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Mouse pups lacking collapsin response mediator protein 4 manifest impaired olfactory function and hyperactivity in the olfactory bulb

Atsuhiro Tsutiya,[1] Masugi Nishihara,[2] Yoshio Goshima[3] and Ritsuko Ohtani-Kaneko[1,4]
[1]Graduate School of Life Sciences, Toyo University, 1-1-1 Itakura, Oura, Gunma 374-0193, Japan
[2]Department of Veterinary Physiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
[3]Department of Molecular Pharmacology and Neurobiology, Yokohama City University Graduate School of Medicine, Yokohama, Japan
[4]Bio-Nano Electronic Research Centre, Toyo University, Kawagoe, Saitama, Japan

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Abstract

Members of the collapsin response mediator protein (CRMP) family are reported to be involved in the pathogenesis of various neuronal disorders, including schizophrenia and autism. One of them, CRMP4, is reported to participate in aspects of neuronal development, such as axonal guidance and dendritic development. However, no physiological or behavioral phenotypes in *Crmp4* knockout (*Crmp4*-KO) mice have been identified, making it difficult to elucidate the *in vivo* roles of CRMP4. Focusing on the olfaction process because of the previous study showing strong expression of *Crmp4* mRNA in the olfactory bulb (OB) during the early postnatal period, it was aimed to test the hypothesis that *Crmp4*-KO pups would exhibit abnormal olfaction. Based on measurements of their ultrasonic vocalizations, impaired olfactory ability in *Crmp4*-KO pups was found. In addition, c-Fos expression, a marker of neuron activity, revealed hyperactivity in the OB of *Crmp4*-KO pups compared with wild-types following exposure to an odorant. Moreover, the mRNA and protein expression levels of glutamate receptor 1 (GluR1) and 2 (GluR2) were exaggerated in *Crmp4*-KO pups relative to other excitatory and inhibitory receptors and transporters, raising the possibility that enhanced expression of these excitatory receptors contributes to the hyperactivity phenotype and impairs olfactory ability. This study provides evidence for an animal model for elucidating the roles of CRMP4 in the development of higher brain functions as well as for elucidating the developmental regulatory mechanisms controlling the activity of the neural circuitry.

Introduction

During the development of complex neural circuits, axonal guidance molecules lead axons to their correct targets through a combination of repulsive and attractive forces. Semaphorin 3A is a typical repulsive guidance molecule for axons, and it is secreted to induce growth cone collapse and axon retraction (Nakamura *et al.*, 2000). The members of the collapsin response mediator protein (CRMP) family (CRMP1–5) were originally identified as intracellular signaling mediators of semaphorin 3A-induced growth cone collapse (Goshima *et al.*, 1995). Since then, CRMPs have been shown to be expressed and function in developing and adult brains (Charrier *et al.*, 2003; Bretin *et al.*, 2005; Veyrac *et al.*, 2005, 2011; Laeremans *et al.*, 2013). Cellular-level studies have revealed that CRMPs are involved in multiple aspects of neuronal development, such as migration (CRMP1, Yamashita *et al.*, 2006; CRMP2, Ip *et al.*, 2011), axonal guidance (CRMP2, Arimura *et al.*, 2005; CRMP4, Khazaei *et al.*, 2014), dendritic organization (CRMP3, Quach *et al.*, 2008; CRMP4, Niisato *et al.*, 2012), dendritic spine development (CRMP1, Yamashita *et al.*, 2007) and synaptic plasticity (CRMP5, Yamashita *et al.*, 2011). These studies indicate that CRMPs play important roles in the formation of neuronal circuits by regulating neuronal development.

In addition, the following studies implicate CRMPs in neurodevelopmental disorders, including schizophrenia and autism spectrum disorder (ASD), which display an endophenotype of abnormal neural activity during social cognition tasks. A gene polymorphism of CRMP2 and altered expression levels of CRMP1 and CRMP2 have been found in schizophrenia (Johnston-Wilson *et al.*, 2000; Hong *et al.*, 2005; Beasley *et al.*, 2006; Bader *et al.*, 2012). Moreover, in patients with ASD, CRMP1 and CRMP2 have been identified as two of the antigens active in maternal autoantibody-related autism (Braunschweig *et al.*, 2013). However, in spite of reviews dealing with the roles of CRMPs at the cellular level, their roles in determining higher brain functions are largely unknown.

There has been only one study reporting that *Crmp1*-deficient mice (*Crmp1*$^{-/-}$) exhibited behavioral abnormalities relevant to schizophrenia (Yamashita *et al.*, 2013). Additional animal models will be necessary to investigate the *in vivo* roles of the CRMPs in

Correspondence: Prof. R. Ohtani-Kaneko, [1]Graduate School of Life Science, as above.
E-mail: r-kaneko@toyo.jp

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higher brain functions. In this study, the focus was on CRMP4, which with CRMP2 synergistically regulates neuronal development (Niisato *et al.*, 2013) and is suggested to play an important role in the function of the cat visual cortex (Cnops *et al.*, 2006). Although a few studies using *Crmp4*-knockout (*Crmp4*-KO) mice have been reported (Niisato *et al.*, 2012; Iwakura *et al.*, 2013; Khazaei *et al.*, 2014), no physiological or behavioral phenotypes of the mutant have been discovered. Because previously strong expression of *Crmp4* mRNA in the olfactory bulb (OB) was found during the early postnatal period (Tsutiya & Ohtani-Kaneko, 2012), suggesting crucial roles for CRMP4 in OB development, it was hypothesized that *Crmp4* deletion would impair olfaction during this period. It was aimed to assess olfactory function in *Crmp4*-KO pups and to study the possible mechanisms underlying any impaired olfactory function found.

Materials and methods

Animals

Crmp4-KO mice (Acc. No. CDB0637K: http://www.cdb.riken.jp/arg/mutant%20mice%20list.html) were established as previously described (Niisato *et al.*, 2012). Briefly, the 5-kb 5′-arm and 1.2-kb 3′-arm of a targeting vector obtained by polymerase chain reaction (PCR) were inserted into the 5′ and 3′ cloning sites of the DT-A/loxP/PGK-Neo-pA/loxP vector. Targeting vectors were linearized and electroporated into TT2 ES cells (Yagi *et al.*, 1993), and homologous recombinant ES clones were used to produce chimeras. After confirming germline transmission, heterozygotes were intercrossed to obtain homozygous mutant mice and maintained on a C57BL/6 × CBA hybrid background. Age-matched wild-type (WT) littermates were used as controls. The mice were maintained on a 12 h light/dark cycle with food and water available *ad libitum*. The use and care of the animals were reviewed and approved by the Institutional Animal Care and Use and Ethical Committee of the University of Tokyo. The total number of animals used was 71.

Ultrasonic vocalizations (UVs)

UVs emitted by WT and *Crmp4*-KO pups, which are one of the easily detectable olfaction-dependent behaviors in pups, were assessed. UV assessment was performed at postnatal day (PD) 7. Detection of UVs was performed according to a previously reported method (Kikusui *et al.*, 2011). A condenser microphone designed to capture frequencies between 10 and 200 kHz (UltraSoundGate CM16; Avisoft Bioacoustics, Glienicke, Germany) was used to detect UVs. The microphone was connected to an A/D converter (UltraSoundGate 116Hn; Avisoft Bioacoustics) with a sampling rate of 375 kHz, and the acoustic signals were transmitted to a sound analysis system (SASLab Pro; Avisoft Bioacoustics).

Male WT and *Crmp4*-KO littermates at PD7 were used for the UV studies. First, whether isolation, physical manipulation or exposure to thermal change (from room temperature to 14 °C) would induce different UV responses between WT (n = 7) and *Crmp4*-KO (n = 4) pups was examined. A pup was isolated in a clean plastic case and UVs were recorded for 5 min at room temperature. After this recording, the pup was immediately moved to a clean glass beaker maintained at 14 °C and UVs were again recorded for 5 min.

Second, olfactory discrimination tests were performed by measuring the number of UVs emitted from pups under two olfactory stimuli: familiar nest odors and unfamiliar ones. For this test, male WT (n = 10) and *Crmp4*-KO pups (n = 13) at PD7 were used. A pup was isolated in a clean glass beaker with nest bedding as a familiar odor, and UVs were recorded for 5 min. After the recording, the pup was immediately returned to its mother in the home cage and left there for 1 h. Subsequently, the pup was again isolated in another clean glass beaker with new bedding (clean, unfamiliar bedding), and UVs were recorded for 5 min. After this recording, the pup was returned to its home cage.

Spectrograms were generated with a fast Fourier transform length of 1024 points and a time-window overlap of 50% (100% frames size, Hamming window) for analysing UVs. A lower cut-off frequency of 20 kHz was used to reduce background noise outside of the relevant frequency band. The number of UVs was counted in each subject and compared between WT and *Crmp4*-KO mice.

Odor-evoked c-Fos expression in the OB

To compare the physiological features of the OB between *Crmp4*-KO and WT pups, the expression of c-Fos, an activity-dependent neuronal marker, in the OB at PD7, was studied after exposure to a simple olfactory stimulus. Because bedding odor is a mixture of odorants, it is not an appropriate stimulus for clear localization of c-Fos-positive neurons in the OB. In the present study, ethyl acetate (EA) was chosen as an olfactory stimulant as it is a standard non-biological odorant used to analyse odor maps in the OB in adult rats and mice (Johnson *et al.*, 2004; Salcedo *et al.*, 2005).

c-Fos expression in the OB induced in WT and *Crmp4*-KO mice by olfactory stimulation at PD7 was investigated. In all, 16 pups were used for this phase of the study. All the following procedures were performed in an incubator at 35 °C. A tight-seal polypropylene box (24.5 × 18.5 × 10.2 cm) containing 270 μL of EA was used for exposing mice to the olfactory stimulus. First, a pup was isolated in a clean case (8 × 8 × 2.5 cm) for 5 min, then it was exposed to EA in the polypropylene box for 5 min, then it was returned to the clean case. This procedure was repeated three times. The control pups were treated in the same way as EA-treated mice but without exposure to EA. The pup was then returned to the clean case and was deeply anesthetized with pentobarbital (50 mg/kg, i.p.) 90 min after the onset of odorant exposure. The pup was transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3). After the brains were removed and fixed with 4% paraformaldehyde in PB overnight at 4 °C, they were rinsed with phosphate-buffered saline (PBS), passed through sucrose solutions of 10, 20 and 30% in PB for 8–12 h each at 4 °C, and then embedded in optimal cutting temperature compound (Sakura Finetek Japan, Tokyo, Japan). Serial frontal cryosections (12 μm thick) of the OB were then cut with a cryostat (CM-3050-S; Leica Microsystems, Wetzlar, Germany) and thaw-mounted onto five sets of MAS-coated glass slides (Matsunami Glass Ind., Osaka, Japan). A series of every fifth section (spaced 60 μm apart) was stained with Cresyl violet (Muto Pure Chemicals, Tokyo, Japan) to determine the neuroanatomical structures. Another series of every fifth section was used for immunohistochemistry to detect c-Fos expression.

For c-Fos immunohistochemistry, sections were treated with 0.3% H_2O_2 in methanol for 30 min (Wako Pure Chemical Industries), followed by incubation with a blocking solution [10% blocking reagent (Takara Bio, Otsu, Japan) and 0.03% Triton-X in PBS] for 1 h at room temperature. The sections were then incubated for 48 h at 4 °C with a 1 : 20 000 dilution of rabbit anti-c-Fos polyclonal antibody (Ab-5; Cat. PC38; Calbiochem, San Diego, CA, USA) in the blocking solution and were then incubated with a biotinylated goat anti-rabbit secondary antibody (BA1000; Vector Laboratories, Burlingame, CA, USA; 1 : 500) for 2 h at room temperature. The sections were placed

TABLE 1. qRT-PCR primer sequences

Target gene	Direction	Primer sequence (5′–3′)	Product size (bp)
Glur1	Forward	CCCTTTACAACGTGGAGGAA	152
	Reverse	GAACAAGGGCGTCTCTTCTG	
Glur2	Forward	ATTTCGGGTAGGGATGGTTC	225
	Reverse	GTTGGGAAGCTTGGTGTGATG	
Glur3	Forward	AACCTTGCTGCTTTCCTGAC	83
	Reverse	GCAATTTCAGTCTGCTTGG	
Glur4	Forward	TACATTGGTGTCAGCGTGG	97
	Reverse	CACTGGGTCCTTCTTTTCC	
Vglut1	Forward	TGGGTTTCTGTATCAGCTTTG	74
	Reverse	TGTGCTGTTGTTGACCATGGACACG	
Vglut2	Forward	CGTGAAGAATGGCAGTATGTCTTC	81
	Reverse	TGAGGCAAATAGTGCATAAAATATGACT	
Gad67	Forward	CTCAGGCTGTATGTCAGATGTTC	111
	Reverse	AAGCGAGTCACAGAGATTGGTC	
Vgat	Forward	CCATTGGCATCATCGTGTT	101
	Reverse	CCAGTTCATCATGCAGTGGAA	
GabaAα1	Forward	AAGGACCCATGACAGTGCTC	149
	Reverse	CAGAGTGCCATCCTCTGTGA	
GabaAβ2	Forward	AACGCCTTCCATCATTGTTC	148
	Reverse	ATCACCACTCCACGACATCA	
GabaBr1	Forward	TCTGGTTGTGCTCTTTGTGC	111
	Reverse	TCCTCATTGTTGTTGGTGGA	
Parvalbumin	Forward	TTCTGGACAAAGACAAAAGTGG	72
	Reverse	CTGAGGAGAAGCCCTTCAGA	
Synapsin1	Forward	CACCGACTGGGCAAAATAC	140
	Reverse	TCCGAAGAACTTCCATGTCC	
β-*actin*	Forward	GCTACAGCTTCACCACCACA	123
	Reverse	TCTCCAGGGAGGAAGAGGAT	

in ABC-peroxidase reagent (Vector Laboratories) and visualized with 3,3′-diaminobenzidine (Wako Pure Chemical Industries).

c-Fos immunohistochemistry was also performed in combination with Nissl staining to examine whether neuronal nuclei are specifically stained with c-Fos antibody. Sections were processed for c-Fos immunohistochemistry as described above without nickel intensification, and then stained with Cresyl violet (Nissl staining) before coverslipping.

Sixteen sections of the OB per animal (interval = 60 μm), located rostral to the front of the accessory OB, were immunohistochemically stained with c-Fos antibody and used for counting. c-Fos-positive cells were detected in the following layers: the glomerular layer (GL); the external plexiform layer (EPL); the mitral cell layer (MCL); and the granule cell layer (GCL). c-Fos-positive cells were then counted exhaustively in each whole layer under a microscope (CX40, Olympus), within each of 16 sections. For each section, the average number of c-Fos-positive cells in each layer was calculated, combining across animals in a given treatment group, and the results statistically compared between a control group and an EA-treated group of the same genotype. Cell counting was performed by investigators blinded to treatments.

Real-time quantitative reverse transcription (qRT)-PCR and Western blotting

To reveal possible mechanisms underlying the exaggerated activity of OB neurons seen in *Crmp4*-KO pups, the expression levels of genes related to excitatory and inhibitory neurotransmission in the OB were studied. The expression of neurotransmitter-related genes that contribute to neural excitation or inhibition in the OB and of a synaptic marker were examined and compared between WT and *Crmp4*-KO mice by real-time qRT-PCR. Male mice (WT, $n = 4$; *Crmp4*-KO, $n = 4$) at PD7 were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and the OBs were dissected out into RNA-later (Qiagen, Valencia, CA, USA). Total RNA was extracted from the OBs using an RNeasy kit (Qiagen), converted to cDNA using the Reverse Transcription Kit (Qiagen) in preparation for real-time qRT-PCR analysis of mRNA. This was performed using a Thermal Cycler Dice Real Time System TP800 (Takara Bio) according to the manufacturer's protocol. The amplification was achieved in a 20-μL volume containing 1 μL cDNA, 200 nM of each primer pair and SYBR Premix Extaq (Takara Bio). The mouse-specific primer sets used are shown in Table 1. The mRNA expression levels of each gene were standardized using simultaneously measured values of β-actin mRNA.

Next, expression levels of the proteins whose levels of mRNAs showed significant differences between WT and *Crmp4*-KO pups were examined with Western blot analysis. Male WT and *Crmp4*-KO mice at PD7 were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and the OBs were dissected out. The OBs were mixed and homogenized in lysis buffer (30 mM Tris, 2 M thiourea, 7 M urea and 4% CHAPS; pH 8.5). Samples were quickly frozen and kept at −80 °C until use. Standardized amounts of protein from the samples were separated on sodium dodecyl sulfate–polyacrylamide gels and

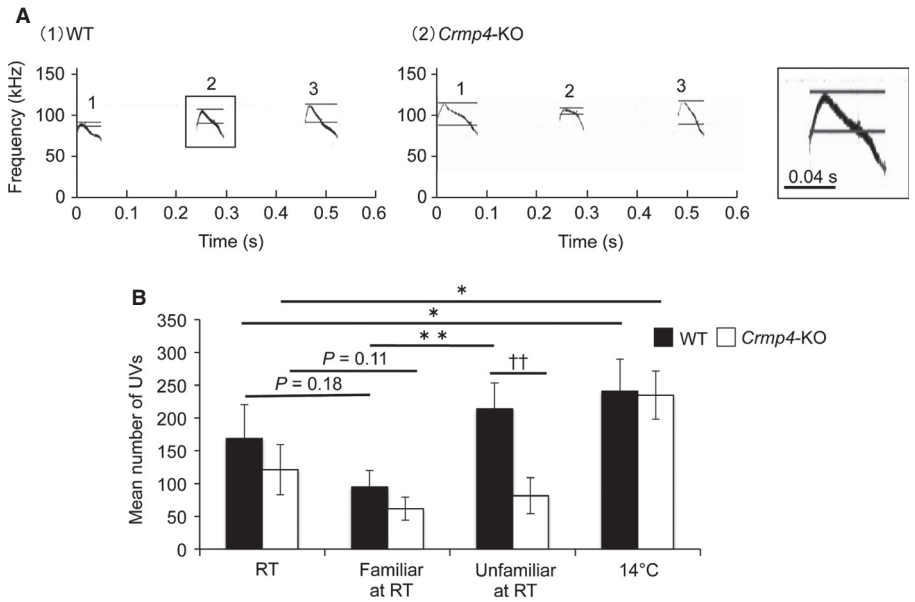

FIG. 1. Olfactory discrimination tests monitoring ultrasonic vocalizations (UVs). (A) Representative sound spectrograms of calls emitted from wild-type (WT) pups and collapsin response mediator protein gene knockout (*Crmp4*-KO) pups, both aged PD7, during isolation at room temperature (RT). A sound spectrogram of a representative call in (A) is shown magnified in the box in the right part of (A), where maximum frequency (upper line), mean frequency (lower line) and duration (length of the lines) of calls are indicated. These three indices were extracted by a sound analysis system (SASLab Pro, Avisoft Bioacoustics), and the number of calls was counted during UV recording. (B) The mean numbers of UVs emitted from WT and *Crmp4*-KO pups were compared at RT and 14 °C without nest bedding present, and at RT in the presence of familiar or unfamiliar nest bedding. The mean numbers of UVs emitted by *Crmp4*-KO and WT pups at 14 °C were significantly different from those at RT (*$P < 0.05$, paired Student's *t*-test). The significant difference in the number of UVs emitted by WT pups is observed between familiar and unfamiliar nest beddings (**$P < 0.01$, paired Student's *t*-test). *Crmp4*-KO pups emitted significantly fewer UVs than WTs when they were exposed to unfamiliar clean bedding from familiar bedding ($^{\dagger\dagger}P < 0.01$, unpaired Student's *t*-test). Values are expressed as means ± SEM.

transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Tokyo, Japan). Each PVDF membrane was divided into two parts after transfer, to provide one part of the membrane for reaction with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody for normalization. The membranes were treated with blocking solution, followed by incubation with a primary antibody overnight at 4 °C. The primary antibodies used in this study were as follows: anti-glutamate receptor (GluR)1 antibody (AB1504, rabbit polyclonal; Millipore; 1 : 1000); anti-GluR2 antibody (MAB397, mouse monoclonal; Millipore; 1 : 1000); anti-GAD67 antibody (MAB5406, mouse monoclonal; Millipore; 1 : 5000); and anti-GAPDH antibody (MAB374, mouse monoclonal; Millipore; 1 : 500). The membranes were incubated with the appropriate species-specific horseradish peroxidase-linked secondary antibody (mouse, 012-23641; Wako; 1 : 5000; rabbit, ab6721; Abcam; 1 : 1000) at room temperature for 1 h, followed by incubation with ECL chemiluminescent reagent (GE Healthcare). The immunoblots were developed using a chemiluminescence detection system (Chemidoc, Bio-Rad), and analysed by Image J software (NIH). Optical density values of protein bands were normalized to the corresponding GAPDH bands to compensate for interlane variability in protein loading. OBs from seven male WT and six *Crmp4*-KO mice were removed for Western blot experiments. The mixture of OBs from two or three mice was used as one sample. Each experiment (WT pups, $n = 3$ samples; *Crmp4*-KOs, $n = 3$) was repeated at least twice.

Statistical analyses

In brief, unpaired or paired Student's *t*-tests were used to analyse recordings from the UV tests. All comparisons of the number of UVs between WT and *Crmp4*-KO pups were performed using unpaired Student's *t*-test. However, when the numbers of UVs were assessed at room temperature and at 14 °C, the same individuals were used. Next, when the olfactory discrimination ability was tested, another group of animals were used. This means that the number of UVs was assessed under familiar and unfamiliar beddings at room temperature using the same individuals within the second group of animals. Therefore, statistical analysis was performed using paired Student's *t*-tests under these two conditions, i.e. the room temperature change and the olfactory stimulant change. For the statistical analysis of c-Fos-positive cells, the number of positive cells in each layer within each section (labeled section numbers 1–16) was statistically compared between control and EA-treated groups of the same genotype by unpaired Student's *t*-test. Unpaired Student's *t*-test was also used for statistical analysis of the expression of mRNA and proteins. In all analyses, *P*-values <0.05 were considered statistically significant.

Results

Crmp4 *deletion disrupts odor discrimination*

There were no significant differences in growth rate between the WT and *Crmp4*-KO mice at any of four time points (PD14, 21, 35 and 56; Table S1), indicating that *Crmp4*-KO pups gained weight normally and received normal nursing care.

Because neonatal mice lack the mobility needed for behavioral tests of olfactory ability such as the 'buried food test', UVs emitted by WT and *Crmp4*-KO pups were assessed, which are one of the easily detectable olfaction-dependent behaviors in pups. UV assessment was performed at PD7 because previous studies have shown that C57/BL6 mice pups produce more UVs at PD7 than at any

other postnatal stage (Fish *et al.*, 2000; Lemasson *et al.*, 2005). It has also been reported that infant rodents emit UVs in a variety of situations, including isolation (Ehret, 2005), physical manipulation (Mällo *et al.*, 2007), exposure to thermal change (Allin & Banks, 1971; Noirot, 1972; Oswalt & Meier, 1975; Blumberg & Alberts, 1990) and exposure to olfactory stimuli (Conely & Bell, 1978; Branchi *et al.*, 1998). Therefore, first, whether *Crmp4*-KO pups produce UV responses similar to WT in cases of isolation, physical manipulation and exposure to thermal change was examined.

When pups were physically manipulated and isolated at room temperature (RT), mean call durations, maximum peak frequencies and mean peak frequencies of the UVs emitted by *Crmp4*-KO pups were comparable to those by WTs (Fig. 1A; Table S2). Mean numbers of UVs emitted by *Crmp4*-KO pups when they were manipulated and isolated at RT or at 14 °C were not significantly different from those emitted by WTs under the same conditions (Fig. 1B). The mean numbers of UVs emitted by *Crmp4*-KO and WT pups at 14 °C were significantly different from those at RT, suggesting that *Crmp4*-KO pups and WTs could respond to decreased temperature by emitting UVs. These studies showed that the ability to emit UVs was not impaired in *Crmp4*-KO mice.

Next, pups were exposed to two different olfactory stimuli (the smell of familiar and unfamiliar nest bedding) and UVs were recorded. The familiar nest bedding and clean bedding were chosen as two different odors in the present study, because it has already been demonstrated in previous studies that these two odor conditions result in different frequencies of UV in WT pups (D'Amato & Cabib, 1987; Branchi *et al.*, 1998; Moles *et al.*, 2004; Zanettini *et al.*, 2010; Wöhr, 2015).

As Fig. 1B shows, mean numbers of UVs emitted by WT and *Crmp4*-KO mice were not significantly different, but tended to be decreased when the pups were exposed to the familiar odor of their nest bedding at RT, compared with those at RT without bedding. These results suggested the possibility that WT and *Crmp4*-KO pups could detect the familiar bedding odor, as previously reported on WT pups in the studies cited above. When the pups were then exposed to the new odor of unfamiliar clean bedding, WT pups produced significantly more UVs than when they were exposed to the familiar odor of their nest bedding ($P < 0.01$, paired Student's *t*-test). However, the mean number of UVs emitted by *Crmp4*-KO pups under exposure to the new odor of unfamiliar bedding was not significantly different from that emitted in the presence of the odor of familiar nest bedding. Hence, *Crmp4*-KO pups emitted significantly fewer UVs than WTs when they were exposed to unfamiliar clean bedding ($P < 0.01$, unpaired Student's *t*-test). These results suggest that *Crmp4*-KO pups have impaired olfactory ability, including possible alteration of discrimination ability and/or altered thresholds for odor detection, compared with WTs.

Crmp4 *deletion induces abnormal c-Fos expression in the OB*

Next, the patterns of c-Fos expression in the OB of WTs were compared in the presence of familiar vs. unfamiliar bedding stimuli. However, a clear difference between these two odors could not be found, because of pervasive c-Fos activation and the complexity of the expression patterns. Therefore, following a previous study (Salcedo *et al.*, 2005), patterns of c-Fos-positive cells were examined due not to the presence of a complex mixture of odorants, but to the presence of a single odorant stimulus (EA).

Therefore, c-Fos expression induced by EA stimulation in the OB of WT and *Crmp4*-KO pups at PD7 was investigated. Male pups of both genotypes were separated into two groups: the control group (WT, $n = 3$; *Crmp4*-KO, $n = 5$); and the EA-treated group (+EA: WT, $n = 3$; *Crmp4*-KO, $n = 5$).

In agreement with Van der Gucht *et al.* (2002), who reported that c-Fos is expressed by neurons upon sensory induction, it was found that c-Fos-positive nuclei in the section of the OB especially stimulated with the odorant EA (Fig. 2A). It was confirmed that c-Fos is expressed in the nuclei of neurons by combined Nissl staining and c-Fos immunohistochemistry (Fig. 2B). Then, 16 frontal sections per animal were immunohistochemically stained (interval = 60 μm) rostral to the front of the accessory OB with c-Fos antibody (Fig. 2C). Then, the expression pattern of c-Fos-positive cells in the OB between WT pups and *Crmp4*-KOs with and without the odorant stimulus EA was compared (Fig. 2D–W). In WT pups without EA stimulation, only a few c-Fos-positive cells were observed in different sections of the OB (Fig. 2D–H). Similarly, only a few c-Fos-positive cells were found in the OB of *Crmp4*-KO pups without odorant stimulation (Fig. 2N–R). On the other hand, many c-Fos-positive cells were detected in WTs treated with EA (Fig. 2I–M), especially in the MCL and GCL on section number 11 (Fig. 2L) and in the MCL on section 13 (Fig. 2M). However, the greatest expression was seen in *Crmp4-KO* pups treated with EA, many c-Fos-positive cells being observed in most of the layers and in most of the sections (Fig. 2S–W). To determine the localization of c-Fos-positive cells more precisely, the exact number of c-Fos-positive cells in layers where positive cells were found were counted, namely the GL, EPL, MCL and GCL of the OB (Fig. 3).

There were few c-Fos-positive cells in any of the layers of the OB in control WT pups (white circles in Fig. 3A, C, E and G). EA significantly increased the number of c-Fos-positive cells compared with the number in control WTs (black circles) only in restricted regions of the OB, specifically, in the 11th section in the EPL, in the 11th and 12th sections in the MCL, and in the 11th section in the GCL (white vs. black circles in Fig. 3C, E, and G; $P < 0.05$, unpaired Student's *t*-test). In the remaining sections, mean numbers of c-Fos-positive cells in the OB of EA-treated WTs were not significantly different from those of controls.

In the OB of control *Crmp4*-KO pups, only a few c-Fos-positive cells were detected, similarly to control WTs (white circles in Fig. 3B, D, F and H). However, after EA stimulation, the numbers of c-Fos-positive cells were dramatically and broadly increased in all four layers in most sections in *Crmp4*-KO mice (black circles in Fig. 3B, D, F, and H), compared with those in the respective sections of control *Crmp4*-KO mice (white circles). Specifically, c-Fos-positive cells were significantly increased in the GL on section nos. 3, 5, 6, 9, 11, 12, 13 and 14 in EA-treated *Crmp4*-KO pups, compared with those in control *Crmp4*-KO pups (Fig. 3B; $P < 0.05$, unpaired Student's *t*-test). In the EPL, the number of c-Fos-positive cells was significantly increased in section no. 9 of EA-treated *Crmp4*-KO pups, compared with *Crmp4*-KO unstimulated controls (Fig. 3D; $P < 0.05$, unpaired Student's *t*-test). In the MCL and GCL of *Crmp4*-KO mice, significant EA-induced increases in c-Fos-positive cells were detected in the MCL on sections nos. 5, 9 and 13, and in the GCL on all sections except nos. 1, 4, 10 and 16 (Fig. 3F and H; $P < 0.05$, unpaired Student's *t*-test).

Therefore, the EA-elicited c-Fos expression in the OB is much greater in *Crmp4*-KO pups than in WTs. While EA-elicited c-Fos-expression was localized in WT pups, such a localization pattern of c-Fos-positive cells was not clear in EA-treated *Crmp4*-KO mice. Instead, EA caused widespread and diffuse expression of c-Fos in the OB of *Crmp4*-KO pups.

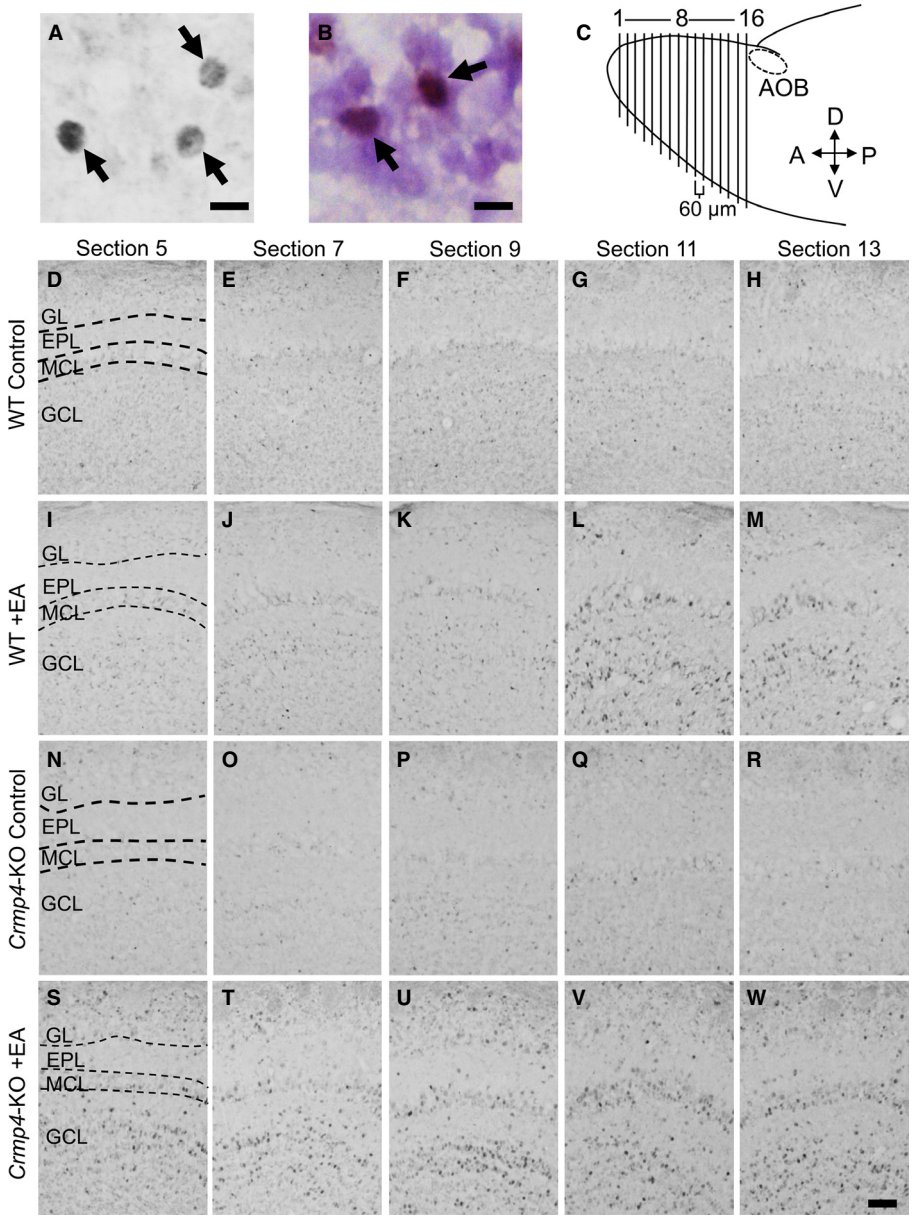

FIG. 2. c-Fos expression in the olfactory bulb (OB) of wild-type (WT) and collapsin response mediator protein gene knockout (*Crmp4*-KO) pups. (A) A representative micrograph showing cells with c-Fos-positive nuclei. The number of cells with strong c-Fos-positive nuclei (arrows) was counted as c-Fos-positive in the following experiments. (B) A representative picture of a section of the OB that has been Nissl- and c-Fos-immunostained. c-Fos-positive cells with black immunoreactivity in the nucleus are identified as neurons by Nissl staining (arrows). (C) Schematic image of sections on which c-Fos-positive cells were counted. Sixteen sections (interval = 60 μm) of the OB located rostral to the front of the accessory OB (AOB) were used. (D–W) Representative micrographs showing c-Fos-positive cells in the OB. Section numbers (5, 7, 9, 11 and 13) indicate the location of cross-sections in the OB (schematically illustrated in C). (D–H) c-Fos expression in the WT pups without ethyl acetate (EA) stimulation. (I–M) c-Fos expression in the WT pups with EA stimulation. (N–R) c-Fos expression in the *Crmp4*-KO pups without EA stimulation. (S–W) c-Fos expression in the *Crmp4*-KO pups with EA stimulation. GL, EPL, MCL and GCL indicate the glomerular layer, external plexiform layer, mitral cell layer and granule cell layer, respectively. c-Fos-positive cells were counted in these layers (Fig. 3). Scale bars: 20 μm (A); 20 μm (B); and 100 μm (W).

Crmp4 *deletion increases the expression of GluR1 and GluR2 in the OB*

Among mRNAs examined in this study (Table 2), real-time qRT-PCR analysis showed significantly higher expression levels of glutamate receptors 1 (*Glur1*) and *Glur2* in *Crmp4*-KO pups compared with WTs (Table 2; $P < 0.05$, unpaired Student's *t*-test). These receptors are members of the AMPA-type receptor family that mediates fast excitatory transmission. However, mRNA expression levels of other members of this family (*Glur3* and *Glur4*) were not significantly different between *Crmp4*-KO and WT pups. The expression levels of glutamate transporters (*Vglut1* and *Vglut2*), glutamate decarboxylase 67 (*Gad67),* vesicular γ-aminobutyric acid (GABA) transporter (*Vgat*), GABA receptors (*GabaAα1*, *GabaAβ2* and *GabaBr1*) and *parvalbumin* were comparable between *Crmp4*-KO and WT pups. Also, mRNA expression levels of *synapsin1*, a synapse-specific marker, were investigated, but no significant difference was detected between *Crmp4*-KO and WT pups.

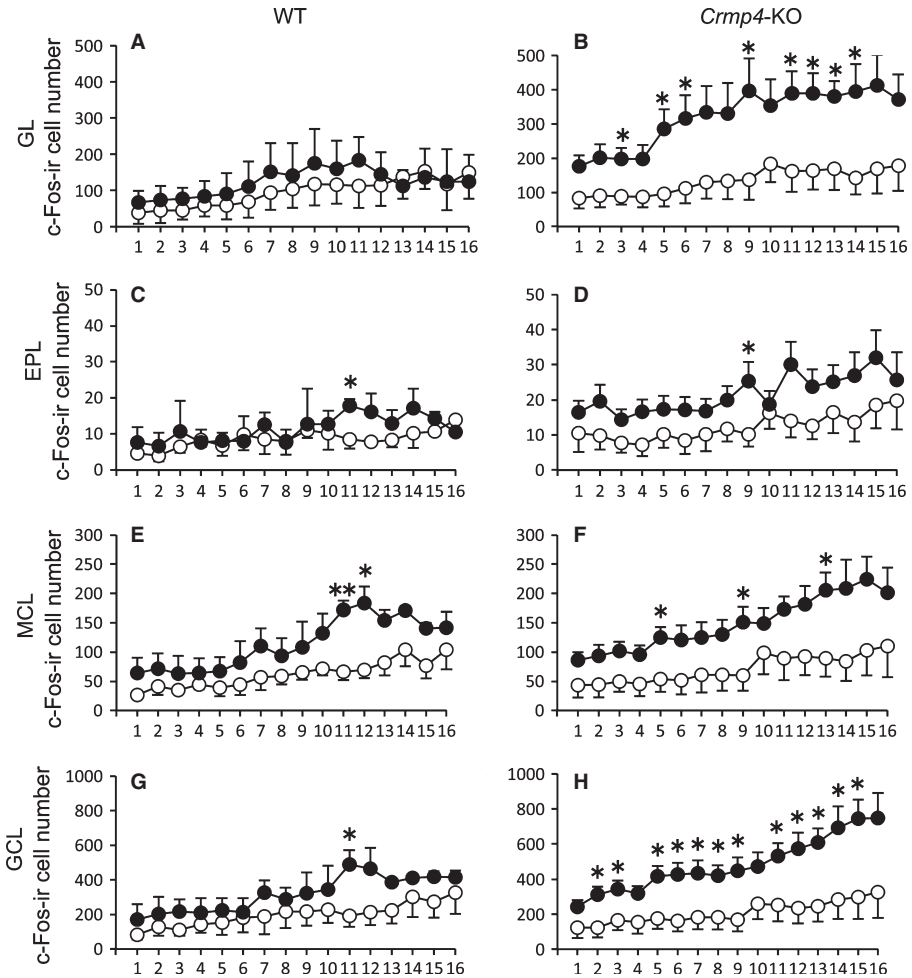

FIG. 3. The number of c-Fos-positive cells in the olfactory bulb (OB). c-Fos-positive cells were counted in the glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL) and granule cell layer (GCL). The average numbers of c-Fos-positive cells in four layers of the wild-type (WT) OB (A, C, E and G) and collapsin response mediator protein gene knockout (*Crmp4*-KO) OB (B, D, F and H) were calculated. Pups were treated with ethyl acetate (+EA, black circles) or were untreated (controls, white circles). Values are expressed as means ± SEM. $*P < 0.05$ and $**P < 0.01$ with unpaired Student's *t*-tests (control vs. EA-treated animals).

TABLE 2. Real-time qRT-PCR measurement of RNA expressed in the OB at PD7

Genes	Difference (*Crmp4*-KO/WT)	*P*-value (*t*-test)
Glur1	1.66	0.03*
Glur2	1.80	0.01*
Glur3	1.31	0.13
Glur4	1.28	0.11
Vglut1	1.13	0.61
Vglut2	1.37	0.30
Gad67	1.22	0.42
Vgat	1.23	0.35
GabaAα1	1.26	0.32
GabaAβ2	1.25	0.25
GabaBr1	1.38	0.10
Parvalbumin	1.03	0.97
Synapsin1	0.99	0.68

Asterisks indicate significant differences between WT and *Crmp4*-KO mice (unpaired Student's *t*-test, $P < 0.05$). KO, knockout; WT, wild-type.

Western blot analysis revealed that expression levels of GluR1 and GluR2 protein were significantly higher in *Crmp4*-KO pups than in WTs, but levels of GAD67 protein were not significantly different between genotypes (Fig. 4; $P < 0.05$, unpaired Student's *t*-test).

Discussion

Three main results were obtained in the present study: (1) PD7 *Crmp4*-KO pups had impaired olfactory function for discriminating between familiar and unfamiliar nest-bedding odors (Fig. 1B); (2) *Crmp4*-KO pups showed neuronal hyperactivity in the OB after a simple odor stimulus, EA (Figs 2 and 3); and (3) *Crmp4*-KO pups exhibited higher levels of GluR1 and GluR2 expressions in the OB, compared with WTs (Fig. 4).

In the present study, UVs emitted from pups were recorded to assess olfactory ability, showing first that the duration time, mean peak frequency and maximum peak frequency of calls by WT pups were comparable to those by *Crmp4*-KO pups. This result suggested that there was no significant difference between WT and *Crmp4*-KO pups in their motor systems for producing UVs. In addition, when pups were isolated, physically manipulated or exposed to thermal change (from room temperature to 14 °C), the number of UVs emitted by *Crmp4*-KO pups was not significantly different from that emitted by WTs (Fig. 1B). However, although WT pups produced increased numbers of UVs when they were exposed to a new, unfa-

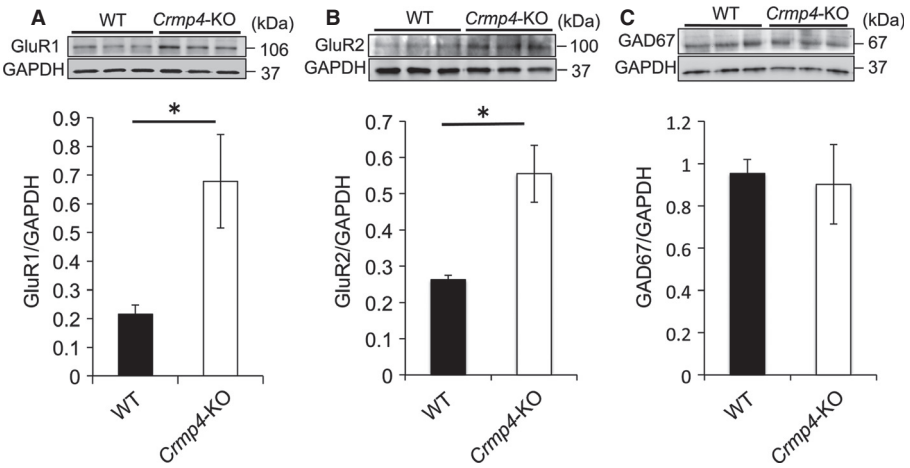

FIG. 4. Immunoblot analysis of GluR1, GluR2 and GAD67 proteins in the olfactory bulb (OB) of wild-type (WT) and collapsin response mediator protein gene knockout (*Crmp4*-KO) pups. Intensity values of (A) GluR1, (B) GluR2 and (C) GAD67 proteins were normalized by dividing them by GAPDH levels. Data are presented as means ± SEM. Asterisks indicate significant differences between WT and *Crmp4*-KO mice (unpaired Student's *t*-test, $P < 0.05$).

miliar odor relative to their performance in the presence of familiar nest-bedding odor, *Crmp4*-KO pups did not respond sensitively to the new odor stimulus (Fig. 1B). These results indicate that *Crmp4*-KO pups could not discriminate the two odors well, suggesting impaired olfactory ability in the mutant.

Aberrant c-Fos expression in the OB of *Crmp4*-KO pups was clearly identified after a simple olfactory stimulus. The EA-elicited c-Fos expression in the OB tended to be much extended in *Crmp4*-KO pups than in WTs, indicating abnormal activity of the OB neurons in *Crmp4*-KO pups. Then, the expression of genes related to neurotransmitters that contribute to neural excitation or inhibition in the OB was examined. As shown in Table 2 and Fig. 4, an increase in mRNA and protein expression levels of GluR1 and GluR2 were found, which belong to a subclass of ionotropic GluRs (GluR1–4) localized to excitatory synapses in the CNS, and which mediate fast excitatory neurotransmission (Traynelis *et al.*, 2010). On the other hand, the mRNA and protein expression levels of GAD67, the primary enzyme for synthesizing the inhibitory neurotransmitter GABA, were not different between *Crmp4*-KO pups and WTs. In addition, mRNA expression levels of a GABA transporter (*Vgat*) and of three GABA receptors (*GabaAα1*, *GabaAβ2* and *GabaBr1*) in *Crmp4*-KO pups were not significantly different from those in WTs. Furthermore, there was no difference between WT and *Crmp4*-KO pups in the mRNA expression level of *parvalbumin*, a marker of the *parvalbumin* neurons that play an important role in inhibiting OB neurons (Kato *et al.*, 2013).

GluR1 and GluR2 have been implicated in many nervous system disorders associated with development, including ASD (Ramanathan *et al.*, 2004; Pilpel *et al.*, 2009) and schizophrenia (Wiedholz *et al.*, 2008; Fitzgerald *et al.*, 2010; Zhang & Abdullah, 2013). Patients with ASD were reported to show elevation of GluR1 expression (Purcell *et al.*, 2001). Some patients with schizophrenia exhibit abnormal elevated expressions of GluR1 and GluR2 (Hu *et al.*, 2014). Mouse models with targeted mutations in candidate genes for ASD also show abnormal expressions of GluR1 and GluR2 (Pilpel *et al.*, 2009). For example, Schmeisser *et al.* (2012) reported an increase of GluR2 expression in ProSAP1/Shank2-KO mice, one of the mouse models of ASD, and concluded that altered glutamatergic neurotransmission could lead to the core symptoms of ASD. In addition, it has been suggested that increased activity in excitatory neurons gives rise to the social and cognitive deficits observed in ASD (Markram & Markram, 2010; Rubenstein, 2010; Vattikuti & Chow, 2010). However, when the mRNA expressions of *Glur3* and *Glur4* were investigated in response to previous reports of expressions of these genes in the OB (Montague & Greer, 1999), no significant difference in expression levels was found between *Crmp4*-KO and WT pups. As far as is known, there have been no reports indicating a relationship between GluR3/4 and ASD, though altered expression levels of GluR3 and GluR4 have been detected in some patients with schizophrenia (Sodhi *et al.*, 2011; Hu *et al.*, 2014).

At present, little is known about the relationship between CRMP4 and neurodevelopmental disorders. However, there are reports showing involvement of CRMPs in ASD and schizophrenia. CRMP2 has been associated with schizophrenia through genetic polymorphisms, changes in protein expression and post-translational modifications (Edgar *et al.*, 2000; Nakata *et al.*, 2003; Beasley *et al.*, 2006), although the involvement of CRMP2 in schizophrenia is still controversial (Ujike *et al.*, 2006; Arai & Itokawa, 2010; Koide *et al.*, 2010). CRMP1 is a novel candidate protein for causing schizophrenic traits (Bader *et al.*, 2012). Braunschweig *et al.* (2013) found that CRMP1 and CRMP2 comprise a portion of the primary antigens of maternal autoantibody-related ASD. Furthermore, they demonstrated that ASD children from mothers with specific reactivity to CRMP1 had elevated stereotypical behaviors compared with ASD children from mothers lacking these antibodies, suggesting that CRMP1 is a candidate protein related to the development of a core behavioral feature of ASD. These previous reports highlight the importance of the CRMP family to the pathogenesis of developmental neuronal disorders such as ASD and schizophrenia. In addition, impaired olfaction has been reported in schizophrenia and ASD (schizophrenia: Malaspina & Coleman, 2003; Moberg & Turetsky, 2003; Moberg *et al.*, 2013; ASD: Suzuki *et al.*, 2003; Bennetto *et al.*, 2007; May *et al.*, 2011).

Based on the current analysis of the c-Fos-positive neurons, the hypothesis that exaggerated neuronal activity in the OB is one of the underlying mechanisms for the impairment of olfactory ability found in *Crmp4*-KO pups is proposed. In WT pups, EA-induced c-Fos-positive neurons appeared only in restricted regions of the OB (Figs 2 and 3). Xu *et al.* (2003) found by functional magnetic resonance imaging that EA induced a localized excitation in the medial and lateral regions of the OB, in the middle part of the rostrocaudal extent. In addition, the localized *c-Fos* mRNA expression in WT adult mice after EA stimulation has been reported previously (Salcedo *et al.*,

2005). In the latter study, however, it was reported that the area with strong *c-Fos* mRNA expression was localized to the ventral portion in the middle part of the rostrocaudal extent of the OB. In the present study, the 11th section, where high c-Fos expression was induced in three layers of the OB by EA, coincided with the middle part of the rostrocaudal extent of the OB in WT pups. Therefore, the longitudinal localization of high numbers of c-Fos-positive cells induced by EA in this study may be coincident with those in the previous reports, although the latter studies were performed in adults, whereas the current study was performed in pups. In *Crmp4*-KO pups, however, rather than a localized increase in c-Fos expression, the EA-induced increase of c-Fos expression took the form of widespread activation throughout the OB. These results indicate that an abnormal odor map develops in the OB of *Crmp4*-KO pups, implicating CRMP4 in the formation of odor maps as well. It is known that the axonal projection pattern of olfactory sensory nerves (OSNs) is crucial for formation of the normal odor map in the OB. Because semaphorin 3A is reported to be a factor important for the convergence and divergence of OSNs (Schwarting *et al.*, 2000, 2004), CRMP4 is possibly involved in the formation of the OSN projection pattern in the OB as an intracellular signaling mediator of semaphorin 3A-induced growth cone collapse. Thus, not only distributional changes in GluR1 and GluR2 but also projection pattern changes in OSNs should be further examined in *Crmp4*-KO mice to clarify the precise roles of CRMP4 in the formation of the odor map. In addition, other brain areas such as the ventral medial hypothalamus and central amygdala, which are reported to be associated with production of UVs after olfactory stimulation (Cox *et al.*, 2012), have a potential for contributing to abnormal UV emission in *Crmp4*-KO pups. It is also interesting to examine whether the observed changes in neuronal activity in the neonatal OB could produce any long-term changes in OB function. To address this question, it is planned to study olfactory ability in older *Crmp4*-KO mice in the near future.

It was shown that *Crmp4* is involved in the regulation of neural activities in the developing OB. Although the *Crmp4* mRNA signal was prominent in the OB, it has been observed to occur throughout the brain during the early postnatal period (Tsutiya & Ohtani-Kaneko, 2012). Therefore, it seems quite likely that increased neuronal activity occurred similarly in other brain regions of *Crmp4*-KO mice, including the cerebral cortex and hippocampus. This study will provide an important animal model for elucidating the roles of CRMP4 in the development of higher brain functions as well as for elucidating the developmental regulatory mechanisms controlling the activity of the neural circuitry.

Supporting Information

Additional supporting information can be found in the online version of this article:

Table S1. Body weight of WT and *Crmp4*-KO mice during development.

Table S2. Isolation-induced UVs emitted by WT and *Crmp4*-KO pups.

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Abbreviations

ASD, autism spectrum disorder; CRMP4, collapsin response mediator protein 4; EA, ethyl acetate; EPL, external plexiform layer; GABA, γ-aminobutyric acid; GCL, granule cell layer; GL, glomerular layer; GluR1, glutamate receptors 1; GluR2, glutamate receptors 2; KO, knockout; MCL, mitral cell layer; OB, olfactory bulb; OSN, olfactory sensory nerve; PB, phosphate buffer; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PD, postnatal day; qRT, quantitative reverse transcription; RT, room temperature; UVs, ultrasonic vocalizations; WT, wild-type.

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