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Disorganized olfactory bulb lamination in mice deficient for transcription factor AP-2 ϵ

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Abstract

Within the olfactory bulb, neurons and their axonal connections are organized into distinct layers corresponding to different functionalities. Here we demonstrate that transcription factor AP-2 ϵ is required for olfactory bulb development, specifically the establishment of appropriate neuronal lamination. During normal mouse embryogenesis, AP-2 ϵ transcripts are one of the earliest markers of olfactory bulb formation, and expression eventually becomes refined to the projection neurons, the mitral and tufted cells. To assess the function of AP-2 ϵ in olfaction, we generated a null allele (the “AK” allele) by inserting a Cre recombinase transgene into the endogenous AP-2 ϵ genomic locus. AP-2 ϵ -null mice exhibited defective olfactory bulb architecture. The mitral cell layer was disorganized, typified by misoriented and aberrantly positioned projection neurons, and the adjacent internal plexiform layer was absent. Despite the significant disruption of olfactory bulb organization, AP-2 ϵ null mice were viable and could discriminate a variety of odors. AP-2 ϵ -null mice thus provide compelling evidence for the robust nature of the mouse olfactory system, and serve as a model system to probe both the regulation of neuronal lamination and the functional circuitry of the olfactory bulb. We also show that Cre recombinase expression directed by the AP-2 ϵ locus can specifically target floxed genes within the olfactory bulb, extending the utility of this AK allele.

Keywords

AP-2 ϵ ; *Tcfap2e*; olfactory bulb; Cre recombinase; mitral cell; internal plexiform layer

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Introduction

The olfactory bulb (OB) is a laminated structure, with layers representing specific limited neuron types, neuronal processes and connections required for processing and relaying odor information to the piriform (olfactory) cortex (Shepherd, 2004; Shepherd, 2007). Starting from the outermost layer, the murine OB contains the olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL), and granule cell layer (GCL). The projection neurons - mitral cells and tufted cells - receive, process, and relay odor information. Mitral cell bodies are normally confined to the MCL, whereas tufted cells bodies are located more superficially in the OB, either in the inner GL or dispersed within the EPL. The development of the ordered structure of the OB requires both innervation by OSN axons and appropriate neural interactions between different OB layers. In this regard, surgical manipulation and explant studies have demonstrated that the olfactory epithelium stimulates outgrowth of mitral and tufted cell projection neurons, possibly via a secreted ligand(s) of the TGF β superfamily (Graziadei and Monti-Graziadei, 1992; Lopez-Mascaraque et al., 2005; Slotnick et al., 2004; Tran et al., 2008). Moreover, two groups of genes have been identified whose deletion in mouse affects the development of the OB layers. Disruption of the first group of genes, including *Fez*, *Pax6*, *Emx1/2* and *Dlx5*, prevents the OSN axons from reaching the OB (Harrison et al., 2008; Hirata et al., 2006; Jimenez et al., 2000; Laub et al., 2006; Long et al., 2003; Nomura and Osumi, 2004; Yoshida et al., 1997). Mutations in the second group of genes, including *Dlx1/2*, *Tbr1*, *Reelin*, *Lis1*, and *Arx*, impact neurogenesis, neural migration, and terminal differentiation of interneurons in the OB (Albrecht et al., 1996; Andrade et al., 2007; Bulfone et al., 1995; Bulfone et al., 1998; Hallonet et al., 1998; Ko et al., 2001; Perez-Garcia et al., 2004; Soria et al., 2004; Tomasiewicz et al., 1993; Toresson and Campbell, 2001; Whitley et al., 2005; Wyss et al., 1980; Yoshihara et al., 2005; Yun et al., 2003).

However, many questions remain with regards to the formation and function of the OB laminar structure. In particular, while recent work has provided information on the migration of principal neuron precursors and formation of the MCL (Blanchart et al., 2006), little is known about the factors contributing to these developmental processes. Disruption of the MCL could potentially alter the development of adjacent layers, the EPL, which includes secondary dendrites from mitral/tufted cells, and the IPL. The IPL contains tufted cell axons, and recent reports suggest that these connect mirror symmetric glomeruli in each OB that receive input from neurons expressing the same olfactory receptor molecule (Liu and Shipley, 1994; Lodovichi et al., 2003; Marks et al., 2006). Since the postsynaptic targets of the tufted cells are inhibitory granule cells it has been hypothesized that the axons within the IPL form intrabulbar inhibitory circuits that are used to coordinate topographically organized neural networks in the OB (Liu and Shipley, 1994). However, as yet there are no suitable animal models to test this hypothesis directly.

The mammalian AP-2 transcription factor family consists of five members: AP-2 α , AP-2 β , AP-2 γ , AP-2 δ and AP-2 ϵ (Eckert et al., 2005; Feng and Williams, 2003; Moser et al., 1995; Tummala et al., 2003; Wang et al., 2004; Zhao et al., 2001a). Three of the mouse AP-2 genes, *Tcfap2a*, *Tcfap2b*, and *Tcfap2c* - encoding AP-2 α , AP-2 β and AP-2 γ , respectively - are essential for embryogenesis and post-natal viability (Auman et al., 2002; Brewer et al., 2004; Nottoli et al., 1998; Satoda et al., 2000; Schorle et al., 1996; Zhao et al., 2001b). Much less is known concerning the function of *Tcfap2d* and *Tcfap2e*, the mouse genes encoding AP-2 δ and AP-2 ϵ . These two genes are more restricted in their expression than the other three family members, with prominent expression detected mainly in the developing midbrain for AP-2 δ and the forebrain for AP-2 ϵ . Expression of *Tcfap2e* is first observed in the OB primordium at E11.5 and then becomes refined to the projection neurons prior to birth, before declining to undetectable levels by P14 (Feng and Williams, 2003; Tummala et al., 2003; Wang et al.,

2004). The dynamic expression pattern of AP-2 ϵ suggested a role for this transcription factor in early OB development (Feng and Williams, 2003). Here, we have investigated this possibility by generating mice containing a *Tcfap2e*-null allele (AK allele). Analysis of homozygous mutant mice demonstrates an essential role for AP-2 ϵ in the establishment of OB lamination as well as for the appropriate orientation and connectivity of individual projection cells.

Results

Generation and Analysis of the *Tcfap2e* Cre recombinase knock-in AK allele

AP-2 ϵ has a restricted expression pattern in the OB and represents the earliest known marker for the developing OB (Feng and Williams, 2003; Tummala et al., 2003; Wang et al., 2004). To understand the function of AP-2 ϵ in OB development, we used ES cell gene targeting to create a null allele in the endogenous mouse *Tcfap2e* locus. This AK allele disrupts exon 4 of the gene, which is essential for the DNA binding activity of AP-2 ϵ (Figure 1A). Subsequent PCR and Southern blot analyses demonstrated that we successfully generated correctly targeted ES cell clones, and could eventually derive mouse embryos heterozygous or homozygous for the AK allele (Figure 1B, C). Analysis of gene expression from the AK allele confirmed that we had disrupted the normal *Tcfap2e* transcript (Figure 2). In these studies we used a probe corresponding to exon 7 of *Tcfap2e*, which lies downstream of the region targeted in the AK allele (Feng and Williams, 2003). In wild-type and AK heterozygous mice, AP-2 ϵ RNA expression was robust in the OB at E12.5 and E18.5 (Figure 2A, C, E, G, I and data not shown), consistent with previously published studies (Feng and Williams, 2003; Tummala et al., 2003; Wang et al., 2004). In contrast, AP-2 ϵ transcripts were greatly reduced in the AK homozygous mutant mice (Figure 2K). These findings indicate that only minimal transcriptional read-through into 3' *Tcfap2e* sequences occurs after the site of Cre insertion, or that the combined *Tcfap2e*/Cre transcript is not as stable as the endogenous *Tcfap2e* mRNA.

We designed the AK allele not only to disrupt AP-2 ϵ production, but also with the intention of driving Cre recombinase expression in the OB so that additional floxed genes might be targeted in this region of the CNS. Therefore, we examined the expression of Cre recombinase transcripts using RNA in situ hybridization. In heterozygous AK mice, Cre transcripts were detected in an analogous pattern to the endogenous *Tcfap2e* RNA. Thus, at E12.5, both *Tcfap2e* and Cre recombinase transcripts were present at the rostral most tip of the telencephalon, the OB primordium (Figure 2A, C-F). Similarly, at E18.5 the expression pattern of both *Tcfap2e* and Cre recombinase was restricted to the MCL of the main OB as well as to a more diffuse layer of tissue internal to the MCL that may represent projection neuron precursors (Fig 2G-J). Equivalent expression of *Tcfap2e* and Cre recombinase was also observed in the anterior portion of the accessory olfactory bulb (AOB; Fig 2I-J). As expected, Cre recombinase was not expressed in wild-type mice (Fig. 2B). These results are consistent with expression of the IRES-Cre cassette being regulated by the endogenous *Tcfap2e* cis-regulatory elements. We next examined *Tcfap2e*-null mice and determined that Cre recombinase expression was observed in the equivalent OB cell layers as endogenous *Tcfap2e* transcripts are observed in control mice (Figure 2L). The Cre expression level was elevated in the AK homozygous compared with heterozygous mice, though, consistent with the presence of two versus one Cre containing allele per cell, respectively (compare Fig. 2J and L). The presence of Cre recombinase transcripts in the *Tcfap2e*-null mice provides strong evidence that the cells that would normally express AP-2 ϵ are still present in the OB.

Analysis of Cre recombinase activity and fate mapping of cells within the OB and other tissues

We next assessed whether *Tcfap2e*-driven Cre recombinase expression could produce specific *LoxP* mediated gene targeting in the OB. Male AK mice were crossed to female Rosa26 reporter (R26R) mice, which only express functional β -galactosidase (β -Gal) protein following Cre-mediated recombination. Resulting offspring were processed as embryos or as isolated OBs at different stages through adulthood and stained for β -Gal activity. At E18.5 and P14, strong β -Gal expression occurred specifically within the main and accessory OBs of brains obtained from heterozygous AK:R26R mice (Fig 3A, B and data not shown). Examination of sectioned material indicated that the β -gal positive cells corresponded almost exclusively to the projection neurons known to express AP-2 ϵ (Fig 3C, cells with large nuclei in the MCL; red arrow). Even though AP-2 ϵ is not expressed in the OB after P14 (Feng and Williams, 2003), strong β -gal activity still occurred in these mitral and tufted cells several months after birth consistent with the long life-span of these post-mitotic projection neurons (Fig. 3D, E). Smaller areas of punctate blue staining were also observed internal to the MCL at E18.5 (Fig 3C, black arrows) but this staining steadily decreased over time so that it was absent by P30 (Fig. 3D, E). Finally, we note that even though AP-2 ϵ is expressed in the normal mouse OB from E11.5 onwards (Feng and Williams, 2003) and that Cre transcripts can also be detected in the OB beginning at E11.5 (Fig 2 and data not shown), the first evidence of β -Gal activity in AK:R26R embryos occurs only around E16.5. The discrepancy between Cre recombinase mRNA expression and β -gal activity is not fully understood at this time, but might conceivably reflect a delay either in the production of active Cre recombinase protein or in the expression of the targeted R26R locus.

The *Tcfap2e* AK allele joins the previously described the *Pcdh21-Cre* transgene (Nagai et al., 2005) as a resource for targeting floxed genes in the OB projection neurons. Moreover, the ability to follow β -gal activity in AK:R26R mice enabled us to detect other domains of AP-2 ϵ expression in embryos and adults. The OB was the major site of Cre recombinase activity in the CNS, while in other tissues strong blue staining was observed in testes and oocytes, with weaker punctate staining in the vicinity of hair follicles, probably corresponding to skin melanocytes (Supplementary Figure 1). These sites of β -gal activity were consistent with the expression pattern of endogenous *Tcfap2e* (Feng and Williams, 2003).

The laminar organization of the OB is disrupted in *Tcfap2e*-null mice

Tcfap2e-null mice were viable and fertile, with a normal external appearance, despite the loss of functional AP-2 ϵ expression in the OB, reproductive system, and skin (Supplementary Figure 2). However, because AP-2 ϵ is specifically expressed at high levels in the developing OB, we performed a detailed examination of this structure to determine whether disruption of *Tcfap2e* caused any associated defects. Initial whole mount analysis indicated that there were no gross morphological defects in the size or shape of the *Tcfap2e*-null OB from E11.5, when AP-2 ϵ is first expressed in this location, through adulthood (Figure 2 and Supplementary Figure 2). However, a more detailed histological analysis of the OB revealed clear defects in its laminar organization. Subtle differences in the organization of the cell layers destined to form the projection neurons first became apparent at E18.5, and were clearly established by P14 (Figure 4 and data not shown). At P14, the cellular organization of the OB was examined using neutral-red staining to demarcate the various OB layers. In wild type controls, the mitral cell bodies were organized in a compact single layer and there was a distinct IPL between the MCL and GCL (Figure 4A, C). However, in the *Tcfap2e* null OB, no IPL was apparent (Figure 4B, D). Concomitantly, mitral cells appeared disorganized and the MCL slightly expanded as compared to wild type littermates (arrows in figure 4D) suggesting defects of neuronal lamination in the mutant OB. Analysis of proliferation and apoptosis did not reveal any major

differences between wild-type and *Tcfap2e*-null mice that could account for these alterations (data not shown).

Specific molecular markers for the various OB layers were employed to obtain a more detailed understanding of the pathology. At P14 in normal mice, expression of procadherin 21 (*Pcdh21*) was associated with periglomerular cells, and was also observed in two layers, corresponding to the cell bodies of the tufted cells and mitral cells (Fig 4E; (Feng and Williams, 2003; Nagai et al., 2005)). Between these two layers of projection neurons, the EPL is a layer of dendritic projections containing relatively few cell bodies. However, in the *Tcfap2e*-null mice, *Pcdh21* staining revealed that the MCL was less compact, and more cell bodies were present within the EPL (Fig 4F, black arrows). It was also notable that, in contrast to the wild-type OB, several *Pcdh21* positive cell bodies were also observed in the *Tcfap2e*-null GCL (Fig 4F, red arrow). Equivalent results were obtained when the expression of a second projection neuron marker, metabotropic glutamate receptor 1 (mGluR1) was examined (Supplementary Figure 3). Next, a R26R allele was bred into the *Tcfap2e*-null mouse background to indelibly mark cells that expressed - or had previously expressed - the associated Cre recombinase transgene. Examination of these mice at 2 months of age revealed blue stained cells, demonstrating that the projection neurons that would normally express *Tcfap2e* can persist in the absence of AP-2 ϵ (Figure 4H). However, in contrast to AK:R26R heterozygous mice, in which blue staining marked two discrete projection neuron layers (Fig 3D, E and 4G), β -gal expressing cells in *Tcfap2e*-null mice were disorganized, with many mislocalized in the EPL and to a lesser extent the GCL (Fig 4H). Together, our findings indicate defects in the appropriate lamination of the MCL, and possibly tufted cells, in the absence of AP-2 ϵ .

We next examined the expression of *Dlx2*, *Dlx5* and *Pax6*, which label interneurons in the GL, as well as in the MCL and GCL (Hirata et al., 2006; Yoshihara et al., 2005). In control mice at P14, these markers render the IPL readily visible as a low-cell-density space between the MCL and GCL (Figure 4I and data not shown). However, in the *Tcfap2e*-null OB, staining of these interneurons is not interrupted by the presence of a lightly stained IPL (Figure 4J), again indicating defects in the formation of correct OB neuronal lamination. In contrast to the defects in the inner layers of the OB, the outer layers - the ONL and the GL - appeared normal in the mutant. Specifically, NCAM expression, which highlights the axons of the OSNs (Hirata et al., 2006; Yoshihara et al., 2005), was similar in both *Tcfap2e*-null and control OBs (Supplementary Figure 4). This latter finding indicates that axons from OSNs were able to reach the OB and correctly synapse with the projection neurons in the GL.

We further probed the disruption of OB laminar organization in the absence of AP-2 ϵ using immunofluorescence detection of NeuN, an interneuron nuclear marker, and PGP9.5, which labels mitral cell bodies and proximal dendrites (Imamura et al., 2006; Nakajima et al., 1998). In the wild type OB, the mitral cell bodies were located immediately above the IPL and were orientated radially - with their primary dendrites projected directly toward the GL (Fig. 5A). However, as shown in Figure 5B, the mutant OB displayed several differences from this normal organization. First, NeuN staining again failed to reveal a distinct IPL. Second, mitral cell bodies were not located in a well-defined layer, but were dispersed more into the lower EPL. Finally, the mitral cell bodies were not properly aligned (Fig 5B, white arrows) and extended misoriented primary dendrites that were not directed radially outward. The absence of a distinct IPL prompted an examination of the axons that normally occupy this location using the 2H3 neurofilament-specific antibody. In the wild type OB, these axons stain as a narrow band corresponding to the IPL (Figure 5C). 2H3 positive axons were still present in the *Tcfap2e*-null mutant OB. However, these axons were no longer confined to a narrow layer, but formed a more diffuse network that extended much deeper into the GCL (Figure 5D). Thus, rather than occupying a layer that was essentially free of nuclei as observed in the wild-type, these axons followed pathways that were interspersed between the cells of the GCL. We also

examined tufted cell organization using immunohistochemical analysis of cholecystokinin (CCK) expression. We determined that although tufted cells were present in their normal positions in the *Tcfap2e*-null OB, a distinct IPL composed of their associated axons was again not apparent (data not shown).

We next tested whether the disruption of normal OB layer formation in the absence of AP-2 ϵ influenced the outgrowth of the OB efferent axons. A crystal of fluorescent dye, DiI, was placed post-mortem in the lateral olfactory tract region for retrograde labeling of the projection neurons via the OB efferent axons. In both control and *Tcfap2e*-null mice, DiI labeling was detected in both mitral and tufted cells. Labeled dendrites could be seen to enter glomeruli and axons could be followed in to the IPL and GCL (Figure 6). However, this technique revealed important defects in the organization of the mutant OB that were consistent with our previous observations. Control mice contained a well-defined layer of axons corresponding to the IPL, but this layer was absent in the mutants (Fig. 6B). In addition, mitral cells were mislocalized in the mutant, and the orientation of their cell bodies and dendritic projections was aberrant (Fig 6C, D, white arrows). Taken together, our data clearly demonstrate that OB neuronal layer formation was disrupted in the *Tcfap2e*-null OB, with a disorganized MCL and loss of a distinct IPL.

Disruption of the OB laminar organization in *Tcfap2e*-null mice did not impair gross olfactory behavior

In mice, normal olfaction is a prerequisite for suckling after birth, and is also an important component of mating behavior (Batterton et al., 2006; Hongo et al., 2000; Murphy and Schneider, 1970; Teicher et al., 1980). The finding that the *Tcfap2e*-null mice are viable after birth, and can be maintained as a fertile colony indicates that olfaction is not fundamentally impaired in the absence of AP-2 ϵ . This finding is consistent with the data presented in Figure 6, that despite the disorganization of the mutant OB, projection neurons can communicate with both OSNs and the piriform cortex. Nevertheless, we considered that the lamination defects in the *Tcfap2e*-null OB might result in more subtle defects in the normal processing of olfactory information. Therefore, several specific methodologies were used to assess whether the *Tcfap2e*-null mice displayed impairment of normal behavior in response to olfactory stimuli. We first performed a buried peanut butter test (Johnston, 1992), and found that both wild type and mutant mice could find the hidden peanut butter within a similar time interval, again suggesting normal odor detection and processing in the *Tcfap2e*-null mice (Supplementary Figure 5). Next, an olfactometer was employed to assess odor discrimination in the *Tcfap2e*-null mutants. Several odor combinations were tested on wild type, AK heterozygous, and *Tcfap2e*-null mice, including Ethyl Acetate (S+) odor compared to odorless mineral oil (S-) (Figure 7). In each instance, *Tcfap2e*-null mice had a similar olfactory learning curve to controls, and reached more than 85% accuracy in their response at the end of the test. The olfactometer was also employed to test for odor sensitivity using a threshold test. Both wild type and *Tcfap2e* mutant mice again displayed a similar concentration threshold for detecting citral odor (data not shown). These findings indicate that the loss of *Tcfap2e* and the associated defects in OB lamination do not result in widespread defects in olfactory behavior in mice housed in laboratory setting. Further specific tests will be required to determine if there are more subtle alterations in odor detection and processing in the *Tcfap2e*-null mice.

Discussion

Previous studies have indicated that *Tcfap2e* expression is one of the earliest events marking the formation of the specialized OB during mouse embryogenesis (Feng and Williams, 2003; Wang et al., 2004). Therefore, in this study, we have generated and characterized *Tcfap2e*-null mice to investigate the role of AP-2 ϵ in the development and function of the olfactory system.

Analysis of these mice reveals a specific requirement for AP-2 ϵ in development of the OB, particularly in the normal lamination of projection neurons and their associated axonal projections. Lamination is a common characteristic of nervous system organization, and occurs in the cortex, hippocampus, and cerebellum in addition to the OB (Beffert et al., 2004). Nevertheless, the molecular mechanisms that regulate lamination, and the importance of lamination for normal brain function, are not fully understood. Due to its simplicity and easy access, the OB is one of the more amenable systems that can be used to investigate neuronal lamination. Previous studies, outlined in the Introduction, have indicated that OB lamination can be influenced by both extrinsic factors, associated with the olfactory epithelium, and intrinsic mechanisms, involving the formation, migration, or connection of OB neurons. Several lines of evidence demonstrate that the loss of AP-2 ϵ results in OB defects belonging to the latter category. First, expression of *Tcfap2e* is normally restricted to the neurons derived from within the OB, beginning at E11.5 in the ventral telencephalon, the precursor territory for the mature OB. As embryogenesis proceeds, *Tcfap2e* expression becomes refined to mitral cells and tufted cells of the main and accessory OB, as well as their presumptive progenitors, until shortly after birth (Feng and Williams, 2003; Wang et al., 2004). Second, the lineage tracing studies we performed using the Cre recombinase cassette present in the AK allele, in combination with the *R26R* transgene, strongly suggest that the defect is cell autonomous. Specifically, our data demonstrate that the β -gal positive projection neurons in which AP-2 ϵ is normally expressed are severely affected in the *Tcfap2e*-null mice.

The loss of AP-2 ϵ results in prominent defects in four OB layers: the EPL, MCL, IPL, and GCL. However, all of the defects presumably originate from inappropriate development of the projection neurons and their associated dendrites and axons. The two types of projection neurons present in the OB are mainly defined based on their position with respect to the EPL. Tufted cells are usually scattered throughout the EPL. In contrast, mitral cell bodies are more centrally located and form a discrete layer that lies between the IPL and their dendrites in the EPL. In *Tcfap2e*-null mice, the MCL is more disorganized and diffuse. Projection neurons are dispersed throughout the EPL and it is not possible to distinguish separate mitral and tufted cell populations. The aberrant location of the projection neurons in the mutant is highlighted by their occasional presence even deeper within the OB, in the GCL. The morphology of the projection neurons is also abnormal. Mature mitral cell bodies are normally oriented perpendicular to the layers of the OB and extend a single apical dendrite towards only one glomerulus. The orientation of the *Tcfap2e*-null projection neuron cell bodies is instead frequently misaligned, which is also reflected in the growth direction of the primary dendrites. Indeed, some dendrites project almost parallel to the lamination of the OB, perpendicular to their normal orientation (see Figs 5B, and 6D for examples). We have also noted that some projection neurons in the P14 mutant OB have more than one primary dendrite arising from their apical surface (see Fig 5B, asterisks). The defects in localization, orientation, and dendritic projection are all characteristics associated with an immature, embryonic, stage of mitral cell development. Tufted cell axonal projections that normally constitute the IPL are also defective in the absence of AP-2 ϵ . No distinct IPL was apparent using standard histology, but neurofilament staining revealed that the axons were still present, although they were more disperse and resided within the GCL. Despite the defects in the location and morphology of the projection neurons, retrograde labeling indicated that they could still make appropriate connections with downstream components of their olfactory relay circuits.

Normal maturation of mitral cells requires an orderly series of events - migration, re-orientation, and the establishment of appropriate neuronal connections (Blanchart et al., 2006). As discussed above, the fate mapping studies support a cell autonomous function for AP-2 ϵ in these projection neurons. Therefore, we postulate that the loss of the AP-2 ϵ transcription factor alters the expression of one or more cell surface molecules or signal transduction components that would normally enable the projection neurons to identify and

respond to their location in the laminar organization of the OB during migration. The inability to recognize their position in the OB would result in the retention of certain immature characteristics, namely their abnormal positions in the OB, the aberrant orientation of their cell bodies, the unusual growth trajectories of the dendrites, and the excess numbers of primary dendrites per cell. Nevertheless, the retrograde labeling studies demonstrate that the projection neurons are not locked into a completely immature state since they are able to establish some relay circuitry. The axons of the projection neurons are also abnormally located, failing to form a distinct IPL, and we hypothesize that this may again occur because of the inability of *Tcfap2e*-null cells to locate or respond to their normal positional cues above the GCL. Alternatively, molecules that facilitate axon fasciculation may be aberrantly expressed in the absence of AP-2 ϵ resulting in more dispersion. Further analysis will be required to determine how AP-2 ϵ dependent gene expression in the projection neurons normally alters their cellular properties to enable appropriate maturation.

Mutations of several other mouse genes, including *Lis1* and *Sp8* (Royal et al., 2002; Waclaw et al., 2006), also affect OB lamination, but none of these display identical features to those observed in the *Tcfap2e*-null mouse. In many of these models there is a significant reduction of OB size (Hirata et al., 2006; Long et al., 2003; Nomura and Osumi, 2004; Waclaw et al., 2006; Yoshida et al., 1997; Yoshihara et al., 2005) - a condition that does not occur in the absence of AP-2 ϵ . Many genetic manipulations altering OB laminar organization also interfere with neuronal lamination in other regions of the brain, especially the cortex. However, AP-2 ϵ expression is mainly restricted to the OB and we have not detected defects in neuronal lamination in other regions of the CNS, including the cortex and cerebellum. In this context, the *Tcfap2e* mutants are viable throughout adulthood and provide an excellent animal model to study the consequences of OB lamination defects on the function of the olfactory system.

There are several possibilities why *Tcfap2e*-null mice are capable of apparently normal odor discrimination and odor concentration threshold assessment under our limited test conditions despite the disruption of OB lamination. One explanation is that orderly lamination is not a prerequisite for major aspects of olfaction. Indeed, although a compact MCL is present in most mammalian species, other arrangements with broad or diffuse MCLs are seen in many vertebrates (Switzer and Johnson, 1977). Alternatively, the normal olfactory behavior we have so far observed in *Tcfap2e*-null mice may also be explained by the robustness of the olfactory system in this rodent. Mice rely considerably on olfaction for their survival, and it might be expected that the OB would have evolved to cope with perturbations in the organization and function of neurons, such as those observed in the absence of AP-2 ϵ . Another possibility is that the AP-2 gene family may have a broader role in OB morphogenesis and function than revealed by the phenotype of the *Tcfap2e*-null mouse. AP-2 α and AP-2 γ are also expressed in the developing OB (Feng and Williams, 2003), however, a detailed understanding of their importance in olfaction remains to be determined because *Tcfap2a* and *Tcfap2c* knockout mice do not survive post-natally (Auman et al., 2002; Nottoli et al., 1998; Schorle et al., 1996; Zhang et al., 1996). Nevertheless, previous studies have implicated AP-2 α in Luteinizing Hormone-Releasing Hormone (LHRH) neuronal development and presumably behavior linked to olfaction (Kramer et al., 2000). Therefore, the disruption of AP-2 ϵ in combination with AP-2 α or AP-2 γ may be required to uncover redundant functions for this family of transcription factors in OB development and function.

Experimental Methods

Generation of the *Tcfap2e* AK allele

The *Tcfap2e* AK allele was generated by targeting IRES-Cre and pGK-Neo sequences into exon 4 of *Tcfap2e* (Figure 1A). Briefly, 5' and 3' homologous DNA fragments (4.8 and 4.5 kb respectively) flanking *Tcfap2e* exon 4 were PCR-derived from 129 ES cell genomic DNA using

primer pairs AK1/2 and AK3/4 (Table 1), respectively. PCR products were sequenced, and cloned into a targeting vector SP73 (Promega). IRES-Cre and a pGK-Neo cassette flanked with *Frt* sites were both inserted between the 5' and 3' homologous fragments using standard cloning procedures (Ausubel et al., 2006). These manipulations result in the interruption of the 226 bp *Tcfap2e* exon 4 sequence by the exogenous sequences 102 bp from the 5' splice site. Next, two copies of a HSV TK negative selection cassette were inserted downstream of the 3' region of homology (Auman et al., 2002; Zhang et al., 1996). The composition and integrity of the plasmid was confirmed by sequence analysis. Following linearization with *NotI*, the final targeting vector was electroporated into CJ7 ES cells, and selected in the presence of G418 using standard procedures (Nagy et al., 2003). Subsequently, ~300 ES clones were selected and screened by PCR analysis.

To identify 5' homologous recombination, we performed PCR with the primer pair FIRES1 and AK8 (Table 1). These primer sequences are located within the IRES-Cre sequence and the genomic sequence directly upstream from the region of 5' homology respectively (Fig. 1A) and produce a 4.8-kb PCR product only when homologous recombination occurred at the 5' end. Primer pair AK8 and AK2 produced a slightly smaller PCR product from the wild type allele, and served as a positive control for PCR screening. PCR screening was performed using Elongase (Invitrogen, Cat No. 10480-010) with the following program: 1 cycle of 95°C 3min; 36 cycles of (95°C 45sec, 58°C 30sec, 68°C 10min); 1 cycle of 68°C 15min. PCR products generated by primer pair AK8 and FIRES1 were sequenced to verify appropriate homologous recombination. A similar strategy was used to identify 3' homologous recombination using primer pair AK6 and Neo3f (Fig. 1A and Table 1). In total, three out of 300 ES cell clones were positive for homologous recombination at both ends.

Southern analysis was used to confirm the results of PCR screening. A Southern probe, located in the genomic sequence upstream from the region of 5' homology (Fig 1A), was PCR amplified using primer pair AK14/15. The probe was then labeled by incorporation of ³²P dCTP in combination with random primers (Ausubel et al., 2006) and used for Southern analysis of genomic DNA digested with *NheI*. This probe detected a 5.7-kb *NheI* fragment from the wild-type allele, and a 9.7-kb product from a correctly targeted allele (Figure 1B). All three PCR-positive ES cell clones were confirmed for appropriate homologous recombination by Southern analysis.

After karyotyping to confirm euploidy, one ES cell clone was microinjected into C57/Bl6 blastocysts (Nagy et al., 2003) and resulting chimeric mice were used to generate AK heterozygous offspring. The AK heterozygous mice were then expanded and maintained by backcrossing with outbred Black Swiss mice (Taconic). Wild-type, heterozygous, and AK homozygous mice were subsequently identified by PCR analysis using primers AK19, AK20, and NeoAK2 (Fig 1C and Table 1) using HotStarTaq Plus DNA Polymerase (Qiagen, Cat# 203603). The PCR cycling parameters used were: 1 cycle of 95°C 3'; 35 cycles of (95°C 45"; 59°C 45"; 72°C 1'); 1 cycle of 72°C 10'. The AK mutant and wild-type *Tcfap2e* alleles produced bands of 400bp and ~250bp in size, respectively. Note that we also derived mice in which the *Frt* flanked *neo* cassette was deleted by breeding mice containing the AK allele with β -actin Flpe mice (Jax Labs, Tg(ACTFLPe)9205Dym/J). We determined that mice homozygous for the *Tcfap2e*-null allele with or without the *neo* cassette exhibited the same gross morphological characteristics. All animal experiments were performed in accordance with protocols approved by the UCD Animal Care and Usage Committee.

β -galactosidase (β -gal) staining in whole mount or frozen sectioned tissues

The timing and tissue specificity of Cre recombinase expression was assessed using the R26R mice (Mao et al., 1999; Soriano, 1999). Mouse embryos or OBs were dissected in ice-cold PBS, and whole-mount stained by X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside)

according to the procedure described previously (Brewer et al., 2004). Alternatively, mouse embryos or OBs were fixed in 0.2% paraformaldehyde (PFA) overnight, dehydrated in 30% sucrose, embedded in OCT compound (Sakura Finetek USA Inc, Torrance CA), and cryosectioned at 20 μ M. β -gal activity was detected on slides using the same protocol as for whole mount analysis. Slides were counterstained by 0.1% nuclear fast red (NFR), dehydrated, treated with Cytoseal (Richard Allen Scientific), coverslipped and photographed.

In situ hybridization (ISH)

Whole mount and cryosection ISH were performed as described previously (Feng and Williams, 2003). The RNA probes used in this study are AP-2 ϵ and mGluR1 (Feng and Williams, 2003), *Dlx2* (Brewer et al., 2004), *Dlx5* (gift of Dr. John L.R. Rubenstein)(Depew et al., 1999), *Pax6* (gift of Dr. Lori Sussel), Cre, and *Pcdh21*. The Cre cDNA probe was derived by PCR from the Nls-Cre plasmid (gift of Dr. Gail Martin) using primers Cre1 and Cre3 (Table 1). The mouse *Pcdh21* probe was derived by PCR from E10.5 FVB mouse head cDNA with primers Pcdh21F and Pcdh21R (Table 1).

Neutral red staining of OB layers

20 μ m OB cryo-sections were stained with 0.1% Neutral red (Sigma N7005) in distilled water for 15 minutes, rinsed 4 times in water, dehydrated, cleared, cover-slipped, and photographed.

Immunohistochemistry (IHC)

Embryos or OBs were dissected in ice-cold PBS, fixed in 10% formalin overnight at 4°C, dehydrated and embedded in paraffin, then cut into 7 μ m sections for IHC. IHC was performed according to manufacturer's instructions using the VECTOR MOM immunodetection kit (Vector Laboratories, Cat# PK-2200). Primary antibodies used in this study were diluted 1:200, including NCAM (5B8 from DSHB), neurofilament (2H3 from DSHB), PGP9.5 (AbD Serotec, cat. No. 7863-0504), NeuN (Chemicon, cat No. MAB377), phospho-histone H3 (Cell signaling, #9706L), L1 (Chemicon, #MAB5272) and GLAST (Chemicon, #AB1782). Appropriate species-specific secondary antibodies conjugated with Alexa-488 or Alexa-568 (Molecular Probes) were diluted at 1:500.

Retrograde labeling of OB projection neurons with Dil

Tcfap2e mutant and control brains were collected and fixed with 4% PFA in PBS overnight. Next, crystalline DiI (Molecular Probes, Eugene, OR) was placed in the region of the lateral olfactory tract with a blunted glass micropipette. The emplaced DiI was covered with 1% agar solution. Tissues were promptly returned to 4% PFA, and the samples were placed in a dark chamber for at least 4 weeks at room temperature to allow retrograde diffusion of the dye. Next, the OB and brain were embedded in 0.3% albumin/0.03 gelatin medium and sectioned at 70 μ m in the coronal plane with a Vibratome. The sections were immediately mounted, coverslipped and photographed with a conventional epifluorescence or confocal microscope (Olympus Fluoview).

Olfactory Behavior Test

This test is a variant of the digging test and was used to assess whether tested mice could sense and locate peanut butter buried 1 cm under the bedding (Johnston, 1992). Peanut butter was placed on the bottom of a disposable petri dish (3cm diameter). After a mouse was removed from his home cage, the petri dish was buried under the bedding and the mouse was returned to his cage. The latency to find and dig the petri dish was recorded for the tested mouse. The trial was repeated twice with six pairs of wild-type and *Tcfap2e*-null mice and the data analyzed using a paired T-test.

Odor detection test with an olfactometer

We used an olfactometer to assess the ability of mice to detect various odors by following the procedure described previously (Clevenger and Restrepo, 2006; Slotnick and Restrepo, 2005). Briefly, tested animals were under water restriction and would obtain extra water by a correct response to a 2.5 sec odor stimulus (S+). For the trial period, S+ and control odor S- (such as odorless mineral oil or a different odor) were presented for equal times and in random order. A correct response was defined as a lick of the water delivery port during the 2 sec response area for S+, but not for S- period. The percentage of correct responses was recorded for up to 200 trials for each mouse. The trials were performed with one to five pairs of wild-type and *Tcfap2e*-null mice and the data analyzed using Anova.

The five different odor pairs utilized in this test are listed below:

1. Limonene 1% (S+) × Mineral Oil (S-)
2. Ethyl Acetate 1% (S+) × Mineral Oil (S-)
3. Citral 1% (S+) × Limonene 1% (S-)
4. Heptanol 1% (S+) × Pentanol 1% (S-)
5. Heptanol 1% (S+) × Hexanol 1% (S-)

Threshold test by MLPest

We used a maximum likelihood adaptive staircase procedure for measurement of the concentration threshold for odor discrimination in individual wild-type and *Tcfap2e*-null mice, MLPest, as previously described by Clevenger and Restrepo (Clevenger and Restrepo, 2006; Slotnick and Restrepo, 2005). In this test, the S+ odorant was 1% citral in mineral oil, and S- is pure mineral oil as a control. Tested concentrations were diluted in log steps, from 1 to 10⁻⁶ % citral in mineral oil. The threshold concentration was defined as the point with maximum slope when the data were fitted by the Weibull psychometric function (Clevenger and Restrepo, 2006).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Albrecht U, Abu-Issa R, Ratz B, Hattori M, Aoki J, Arai H, Inoue K, Eichele G. Platelet-activating factor acetylhydrolase expression and activity suggest a link between neuronal migration and platelet-activating factor. *Dev Biol* 1996;180:579–593. [PubMed: 8954729]
- Andrade N, Komnenovic V, Blake SM, Jossin Y, Howell B, Goffinet A, Schneider WJ, Nimpf J. ApoER2/VLDL receptor and Dab1 in the rostral migratory stream function in postnatal neuronal migration independently of Reelin. *Proc Natl Acad Sci U S A* 2007;104:8508–8513. [PubMed: 17494763]

- Auman HJ, Nottoli T, Lakiza O, Winger Q, Donaldson S, Williams T. Transcription factor AP-2 γ is essential in the extra-embryonic lineages for early postimplantation development. *Development* 2002;129:2733–2747. [PubMed: 12015300]
- Ausubel, FM.; Moore, DD.; Brent, R.; Kingston, RE.; Seidman, JG.; Smith, JA.; Struhl, K. *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc.; 2006.
- Batterton MN, Robarts D, Woodley SK, Baum MJ. Comparison of odor and mating-induced glomerular activation in the main olfactory bulb of estrous female ferrets. *Neurosci Lett* 2006;400:224–229. [PubMed: 16530960]
- Beffert U, Weeber EJ, Morfini G, Ko J, Brady ST, Tsai LH, Sweatt JD, Herz J. Reelin and cyclin-dependent kinase 5-dependent signals cooperate in regulating neuronal migration and synaptic transmission. *J Neurosci* 2004;24:1897–1906. [PubMed: 14985430]
- Blanchart A, De Carlos JA, Lopez-Mascaraque L. Time frame of mitral cell development in the mice olfactory bulb. *J Comp Neurol* 2006;496:529–543. [PubMed: 16572431]
- Brewer S, Feng W, Huang J, Sullivan S, Williams T. Wnt1-Cre-mediated deletion of AP-2 α causes multiple neural crest-related defects. *Dev Biol* 2004;267:135–152. [PubMed: 14975722]
- Bulfone A, Smiga SM, Shimamura K, Peterson A, Puellas L, Rubenstein JL. T-brain-1: a homolog of Brachyury whose expression defines molecularly distinct domains within the cerebral cortex. *Neuron* 1995;15:63–78. [PubMed: 7619531]
- Bulfone A, Wang F, Hevner R, Anderson S, Cutforth T, Chen S, Meneses J, Pedersen R, Axel R, Rubenstein JL. An olfactory sensory map develops in the absence of normal projection neurons or GABAergic interneurons. *Neuron* 1998;21:1273–1282. [PubMed: 9883721]
- Clevenger AC, Restrepo D. Evaluation of the validity of a maximum likelihood adaptive staircase procedure for measurement of olfactory detection threshold in mice. *Chem Senses* 2006;31:9–26. [PubMed: 16306319]
- Depew MJ, Liu JK, Long JE, Presley R, Meneses JJ, Pedersen RA, Rubenstein JL. Dlx5 regulates regional development of the branchial arches and sensory capsules. *Development* 1999;126:3831–3846. [PubMed: 10433912]
- Eckert D, Buhl S, Weber S, Jager R, Schorle H. The AP-2 family of transcription factors. *Genome Biol* 2005;6:246. [PubMed: 16420676]
- Feng W, Williams T. Cloning and characterization of the mouse AP-2 ϵ gene: a novel family member expressed in the developing olfactory bulb. *Mol Cell Neurosci* 2003;24:460–475. [PubMed: 14572467]
- Graziadei PP, Monti-Graziadei AG. The influence of the olfactory placode on the development of the telencephalon in *Xenopus laevis*. *Neuroscience* 1992;46:617–629. [PubMed: 1545913]
- Hallonet M, Hollemann T, Wehr R, Jenkins NA, Copeland NG, Pieler T, Gruss P. Vax1 is a novel homeobox-containing gene expressed in the developing anterior ventral forebrain. *Development* 1998;125:2599–2610. [PubMed: 9636075]
- Harrison SJ, Nishinakamura R, Monaghan AP. Sall1 regulates mitral cell development and olfactory nerve extension in the developing olfactory bulb. *Cereb Cortex* 2008;18:1604–1617. [PubMed: 18024993]
- Hirata T, Nakazawa M, Yoshihara S, Miyachi H, Kitamura K, Yoshihara Y, Hibi M. Zinc-finger gene Fez in the olfactory sensory neurons regulates development of the olfactory bulb non-cell-autonomously. *Development* 2006;133:1433–1443. [PubMed: 16540508]
- Hongo T, Hakuba A, Shiota K, Naruse I. Suckling dysfunction caused by defects in the olfactory system in genetic arhinencephaly mice. *Biol Neonate* 2000;78:293–299. [PubMed: 11093009]
- Imamura F, Nagao H, Naritsuka H, Murata Y, Taniguchi H, Mori K. A leucine-rich repeat membrane protein, 5T4, is expressed by a subtype of granule cells with dendritic arbors in specific strata of the mouse olfactory bulb. *J Comp Neurol* 2006;495:754–768. [PubMed: 16506198]
- Jimenez D, Garcia C, de Castro F, Chedotal A, Sotelo C, de Carlos JA, Valverde F, Lopez-Mascaraque L. Evidence for intrinsic development of olfactory structures in Pax-6 mutant mice. *J Comp Neurol* 2000;428:511–526. [PubMed: 11074448]
- Johnston RE. Vomeronasal and/or olfactory mediation of ultrasonic calling and scent marking by female golden hamsters. *Physiol Behav* 1992;51:437–448. [PubMed: 1523219]

- Ko J, Humbert S, Bronson RT, Takahashi S, Kulkarni AB, Li E, Tsai LH. p35 and p39 are essential for cyclin-dependent kinase 5 function during neurodevelopment. *J Neurosci* 2001;21:6758–6771. [PubMed: 11517264]
- Kramer PR, Krishnamurthy R, Mitchell PJ, Wray S. Transcription factor activator protein-2 is required for continued luteinizing hormone-releasing hormone expression in the forebrain of developing mice. *Endocrinology* 2000;141:1823–1838. [PubMed: 10803593]
- Laub F, Dragomir C, Ramirez F. Mice without transcription factor KLF7 provide new insight into olfactory bulb development. *Brain Res* 2006;1103:108–113. [PubMed: 16814267]
- Liu WL, Shipley MT. Intrabulbar associational system in the rat olfactory bulb comprises cholecystokinin-containing tufted cells that synapse onto the dendrites of GABAergic granule cells. *J Comp Neurol* 1994;346:541–558. [PubMed: 7983243]
- Lodovichi C, Belluscio L, Katz LC. Functional topography of connections linking mirror-symmetric maps in the mouse olfactory bulb. *Neuron* 2003;38:265–276. [PubMed: 12718860]
- Long JE, Garel S, Depew MJ, Tobet S, Rubenstein JL. DLX5 regulates development of peripheral and central components of the olfactory system. *J Neurosci* 2003;23:568–578. [PubMed: 12533617]
- Lopez-Mascaraque L, Garcia C, Blanchart A, De Carlos JA. Olfactory epithelium influences the orientation of mitral cell dendrites during development. *Dev Dyn* 2005;232:325–335. [PubMed: 15614760]
- Mao X, Fujiwara Y, Orkin SH. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. *Proc Natl Acad Sci U S A* 1999;96:5037–5042. [PubMed: 10220414]
- Marks CA, Cheng K, Cummings DM, Belluscio L. Activity-dependent plasticity in the olfactory intrabulbar map. *J Neurosci* 2006;26:11257–11266. [PubMed: 17079653]
- Moser M, Imhof A, Pscherer A, Bauer R, Amselgruber W, Sinowatz F, Hofstadter F, Schule R, Buettner R. Cloning and characterization of a second AP-2 transcription factor: AP-2 β . *Development* 1995;121:2779–2788. [PubMed: 7555706]
- Murphy MR, Schneider GE. Olfactory bulb removal eliminates mating behavior in the male golden hamster. *Science* 1970;167:302–304. [PubMed: 5409714]
- Nagai Y, Sano H, Yokoi M. Transgenic expression of Cre recombinase in mitral/tufted cells of the olfactory bulb. *Genesis* 2005;43:12–16. [PubMed: 16106355]
- Nagy, A.; Gertsenstein, M.; Vintersten, K.; Behringer, R. *Manipulating the Mouse Embryo*. Vol. 3rd ed.. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, New York: 2003.
- Nakajima T, Murabayashi C, Ogawa K, Taniguchi K. Immunoreactivity of protein gene product 9.5 (PGP 9.5) in the developing hamster olfactory bulb. *Anat Rec* 1998;250:238–244. [PubMed: 9489784]
- Nomura T, Osumi N. Misrouting of mitral cell progenitors in the Pax6/small eye rat telencephalon. *Development* 2004;131:787–796. [PubMed: 14736749]
- Nottoli T, Hagopian-Donaldson S, Zhang J, Perkins A, Williams T. AP-2-null cells disrupt morphogenesis of the eye, face, and limbs in chimeric mice. *Proc Natl Acad Sci U S A* 1998;95:13714–13719. [PubMed: 9811866]
- Perez-Garcia CG, Tissir F, Goffinet AM, Meyer G. Reelin receptors in developing laminated brain structures of mouse and human. *Eur J Neurosci* 2004;20:2827–2832. [PubMed: 15548227]
- Royal SJ, Gambello MJ, Wynshaw-Boris A, Key B, Clarris HJ. Laminar disorganisation of mitral cells in the olfactory bulb does not affect topographic targeting of primary olfactory axons. *Brain Res* 2002;932:1–9. [PubMed: 11911856]
- Satoda M, Zhao F, Diaz GA, Burn J, Goodship J, Davidson HR, Pierpont ME, Gelb BD. Mutations in TFAP2B cause Char syndrome, a familial form of patent ductus arteriosus. *Nat Genet* 2000;25:42–46. [PubMed: 10802654]
- Schorle H, Meier P, Buchert M, Jaenisch R, Mitchell PJ. Transcription factor AP-2 essential for cranial closure and craniofacial development. *Nature* 1996;381:235–238. [PubMed: 8622765]
- Shepherd, GM., editor. *The Synaptic Organization of the Brain*. Vol. 5th ed. Oxford University Press; New York: 2004.
- Shepherd GM. Perspectives on olfactory processing, conscious perception, and orbitofrontal cortex. *Ann N Y Acad Sci* 2007;1121:87–101. [PubMed: 17872397]

- Slotnick B, Cockerham R, Pickett E. Olfaction in olfactory bulbectomized rats. *J Neurosci* 2004;24:9195–9200. [PubMed: 15483138]
- Slotnick B, Restrepo D. Olfactometry with mice. *Curr Protoc Neurosci* 2005;20. [PubMed: 18428626] Chapter 8, Unit 8
- Soria JM, Taglialatela P, Gil-Perotin S, Galli R, Gritti A, Verdugo JM, Bertuzzi S. Defective postnatal neurogenesis and disorganization of the rostral migratory stream in absence of the Vax1 homeobox gene. *J Neurosci* 2004;24:11171–11181. [PubMed: 15590934]
- Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999;21:70–71. [PubMed: 9916792]
- Switzer RC 3rd, Johnson JI Jr. Absence of mitral cells in monolayer in monotremes. Variations in vertebrate olfactory bulbs. *Acta Anat (Basel)* 1977;99:36–42. [PubMed: 899683]
- Teicher MH, Stewart WB, Kauer JS, Shepherd GM. Suckling pheromone stimulation of a modified glomerular region in the developing rat olfactory bulb revealed by the 2-deoxyglucose method. *Brain Res* 1980;194:530–535. [PubMed: 7388629]
- Tomasiewicz H, Ono K, Yee D, Thompson C, Goridis C, Rutishauser U, Magnuson T. Genetic deletion of a neural cell adhesion molecule variant (N-CAM-180) produces distinct defects in the central nervous system. *Neuron* 1993;11:1163–1174. [PubMed: 8274281]
- Toresson H, Campbell K. A role for Gsh1 in the developing striatum and olfactory bulb of Gsh2 mutant mice. *Development* 2001;128:4769–4780. [PubMed: 11731457]
- Tran H, Chen H, Walz A, Posthumus JC, Gong Q. Influence of olfactory epithelium on mitral/tufted cell dendritic outgrowth. *PloS one* 2008;3:e3816. [PubMed: 19043569]
- Tummala R, Romano RA, Fuchs E, Sinha S. Molecular cloning and characterization of AP-2ε, a fifth member of the AP-2 family. *Gene* 2003;321:93–102. [PubMed: 14636996]
- Waclaw RR, Allen ZJ 2nd, Bell SM, Erdelyi F, Szabo G, Potter SS, Campbell K. The zinc finger transcription factor Sp8 regulates the generation and diversity of olfactory bulb interneurons. *Neuron* 2006;49:503–516. [PubMed: 16476661]
- Wang HV, Vaupel K, Buettner R, Bosserhoff AK, Moser M. Identification and embryonic expression of a new AP-2 transcription factor, AP-2ε. *Dev Dyn* 2004;231:128–135. [PubMed: 15305293]
- Whitley M, Treloar H, De Arcangelis A, Georges Labouesse E, Greer CA. The α6 integrin subunit in the developing mouse olfactory bulb. *J Neurocytol* 2005;34:81–96. [PubMed: 16374711]
- Wyss JM, Stanfield BB, Cowan WM. Structural abnormalities in the olfactory bulb of the Reeler mouse. *Brain Res* 1980;188:566–571. [PubMed: 7370778]
- Yoshida M, Suda Y, Matsuo I, Miyamoto N, Takeda N, Kuratani S, Aizawa S. Emx1 and Emx2 functions in development of dorsal telencephalon. *Development* 1997;124:101–111. [PubMed: 9006071]
- Yoshihara S, Omichi K, Yanazawa M, Kitamura K, Yoshihara Y. Arx homeobox gene is essential for development of mouse olfactory system. *Development* 2005;132:751–762. [PubMed: 15677725]
- Yun K, Garel S, Fischman S, Rubenstein JL. Patterning of the lateral ganglionic eminence by the Gsh1 and Gsh2 homeobox genes regulates striatal and olfactory bulb histogenesis and the growth of axons through the basal ganglia. *J Comp Neurol* 2003;461:151–165. [PubMed: 12724834]
- Zhang J, Hagopian-Donaldson S, Serbedzija G, Elsemore J, Plehn-Dujowich D, McMahon AP, Flavell RA, Williams T. Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. *Nature* 1996;381:238–241. [PubMed: 8622766]
- Zhao F, Satoda M, Licht JD, Hayashizaki Y, Gelb BD. Cloning and characterization of a novel mouse AP-2 transcription factor, AP-2δ, with unique DNA binding and transactivation properties. *J Biol Chem* 2001a;276:40755–40760. [PubMed: 11522791]
- Zhao F, Weismann CG, Satoda M, Pierpont ME, Sweeney E, Thompson EM, Gelb BD. Novel TFAP2B mutations that cause Char syndrome provide a genotype-phenotype correlation. *Am J Hum Genet* 2001b;69:695–703. [PubMed: 11505339]

Figure 1. Derivation and identification of cells and mice containing the *Tcfap2e* AK allele
(A) Graphic representation of the wild-type *Tcfap2e* allele (top), targeting vector (middle), and recombined AK allele (bottom). Black boxes denote, from left to right, exons 4, 5 and 6. Light grey box in wild-type allele indicates location of the southern probe used to confirm homologous recombination. "Cre" represents the IRES Nls Cre SV40 poly A addition cassette. "Neo" is the neomycin resistance selection cassette. "TK" is the HSV thymidine kinase cassette. White triangles represent Frt sites. Arrows indicate the locations of the primers used in screening for homologous recombination and for latter mouse genotyping. Primers shown with circles attached or with no attachment were used to screen for appropriate 5' and 3' homologous recombination in ES cell clones, respectively. **(B)** Southern blot analyses of genomic DNA extracted from wild-type (+/+) and AK heterozygous (+/ak) mouse tails and digested with NheI. Product for wild allele is 5.7kb, and targeted AK allele is 9.7kb. **(C)** PCR genotyping results for wild-type, AK heterozygous, and AK homozygous (ak/ak) mice by using primers AK19, AK20 and NeoAK2 (primers marked with diamonds in A). The wild type and AK PCR products are 252 bp and 400 bp respectively. M, DNA size markers.

Figure 2. The expression of Cre recombinase transcripts parallels *Tcfap2e* in AK heterozygous mice (A-F) In situ hybridization analysis on E12.5 mouse head sections using either a probe for *Tcfap2e* exon 7 (A, C, E) or Cre recombinase (B, D, F). Wild-type (A and B) or AK heterozygous (C-F) heads are shown in sagittal (A-D) or oblique horizontal (E, F) sections. **(G-L)** In situ hybridization on E18.5 OBs using either a probe for *Tcfap2e* exon 7 (G, I, K) or Cre recombinase (H, J, L). AK heterozygous (G-J) or AK homozygous (K, L) OBs are displayed as coronal (G, H) or sagittal (I-L) sections. AOB: accessory olfactory bulb; GL: glomerular layer; MCL: mitral cell layer; OB: olfactory bulb; OE: olfactory epithelium.

Figure 3. *Tcfap2e*-directed Cre-mediated recombination of the *Rosa26* reporter allele in AK heterozygous mice identifies distinct neuronal layers during OB development

(A-C) E18.5 wild type (A) or AK heterozygous (B, C) OBs stained for β -gal activity in whole mount (A and B) or sagittal section (C). Red boxed area in C is shown in greater detail in C' with stained presumptive mitral cells and punctate staining within the GCL indicated by a red and black arrows, respectively. The staining in the GCL may represent immature projection neurons or cellular remnants that are no longer present in this layer by 2 months of age. (D-E) 2 month old (P2M) AK heterozygous OBs stained for β -gal activity. (D) A frontal view of an OB that was stained in whole mount prior to removal of the most rostral tissue to provide a better view of the internal cell layers. (E) Sagittal section counterstained with nuclear fast red. AOB, accessory olfactory bulb; EPL, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; IPL, internal plexiform layer; MC, mitral cell; MOB, main olfactory bulb; TC: Tufted cell.

Figure 4. Disruption of neuronal layer formation in AK homozygous OBs

(A-D) Neutral red staining of low power coronal (A, B; dorsal at top) and higher power horizontal sections (C, D) of P14 OBs from wild-type (A, C) and AK homozygous mice (B, D). (E-J) Marker analysis of OB organization using horizontal sections in P14 (E, F, I and J) and 2 month old (G, H) wild-type (E, I), heterozygous (G), and *Tcfap2e*-null (F, H, and J) mice. Mice in panels (G, H) also contain the R26R allele and were stained for β -gal activity. RNA in situ hybridizations were performed using a *Pcdh21* probe (E, F) or a *Dlx5* probe (I, J). Black arrows in panels (D, F, G and H) show examples of projection neurons present in the EPL. Note that an occasional such cell is also seen in the EPL of heterozygous mice (G). Red arrows in (F and H) illustrate projection neurons misplaced in the GCL of the *Tcfap2e*-null mice. EPL: external plexiform layer; GCL: granule cell layer; GL: glomerular layer; IPL: internal plexiform layer; MCL: mitral cell layer.

Figure 5. Disorganization of the MCL and absence of the IPL in P14 AK homozygous OBs
 (A, B) Immunohistochemical analysis of expression of NeuN (red: interneurons) and PGP9.5 (green: mitral cells and their proximal dendrites) on P14 horizontal OB sections from wild-type (A) and AK homozygous (B) mice. White arrows in (B) show examples of misoriented mitral cells inappropriately present within the EPL. White asterisks in (B) show mitral cells that appear to have two primary dendrites. (C-D) Immunostaining using the 2H3 antibody of neurofilament (green: axons from projection neurons) on horizontal sections of P14 OBs from wild-type (C) and AK homozygous (D) mice. Hoechst (blue) was used to stain cell nuclei. EPL: external plexiform layer; GCL: granule cell layer; GL: glomerular layer; IPL: internal plexiform layer; MCL: mitral cell layer.

Figure 6. Olfactory circuitry is intact in *Tcfap2e*-null mice despite the alterations in OB neuronal lamination

DiI immunofluorescent images following retrograde labeling of the olfactory circuitry in P14 OBs from wild type (A) and *Tcfap2e*-null mice (B-D). White arrows in (C, D) highlight mitral cells with an aberrant direction of projection of the primary dendrites. EPL: external plexiform layer; GL: glomerular layer; IPL: internal plexiform layer; MCL: mitral cell layer.

Figure 7. Similar learning performance in an odor-detection task by adult AK homozygous mice and control littermates

Each point on the curve represents the percent of correct responses at each subsequent period of testing (block number). The odor used in this experiment was 1% Ethyl Acetate (S+) and mineral oil solvent served as a control (S-). The black line marks the accuracy criterion of 85%. Error bars represent the standard deviation for n=5 sessions.

Table 1

List of PCR primers employed

AK1	5'-ATTGCGCCGCCTGACTCAGAGATCAGCCTGCTTCTGC-3'
AK2	5'-TAACTCGAGCCAGGCACGGAGCAGAAGACCTCACTG-3'
AK3	5'-ATTGTCGACGGCTGTCGTTGCTCAGCTCAACATC-3'
AK4	5'-AACTAGTAGCTAGCCATTCTTTGTGAGTCAGATAAGGG-3'
AK6	5'-CCAGAGTGACTGAGATGAGACAAACC-3'
AK8	5'-GGGATACTGTTTGTGAGCACTGTTAG-3'
AK14	5'-CTAGAAATGCAGAAGTTCTGAGTTTC-3'
AK15	5'-CCAGCCAGGACTGCATAGTGAGAC-3'
AK19	5'-GCTGGTGAGTCAACCTGCCTGCAG-3'
AK20	5'-GGTCACCTTGTAAGTGGATGTTGAG-3'
Neoak2	5'-GATAAATGCCTGCTCTTACTGAAGG-3'
Neo3f	5'-CCATCAGAAGCTTATCGATACCGTCG-3'
FIRES1	5'-CTAGACTAGTCTAGCTAGAGCGGCCCG-3'
Cre1	5'-GCTGGTTAGCACCGCAGGTGTAGAG-3'
Cre3	5'-CGCCATCTTCCAGCAGGCGCACC-3'
Pcdh21F	5'-CCTGATGGTAGGTGTAAGGTGAC-3'
Pcdh21R	5'-CCTGCCACCAATCACTGCATG-3'