Retinoic acid receptor and CNGA2 channel signaling are part of a regulatory feedback loop controlling axonal convergence and survival of olfactory sensory neurons

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ABSTRACT Little is known about the identities and functions of extracellular signaling molecules that work in concert with neuronal activity to regulate refinement and maintenance of the mouse olfactory sensory map. We show that expression of a dominant negative retinoic acid receptor (RAR) in olfactory sensory neurons (OSNs) increased the number of glomeruli that incorrectly contained OSN axons expressing different odorant receptors. This phenotype became apparent postnatally, coincided with increased cell death, and was preceded by increased Neuropilin-1 and reduced Kirrel-2 expressions. Kirrel-2-mediated cell adhesion influences odorant receptor-specific axonal convergence and is regulated by odorant receptor signaling via the olfactory cyclic nucleotide-gated (CNG) ion channel. Accordingly, we found that inhibited RAR function correlated with reduced CNG channel expression. Noris occlusion experiments and analysis of CNG channel-deficient mice further indicated that RAR-regulated CNG channel levels influenced the intrinsic neuronal activity required for cell survival in the absence of odor stimulation. Finally, we showed that CNG channel activity regulated expression of the retinoic acid-degrading enzyme Cyp26B1. Combined, these results identify a novel homeostatic feedback mechanism involving retinoic acid metabolism and CNG channel activity, which influences glomerular homogeneity and maintenance of precisely connected OSNs.—Öztokatli, H., Hörnberg, M., Berghard, A., Bohm, S. Retinoic acid receptor and CNGA2 channel signaling are part of a regulatory feedback loop controlling axonal convergence and survival of olfactory sensory neurons. *FASEB J.* 26, 617–627 (2012). www.fasebj.org

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The interplay between extracellular signaling molecules and neuronal activity during development of precise neuronal connections remains poorly understood. Vitamin A derivatives (retinoids), particularly all-trans retinoic acid (RA), are potent signaling molecules that regulate neuronal plasticity and both activity-dependent and activity-independent phases of neuronal circuit formation (1). RA exerts these effects by regulating gene expression via heterodimers of nuclear transcription factors, termed retinoic acid receptors (RARs) and retinoid X receptors (RXRs; ref. 2). Inhibition of RAR-dependent gene expression in olfactory sensory neurons (OSNs) and vomeronasal neurons increases cell death during a postnatal and neural activity-dependent phase of sensory map formation (3, 4). The olfactory sensory map is formed as a consequence of the selective axonal convergence of ~1200 OSN subpopulations into topographically positioned spherical neuropils, the olfactory glomeruli (5–7). Each OSN subpopulation expresses one defined odorant receptor (OR) gene, and each mature glomerulus contains axon terminals of “OR-like” OSNs, i.e., OSNs expressing the same OR (ref. 8; see Fig. 1G).

The binding of an odorant to an OR activates a specific G protein (Golf) and adenylyl cyclase type 3 (AC3), increasing intracellular cAMP. Peak cAMP levels induce a transient influx of Ca2+ through the cyclic nucleotide-gated (CNG) channel, which can lead to depolarization of an OSN to above the threshold of excitation (9, 10). The CNG channel also contributes to resting conductance at basal cAMP levels (11). In addition to sensory transduction in olfactory cilia, CNG channels are also located in OSN axon terminals, where they regulate both local intracellular Ca2+ levels and neurotransmitter release (12, 13).

According to the current model of olfactory sensory map development, the activity determined by the OR expressed in a given OSN modulates the expression profile of downstream genes, thus influencing axonal convergence and glomerular positioning (7, 8, 14).

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Coinciding with the map assembly, both odor-induced CNG channel activity and cell-intrinsic neuronal activity regulate cell survival and maintenance of connected OSNs (15, 16). The effect of OR signaling on axonal coalescence is exemplified by the severely perturbed sensory map in AC3-deficient mice (17–20); this phenotype coincides with reduced expression of the Semaphorin receptor Neuropilin-1 (Nrp1; ref. 19). In contrast, a virtually normal sensory map is observed in mice that lack a functional cyclic nucleotide-gated channel A2 (CNGA2) gene (21–23), which codes for a subunit that is essential for CNG channel function and olfactory sensory transduction (24). Such results have been interpreted as indicating that OR-dependent cAMP signaling, but not CNGA2-dependent neuronal activity, plays a major role in olfactory sensory map formation (8). However, Serizawa et al. (25) recently reported that convergence of OR-like axons is regulated in an OR- and CNGA2-dependent manner by genes encoding the homophilic adhesive proteins Kirrel-2/Kirrel-3 and the repulsive proteins Ephrin-A5/Eph-A5.

Here, we show that inhibition of RAR function in OSNs results in reduced CNGA2 and Kirrel-2 expressions and a subsequent increase in the number of glomeruli with axons of >1 OR identity. Unilateral naris closure and analysis of CNGA2-deficient mice further suggest that the CNG channel regulates expression of the RA-degrading enzyme cytochrome Cyp26B1 and thus negatively regulates RA signaling. Our results indicate that odor stimulation might terminate the period during which a given OSN is dependent on locally synthesized RA to accomplish axonal convergence with full precision and to prevail during competition for cell survival in the absence of odor stimulation.

MATERIALS AND METHODS

Generation of transgenic mice

The generation of the olfactory marker protein (OMP)-dominant-negative retinoic acid receptor (dnRAR) transgenic mouse lines has been previously described (3). Briefly, the transgenics were generated using F1 embryos of C57BL/6 × CBA crosses and genomic OMP DNA (∼6000 to −10 relative to translational start), the coding sequence of dnRAR (RARo403; ref. 26), and an SV40 polyadenylation site. Analyses were carried out using mice from at least the sixth generation of dnRAR transgenic × C57BL/6 backcrosses. Tissue phenotype characterization was performed using the F1 generations from crosses between dnRAR transgenics and OR-reporter mice, including P2-IRES-tauLacZ, MOI2.3-IRES-GFP-IRES-tauLacZ, and OMP-tauLacZ mice on a C57BL/6 genetic background (7, 27). Mice were killed by cervical dislocation. Animal experiments were approved by the Ethical Committee for Animal Research at Umeå University.

In situ hybridization

Tissue samples were dissected, immersed in 4% paraformaldehyde in PBS (pH 7.4) at 4°C overnight, and then cryoprotected in 20% w/v sucrose in PBS at 4°C for 16–24 h. Tissue from >3-wk-old mice was treated with RDO Rapid Decalcifier (ApexEngineering Products, Aurora, IL, USA) before protection and freezing in Tissue-Tek O.C.T. (Sakura Fine Tek, Torrance, CA, USA) and cryosectioning (12 μm). In situ hybridization using digoxigenin-labeled probes was performed as described previously (28). cRNA probes specific for OMP, Gaolf, Gaoc, AC3, CNGA2, K21, Kirrel-2, and Cyp26B1 were described previously (3, 4, 29–31). Nrp1-, Ephrin-A5-, MOR256-17-, and Olfactorin-specific probes corresponded to EST clones with GenBank accession nos. AA036088, BG921710, BC115838, and BC100685, respectively.

Immunohistochemistry

Tissues were fixed in PFA for 1–2 h, decalcified, cryoprotected, embedded, and sectioned as described above. Background was blocked by incubation for 1 h in 2% BSA in PBS with 0.3% Triton X-100 (Tx), followed by overnight incubation at 4°C in PBS with 0.3% Tx containing anti-Kirrel2 (1:500; R&D Systems, Minneapolis, MN, USA), anti-OMP (1:500; Wako Chemicals, Osaka, Japan), anti-GAP-43 (1:1000; Chemicon, Temecula, CA, USA), anti-cleaved caspase 3 (1:500; Becton-Dickinson, Franklin Lakes, NJ, USA), anti-CNGA2 (1:500; Alomone Labs, Jerusalem, Israel), anti-AC3 (1:500; C20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-MOR256-17 (1:1,000; ref. 32), anti-GFP (1:500; Molecular Probes, Eugene, OR, USA), anti-Nrp1 (1:150; Ab-1 Oncogene Research Products, Cambridge, MA, USA), or anti-tyrosine hydroxylase (1:500; Pel-Freez Biologicals, Rogers, AR, USA). After being washed, specific immunoreactions were visualized using a Cy3-conjugated anti-rabbit antisera (Jackson ImmunoResearch, West Grove, PA, USA) or Alexa488-conjugated anti-goat antisera (Molecular Probes). Alternatively, biotinylated anti-rabbit antisera and avidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) were used with 3,3′-diaminobenzidine as a substrate. Double activated caspase-3/Kirrel-2, βgal/Kirrel-2, and MOR256-17/Kirrel-2 images were produced using a Leica DM IRB confocal microscope with Ar and He/Ne lasers (Leica Microsystems, Wetzlar, Germany). Sections were counterstained with Hoechst 33258 (Sigma, St. Louis, MO, USA).

Western blot

Olfactory epithelial tissue was harvested in RIPA buffer (0.5 M Tris-HCl, pH 7.4; 1.5 M NaCl; 2.5% deoxycholic acid; 10% Tx; and 10 mM EDTA). Samples were boiled in Laemmli buffer and sonicated. Protein aliquots (25–100 μg) were separated by gel electrophoresis (Bio-Rad Ready Tris-HCl Gel, 4–20%; Bio-Rad, Hercules, CA, USA) and electroblotted onto nitrocellulose filters (Whatman, Clifton, NJ, USA). Filters were blocked in 5% w/v nonfat powdered milk in Tris-buffered saline (pH 8.0) with 0.1% Tween-20 and incubated overnight at 4°C with primary antibodies, rabbit polyclonal anti-OCNC1 (1:200; Alomone Labs), and rabbit polyclonal anti-β tubulin (1:2000; Biosite, San Diego, CA, USA), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000; Pierce, Rockford, IL, USA) for 30 min. Blots were developed with the ECL detection system (SuperSignal West Dura; Pierce). Quantity One 1-D analysis software (Bio-Rad) was used to quantify signals from images taken by ImageQuant LAS 4000 (GE Healthcare, Little Chalfont, UK).
Quantification of homogeneous/heterogeneous glomeruli and OR-positive OSNs

Slides were rinsed in 0.1 M phosphate buffer, 2 mM MgCl₂, 5 mM EGTA, 0.02% Nonidet-P40, and 0.001% sodium deoxycholate. The staining reaction took place at 37°C, overnight in the same buffer with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside, 5 mM K₃Fe(CN)₆ and 5 mM K₄Fe(CN)₆. After OMP immunohistochemistry, we counted the numbers of βgal-positive homogenous and heterogenous glomeruli for every fourth (2 to 6 wk old), every third (1 wk old), or every second (newborn mice) section. A glomerulus was defined by its ring of juxtaglomerular nuclei. Homogenous and heterogenous glomeruli were defined as described previously (33). To control for the possibility that reduced RA signaling inhibited axonal transport of the reporter proteins, which in theory could have resulted in heterogeneous glomeruli, we used an OR-specific antibody (anti-MOR256-17) and olfactory bulb sections of heterozygous OMP-tauLacZ mice that express βgal in all OSN axons (7, 32). To quantify the numbers of MOR256-17 and K21 expressing OSNs, nonradioactive in situ was used and the number of positive cells was determined for every fifth section throughout the rostrocaudal extent of the nasal cavities of 4-wk-old mice.

Unilateral naris closure

In anesthetized animals, the left or right naris was cauterized (System 3000 Electrosurgical Unit; Bard Norden AB, Helsingborg, Sweden). Scar formation resulted in permanent unilateral naris closure. Complete closure was confirmed by applying a drop of water to the cauterized nostril before the animals were killed and by postmortem examination under a dissecting microscope.

RESULTS

dnRAR in OSNs interferes with postnatal development of homogeneous glomeruli

To study the role of RA signaling in OSNs, we used transgenic mice that carry a dnRAR transgene under transcriptional control of the OMP promoter, which selectively drives expression in OSNs and vomeronasal neurons, but not in their dividing precursor cells (3, 4). The dnRAR transgene (hRARα403) forms inactive dnRAR-RXR heterodimers and inhibits transcription mediated by all RAR isoforms (RARα, β, and γ; ref. 26). We have previously shown that OMP promoter-driven dnRAR expression results in reduced RA-responsive element-dependent transcription during the first postnatal week and that this is followed by increased OSN death during the second week when OSN axons had formed glomeruli at apparently correct topographic positions (3). To study whether inhibition of RAR function interfered with glomerular maturation, i.e., the postnatal elimination of heterogeneous (“immature”) glomeruli formed by axons of >1 OR identity and/or elimination of supernumerary glomeruli (33, 34), we analyzed dnRAR mice bred to the P2 OR-reporter mouse line (7). In this reporter line, β-galactosidase (βgal) was selectively expressed in axons of OR-like OSNs, e.g., the P2 OR. OMP was expressed in virtually all OSNs, and thus the immunoreactivities for OMP and βgal overlapped completely in homogeneous P2

Figure 1. Axons of OSNs expressing a dnRAR generate heterogeneous glomeruli. A, B) Double OMP immunohistochemical (red) and βgal histochemical (yellow) analysis showing βgal-positive P2 axons in a homogeneous P2 glomerulus in control (A) and a heterogeneous P2 glomerulus in dnRAR transgenic (B). C, D) Double OMP (red) and EGFP (green) immunohistochemical analysis showing EGFP-positive MOL2.3 axons terminals in a homogeneous MOL2.3 glomerulus in control (C) and a heterogeneous MOL2.3 glomerulus in dnRAR (D). E, F) Double MOR256-17 and βgal immunohistochemistry analysis of an OSN subpopulation expressing the MOR256-17 OR (yellow) in a heterozygous OMP-tauLacZ reporter mouse that expresses βgal (green) in all OSNs. Glomeruli are shown that are homogeneous with regard to both OMP-promoter expressed βgal and MOR256-17 immunoreactivity in control (E) and a heterogeneous glomerulus in a dnRAR (F). Dashed outlines delineate glomeruli with axons of defined OR identities. G) Schematic representation of OSNs in the olfactory epithelium that project to target neurons in olfactory bulb. Axons of neurons expressing the same odorant receptor converge and form synapses in discrete structures called glomeruli.
TABLE 1. Increased number of heterogeneous glomeruli in dnRAR transgenic mice

| OR/age | Genotype | Half-bulbs (n) | Total [n (min–max)] | Homogeneous (n) | Heterogeneous (n) |
|--------|----------|---------------|---------------------|----------------|------------------|
| P2/PD1 | Cont 12  | 1.92 ± 0.26 (1–4) | ND | ND |
|        | dnRAR 16 | 1.56 ± 0.16 (1–3) | ND | ND |
| P2/PD7 | Cont 12  | 1.92 ± 0.26 (1–3) | 1.25 ± 0.13 | 0.67 ± 0.26 |
|        | dnRAR 12 | 1.58 ± 0.24 (1–3) | 0.92 ± 0.15 | 0.67 ± 0.19 |
| P2/PD14 | Cont 12  | 1.62 ± 0.18 (1–3) | 1.25 ± 0.11 | 0.37 ± 0.15 |
|        | dnRAR 12 | 1.50 ± 0.29 (1–3) | 0.25 ± 0.13*** | 1.25 ± 0.25* |
| MOL2.3/PD14 | Cont 16 | 1.25 ± 0.45 (1–2) | 1.25 ± 0.45 | 0.00 ± 0.00 |
|        | dnRAR 16 | 0.94 ± 0.44 (1–2) | 0.00 ± 0.00*** | 0.94 ± 0.44*** |
| P2/PD28–42 | Cont 16 | 1.69 ± 0.20 (1–3) | 1.44 ± 0.20 | 0.25 ± 0.14 |
|        | dnRAR 20 | 2.75 ± 0.22*** (1–5) | 0.25 ± 0.10*** | 2.50 ± 0.21*** |

Organization of OSN axons within P2 and MOL2.3 glomeruli was examined by double OMP immunohistochemical/β-gal histochemical staining of serial coronal sections through the olfactory bulbs of both control (cont) and dnRAR transgenic mice. Homogeneous and heterogeneous glomeruli were not determined (ND) at postnatal day 1 (PD1). All values are given as means ± sd. **P < 0.01, ***P < 0.001, Mann-Whitney test, using the Minitab 15 program.

glomeruli. In contrast, in heterogeneous glomeruli, a substantial fraction of the OMP-positive axons will be β-gal-negative since these correspond to OSNs expressing other ORs, different from P2 (33). Double immunohistochemistry of serial coronal sections through the olfactory bulbs of 4-wk-old mice revealed that the majority of P2-glomeruli were heterogeneous in dnRAR transgenic mice (Fig. 1A, B and Table 1). Thus, glomeruli in dnRAR transgenics differed from those in controls in that each glomerulus more often contained an OMP-positive neurophil divided into β-gal-positive and β-gal-negative domains. At 1 wk of age, there was no difference in dnRAR transgenic mice compared with littermate controls (Table 1). Between postnatal weeks 1 and 2, the ratio of heterogeneous to homogeneous glomeruli diverged in OMP-dnRAR mice compared with controls. The increased ratio in dnRAR transgenic mice was a consequence of a reduction in homogeneous glomeruli and a concomitant increase in heterogeneous glomeruli. In 2-wk-old dnRAR transgenic mice, 83% of the P2 glomeruli were heterogeneous compared with 23% in controls. A 1.6-fold increase in the total number of P2 glomeruli was seen in 4- to 6-wk-old dnRAR mice. We also analyzed the progeny of dnRAR mice bred to an OR-reporter mouse line that selectively expresses enhanced green fluorescent protein (EGFP) in axons of OSNs specified to express another OR gene (MOL2.3; ref. 27). In 2-wk-old MOL2.3 mice expressing the dnRAR transgene, 100% of the glomeruli were heterogeneous compared with 0% in control (Fig. 1C, D and Table 1). We also analyzed OSNs expressing the OR MOR256-17 (32). An anti-MOR256-17 specific antibody was used for heterozygous OMP-tauLacZ mice that express tauβgal in all OSN axons (7). Double MOR256-17 and β-gal immunohistochemical analyses revealed an increased number of heterogeneous MOR256-17 glomeruli in 1-mo-old dnRAR transgenic mice compared with controls (Fig. 1E, F; see also Supplemental Fig. S2B). The increase in heterogeneous MOR256-17 glomeruli was, however, less dramatic than for MOL2.3- and P2-positive OSNs. Collectively, the analyses of OSNs with defined OR identities indicated that RAR normally regulates genes required for glomerular homogeneity, even though all OSN subpopulations did not show equal sensitivity to the effects of reduced RAR function.

Altered expression of genes known to regulate OR-specific axonal convergence

We next analyzed whether the increased number of heterogeneous glomeruli coincided with altered expression of genes known to influence axon sorting and OR-specific convergence, i.e., Kirrel-2, Ephrin-A5, and Nrp1 (25, 35–37). We used consecutive sections of olfactory epithelium from postnatal day 4 (PD4), which is before the glomerular phenotype and the increased death of OSNs in dnRAR transgenics (Table 1 and ref. 3). In situ hybridization analyses showed reduced Kirrel-2 mRNA expression in dnRAR mice compared with controls, while Ephrin-A5 expression was unaltered (Fig. 2A). We also observed increased Nrp1 mRNA in OSNs of dnRAR transgenic mice (Fig. 2A). Immunohistochemical analyses of sections, consecutive to the sections used for the in situ hybridization analyses, showed that the distribution and intensity of immunoreactivity corresponding to markers for mature (OMP) and immature (GAP43) OSNs were unaltered (Supplemental Fig. S1 and ref. 38). In accordance with the in situ hybridization results, we found increased Nrp1 and decreased Kirrel-2 immunoreactivities in the nerve and glomerular layers of dnRAR transgenics (Fig. 3A). An analysis of Kirrel-2 expression in glomeruli with known OR identities (P2, MOR256-17) also showed reduced Kirrel-2 immunoreactivity in the transgenic mice (Supplemental Fig. S2).

Taken together, these results showed that altered levels of Nrp1 and Kirrel-2 (regulators of selective OSN axonal fasciculation) correlated with an increased postnatal number of heterogeneous glomeruli.

Interestingly, analysis of Kirrel-2 immunoreactivity not only confirmed reduced Kirrel-2 on OSN axons in dnRAR transgenics but also revealed a heterogeneous
distribution of Kirrel-2 within glomeruli on virtually every section (Fig. 3; Supplemental Fig. S2). Since the Kirrel-2 level varies with the OR type (25), it is likely that the heterogeneous Kirrel-2 levels in a single glomerulus were a consequence of the axon OR heterogeneity.

We have previously shown an inverse relationship between increased caspase-3 activation and Kirrel-2 levels among subpopulations of vomeronasal neurons in dnRAR transgenic animals (4). Confocal microscopy of Kirrel-2 and activated caspase-3 double immunohistochemical analyses of OSN axons revealed a similar relationship for glomeruli in the olfactory bulb. Individual glomeruli with high levels of activated caspase-3 expressed low levels of Kirrel-2 (Fig. 3Ba–c). This inverse relationship was also evident in heterogeneous glomeruli in dnRAR transgenics (Fig. 3Bd–f). Since different OR-like OSN subpopulations differentially expressed Kirrel-2, this result suggested that caspase-3 may be activated in axons of OR-like subpopulations that express relatively low levels of the neural activity-regulated gene Kirrel-2.

Low Kirrel-2 correlates with reduced CNGA2 channel expression

Levels of Kirrel-2 and Nrp1 are regulated by OR signaling. To address the possibility that altered Kirrel-2 and Nrp1 levels in dnRAR mice were caused by altered expression of proteins mediating sensory transduction in OSNs, we analyzed for Gaolf, AC3, and CNGA2. In situ hybridization analysis demonstrated that Gaolf and AC3 mRNA levels were equal in newborn dnRAR transgenics and controls, while CNGA2 expression was significantly reduced in dnRAR (Fig. 2B). Immunohistochemistry confirmed this result, showing reduced CNGA2 in cilia (Fig. 4A) and axon terminals of dnRAR transgenic mice (Supplemental Fig. S3). To estimate the reduction of CNGA2 during the first postnatal week, we used Western blot analyses of PD5–7 mice. The anti-CNGA2 antibody generated a 130-kDa band in total protein fractions from the olfactory epithelium (39). The intensity of the 130-kDa band was reduced in extracts from dnRAR transgenic mice, while a 50-kDa band corresponding to neuron-specific β-tubulin (class III) was not (Fig. 4B). Quantification showed that the relative intensities of CNGA2 and β-tubulin signals were 49 ± 19 and 103 ± 33%, respectively (means ± SD; P=0.019, Student’s test, 2-tailed), in dnRAR transgenic mice (n=4) compared with controls (n=6). Collectively, the results of in situ hybridization, immunohistochemical, and Western blot analyses suggested that CNGA2 was reduced but not completely absent in OSNs of dnRAR transgenics.

dnRAR modulates gene expression by both CNGA2-dependent and -independent mechanisms

To analyze whether the effects of reduced CNG channel levels on gene expression differed from those of reduced activity of the CNG channel, we utilized the unilateral naris closure model to reduce airflow and odor input to OSNs in one nasal cavity (40). Tissue from 3-wk-old mice was analyzed 2 d after naris closure. In agreement with previous re-
and no activated caspase-3 (arrows), whereas the other glomerulus contains axons that express low levels of Kirrel-2 and high levels of activated caspase-3 (arrowheads). d–f) Two glomeruli are shown; one contains axons with high levels of Kirrel-2 and no activated caspase-3 (arrows), whereas the other is a heterogeneous glomerulus with regions that show an inverse relationship between axonal Kirrel-2 expression and caspase-3 activation (arrowhead). Dashed outlines delineate individual glomeruli.

Figure 3. Nrp1, Kirrel-2, and activated caspase-3 expression in the glomerular layer of the olfactory bulb. A) Immunohistochemical analysis of the glomerular layer in PD14 mice with antibodies specific for OMP, Nrp1, and Kirrel-2. Axonal OMP is unaltered, whereas Nrp1 is higher, in glomeruli of dnRAR transgenics. Kirrel-2 immunoreactivity is reduced in OSN axons of dnRAR transgenics. Arrow indicates a glomerulus in a dnRAR transgenic mouse that contains a neuropil with mixed high and low Kirrel-2 regions. B) Double-activated caspase-3 (Casp-3; red) and Kirrel-2 (green) immunohistochemical analysis of glomeruli of PD14 dnRAR transgenic mice. a–c) Two glomeruli are shown; one contains OSN axons with high levels of Kirrel-2 and no activated caspase-3 (arrows), whereas the other is a heterogeneous glomerulus with regions that show an inverse relationship between axonal Kirrel-2 expression and caspase-3 activation (arrowhead). Dashed outlines delineate individual glomeruli.

Figure 4. Inhibition of RAR results in reduced expression of CNGA2 protein. A) Immunohistochemical analyses of olfactory epithelium from PD2 and PD35 mice using anti-CNGA2 and anti-AC3 antibodies. CNGA2 protein levels are reduced in cilia in dnRAR transgenic mice (arrow) compared with control (arrow). Olfactory epithelium is thinner in PD35 dnRAR mice due to the increased postnatal cell death (3). Dashed lines indicate basal lamina. Scale bar = 100 μm. B) Western blots of olfactory epithelia showing 130- and 50-kDa proteins corresponding to CNGA2 and neuron-specific β-tubulin (βTub), respectively. Intensity of the CNGA2 band is reduced in dnRAR transgenic mice compared with littermate control mice, whereas the intensity of the β-tubulin band is unaltered in the same protein preparation.
Olfactorin is an extracellular membrane-bound protein of unknown function (41). Nasal occlusion did not influence Olfactorin expression (Fig. 5E), nor was Olfactorin altered in CNGA2-null mice (Fig. 6A). These results indicated that Olfactorin normally is positively regulated by RAR by a mechanism that is independent of CNGA2 and channel activation. Analyses of CNGA2-null mice also confirmed previous results by Serizawa et al. (25), showing that a functional CNGA2 gene is required for Kirrel-2 to be expressed in OSNs (Fig. 6A). The reduced, but detectable, expression of Kirrel-2 in the transgenics thus indicated that dnRAR did not abolish CNG channel function completely. These results are compatible with a model in which RA regulates Kirrel-2 expression indirectly by influencing the level of CNG channel in cilia and/or axon terminals.

**RA-degrading enzyme Cyp26B1 is, like Kirrel-2, regulated by a CNGA2-dependent mechanism**

The finding that decreased CNG stimulation by nasal closure as well as CNGA2 deficiency, influenced Kirrel-2 and Olfactorin expression in OSNs, suggests that RA signaling in OSNs is regulated by the CNGA2-dependent mechanism.
rel-2 but not Olfactorin expression, was interesting in light of our previous finding that RAR signaling positively regulates the expression of the RA degrading enzyme Cyp26B1 in OSNs (3). To analyze the interesting possibility that Cyp26B1 expression might also be regulated by neuronal activity in a CNGA2-dependent manner, we compared the regulation of Cyp26B1 expression to that of Kirrel-2 and Olfactorin. In situ hybridization analysis of CNGA2 null mice and after naris closure showed that the regulation of Cyp26B1 expression resembled that of Kirrel-2, i.e., Cyp26B1 was reduced in the absence of CNGA2 and in response to naris closure (Fig. 6B, C). Thus, Cyp26B1-mediated negative feedback control of RA signaling might involve CNG channel activity.

**Variable sensitivity to dnRAR among OR-like OSN subpopulations**

Next, we addressed whether low Kirrel-2 expression also correlated to increased caspase-3 activation in CNGA2−/− mice. The CNGA2 gene is on the X chromosome, and, due to dosage compensation, female CNGA2−/− mice have an olfactory epithelium that is a mosaic of CNGA2-positive and dying CNGA2-negative OSNs (15). We observed increased caspase-3 activation at PD10 in OSNs of CNGA2−/− female mice in comparison to littermate controls (Fig. 7A). In similarity to the dnRAR transgenics, double immunohistochemistry of CNGA2−/− olfactory bulb sections showed that caspase-3 was activated in Kirrel-2-negative glomeruli (Fig. 7B). The correlation between caspase-3 activation and low levels of CNGA2 and Kirrel-2 in distinct glomeruli was interesting in light of the fact that Kirrel-2 expression varies among OR-like OSN subpopulations in an OR- and CNGA2-dependent manner (25). To directly determine whether OR-like subpopulations showed differential sensitivity to the effect of reduced CNGA2 expression in the dnRAR transgenics, we performed in situ hybridization analyses with probes specific for different ORs. While 40% of P2 and 38% MOR2.3 OSNs survived in 1-mo-old dnRAR transgenics (3), the corresponding numbers for MOR256-17 OSNs and K21 OSNs were 85 and 73%, respectively (Fig. 7C). Thus, all subpopulations of OSNs did not show equal sensitivity to dnRAR. In homozygous dnRAR transgenics, we found a further 14% reduction in the numbers of MOR256-17 and K21 OSNs compared with hemizygous mice (Fig. 7C). This copy number dependence suggested that the dnRAR line carried a hypomorph mutation, which is in line with the possibility that the differences in sensitivity among OSN subpopulations may depend on local variations in RA concentration.

**RAR has prosurvival effects independent of odor stimulation**

The mechanism mediating the selective death of CNGA2-negative OSNs in CNGA2−/− mice appears to depend on sensory stimulation since these neurons survive after naris closure, which prevents odor-induced activity in the CNGA2-positive OSNs (15). As the increased death of OSNs in postnatal dnRAR mice was associated with reduced CNGA2 expression, we ana-
analyzed whether the increase in caspase-3 activation was driven by odor-induced activity. An alternative possibility was that reduced CNGA2 expression decreased cell-intrinsic excitability, which appears to positively influence survival of OSNs (16). Newborn mice (PD1–2) were deprived of sensory input by unilateral naris closure and examined for caspase-3 activation at PD11–12. The result showed that activated caspase-3 was not reduced ipsilateral to the closed naris in dnRAR transgenic mice (Fig. 7D, E). Moreover, the difference between the numbers of activated caspase-3-positive cells in control compared with dnRAR transgenics was most pronounced in the absence of odor stimulation. Thus, the cell death in the dnRAR transgenics appeared to primarily be mediated by a cell-intrinsic mechanism that was independent of odor-induced CNGA2 channel activity.

Tyrosine hydroxylase is reduced in postsynaptic periglomerular cells in the olfactory bulb in absence of odor stimulation by naris closure as well as in CNGA2 deficient mice (21). We found that the reduction of glomerular tyrosine hydroxylase immunoreactivity ipsilateral to the closed naris was similar in dnRAR transgenic and control mice (Fig. 7E). This result indicates that the level of CNGA2 channel in dnRAR transgenic mice was sufficient to mediate some level of odor-induced neuronal activity.

**DISCUSSION**

Our results show that inhibition of RAR-mediated gene regulation in postmitotic OSNs significantly reduces CNGA2 expression. As the CNGA2 subunit is essential for CNG channel function, this finding indicates that RA can modulate the number of functional CNG channels in the membranes of dendritic cilia and axon terminals. CNG channels contribute to resting conductance at basal cAMP levels (11), so it is conceivable that a RAR-dependent change in the number of channels in cilia and/or axon terminals could alter the influx of ions sufficiently to influence basal expression of a set of neural activity-regulated genes. The finding that Kirrel-2 is down-regulated in response to inhibited RAR function supports this model, since variations in Kirrel-2 expression between different OR-like OSN subpopulations are a direct readout of OR-dependent CNG channel activity (25). Analysis of Ephrin-A5 indicated that not all activity-dependent genes are sensitive to the dnRAR-induced CNGA2 decrease. While the mechanisms for CNGA2-dependent gene expression in OSNs remain to be determined, note that much of the inward CNG channel current is carried by Ca$^{2+}$, which, in turn, can regulate numerous enzymes and transcription factors with different activation properties that allow for discrimination between intracellular free Ca$^{2+}$ concentrations that differ in strength, frequency, duration, and/or localization of Ca$^{2+}$ influx (42).

We showed that lowering CNG channel activity by CNGA2 gene deletion or naris closure decreased the expressions of both Kirrel-2 and Cyp26B1. Since Cyp26B1 degrades and inactivates RA, these findings suggest that RA and CNG channel signaling are parts of a novel regulatory feedback loop. As expected, RAR also regulates gene expression in OSNs independent of CNG channel activity, as demonstrated for Olfactoerin and Nrp1. A gene regulatory model based on the obtained results is outlined in Fig. 8. Interestingly, the model includes an unanticipated crosstalk between activity-dependent and activity-independent gene regulation that may explain the influence of inhibited RAR function on olfactory bulb glomerular homogeneity and postnatal OSN survival.

**Influence of RAR on glomerular homogeneity**

Reduced RAR function resulted in an increased fraction of heterogeneous glomeruli. Heterogeneous glomeruli accumulate during the first 6 wk of postnatal development, likely as a consequence of continuous addition of new axons. Interestingly, the increase in glomerular heterogeneity coincided with an increase in OSN death beginning in the second postnatal week (3). Conceivably, OSNs may die because altered levels of proteins such as Kirrel-2 and Nrp1 interfere with the mechanisms regulating establishment and maintenance of glomeruli with axons of defined OR identities. An additional, nonexclusive, possibility is that OSN death frees “glomerular space” for axons representing OSN subpopulations that are less sensitive to the effects of reduced RAR signaling. During normal postnatal development, supernumerary heterogeneous glomeruli disappear during the first ~2 mo of postnatal life (33, 34). The mechanism for this glomerular refinement process is unknown but appears to depend on OSN turnover (33). In dnRAR mice, mature OSNs die without a compensatory increase in OSN generation (3). Thus, OSN death caused by inhibited RAR-dependent transcription appears to bypass the unknown homeostatic...
mechanism controlling OSN numbers in the epithelium, i.e., the quantitative matching of OSNs and olfactory bulb target neurons. This may explain why supernumerary of glomeruli is not a prominent phenotype in dnRAR transgenics. Moreover, given that glomerular refinement appears to depend on OSN turnover, it is conceivable that the lack of a compensatory increase in neurogenesis allows for prolonged maintenance of a large fraction of heterogeneous glomeruli in the dnRAR transgenics.

It has been shown that axons of CNGA2-positive and CNGA2-negative OR-like OSNs are in distinct glomeruli (23), seemingly dependent on different expression levels of CNG channel-regulated genes, such as Kirrel-2 (25). Decreased adhesion between OSN axons (for example, due to reduced Kirrel-2) may thus contribute to the reduced axonal homogeneity in glomeruli of dnRAR transgenic mice. However, we cannot exclude a role for the increased expression of Nrp1, which plays a role in repulsive interactions between OSN axons in the nerve and between axons and glial cells (36, 37). Interestingly, it has been shown that inactivation of the RA-degrading enzyme Cyp26A1 results in reduced expression of Nrp1 in ES cells (43). This indicates that Nrp1 is also negatively regulated by RA in other cell types.

RA-regulated survival of OSNs

The overall level of neuronal activity (as determined by the synaptic input received, combined with cell-intrinsic membrane properties) can promote neuronal survival during integration of a neuron into a circuit (44). We found that naris closure did not influence dnRAR-induced caspase-3 activation, indicating a possibility that OSN death in dnRAR transgenic mice is driven by an odor-independent mechanism. We hypothesize that a decreased number of functional CNG channels in an OSN will increase its dependency on OR activity to achieve the Ca\(^{2+}\) concentration dynamics required for cell survival. Thus, reduced CNG channel expression may, in effect, be equal to diminished cell-intrinsic basal neuronal activity. Analyses of mice that overexpress the Kir2.1 potassium channel have provided evidence that cell-intrinsic activity plays a pivotal role in establishment and maintenance of glomeruli as well as OSN survival (16). Since cell-intrinsic activity is required to maintain OR-specific OSN subpopulations, one straightforward scenario is that the OSNs that are most sensitive to reduced CNG channel expression correspond to OR subpopulations with relatively low basal levels of cAMP, or alternatively, neurons that are activated by infrequently encountered odors. Analyses of CNGA2\(^{+/−}\) mice showed that CNGA2 deficiency correlated with increased caspase-3 activation. Conceivably, dnRAR may reduce CNG channel activity in a large fraction of OSNs to below a cell-intrinsic activity threshold required for prolonged survival. In support of this, we found increased caspase-3-activation in OSNs that express relatively low levels of Kirrel-2, indicating that the OSNs express ORs that mediates low CNG channel activity (25). Moreover, we found that MOR256-17 OSNs, a subpopulation that normally expresses relatively high levels of Kirrel-2 (25), is relatively refractory to the effect of reduced CNGA2 expression.

Function of the RA-RAR-CNGA2-Cyp26B1 feedback loop

Taken together, our results reveal the possibility that RA-regulated CNG channel expression in OSNs contributes to the cell-intrinsic excitability required for maintaining precisely connected OSNs in the absence of odor-stimulated CNG channel activity. According to this model, the RA-RAR-CNGA2-Cyp26B1 feedback loop could adjust to global inactivity, e.g., in response to naris occlusion caused by infection. Such a role resembles the function of RA in the homeostatic synaptic plasticity of hippocampal neurons (45). Inhibition of activity in these neurons by tetrodotoxin triggers an RA- and RARα-dependent compensatory increase in synaptic strength through increased expression of GluR1 receptors. Another nonmutually exclusive possibility is that the feedback loop provides a mechanism by which odor stimulation can terminate a period during which an OSNs responds to RA. Odor stimulation would thus inhibit the RA-regulated gene program by inducing Cyp26B1-mediated degradation of RA and, at the same time, initiate an odor-regulated gene program that may be required, e.g., for long-term maintenance of connected OSNs and/or learning-dependent neuronal plasticity (34, 46). The duration for which an OSN depends on RA for survival would depend on both odor environment and RA availability. An important role for the level of RA availability is indicated by the finding that the number of OSNs expressing a specific OR was reduced in homozygous compared with hemizygous dnRAR transgenic mice. Moreover, RA-synthesizing enzymes (RALDHs) are both developmentally and spatially regulated in cells adjacent to OSNs (31, 47). Future analyses of genetically modified mice will address the exciting possibility that spatial and temporal variations in RA metabolism work in concert with the odor environment to influence the numbers of connected OSNs expressing a given OR.

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