Genetic disruptions of *O/E2* and *O/E3* genes reveal involvement in olfactory receptor neuron projection

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Summary

The mammalian Olf1/EBF (O/E) family of repeated helix-loop-helix (rHLH) transcription factors has been implicated in olfactory system gene regulation, nervous system development and B-cell differentiation. *Ebf* (*O/E1*) mutant animals showed defects in B-cell lineage and brain regions where it is the only O/E family member expressed, but the olfactory epithelium appeared unaffected and olfactory marker expression was grossly normal in these animals. In order to further study the mammalian O/E proteins, we disrupted *O/E2* and *O/E3* genes in mouse and placed *tau-lacZ* and *tau-GFP* reporter genes under the control of the respective endogenous *O/E* promoters. Mice mutant for each of these genes display reduced viability and other gene-specific phenotypes. Interestingly, both *O/E2* and *O/E3* knockout mice as well as *O/E2/O/E3* double heterozygous animals share a common phenotype: olfactory neurons (ORN) fail to project to dorsal olfactory bulb. We suggest that a decreased dose of O/E protein may alter expression of O/E target genes and underlie the ORN projection defect.

Supplementary data available online

Key words: Olfactory, Axon targeting, Transcription factor

Introduction

The sensory neurons of the mammalian olfactory system are remarkable in their ability to undergo continuous replacement throughout the lifespan of the animal (Mackay-Sim and Kittel, 1991). This continual replenishment of olfactory receptor neurons (ORNs) recapitulates many aspects of embryonic neural development; neuroblast-like cells in the basal cell layer divide, differentiate into ORNs and migrate to an apical position in the neuroepithelium. Subsequently, mature ORN proteins including those essential for transducing odorant signals are induced as immature ORNs differentiate into functional neurons.

Analysis of the promoter regions of several ORN enriched genes revealed a conserved sequence, the Olf1 sites, that bound a factor present in olfactory nuclear extracts (Kudrycki et al., 1993; Wang et al., 1993). Studies in transgenic animals suggested that this site contributes to specific expression of an olfactory-specific gene in vivo (Kudrycki et al., 1998; Walters et al., 1996). A family of related factors (Olf1/EBF or O/E proteins; Ebf1 – Mouse Genome Informatics) encoding a novel repeated helix-loop-helix (rHLH) domain that bound to the Olf1 site was identified by the yeast 1-hybrid screen and subsequent homology screens (Wang and Reed, 1993; Wang et al., 2002; Wang et al., 1997). In vitro analysis of O/E1, O/E2, O/E3 and O/E4 revealed that they activate transcription of a reporter gene and bind the Olf1 site as dimers consisting of any two O/E subunits. Although the *O/E1* mRNA is expressed in several adult tissues examined, expression of *O/E2*, *O/E3* and *O/E4* are more restricted with highest levels of expression of each member detected in olfactory epithelium (Wang et al., 2002; Wang et al., 1997).

O/E proteins are present in the central and peripheral nervous system during development. In the CNS, O/E messages were detected as early as embryonic day nine (E9) in post-mitotic cells in distinct but overlapping patterns (Davis and Reed, 1996; Garel et al., 1997; Wang et al., 1997). In the spinal cord, O/E messages were present in post-mitotic cells of the subventricular mantle layer. As these cells migrate away from the ventricular zone, they first cease O/E3 expression, and after terminal differentiation, the neurons cease expression of O/E1 and O/E2. The O/E mRNAs are also observed in sensory structures including the olfactory epithelium, vomeronasal organ (VNO), retina, dorsal root ganglia (DRG) and some cranial nerve ganglia. Expression in most regions is transient and only detected during embryonic development, implying a role in neuronal differentiation. The olfactory epithelium and VNO continue to express O/E proteins into adulthood, consistent with the continual neuronal differentiation in these tissues and an independent role for the O/E proteins in regulating mature ORN gene expression.

Homologs of the O/E genes have been characterized in *C. elegans*, *Drosophila* and *Xenopus* (Bally-Cuif et al., 1998; Crozatier et al., 1996; Dubois et al., 1998; Gisler et al., 2000; Pozzoli et al., 2001; Prasad et al., 1998). The overexpression of *Xenopus* Xcoe2 (orthologous to mouse O/E3) promoted ectopic neuronal differentiation whereas expression of a dominant-negative allele showed that it was required for neuronal development. Additional experiments suggested a role for Xcoe2 in Notch-Delta signaling and placed Xcoe2 between neurogenin 1 (Ngn1) and NeuroD in a transcriptional regulation cascade. Studies of *Xenopus* Xebf3 (orthologous to mouse O/E2) showed that this protein promoted neuronal differentiation and functions downstream of NeuroD. The *Drosophila* O/E-like protein, *Collier*, may mediate Notch and Hedgehog signaling (Crozatier and Vincent, 1999; Vervoort et al., 1999). Mutations in the *C. elegans* O/E homolog, UNC-3, leads to aberrant cell fate in the motoneuron lineage and an uncoordinated phenotype. Additionally, *unc-3* worms display specific defects in ASI chemosensory neurons.

The O/E1 protein also contributes to gene regulation outside of the nervous system. Mice lacking functional O/E1 protein, independently identified and cloned as EBF (early B-cell factor), exhibit a profound defect in B-cell development (Hagman et al., 1993; Lin and Grosschedl, 1995; Travis et al., 1993). However, disruption of the *Ebf* (*O/E1*) gene did not alter tissue morphology or gene expression in the olfactory epithelium, presumably owing to redundancy and functional rescue by other *O/E* family members (Wang et al., 1997). Interestingly, in the striatum and cranial nerve nuclei where only O/E1 is expressed, absence of the O/E1 protein leads to atrophy and abnormal cellular migration, axonal fasciculation and projection (Garel et al., 2000; Garel et al., 1999; Garel et al., 2002). The apparent functional redundancy of the multiple O/E proteins expressed in olfactory epithelium might obscure efforts to elucidate the function of single family members by gene disruption.

Recently, a genetic disruption of the Ebf2 (O/E3) gene has been generated and the consequences of O/E3 loss examined in neuronal and non-neuronal tissues (Corradi et al., 2003). In addition to abnormalities of the neuroendocrine axis resulting in hypogonadism, there were specific defects in peripheral motor nerves. These neuronal phenotypes appeared to derive, at least in part, from hypomyelination and segmental dysmyelination. The expression of additional O/E family members in these neurons were not specifically examined.

We have used homologous recombination to generate mice in which *tau-lacZ* and *tau-GFP* reporters replaced multiple coding exons of *O/E2* and *O/E3* genes, respectively. The directed expression of a reporter such as β-galactosidase (β-gal) or GFP in O/E-expressing cells permits the analysis of the role of O/E proteins in cell fate decision and axonal projection. Neonatal lethality was observed before postnatal day 2 in $O/E2^{lacZ/lacZ}$-null animals, and frequent postnatal lethality was observed in $O/E3^{GFP/GFP}$-null animals, although approximately half survived into adulthood. The expression of putative O/E-target genes and the morphological appearance of the olfactory epithelium are normal in both mutant lines. However, there is a marked defect in the projection of olfactory axons to the dorsal olfactory bulb (OB) surface in $O/E2^{lacZ/lacZ}$- and $O/E3^{GFP/GFP}$-null animals, and a similar phenotype was observed in $O/E2^{lacZ/+}/O/E3^{GFP/+}$ double heterozygous mice. Together, our observations suggest that O/E proteins are only partially redundant in the olfactory system and make both distinct and dose-dependent contributions to ORN targeting in the OB.

Materials and methods

Generation of *O/E2* and *O/E3*-mutant mice

O/E2 and O/E3 cDNAs were used to screen a 129/SvJ mouse lambda genomic library (Stratagene, La Jolla, CA), and clones carrying 5′ exons were identified by PCR. *Tau-lacZpA (TL)* and *tau-GFPpA (TEG)* replacement cassettes were introduced into the *Not*I and *Pst*I restriction sites within the 5′ untranslated regions (UTRs) of *O/E2* and *O/E3* genes and replaced the first 6 and 5 exons, respectively. The *TEG* replacement cassette was based on the *TL* cassette (Mombaerts et al., 1996) in which the *lacZpA* was replaced with the *GFPpA* from the pEGFP-N1 plasmid (CLONTECH, Palo Alto, CA). The *loxP*-flanked *pgk-neo* selection cassette, *LΔTNL*, required for selection in ES cells, was removed after generation of chimeric mice (Zhao and Reed, 2001).

Linearized replacement constructs were electroporated into R1 129 embryonic stem (ES) cells, and G418-resistant clones with homologous recombination events were identified by Southern hybridization. The genetically modified ES cells were expanded and injected into C57BL/6 blastocysts, and the resulting 129/Sv-C57BL/6 chimeric mice and their *O/E* heterozygous progeny were mated with cre-expressing transgenic mice to generate *O/E* mutant alleles that deleted the Neo selection cassette. The genotypes of subsequent generations of *O/E* mutant mice were determined by PCR analysis.

In situ hybridization

Digoxigenin-labeled riboprobes (Wang et al., 1997) synthesized from plasmids containing the cDNA sequences of OMP, CNGA2, O/E1, O/E2 and O/E3 were hybridized to paraformaldehyde or Bouin's solution-fixed embryonic and adult olfactory tissues sections (14-20 μm). In situ hybridization was performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993) with the following modification in pre-prehybridization treatment. Tissue sections were post-fixed in Bouin's solution (Sigma, St Louis, MO, USA) or 4% paraformaldehyde (PFA) for 10 minutes at room temperature and washed in PBS (three times, 5 minutes each). Proteinase K treatment (20 μg/ml) was carried out at room temperature for 10 minutes followed by PBS wash and post-fixation in Bouin's solution or 4% PFA (10 minutes at room temperature). The tissue sections were then washed in PBS (three times, 5 minutes each) followed by water (5 minutes) and acetylation in triethanolamine/acetic anhydride/HCl solution. Finally, the tissue sections were washed three times in PBS before prehybridization and probe addition.

Immunohistochemistry

Immunohistochemistry was performed on PFA-fixed embryonic and adult olfactory tissue sections (14-20 μm). Sections were washed in Buffer T (0.1 M Tris, pH 7.5, 0.15 M NaCl, 0.1% Triton X-100, three time, 5 minutes each) before incubation in buffer T+10% normal serum (buffer T1). Incubation with primary antibody was diluted in buffer T1 and incubated at 4°C overnight. Sections were washed in Buffer T three times (5 minutes each) at room temperature and antibody binding visualized with Cy3- or HRP- (horse radish peroxidase) conjugated secondary antibodies. A monoclonal antibody to Map2 (Sigma) was used to visualize relay neuronal dendrites. Fluorescent Nissl stain (Neurotrace 546, Molecular Probes) was used according to manufacturer's directions.

X-gal staining

The X-gal staining for whole-mount preparation and on sections was performed essentially as described (Mombaerts et al., 1996). Fixed

tissues were isolated following intracardiac perfusion of anesthetized mice (ketamine-xylazine, RBI, Natick, MA). For cryosections, dissected tissues were treated with 20% sucrose plus 250 mM EDTA for 24 hours at 4°C, frozen in OCT compound (Sukura Finetek, Torrance, CA), and sections cut (10-20 μm) in a cryostat.

Images of whole-mount X-gal staining were acquired using a Leica ZFIII stereomicroscope and Zeiss Axiocam color digital camera. GFP fluorescent images in whole-mount tissues were obtained as flattened *z*-stack images collected on a Zeiss LSM510 confocal microscope.

Results

Generation of *O/E2* and *O/E3*-mutant mice

O/E2 and *O/E3* gene-targeted mutations were generated by inserting *tau-lacZ* and *tau-GFP* reporter genes with polyadenylation signals into the 5′ UTRs (untranslated regions) and replacing the first six and five coding exons, respectively (Fig. 1A,B). These exons (encoding the first 185 and 162 amino acids) contain domains of O/E proteins that are crucial

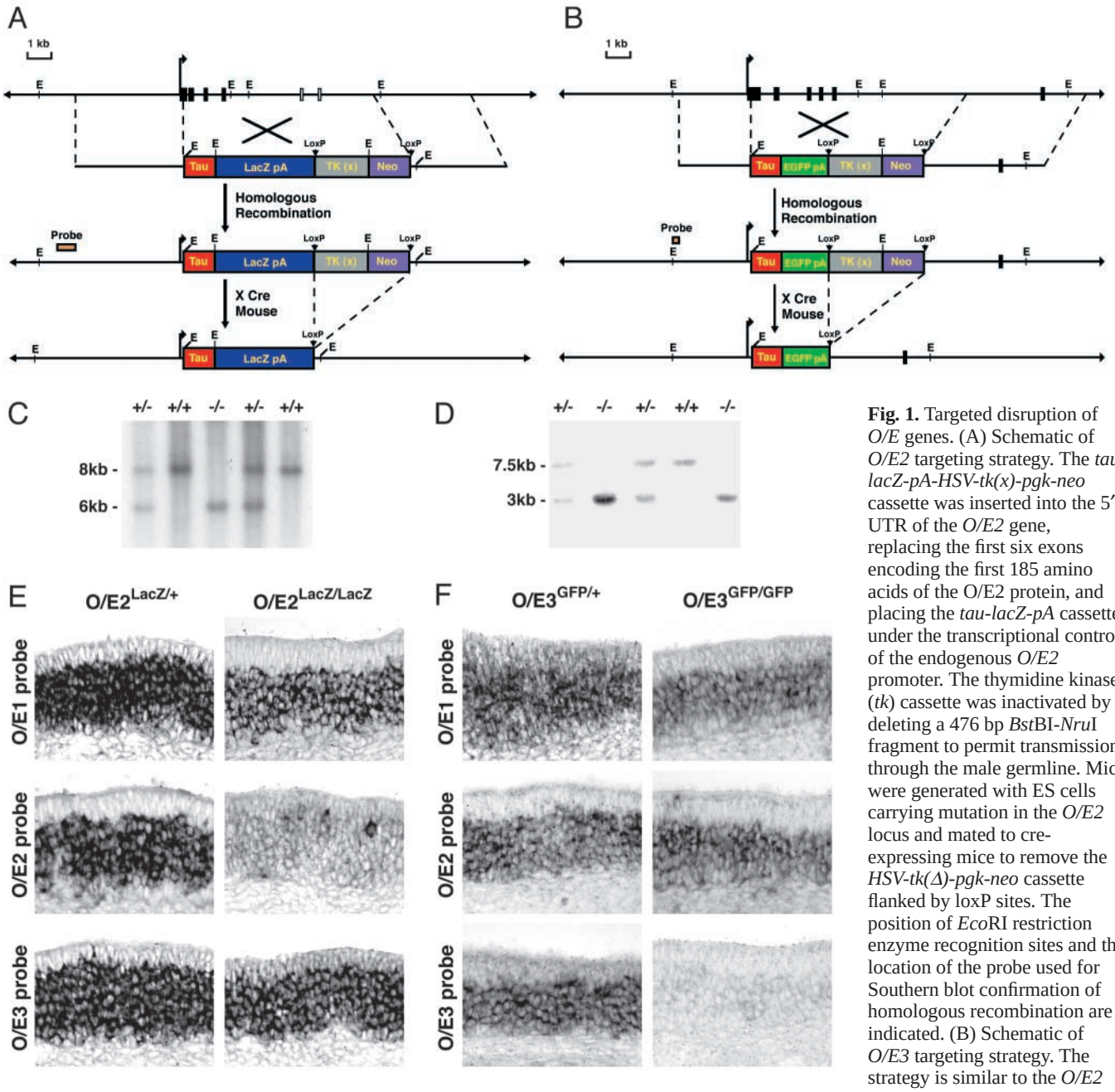

Fig. 1. Targeted disruption of *O/E* genes. (A) Schematic of *O/E2* targeting strategy. The *tau-lacZ-pA-HSV-tk(x)-pgk-neo* cassette was inserted into the 5′ UTR of the *O/E2* gene, replacing the first six exons encoding the first 185 amino acids of the O/E2 protein, and placing the *tau-lacZ-pA* cassette under the transcriptional control of the endogenous *O/E2* promoter. The thymidine kinase (*tk*) cassette was inactivated by deleting a 476 bp *Bst*BI-*Nru*I fragment to permit transmission through the male germline. Mice were generated with ES cells carrying mutation in the *O/E2* locus and mated to cre-expressing mice to remove the *HSV-tk(Δ)-pgk-neo* cassette flanked by loxP sites. The position of *Eco*RI restriction enzyme recognition sites and the location of the probe used for Southern blot confirmation of homologous recombination are indicated. (B) Schematic of *O/E3* targeting strategy. The strategy is similar to the *O/E2* targeting strategy with the following changes. A *tau-GFP-pA* reporter gene was used in the place of *tau-lacZ-pA* cassette, and five exons of the *O/E3* gene encoding the first 162 amino acids of the O/E3 protein were replaced. (C) Southern blot analysis of the *O/E2* alleles. Genomic DNA of *O/E2* mutant littermates was digested with *Eco*RI restriction enzyme, separated by agarose-gel filtration and subjected to Southern blot analysis. A wild-type *O/E2* allele yields a 8 kb hybridization signal and a mutated *O/E2* allele gives a 6 kb signal. (D) Southern blot analysis of the *O/E3* alleles. A wild-type *O/E3* allele gives a 7.5 kb hybridization signal and a mutated *O/E3* allele gives a 3 kb hybridization signal. (E) In situ hybridization of olfactory epithelium sections of neonatal *O/E2* heterozygous and homozygous animals with O/E1, O/E2 and O/E3 probes. (F) In situ hybridization of olfactory epithelium sections of neonatal *O/E3* heterozygous and homozygous animals with O/E1, O/E2 and O/E3 probes.

for DNA binding and transactivation at the O/E-binding site. These reporter-tagged, genetic disruptions of the respective O/E genes are referred to as *O/E2-lacZ* ($O/E2^{lacZ}$) and *O/E3-GFP* ($O/E3^{GFP}$). Mice generated from these constructs express the tau-reporter expression cassettes and the polyadenylation signals should prevent the transcription of downstream exons. Homologous recombination events were confirmed by Southern blot analysis (Fig. 1C,D). In situ hybridization with probes complementary to 3′ O/E mRNAs indicated that the reporter insertion and gene disruptions abolished *O/E2* and *O/E3* expression in $O/E2^{lacZ/lacZ}$ and $O/E3^{GFP/GFP}$ mice, respectively (Fig. 1E,F). It was possible that mutation of one *O/E* family member could lead to aberrant expression of other family members as has been described for the MyoD-related transcription factors (Weintraub, 1993). However, in the $O/E2^{lacZ/lacZ}$ and $O/E3^{GFP/GFP}$ animals, the expression of other *O/E* genes was not abolished (Fig. 1E,F), suggesting that mutations in the *O/E2* and *O/E3* genes did not lead to gross misregulation of the entire *O/E* gene family, and the phenotypes associated with each mutation are specific to the loss of individual *O/E* genes.

Reporter expression mimics endogenous O/E pattern

The gene replacement strategy that disrupted the *O/E2*- and *O/E3*-coding regions placed *tau-lacZ* and *tau-GFP* reporter genes under the control of the respective promoters. Studies in *C. elegans* and *Xenopus* suggested that loss of an O/E protein resulted in defects in axon fasciculation and targeting and altered cell fate determination. This gene replacement strategy allowed an analysis of the location and projection of O/E-expressing neurons by following reporter expression. In agreement with previously described O/E expression patterns, tau-β-gal and tau-GFP reporters exhibit prominent expression in the adult olfactory epithelium and vomeronasal organ of heterozygous mice (Fig. 2A-D). Fusion to the microtubule-associated protein tau specifically localizes the β-gal and GFP reporters, and allowed visualization of axons and nerve termini of cells that normally express O/E2 and O/E3, respectively. The β-gal and GFP reporters were detected in the neuronal cell layer of olfactory epithelium and in olfactory axon bundles (Fig. 2E,G). A small number of O/E3-positive cells near the basal lamina do not stain with a neuron-specific tubulin antibody and presumably reflect an olfactory progenitor population that precedes the expression of ORs (Iwema and Schwob, 2003). We have not examined whether these O/E3-labelled cells are proliferative (Fig. 2K). ORNs project their axons several millimeters to glomerular structures within the adult OB. In heterozygous animals, olfactory glomeruli can be visualized by X-gal staining or intrinsic GFP fluorescence (Fig. 2F,H). We do not observe intrinsic O/E2 or O/E3 expression within the OB during embryonic development or in the adult (Fig. 2F,H, and see Fig. S1 at http://dev.biologists.org/supplemental).

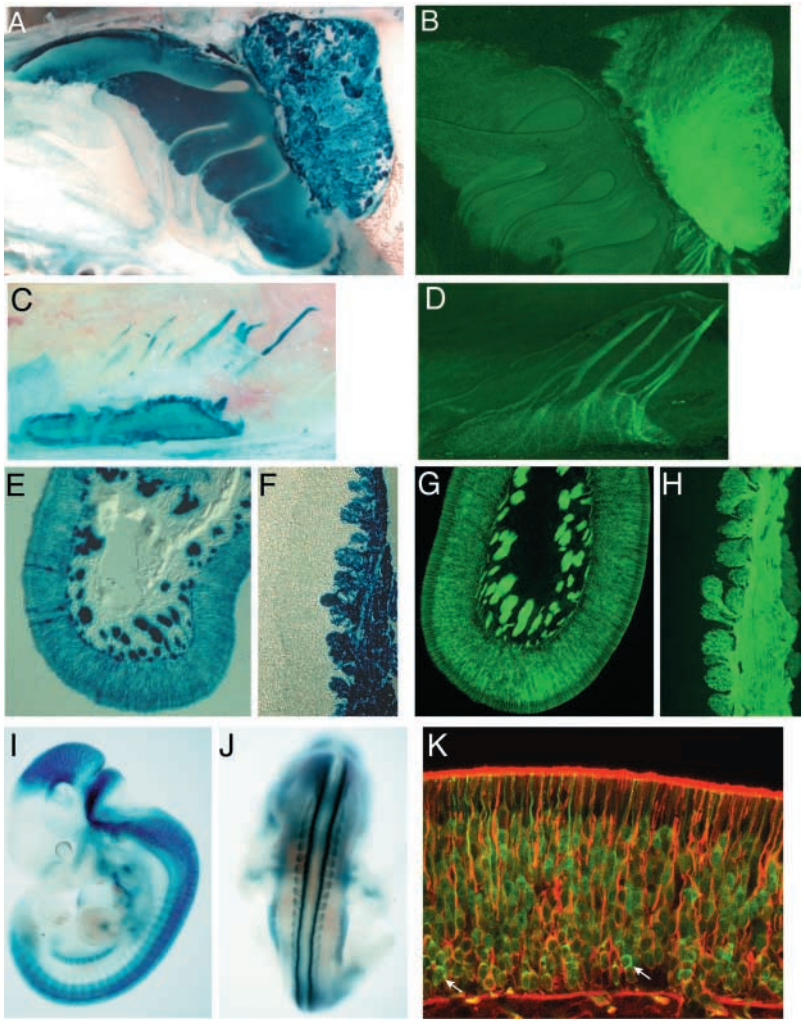

Fig. 2. Expression of Tau-reporters mimic endogenous O/E pattern. (A,C) Mid-sagittal view of an adult *O/E2* heterozygous animal after X-gal staining. β-galactosidase activity was present in the olfactory epithelium and bulb (A), vomeronasal organ (C) and axon fibers projecting from these two structures. (B,D) Mid-sagittal view of an adult *O/E3* heterozygous animal. GFP fluorescence was visible in the olfactory epithelium and bulb (B), vomeronasal organ (D), and axon fibers projecting from these two structures. (E) X-gal staining of olfactory epithelium section of an $O/E2^{lacZ/lacZ}$ animal showing β-gal activity in the ORN cell bodies and axon bundles. (F) X-gal staining of OB section of $O/E2^{lacZ/+}$ animal showing β-gal activity in ORN axons projecting to the olfactory glomeruli. (G) An olfactory epithelium section of an $O/E3^{GFP/+}$ animal showing GFP fluorescence in the ORN cell bodies and axon bundles. (H) An OB section of $O/E3^{GFP/+}$ animal showing GFP fluorescence in ORN axons projecting to the olfactory glomeruli. (I,J) Whole-mount X-gal staining of an E11 $O/E2^{lacZ/+}$ mouse embryo showing β-gal expression in the neural tissues. (K) Two-color confocal image of OE from heterozygous OE3 mouse. Intrinsic GFP localization is shown in green and neuronal specific tubulin shown in red. Two cells near the basal lamina that express only the GFP reporter are indicated by arrows.

Tau-reporters are expressed in developing neural structures

We next examined the patterns of the reporter gene products in embryos to determine whether they mimicked endogenous O/E expression. In E11.5

Table 1. Summary of *O/E* genotype/survival at various ages

Parents	Age genotyped	Number of litters	Genotype +/+	Genotype +/–	Genotype –/–
O/E2 (+/LacZ) × O/E2 (+/LacZ)	E10.5 to E19	19	31 (22%)	73 (50%)	41 (28%)
O/E2 (+/LacZ) × O/E2 (+/LacZ)	P0	16	29 (25%)	58 (49%)	31 (26%)
O/E2 (+/LacZ) × O/E2 (+/LacZ)	Three weeks	8	20 (42%)	28 (58%)	0
O/E3 (+/GFP) × O/E3 (+/GFP)	Embryos + neonates	19	36 (25%)	72 (50%)	37 (25%)
O/E3 (+/GFP) × O/E3 (+/GFP)	Three weeks	41	73 (26%)	156 (55%)	56 (19%)

heterozygous embryos, we detected *O/E2* driven β-gal expression in the DRG and along the spinal cord in whole-mount X-gal staining, and prominent reporter expression was also detected in the midbrain hindbrain region (Fig. 2I,J). We also detected GFP fluorescence in specific developing neural structures of E11.5 $O/E3^{GFP/+}$ embryos. However, this GFP fluorescence was too weak to easily discern the details of *O/E3* expression in whole-mount preparations of heterozygous embryos. These observations indicate that we have successfully generated *O/E2* and *O/E3* mutant mice, and the reporter expression agrees with the previously described O/E pattern (Wang et al., 1997).

Phenotype of O/E2-deficient mice

Neonatal lethality was observed in *O/E2* mutant animals. An expected 1:2:1 Mendelian inheritance pattern was observed in embryonic and newborn litters of heterozygous crosses but none survived to adulthood (Table 1). At birth, the $O/E2^{lacZ/lacZ}$ neonates were indistinguishable from heterozygous and wild-type littermates, but most $O/E2^{lacZ/lacZ}$ mutants died within 1 day, and all homozygous mutant animals died by postnatal day 2 (P2).

An ORN projection defect and a modest reduction of the OB size were observed in newborn $O/E2^{lacZ/lacZ}$ animals. This phenotype was apparent in whole-mount X-gal staining and clearly demonstrated in the coronal sections of neonatal OB (Fig. 3). By P1 in $O/E2^{lacZ/+}$ mice, ORN axons, visualized by whole-mount X-gal staining, have already extended across the dorsal OB. The bulb was smaller in $O/E2^{lacZ/lacZ}$ neonates and X-gal-labeled olfactory axons failed to project to the dorsal and lateral surfaces of the OB (Fig. 3A). These phenotypes were also observed in immunohistochemical staining of coronal sections of OB with antibodies to β-galactosidase and OMP, a mature neuronal marker (Fig. 3B). At a position along the rostrocaudal axis where the OB is first visible in the heterozygous animal, the corresponding coronal section in the homozygous mutant animal showed an accumulation of axon fibers but no OB tissue. At a more caudal position where the

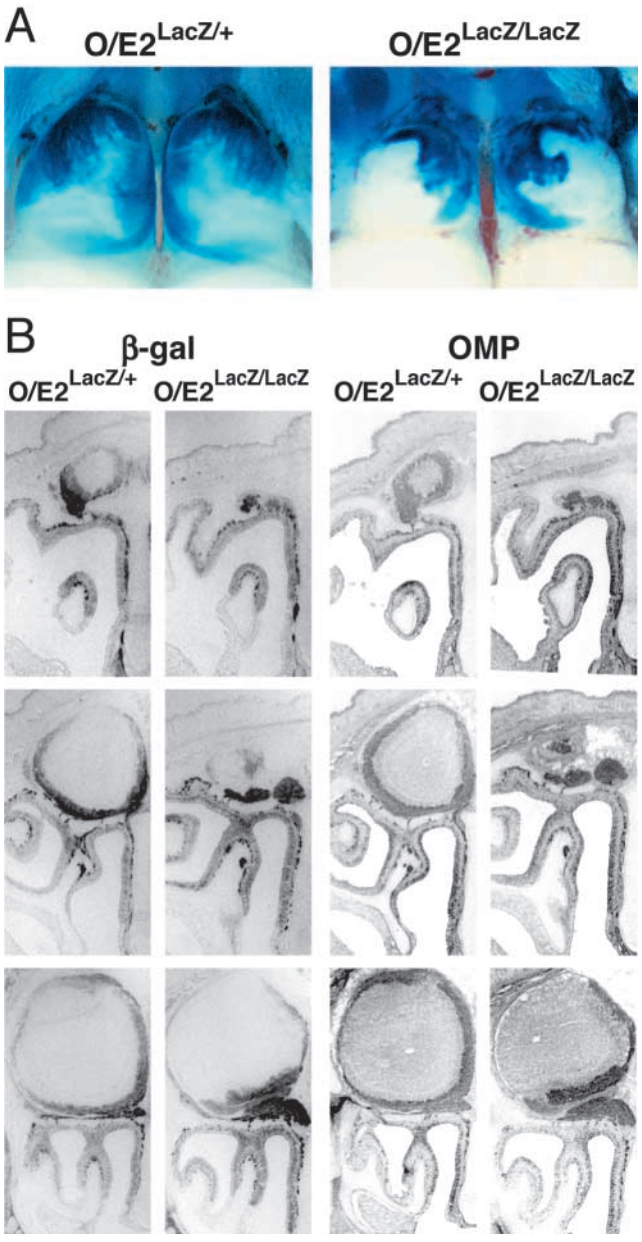

Fig. 3. Neonatal $O/E2^{lacZ/lacZ}$ mice exhibit ORN projection defect. (A) Whole-mount X-gal staining of $O/E2^{lacZ/+}$ and $O/E2^{lacZ/lacZ}$ neonates showing the ORN projection patterns to the OB. At P1 in $O/E2^{lacZ/+}$ animals, the β-gal positive ORN axons cover most of the OB surface, but the $O/E2^{lacZ/lacZ}$ mice lack projections to the lateral and dorsal regions of their OB. In addition to the projection phenotype, the OB of neonatal $O/E2^{lacZ/lacZ}$ animals are significantly smaller and rounder in shape than those of the heterozygous littermates. Similar results were observed in additional $O/E2^{lacZ/lacZ}$ mice (n=8). (B) β-Galactosidase staining and OMP immunohistochemistry of coronal sections through the OB of $O/E2^{lacZ/+}$ and $O/E2^{lacZ/lacZ}$ neonates showing the ORN projection patterns to the OB. The sections are matched by the morphology of the olfactory turbinates. In the sections of *O/E2* heterozygous neonate, the β-gal and OMP-positive ORN axons covers most of the OB surface, but the $O/E2^{lacZ/lacZ}$ animals display no apparent projections to the lateral and dorsal regions of their OB. In addition, the sections also demonstrate the shortening of the OB in the $O/E2^{lacZ/lacZ}$ animals. In all panels, dorsal is at the top and medial is towards the right.

ORN axons of an *O/E2* heterozygous animal completely cover the surface of the OB, the ORN axons of an *O/E2* mutant accumulated ventromedially and failed to innervate the dorsolateral surface of the OB. These results showed that the *O/E2* mutant neonates have a reduction in the OB size and clearly demonstrated an ORN projection defect associated with *O/E2* loss.

Phenotype of O/E3-deficient mice

The $O/E3^{GFP/GFP}$ mice display neuronal defects but in contrast to $O/E2^{lacZ/lacZ}$ mutants, a majority of the *O/E3* null mice survive to adulthood. A1:2:1 Mendelian distribution of genotypes was observed at birth, but ~25% of *O/E3* mutant neonates died by P21 (Table 1). At P14, $O/E3^{GFP/GFP}$ mice were smaller and these animals required additional nursing time before weaning. An additional 25% of $O/E3^{GFP/GFP}$ mutants died by P30. The $O/E3^{GFP/GFP}$ animals that survived to adulthood grew to full size. The females were fertile with a slightly reduced mating efficiency and nurse normal-sized litters. However, the $O/E3^{GFP/GFP}$ males had a dramatically reduced mating efficiency. These growth and fertility defects are consistent with the recent report by Corradi et al. (Corradi et al., 2003).

The ORN axons in adult *O/E3* null mice displayed a stereotyped abnormal projection in the OB. When visualized by whole-mount GFP fluorescence, the dorsal aspect of the OB in $O/E3^{GFP/GFP}$ mice were void of olfactory glomeruli, and this region possessed few if any axon fibers (Fig. 4A). In coronal sections of adult OB, glomeruli were clearly absent on the dorsal surface, and GFP fluorescence and OMP staining showed a reduction of fibers in this region (Fig. 4A, Fig. 5A). In contrast to P1 $O/E2^{lacZ/lacZ}$ mice, OMP immunoreactivity was detected on the entire surface of rostral OB sections in $O/E3^{GFP/GFP}$ neonates (Fig. 4B). In more caudal sections, OMP staining diminishes in intensity and extent. In all sections, the thinner external plexiform layer on the dorsal aspect of the OB in $O/E3^{GFP/GFP}$ mice was consistent with the eventual absence of axons and glomeruli in this region seen in the adult.

The expression of tyrosine hydroxylase (Th) in the periglomerular neurons of the OB is dependent on innervation and sensory activity by the olfactory epithelium. (Baker et al., 1999; Cho et al., 1996; Nadi et al., 1981). Th expression was detected around the olfactory glomeruli of $O/E3^{GFP/GFP}$ animals, but its expression in dorsal OB was greatly attenuated due to the absence of olfactory glomeruli and reduction of ORN axons in this region (Fig. 5B). These results suggest that ORN axons are able to form functional connections with the OB of *O/E*-deficient animals, and these connections propagate sensory signals in the absence of O/E3.

We next asked whether the dorsal OB that lacks innervation by ORNs retains cells that comprise the normal neuronal circuitry. Nissl staining of OB from $O/E3^{GFP/GFP}$ mice revealed the characteristic mitral cell layer and scattered neuronal cells in the external plexiform region (Fig. 5C). Additional Nissl stained cell bodies were observed where periglomerular cells normally reside, but are not organized around discrete glomeruli in the absence of ORN axons. Moreover, the dramatically reduced Map2 staining in the dorsal bulb region (Fig. 5C) suggests that there are significant perturbations in dendritic organization.

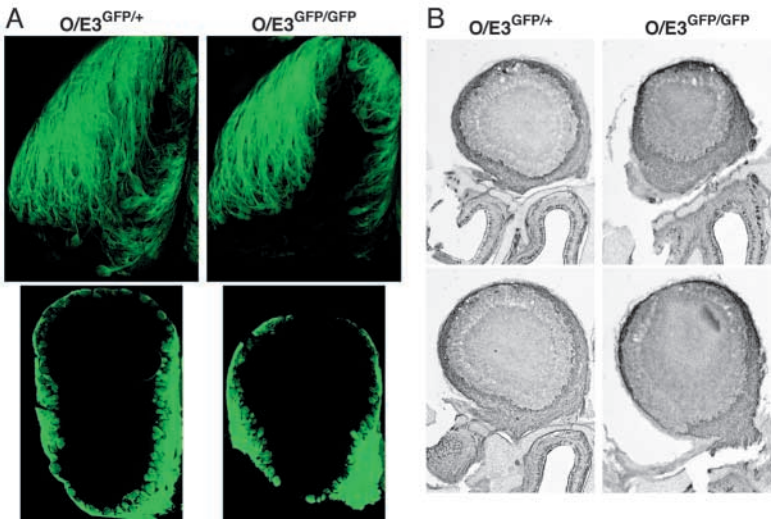

Fig. 4. Adult *O/E3* mutants exhibit ORN projection defect. (A) The ORN projection to the OB was visualized by *O/E3*-directed GFP fluorescence in heterozygous and $O/E3^{GFP/GFP}$ mice. The absence of ORN axons on the dorsal surface of $O/E3^{GFP/GFP}$ OB can be seen in the whole-mount view and in coronal sections. Similar results were obtained in five independent mice of each genotype. Similar to the observation in *O/E2* mutant animals, the OBs of adult $O/E3^{GFP/GFP}$ mice are slightly smaller and rounder in shape than that of their heterozygous littermates. (B) OMP immunohistochemistry of coronal sections through the OB of *O/E3* heterozygous and homozygous neonates showing the ORN projection patterns to the OB. Sections from both anterior and posterior OB are shown. In both animals, the ORN axons cover the entire OB. However, stronger anti-OMP immunoreactivity was seen on the dorsal and lateral OB of the $O/E3^{GFP/GFP}$ neonate, and thinning of the external plexiform layer on the dorsal aspect of $O/E3^{GFP/GFP}$ OB was observed, indicating a possible prelude to the adult phenotype. In all panels, dorsal is towards the top and medial is towards the right.

The pattern of olfactory enriched genes and guidance molecules appear normal

Semaphorin (Sema) family of guidance molecules and their receptors, neuropilins (Npns), have been implicated in the projection of olfactory axons and proper formation of the olfactory circuitry (Pasterkamp et al., 1999; Renzi et al., 2000; Schwarting et al., 2000). The levels of Npn1, Npn2, Sema3a and Sema3f message expression and their patterns along the anterior-posterior axis were grossly normal in E16.5 *O/E2*- and *O/E3*-null animals (data not shown). This observation suggests that the ORN projection defect was not caused by misexpression of these guidance molecules. In addition, the expression of CNGA2, OMP and two olfactory receptors (M72 and M4) were not significantly altered in the *O/E2* and *O/E3* mutant animals (see Fig. S2 at http://dev.biologists.org/supplemental). Finally, electro-olfactogram (EOG) recordings revealed normal responses to short single-pulse odorant applications in $O/E3^{GFP/GFP}$ mice (data not shown). This indicates that olfactory gene expression is grossly normal and the odorant transduction machinery is present in the absence of O/E2 or O/E3.

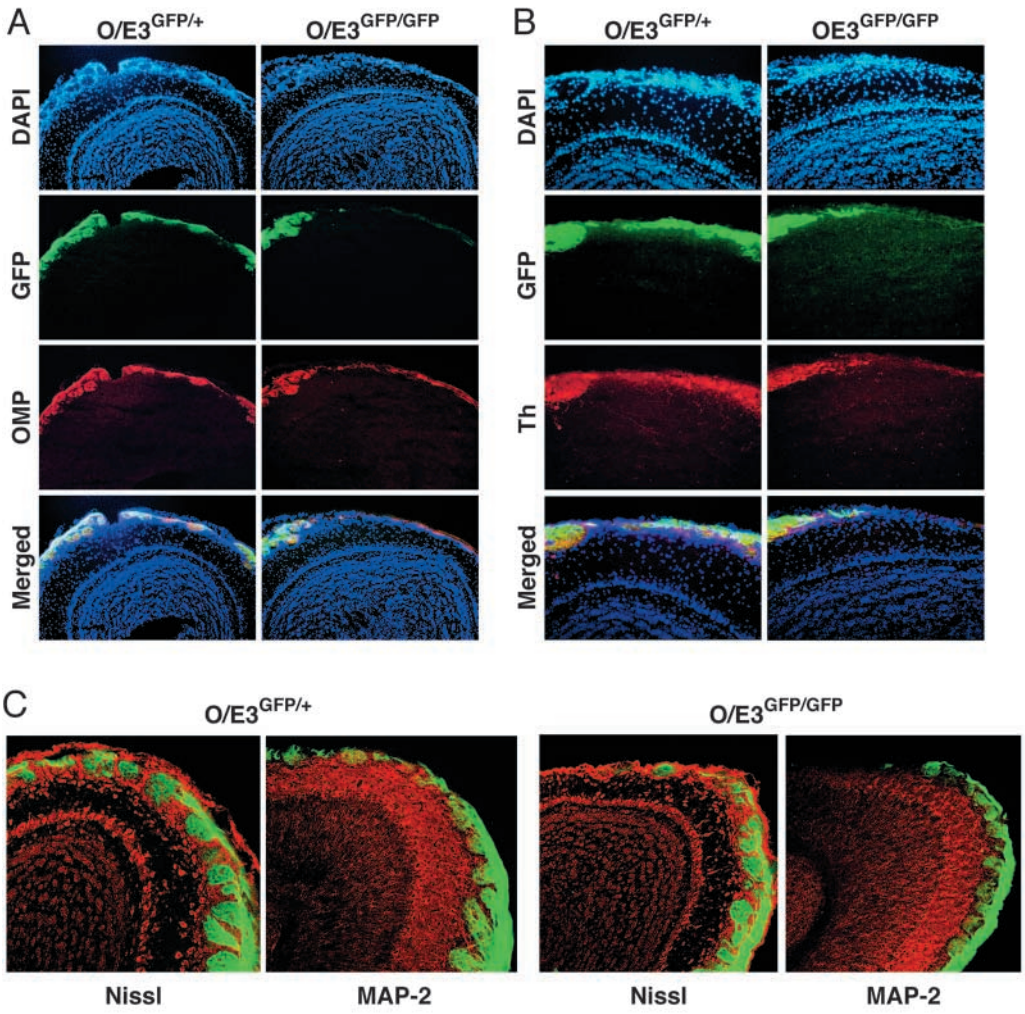

Fig. 5. Functional connections are established in the OB of adult *O/E3*$^{GFP/GFP}$ mutants. (A) The ORN projection to dorsal OB and olfactory glomeruli formation in this region were visualized by GFP fluorescence and OMP immunofluorescence. The patterns of GFP and OMP in the coronal sections clearly show the absence of olfactory glomeruli in the dorsal aspect of *O/E3*$^{GFP/GFP}$ mutant OB. (B) Tyrosine hydroxylase (Th) expression in the periglomerular neurons is dependent on innervation and activity from the olfactory epithelium. Immunofluorescence demonstrates that Th expression overlaps the GFP-positive glomeruli and suggests that ORNs transduce signals and form functional connections within the OB of *O/E3*$^{GFP/GFP}$ animals. (C) Characterization of OB neurons in *O/E3*$^{GFP/GFP}$ mice. The soma of mitral, tufted and periglomerular cells are visualized with a fluorescent Nissl stain in heterozygous and homozygous *O/E3*$^{GFP/GFP}$ mice. Although there is considerable disorganization of the glomerular layer, many neurons are present. The intensity of dendritic marker Map2 staining is considerably diminished in *O/E3*$^{GFP/GFP}$ animals, consistent with the thinning of the external plexiform layer on the dorsal surface. The intrinsic GFP labeling of ORN axons and their convergence into glomeruli is shown in green.

Phenotype of *O/E2* / *O/E3* double heterozygous animals

The mouse O/E proteins exhibit largely overlapping expression in the olfactory epithelium during development and into adulthood, and share a similar ability to bind DNA and activate transcription in vitro. Although there are phenotypic differences between *O/E2*- and *O/E3*-null animals, common ORN projection defects are shared among these animals. In order to investigate whether the observed projection phenotype is specific to each *O/E* mutation or the effect of reduced total O/E dose, we studied the ORN projection pattern in *O/E2*$^{lacZ/+}$/*O/E3*$^{GFP/+}$ double heterozygous animals.

The *O/E2/O/E3* double heterozygous animals are viable, and their gross appearance is similar to that of their *O/E2* or *O/E3* single heterozygous and wild-type littermates. The *O/E2*$^{lacZ/+}$/*O/E3*$^{GFP/+}$ double heterozygous animals exhibit an ORN projection defect that is similar to *O/E3*$^{GFP/GFP}$ littermates: failure to innervate dorsal OB. In contrast to homozygous *O/E2*- and *O/E3*-null animals, the OB of the double heterozygous animals is similar in shape and size to *O/E* heterozygous and wild-type animals (Fig. 6A,B). Thus, the presence of projection defects in ORNs of *O/E2*$^{lacZ/+}$/*O/E3*$^{GFP/+}$ double heterozygous animals but normal OB size suggests that total O/E gene dose accounts for some, but not all of the observed phenotypes.

Tau-reporter overexpression does not cause the ORN projection defect

The microtubule-associated protein tau stabilizes microtubule assembly and has been implicated in various cellular processes including neurodegenerative disorders (Garcia and Cleveland, 2001; Grant et al., 2001; Nagy et al., 2000; Paglini et al., 2000). Overexpression of tau in transgenic animals can lead to cytoskeletal disruption, vesicle trafficking defects and neuronal abnormalities (Ebneth et al., 1998; Grundke-Iqbal and Iqbal, 1999; Probst et al., 2000), although high levels of tau-reporter expression have not been implicated in olfactory system abnormalities (Belluscio et al., 1999; Mombaerts et al., 1996; Strotmann et al., 2000; Wang et al., 1998; Zheng et al., 2000). To eliminate the possibility that high level tau-reporter expression caused the observed defects, we generated mice carrying both *O/E3-tau-GFP* and *OMP-tau-lacZ* alleles. In these animals, tau-β-galactosidase reporter is expressed in mature olfactory neurons under the control of *OMP*, a strong

olfactory-specific promoter, and the level of tau-reporter expression, which is based on intensity of β-galactosidase staining in mature ORNs, exceeded that present in either *O/E2* or *O/E3* homozygous animals.

The patterns of ORN projection and glomeruli formation on the OB of mice carrying *O/E3-tau-GFP* and *OMP-tau-lacZ* alleles was dependent on the *O/E3* locus and not on the amount of tau-reporter (Fig. 6). As expected, $O/E3^{GFP/GFP}$ animals carrying one *OMP-tau-lacZ* allele exhibited the same ORN projection defect seen in *O/E3* mutant animals with wild-type *OMP* loci (Fig. 6E). In mice carrying one *O/E3-tau-GFP* ($O/E3^{GFP/+}$) and two *OMP-tau-lacZ* alleles ($OMP^{lacZ/lacZ}$), the patterns of ORN projection and olfactory glomeruli appeared normal indicating that the high level of tau-reporter present in these animals did not cause ORN projection defects (Fig. 6C,D). Based on our results and the normal ORN projection in animals expressing tau-reporters under *OMP* and *OR* promoters (Belluscio et al., 1999; Mombaerts et al., 1996; Strotmann et al., 2000; Wang et al., 1998; Zheng et al., 2000), we conclude that the observed ORN projection phenotype is caused by mutations in the *O/E* genes.

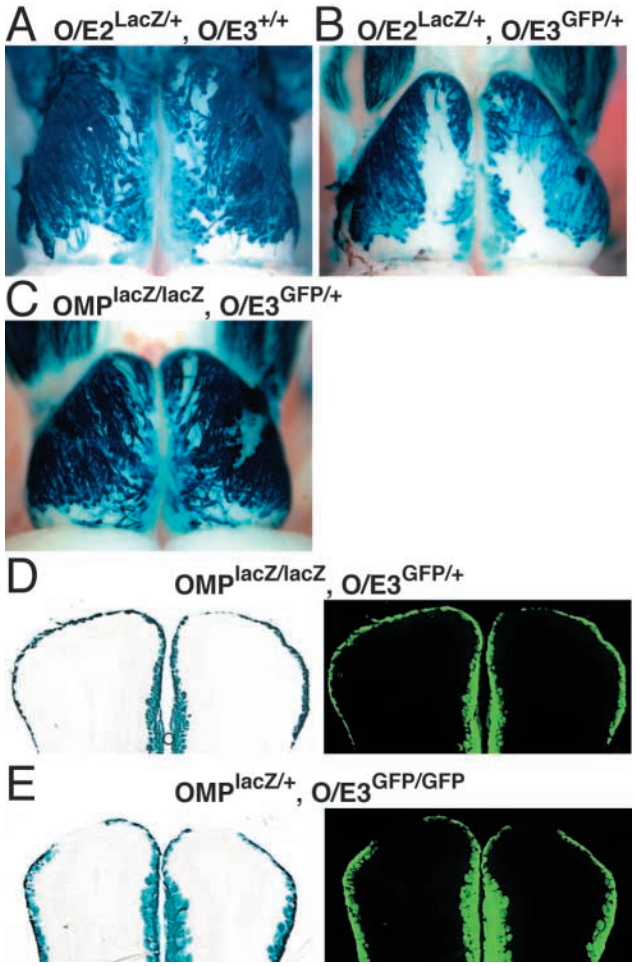

Fig. 6. $O/E2^{lacZ/+}/O/E3^{GFP/+}$ double heterozygous animals exhibit similar projection defects independent of Tau overexpression. (A) The ORN projection patterns on dorsal OB of adult $O/E2^{lacZ/+}$ and $O/E3^{GFP/+}$ mice were visualized by whole-mount X-gal staining. ORN axons and glomeruli are visible on the entire dorsal surface of the OB. (B) X-gal staining pattern of an $O/E2^{lacZ/+}/O/E3^{GFP/+}$ animal show areas where ORN axons and glomeruli are absent. (C) X-gal staining pattern of an $O/E3^{GFP/+}$ animal carrying two *OMP-tau-lacZ* alleles reveals a normal projection pattern, indicating that the extra gene dose of tau-β-galactosidase from the *OMP-tau-lacZ* alleles does not cause a projection defect. (D) Coronal sections of the OB from an animal with the same genotype as the one described in C show normal projections to the dorsal surface. (E) Coronal sections of the OB from an $O/E3^{GFP/GFP}$ animal carrying one *OMP-tau-lacZ* allele show the same projection defect seen in $O/E3^{GFP/GFP}$ homozygous animals.

Odorant receptor-dependent axonal convergence in $O/E^{GFP/GFP}$ mice

Odorant receptors (ORs) are expressed in one of the four spatial zones in the olfactory epithelium, and cells expressing a particular OR project their axons to spatially defined glomeruli within the OB (Mombaerts, 1999; Mori et al., 1999; Ressler et al., 1993; Vassar et al., 1993). Neurons expressing a particular OR project axons to two regions of each OB, one on the medial surface and the other on the lateral surface (Mombaerts et al., 1996; Royal and Key, 1999; Wang et al., 1998). We first examined whether OR-dependent axonal convergence occurs normally in *O/E3* null animals. Mice were generated that carried *O/E3-tau-GFP* alleles and either *P2-IRES-tau-lacZ* ($P2^{lacZ}$) or *M72-IRES-tau-lacZ* ($M72^{lacZ}$) tagged odorant receptor genes. These mice allowed us to examine the projection of both ventral and dorsal-targeting ORN axons in an O/E3 mutant background.

The axons of P2-expressing neurons converge to characteristic locations on the medial surface of the OB in *O/E3* heterozygous animals (Fig. 7A). In $O/E3^{GFP/GFP}$ homozygous animals, however, few fibers are visible on the medial surface, and most fibers terminate on the ventral surface in close juxtaposition to the cribriform plate (Fig. 7B). We next performed X-gal staining on coronal sections of the OB of $O/E3^{GFP/GFP}$ mice carrying $P2^{lacZ}$ alleles in order to address whether P2 axons converge to defined glomeruli and to determine the positions of the labeled glomeruli. We observed convergence of P2 ORN axons to defined glomeruli on the lateral and medial aspects of each OB, indicating that absence of the *O/E3* gene product did not affect the ability of axons to converge (Fig. 7D,F). The different shape of the olfactory bulb in $O/E3^{GFP/GFP}$ mice make it difficult to assign specific perturbations to the projection pattern of lacZ-labeled axons. However, consistent with the patterns observed in whole-mount staining, P2 axons in $O/E3^{GFP/GFP}$ animals converged to more ventral positions in the medial (Fig. 7C,D) and lateral (Fig. 7E,F) aspects of the OB relative to their heterozygous littermates. The ventral shift of P2 axon projection pattern suggests that there may be an overall ventral shift of olfactory glomerular positions in *O/E3* null mice, resulting in the absence of ORN fibers and olfactory glomeruli on the dorsal surface of the OB. Alternatively, the apparent shift in the position of the P2 glomerulus may simply result from changes in bulb shape and the dorsal projection defect in $O/E3^{GFP/GFP}$ animals derived from an independent primary defect, elimination of dorsal glomeruli.

To investigate the retention and location of dorsal glomeruli in *O/E3*-null mice, we examined the projection pattern of $M72^{lacZ}$ axons that normally target to the dorsal surface of the bulb. If dorsal glomeruli are eliminated in $O/E3^{GFP/GFP}$ mice,

the targeting of M72-labelled axons may be significantly perturbed. However, we observe stereotypic convergence of *lacZ*-labeled axons in *O/E3* mutant mice in spite of the broad region of the dorsal bulb that is devoid of GFP-positive fibers and glomeruli (Fig. 8). In *O/E3*$^{GFP/GFP}$ mice, the position of the *lacZ*-labeled glomerulus may be displaced from the dorsal surface, but changes in bulb morphology complicate the quantification of this displacement.

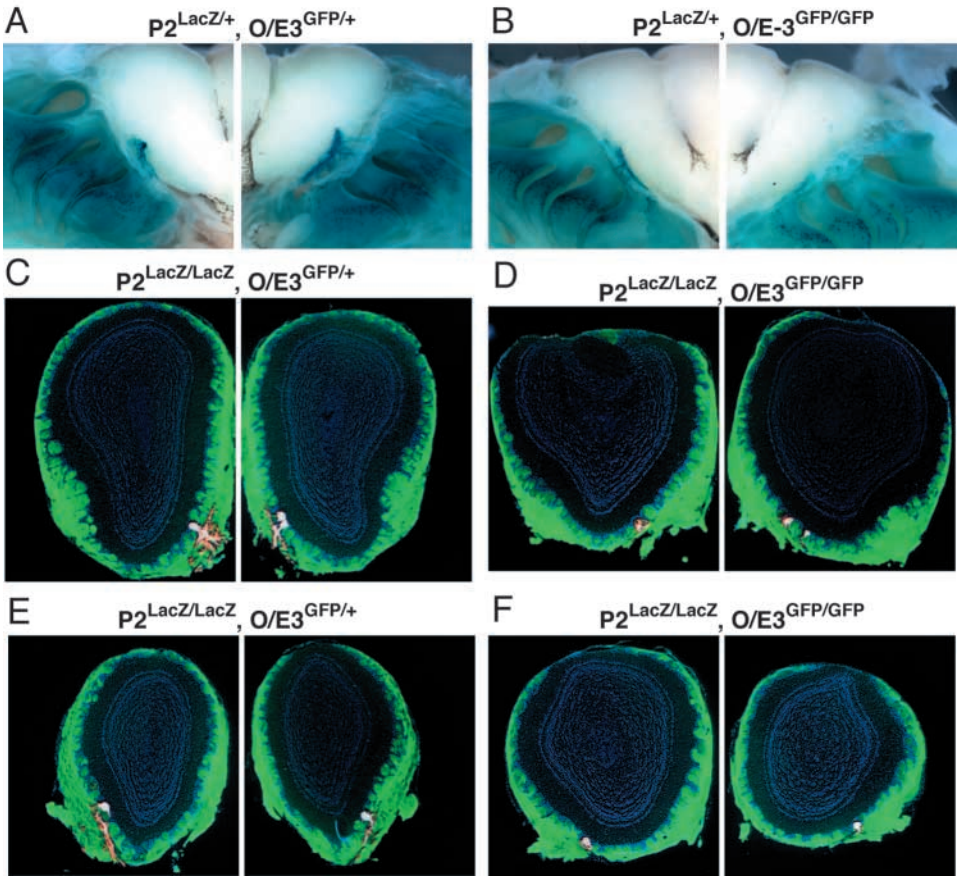

Fig. 7. Positions of P2 glomeruli are shifted ventrally. (A) Projections of ORNs expressing the P2 odorant receptor were visualized by X-gal staining in an *O/E3*$^{GFP/+}$ mouse carrying a *P2*^{lacZ} allele. (B) In agreement with the previously described pattern, axons from P2-expressing ORNs project dorsally before converging to a defined location on the medial surface of the OB in *O/E3*$^{GFP/+}$ animals. In an *O/E3*$^{GFP/GFP}$ mouse carrying a *P2*^{lacZ} allele, very few axons were visible on the medial surface of the OB indicating a change in projection pattern. (C,D) X-gal staining on coronal sections of OB showed convergence of P2-expressing axons to defined glomeruli on the medial aspect of OB. X-gal signal was pseudocolored in white for clear visualization. The sections were also stained with DAPI (blue) to mark the layers of the OB, and ORN axons were visualized by GFP fluorescence. P2-expressing axons in *O/E3*$^{GFP/GFP}$ homozygous animals converge to a more ventral position relative to their heterozygous littermates. (E,F) Coronal sections of the OB were treated as described in C to show convergence of P2-expressing axons to defined glomeruli on the lateral aspect of OB. Similarly, P2-expressing axons in *O/E3* homozygous animals converge to a more ventral position relative to their heterozygous littermates.

Discussion

Subtle variations in the complex intrinsic and extrinsic developmental cues can alter cell fate and identity. As an immature ORN starts to differentiate and emerge from the basal region of the olfactory epithelium, expression of genes required for mature ORN functions, including the signal transduction components (odorant receptors, *Golf*, *ACIII* and *CNGA2*) and other mature ORN markers (*OMP*, *50.06* and *50.11*), are coordinately regulated. A DNA sequence (the Olf1 site) is found in the promoters of these genes (Vassalli et al., 2002; Wang et al., 1993). The O/E proteins that bind to this site may act as a genetic switch for ORN development and regulate the expression of odorant transduction components and other mature ORN markers. The presence of Olf1 sites in odorant receptor promoters and their potential dependence on O/E proteins for receptor expression (Vassalli et al., 2002) is especially intriguing in light of the defects in axonal targeting observed here. The expression of multiple *O/E* genes in the mouse olfactory system may account for the absence of gross phenotypic defects like those reported in B cells where O/E1 (EBF) is uniquely expressed.

We describe the generation and analysis of mice carrying *O/E2* and *O/E3* null mutations. Replacement of the 5′ coding exons of *O/E2* and *O/E3* with *tau-lacZ* and *tau-GFP* reporter genes, respectively, allow the visualization of the neurons that normally express these *O/E* genes and examination of their axonal projections. Many CNS and PNS neurons express O/E proteins during development, and the expression of individual O/E proteins follows distinct temporal and spatial patterns. Defects in brain regions that express only O/E1 were reported in *O/E1* knockout animals (Garel et al., 2000; Garel et al., 1999; Garel et al., 2002). These studies relied on the expression of additional markers, and were limited in their ability to trace axon projection, cellular lineage and migration. Although we have not analyzed regions outside of the olfactory system, *O/E2*- and *O/E3*-tagged animals could assist such studies by providing reporters for monitoring cell lineage, cellular migration and axonal projection.

Although our findings strengthen the notion that O/E proteins are at least partially redundant in maintaining the expression of target genes, O/E expression in developing neuronal systems suggest that *O/E* genes are activated in distinct stages of development (Garel et al., 1997). In *Xenopus* primary neurogenesis, *Ngn1*, *O/E3*, *NeuroD* and *O/E2* are proposed to participate in a sequential transcriptional cascade where Ngn1 can bypass O/E3 and regulate NeuroD expression, and a similar transcriptional cascade may be present in the

mouse olfactory system (Cau et al., 2002; Dubois et al., 1998; Pozzoli et al., 2001). Therefore, the overall phenotype associated with each *O/E* genotype is likely to be a combination of both *O/E* gene-specific and O/E dose effects. An alternative yet complementary transcriptional cascade involving Mash1, Ngn1 and NeuroD has been proposed for the development of ORNs (Cau et al., 2002; Cau et al., 2000; Cau et al., 1997). However, the contributions of O/E2 and O/E3 proteins were not specifically addressed, and further studies are necessary to elucidate the interactions of HLH factors during ORN development.

Mutation in *O/E* genes leads to abnormalities in olfactory bulb innervation

The failure of axons to extend to the most dorsal aspects of the bulb in *O/E2*- or *O/E3*-null mice could arise from the elimination of target glomeruli in an intrinsic map of the bulb, from a global shift in the underlying bulb organization, or from an intrinsic failure in axon extension by ORNs. The glomeruli in the dorsal OB receive innervation from the most dorsal zone (zone-I) of the four spatial zones in the epithelium (Mori et al., 2000). If the phenotype observed in *O/E3* mutants results from elimination of glomeruli on the dorsal surface of the OB, one would expect that specific ORs from zone I of the olfactory epithelium would not be expressed.(Konzelmann et al., 1998). ORs in zone I and zone II (M72 and M4, respectively) (Qasba and Reed, 1998; Zheng et al., 2000) were present in their normal domains of the epithelium in adult $O/E3^{GFP/GFP}$ mice (see Fig. S2 at http://dev.biologists.org/supplemental), demonstrating that zone I OR expression for at least one receptor is largely normal. In addition, the observed patterns of dorsal projecting M72 axons and ventral targeting P2 axons are consistent with a global shift in the glomerular map in $O/E3^{GFP/GFP}$ mice. However, axons of M72- and P2-expressing cells do not target to the region of the OB where glomeruli are absent in O/E3-null mice. A specific perturbation in expression of ORs whose axons target to the dorsal-most glomeruli may also be responsible for the observed projection defect. This analysis must await the identification and genetic targeting of this subset of receptors.

Primary olfactory axons play an important role in glomerular definition and formation (Belluscio et al., 2002; Bozza et al., 2002; Carr and Farbman, 1993; Couper Leo et al., 2000; Fiske and Brunjes, 2001; Schwob et al., 1992). The aberrant axonal projection patterns could derive from a misreading of the gradients and cues present in the olfactory bulb. These axons would then induce glomeruli at inappropriate locations. The stereotyped location of glomeruli in the bulb are established by ORN axons perinatally and are retained in position in spite of continual replacement of the neurons. An inability of the ORN axons to extend as far on the bulb surface would lead to a similar reorganization of glomerular position.

The defects in ORN projection to dorsal OB, although distinct, are very similar in $O/E2^{lacZ/lacZ}$ and $O/E3^{GFP/GFP}$ mutants. The inability of ORNs to innervate dorsal OB in both mutants suggests that reduced O/E dose levels in these cells may contribute to the observed phenotype. Consistent with this notion, the ORN axons of $O/E2^{lacZ/+}/O/E3^{GFP/+}$ double heterozygous animals also fail to innervate the dorsal bulb. These gene dose effects, combined with high O/E expression in olfactory epithelium and absence from the OB, suggests an alternative mechanism to account for the projection defects. The expression of OR protein is essential to proper axon targeting (Mombaerts et al., 1996; Wang et al., 1998) and receptor expression levels could represent a crucial determinant in the length of axon extension. The presence of O/E consensus binding sites in the minimal promoter region for several ORs (Vassalli et al., 2002) (R.R.R., unpublished) further implicates in these transcription factors in receptor expression. The overlapping expression of each of the O/E family members in all olfactory neurons is consistent with the total dose of O/E gene expression rather than the particular identity that results in a failure to project all of the way to the most dorsal regions of the bulb.

In summary, although *O/E2* and *O/E3* mutant animals have distinct gross phenotypes, they share similar abnormal ORN projection patterns. We have demonstrated that the functions of *O/E* genes are not completely redundant, and the olfactory phenotype in each mutant probably derives from O/E dose dependent and individual *O/E* gene-specific effects. Although no olfactory phenotype has been reported in *O/E1* knockout mice (Lin and Grosschedl, 1995), our results suggest these mice may have similar defects that have not been detected in these untagged *O/E1* mutant animals. O/E proteins are

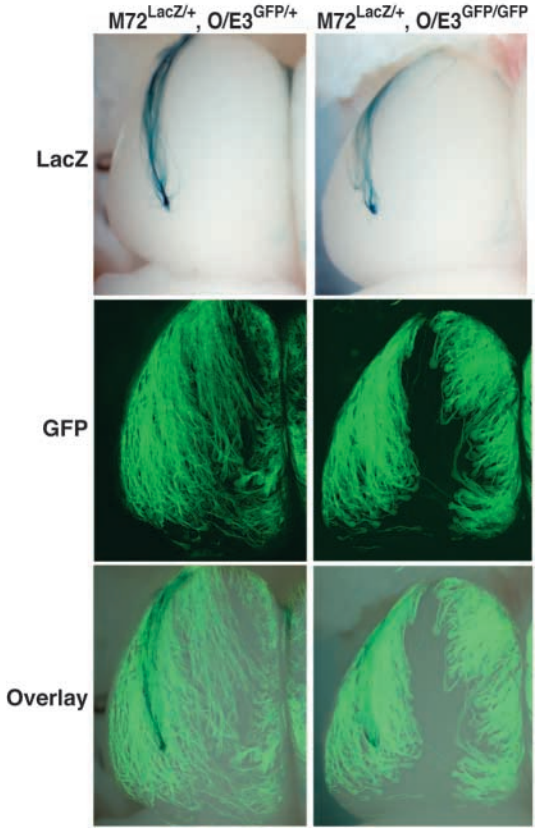

Fig. 8. The dorsal M72 glomeruli are retained in $O/E3^{GFP/GFP}$ homozygous mice. The tau-lacZ reporter-tagged receptor M72 converges to a single lateral glomerulus in $O/E3^{GFP/+}$ and $O/E3^{GFP/GFP}$ homozygous animals. The bulb was mounted, GFP was imaged by confocal microscopy and the bulb stained to visualize *lacZ*-containing axons. An overlay of the two images, registered with landmarks in the bulb, reveals that the M72 glomerulus is present in $O/E3^{GFP/GFP}$ homozygous mice.

potential regulators of ORN gene expression, and their expression in embryos suggests a broader function in the development of the nervous system. Detailed investigation of the brains of *O/E1* mutant mice have indicated a role for O/E1 in neurodevelopment, and similar analysis of *O/E2* and *O/E3* mutant animals may reveal additional functions for these O/E family members.

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