Kirrel3 is required for the coalescence of vomeronasal sensory neuron axons into glomeruli and for male-male aggression

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SUMMARY
The accessory olfactory system controls social and sexual interactions in mice that are crucial for survival. Vomeronasal sensory neurons (VSNs) form synapses with dendrites of second order neurons in glomeruli of the accessory olfactory bulb (AOB). Axons of VSNs expressing the same vomeronasal receptor coalesce into multiple glomeruli within spatially conserved regions of the AOB. Here we examine the role of the Kirrel family of transmembrane proteins in the coalescence of VSN axons within the AOB. We find that Kirrel2 and Kirrel3 are differentially expressed in subpopulations of VSNs and that their expression is regulated by activity. Although Kirrel3 expression is not required for early axonal guidance events, such as fasciculation of the vomeronasal tract and segregation of apical and basal VSN axons in the AOB, it is necessary for proper coalescence of axons into glomeruli. Ablation of Kirrel3 expression results in disorganization of the glomerular layer of the posterior AOB and formation of fewer, larger glomeruli. Furthermore, Kirrel3−/− mice display a loss of male-male aggression in a resident-intruder assay. Taken together, our results indicate that differential expression of Kirrels on vomeronasal axons generates a molecular code that dictates their proper coalescence into glomeruli within the AOB.

KEY WORDS: Kirrel, Axon guidance, Olfactory system, Mouse

INTRODUCTION
The flow of information within the nervous system relies on the organization of neurons into an intricate array of nuclei in which axons converge to form connections with their synaptic partners. The accurate formation of these circuits is especially important in the sensory systems, where sensory neurons respond to stimuli from the environment and relay information to the central nervous system (CNS). The encoding of this information is dependent on the formation of neural maps that can be described as either continuous or discrete based on the type of representation they generate in the CNS (reviewed by Luo and Flanagan, 2007). The formation of these maps relies on both short- and long-range cues that direct the growth of axons into specific areas within target fields. In addition, in regions of the nervous system where discrete maps are formed, axons must converge or coalesce into discrete synaptic units. Our understanding of the molecular mechanisms that regulate the coalescence of axons into such synaptic units remains fairly limited.

The olfactory systems have provided us with interesting insight into some of the processes that regulate discrete map formation and their importance for sensory coding. Olfactory sensory neuron (OSN) axons expressing a specific olfactory receptor (OR) innervate on average two spatially conserved glomeruli, containing dendrites of second-order neurons, out of the ~1800 glomeruli present in the olfactory bulb (Malnic et al., 1999; Mombaerts et al., 1996; Ressler et al., 1994; Serizawa et al., 2003; Vassar et al., 1994). The spatial arrangement of these glomeruli contributes to the coding of odor information (Cho et al., 2011; Johnson and Leon, 2007; Mori et al., 2006).

In contrast to the main olfactory system, the glomerular map generated in the accessory olfactory system appears to be more complex. Vomeronasal sensory neurons (VSNs) express one or more vomeronasal receptors (VRs) to detect chemical compounds that regulate a variety of innate behaviors, including social dominance and maternal care (Boschat et al., 2002; Chamero et al., 2011; Dulac and Wagner, 2006; Haga et al., 2010; Hasen and Gammie, 2009; Leinders-Zufall et al., 2009; Martini et al., 2001; Papes et al., 2010; Stowers and Logan, 2010; Zufall and Leinders-Zufall, 2007). VSNs expressing the same VR project axons that coalesce into 10 to 30 different glomeruli in spatially conserved regions of the accessory olfactory bulb (AOB) (Belluscio et al., 1999; Del Punta et al., 2002; Rodriguez et al., 1999). VSNs located in the apical region of the vomeronasal organ (VNO) express members of the V1R family of receptors and project their axons to the anterior region of the AOB. By contrast, basally located VSNs express members of the V2R family of receptors and innervate the posterior region of the AOB (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Pantages and Dulac, 2000; Rodriguez et al., 2002; Ryba and Tirindelli, 1997). Although we have a good understanding of the processes that regulate the segregation of basal and apical VSN axons within the AOB (Cloutier et al., 2002; Cloutier et al., 2004; Knöll et al., 2003; Prince et al., 2009; Walz et al., 2007; Walz et al., 2002), little is known about the mechanisms that control the coalescence of VSN axons and the formation of glomeruli in this information relay structure.

Members of the Kirrel (or Neph) family of transmembrane proteins are immunoglobulin domain-containing cell-adhesion-like
molecules that are widely expressed in the nervous system (Gerke et al., 2006; Völker et al., 2012). In C. elegans, the Kirrel homolog SYG-1 regulates specificity of synapse formation in hermaphrodite-specific neurons (Shen and Bargmann, 2003; Shen et al., 2004). In Drosophila, the Kirrel homolog IrreC-rst (Roughest – FlyBase) regulates sense organ patterning, ommatidia formation, and the accurate projection of columnar visual neuron axons (Bao et al., 2010; Schneider et al., 1995; Venugopala Reddy et al., 1999). In addition, Kirrels have been implicated in the regulation of axonal coalescence into glomeruli of the mouse olfactory bulb (OB). Overexpression of Kirrel2 or Kirrel3 leads to improper targeting of axons within the OB (Serizawa et al., 2006). However, whether Kirrels are required for axonal coalescence remains to be established using loss-of-function approaches.

Here we have examined the role of the Kirrel family of proteins in regulating the coalescence of VSN axons into glomeruli in the AOB. We show that Kirrel2 and Kirrel3 are differentially expressed within subpopulations of VSNs. Although Kirrel3 expression is dispensable for the fasciculation of the vomeronasal tract and the segregation of VSN axons within the OB, it is required for the coalescence of VSN axons into glomeruli in the AOB. Furthermore, Kirrel3 mutant mice display impaired male-male aggression. We propose that the differential expression of Kirrels in VSNs generates a molecular diversity in VSN axons that is necessary for proper coalescence and formation of glomeruli.

MATERIALS AND METHODS

Animals

For in situ hybridization experiments, embryonic day (E) E18 embryos were obtained from timed-pregnant CD1 females purchased from Charles River (Saint-Constant, Canada). Trp2 (Stowers et al., 2002), VN12-tau-lacZ (Belluscio et al., 1999) and EC2-tau-lacZ mice (Cloutier et al., 2004) mice have been described. The Animal Care Committee of the Montreal Neurological Institute and McGill University has approved all animal procedures.

Generation of Kirrel3−− mice

Kirrel3 mutant mice were generated by standard homologous recombination methods to introduce a farnesylated eGFP cassette, 3′ UTR and poly(A) signal (from the pEGFP-F vector, Clontech) into the first exon of the Kirrel3 gene, in frame with the ATG start site but deleting the endogenous 18 amino acid signal sequence of Kirrel3. This was followed by the self-excision ACN cassette used for G-418 selection of homologously targeted embryonic stem cells (ESC) clones, to remove the neo marker during germline transmission in male chimera (Bunting et al., 1999). Thus, the membrane-associated eGFP should be expressed under the control of endogenous Kirrel3 regulatory sequences in this allele, while all Kirrel3 function is concomitantly removed.

In situ hybridization

Dioxygenin or fluorescein-labeled cRNA probes were synthesized by in vitro transcription using DIG or Flu labeling mix (Roche, Mannheim, Germany) according to the manufacturer’s recommendations. Probes were synthesized as cDNA clones encoding Kirrel1, Kirrel2 and Kirrel3 (kind gifts of Dr David Ginty, Johns Hopkins School of Medicine, Baltimore, USA), and Gαd and Gαi2 (Cloutier et al., 2002). Fresh frozen brains or nasal cavities were cryosectioned at 20 μm, fixed, and processed as previously described (Cho et al., 2007).

Reverse transcription PCR

Brain, OB and VNO samples were isolated from adult Kirrel3+/− and Kirrel3−− mice. RNA was purified using the Qiazol reagent (Qiagen) followed by the RNeasy Lipid Tissue Mini Kit (Qiagen). DNase treatment was performed using DNase I (Invitrogen). cDNA was generated using the SuperScript II reverse transcriptase system (Invitrogen). PCR oligonucleotides for Gapdh, 5′-GCTTCTGCAACCACCTG-3′ and 5′-CCGACGCCTGTACACCTTCT-3′; and Kirrel3, 5′-CTGGAGGATGAGC-3′ and 5′-GTCTGACACCACTGTC-3′. The Kirrel3 PCR reaction was conducted for 30 cycles using the GoTaq system (Promega).

Immunohistochemical procedures

Adult mice and postnatal pups were anesthetized and perfused transcardially with ice-cold PBS containing 4% paraformaldehyde and processed as previously described (Prince et al., 2009). The sections were incubated with primary antibody using the following dilutions: anti-GF (1:500; Invitrogen), anti-Kirrel2 (1:500; R&D Systems), anti-Gαi2 (1:500; WAKO Chemicals, USA), anti-Gαo (1:500; MBL), anti-β-galactosidase (1:500; Abd Serotech), anti-olfactory marker protein (OMP) (1:1000; WAKO Chemicals, USA), anti-SV2 (synaptic vesicle protein 2) (1:1000; DHSH, University of Iowa, Iowa City, IA), anti-VGLUT2 (1:500; Synaptic Systems, Goettingen, Germany), and anti-PSD-95 (NeuroMab, UC Davis/NIH NeuroMab Facility, Davis, CA). After rinsing in TBS, primary antibody was detected with the appropriate Alexa-488 or Alexa-546-conjugated secondary antibody (1:500; Molecular Probes). Bandeiraea simplicifolia (BS) lectin (1:1500; Vector Laboratories, Burlingame, USA) was applied with the secondary antibody.

X-Gal staining

For whole-mount X-Gal staining, 2- to 3-month-old adult mice were perfused transcardially with ice-cold PBS containing 4% paraformaldehyde, 2 mM MgSO4, and 5 mM EGTA. Whole olfactory bulbs were processed for X-Gal staining as previously described (Mombaerts et al., 1996).

Analysis of glomeruli in adult accessory olfactory bulbs

Two- to three-month-old adult mice were perfused as described above. Both olfactory bulbs were cryosectioned (20 μm) in the sagittal plane and all sections were collected on microscope slides. Sections were immunostained with primary antibodies (OMP, Kirrel2 or GFP), incubated with BS lectin, and counterstained with Hoechst. The border between the anterior and posterior regions of the AOB was identified by the intensity of BS lectin as previously described (Cloutier et al., 2002). The number of OMP-positive glomeruli was counted on each section of both AOBs from each mouse examined. As glomeruli in the AOB are not as well defined by the presence of peripheral cells as in the OB, we defined a glomerulus as an OMP-positive structure completely surrounded by a region of non-innervated neuropil. For the glomerular size analysis, the longest diameter of OMP-, Kirrel2- and GFP-positive glomeruli was measured and averaged. For analysis of the expression of Kirrel2 and GFP in glomeruli, glomeruli were hand drawn according to the criteria described above and the average fluorescence inside the region defined as a glomerulus was measured using ImageJ software. Hierarchical clustering analysis of the expression dataset was performed using the Ward method with software available at http://www.wessa.net/rwasp_hierarchicalclustering.wasp/.

Analysis of the number of VSNs in adult VNOs

Coronal sections through the VNO of adult mice were immunostained with OMP antibodies and the number of OMP-positive VSNs within a defined area in the VNO was counted on ten sections of each VNO examined. The average number of VSNs/mm2 per genotype examined was determined.

Resident-intruder assay

The resident-intruder assay was adapted from territorial aggression assays (Stowers et al., 2002) and the resident intruder paradigm (Buck, 1996). Castrated 8-week-old male mice (Charles River) were used as intruders. Littermate resident control and Kirrel3−− mice were singly housed for at least 7 days. The resident’s cage was placed in the test room and, following a 10-minute period of habituation, an intruder mouse was added to the resident’s cage for a 10-minute testing period, then immediately removed. The testing paradigm was repeated 24-48 hours later, this time with the intruder mouse swabbed with freshly collected urine obtained from sexually mature mixed C57BL/6J129 male mice housed in another location. Control and Kirrel3−− mice were kept in a C57BL/6J129 mixed background and
littermates were used for testing. Trials were performed using both CD1 and C57BL/6 castrated intruder males and no significant difference in the response of either control or Kirrel3−/− mice to these two strains of mice was observed. The behavioral analyses presented in Fig. 9A-E therefore include all trials performed with both strains of intruder mice. Testing occurred during the last 4 hours of the resident’s light phase and was video recorded using a digital camera.

Food-finding test
The food-finding test was adapted from Stowers et al. (Stowers et al., 2002). Following a 16-20 hour food-deprivation period, the time required for control and Kirrel3−/− mice to find half of a cookie buried in fresh bedding was measured.

RESULTS
Expression of Kirrel family members in vomeronasal sensory neurons
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Food-finding test
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To begin to assess the role of Kirrel family members in the coalescence of VSN axons in the AOB, we examined their patterns of expression using in situ hybridization during late embryogenesis, when VSN axons have begun to innervate the AOB. Although Kirrel2 and Kirrel3 transcripts are detected in VSNs at E18 (Fig. 1B,C), Kirrel1 transcript is not observed in VSNs even though it is readily detectable in OSNs in the olfactory epithelium (OE) (Fig. 1A; data not shown). We examined more precisely the expression of Kirrel2 and Kirrel3 in the VNO of postnatal (P) day 15 mice, when V1R and V2R-expressing VSNs are fully segregated in the VNO. At that stage of development, Kirrel2 expression is mostly restricted to Gαo2-positive VSNs that express V1Rs and that are located in the apical region of the VNO (Fig. 1D-F). By contrast, Kirrel3 is mainly expressed in Gαo2-positive VSNs that express V2Rs and that are located in the basal region of the VNO (Fig. 1M-O). However, a subset of VSNs located in the basal region of the VNO and expressing Gαo2 also expresses Kirrel2 (Fig. 1G-I), whereas a subset of apical VSNs also express Kirrel3 (Fig. 1J-L). A comparison of Kirrel2 and Kirrel3 expression in the VNO revealed that VSNs could be classified into at least three different populations based on their expression of Kirrel genes: VSNs expressing (1) Kirrel2 alone, (2) Kirrel3 alone, or (3) both Kirrel2 and Kirrel3 (Fig. 1R).

To further assess the coexpression of Kirrel2 and Kirrel3 in subsets of VSNs, we examined the projections of Kirrel2- and Kirrel3-expressing VSNs to the AOB using immunohistochemistry to detect axons of Kirrel-expressing VSNs. In the case of Kirrel2 we used a specific antibody, but as one was not available for Kirrel3, we instead examined mice carrying a Kirrel3 allele targeted with a GFP cassette within the first exon. In the VNO of these mice, GFP expression faithfully recapitulates Kirrel3 expression (see Fig. 4G-I'). Immunostaining of sagittal sections of AOB from adult mice with Kirrel2 and GFP antibodies revealed that a large proportion of glolemuli within the anterior region of the AOB are innervated by Kirrel2 positive axons, whereas most glolemuli within the posterior AOB are innervated by Kirrel3-positive axons (Fig. 2A,C,E). A quantification of the fluorescence signals in the glolemular layer revealed that most glolemuli can be classified into one of four different populations of glolemuli based on the levels of Kirrel2 and Kirrel3 expressed by innervating axons: (1) high Kirrel2 expression; (2) high Kirrel3 expression; (3) high Kirrel2 and low Kirrel3 expression; and (4) low Kirrel2 and high Kirrel3 expression (Fig. 2B,D,F,G). The differential expression of Kirrel2 and Kirrel3 on VSN axons may provide a molecular diversity that regulates their coalescence into distinct glolemuli within the AOB (Fig. 2H).

Activity-regulated expression of Kirrel2 and Kirrel3 in VSNs
The coalescence of VSN axons into glolemuli requires expression of a VR and is dependent on activity (Belluscio et al., 1999; Hovis et al., 2012; Rodriguez et al., 1999). It is possible that VR-induced
activity controls the expression of cell-surface receptors that control coalescence of VSN axons. We therefore assessed whether the expression of Kirrels in VSNs is regulated by activity. We examined their expression by in situ hybridization in the VNO of mice containing a deletion of the \textit{Trp2} (\textit{Trpc2} – Mouse Genome Informatics) gene, which abolishes pheromone-evoked neural activity in VSNs (Stowers et al., 2002). At P0, Kirrel2 is expressed in a subset of cells in the VNO in \textit{Trp2}+/+ mice (Fig. 3A,C) but its expression is drastically reduced in the VNO of \textit{Trp2}−/− mice (Fig. 3B,D). By contrast, the expression of Kirrel3 in VSNs is upregulated in \textit{Trp2}−/− mice compared with \textit{Trp2}+/+ mice (Fig. 3E-H), whereas its expression in the OE is unchanged (G,H). Scale bars: 250 μm in A,B,E,F; 500 μm in C,D,G,H.

Kirrel3 is dispensable for fasciculation of the vomeronasal tract and for segregation of apical and basal VSN axons in the AOB

Our experiments showing differential expression of Kirrels in VSNs suggest their expression may provide a molecular diversity to VSN axons that is necessary for correct innervation of the AOB. To test whether Kirrels contribute to the coalescence of axons in the AOB, we generated a \textit{Kirrel3} null allele by inserting a GFP cassette within the first exon of the \textit{Kirrel3} gene (Fig. 4A). Combined in situ hybridization and RT-PCR analyses showed that \textit{Kirrel3} transcript is absent in the VNO and OE of \textit{Kirrel3}−/− mice (Fig. 4B-F). These mice are viable and their size is similar to control mice. Furthermore, the localization of apical and basal VSNs within the VNO is unaffected in these mice (Fig. 4J,K).
We first examined whether ablation of Kirrel3 expression affects the projection of VSN axons to the AOB by examining the state of fasciculation and their segregation inside the AOB. To examine the state of fasciculation and the organization of the vomeronasal tract, we crossed the Kirrel3 mutant mice with mice expressing tau-lacZ in a specific population of basal VSNs that expresses the V2R EC2. Kirrel3 is highly expressed in EC2-positive neurons as assessed by GFP and β-galactosidase immunohistochemistry in VNOs of Kirrel3; EC2-tau-lacZ mice (Fig. 7A-C). Whole-mount X-Gal staining of the OB revealed that EC2-positive axons remain tightly fasciculated and project accurately to innervate the AOB in Kirrel3−/− mice (Fig. 5I,M). Furthermore, EC2-expressing axons are evenly distributed across the whole width of the vomeronasal tract in both Kirrel3+/+ and Kirrel3−/− mice, indicating that ablation of Kirrel3 expression does not affect the overall organization of this tract (Fig. 5J-L,N-P).

We next examined whether the segregation of apical and basal VSN axons into the anterior and posterior regions of the AOB, respectively, is maintained in Kirrel3−/− mice. As observed in Kirrel3+/+ mice, Gαi2 and Gαo-expressing axons project exclusively to the anterior and posterior halves of the AOB, respectively, in Kirrel3−/− mice (Fig. 5A-D). Furthermore, the segregation of axons from specific subpopulations of apical and basal VSNs expressing the VN12 and EC2 vomeronasal receptors, respectively, are unaffected in Kirrel3−/− mice (Fig. 5E-H). Taken together, these results demonstrate that VSN axons reach the AOB and segregate accurately in the absence of Kirrel3, suggesting that Kirrel3 may function in later stages of circuit formation such as in the coalescence of axons into specific glomeruli.

**Kirrel3 is required for the coalescence of axons in the AOB**

The differential expression of Kirrel2 and Kirrel3 we observed on VSN axons may serve to promote the accurate coalescence of like-axons into spatially conserved glomeruli within the AOB. If so, we would predict that disrupting the expression of Kirrel3 may lead to decreased molecular diversity between VSN axons and improper formation of glomeruli in the AOB. To examine whether Kirrel3 is necessary for axonal coalescence, we performed a detailed analysis of the glomerular layer in Kirrel3−/− mice. Sagittal sections of the AOB of control and Kirrel3−/− mice were stained with an anti-OMP to mark all glomeruli and with BS lectin to define the anterior-posterior border of the AOB. In the anterior region of the AOB, many small glomeruli of a mainly uniform shape can be seen both in control and Kirrel3−/− mice, although some regions within the anterior AOB appear to have slightly larger glomeruli in Kirrel3−/− mice (Fig. 6A,B). In the posterior AOB, glomeruli are fairly uniform in size in control mice while they are misshapen and on average twice as large in Kirrel3−/− mice (Fig. 6A-B″,Q). The larger glomeruli are positive for the presynaptic markers SV2 and VGLUT-2, as well as for the postsynaptic marker PSD-95, which is consistent with the presence of synapses within these glomeruli (Fig. 6C-J).

The increase in glomerular size observed in the posterior region of the AOB in Kirrel3−/− mice could result from the inability of axons to coalesce into multiple glomeruli within specific regions of the posterior AOB or could be due to an increase in the size of VSN axon terminals. An increase in axon termini size would be expected to result in the formation of larger glomeruli but should not affect
Kirrel3 controls axonal coalescence

Kirrel3 is required for the appropriate coalescence of VSN axons within the posterior region of the AOB, as the number of glomeruli in the posterior region of the AOB in Kirrel3−/− mice is not a consequence of a reduced number of VSNs innervating the AOB, as the number of VSNs in the VNO of wild-type and Kirrel3−/− mice is comparable (Fig. 6R).

We then examined the coalescence of axons from EC2-expressing VSNs, which express Kirrel3, during development of the glomerular layer in Kirrel3+/+; EC2-tau-lacZ and Kirrel3−/−; EC2-tau-lacZ mice at postnatal days 5, 15, 30 and 90. In Kirrel3+/+ mice, EC2-expressing axons coalesce into small well-defined glomeruli that are innervated by a single population of VSN axons (Fig. 7D,E,H,I,L,M,P,Q). By contrast, EC2-expressing axons form more diffuse glomeruli in the AOB of early postnatal Kirrel3−/− mice (Fig. 7F,G,J,K). Furthermore, EC2-positive glomeruli are heterogeneous and contain additional populations of VSN axons in Kirrel3−/− mice (Fig. 7R,S).

As four main populations of glomeruli expressing different levels of Kirrel2 and Kirrel3 are usually observed in the posterior region of the AOB, we would predict that ablating Kirrel3 expression may reduce the molecular diversity that exists between axons and affect the size of both Kirrel2-positive and Kirrel3-positive glomeruli that are formed in this region. To examine the size of Kirrel3-positive and Kirrel2-positive glomeruli in the posterior region of the AOB, we stained sagittal sections of the AOB showing EC2-expressing VSN axons with X-Gal in adult Kirrel3+/+, Kirrel3−/−; EC2-tau-lacZ (I) and Kirrel3−/−; EC2-tau-lacZ mice (M). Tightly fasciculated axons bundles projecting to the AOB are observed in all mice (n=3). (J-L,P) Parasagittal sections through the vomeronasal nerve of adult Kirrel3+/+, Kirrel3−/−; EC2-tau-lacZ (J-L) and Kirrel3−/−; EC2-tau-lacZ mice (P) were stained with anti-β-galactosidase (J,N,L,P) and BS lectin (K,L,O,P). EC2-expressing fibers within the nerve of both Kirrel3+/+, Kirrel3−/−; EC2-tau-lacZ (J-L) and Kirrel3−/−; EC2-tau-lacZ mice (P) are evenly distributed and maintain a linear organization within nerve bundles (n=4). Scale bars: 125 μm in A, 250 μm in J, anterior; D, dorsal; P, posterior; V, ventral.
in Kirrel expression profiles on VSN axons is essential for glomerular formation.

**Loss of male-male aggression in Kirrel3−/− mice**

The interpretation of signals detected from the environment is dependent on the formation of accurate synaptic connections in the nervous system. The disrupted organization of circuits in the AOB or in other regions of the brain associated with VNO-regulated behaviors may affect the ability of mice to interpret signals from pheromonal cues detected by the VNO. We therefore examined whether Kirrel3−/− mice, glomeruli located in the posterior AOB are medium sized and maintain a uniform shape (A−A′), whereas they are much larger and have distorted shapes (A′−A″) in Kirrel3−/− mice (B−B′). The larger glomeruli observed in Kirrel3−/− mice are positive for the synaptic markers SV2 (D), VGLUT-2 (H) and PSD-95 (I), suggesting that synapses are formed in these glomeruli. (O-R) Quantification of the average number of glomeruli (O,P) and glomerular diameter (Q) in the AOB per section (n=3 mice). Data were analyzed using a two-tailed t-test. *P<0.02, ***P<0.001. (R) Number of VSNs/mm² in the vomeronasal organ of Kirrel3+/+ and Kirrel3−/− mice. The number of VSNs is similar in Kirrel3+/+ and Kirrel3−/− mice (n=3 mice). Data were analyzed using a two-tailed t-test. Scale bar: 125 μm in A,B,K-N; 25 μm in A′,A″,B′,B″,E−I; 12.5 μm in C,D.

Fig. 6. Glomeruli formation in the posterior region of the AOB is disrupted in Kirrel3−/− mice. (A−N) Parasagittal AOB sections of adult Kirrel3+/+ (A−C,E,G,K,M) and Kirrel3−/− mice (B−B′,D,H,L,N) were stained with anti-OMP (A−B,H,K,N), BS lectin (K,N), Hoechst (A−B″,E−N), anti-SV2 (C,D), anti-VGLUT-2 (E,G,H,J) and anti-PSD-95 (F,G,L). Glomeruli positioned in the anterior region of the AOB in Kirrel3+/+ (A,M) and Kirrel3−/− mice (B,N) are small and mainly uniform in shape. In Kirrel3−/− mice, glomeruli located in the posterior AOB are medium sized and maintain a uniform shape (A−A′), whereas they are much larger and have distorted shapes (A′−A″) in Kirrel3−/− mice (B−B′). The larger glomeruli observed in Kirrel3−/− mice are positive for the synaptic markers SV2 (D), VGLUT-2 (H) and PSD-95 (I), suggesting that synapses are formed in these glomeruli. (O−R) Quantification of the average number of glomeruli (O,P) and glomerular diameter (Q) in the AOB per section (n=3 mice). Data were analyzed using a two-tailed t-test. *P<0.02, ***P<0.001. (R) Number of VSNs/mm² in the vomeronasal organ of Kirrel3+/+ and Kirrel3−/− mice. The number of VSNs is similar in Kirrel3+/+ and Kirrel3−/− mice (n=3 mice). Data were analyzed using a two-tailed t-test. Scale bar: 125 μm in A,B,K-N; 25 μm in A′,A″,B′,B″,E−I; 12.5 μm in C,D.

To examine the response of the resident male to urine pheromones, castrated males were then swabbed with urine from a sexually mature male mouse before being introduced into the resident’s cage. The swabbing of male urine on the intruder dramatically increased the level of aggression of control resident mice and attacks towards the intruder mice were observed in almost all trials (14/15) (Fig. 9C; supplementary material Movie 1). These attacks were much more frequent and tended to escalate in duration and intensity during the last minutes of each trial, as quantified by the average time the resident engages in aggressive behavior (46.0±8.3 seconds versus 16.6±6.7 seconds, n=15) (Fig. 9A,C,E). By contrast, addition of intruder mice swabbed with urine to the Kirrel3−/− resident cage did not lead to an increase in the number or duration of attacks against the intruder males (Fig. 9D; supplementary material Movie 2). A few attacks were noted in approximately half the trials (8/15 trials) but these attacks remained minimal and did not escalate in frequency or intensity (9.8±3.2 seconds, n=15) (Fig. 9B,D,E). Hence control mice fought almost five times longer than Kirrel3−/− mice per trial when an intruder swabbed with urine was introduced into their cage (Fig. 9E).
Taken together, our results demonstrate that Kirrel3 is essential for the coalescence of VSN axons in the AOB and that ablation of Kirrel3 expression leads to a loss of male-male aggression and improper gender discrimination.

**DISCUSSION**

**Establishment of a functional glomerular map in the AOB**

The development of sensory maps in the nervous system is essential for responses to signals in the environment. In the olfactory systems the coalescence of sensory neuron axons into specific synaptic units is a critical process during formation of discrete sensory maps. In the accessory olfactory system, axon guidance cues promote the initial segregation of apical and basal VSN axons to the anterior and posterior regions of the AOB, respectively. Our results show that once axons have segregated to these two regions, they are dependent on the expression of Kirrel family members to coalesce and form distinct glomeruli within the glomerular layer of the AOB. In contrast to the main olfactory system, where most OSNs express both Kirrel2 and Kirrel3 at complementary levels (Serizawa et al., 2006), four distinct populations of VSNs can be identified in the VNO based on their expression levels of Kirrel2 and Kirrel3. We propose that the molecular diversity generated by the expression of Kirrel2 and Kirrel3 on VSN axons is necessary to promote the coalescence of like axons into distinct glomeruli within clusters of glomeruli in the AOB. Our results suggest that removal of Kirrel3 expression decreases the molecular diversity between axons, which leads to the formation of larger, less defined, and heterogeneous glomeruli. In the absence of Kirrel3 expression, two main populations of axons would exist and coalesce together into larger glomeruli, with one population expressing mainly high levels of Kirrel2 and one population of axons expressing low levels or no Kirrel2. This model is supported by our observation that removal of Kirrel3 expression leads to the formation of both larger GFP-positive and Kirrel2-positive glomeruli in the posterior AOB of Kirrel3−/− mice (Fig. 8H,I). Although we favor the loss of diversity as a model to explain the phenotype observed in these mice, alternative mechanisms of action for Kirrel3 must be considered. For example, Kirrel3 may regulate the self-avoidance of subsets of axons, by masking intrinsic adhesion proteins, so that they can disperse into multiple smaller glomeruli in the AOB. The transmembrane molecules DSCAM and DSCAML1 have been proposed to promote self-avoidance through such a mechanism in the mouse retina (Fuerst et al., 2009).

**Activity-dependent regulation of Kirrel expression in VSNs**

Early after birth, VSN axonal targeting appears imprecise and is then refined with time during postnatal development. Sensory activity can regulate the coalescence and refinement of axons in the AOB (Hovis et al., 2012). The activity-regulated expression of Kirrel2 and Kirrel3 in VSNs may contribute to the control of axonal coalescence in the AOB. In keeping with this possibility, we observed that ablation of the TRP2 channel leads to changes in the expression of Kirrel2 and Kirrel3 in VSNs (Fig. 3). Whereas Kirrel3 expression is upregulated in the absence of activity, Kirrel2 expression is decreased. In the main olfactory system, expression of Kirrel2 and Kirrel3 is modulated by the levels of cAMP and is dependent on the expression of the cAMP-regulated ion channel CNGA2 (Serizawa et al., 2006). Our results indicate that a TRP2-mediated increase in Ca2+ inside VSNs can affect gene expression, including that of Kirrel2 and Kirrel3. It is possible that expression...
of other cell-adhesion molecules, such as Big-2, whose expression is regulated by activity in OSNs may also be altered in VSNs from Trp2<sup>−/−</sup> mice (Kaneko-Goto et al., 2008). Hence regulation of gene expression downstream of TRPC2 channels has the potential to regulate a variety of processes in VSNs, including axonal coalescence and refinement.

**AOB glomerular map and the control of pheromonal responses**

Although the spatial arrangement of glomeruli within the OB contributes to the coding of odor information (Cho et al., 2011; Johnson and Leon, 2007; Mori et al., 2006), it remains unclear whether the formation of glomerular maps in the AOB is essential to mediate stereotypic behaviors regulated by the accessory olfactory system. In the main olfactory system, relatively simple glomerular maps are formed with OSNs expressing the same OR coalescing into an average of two conserved glomeruli per OB. By contrast, a greater complexity of glomerular innervation is observed in the AOS, where VSNs expressing the same VR can innervate up to 30 different glomeruli in spatially conserved regions of the AOB (Belluscio et al., 1999; Del Punta et al., 2002; Wagner et al., 2006). Within these conserved domains, glomeruli innervated by VSNs expressing different VRs are intermingled, suggesting that accurate coalescence of VSN axons may be particularly important for the formation of a glomerular map. It has been proposed that the seemingly divergent nature of information input in the AOB is rendered convergent through the homotypic innervation of glomeruli by mitral cells of the AOB, whereby dendrites of a single mitral cell project to multiple glomeruli innervated by VSNs expressing a single type of VR (Del Punta et al., 2002). However, selective heterotypic connectivity between mitral cells and glomeruli has also been reported (Wagner et al., 2006). These multiple modes of connectivity may underlie the functional diversity observed in AOB mitral cell populations associated with the processing of sensory input through VSNs (Meeks et al., 2010). It is therefore possible that the formation of precise glomeruli within specific regions of the AOB is critical for responses to pheromones. Fusion of glomeruli could lead to the activation of improper subsets of AOB mitral cells in response to chemical compounds and improper processing of information at relay centers in the brain. In keeping with this possibility, we observe a loss of male-male aggression in Kirrel3<sup>−/−</sup> mice (Fig. 9). However, we cannot exclude the possibility that defects in the wiring of other brain regions could contribute to this behavioral phenotype. Indeed, Kirrel3 is widely expressed in the developing brain, including in structures associated with the regulation of aggressive behaviors such as the hypothalamus (Gerke et al., 2006; Völker et al., 2012) (data not shown). Furthermore, although basic function of the main olfactory system is intact in Kirrel3<sup>−/−</sup> mice, we cannot exclude the possibility that defects in this system could lead to the loss of male-male aggression in these mice. Mice lacking sensory signaling in OSNs following deletion of the type 3 adenylyl cyclase gene lack responsiveness to pheromones and do not display male-to-male aggression, indicating that the main olfactory system also contributes to the processing of pheromonal information (Wang et al., 2006). In the future, selective ablation of Kirrel family members in VSNs should provide further information on the contribution of an AOB glomerular map to responsiveness to pheromones and the regulation of social behaviors in mice.
Fig. 9. Loss of Kirrel3 expression leads to reduced aggression in male mice. (A-D) Attack behavior observed during a resident–intruder paradigm of resident Kirrel3+/+ (A,C) and Kirrel3−/− mice (B,D) when a castrated male alone (A,B) or swabbed with sexually mature male urine (C,D) is introduced into the resident’s cage. Kirrel3+/+ and Kirrel3−/− resident mice display a background level of aggression when castrated male mice are introduced in their cage (A,B); however, increased attacks are only observed in Kirrel3+/+ mice, but not Kirrel3−/− mice, in response to the introduction of a swabbed castrated male intruder mouse (C,D). Each black cross represents time (s) at which attack behavior occurred over 15 trials. Mounting behavior by Kirrel3−/− was also recorded and represented using red crosses. (E) Quantification of the average total number of seconds fought in each resident–intruder paradigm (n=15). Data were analyzed by a Dunnett one-way ANOVA. **P<0.01. (F) Quantification of the latency to find a hidden cookie in a food-finding assay. The time required to locate (latency) a hidden cookie is similar for both Kirrel3+/+ and Kirrel3−/− mice (n=7). Int, intruder.

Taken together, our results demonstrate a critical role for Kirrel3 in the coalescence of VSN axons into glomeruli of the AOB and in the regulation of social behaviors in mice.

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