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Grueneberg Ganglion Neurons Are Activated by a Defined Set of Odorants

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Abstract

Based on a variety of recent findings, the Grueneberg ganglion (GG) in the vestibule of the nasal cavity is considered as an olfactory compartment. However, defined chemical substances that activate GG neurons have not been identified. In this study, the responsiveness of murine GG cells to odorants was examined by monitoring the expression of the activity-dependent gene c-Fos. Testing a number of odorous compounds, cells in the GG were found to respond to dimethylpyrazine (DMP) and a few related substances. These responses were dose-dependent and restricted to early postnatal stages. The DMP-responsive GG cells belonged to the subset of GG neurons that coexpress the signaling elements V2r83, GC-G, and CNGA3. These cells have been previously reported to respond to cool ambient temperatures as well. In fact, cool temperatures enhanced DMP-evoked responses of GG cells. These findings support the concept that the GG of neonatal mice operates as a dual sensory organ that is stimulated by both the odorous compound DMP and cool ambient temperatures.

Key words: c-Fos, chemosensory, dimethylpyrazine, olfaction, thermosensory

Introduction

The Grueneberg ganglion (GG) is a small cluster of neuronal cells localized to the nasal vestibule in several mammalian species (Grüneberg 1973; Tachibana et al. 1990; Fuss et al. 2005; Koos and Fraser 2005; Fleischer, Hass, et al. 2006; Roppolo et al. 2006; Storan and Key 2006). The function of this ganglion is still elusive although recent studies have shown that murine GG neurons are activated by cool ambient temperatures (Mamasuew et al. 2008; Mamasuew et al. 2010; Schmid et al. 2010). However, in several respects, GG neurons resemble olfactory sensory neurons in the main olfactory epithelium (MOE), the vomeronasal organ (VNO), and the septal organ, including the expression of the olfactory marker protein (OMP) and the projection of axonal processes to the olfactory bulb of the brain (Fuss et al. 2005; Koos and Fraser 2005; Fleischer, Hass, et al. 2006; Roppolo et al. 2006; Storan and Key 2006). Based on these findings, along with the expression of chemosensory receptors (Fleischer, Schwarzenbacher, et al. 2006; Fleischer et al. 2007), it has been proposed that GG neurons may function as chemosensory cells. Yet, no defined chemical substances activating GG neurons have been identified, although it has been reported that GG neurons respond to aqueous solutions of compounds of unknown identity and concentration emitted from stressed mice (Brechbühl et al. 2008). The lack of knowledge about chemical compounds eliciting olfactory responses in the GG is a major restraint of more straightforward investigations about the chemosensory features of GG neurons, such as exploration of the molecular transduction pathway(s) that might underlie putative odor-induced responses of the GG. In this regard, the number of odorous or pheromonal molecules which theoretically could activate GG neurons appears to be almost unlimited. Therefore, in the present study, an approach was chosen based on the previous observation that cells in the GG project their axons to a given type of glomeruli in the olfactory bulb designated as "necklace" glomeruli (Fuss et al. 2005; Koos and Fraser 2005; Roppolo et al. 2006; Storan and Key 2006). Besides innervation from the GG, necklace glomeruli also receive axonal input from the so-called GC-D neurons in the MOE (Juilfs et al. 1997; Walz et al. 2007; Leinders-Zufall et al. 2007). Detailed analyses of necklace glomeruli have shown that they are activated in mice exposed to the odorants 2-heptanone and 2,5-dimethylpyrazine (2,5-DMP), respectively (Lin et al. 2004); both substances apparently do not activate GC-D neurons (Leinders-Zufall et al. 2007). Therefore, we hypothesized that this activation of necklace glomeruli could be due to stimulation of GG neurons by these odorants. Thus, as an initial step to explore a putative

chemosensory role of GG neurons and to identify exogenous chemical compounds capable of activating these cells, 2-heptanone and 2,5-DMP were investigated for their potential to evoke olfactory responses in cells of the GG.

Because GG neurons lack any direct access to the nasal lumen (Roppolo et al. 2006; Brechbühl et al. 2008), it is unclear whether they can be reached by odors in vivo. Therefore, potential activation of these cells by odorants was assessed in living animals by monitoring the expression of the immediate early gene c-Fos. Previously, this approach has turned out to be particularly useful to visualize odor-induced responses of OMP-positive neurons in other chemosensory compartments of the nose (Halem et al. 1999; Kimoto and Touhara 2005; Kimoto et al. 2005; Norlin et al. 2005).

Materials and methods

Mice

This study was performed on mice of wild-type strains C57/BL6J or C57/BL6N purchased from Charles River. All experiments comply with the *Principles of animal care*, publication no. 85-23, revised 1985, of the National Institutes of Health and with the current laws of Germany.

Stimulation experiments

Mice were housed under a 12 h light:dark cycle (light on at 7:00 AM). Experiments were carried out between 9:00 AM and 4:00 PM. For exposure, animals were placed in a sealed plastic box containing a filter paper soaked with a small quantity of the relevant odorant or with water in control experiments. For neonatal (postnatal stage P0 to P4) or older (P7 to P14) pups, boxes with a volume of 0.56 L were used; for adults, boxes had a volume of 3.6 L. If not explicitly stated otherwise, 10 µL of the odorous substances were placed on the filter paper for pups and (due to the larger size of the box) 60 µL for adults. For solid substances (2,6-DMP and pyrazine), a small amount was filled in an open Eppendorf cup, which was placed into the plastic box. All odorants were purchased from Sigma-Aldrich at the highest purity available. Unless indicated otherwise, exposure to odorants lasted for 1 h. During odor exposure, the plastic boxes with the animals inside were transferred into an incubator (CERTOMAT BS-1; B. Braun Biotech International) adjusted to a temperature of 30 °C to avoid the previously described c-Fos expression evoked by cool ambient temperatures in neonatal stages (Mamasuew et al. 2008). However, in experiments in which pups were exposed to cool temperatures (Figure 10), plastic boxes with the animals inside were transferred for 10 minutes (min) to an incubator adjusted to 15 °C.

With the exception of short (5, 10, or 30 min) exposures to stimuli (Figures 9 and 10), mice were sacrificed by cervical dislocation and decapitation directly after exposure. In case of short exposures to 2,3-DMP and/or cool temperatures, after exposure and before sacrificing the animals, they were transferred into a plastic box without 2,3-DMP and kept for further 30 min in an incubator adjusted to 30 °C because expression of a detectable amount of messenger RNA encoding c-Fos takes a while after initial stimulus application.

Tissue preparation

Heads of mice were dissected and embedded in Leica OCT Cryocompound "tissue freezing medium" (Leica Microsystems) and quickly frozen on dry ice. Sections (12 µm) through the nose were cut on a CM3000 cryostat (Leica Microsystems) and adhered to Star Frost microslides (Knittel Gläser) for in situ hybridization or to Polysine microslides (Menzel Gläser) for double-staining in situ hybridization.

In situ hybridization

Digoxigenin- and biotin-labeled antisense riboprobes were generated from partial cDNA clones in pGem-T plasmids encoding mouse c-Fos, TAAR6, TAAR7D, GC-G, or OMP using the T7/SP6 RNA transcription system (Roche Diagnostics) as recommended by the manufacturer. With these antisense RNA probes, in situ hybridization experiments were conducted as described recently (Mamasuew et al. 2008; Mamasuew et al. 2010) and as detailed in the Supplementary Material. From each animal investigated, all sections along the rostrocaudal extent of the GG were analyzed. For statistical analyses (Table 1 as well as Figures 7E and 10E), all c-Fos-positive cells on these sections were counted.

Microscopy and photography

Sections were photographed using a Zeiss Axiophot (Carl Zeiss MicroImaging). Fluorescence was examined with a SensiCam CCD camera (PCO) and the Zeiss Axiovision imaging system (Zeiss) with appropriate filter sets.

Results

Identification of odorants activating GG cells

As an initial step to explore whether chemical substances might activate GG neurons, a number of odorants were

Table 1 Number of GG cells responding to given odorants

	2,5-DMP	2,3-DMP	2,3,5-TMP	2,3-Lutidine
Stained GG cells	155 (±16)	334 (±6)	201 (±5)	109 (±16)

After 1-h exposure to different odorous substances (2,5-DMP, 2,3-DMP, 2,3,5-TMP, or 2,3-lutidine), coronal sections through the GG of early postnatal pups were assessed by in situ hybridization with a c-Fos-specific antisense probe. All stained cells on every section along the rostrocaudal extent of the GG were counted; means of values derived from 3 to 4 animals are shown (the standard error of the mean is given). The number of 2,6-DMP-reactive cells was not determinable due to the weakness of the in situ hybridization signals (Figure 3B). In total, the GG of mouse pups comprises about 785 OMP-positive neurons (Fleischer et al. 2007).

tested, including 1-heptanol (an alcohol), citral (an aldehyde), and 4-heptanone (a ketone). To reveal potential responses of GG cells to these odorants, the expression of c-Fos was monitored by in situ hybridization using a c-Fos-specific antisense riboprobe. As a control, to ensure the suitability of these experimental conditions (i.e., concentration of odorants and their accessibility to nasal compartments), c-Fos expression in the MOE was visualized concomitantly. These experiments were carried out with early postnatal mouse pups since expression of chemosensory receptors in the GG is most prominent in perinatal stages (Fleischer et al. 2007). For exposure, pups were placed for 1 h in a sealed plastic box containing a filter paper soaked with 10 μL of the relevant odorant or with water in control experiments. In the absence of 1-heptanol, citral or 4-heptanone, no c-Fos expression was detectable in the GG or in the MOE, respectively (Figure 1A–D). Upon exposure to these odorants, intense c-Fos expression was visible in a subset of MOE cells, indicating activation of sensory cells in the MOE (Figure 1F,H,J). By contrast, no c-Fos expression was detectable in the GG (Figure 1E,G,I). The observation that these odorants did not elicit c-Fos expression in the GG suggests that GG cells may respond only to a limited set of distinct chemical compounds. As the axons of GG neurons terminate in necklace glomeruli, we hypothesized that chemical substances which elicit responses in necklace glomeruli might be candidate ligands for GG neurons. In a previous study, it has been reported that some necklace glomeruli were activated by 2-heptanone or 2,5-DMP (Lin et al. 2004). Therefore, early postnatal pups were exposed for 1 h to 2-heptanone, and the GG was subsequently analyzed for c-Fos expression. Although numerous cells in the MOE were c-Fos-positive (Figure 2B), no c-Fos expression was visible in the GG (Figure 2A), indicating that in contrast to a subpopulation of cells in the MOE, neurons in the GG are not responsive to 2-heptanone. Testing 2,5-DMP, it was found that exposure to this compound elicited c-Fos expression not only in the MOE (Figure 2D) but additionally also in a subset of GG cells (Figure 2C and Table 1). These results provide in fact the first indication for responsiveness of GG neurons to a distinct chemical substance (2,5-DMP).

The chemical structure of 2,5-DMP comprises a pyrazine nucleus with 2 para-oriented tertiary nitrogen atoms. To explore the specificity of 2,5-DMP as a ligand for GG cells, we assessed whether chemically related substances might also evoke GG responses. In this regard, 2 isomers of 2,5-DMP were tested: 2,3-DMP and 2,6-DMP. Following exposure to these compounds, numerous c-Fos-positive cells were visualized in the MOE, demonstrating activation of sensory cells (Supplementary Figure 1A,B). Analyses of the GG revealed that 2,3-DMP evoked intense c-Fos expression in numerous cells of the GG (Figure 3A and Table 1), apparently even more intense than 2,5-DMP. The isomer 2,6-DMP induced only rather weak responses

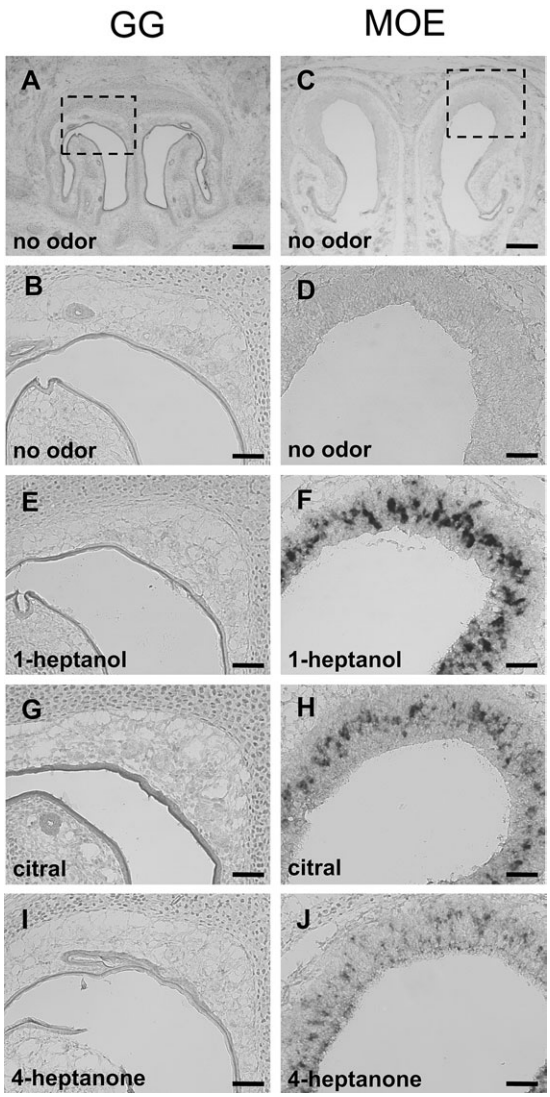

Figure 1 Exposure to 1-heptanol, citral, or 4-heptanone does not induce c-Fos expression in the GG of neonates. **(A–D)** In situ hybridization with an antisense riboprobe specific for c-Fos on coronal sections through the GG and the MOE of a neonatal mouse (stage P1) which was kept in a sealed plastic box for 1 h in the absence of odorants. **(B, D)** Higher magnification of the boxed areas in A and C. No c-Fos signals are detectable in the GG and the MOE. **(E–J)** After a 1-h exposure to 1-heptanol (E–F), citral (G, H) or 4-heptanone (I–J), intense expression of c-Fos was induced in the MOE of pups (stage P0–P2) (F, H, J), whereas no c-Fos expression was detectable in the GG (E, G, I). All data shown are representative of 3–6 independent experiments each. For each of these experiments, a "novel" litter of pups was used. From each of these litters, 1–3 animals were used for each odorant tested. Scale bars: A, C: 200 μm; B, D–J: 50 μm.

in a few GG cells (arrows in Figure 3B). Subsequently, 2,3,5-trimethylpyrazine (2,3,5-TMP) was investigated because it combines structural features of both 2,3-DMP and 2,5-DMP: besides the heterocyclic pyrazine ring, it comprises all methyl side chains of 2,3-DMP and 2,5-DMP. In these experiments, it was found that 2,3,5-TMP also elicited expression of c-Fos in the GG (Figure 3C and Table 1). In

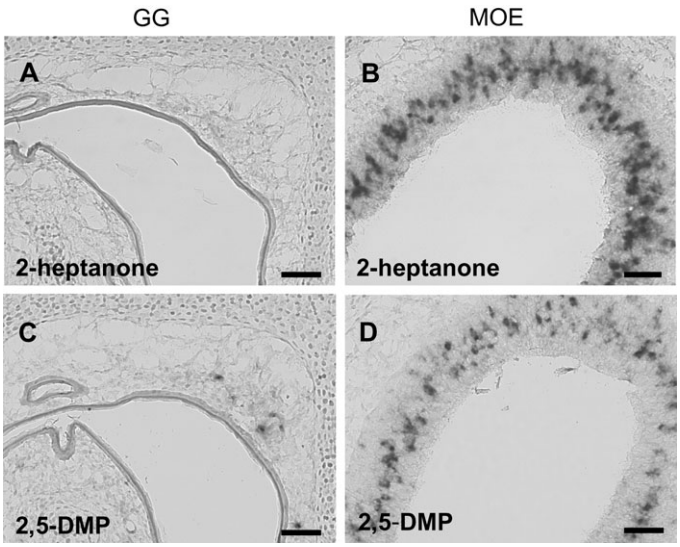

Figure 2 Expression of c-Fos is induced in the GG of pups after exposure to 2,5-DMP but not after exposure to 2-heptanone. **(A, B)** In situ hybridization with a c-Fos-specific antisense probe on coronal sections through the GG (A) and the MOE (B) of a newborn mouse (stage P1) which was exposed to 2-heptanone for 1 h. Strong expression of c-Fos was detectable in the MOE (B) but not in the GG (A). **(C, D)** 1-h exposure of a neonatal pup (stage P3) to the odorant 2,5-DMP elicited expression of c-Fos in the MOE (D); however, 2,5-DMP also induced expression of c-Fos in a subset of cells in the GG (C). All figures depicted are representative of 4–8 independent experiments each. For each of these experiments, a "novel" litter was used. From each of these litters, 1–3 animals were used for each odorant tested. Scale bars: 50 μm.

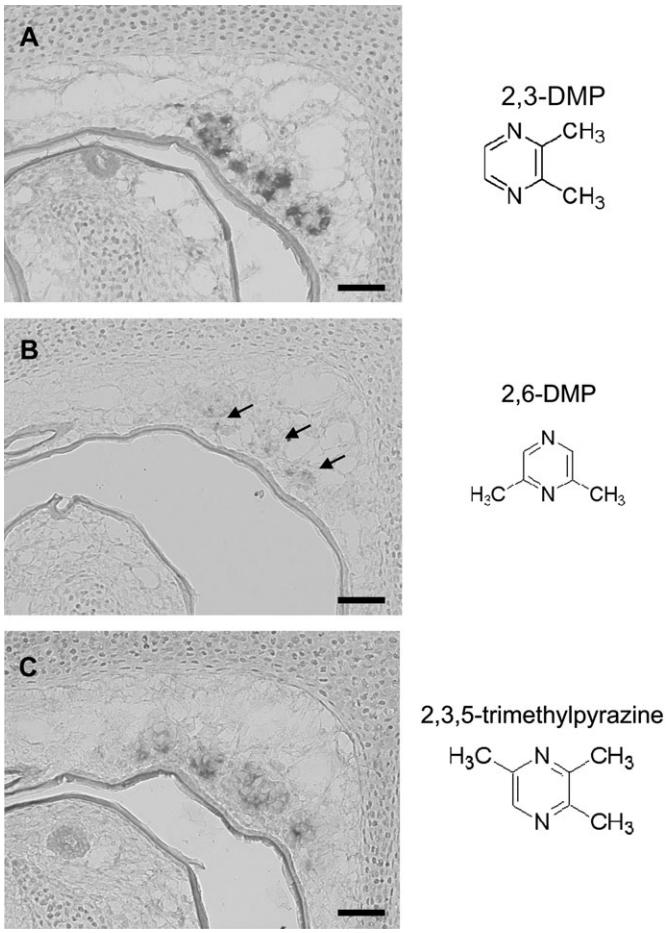

Figure 3 Molecules closely related to 2,5-DMP induce intense c-Fos expression in the GG of neonates. **(A–C)** Expression of c-Fos in the GG of neonates (stage P1–P2) exposed for 1 h to 2,3-DMP (A), 2,6-DMP (B), or 2,3,5-TMP (C) was analyzed by in situ hybridization using a c-Fos-specific antisense probe on coronal sections through the anterior nasal region. 2,3-DMP and 2,3,5-TMP evoked intense c-Fos expression in the GG (A, C). Only weakly labeled cells could be visualized after exposure to 2,6-DMP (arrows in B). All images shown are representative of 6 independent experiments each. For each of these experiments, a "novel" litter was used. From each of these litters, 1–3 animals were used for each odorant tested. Scale bars: 50 μm.

summary, these findings reveal that pyrazine derivates stimulate GG cells. To further explore this issue, attempts were made to determine which part(s) of the DMP molecules may be essential for eliciting GG responses. Toward this goal, substances chemically related to DMP were assessed. As a first candidate, pyrazine was used. Exposure to pyrazine did not induce c-Fos signals in the GG (Figure 4A), whereas c-Fos staining in the MOE was visible (Figure 4B). Thus, the pyrazine nucleus of DMP alone is not sufficient to stimulate GG cells. Testing 2-methylpyrazine, which comprises a pyrazine ring supplemented with a single methyl side chain, revealed that this compound induced c-Fos expression in the MOE (Figure 4D) but not in the GG (Figure 4C). Consequently, a pyrazine ring combined with a single methyl side chain (2-methylpyrazine) does not seem to be sufficient to activate GG cells, suggesting that at least 2 methyl side chains, like in 2,5-DMP and 2,3-DMP, are necessary for eliciting GG responses. To investigate the importance of the side chain length, experiments were performed using 2,3-diethylpyrazine. As shown in Figure 4E, 2,3-diethylpyrazine did not induce visible c-Fos expression in GG neurons, although an intense c-Fos staining was found in the MOE (Figure 4F). Taken together, these findings demonstrate the importance of the number and the length of the alkyl side chains for GG responsiveness.

Next, to assess the role of the nitrogen atoms in the pyrazine ring of DMP for GG responsiveness, exposure to 2,3-lutidine and *o*-xylene was conducted. Compared with DMP, these molecules lack one (2,3-lutidine) or 2 (*o*-xylene) nitrogen atoms, whereas the residual molecular structure is identical to 2,3-DMP. Both substances elicited responses in the MOE (Figure 4H,J), whereas only 2,3-lutidine evoked c-Fos expression in a subset of cells in the GG (Figure 4G,I and Table 1). Thus, it seems that at least one nitrogen atom in the aromatic ring is required to elicit responses of GG cells.

Identification of 2,3-DMP-responsive GG cells

The anterior nasal region harboring the GG comprises several cell types, including GG neurons, glia, and mesenchyme

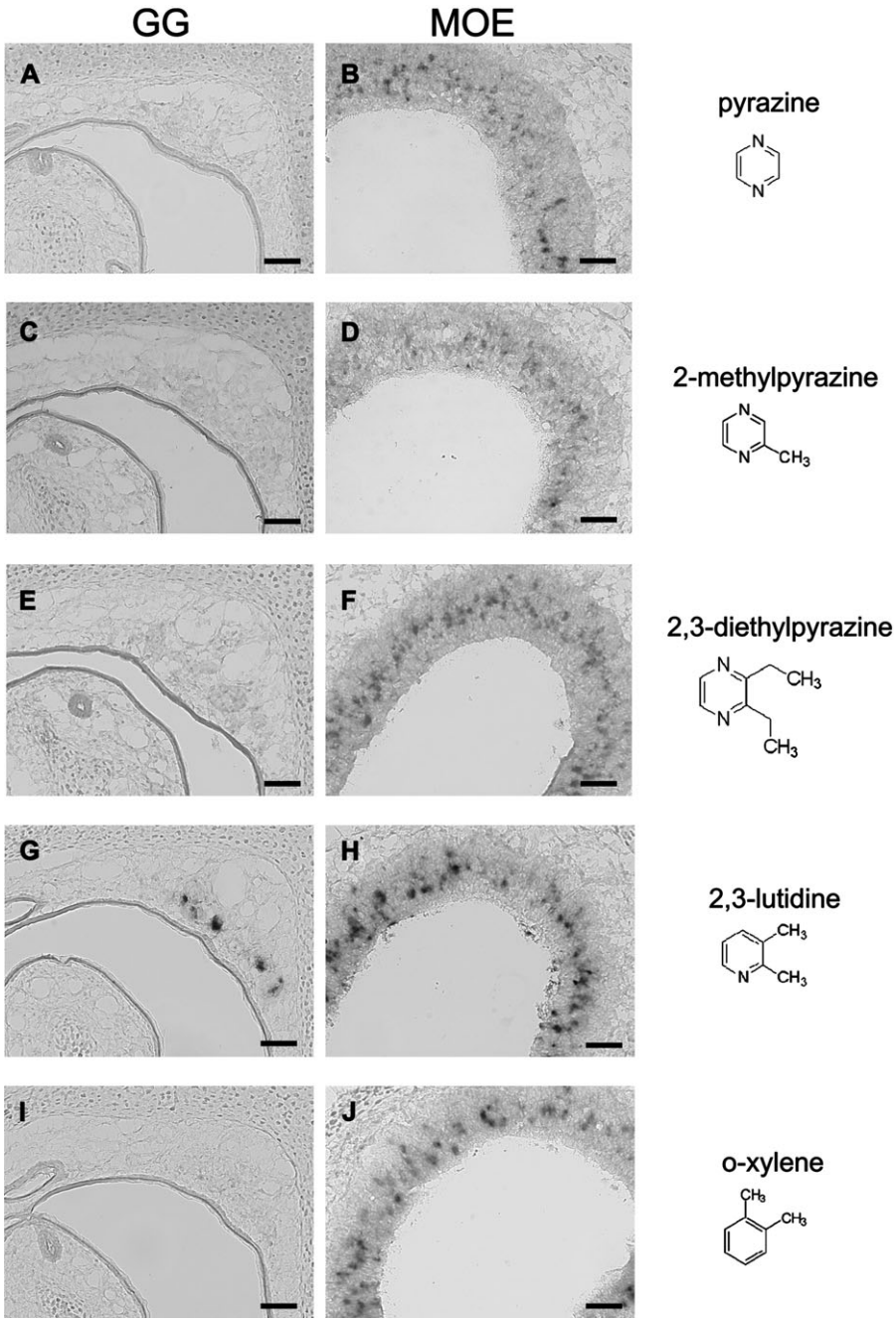

Figure 4 No expression of c-Fos in the GG of pups after exposure to DMP-related substances lacking methyl side chains or the 2 nitrogen atoms of DMP. **(A–J)** Coronal sections through the GG and the MOE of newborn mice (stage P0–P3) which were exposed for 1 h to pyrazine (A, B), 2-methylpyrazine (C, D), 2,3-diethylpyrazine (E, F), 2,3-lutidine (G, H), or *o*-xylene (I–J). In situ hybridization with a c-Fos-specific antisense probe revealed that pyrazine, 2-methylpyrazine, 2,3-diethylpyrazine, and *o*-xylene evoked expression of c-Fos only in the MOE (B, D, F, J) but not in the GG (A, C, E, I). 2,3-lutidine, however, induced c-Fos expression in the MOE and in a subset of GG cells (G, H). All data shown are representative of 4–5 independent experiments each. For each of these experiments, a "novel" litter was used. From each of these litters, 1–3 animals were used for each odorant tested. Scale bars: 50 μm.

cells (reviewed by Fleischer and Breer 2010). To identify the DMP-responsive cell type(s), 2-color in situ hybridization experiments were conducted. These experiments revealed that the 2,3-DMP-induced c-Fos expression occurred in OMP-positive cells of the GG (Figure 5A–C); thus indicating that 2,3-DMP indeed stimulates GG neurons. The GG comprises at least 2 distinct subsets of neurons which are characterized by the expression of different chemosensory receptor types: although a smaller subset of GG neurons is endowed with receptors of the trace amine-associated receptor (TAAR) family, most notably TAAR6 or TAAR7, the overwhelming majority of GG neurons expresses the receptor V2r83

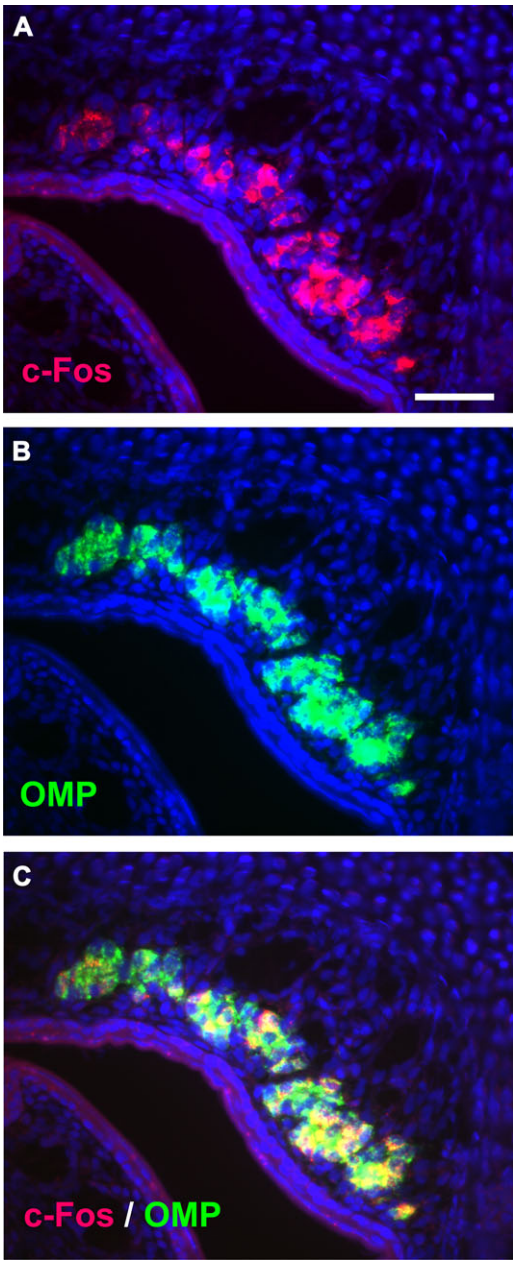

Figure 5 Expression of c-Fos in OMP-positive GG neurons of neonates after exposure to 2,3-DMP. **(A–C)** Double-fluorescence in situ-hybridization on a coronal section through the GG of a pup (stage P2) after exposure to 2,3-DMP for 1 h. The section was incubated with antisense probes for c-Fos (A) and OMP (B). The overlay (C) revealed expression of c-Fos in OMP-positive GG neurons. Counterstaining was conducted with DAPI. Scale bar: 50 μm.

(Fleischer et al. 2007). The V2r83-positive GG cells coexpress the transmembrane guanylyl cyclase GC-G and the cyclic nucleotide-gated ion channel CNGA3 (Fleischer et al. 2009; Mamasuew et al. 2010). To approach the question whether a receptor-specific subpopulation of GG neurons responds to 2,3-DMP, double-staining in situ hybridization experiments were performed. The results indicate that 2,3-DMP did not evoke responses in TAAR-positive GG neurons (Figure 6A–D). By contrast, c-Fos signals elicited by exposure to 2,3-DMP were detectable in numerous V2r83-/GC-G-/CNGA3-positive GG neurons (Figure 6E,F), indicating that this subpopulation of GG cells is responsive to 2,3-DMP.

Dose dependency of GG responses to 2,3-DMP

In order to determine the sensitivity of GG neurons to 2,3-DMP, experiments were performed in which different amounts of 2,3-DMP were spotted onto the filter paper in the plastic box with the pups. It was found that exposure to higher amounts (20 or 10 μL) of 2,3-DMP elicited intense c-Fos expression in the GG (Figure 7A,B), whereas exposure to lower amounts (5 or 2.5 μL) of 2,3-DMP induced a weaker expression of c-Fos (Figure 7C,D). In fact, compared with higher amounts of 2,3-DMP, at lower amounts, the number of c-Fos-positive GG cells was reduced by approximately 50% (Figure 7E), indicating a dose dependence of the odor-evoked responses. Because 2,3-DMP induced c-Fos expression in the GG as well as in the MOE, the sensitivity of these 2 chemosensory compartments was compared. It was found that upon exposure to 1 μL of 2,3-DMP, c-Fos-specific labeling was observed in the GG and in the MOE (Figure 8A,B). Applying only 0.1 μL of 2,3-DMP led to a significantly weaker c-Fos expression in both the GG (arrows in Figure 8C) and the MOE (Figure 8D). After exposure to 0.01 μL of 2,3-DMP, c-Fos staining was hardly detectable in these 2 compartments (Figure 8E,F); however, in some regions of the MOE, a few c-Fos signals were visible (data not shown). Thus, the threshold of 2,3-DMP-induced responses in the GG and in the MOE seems to be in the same concentration range although the GG lacks direct access to the lumen of the nasal cavity.

Time course of the odor-induced signal

In the preceding experiments, pups were exposed to odorants for 1 h. This exposure time was chosen because GG neurons lack direct access to the nasal cavity; accordingly, it may take some time for odorous molecules to reach GG cells. To explore the time dependence of odor-evoked GG responses, c-Fos expression was determined upon exposure to 2,3-DMP for 5, 30, or 60 min. The results depicted in Figure 9 indicate that an odor exposure of 5 min is already sufficient to induce detectable c-Fos signals in GG neurons (Figure 9B). In comparison, an odor exposure of 30 or 60 min elicited stronger signals. In this context, response intensities evoked by stimulation for 30 or 60 min seemed to be similar (Figure 9C,D).

Dual stimulation of GG neurons with odorant and coolness

In addition to DMP, the V2r83-/GC-G-/CNGA3-positive GG neurons in neonatal pups are also activated by cool ambient temperatures (Mamasuew et al. 2008; Mamasuew et al. 2010). Therefore, we hypothesized that temperature-related

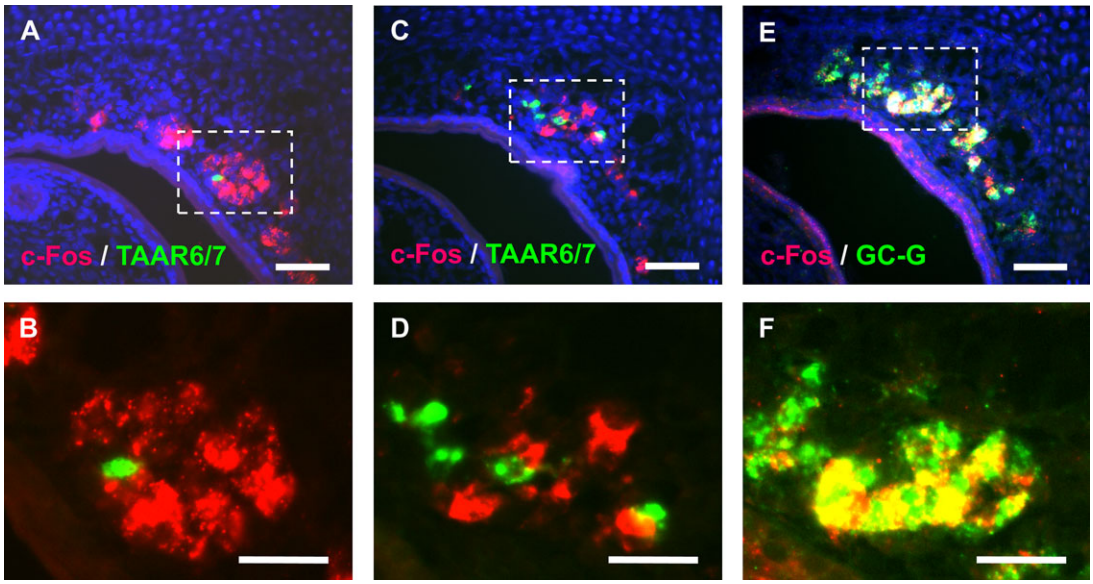

Figure 6 The 2,3-DMP-induced c-Fos expression is restricted to a defined subset of GG neurons in pups. **(A–D)** Coronal sections through the GG of a neonatal mouse (stage P1) exposed to 2,3-DMP for 1 h and hybridized with antisense riboprobes for c-Fos, TAAR6 and TAAR7. High magnification images (B, D) demonstrated that TAAR-positive GG neurons did not co-express c-Fos upon exposure to 2,3-DMP. **(E–F)** Performing the same experiment and incubating the sections with probes for c-Fos and GC-G revealed that c-Fos was expressed by GC-G-positive GG neurons. The images depicted are representative of 3–6 independent experiments each. For each of these experiments, a 'novel' litter was used. From each of these litters, 2–5 animals were used. Sections were counterstained with DAPI. Scale bars: A, C, E 50 μm; B, D, F 25 μm.

and chemical stimuli might be integrated by these cells. To test this concept, pups were exposed to a short (10 min) pulse of cool temperature (15 °C) or odorant (2,3-DMP), respectively. In both cases, these stimulations led to c-Fos signals in the GG. However, when pups received a short and simultaneous pulse of cool temperature and 2,3-DMP, c-Fos signals in the GG were clearly enhanced (Figure 10), suggesting that responses induced by coolness and 2,3-DMP are indeed integrated in GG neurons activated by these 2 stimuli.

Age-dependent responses of GG neurons to 2,3-DMP

Based on the downregulation of chemosensory receptor expression in older stages compared with pups (Fleischer et al. 2007), it was assessed whether the responsiveness of GG neurons to 2,3-DMP may change during postnatal development. To address this issue, mice of different developmental stages (ranging from postnatal day 1 (P1) to adults) were exposed to 2,3-DMP, and odor-induced c-Fos expression in the GG was subsequently determined. These experiments revealed that in comparison with the intense expression of c-Fos in stages P1 and P7 (Figure 11A,B), c-Fos signals were much weaker in the GG of P8 and P10 animals (Figure 11C,D). In GG neurons of P14 and adult mice, c-Fos labeling was undetectable after exposure to 2,3-DMP (Figure 11E,F). These observations indicate that the responsiveness of the GG to this compound in pups is diminished or even absent in older and adult mice. In contrast to the GG, c-Fos signals following exposure to 2,3-DMP were clearly detectable in the MOE of both the neonatal (Figure 3) and the adult (Supplementary Figure 2) stage.

Discussion

Albeit it has been reported that GG neurons respond to aqueous solutions containing unknown chemical substances apparently emitted from stressed mice (Brechbühl et al. 2008), so far, no defined chemical compounds have been identified which activate GG neurons. In the present study, for the first time, GG neurons were found to respond to a defined group of chemicals—including DMP and some related compounds. This observation strongly substantiates the notion that the GG may operate as an olfactory subsystem in the nose.

Unlike DMP, a series of intensely smelling odorants, such as 1-heptanol, citral, 4-heptanone, or 2-heptanone, which elicited strong responses in the MOE, did not activate the GG. This rather selective response of GG neurons is consistent with the finding that only a very limited number of chemosensory receptor types appears to be expressed in the GG (Fleischer, Schwarzenbacher, et al. 2006; Fleischer et al. 2007). Moreover, the GG responses elicited by DMP are restricted to the V2r83-/GC-G-/CNGA3-expressing subset of GG neurons (Figure 6). Because V2r83 is the only chemosensory receptor identified in these cells, it has to be considered that this receptor might be relevant for mediating DMP-induced stimulation of GG neurons. A functional role of V2r83 in the responsiveness of GG neurons to DMP would be consistent with the observation that the decay of V2r83

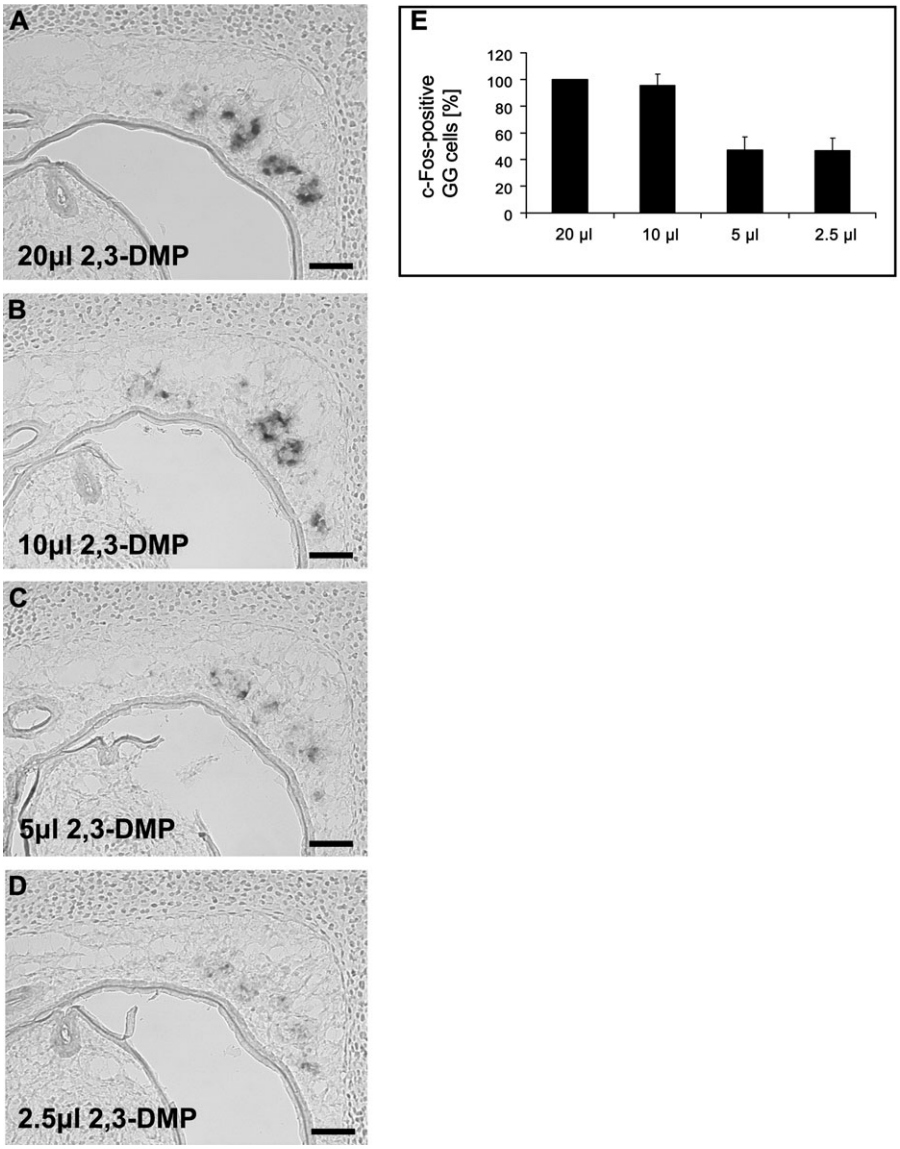

Figure 7 Dose-dependent activation of GG neurons in neonates by 2,3-DMP. **(A–D)** Expression of c-Fos in the GG of pups (stage P0) after exposure to different quantities of 2,3-DMP for 1 h was monitored on coronal sections by in situ hybridization with an antisense probe specific for c-Fos. Exposure to 20 or 10 µL of 2,3-DMP evoked intense c-Fos expression in the GG (A, B), whereas 5 or 2.5 µL of 2,3-DMP induced a weaker expression of c-Fos only (C, D). All images depicted are representative of 4 independent experiments each. For each of these experiments, a "novel" litter was used. From each of these litters, 1–3 animals were used for each DMP quantity tested. Scale bars: 50 µm. **(E)** Quantification of the c-Fos-positive GG cells after exposure to different amounts of 2,3-DMP. All stained cells on every section along the rostrocaudal extent of the GG were counted; means of values derived from 4 experiments are shown. For these experiments, the number of c-Fos-positive cells following exposure to 10, 5, or 2.5 µL of 2,3-DMP is given relative to the number of GG cells responding to 20 µL of 2,3-DMP; the latter was set as 100%. The standard error of the mean is indicated.

expression in older stages (Fleischer et al. 2007) coincides with a diminished responsiveness to DMP (Figure 11). In addition to GG cells, V2r83 is also present in basal neurons of the VNO (Silvotti et al. 2007; Mamasuew et al. 2008). Exposing mice to 2,3-DMP, no c-Fos expression was detectable in the basal layer of the VNO (Supplementary Figure 3). Albeit this finding seems to argue against an involvement of V2r83 in DMP-induced activation of the GG, it has to be taken into account that different splicing variants of V2r83 exist in the GG (Fleischer, Schwarzenbacher, et al. 2006), which could be distinct from the V2r83 isoform expressed in the VNO. Accordingly, potential binding of DMP to V2r83 has to be investigated in future studies, such as analyses of heterologously expressed variants of V2r83 receptor. Moreover, it cannot be ruled out that DMP activates an unidentified chemoreceptor in GG cells.

The chemical compounds stimulating the GG also evoked responses in the MOE (Figures 2–4 and Supplementary Figure 1); thereby, the sensitivity of both systems seems to be similar (Figure 8). It is unclear why DMP and related

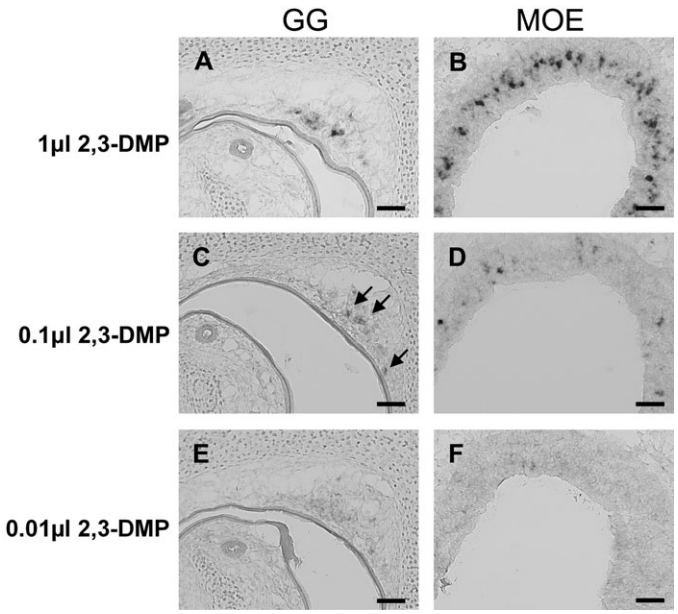

Figure 8 Determination of the detection threshold for 2,3-DMP in the GG and the MOE. **(A–F)** Coronal sections of the GG and the MOE from neonatal mice (stage P1) exposed to different quantities of 2,3-DMP were incubated with an antisense probe for c-Fos. (A, B) 1 µL of 2,3-DMP evoked intense c-Fos expression in the MOE (B) as well as clear signals in the GG (A). (C, D) c-Fos expression was also clearly detectable in the MOE of pups exposed to 0.1 µL 2,3-DMP (D), whereas only weak c-Fos expression was observed in the GG (arrows in C). (E, F) After exposure to 0.01 µL, in the GG (E) as well as in the MOE (F), almost no c-Fos expression was detectable any more. The figures shown are representative of 3 independent experiments. For each of these experiments, a "novel" litter was used. From each of these litters, 1–3 animals were used for each odor quantity tested. Scale bars: 50 µm.

chemical compounds stimulate neurons in both the GG and the MOE. These findings might suggest that the GG serves a redundant function; however, it is important to note that GG neurons project their axonal processes to a special set of glomeruli—the necklace glomeruli—which are distinct from the glomeruli typically innervated by MOE neurons. Therefore, based on the different axonal projection pattern, it can be assumed that activation of DMP-reactive cells in the GG on the one hand and in the MOE on the other hand might evoke distinct physiological or behavioral responses of the animal. Responsiveness to the same odorants has been also described for subsets of olfactory sensory neurons in the septal organ and in the MOE (Ma et al. 2003). Moreover, neurons in the septal organ and in the MOE innervate distinct glomeruli in the olfactory bulb (Levai and Strotmann 2003). Accordingly, sensory cells in different nasal compartments might share a similar response spectrum but differ in their axonal wiring pattern that determines the neuronal processing of olfactory information.

The functional implications of GG responsiveness to DMP are unclear. The finding that DMP-induced responses of GG neurons only occur in early postnatal stages suggests that

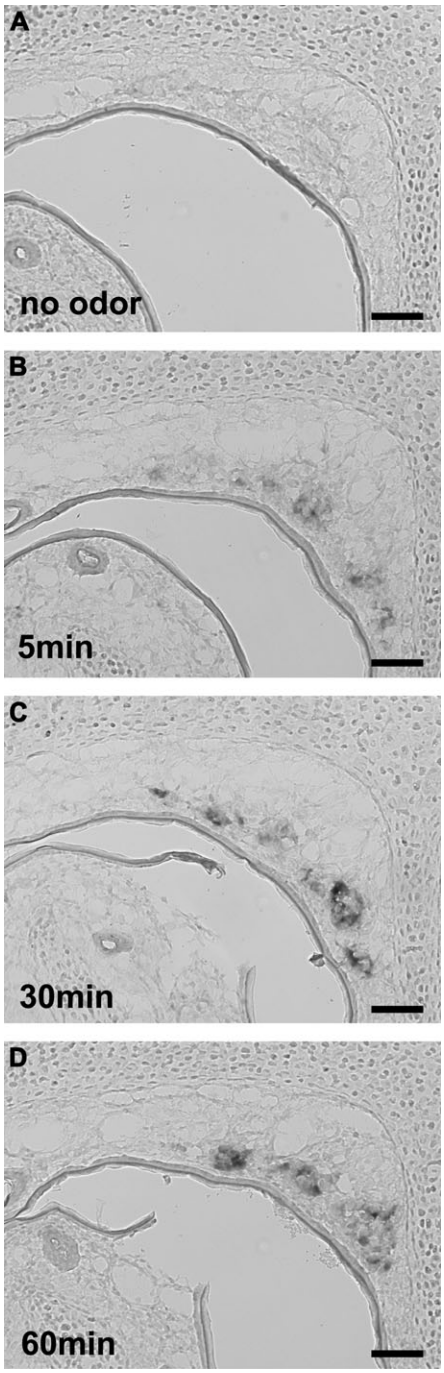

Figure 9 Short exposure to 2,3-DMP is sufficient to elicit c-Fos expression in the GG of neonates. **(A–D)** Following exposure of P2 mice to 2,3-DMP for different time intervals, coronal sections through the GG were hybridized with antisense riboprobes for c-Fos. In contrast to the absence of odorant (A), already a 5-min (B) exposure to 2,3-DMP led to detectable c-Fos expression in the GG. An exposure to 2,3-DMP for 30 min (C) or 60 min (D) elicited intense c-Fos staining in GG neurons. Exposure to 2,3-DMP for 5 or 30 min was followed by a 30-min period without odor exposure because expression of a detectable amount of messenger RNA encoding c-Fos takes a while after initial stimulus application. All images depicted are representative of 4 independent experiments each. For each of these experiments, a "novel" litter was used. From each of these litters, 1–3 animals were used for each exposure time tested. Scale bars: 50 µm.

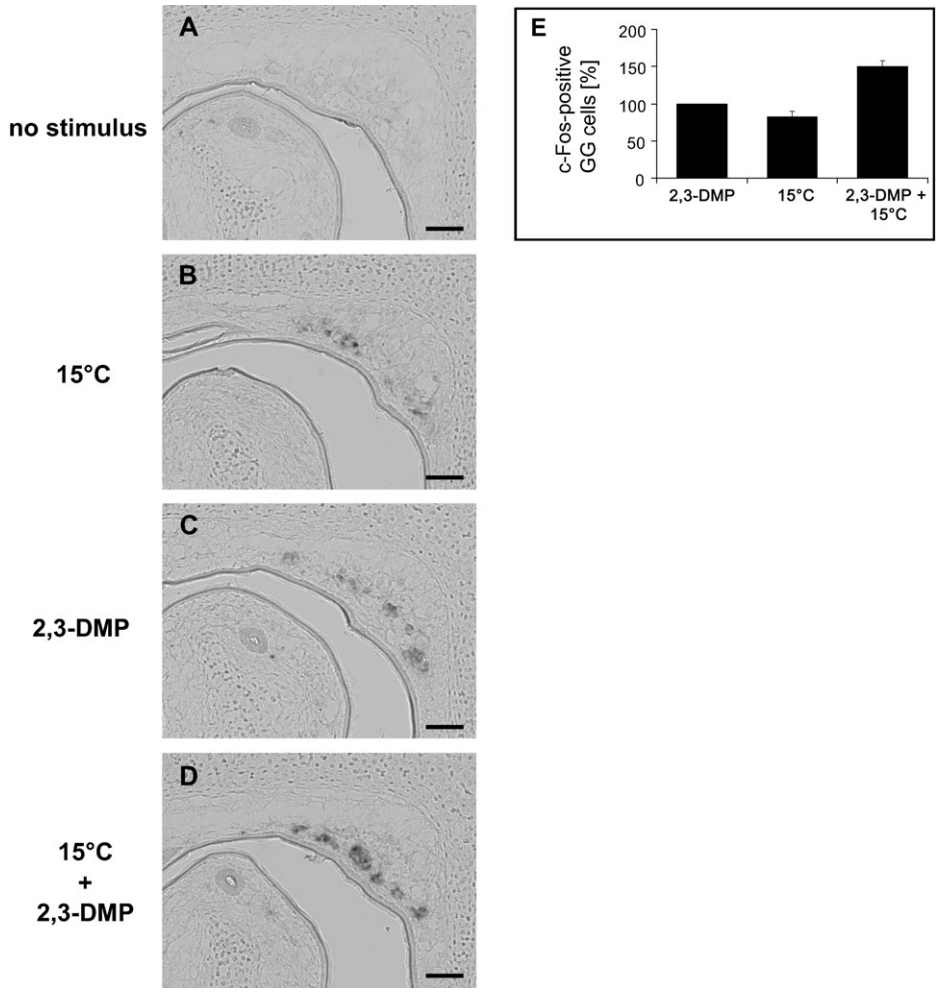

Figure 10 Odor-induced activation of GG neurons is enhanced by cool ambient temperatures. **(A–D)** Exposure of neonatal pups to 2,3-DMP and/or to cool ambient temperatures (15 °C) for 10 min was followed by a 30-min period without stimulus (30 °C, without odorant) to allow generation of a detectable amount of c-Fos mRNA. Subsequently, c-Fos expression in the GG was analyzed on coronal sections through the GG by in situ hybridization with an antisense riboprobe for c-Fos. Compared with controls without stimulus (30 °C, no 2,3-DMP) (A), exposure to a cool ambient temperature of 15 °C (B) or to 2,3-DMP (C) led to clearly detectable c-Fos signals in the GG. When pups were simultaneously exposed to coolness and 2,3-DMP, expression of c-Fos was enhanced (D). The figures shown are representative of 5 independent experiments. For each of these experiments, a "novel" litter was used. From each of these litters, 1–3 animals were used for each experimental condition tested. Scale bars: 50 μm. **(E)** Quantification of the c-Fos-positive GG cells after exposure to 2,3-DMP, coolness (15 °C) or both stimuli. All stained cells on every section along the rostrocaudal extent of the GG were counted; means of values derived from 4 experiments are shown. For these experiments, the number of c-Fos-positive cells following exposure to coolness or coolness plus 2,3-DMP is given relative to the number of GG cells responding to 2,3-DMP; the latter was set as 100%. The standard error of the mean is indicated.

a potential functional relevance associated with GG responsiveness to DMP is restricted to neonates. However, as there is nothing known about any specific effects of DMP in neonatal mice, no immediate implication of GG responses to DMP is deducible. Moreover, the observation that only the GG of neonates responds to DMP does not rule out the possibility that the GG serves a distinct function in adults.

During the early postnatal phase, GG neurons are also activated by cool ambient temperatures (Mamasuew et al. 2008). In this context, it has been found that cool temperatures activate only the V2r83-/GC-G-/CNGA3-positive GG neurons (Mamasuew et al. 2008; Mamasuew et al. 2010). In fact, about 88% of the V2r83-/GC-G-/CNGA3-expressing GG cells respond to coolness (Mamasuew et al. 2010). Given that there are about 650 V2r83-/GC-G-/CNGA3-expressing GG neurons in mouse pups (Fleischer et al. 2007; Mamasuew et al. 2010) and that 2,3-DMP only stimulates this subset of GG cells (Figure 6), exposure to 2,3-DMP elicits responses in about 51% of these cells (Table 1). Accordingly, a considerable number of the V2r83-/GC-G-/CNGA3-expressing GG neurons responds to both coolness and 2,3-DMP. This concept is further substantiated by the observation that an exposure to both stimuli does not only increase the number of responding cells (Figure 10E) but—more importantly—also enhances the signal intensity in these cells (Figure 10D). Consequently,

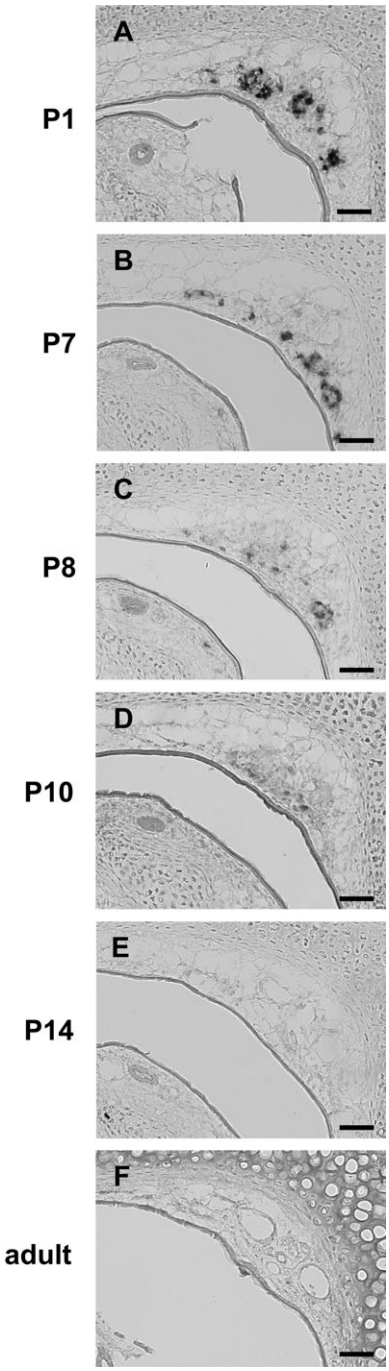

Figure 11 Decreased odor-induced c-Fos expression in the GG of older developmental stages. **(A–F)** Coronal sections through the GG of individuals from different developmental stages (P1, P7, P8, P10, P14, and adult) exposed to 2,3-DMP for 1 h. Sections were hybridized with a c-Fos-specific riboprobe. In stage P7 (B), similar to newborn pups (A), intense c-Fos signals were observed in GG neurons, whereas c-Fos expression was already diminished at P8 (C) and hardly detectable at P10 (D). At stage P14 (E) and in adults (F), no expression of c-Fos in the GG was visible. All figures depicted are representative of 3–4 independent experiments each. For each of these experiments, a "novel" litter of pups was used. From each of these litters, 1–3 animals were used for each developmental stage tested. Scale bars: 50 μm.

these cells can be considered as dual sensory neurons. A similar sensory feature has been previously reported for the so-called AWC neurons in the nematode *Caenorhabditis elegans* which also respond to both temperature and chemical stimuli (Bargmann et al. 1993; Kuhara et al. 2008). Furthermore, dual sensory cells have been already described in the olfactory system of mice: subsets of sensory neurons in the septal organ and in the MOE have been found to respond to chemical and mechanical stimuli. For these cells, it has been proposed that an increased airflow in the nose during sniffing could mechanically sensitize these neurons for a chemical stimulus (Grosmaitre et al. 2007). Accordingly, cool temperatures might enhance the sensitivity of GG neurons for appropriate odorants. This view seems to be supported by the observation that coolness and DMP mutually enhance activation of GG neurons (Figure 10). Hence, coolness may indeed sensitize GG neurons, thus increasing responsiveness to appropriate chemical compounds. Such an increased sensitivity for odorants might be particular relevant at cool ambient temperatures when the volatility of odorous molecules is reduced. Interestingly, in this context, it has been recently reported that cool temperatures also enhance olfactory sensitivity in Drosophila (Riveron et al. 2009).

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/.

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