cells can be identified (Shepherd and Oxford University Press., 2004). Neurons in the piriform cortex are important for odor representation and behavioral learning (Choi et al., 2011, Chapuis and Wilson, 2012). Disruption of the piriform cortex caused impaired odor identification (Li et al., 2010). However, the relationship between dysfunction in the olfactory cortex and impaired social behavior is still not clear.

I-2-2 Smell test

In clinical research, olfactory deficits is an endophenotype for neurological or psychiatry disorders, such as Alzheimer's Disease, Parkinson's disease, schizophrenia, and autism (Moberg et al., 1999, Suzuki et al., 2003, Wang et al., 2011a). Estimation of olfactory function might be a diagnostic parameter for early of diseases (Tissingh et al., 2001). Olfactory function can be evaluated in three dimensions, namely, odor detection, odor discrimination and odor identification (Hummel et al., 1997). The odor threshold is estimated as the lowest concentration of a specific odor perceived by subjects. The odor discrimination is tested while the subject is able to distinguish different kinds of odors. The odor identification is examined if the scent is matched to its characteristic. In rodents, the olfactory functions can be evaluated using behavioral methods. Buried food test can be used for odor detection (Yang and Crawley, 2009) and the ability of odor discrimination can be examined by repetitive exposures of odors i.e. olfactory habituation dishabituation test (Yang and Crawley, 2009).

I-3 Dlgap2, a potential link the olfactory system and social behavior

I-3-1 Location and function of Dlgap2

Discs, large (Drosophila) homolog-associated protein 2 (DLGAP2, Gene ID: 9228), also known as Synapse-associated protein (SAP) 90/Postsynaptic density (PSD)-95-associated protein 2 (SAPAP2) or guanylate kinase-associated

protein/hDLG-associated protein 2 (GKAP2), is located in the postsynaptic density (Fig. B). DLGAP2 contains PDZ, guanlyate kinase (GK) domain and SH3 domains that interacts with various postsynaptic proteins including Shank and PSD-95 (Kim et al., 1997, Takeuchi et al., 1997, Feng and Zhang, 2009). It is believed the

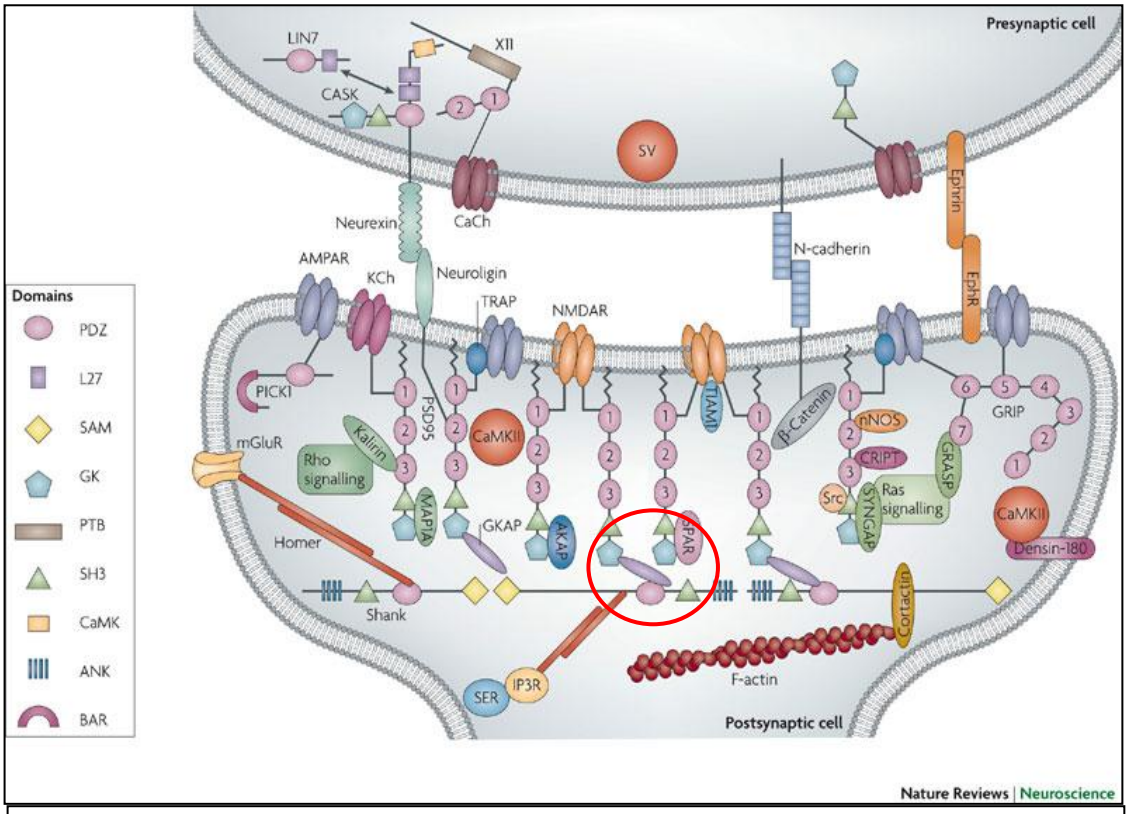

Figure B. Organizations in the postsynaptic density. DLGAP (GKAP) interacted with PSD-95 and SHANK highlighted by the red circle (Adopted from Feng and Zhang, 2009).

PSD95-DLGAP-SHANK complex scaffolds the excitatory glutamatergic synapses and play important roles in synaptic transmission and postsynaptic signaling (Ting et al., 2012). Recently, it was found as an autistic spectrum disorder candidate gene discovered by copy number variation clinical studies (Pinto et al., 2010). Furthemore, a deletion in 8p23, where DLGAP2 gene is located, has been indentified in a patient

with autism in Taiwan (Chien et al., 2010).

I-3-2 DLGAP family and its related protein

Dysregulation of DLGAPs (SAPAPs) would cause neurological deficits. For example, mice lacking Dlgap3 showed altered synaptic transmission in corticostriatal pathway and obsessive-compulsive like behaviors (Welch et al., 2007). As for DLGAP2, it has been linked with epigenetic vulnerability to post-traumatic stress disorder (Chertkow-Deutsher et al., 2010) and found altered in a mouse model of Fragile X syndrome (Schutt et al., 2009). Also, other postsynaptic proteins may be related to social functions. Shank3 is a SHANK family protein, which is also a postsynaptic scaffolding protein enriched in excitatory synapse just like DLGAP and interacts with it. When Shank3 is being knockout, the mice exhibit abnormal social behaviors (Peca et al., 2011, Wang et al., 2011b). Neuroligin-3 is a cell adhesion protein in the postsynaptic membrane, and altered synaptic transmission and social withdraw phenotypes have been reported in Neuroligin-3 mutant mice (Tabuchi et al., 2007). Together, these findings pinpoint that proper synaptic transmissions are necessary for processing normal social functions.

I-4 Experimental design

We have generated a *Dlgap2* mutant mouse line in which the exon 6 is deleted and expression of Dlgap2 protein is abolished. Homozygote *Dlgap2* knockout (KO) mice exhibited abnormal social interaction, including aggression behaviors (unpublished observations). In the studies of "Allen Institute for Brain Science",

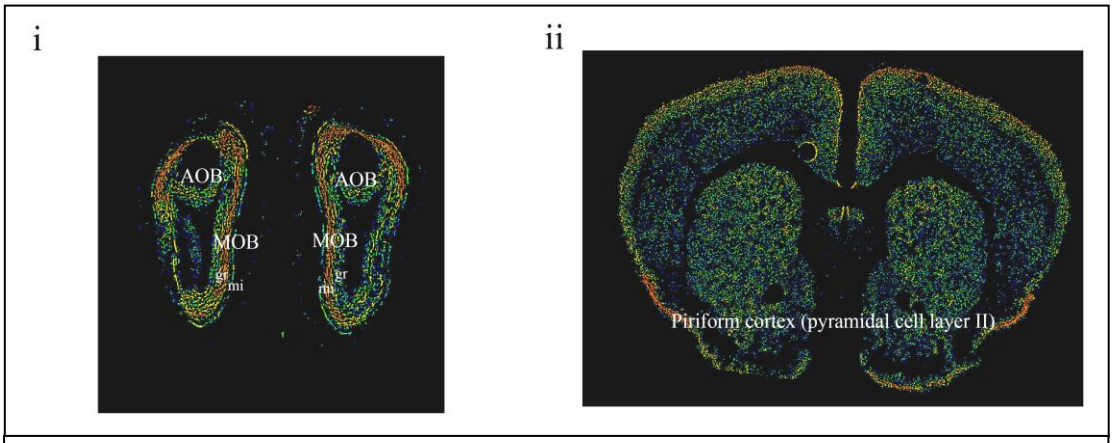

Figure C. mRNA expression of *Dlgap2* in the olfactory system. (i) Dlgap2 mRNA was highly expressed in the granule cell layer (gr) and mitral cell layer (mi) of the main olfactory bulb (MOB). (ii) Dlgap2 mRNA was highly expressed in the pyramidal cell layer II of the piriform cortex (Modified and adopted from Allen Brain Atlas [http://www.brain-map.org/]).

Dlgap2 mRNA was highly expressed in the MOS especially in the mitral and granule cell layer of the olfactory bulb (Fig. Ci) and layer II of the piriform cortex (Fig. Cii), indicating its roles in olfactory function. Therefore the roles of Dlgap2 in olfaction function such as odor detection and discrimination, in relation to social behavior, were elucidated using *Dlgap2* KO mice. Furthermore, morphological features of neurons in the piriform cortex, an integration center of odor information, were examined using Golgi-Cox stain and neuromorphometric methods. The role of Dlgap2 in the structure and function of the olfactory system in mice could be revealed.

II. Materials and Methods

II-1 Subjects

Adult male (8-12 weeks) *Dlgap2* homozygous knockout (-/-) and wild-type control mice were used in this experiments. *Dlgap2* mutant mice were created using the Cre/loxP system. Exon 6 of Dlgap2 gene was flanked by two loxP sites, then exon 6 of Dlgap2 gene was deleted by Cre-induced recombination (Fig. 1A). After the initial Dlgap2 mutants were generated, *Dlgap2* heterozygous knockout mice were backcrossed to C57BL/6 mice for at least 8 generations to alleviate S129 background. Genotypes were determined by PCR of mouse tail DNA; primer F1 (GCCACATTCATAACATAGCTAC) and R1 (ACCTCTGCTACATACCCACTC) for the wild-type allele (600 base pairs), and primer F1 and R2 (ACACATGGGATGCTGTACGC) for the mutant allele (800 base pairs) (Fig. 1B). In this study, adult (>8 weeks) male *Dlgap2* homozygote knockout (*Dlgap2* KO) mice and wildtype (WT) mice were used.

II-2 Behavior tests

II-2-1 Buried Food Test

Prior to the test, a piece of novel food (chocolate cereal) was put in the home

cage. If the food was not consumed, the mouse was excluded. Before the buried food test, subjects were food-deprived for 24 hours. For the test, individual mouse was placed in a cage in which the base was covered with clean bedding of 3 cm-thick, allowed for exploration in the cage for 5 minutes and then removed. A piece of chocolate cereal was buried 1cm beneath the surface at a cage corner randomly. The subject mouse was then re-placed in the cage and the time latency to find the chocolate cereal was recorded.

II-2-2 The Olfactory Habituation/Dishabituation Tests

Two non-social odors (use distilled water as solvent), almond extract (McCormick; 1:100 dilution) and imitation banana extract (McCormick; 1:100 dilution) will be used. Social odors are obtained from two unfamiliar mice home cages. For non-social odors, the cotton tip part of the applicator will be dipped into the odor solutions; for social odors, the applicator will be swiped the cage bottom. After the acclimatization to dry applicator for 30 minutes, olfactory habituation/dishabituation test was conducted. Same odor stimulus was applied for 2 minutes for 3 times with 1 minute interval. Another odor stimulus was then used. The order of presentations was fixed: water, water, water, almond, almond, almond, banana, banana, banana, cage A, cage A, cage A, cage B, cage B, cage B.

II-3 Western blot

The brains were rapidly removed and immediately dissected on ice. The olfactory bulbs were collected and homogenized in lysis buffer and centrifuged. The supernatant was assayed for protein concentration by the Bradford method using Protein Assay kit. Protein samples mixed with protein dye were heated to 95℃ for denaturing, loaded in gel composed of 10 % SDS-polyacrylamide gel and 5% stacking gel, and separated by electrophoresis. The gel was then transferred electrophoretically to a methanol actived nitrocellulose membrane and the membrane was proceeded for blocking for 1 hr. Subsequently the membrane was incubated with antibodies against synaptic proteins including Dlgap2, GluR1, NR2A, NR2B, CaMKIIα, CaMKIIβ and PSD-95. Antibody against β-actin was used as internal control HRP-conjugated secondary antibodies were applied and the signaling was developed and detected using ECL reagent and UVP AutoChemi Image System, and quantified with ImageJ software.

The expression of given protein was analyzed by measuring the density of selected band and adjusted with β-actin band of the same sample. The level of given protein in WT mice was adjusted as 1 while comparing with the same protein in KO mice.

II-4 Histology

Adult WT and KO mice were deeply anesthetized by intraperitoneal injection of 2.5% Avertin and perfused transcardially with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Whole brains were taken and postfixed overnight in the same fixative at 4 ℃.

II-4-1 Hematoxylin stain

Coronal sections of 50 μm thick containing the olfactory bulb and the piriform cortex are collected, mounted on the glass slides and stained with 2% Hematoxylin. After dehydration, sections were covered and examined.

II-4-2 Golgi-Cox stain

Morphological features of superficial pyramidal cells in the piriform cortex were revealed using Golgi-Cox stain. In brief, brain tissues were taken and placed in the impregnation solution (solutions A and B in FD Rapid GolgiStain kit, FD NeuroTechnologies, Ellicott City, MD, USA) for 17 days. After several washes, brain tissues were cut into sagittal sections with a vibration microtome at the thickness of 150 μm from Lateral 3.00 mm to 2.00 mm. Brain sections were collected and reacted

with the mixture of developer and fixer in FD Rapid GolgiStain kit (solution C and D, 1:1) for 2 minutes. Dendrites morphology was then examined under a light microscope with 20X objective lens, captured with Stereoinvestigator system (Microbrightfield, Williston, VT, USA) and reconstructed with Neurolucida software (Microbrightfield). Morphometric analyses were conducted using Neurolucida explore (Microbrightfield). Dendritic spines were examined with 100X objective oil immersion lens.

II-5 Data analysis

All values and bars represent means ± s.e.m. Statistical significance was analyzed by Student's t-test for buried food test, neuronal morphology and spine density; furthermore, repeat measurement ANOVA was for the olfactory habituation/ dishabituation test. Asterisks were used to indicate significant differences (*, $p<0.05$; **, $p<0.001$).

III. Results

III-1 Generation of *Dlgap2* KO mice

In adult *Dlgap2* KO mice, the overall brain structure was comparable to that in age-matched WT mice (Fig. 2A). It has been reported that Dlgap2 mRNA is expressed in the olfactory system (Lein et al., 2007).We checked the expression of Dlgap2 protein in the olfactory bulb using immunoblotting. In WT mice, full length Dlgap2 was detected; whereas its expression was abolished in *Dlgap2* KO mice (Fig. 2B). The cytoarchitectures of the olfactory bulb and olfactory cortex were identical between WT and KO mice in macroscopic level (Fig. 3). These results suggested the gross structure of the olfactory system and the whole brain is not affected by Dlgap2 deletion.

III-2 Altered synaptic protein expression in Dlgap2 KO mice

Dlgap2 has been classified as a scaffold protein in the postsynaptic density of excitatory synapses (Takeuchi et al., 1997). The expressions of synaptic protein were then examined. We checked different categories of proteins namely, glutamatergic receptors (GluR1, NR2A and NR2B) signaling molecules (CaMKIIα and CaMKIIβ) and scaffold protein, PSD95. GluR1 is a key component of AMPA receptor while

NR2A and NR2B are components of NMDA receptor. CaMKIIs are known for Ca^{2+}-dependent signal transduction. PSD95 is a major anchor site for various synaptic proteins (Kim et al., 1997, Kim and Sheng, 2004). Protein samples were collected from the total lysates of the olfactory bulbs of WT and KO mice and analyzed by immunoblotting (Fig. 4A). Notably, the level of GluR1 was up-regulated whereas the levels of other proteins were not altered (Fig. 4B). Increased GluR1 level in the olfactory bulb of KO mice implied that the glutamatergic synaptic transmission might be affected.

III-3 Dlgap2 KO mice displayed abnormal olfactory responses

In the olfactory bulb of KO mice, Dlgap2 was deficient, indicating altered structural integrity of postsynaptic density and dysregulated excitatory neurotransmission. Consequently, the olfactory function might be affected. To test this hypothesis, a test for odor detection, buried food test, was conducted. Mice were food-restricted in order to increase the motivation for food searching. A small piece of chocolate cereal was buried 1 cm beneath the surface at a cage corner (Fig. 5A). The only way for a mouse to retrieve the food was to dig out the buried cereal using its olfactory function. In this test, both WT and KO mice quickly retrieved the food (Fig. 5B), indicating the odor detection function was not affected by deletion of Dlgap2 in

the olfactory bulb.

Next, we examined the ability of odor discrimination by repetitively administering odor stimuli to the tested mouse using a cotton odor applicator (Fig. 6A). While a novel odor was exposed to mouse, it frequently sniffed the odor applicator but the sniffing frequency declined with time quickly because of olfactory habituation. However, while another novel odor was demonstrated, the sniffing time increased exhibiting a pattern of dishabituation. Such habituation/dishabituation phenomenon indicates the ability of odor discrimination. It was the case for both WT and KO mice while natural odors, like almond and banana were provided (Fig. 6B left). However, compared with WT mice, KO mice displayed less interest toward such odor stimuli. On the other hand, while social odors (scent of unfamiliar home cages) were provided, patterns of habituation/dishabituation were also evident in both WT and KO mice while KO mice exhibited greater interest toward social odors (Fig. 6B right).

Together, these results indicated that although the fundamental ability of olfactory function was preserved in *Dlgap2* KO mice, odor preference was noticed in KO mice. Reduced interests against nonsocial odors and hyper-reactivity toward social scent in KO mice were characterized, suggesting that deletion of Dlgap2 might affect the higher olfactory functions such as discrimination and interpretation of odor

stimuli.

III-4 Altered dendritic architectures in the piriform cortex of Dlgap2 KO mice.

To further elucidate the phenotypes of these mutant mice in the olfactory system, we next checked the dendritic architectures of superficial pyramidal neurons of the piriform cortex. The piriform cortex directly receives olfactory inputs from the main olfactory bulb and is an integration center for olfactory information. Using Golgi-Cox impregnation method, the morphology of superficial pyramidal neurons was revealed (Fig. 7). Usually, one apical and several basilar dendrites were presented.

The dendritic structures were then analyzed using neurolucida system. For apical dendrites, 55 cases were obtained from 10 WT mice and 64 from 10 KO mice. For basilar dendrites, 25 cases from 7 WT and 40 from 8 KO mice, respectively. Compared to neurons in WT mice, the total dendritic length and number of segments were increased in both apical and basilar dendrites of neurons obtained from KO (Fig. 8). The extended dendritic profile was also noticed as increased number of intersections, nodes and endings in sholl analysis (Fig. 9). Interestingly, greater dendritic complexity was evident in the distal regions of the apical dendrites (Fig. 9A, 9B) and proximal regions of the basilar dendrites (Fig. 9D, 9E), but there were no difference between WT and KO mice in endings of sholl analysis (Fig. 9C, 9F).

Next, for more detailed examination in dendrites, dendritic segments were classified into terminal and internodal segments and analyzed. In apical dendrites, number of segments were increased in KO mice when dendritic order was higher (Fig. 10 A); in basilar dendrites, the highest order was sixth in WT mice but tenth in KO mice (Fig. 10B). Although the dendritic segments were increased in higher order of dendrites, both in apical and basilar dendrites the internodal and terminal parts of dendritic segments lengths were no difference between WT and KO mice (Fig. 10C, 10D).

The density of dendritic spines was also examined (Fig. 11). In apical dendrites, the number of spines was counted in proximal (less than 80 μm from the soma) and distal (greater than 80 μm from the soma) regions, respectively. For basilar dendrites, spines were counted in different orders. The density of dendritic spine was lower in the proximal regions of apical dendrites in neurons obtained from *Dlgap2* KO mice (Fig. 11B). However, the spine density in basilar dendrites of superficial pyramidal cells of the piriform cortex was comparable between WT and KO mice (Fig. 11D).

IV. Discussion

IV-1 Postsynaptic protein Dlgap2 plays important roles in the structure and function of the olfactory system

In the rodent brain, *Dlgap2* mRNA is highly expressed in the MOS including the OB and the piriform cortex (Chien et al., 2007, Allen Brain Atlas [http://www. brain-map.org/]). In present study, deletion of *Dlgap2* in mice resulted in impaired olfactory function, changes of postsynaptic proteins and altered dendritic morphology in the MOS. To the best of our knowledge, it was the first attempt to link the expression Dlgap2 with the structure and function of the olfactory system. In *Dlgap2* KO mice, increased expression of Glu1R, a component of AMPA receptor in the OB and changed dendritic architectures in superficial pyramidal cells in the piriform cortex were noticed. These molecular and structural abnormality might explain the altered olfactory responses in *Dlgap2* KO mice.

IV-2 Increased protein level and dendritic lengths may be compensatory effects

In the nervous system, synaptic homeostasis is important mechanism for maintaining the function of neural network. In particular, the neuronal activities can be adjusted corresponding to the chronic changes (Turrigiano and Nelson, 2004). A

recent study revealed that DLGAP plays an important role in homeostatic scaling of postsynaptic proteins (Shin et al., 2012). In hippocampal neurons, tetrodotoxin -induced inactivity results in synaptic accumulation of DLGAP while bicuculline-produced overexcitation removes synaptic DLGAP. These events are controlled by the activity of CaMKII. However, both tetrodotoxin- and bicuculline-driven synaptic alterations are abolished while the turnover of DLGAP is disrupted. On the other hand, when the turnover of DLGAP is diminished, synaptic scaling of AMPA receptor miniature excitatory postsynaptic current (mEPSC) is also impaired (Shin et al., 2012). These results indicate the significance of DLGAP in maintaining the integrity of postsynaptic density and synaptic functions. In the present study, deletion of *Dlgap2* can be considered as a chronic manipulation and it directly reduced spine density, diminishing the efficiency of synaptic transmission. Consequently, a compensatory mechanism might be elicited. To maintain the efficacy of synaptic transmission, the expression glutamate receptor, especially the AMPA receptor, such as GluR1, was elevated. Furthermore, the number and length of dendritic segments were also increased, augmenting the probability of synaptic contacts to balance the system. However, since DLGAP is an essential component for homeostatic scaling (Shin et al., 2012), when Dlgap2 was deleted, the compensatory mechanism might not be efficient. As a consequence, the function of the olfactory system was not fully rescued.

IV-3 The hyper-social activities in *Dlgap2* KO mice are associated with aggression

In *Dlgap2* KO mice, the abnormal strong preference of social odor can be viewed as an autistic behavioral phenotype, and lead to their aggressive behaviors. In *Dlgap2* KO mice defects in synaptic structure and function were characterized in the orbitofrontal cortex including altered spine density, expression of postsynaptic proteins, miniature excitatory postsynaptic current and the dense of postsynaptic density. The orbitofrontal cortex is well-known for its participation in aggressive social behaviors (Nelson and Trainor, 2007), and the important roles in the integration of olfactory information (Rolls and Baylis, 1994). To better clarify the links between Dlgap2 function, olfactory perception and social behaviors, further studies of the orbitofrontal cortex are need.

IV-4 Olfactory deficit might be an endophenotype of autism

The abnormal olfaction function such as odor perception in *Dlgap2* KO mice also corresponded to the olfactory deficits in patients with autism (Bennetto et al., 2007, Baron-Cohen et al., 2009). These clinical findings as well as our observations implied that the olfactory problem might be considered as an autistic endophenotype

which may advance the understanding of the etiology of autism. These finding might facilitate the development of diagnostic technique for the early detection of autism.

V. Conclusion

Using this autism-related gene *Dlgap2* KO mice model, the association between olfaction and social behaviors was established. Our results also revealed the first time that Dlgap 2, a postsynaptic protein is required for other synaptic proteins formation in the olfactory bulb and the dendritic development of neurons in the piriform cortex. In *Dlgap2* KO mice, dendritic abnormality in the olfactory system might disturb the detection or interpretation of social signals which might account for social odor-induced hyperactivity. However; further studies are needed to establish the causal link between olfactory impairments and autistic phenotypes.

Figure legends

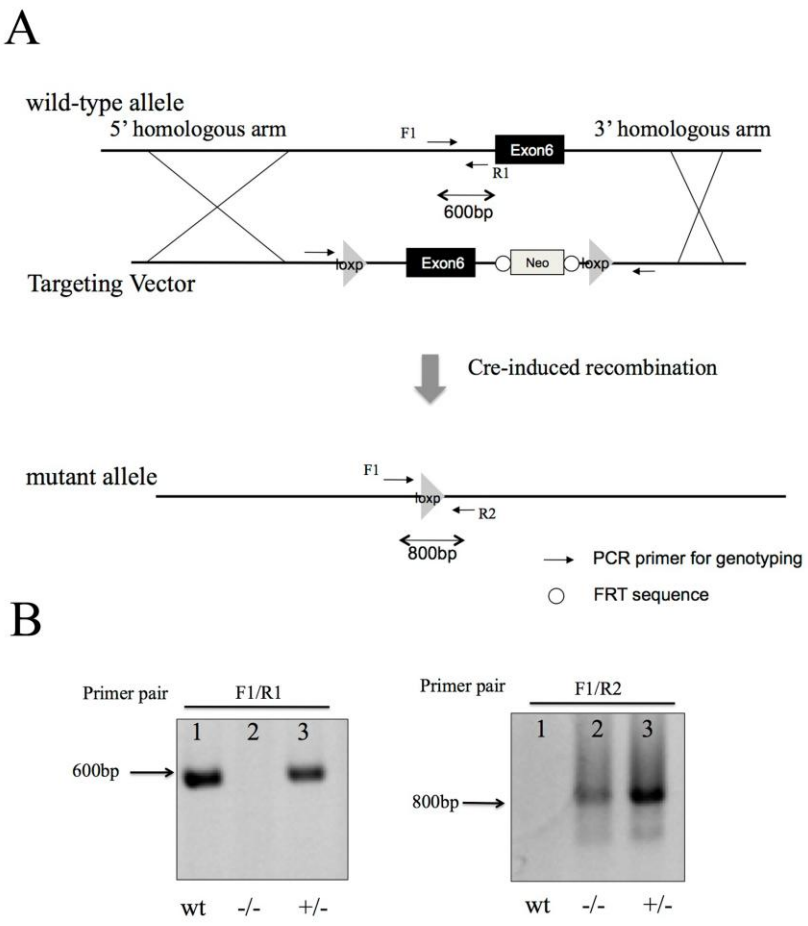

Figure 1. Generation and genotyping of *Dlgap2* knockout (KO) mice.

(A) Generation of *Dlgap2* knockout (KO) mice. Exon 6 of Dlgap2 gene of wild-type allele was replaced by targeting vector containing Neo cassette and flanked by two loxP sites. After induced Cre recombination, exon 6 of Dlgap2 gene was deleted. (B) Genotypes of mice were determined by PCR.

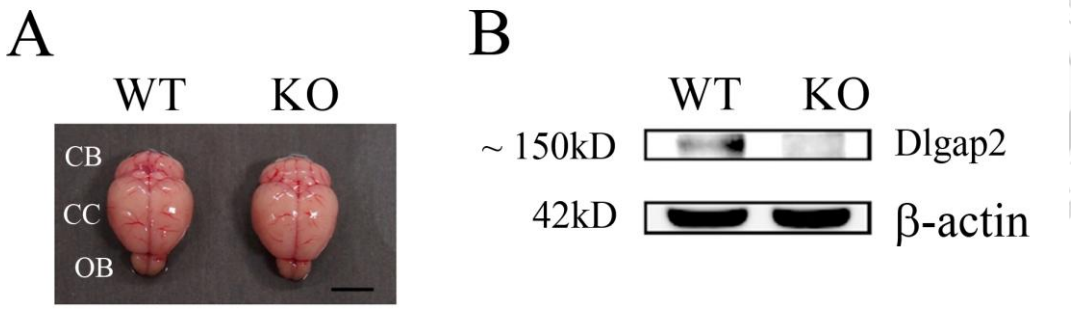

Figure 2. Gross brain morphology and Dlgap2 protein expression.

(A) The size of the olfactory bulb (OB), cerebral cortex (CC) and cerebellum (CB) were comparable between adult WT and *Dlgap2* KO mice. Scale bar = 5 mm. (B) Dlgap2 protein expression was revealed by immunoblotting. Dlgap2 protein was expressed in the olfactory bulb of WT mice but not in KO mice. β-actin was used as internal control.

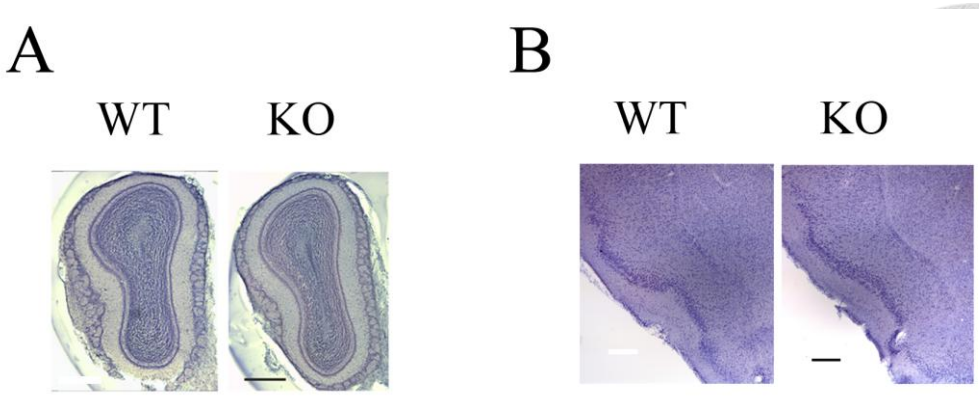

Figure 3. Gross structures of the olfactory system.

(A) Hematoxylin stain of the olfactory bulb and the cellular patterns were comparable between WT and *Dlgap2* KO mice. (B) Hematoxylin stain of the piriform cortex and the cellular patterns were comparable between WT and KO mice. Scale bar = 2.5 mm.

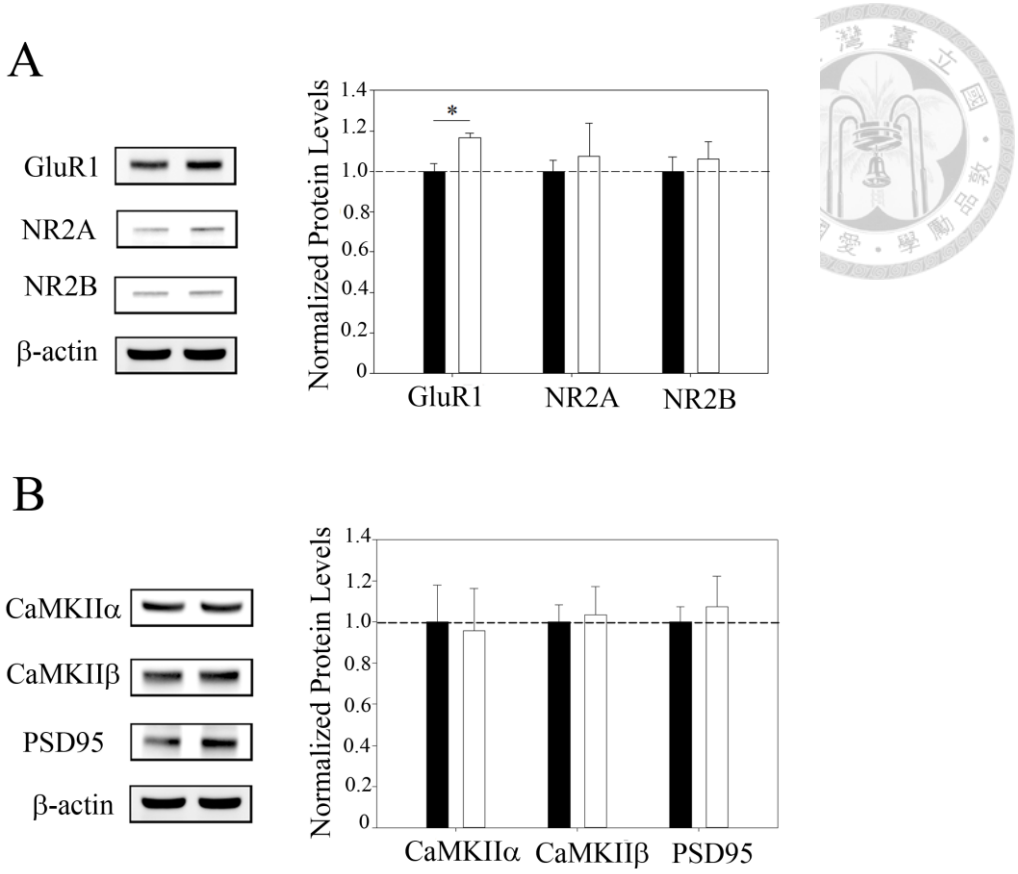

Figure 4. Expression of synaptic proteins in the olfactory bulb.

Expression of synaptic proteins including (A) receptor proteins, such as GluR1, NR2A, NR2B and (B), signaling and scaffold proteins such as CaMKIIα, CaMKIIβ and PSD95 was examined in the olfactory bulb (OB) of WT (n=3) and *Dlgap2* KO (n=3) mice using western blot analysis. Compared to WT (expression was adjusted as 1), GluR1 was up-regulated in KO whereas other proteins were not altered. Data are mean ± SEM. Asterisks are significant differences comparing to WT (*, $p<0.05$).

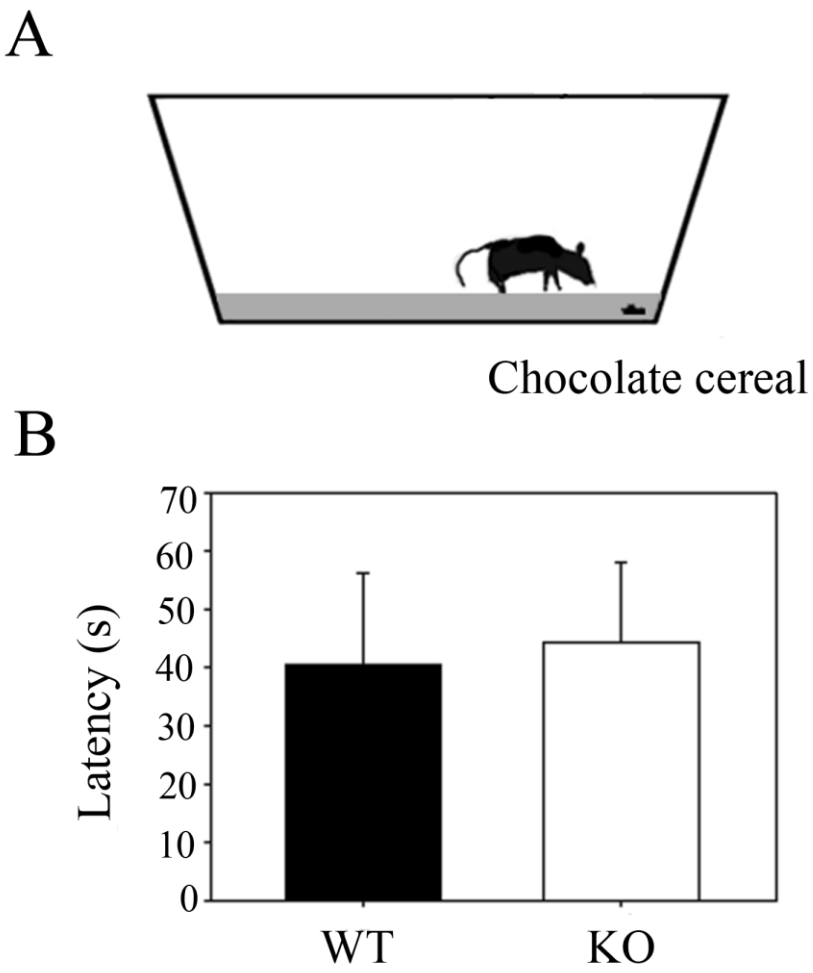

Figure 5. Buried food test.

(A) The setup of buried food test. After food-deprivation, each mouse was released in to a cage in which a small piece of chocolate cereal was buried. (B) The latency to find the buried food in WT (n = 6) and *Dlgap2* KO (n = 6) mice was not distinguishable, indicating the ability of olfactory detection in WT and KO mice was comparable.

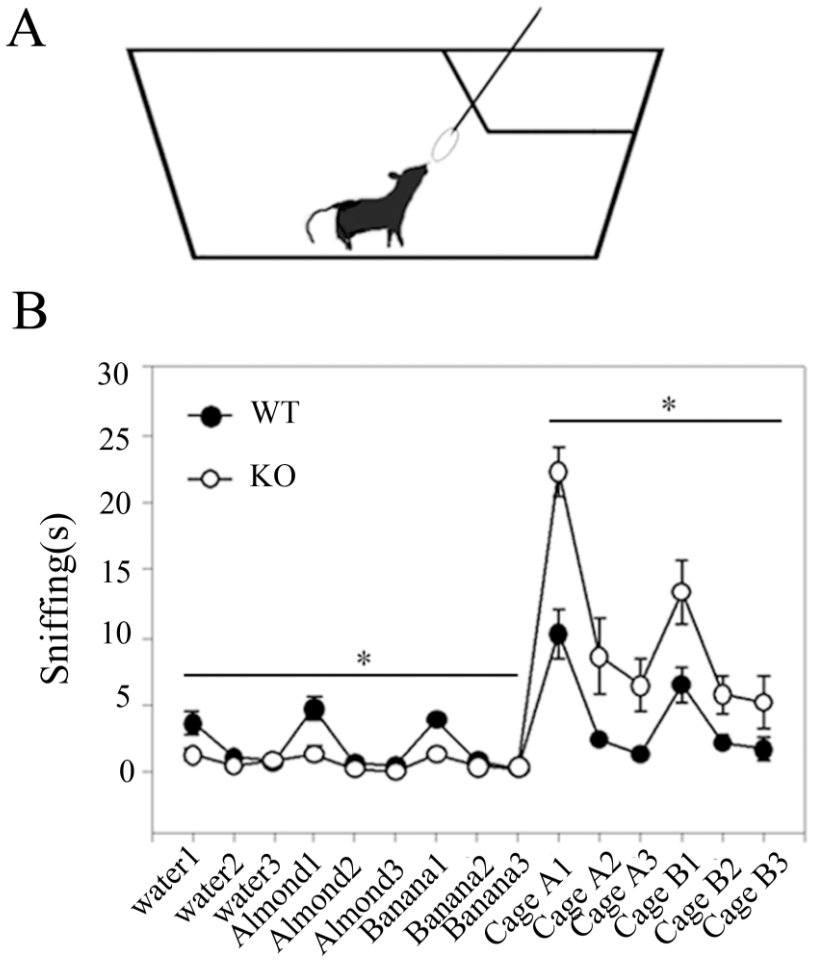

Figure 6. Olfactory habituation/ dishabituation test.

(A) The setup of the habituation/dishabituation test. (B) Left. While exposing to natural odors (water, almond and banana), both WT and *Dlgap2* KO mice exhibited patterns of habituation/dishabituation in the sniffing time, indicating that mice in two groups were able to discriminate different natural odors. However, KO mice sniffed fewer amount of time toward natural odors. Right. While exposing to social odors (beddings from cage A and cage B), KO mice exhibited hyperactivity toward social odors. Data are mean ± SEM. *Asterisks* are used to indicate significant difference between WT and KO mice (*$p < 0.05$).

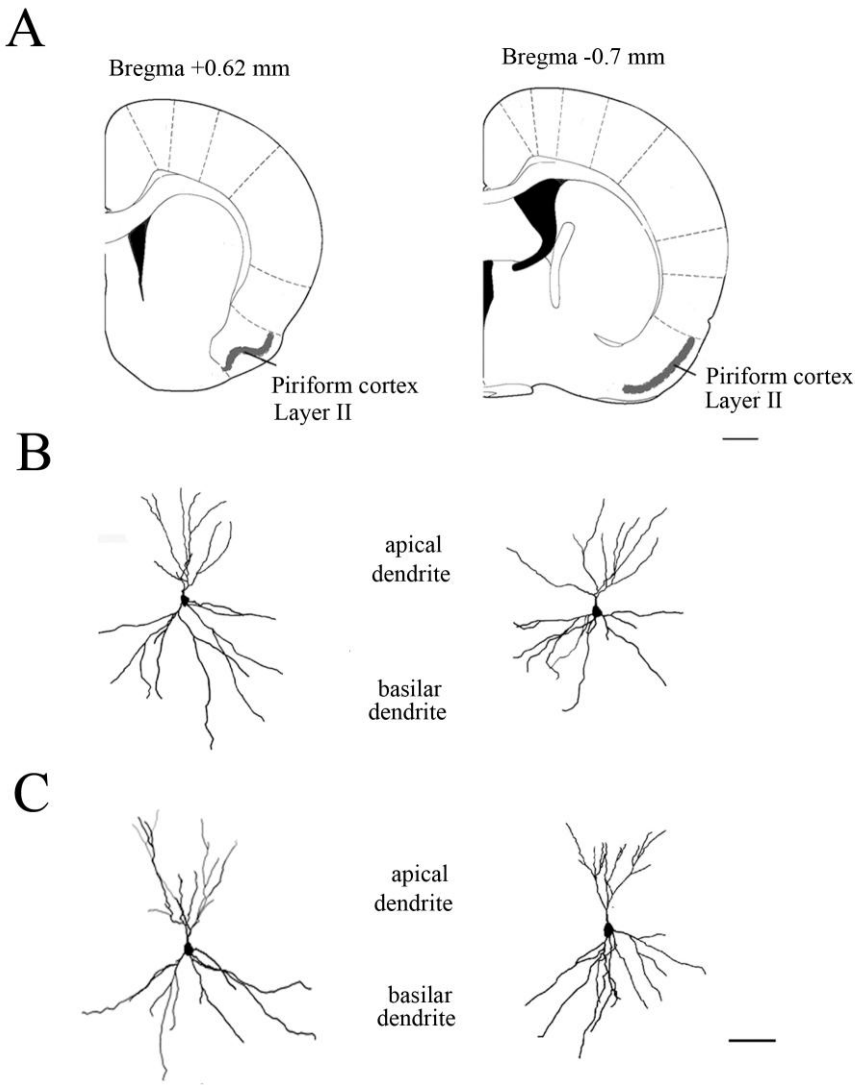

Figure 7. Superficial pyramidal cells in the piriform cortex.

(A) The superficial layer, layer II is marked gray in the illustration Scale bar= 1 cm. Examples of Golgi-stained layer II pyramidal neurons in WT (B) and Dlgap2 KO (C) mice. Note the apical and basilar dendrites. Usually, superficial pyramidal neurons have one apical and several basilar dendrites. Scale bar = 100 μm.

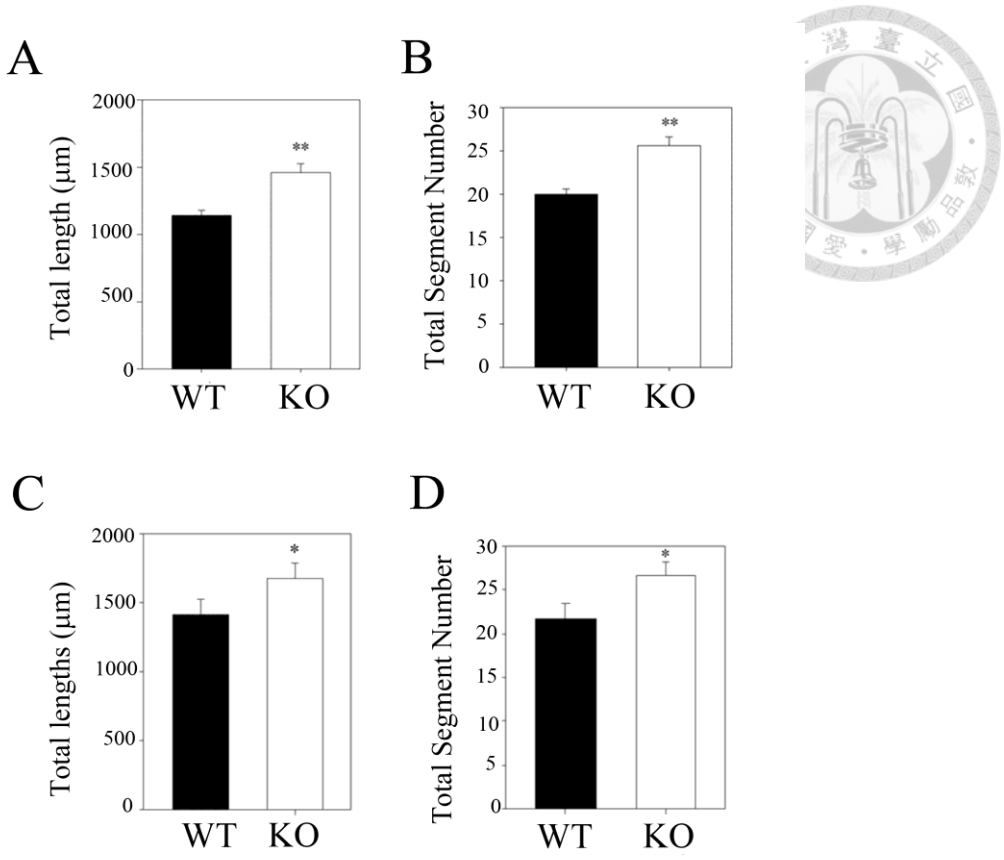

Figure 8. Morphometric analyses of superficial pyramidal cells in piriform cortex. In both apical dendrites (A and B) and basilar dendrites (C and D), neurons in *Dlgap2* KO mice had longer total dendritic length (A and C) and greater number of total dendritic segments (B and D) compared with those in WT mice. Data are mean ± SEM. *Asterisks* are used to indicate significant difference (*, $p<0.05$**, $p<0.001$). For apical dendrites, n = 55 cases from 10 WT and n = 64 cases from 10 KO mice; for basilar dendrites, n = 25 cases from 7 WT and n = 40 cases from 8 KO mice.

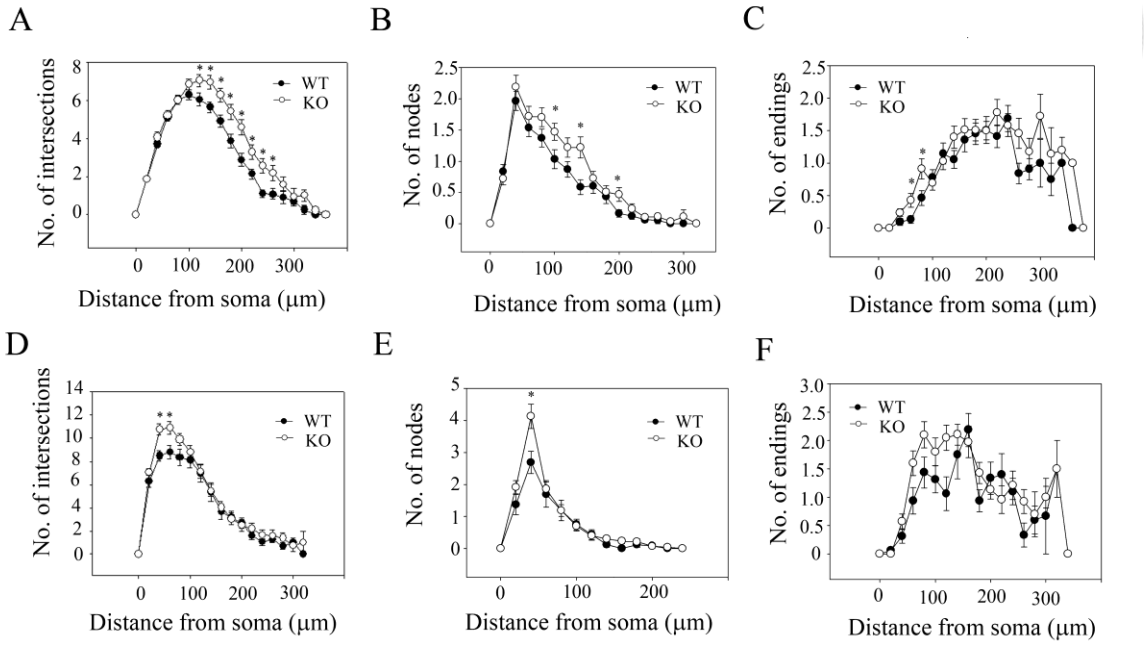

Figure 9. Sholl analyses of superficial pyramidal cells in piriform cortex.

The complexity of dendritic arbors was analyzed using the concentric-ring method of Sholl. The dendritic complexity was reflected by the numbers of intersections, nodes and endings between the dendritic branches and concentric rings. In both apical and basilar dendrites greater intersection and nodes were noticed in neurons from *Dlgap2* KO mice, especially in the distal regions of the apical dendrites (A and B) and proximal regions of the basilar dendrites (D and E). However, the number of endings was comparable between genotypes (C and F). Data are mean ± SEM. *Asterisks* are used to indicate significant difference (*, $p<0.05$). (For apical dendrites, n = 55 neurons in WT and n = 64 neurons in KO mice; for basilar dendrites, n = 25 neurons in WT and n = 40 neurons in KO mice.)

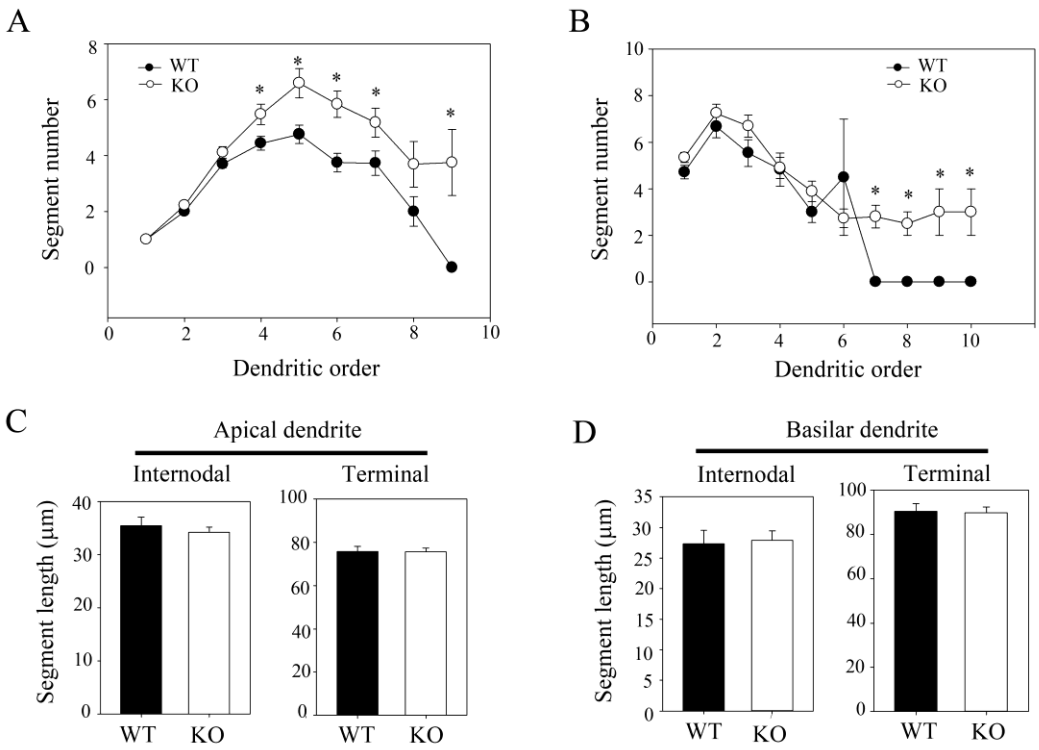

Figure 10. Segment analyses of superficial pyramidal cells in piriform cortex.

(A) The segment numbers were increased as dendritic orders were higher in apical dendrites. (B) There were no segments when dendritic orders were higher than seventh in basilar dendrites of *Dlgap2* KO mice. (C and D) Differentiate dendrites into internodal and terminal parts. (C) The segments of apical dendrites were no different between WT and KO mice. (D) The segments of basilar dendrites were no different between WT and KO mice. Data are mean ± SEM. *Asterisks* are used to indicate significant difference (*, *p<0.05;***,p<0.01).*

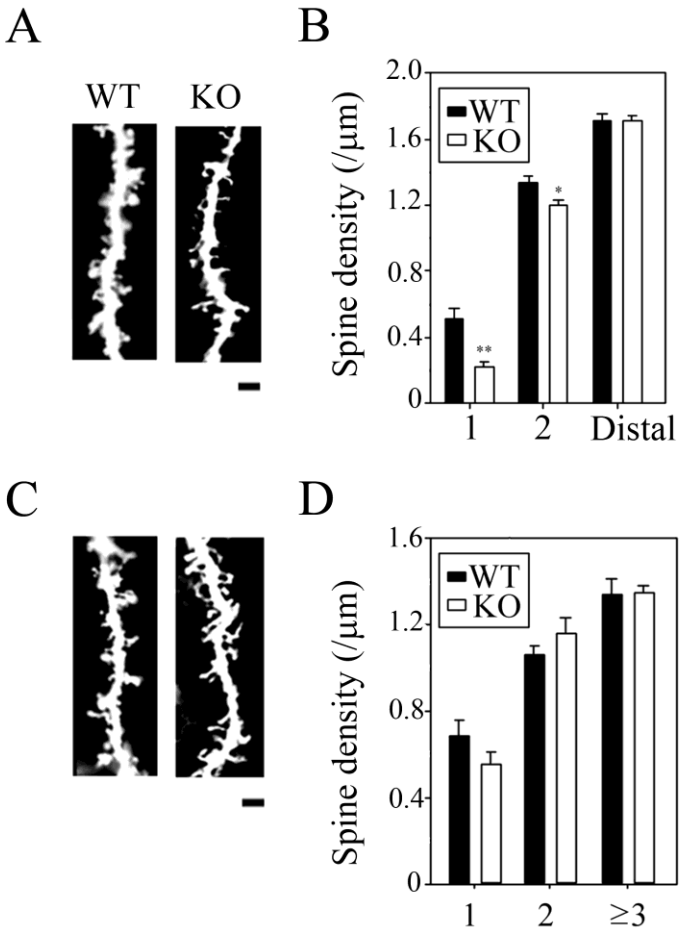

Figure 11. Spine density of superficial pyramidal cells in the piriform cortex.

(A) Images of proximal apical dendrites from neurons of WT and *Dlgap2* KO mice. (B) In apical dendrite, the spine density of was lower in KO mice particularly in the proximal regions (orders 1 and 2). (C) Images of proximal basilar dendrites. (D) The spine density in the basilar dendrites was comparable between two genotypes. Data are mean ± SEM. *Asterisks* are used to indicate significant difference (*, *p<0.05;**, *p<0.001*). Scale bar = 2.5 μm.

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