

# Receptor guanylyl cyclases in mammalian olfactory function

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**Abstract** The contributions of guanylyl cyclases to sensory signaling in the olfactory system have been unclear. Recently, studies of a specialized subpopulation of olfactory sensory neurons (OSNs) located in the main olfactory epithelium have provided important insights into the neuronal function of one receptor guanylyl cyclase, GC-D. Mice expressing reporters such as  $\beta$ -galactosidase and green fluorescent protein in OSNs that normally express GC-D have allowed investigators to identify these neurons in situ, facilitating anatomical and physiological studies of this sparse neuronal population. The specific perturbation of GC-D function in vivo has helped to resolve the role of this guanylyl cyclase in the transduction of olfactory stimuli. Similar approaches could be useful for the study of the orphan receptor GC-G, which is expressed in another distinct subpopulation of sensory neurons located in the Grueneberg ganglion. In this review, we discuss key findings that have reinvigorated the study of guanylyl cyclase function in the olfactory system.

**Keywords** Olfactory epithelium · Chemosensory neurons · Natriuretic peptides · Uroguanylin · Guanylin · Carbon dioxide · Necklace glomeruli · Grueneberg ganglion · GC-D · GC-G · cGMP · Cyclic nucleotide · Cyclic nucleotide-gated channel

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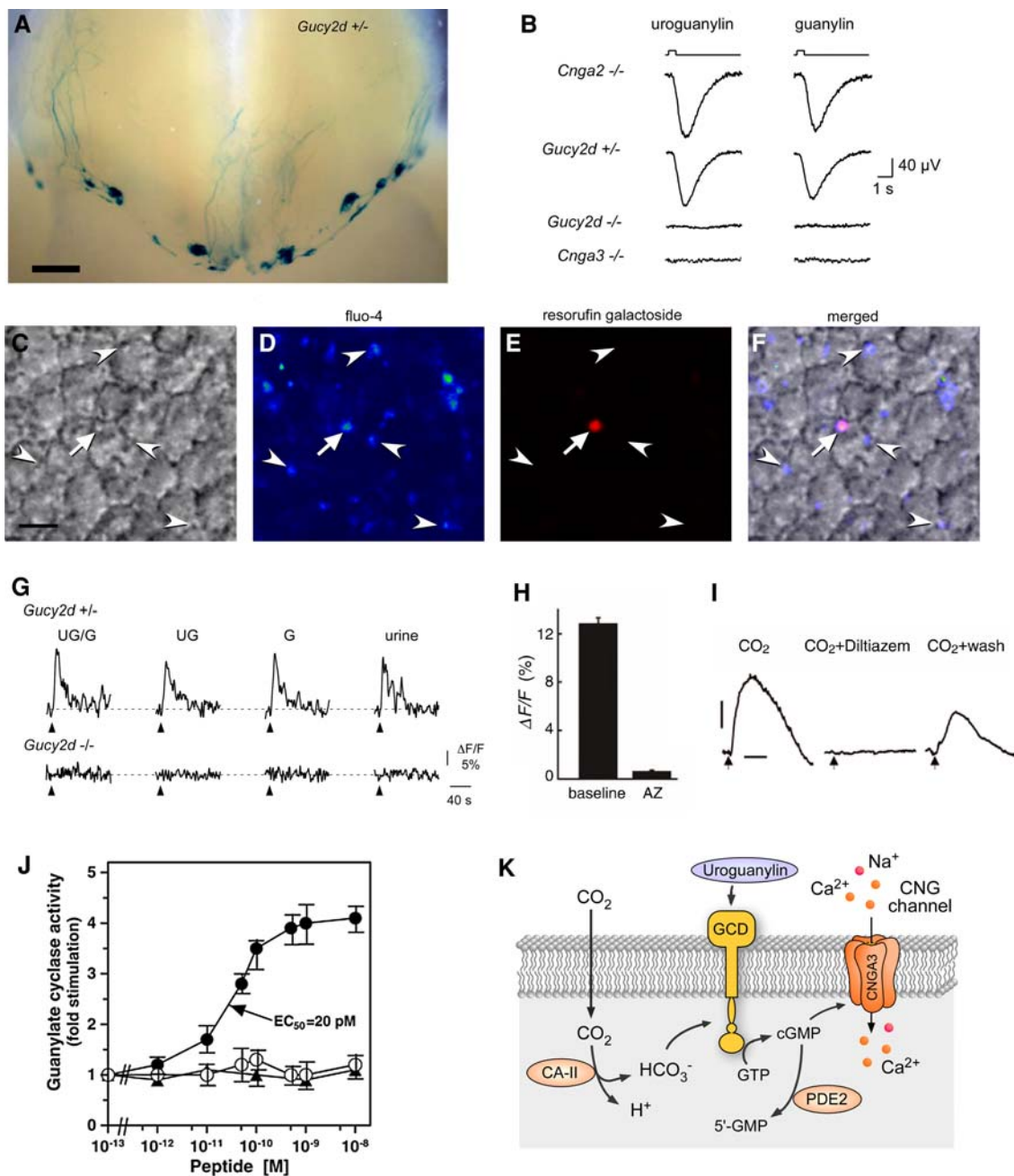
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## Introduction

Sensory neurons in the mammalian nose, which detect a vast range of molecular cues and transduce them into electrical membrane signals, have emerged as excellent model systems to investigate the function of guanylyl cyclases as critical components of neuronal cGMP-dependent signaling cascades. In the olfactory system, current interest has focused on members of the receptor guanylyl cyclase family, specifically the receptor guanylyl cyclase GC-D [1], and their downstream signaling components (Fig. 1). In order to appreciate recent developments in this field, it is necessary to gain some understanding on the cellular and molecular organization of the mammalian sense of smell.

The murine olfactory system is composed of four anatomically segregated sensory organs: the main olfactory epithelium (MOE), the vomeronasal organ (VNO), the septal organ of Masera (SOM), and the Grueneberg ganglion (GG) [2]. It is now clear that each of these organs contains structurally and functionally distinct chemosensory subsystems [2]. Sensory neurons in each subsystem make distinct neural connections to specific regions of the olfactory forebrain (i.e., the main olfactory bulb or the accessory olfactory bulb), and are distinguished by the receptors, signal transduction molecules, and ion channels they express [2]. These diverse sensory neuron populations also vary in the sensory cues they detect [2]. Although a detailed discussion of this complex organization is beyond the scope of the present article and the reader is referred to several recent reviews [2–4], it is important to note that the vast majority of olfactory sensory neurons (OSNs) in the MOE are classical, or canonical, OSNs. Each canonical OSN expresses a member of the odor receptor (OR) gene family and a cAMP signaling cascade that consists of the G



**Fig. 1** The receptor guanylyl cyclase GC-D and its role in olfactory function. **a** Whole-mount X-gal staining of olfactory bulbs from a *Gucy2d-Mapt-lacZ*<sup>+/-</sup> mouse shows axons of GC-D+ OSNs innervating the necklace glomeruli (blue). **b** Recordings of stimulus-evoked field potentials in response to MOE stimulation by uroguanylin (UG) or guanylin (G) in *Cnga2*<sup>-/-</sup>, *Gucy2d*<sup>+/-</sup> and *Cnga3*<sup>-/-</sup> gene-targeted mice. **c–f** Imaging of intracellular Ca<sup>2+</sup> in an identified dendritic knob of a GC-D+ OSN: **c** en face view of the MOE surface, visualized with transmitted light; **d** Ca<sup>2+</sup> signals at rest in canonical (some shown with arrowheads) and GC-D+ (arrow) OSNs; **E** GC-D+ OSN dendritic knob visualized with the fluorescent  $\beta$ -gal substrate resorufin galactoside; **f** merged image. **g** Examples of Ca<sup>2+</sup> responses to UG (1  $\mu$ M), G (1  $\mu$ M) a mixture of both peptides (1  $\mu$ M

each) or dilute urine (1:100) in dendritic knobs from *Gucy2d-Mapt-lacZ*<sup>+/-</sup> or <sup>-/-</sup> mice. Panels A–G reprinted with permission from reference 16. Copyright 2007, National Academy of Sciences. **h** CO<sub>2</sub>-dependent Ca<sup>2+</sup> signals in GC-D+ OSNs are blocked by the carbonic anhydrase inhibitor acetazolamide (AZ). **i** Representative example of l-cis-diltiazem inhibition of CO<sub>2</sub>-dependent Ca<sup>2+</sup> signals in a GC-D+ OSN, implicating cyclic nucleotide-gated channels in the transduction of CO<sub>2</sub>. Panels H and I reprinted with permission from reference 17. **j** Cyclase activity of rat GC-D expressed in COS-7 cells is stimulated by human uroguanylin but not rat guanylin. Reprinted with permission from reference 22. **k** A possible mechanism for the transduction of uroguanylin (or guanylin) and CO<sub>2</sub> by GC-D+ OSNs

protein subunit  $G\alpha_{\text{olf}}$ , type III adenylyl cyclase (ACIII), and the cyclic nucleotide-gated channel subunits CNGA2, CNGA4, and CNGB1b, which form a cAMP-gated cation channel. In contrast, GC-D is expressed in a relatively small subpopulation of MOE neurons, less than 0.1% [1]. These cells lack the signal transduction elements associated with a cAMP-mediated signaling cascade found in canonical OSNs [2, 5, 6]. Instead, they express the cGMP-specific CNG channel subunit CNGA3 [6] (which is also crucial for color vision in cone photoreceptors) and a cGMP-stimulated phosphodiesterase, PDE2 [5]. GC-D-expressing (GC-D+) OSNs exhibit typical OSN bipolar morphology including a cell body, a single dendrite containing sensory cilia at its tip, and a single axon projecting from the soma toward the basal lamina of the sensory epithelium [1, 5]. The axons of GC-D+ OSNs terminate in distinct regions of the main olfactory bulb known as necklace glomeruli [5], where they synapse onto second order neurons. Before we begin to discuss the functional properties of GC-D and GC-D+ OSNs, we will provide a brief historical account of cGMP signaling in vertebrate olfaction.

### cGMP signaling in olfaction: early results

Studies of cGMP signaling in the vertebrate nose began in the late 1980s and early 1990s and focused initially on canonical OSNs. This interest was triggered, in part, by the finding that the cAMP-sensitive CNG channel in these cells is gated by both cAMP, the primary odor-evoked second messenger of canonical OSNs, and by cGMP [7]. In parallel, early biochemical studies using broken cell preparations had demonstrated that odor stimuli are capable of inducing elevated cGMP levels [8]. As compared with the relatively rapid elevations of cAMP, odor-evoked cGMP signals showed rather slow onset kinetics that outlast the cAMP response by several minutes. The search for molecular pathways underlying cGMP production in canonical OSNs centered initially on soluble guanylyl cyclase (sGC) and its gaseous stimulators: nitric oxide (NO) and carbon monoxide (CO) [8, 9]. There are indications that CO and cGMP contribute to long-term adaptation in canonical OSNs. This research has been reviewed elsewhere [10, 11].

Other more recent studies investigated a role for cGMP as a modulator of cAMP signaling in canonical OSNs [12, 13]. In light of new insights into the subsystem organization of the sense of smell [2–4], and as these studies employed broken membrane preparations or primary cultures of OSNs, the molecular identity of cGMP-producing cells in these experiments could not be determined with certainty.

### The guanylyl cyclase GC-D mediates chemosensory transduction in a specialized subpopulation of OSNs

Although several studies supported a role for cGMP in the transduction and/or modulation of olfactory signals (see above), little was known about the cellular or molecular basis of these functions. The cloning of a receptor guanylyl cyclase, GC-D, from rat MOE by David Garber's laboratory [1] provided a key molecular target for further investigation of cGMP-signaling mechanisms in this tissue. Like other receptor guanylyl cyclases (also called particulate or membrane guanylyl cyclases), GC-D is an integral membrane protein with an extracellular receptor domain and an intracellular kinase-homology domain joined by a single transmembrane linker; a guanylyl cyclase domain is found near the carboxy-terminus [1, 14, 15]. Other mammalian receptor guanylyl cyclases are receptors for various natriuretic peptides (e.g., GC-A, GC-B, and GC-C) or are components of a G protein-coupled receptor-dependent transduction mechanism (e.g., the photoreceptor-specific GC-E and GC-F) [15]. However, the functional role of GC-D in the olfactory system was unknown.

In situ hybridization [1] and immunohistochemical [5] studies indicated that GC-D is expressed in a small subset of MOE neurons. GC-D+ OSNs are scattered amongst canonical OSNs, although within the dorsal recesses of the nasal cavity they do appear in clusters [1, 5, 16–18]. This punctate expression pattern, reminiscent of that seen for canonical odorant receptors [19], suggested that GC-D is a marker for a functionally distinct subpopulation of OSNs. Identification of two other molecular markers specifically expressed in GC-D+ OSNs and implicated in cGMP signaling—the cGMP-stimulated phosphodiesterase (PDE) PDE2 [5] and the cGMP-specific cyclic nucleotide-gated (CNG) channel subunit CNGA3 [6]—supported this interpretation. Also, GC-D+ OSNs do not express critical components of the canonical cAMP-mediated odor transduction cascade, including adenylyl cyclase III, PDE4A, or CNGA2 [5, 6], emphasizing that GC-D+ OSNs are functionally distinct from canonical OSNs and that GC-D is not a component of the canonical odor transduction mechanism. Indeed, GC-D+ OSN activity is maintained in gene-targeted mice that lack odor responses in canonical OSNs [16, 20].

Little progress was made in elucidating the olfactory role of these novel OSNs for several years. However, two articles published contemporaneously in 2007 shed new light on the chemosensory function of GC-D+ OSNs [16, 17]. A study from our groups [16] indicated that GC-D+ OSNs are stimulated by the natriuretic peptides uroguanylin and guanylin, as well as components of urine (a rich source of social signals for mice). Furthermore, these chemosensory stimuli are transduced by an excitatory,

cGMP-mediated signaling cascade that requires both GC-D and CNGA3. The other study [17] found that GC-D+ OSNs respond to CO<sub>2</sub>, and that these responses depend on the CO<sub>2</sub>-metabolizing enzyme carbonic anhydrase II (CAII; another specific marker of GC-D+ OSNs). Both studies are discussed below.

### Natriuretic peptide sensitivity of GC-D-expressing OSNs

In order to determine the chemosensory function of GC-D+ OSNs, we combined immunohistochemical, electrophysiological, and Ca<sup>2+</sup>-imaging approaches to analyze chemosensory responses in the MOE of several lines of gene-targeted mice [16] (Fig. 1). We found that two natriuretic peptide hormones that serve as ligands for GC-C, uroguanylin and guanylin, elicited responses in field potential recordings from the MOE of *Cnga2* null mice, which lack odor responses in canonical OSNs. However, neither urodilatin (a GC-A ligand) nor the heat stable enterotoxin STp (a GC-C ligand) elicited any response. A combination of pharmacological and genetic approaches indicated that MOE responses to uroguanylin and guanylin were dependent on a cGMP-mediated signaling cascade that includes both GC-D and CNGA3: peptide responses present in wildtype mice were attenuated by the CNG channel blocker 1-cis-diltiazem but not the adenylyl cyclase inhibitor SQ22536, but were absent in *Cnga3*<sup>-/-</sup> and *Gucy2d* (i.e., *Gucy2d-Mapt-lacZ*)<sup>-/-</sup> mice (Fig. 1).

*Gucy2d-Mapt-lacZ* mice were generated by replacing a portion of the *Gucy2d* gene (MGI:106030), which encodes GC-D, with a reporter construct encoding a tau- $\beta$ -galactosidase ( $\beta$ -gal) fusion protein through gene targeting in embryonic stem cells [16]. The reporter follows an internal ribosomal entry site (IRES), permitting the transcription of a bicistronic message under the control of the *Gucy2d* promoter. Thus, OSNs normally expressing GC-D will express  $\beta$ -gal protein from one allele (and GC-D from the other) in +/- mice and from both alleles in -/- (i.e., GC-D null) mice. Taking advantage of this reporter, we were able to identify and functionally characterize GC-D+ OSNs in situ using fluorescent  $\beta$ -gal substrates and an *en face* MOE preparation [16] (Fig. 1). Loose patch clamp recordings from dendritic knobs of  $\beta$ -gal+ OSNs in *Gucy2d-Mapt-lacZ*<sup>+/-</sup> mice showed increases in action potential firing upon stimulation with uroguanylin, guanylin, dilute urine, or the membrane permeable cGMP analog 8-Br-cGMP. In contrast, while  $\beta$ -gal+ OSNs in *Gucy2d-Mapt-lacZ*<sup>-/-</sup> mice responded robustly to 8-Br-cGMP, they showed no responses to uroguanylin, guanylin, or urine. These experiments showed that in contrast to the mammalian phototransduction cascade, which exhibits a stimulus-dependent

hyperpolarization [21], GC-D+ OSNs transduce stimuli through a novel excitatory cGMP-mediated signaling mechanism.

However, GC-D+ OSNs are not functionally homogeneous. Using confocal microscopy to image Ca<sup>2+</sup> signals in dendritic knobs of  $\beta$ -gal+ OSNs from *Gucy2d-Mapt-lacZ*<sup>+/-</sup> mice, we found evidence for three functionally distinct populations of GC-D+ OSNs: one responsive to only uroguanylin (~25%), one to only guanylin (~25%), and one to both peptides (~50%) [16]. Although the molecular basis of these differences in stimulus tuning is unknown, these results could suggest receptors that differ in splice forms, post-translational modifications, or the presence of different receptor subunits. The identity of the peptide-sensitive chemoreceptor in GC-D+ neurons is yet to be established, but clearly GC-D itself is a leading candidate. Several other mammalian receptor GCs are receptors for natriuretic peptides [15], and rat GC-D is responsive to uroguanylin (although not guanylin) at picomolar concentrations when expressed in heterologous cells [22] (Fig. 1). This exquisite sensitivity is similar to that seen for GC-D+ OSNs, which exhibit an EC<sub>50</sub> of 66 pM for uroguanylin [16].

### CO<sub>2</sub> sensitivity of GC-D-expressing OSNs

A study from Minmin Luo's group reached a very different conclusion: that GC-D+ OSNs act as sensors of near-atmospheric concentrations of CO<sub>2</sub> [17]. They found that single OSNs expressing PDE2 (a marker for GC-D+ OSNs) also expressed the enzyme carbonic anhydrase II (CAII), which can metabolize CO<sub>2</sub> and related compounds. Recognizing that rodents can be trained to avoid CO<sub>2</sub> at concentrations as low as 0.5% [23, 24], these researchers explored the possibility that CAII+ (i.e., GC-D+) neurons mediate this avoidance behavior. In order to do so, they first employed a line of gene-targeted mice in which a fusion protein composed of tau and green fluorescence protein (GFP) is cotranslated with GC-D from a bicistronic message (GCD-ITG mice), thus specifically labeling GC-D+ OSNs but leaving GC-D function intact. Ca<sup>2+</sup>-imaging of GFP+ dendritic knobs in these mice showed concentration-dependent responses to CO<sub>2</sub>. These responses were inhibited by both acetazolamide, a CA inhibitor, and by the CNG channel blocker 1-cis-diltiazem, thereby implicating both types of proteins in CO<sub>2</sub> transduction by these OSNs (Fig. 1). A role for CAII and GC-D+ neurons in CO<sub>2</sub> detection was further supported by electrophysiological recordings from olfactory bulb neurons associated with necklace glomeruli, the central nervous system target of GC-D+ OSN axons, and by behavioral experiments showing that *Car2* null mice (in which the gene encoding

CAII is disrupted) show deficits in behavioral responses to CO<sub>2</sub> as compared to wildtype mice.

How can these two very different models for GC-D+ OSN function be reconciled? The different gene-targeting strategies used to generate the two mouse lines could lead to functional differences, as could the expression level and type of reporter used. For example, the GCD-ITG mice (and similar GCD-ITL mice, which express  $\beta$ -gal instead of GFP) show numerous small glomeruli anterior to the necklace region [17, 18] that we do not observe in the *Gucy2d-Mapt-lacZ* mice [16, 25, 26]. However, a more intriguing explanation is that these cells may act as multimodal chemosensors that are responsive to both uroguanylin/guanylin and to CO<sub>2</sub>. The ability of the CNG channel blocker l-cis-diltiazem to reduce responses to both types of stimuli [16, 17] suggests that the CNGA3 channel is part of a common transduction pathway. However, what about GC-D? We already discussed that the cyclase activity of GC-D is required for OSN sensitivity to uroguanylin and guanylin [16] and that uroguanylin can activate GC-D in vitro [22]. Therefore, GC-D could act as both a receptor and effector in the transduction of peptide stimuli by these cells. As we shall discuss in detail below, GC-D is also a target of intracellular modulators including bicarbonate ion, which is a product of CO<sub>2</sub> metabolism by carbonic anhydrase. Thus, GC-D could serve as the point of convergence between two seemingly disparate sensory signaling cascades. In this case, GC-D+ OSNs could act to integrate multiple chemosignals, perhaps semiochemicals that act as social cues. Intriguingly, GC-D+ OSNs may be part of a coincidence detection system. Individual necklace glomeruli, the olfactory bulb targets of GC-D+ OSNs, are also innervated by GC-D-negative neurons [26]. This heterogeneous sensory innervation, which differs dramatically from the functionally homogeneous innervation of canonical olfactory bulb glomeruli, suggests that necklace glomeruli could integrate more than one type of sensory input (e.g., social signals and general odors).

### Modulation of GC-D activity by intracellular signals

In mammalian photoreceptors, receptor guanylyl cyclase activity is modulated by a class of Ca<sup>2+</sup>-sensitive proteins known as GCAPs (guanylyl cyclase-activating proteins) [15, 27]. Ca<sup>2+</sup>-bound GCAPs inhibit photoreceptor GCs, while Ca<sup>2+</sup>-deficient GCAPs activate these same GCs. Thus, photoreceptor guanylyl cyclase activity is high when intracellular Ca<sup>2+</sup> is low. In contrast, Ca<sup>2+</sup>-binding proteins, including GCAP1, neurocalcin  $\delta$  and hippocalcin, may positively regulate receptor guanylyl cyclase activity in the rodent MOE. A guanylyl cyclase activity present in cultured OSNs and in membrane preparations containing

OSN cilia could be activated by Ca<sup>2+</sup> and GCAP1 [13] or by hippocalcin [28]. Subsequent studies of heterologously expressed GC-D (as well as of membrane preparations isolated from MOE) have implicated GCAP1, the myristolated form of neurocalcin  $\delta$ , and hippocalcin in the Ca<sup>2+</sup>-dependent activation of this particular guanylyl cyclase [29–33]. Both groups concluded that regulation of receptor guanylyl cyclases by Ca<sup>2+</sup>-binding proteins provides an opportunity to amplify or otherwise impact the canonical odor-dependent cAMP signaling cascade [e.g., 13, 22]. However, a role for receptor guanylyl cyclases in odor transduction by canonical OSNs seems unlikely. Of the known mammalian receptor guanylyl cyclases, only GC-D has been identified in MOE neurons [1]. GC-D expression is restricted to a subpopulation of OSNs, as evidenced by extensive in situ hybridization, immunohistochemistry, single-cell molecular profiling, and gene-targeting studies [1, 5, 6, 16–18, 25, 26]. Furthermore, GC-D-expressing OSNs do not express components of the canonical OSN odor transduction cascade [5, 6]. Thus, Ca<sup>2+</sup>-dependent regulation of GC-D activity is not part of the canonical odor transduction mechanism, but may play a positive modulatory role in GC-D+ OSNs (e.g., [31, 32]).

A particularly novel mechanism for intracellular modulation of GC-D activity has recently been described. As discussed earlier, activation of GC-D+ OSNs by CO<sub>2</sub> requires the enzyme CAII [17]. The metabolism of CO<sub>2</sub> by carbonic anhydrases produces protons and bicarbonate ion, but it was not known how this process could lead to activation of GC-D+ neurons. Research from two groups now suggests that bicarbonate ion could activate these cells through the direct stimulation of the cyclase domain of GC-D. Treatment of cultured cells expressing GC-D with either sodium or potassium bicarbonate elicits an increase in cGMP production [34, 35]. This activation appears specific to GC-D: bicarbonate does not activate other receptor guanylyl cyclases tested [34, 35]. Purified GC-D can be directly stimulated by bicarbonate [34, 35]; this stimulation increases GC-D activity by increasing  $V_{max}$  without affecting the  $K_m$  for GTP [35]. Together, these data suggest that CO<sub>2</sub> indirectly activates GC-D, through bicarbonate ion, through its metabolism by CAII. However, it remains to be demonstrated that bicarbonate stimulates GC-D activity in vivo or that CO<sub>2</sub>-dependent activation of GC-D+ OSNs requires this guanylyl cyclase.

### Neurons in the Grueneberg ganglion express cGMP-related signaling elements, including the guanylyl cyclase GC-G

Although we have focused this review on the function of GC-D and GC-D+ OSNs, this particular receptor guanylyl

cyclase may not be the only one important for olfactory function. At least one additional olfactory subsystem in the mouse nose—the Grueneberg ganglion—expresses specific cGMP-signaling elements including the orphan receptor guanylyl cyclase GC-G [36, 37] and cGMP-dependent kinase II [37]. Therefore, sensory cells of the Grueneberg ganglion might employ a cGMP-mediated second messenger cascade for signal detection. Hence, knowledge gleaned from the analysis of GC-D+ OSNs might serve as a model for understanding the function of Grueneberg ganglion cells.

The Grueneberg ganglion [38], which was re-discovered just a few years ago [38–43], is located at the rostral tip of the nasal cavity and consists of sensory neurons that project their axons to a small number of glomeruli in the dorso-medial aspect of the caudal main olfactory bulb. Interestingly, this region overlaps to some degree with the necklace glomeruli innervated by the GC-D+ OSNs. Two recent reports implicate the Grueneberg ganglion cells in the detection of very different types of sensory stimuli [44, 45]. One study reported that neurons of the Grueneberg ganglion detect yet unidentified chemicals that might function as alarm pheromones [44]. A second study reported that Grueneberg ganglion cells respond to cool ambient temperature, indicating a role of the Grueneberg ganglion in thermosensation [45]. As in the GC-D+ OSNs, a possible explanation for these seemingly disparate findings is that neurons of the Grueneberg ganglion function to integrate different types of sensory signals, maybe even different sensory modalities.

Another intriguing parallel between cells of the Grueneberg ganglion and GC-D+ OSNs is the finding that both express a member of the receptor guanylyl cyclase family. However, in case of the Grueneberg ganglion cells, it is not GC-D but the orphan receptor guanylyl cyclase GC-G [36, 37]. GC-G seems to be expressed in a large majority of Grueneberg ganglion cells, which are also characterized by the co-expression of olfactory marker protein (OMP) and PDE2 but lack CAII [36, 37]. Together, these findings imply that a cGMP-mediated signaling cascade operates in the Grueneberg ganglion. It should be possible in the near future to define the role of GC-G in neuronal sensing in the mouse nose.

## Conclusions

The identification of GC-D was a critical step toward deciphering the roles of guanylyl cyclases and cGMP signaling in the mammalian olfactory system. However, introduction of new gene-targeted mice [16, 17] has rapidly advanced the studies of GC-D and the neurons that express it. Mice expressing reporters such as  $\beta$ -gal and GFP under

the control of the *Gucy2d* promoter have allowed investigators to easily identify GC-D+ OSNs in situ, facilitating anatomical and physiological studies of this sparse OSN subpopulation [16–18, 26]. Furthermore, the specific perturbation of GC-D function in vivo [16] has helped to resolve the role of this guanylyl cyclase in the transduction of chemosensory stimuli (similar approaches could be useful for the study of the orphan receptor GC-G in the Grueneberg ganglion). In the case of GC-D, future studies that build on the observation that this receptor guanylyl cyclase may serve to integrate diverse sensory signals should help elucidate the contributions of GC-D+ OSNs to mammalian chemosensation.

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