Original Paper

Neuro-Signals

Neurosignals 2011;19:198–209
DOI: 10.1159/000329333

Received: December 8, 2010
Accepted after revision: May 11, 2011
Published online: August 31, 2011

Chemo- and Thermosensory Responsiveness of Grueneberg Ganglion Neurons Relies on Cyclic Guanosine Monophosphate Signaling Elements

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Key Words
Olfaction · Cyclic nucleotide-gated ion channel A3 · Guanylate cyclase-G · Cyclic guanosine monophosphate · c-Fos · 2,3-Dimethylpyrazine

Abstract
Neurons of the Grueneberg ganglion (GG) in the anterior nasal region of mouse pups respond to cool temperatures and to a small set of odorants. While the thermosensory reactivity appears to be mediated by elements of a cyclic guanosine monophosphate (cGMP) cascade, the molecular mechanisms underlying the odor-induced responses are unclear. Since odor-responsive GG cells are endowed with elements of a cGMP pathway, specifically the transmembrane guanylyl cyclase subtype GC-G and the cyclic nucleotide-gated ion channel CNGA3, the possibility was explored whether these cGMP signaling elements may also be involved in chemosensory GG responses. Experiments with transgenic mice deficient for GC-G or CNGA3 revealed that GG responsiveness to given odorants was significantly diminished in these knockout animals. These findings suggest that a cGMP cascade may be important for both olfactory and thermosensory signaling in the GG. However, in contrast to the thermosensory reactivity, which did not decline over time, the chemosensory response underwent adaptation upon extended stimulation, suggesting that the two transduction processes only partially overlap.

Introduction

In the nose of mammals, olfactory sensory neurons (OSNs) are localized in distinct nasal compartments, including the main olfactory epithelium (MOE), the vomeronasal organ and septal organ [for review see 1–3]. In addition, neurons of the Grueneberg ganglion (GG) in the anterior nasal region also share characteristic features of OSNs; therefore, it was proposed that they may serve as chemosensors as well [4–8; for review see 9]. In fact, most recent studies suggest that these cells – in addition to activation by cool ambient temperatures [10, 11] – are indeed activated by chemical compounds [12, 13]. However, it is currently unclear which transduction pathways enable GG neurons to respond to odorants since little is known about the signaling elements contributing to odor-induced reactivity in these cells. In this

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regard, a few olfactory receptor types, namely receptor V2r83 and some members of the trace amine-associated receptor family, have been found to be expressed in GG neurons [14, 15]. Moreover, most GG neurons express distinct signaling elements associated with the second messenger substance cyclic guanosine monophosphate (cGMP), including the transmembrane guanylyl cyclase subtype GC-G, the cGMP-dependent phosphodiesterase PDE2A and the cGMP-activated cyclic nucleotide-gated (CNG) ion channel CNGA3 [16–18]. GG neurons expressing such cGMP-associated transduction components were found to be activated by the odorant 2,3-dimethylpyrazine (2,3-DMP) [13]. Thus, cGMP signaling may contribute to the responsiveness of GG cells to odorants. To scrutinize this hypothesis, in the present study, the responsiveness of GG neurons to 2,3-DMP was investigated in transgenic mice deficient for CNGA3 or GC-G. In these approaches, odor-evoked activation of GG cells was monitored by visualizing the expression of c-Fos, an activity-dependent immediate early gene, which has previously been used as a reliable marker for odorant-induced responses in chemosensory neurons [13, 19–22].

Animals and Methods

Mice

This study was performed on mice of wild-type strains C57BL/6J or C57BL/6N purchased from Charles River (Sulzfeld, Germany). The generation of the CNGA3-deficient (CNGA3–/–) and the GC-G-deficient (GC-G–/–) mouse strain has been described previously [23, 24]. 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-hour light/dark cycle (light on at 7:00 a.m.). Experiments were carried out between 9:00 a.m. and 4:00 p.m. For exposure, individual pups were placed in a sealed plastic box (with a volume of 0.56 liter) containing a filter paper soaked with a small quantity (10 μl) of the relevant odorant (2,3-DMP or 2,3-lutidine) or with water in control experiments. The odorants were purchased from Sigma-Aldrich (St. Louis, Mo., USA) 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, Melsungen, Germany) adjusted to a temperature of 30°C to avoid the previously described c-Fos expression evoked by cool ambient temperatures in neonatal stages [10]. However, in experiments in which pups were exposed to coolness (fig. 5–7), plastic boxes with the animals inside were transferred to an incubator adjusted to the desired cool temperature (22 or 26°C) for the indicated period of time.

Mice were sacrificed by cervical dislocation and decapitation directly after exposure.

Tissue Preparation

Heads of mice were dissected and embedded in Leica OCT Cryocompound 'tissue freezing medium' (Leica Microsystems, Bensheim, Germany) and quickly frozen on dry ice. Sections (12 μm) through the nose were cut on a CM3050S cryostat (Leica Microsystems) and adhered to Star Frost microslides (Knittel Gläser, Braunschweig, Germany) for in situ hybridization. For immunohistochemistry, heads of mice were prepared as described above, fixed in 4% paraformaldehyde in 150 mM phosphate buffer (pH 7.4) for 5 h at 4°C followed by cryoprotection in 25% sucrose [in 1×PBS (0.85% NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4)] at 4°C overnight. Sections (10–15 μm) were cut on a CM3050S cryostat (Leica Microsystems) and adhered to Superfrost Plus microscope slides (Menzel Gläser, Braunschweig, Germany).

In situ Hybridization

Digoxigenin-labeled antisense riboprobes were generated from partial cDNA clones in pGem-T plasmids encoding mouse c-Fos, olfactory marker protein (OMP), V2r83 or CNGA3 [18] using the T7/SP6 RNA transcription system (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer. With these antisense RNA probes, in situ hybridization experiments were conducted as described recently [10, 13, 18].

Statistical Analyses

From each animal investigated, all sections along the rostrocaudal extent of the GG were analyzed. For statistical analyses (fig. 1, 2, 4–7; as well as supplemental fig. 1 and 4; for all online supplementary material, see www.karger.com/doi/10.1159/000329333), all c-Fos-positive cells on these sections were counted. In figures with bar charts, the standard deviation is indicated. p values were determined by two-tailed paired t tests (confidence interval 0.95) using GraphPad Prism 5.0 software (***$p < 0.001$; **$0.001 < p < 0.01$; *$0.01 < p < 0.05$; $p > 0.05$ = not significant).

Immunohistochemistry

To visualize the localization of GC-G, tissue sections were incubated overnight at 4°C with a specific polyclonal antibody (PGCG-701AP; FabGennix Inc., Frisco, Tex., USA) generated in rabbits. The antibody was used at a dilution of 1:500 in 1×PBS containing 0.3% Triton X-100. Secondary detection was carried out using appropriate secondary antibodies coupled to Alexa dyes (Invitrogen, Carlsbad, Calif., USA). Counterstaining was performed for 3 min with propidium iodide (1 μg/ml in 1×PBS). Finally, sections were rinsed with H_2O and subsequently mounted in 66% glycerol/1×PBS.

Microscopy and Photography

Sections were photographed using a Zeiss Axiophot (Carl Zeiss MicroImaging, Göttingen, Germany). For confocal microscopy of immunohistochemical staining experiments, a Zeiss LSM 510 META system was used.

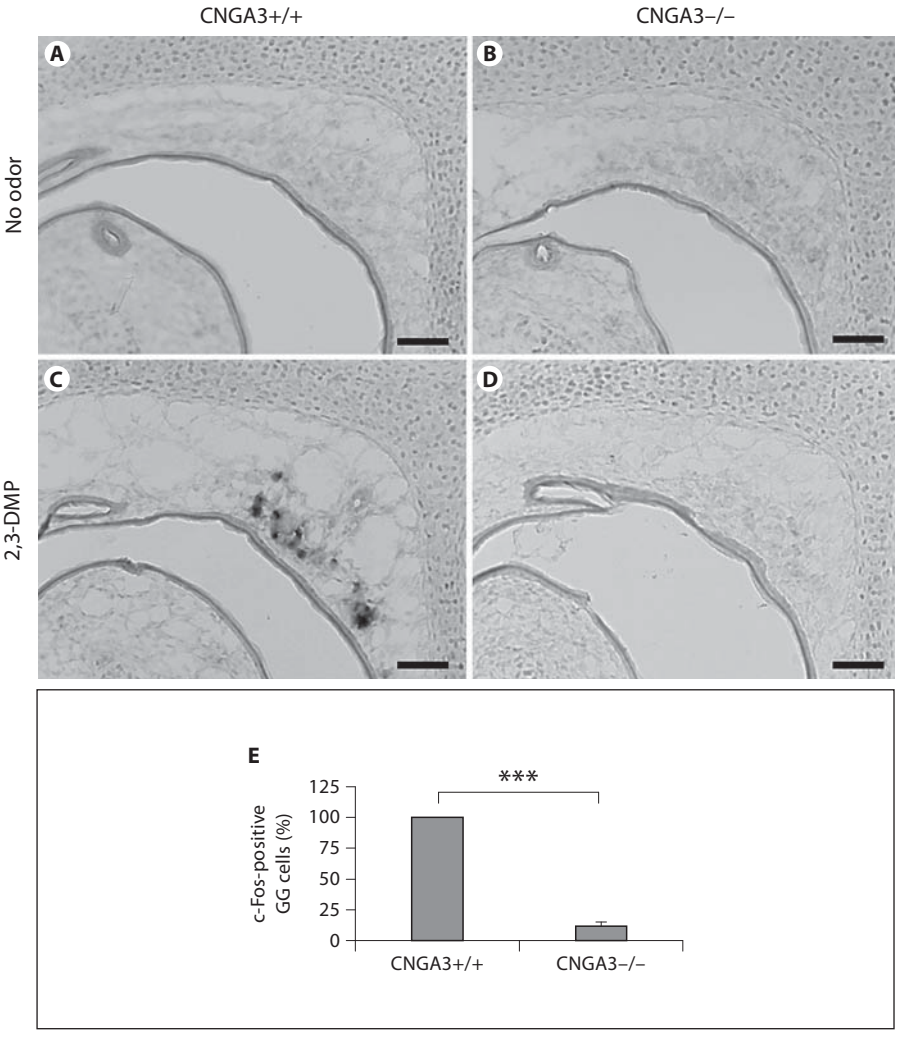

Fig. 1. Attenuation of 2,3-DMP-evoked responses in the GG of CNGA3-deficient mice. **A**, **B** In situ hybridization experiments with an antisense probe for c-Fos on coronal sections through the GG of wild-type (**A**) or CNGA3-deficient (**B**) neonatal pups which were kept in sealed plastic boxes in the absence of 2,3-DMP. In these animals, no c-Fos signals in the GG were detectable. **C** Exposing wild-type pups to 2,3-DMP for 1 h, intense signals were observed in the GG. **D** In the GG of CNGA3-deficient pups, however, responsiveness to 2,3-DMP was significantly lower. The data shown are representative of 5 independent experiments. For each of these 5 independent experiments, a 'novel' litter was used. Scale bars: 50 μm. **E** Quantification of the c-Fos-positive GG cells in wild-type and CNGA3-deficient pups upon exposure to 2,3-DMP for 1 h. All stained cells on every section along the rostrocaudal extent of the GG were counted; the results shown are based on 5 experiments. In each of these experiments, the number of c-Fos-positive cells in the GG of a CNGA3–/– mouse was determined relative to that in a concomitantly processed CNGA3+/+ pup; the latter was set as 100%. A mean of values, the standard deviation and the p value were calculated ($p < 0.0001$).

Results

CNGA3 and GC-G Contribute to Odor-Induced GG Responses

As a first step to evaluate the potential relevance of cGMP signaling for the odor-induced responsiveness of GG neurons, activation of these cells by 2,3-DMP was analyzed in wild-type (CNGA3+/+) and in CNGA3-deficient (CNGA3–/–) mice. It has been demonstrated previously that GG neurons in CNGA3–/– animals indeed lack expression of CNGA3; nevertheless, they are still endowed with other characteristic molecules of GG neurons, including the OMP, the olfactory receptor V2r83 and the guanylyl cyclase GC-G [18]. To monitor responses of GG cells elicited by 2,3-DMP, c-Fos expression was visualized by in situ hybridization using a c-Fos-specific antisense riboprobe. These experiments were carried out with early postnatal pups since activation of GG neurons by 2,3-DMP is particularly prominent at this age [13]. Without exposure to 2,3-DMP, no significant c-Fos signals were visible either in wild-type (fig. 1A) or in CNGA3–/– pups (fig. 1B). Following exposure of pups to 2,3-DMP for 1 h, a marked c-Fos expression was detectable in a considerable number of GG neurons in wild-type individuals (fig. 1C), whereas in CNGA3–/– animals, expression of c-Fos was very weak or even absent (fig. 1D). In fact, in CNGA3-deficient pups, after an exposure to 2,3-DMP for 1 h, the number of c-Fos-positive GG cells was reduced by more than 88% compared to wild-type conspecifics (fig. 1E). In CNGA3-heterozygous animals (CNGA3+/–), c-Fos expression was clearly visible albeit the hybridization signals seemed to be some-

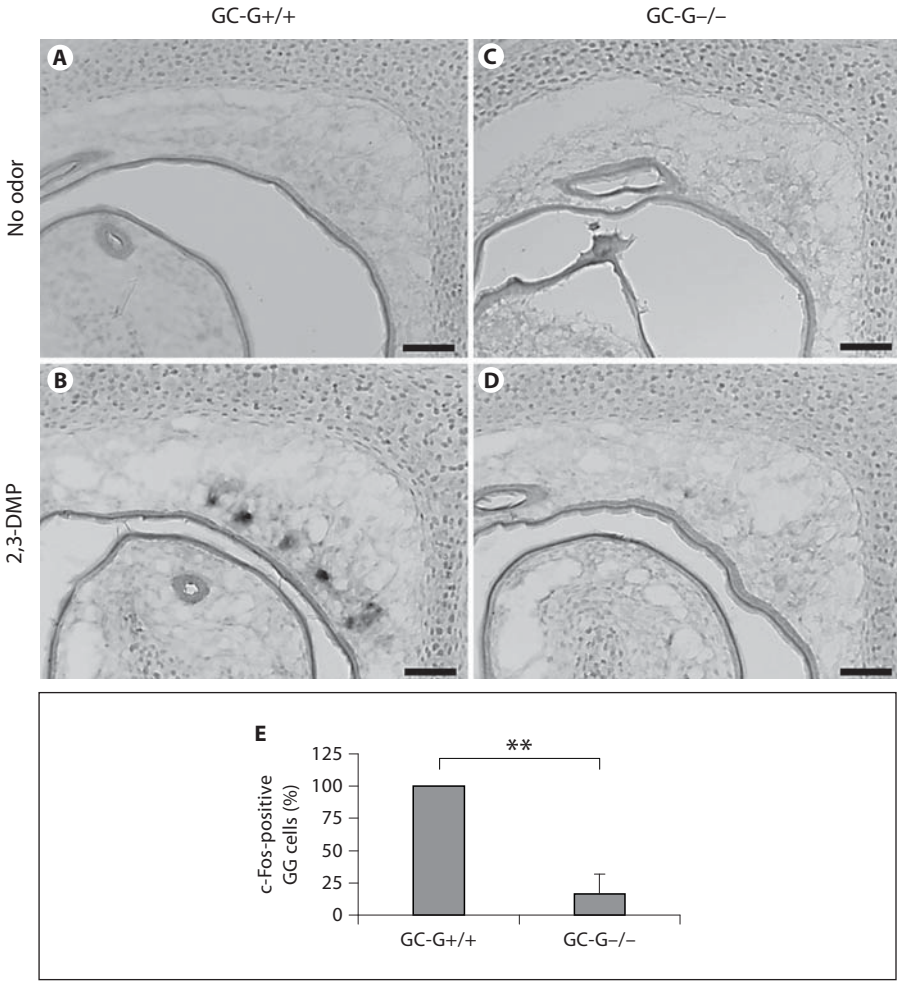

Fig. 2. Decreased responsiveness to 2,3-DMP in the GG of GC-G-deficient mice. **A–D** Expression of c-Fos in the GG of early postnatal pups was analyzed by in situ hybridization using a c-Fos-specific antisense probe. **A**, **C** In the GG of wild-type (**A**) and GC-G-deficient (**C**) pups kept in sealed plastic boxes without 2,3-DMP for 1 h, c-Fos expression was undetectable. **B** Following exposure to 2,3-DMP for 1 h, c-Fos signals were clearly observable in the GG of wild-type mice. **D** Compared to wild-type conspecifics, 2,3-DMP-evoked c-Fos expression was strongly reduced or even absent in the GG of GC-G-deficient pups. All figures depicted are representative of 6 independent experiments. For each of these experiments, a 'novel' litter was used. Scale bars: 50 μm. **E** Counting the c-Fos-expressing GG cells after exposure to 2,3-DMP for 1 h revealed that the number of these cells is strikingly lower in GC-G-deficient than in wild-type pups. The results are derived from 4 experiments. In each experiment, the number of c-Fos-positive GG cells in a GC-G–/– pup was determined relative to that in a concomitantly processed GC-G+/+ conspecific, which was set as 100%. A mean of values, the standard deviation and the p value were calculated ($p < 0.0024$).

what weaker than in wild-type conspecifics (online suppl. fig. 1). Moreover, in comparison to wild-type pups, the number of c-Fos-positive GG neurons was decreased by about 25% (online suppl. fig. 1E).

To further explore the relevance of cGMP signaling elements for odor-induced GG responses, pups deficient for the transmembrane guanylyl cyclase GC-G were investigated. In this transgenic GC-G–/– mouse line, an exon coding for a portion of the N-terminal extracellular region of GC-G has been deleted [24]. Importantly, the catalytic domain of GC-G – similar to other transmembrane guanylyl cyclases – is located at the C-terminal region [25, 26]. Therefore, it would be conceivable that a truncated GC-G protein, which might still comprise guanylyl cyclase activity, is expressed in the GG of GC-G–/– animals. Immunohistochemical experiments using a specific antibody directed against the C-terminal region of GC-G revealed, however, that the catalytic domain of GC-G is absent from the GG of GC-G–/– mice (online suppl. fig. 2). Furthermore, in view of a possible reactivity of GG neurons in GC-G–/– pups to the odorant 2,3-DMP, it was verified that characteristic elements of 2,3-DMP-responsive GG cells (OMP, the receptor V2r83 and the ion channel CNGA3) are still expressed in the GG of GC-G–/– mice (online suppl. fig. 3). To assess the responsiveness of GG neurons in GC-G–/– mice to chemical stimuli, neonatal pups were exposed to 2,3-DMP for 1 h. As documented in figure 2, intense c-Fos expression was observed in the GG of wild-type animals, whereas in GC-G–/– mice, c-Fos expression was very weak or absent: in comparison to wild-type conspecifics, the number of c-Fos-expressing GG cells was decreased by approximately 85% in GC-G–/– pups (fig. 2E), supporting the concept that GC-G contributes to odor-evoked responses in the GG. Consistent with this notion, in heterozygous (GC-G+/–) pups, c-Fos expression in the GG was clearly

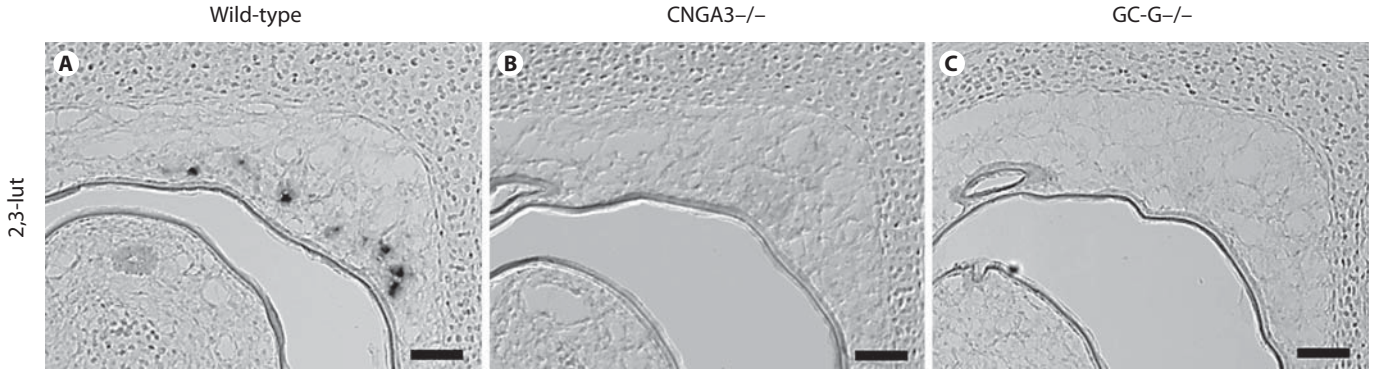

Fig. 3. Expression of c-Fos in the GG of wild-type, CNGA3- or GC-G-deficient animals upon stimulation with the odorant 2,3-lutidine. Visualization of c-Fos expression by in situ hybridization with a c-Fos-specific probe on coronal sections through the GG of early postnatal pups. Upon exposure to 2,3-lutidine (2,3-lut) for 1 h, c-Fos expression was clearly detectable in wild-type individuals (**A**). In comparison, c-Fos expression was weak or absent in the GG of animals deficient for CNGA3 (**B**) or GC-G (**C**). The figures shown are representative of 5 independent experiments. For each of these experiments, a 'novel' litter was used. Scale bars: 50 μm.

detectable, although the signals were somewhat weaker than in wild-type animals (online suppl. fig. 4). Furthermore, the number of c-Fos-positive GG cells was reduced by about 50% in GC-G+/– pups as compared with wild-type animals (online suppl. fig. 4E).

2,3-DMP is not the only odorant capable of activating GG cells [13]. Therefore, it was assessed whether GG responsiveness to other odorants – which could be detected by a subpopulation of GG neurons distinct from the 2,3-DMP-reactive cells – might also rely on cGMP signaling. For this purpose, the odorant 2,3-lutidine was employed since this compound – similar to 2,3-DMP – strongly activates cells in the GG; however, the number of 2,3-lutidine-responsive GG cells is smaller than that activated by 2,3-DMP [13]. Exposure to 2,3-lutidine for 1 h resulted in a strong expression of c-Fos in a number of GG neurons in wild-type pups. In comparison, in the GG of CNGA3–/– and GC-G–/– pups, c-Fos signals were hardly detectable (fig. 3). In summary, these findings indicate that deficiency of CNGA3 or GC-G suppresses responsiveness of GG neurons to odorants such as 2,3-DMP and 2,3-lutidine, supporting the notion that signaling elements of a cGMP-mediated pathway contribute to chemosensory transduction processes in these cells.

Odor-Evoked but Not Coolness-Induced GG Responses Undergo Adaptation

Although GG responses induced by the odorants 2,3-DMP or 2,3-lutidine were weak or absent in pups deficient for CNGA3 or GC-G, it cannot be ruled out that intense odor-evoked signals might still occur in the GG of these animals, though with a certain delay. To test the latter notion, in subsequent experiments, pups were exposed to 2,3-DMP for 2 h. These approaches revealed that even after such an elongated exposure time, c-Fos signals in the GG of animals deficient for CNGA3 or GC-G were either weak or absent (data not shown); more interestingly, following a prolonged exposure, in wild-type pups, responsiveness to 2,3-DMP seemed to be reduced as well. Therefore, to evaluate the time course of odor-induced GG responses more systematically, c-Fos expression in the GG of wild-type pups was analyzed following exposure to 2,3-DMP for 1, 2 or 3 h. It was found that the c-Fos signals were strongest after 1-hour exposure but could hardly be detected after 3-hour exposure (fig. 4). Accordingly, the number of c-Fos-expressing GG cells was approximately 84% lower in pups exposed to 2,3-DMP for 3 h than in those exposed to this substance for only 1 h (fig. 4D). Thus, these experiments indicate that GG neurons seem to undergo adaptation following an extended exposure to 2,3-DMP.

Because coolness-evoked responses of GG neurons – similar to odorant-induced activation – also appear to rely on cGMP signaling [18], we next assessed whether an extended exposure to cool temperatures would also result in diminished c-Fos signals. Therefore, wild-type pups were exposed to a cool ambient temperature (22°C) for 1, 2 or 3 h and the GG was subsequently analyzed for c-Fos expression. These approaches revealed that the intensity and the number of c-Fos signals increased with longer

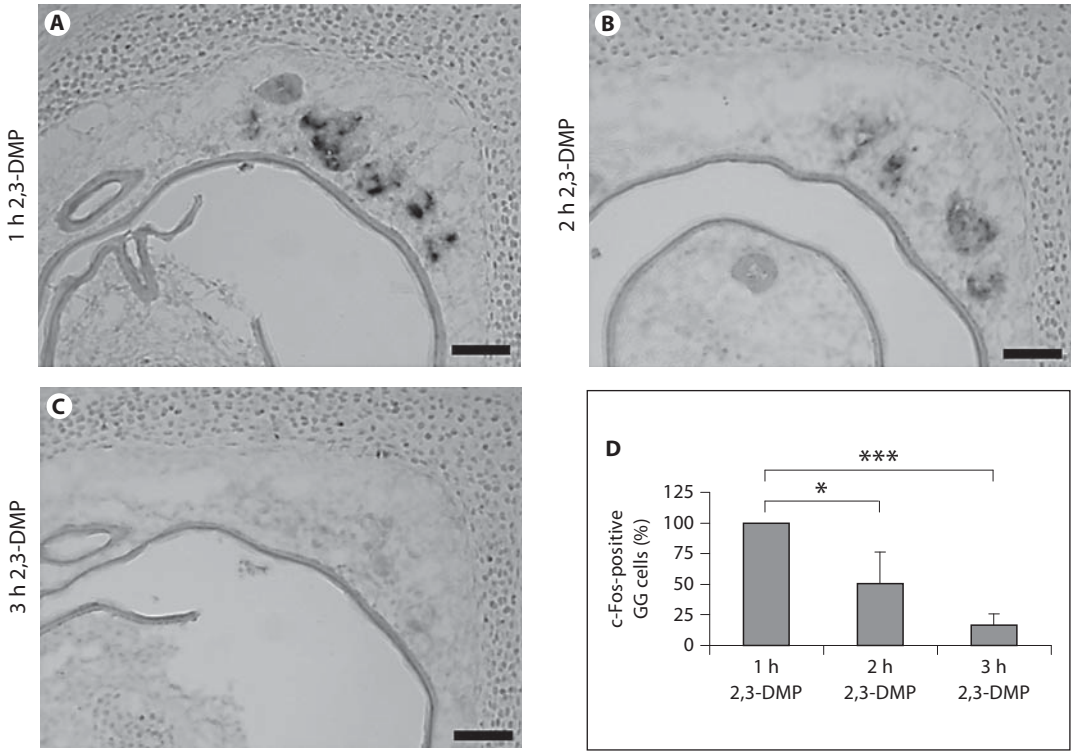

Fig. 4. Long-term exposure to the odorant 2,3-DMP attenuates odor-induced c-Fos expression in the GG. **A–C** Following exposure to 2,3-DMP for 1 (**A**), 2 (**B**) or 3 h (**C**), expression of c-Fos in the GG of early postnatal pups was monitored by in situ hybridization. In the GG, c-Fos expression was highest after a 1-hour exposure (**A**) and decreased in case of longer exposure times (**B**, **C**). The images shown are representative of 5 independent experiments. For each of these experiments, a 'novel' litter was used. Scale bars: 50 μm. **D** Quantification of the c-Fos-positive GG cells after exposure to 2,3-DMP for the above-mentioned time intervals. The results are based on 4 experiments. In each experiment, the number of c-Fos-positive GG cells after exposure to 2,3-DMP for 2 or 3 h was determined relative to that in a concomitantly processed pup, which was exposed to 2,3-DMP for 1 h only; the latter number was set as 100%. Means of values, the standard deviations and p values were calculated (* $p = 0.0455$ for 1 vs. 2 h and *** $p = 0.0006$ for 1 vs. 3 h).

exposure time to cool temperatures (fig. 5). Consequently, although odor- and coolness-induced responses of GG neurons seem to be mediated by cGMP signaling, the odor-evoked activation diminishes over time, whereas the coolness-induced response does not. In this regard, since 2,3-DMP-reactive GG neurons also respond to coolness [13], we investigated whether odor-adapted GG neurons are still activated by coolness. For this purpose, coolness-induced GG responses were assessed in wild-type animals which had been adapted to 2,3-DMP as well as in littermates which had not been exposed to this odorant. In these approaches, it was found that in the GG of odor-adapted pups, similar to the GG of non-adapted littermates, following exposure to coolness c-Fos signals were clearly visible (fig. 6). Moreover, the number of c-Fos-expressing GG cells was even somewhat higher in the odor-adapted pups (fig. 6D). Thus, these experiments demonstrate that odor-induced adaptation of GG neurons does not inhibit coolness-evoked responses in these cells.

Adaptation of Odor-Evoked GG Responses at Cool Temperatures?

Recently, it has been described that cool temperatures enhance GG responses evoked by short-term exposure to odorants [13]; yet, it is unclear whether coolness might also affect GG adaptation to odors which occurs after long-term exposure to odorants (fig. 4). To address this issue, pups were exposed to 2,3-DMP for 1, 2 or 3 h at a cool ambient temperature. For these experiments, a moderate cool temperature (26°C), which itself induces only weak c-Fos expression (fig. 7A), was chosen because signals induced by colder temperatures might strongly superimpose odorant-evoked responses. It turned out that

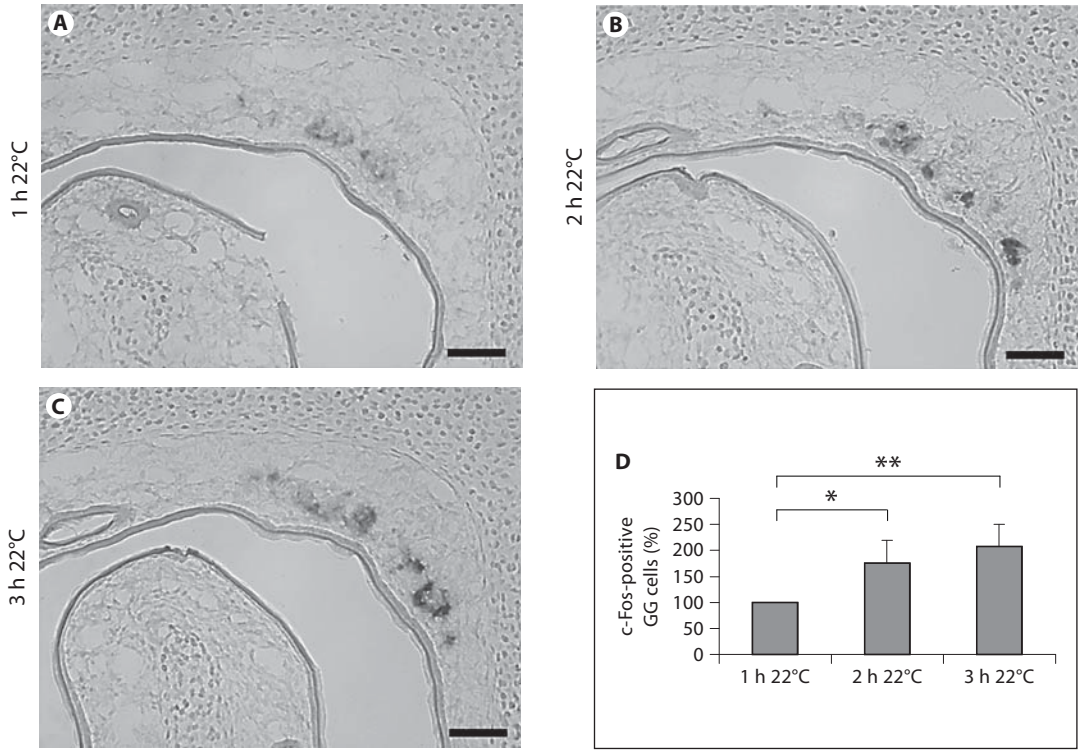

Fig. 5. Long-term exposure to coolness evokes an increased expression of c-Fos in the GG. **A–C** Coronal sections through the GG of neonatal mice exposed to a cool ambient temperature (22°C) for 1, 2 or 3 h were hybridized with an antisense riboprobe for c-Fos. Longer exposure to coolness (2 or 3 h; **B**, **C**) induced an enhanced c-Fos expression compared to a shorter exposure time (1 h; **A**). The figures depicted are representative of 6 independent experiments. For each of these experiments, a 'novel' litter was used. Scale bars: 50 μm. **D** Counting the c-Fos-expressing GG cells demonstrated that the number of these cells increased with exposure time to 22°C. The numbers are based on 6 experiments. In each experiment, the number of c-Fos-positive GG cells after exposure to 22°C for 2 or 3 h was determined relative to that in a concomitantly processed pup, which was exposed to 22°C for 1 h only; the latter number was set as 100%. Means of values, the standard deviations and p values were calculated (* $p = 0.0126$ for 1 vs. 2 h and ** $p = 0.0024$ for 1 vs. 3 h).

at 26°C, the response to 2,3-DMP did not diminish over time; in fact, the odorant-evoked response was even stronger after 3 h than 1 h of odor exposure (fig. 7B–D), i.e., the number of c-Fos-expressing cells in the GG was clearly higher following a longer (3 h) exposure to 2,3-DMP than after a shorter (1 h) one (fig. 7E). Based on these results, adaptation of odor-induced GG responses is not observed at cool ambient temperatures.

Discussion

Recently, it has been found that a small number of given odorants (most notably 2,3-DMP and some related substances) activate neurons in the GG [13]. The molecular mechanisms underlying these responses are entirely elusive; yet, it was observed that 2,3-DMP elicited responses only in those GG neurons which express elements of a cGMP cascade [13]. It was therefore hypothesized that cGMP-associated signaling elements might be involved in odor-induced responses of these cells. In the present study, it was found that the transmembrane guanylyl cyclase GC-G and the cyclic nucleotide-gated ion channel CNGA3 seem to be part of the odor-evoked transduction processes in the GG (fig. 1, 2). However, even in the absence of CNGA3 or GC-G, a smaller number of GG cells was still responsive to 2,3-DMP (fig. 1E, 2E). This could be due to the expression of another cGMP-activated CNG channel (CNGA2), which has recently been observed in a minor subset of GG cells [18]. Analogously, in addition to GC-G, another transmembrane guanylyl cyclase subtype (GC-A) has also been reported to be expressed in a small subpopulation of cells in the GG [17]. Although our own experimental

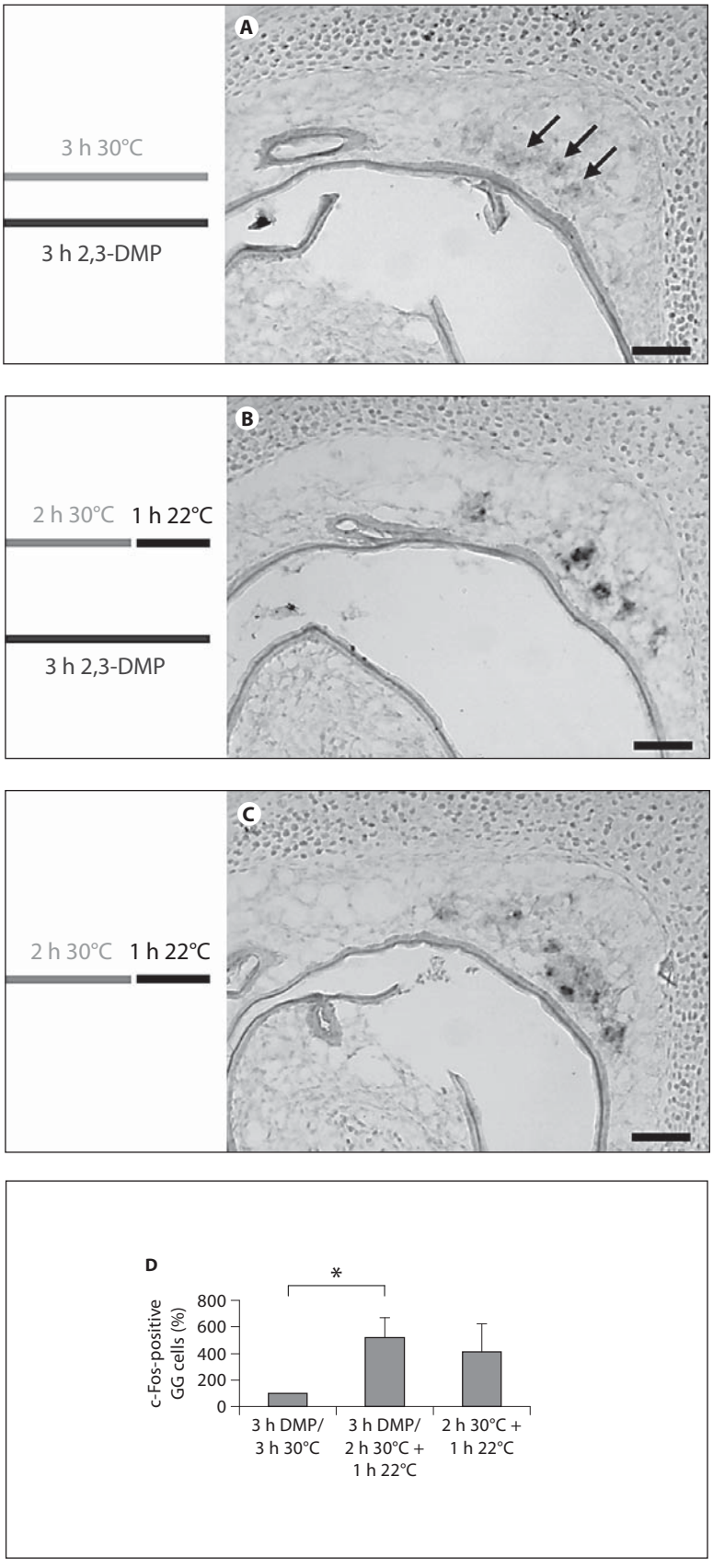

Fig. 6. Coolness-induced c-Fos expression in the GG is not attenuated by adaptation to 2,3-DMP. **A–C** Expression of c-Fos in early postnatal pups was visualized by in situ hybridization with a c-Fos-specific antisense probe on coronal sections through the GG. **A** Following an exposure to 2,3-DMP for 3 h (at 30°C), signals (arrows) were hardly detectable in the GG. **B**, **C** In pups exposed to 2,3-DMP for 3 h, whereby these animals were kept at a cool ambient temperature (22°C) for the last hour (**B**), c-Fos signals were clearly visible, similar to those in coolness-exposed pups (22°C for 1 h) without exposure to 2,3-DMP (**C**). All images depicted are representative of 4 independent experiments. For each of these experiments, a 'novel' litter was used. Scale bars: 50 μm. **D** Comparative quantification of the c-Fos-positive GG cells in the above-mentioned experimental approaches. The numbers are derived from 4 experiments. In each experiment, 3 pups were concomitantly processed; one pup each was subjected to 1 of the 3 above-described exposure paradigms (3 h 2,3-DMP/3 h 30°C or 3 h 2,3-DMP/2 h 30°C + 1 h 22°C or 2 h 30°C + 1 h 22°C, respectively). In each of the 4 experiments, the number of c-Fos-positive GG cells for the pup exposed to 2,3-DMP for 3 h at 30°C was set as 100%. Means of values, the standard deviations and p values were calculated (* p = 0.0165 for 3 h 2,3-DMP/3 h 30°C vs. 3 h 2,3-DMP/2 h 30°C + 1 h 22°C).

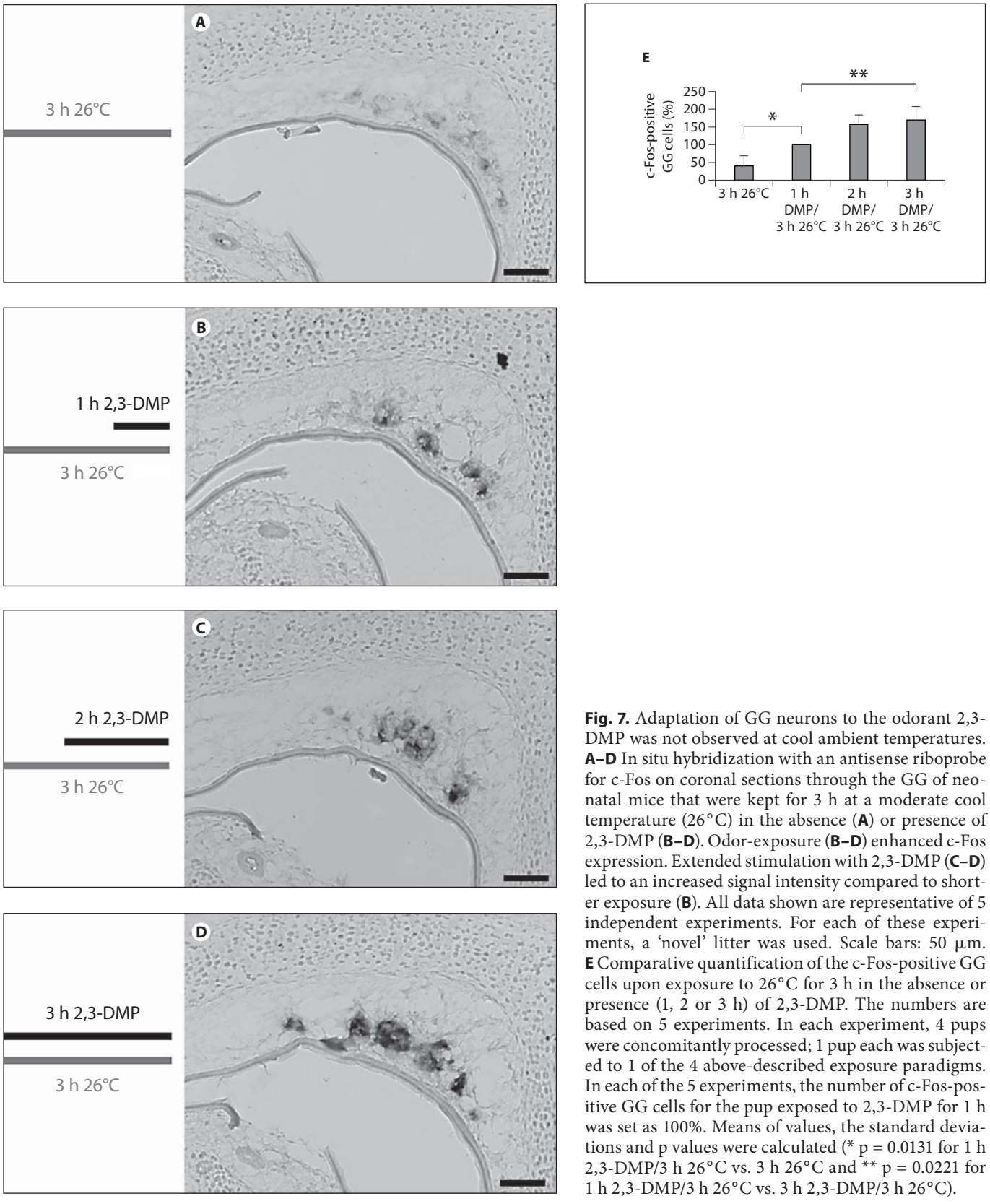

Fig. 7. Adaptation of GG neurons to the odorant 2,3-DMP was not observed at cool ambient temperatures. **A–D** In situ hybridization with an antisense riboprobe for c-Fos on coronal sections through the GG of neonatal mice that were kept for 3 h at a moderate cool temperature (26°C) in the absence (**A**) or presence of 2,3-DMP (**B–D**). Odor-exposure (**B–D**) enhanced c-Fos expression. Extended stimulation with 2,3-DMP (**C–D**) led to an increased signal intensity compared to shorter exposure (**B**). All data shown are representative of 5 independent experiments. For each of these experiments, a 'novel' litter was used. Scale bars: 50 μm. **E** Comparative quantification of the c-Fos-positive GG cells upon exposure to 26°C for 3 h in the absence or presence (1, 2 or 3 h) of 2,3-DMP. The numbers are based on 5 experiments. In each experiment, 4 pups were concomitantly processed; 1 pup each was subjected to 1 of the 4 above-described exposure paradigms. In each of the 5 experiments, the number of c-Fos-positive GG cells for the pup exposed to 2,3-DMP for 1 h was set as 100%. Means of values, the standard deviations and p values were calculated (* $p = 0.0131$ for 1 h 2,3-DMP/3 h 26°C vs. 3 h 26°C and ** $p = 0.0221$ for 1 h 2,3-DMP/3 h 26°C vs. 3 h 2,3-DMP/3 h 26°C).

findings contradict a substantial expression of transmembrane guanylyl cyclases other than GC-G in the GG [16], a low-level expression of another transmembrane guanylyl cyclase subtype in some GG neurons might account for the residual odor responsiveness in GC-G-deficient animals.

An apparent role of a cGMP cascade in the response of chemosensory cells in the GG is reminiscent of the so-called GC-D neurons in the MOE of rodents. In these cells, the guanylyl cyclase subtype GC-D together with the CNGA3 channel mediate the response to given chemical compounds, notably to CO_2, CS_2 and some peptides [27–29]. GC-D neurons appear to be activated by appropriate chemical compounds via the transmembrane protein GC-D which seems to serve as a receptor [30–32]. For GG neurons, it is yet unclear whether activating compounds (such as 2,3-DMP) directly bind to the transmembrane protein GC-G. The observation that 2,3-DMP and 2,3-lutidine also activate a considerable number of cells in the MOE [13] may argue against this view, since the enzyme GC-G is completely absent from the MOE [16]. Alternatively, it is conceivable that neurons of the GG – similar to OSNs in other chemosensory nasal compartments – may be activated by odorants via G protein-coupled olfactory receptors. Accordingly, GG neurons might express appropriate olfactory receptor proteins. Consistent with this notion, neurons in the GG express α subunits of trimeric G proteins, notably G_o and G_i [14], which are considered to be involved in chemosensory signaling processes in vomeronasal neurons [33–37]. Interestingly, the overwhelming majority of GG neurons is endowed with a distinct vomeronasal receptor (V2r83) [14]; whether this receptor is indeed involved in the activation of GG cells by odorants is unclear. Based on the expression of G protein subunits and an olfactory receptor subtype in GG neurons, it is possible that odorous substances may activate a G protein-coupled signaling pathway which subsequently may lead to activation of the cGMP cascade. Such a scenario would be reminiscent of the transduction processes in some chemosensory neurons of the nematode *Caenorhabditis elegans,* including the so-called AWC neurons [for review see 38]. In these cells, odorous substances are supposed to activate via G protein-coupled receptors a G protein of the G_i family, which via an unknown process results in an increased intracellular concentration of cGMP that is synthesized by transmembrane guanylyl cyclases. The elevated cGMP level ultimately activates CNG channels which cause a depolarization of the cell [for review see 38]. Intriguingly, the *C. elegans* AWC neurons also respond to thermal stimuli [39, 40] and both the chemosensory as well as the thermosensory transduction process in these cells is mediated via transmembrane guanylyl cyclases and CNG channels [39]. Similarly, in the GG, cGMP-mediated signaling is not only important for olfactory (this study) but also for thermosensory transduction since CNGA3 [18] and GC-G (our unpublished observations) contribute to the activation of these cells at cool ambient temperatures as well. Consequently, in these dual sensory neurons, the transduction processes for both modalities (temperature and odorant) appear to converge on cGMP signaling. This concept is in line with our recent observation that coolness can enhance odor-induced responses in the GG [13]. Interestingly, in the mammalian olfactory system, not only GG neurons seem to be dual sensory: it has been reported that neurons in the MOE and in the septal organ respond to chemical as well as to mechanical stimuli, using a molecular cascade mediated by the second messenger substance cyclic adenosine monophosphate [41]. It has been proposed that mechanical stimulation might sensitize OSNs for odorous compounds [41]. Based on this concept, it can be speculated that activation of GG neurons by cooler temperatures may sensitize the cells for a chemical stimulus. In this context, it may be noteworthy that in living mice, GG responses induced by cool temperatures or appropriate odorous compounds were mainly (coolness) or even exclusively (odorants) observed in very young pups [10, 13]. Usually, neonatal pups of rodents are kept warm by their dam for most of the time [42]; consequently, they are exposed to cool temperatures only in the absence of their mother. Thus, detection of given odorous compounds from the environment by GG neurons might be most relevant for pups in the absence of their dam. In line with a potential physiological relevance of the interplay between coolness-induced and chemical stimulation, odorant-evoked responsiveness of GG neurons was not observed to adapt at cool ambient temperatures (fig. 7). Conversely, at warm temperatures, i.e. in the presence of the warmth-giving dam, odor-induced responses of GG neurons rapidly adapt (fig. 4).

Although both chemo- and thermosensory responses of GG neurons rely on a cGMP cascade, the relevant transduction pathways seem to be partially distinct: while odor-induced responses adapt (at warm ambient temperatures; fig. 4), coolness-induced responses do not (fig. 5). Based on the finding that odor-adapted GG neurons are still responsive to coolness (fig. 6), it can be assumed that odorant-induced adaptation does not occur

on the level of cGMP-associated signaling elements since GC-G and CNGA3 are crucial for the coolness-evoked response of these cells [18, unpublished observations]. Accordingly, adaptation seems to be due to elements of the odorant-induced pathway which are apparently operating upstream of cGMP signaling.

Acknowledgements

The authors would like to thank Anne Ullrich for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (Br712/24-1).

References

1 Breer H, Fleischer J, Strotmann J: The sense of smell: multiple olfactory subsystems. Cell Mol Life Sci 2006;63:1465–1475.
2 Ma M: Encoding olfactory signals via multiple chemosensory systems. Crit Rev Biochem Mol Biol 2007;42:463–480.
3 Munger SD, Leinders-Zufall T, Zufall F: Subsystem organization of the mammalian sense of smell. Annu Rev Physiol 2009;71: 115–140.
4 Fuss SH, Omura M, Mombaerts P: The Grueneberg ganglion of the mouse projects axons to glomeruli in the olfactory bulb. Eur J Neurosci 2005;22:2649–2664.
5 Koos DS, Fraser SE: The Grueneberg ganglion projects to the olfactory bulb. Neuroreport 2005;16:1929–1932.
6 Fleischer J, Hass N, Schwarzenbacher K, Besser S, Breer H: A novel population of neuronal cells expressing the olfactory marker protein (OMP) in the anterior/dorsal region of the nasal cavity. Histochem Cell Biol 2006; 125:337–349.
7 Roppolo D, Ribaud V, Jungo VP, Lüscher C, Rodriguez I: Projection of the Gruneberg ganglion to the mouse olfactory bulb. Eur J Neurosci 2006;23:2887–2894.
8 Storan MJ, Key B: Septal organ of Gruneberg is part of the olfactory system. J Comp Neurol 2006;494:834–844.
9 Fleischer J, Breer H: The Grueneberg ganglion: a novel sensory system in the nose. Histol Histopathol 2010;25:909–915.
10 Mamasuew K, Breer H, Fleischer J: Grueneberg ganglion neurons respond to cool ambient temperatures. Eur J Neurosci 2008;28: 1775–1785.
11 Schmid A, Pyrski M, Biel M, Leinders-Zufall T, Zufall F: Grueneberg ganglion neurons are finely tuned cold sensors. J Neurosci 2010; 30:7563–7568.
12 Brechbühl J, Klaey M, Broillet MC: Grueneberg ganglion cells mediate alarm pheromone detection in mice. Science 2008;321: 1092–1095.
13 Mamasuew K, Hofmann N, Breer H, Fleischer J: Grueneberg ganglion neurons are activated by a defined set of odorants. Chem Senses 2011;36:271–282.
14 Fleischer J, Schwarzenbacher K, Besser S, Hass N, Breer H: Olfactory receptors and signalling elements in the Grueneberg ganglion. J Neurochem 2006;98:543–554.
15 Fleischer J, Schwarzenbacher K, Breer H: Expression of trace amine-associated receptors in the Grueneberg ganglion. Chem Senses 2007;32:623–631.
16 Fleischer J, Mamasuew K, Breer H: Expression of cGMP signaling elements in the Grueneberg ganglion. Histochem Cell Biol 2009;131:75–88.
17 Liu CY, Fraser SE, Koos DS: Grueneberg ganglion olfactory subsystem employs a cGMP signaling pathway. J Comp Neurol 2009;516: 36–48.
18 Mamasuew K, Michalakis S, Breer H, Biel M, Fleischer J: The cyclic nucleotide-gated ion channel CNGA3 contributes to coolness-induced responses of Grueneberg ganglion neurons. Cell Mol Life Sci 2010;67:1859–1869.
19 Halem HA, Cherry JA, Baum MJ: Vomeronasal neuroepithelium and forebrain Fos responses to male pheromones in male and female mice. J Neurobiol 1999;39:249–263.
20 Kimoto H, Touhara K: Induction of c-Fos expression in mouse vomeronasal neurons by sex-specific non-volatile pheromone(s). Chem Senses 2005;30(suppl 1):i146–i147.
21 Kimoto H, Haga S, Sato K, Touhara K: Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. Nature 2005;43:898–901.
22 Norlin EM, Vedin V, Bohm S, Berghard A: Odorant-dependent, spatially restricted induction of c-fos in the olfactory epithelium of the mouse. J Neurochem 2005;93:1594–1602.
23 Biel M, Seeliger M, Pfeifer A, Kohler K, Gerstner A, Ludwig A, Jaissle G, Fauser S, Zrenner E, Hofmann F: Selective loss of cone function in mice lacking the cyclic nucleotide-gated channel CNG3. Proc Natl Acad Sci USA 1999;96:7553–7557.
24 Lin H, Cheng CF, Hou HH, Lian WS, Chao YC, Ciou YY, Djoko B, Tsai MT, Cheng CJ, Yang RB: Disruption of guanylyl cyclase-G protects against acute renal injury. J Am Soc Nephrol 2008;19:339–348.
25 Kuhn M: Function and dysfunction of mammalian membrane guanylyl cyclase receptors: lessons from genetic mouse models and implications for human diseases. Handb Exp Pharmacol 2009;191:47–69.
26 Chao YC, Cheng CJ, Hsieh HT, Lin CC, Chen CC, Yang RB: Guanylate cyclase-G, expressed in the Grueneberg ganglion olfactory subsystem, is activated by bicarbonate. Biochem J 2010;432:267–273.
27 Hu J, Zhong C, Ding C, Chi Q, Walz A, Mombaerts P, Matsunami H, Luo M: Detection of near-atmospheric concentrations of CO_2 by an olfactory subsystem in the mouse. Science 2007;317:953–957.
28 Leinders-Zufall T, Cockerham RE, Michalakis S, Biel M, Garbers DL, Reed RR, Zufall F, Munger SD: Contribution of the receptor guanylyl cyclase GC-D to chemosensory function in the olfactory epithelium. Proc Natl Acad Sci USA 2007;104:14507–14512.
29 Munger SD, Leinders-Zufall T, McDougall LM, Cockerham RE, Schmid A, Wandernoth P, Wennemuth G, Biel M, Zufall F, Kelliher KR: An olfactory subsystem that detects carbon disulfide and mediates food-related social learning. Curr Biol 2010;20: 1438–1444.
30 Duda T, Sharma RK: ONE-GC membrane guanylate cyclase, a trimodal odorant signal transducer. Biochem Biophys Res Commun 2008;367:440–445.
31 Guo D, Zhang JJ, Huang XY: Stimulation of guanylyl cyclase-D by bicarbonate. Biochemistry 2009;48:4417–4422.
32 Sun L, Wang H, Hu J, Han J, Matsunami H, Luo M: Guanylyl cyclase-D in the olfactory CO_2 neurons is activated by bicarbonate. Proc Natl Acad Sci USA 2009;106:2041–2046.
33 Halpern M, Shapiro LS, Jia C: Differential localization of G proteins in the opossum vomeronasal system. Brain Res 1995;677: 157–161.
34 Berghard A, Buck LB: Sensory transduction in vomeronasal neurons: evidence for G alpha o, G alpha i2, and adenylyl cyclase II as major components of a pheromone signaling cascade. J Neurosci 1996;16:909–918.

35 Jia C, Halpern M: Subclasses of vomeronasal receptor neurons: differential expression of G proteins (Gi alpha 2 and G(o alpha)) and segregated projections to the accessory olfactory bulb. Brain Res 1996;719:117–128.
36 Krieger J, Schmitt A, Löbel D, Gudermann T, Schultz G, Breer H, Boekhoff I: Selective activation of G protein subtypes in the vomeronasal organ upon stimulation with urine-derived compounds. J Biol Chem 1999;274:4655–4662.
37 Matsuoka M, Yoshida-Matsuoka J, Iwasaki N, Norita M, Costanzo RM, Ichikawa M: Immunocytochemical study of G(i)2alpha and G(o)alpha on the epithelium surface of the rat vomeronasal organ. Chem Senses 2001;26:161–166.
38 Bargmann CI: Chemosensation in *C. elegans*; in The *C. elegans* Research Community (ed): WormBook, 2006, doi/10.1895/wormbook.1.123.1.
39 Kuhara A, Okumura M, Kimata T, Tanizawa Y, Takano R, Kimura KD, Inada H, Matsumoto K, Mori I: Temperature sensing by an olfactory neuron in a circuit controlling behavior of *C. elegans*. Science 2008;320:803–807.
40 Biron D, Wasserman S, Thomas JH, Samuel AD, Sengupta P: An olfactory neuron responds stochastically to temperature and modulates *Caenorhabditis elegans* thermotactic behavior. Proc Natl Acad Sci USA 2008;105:11002–11007.
41 Grosmaitre X, Santarelli LC, Tan J, Luo M, Ma M: Dual functions of mammalian olfactory sensory neurons as odor detectors and mechanical sensors. Nat Neurosci 2007;10:348–354.
42 Grota LJ, Ader R: Continuous recording of maternal behavior in *Rattus norvegicus*. Anim Behav 1969;17:722–729.