*--- Chapter 5 ---*

# Weaning induces neuronal activity in the Grueneberg ganglion olfactory subsystem

Cambrian Y. Liu, Scott E. Fraser, David S. Koos

ABSTRACT

The Grueneberg ganglion (GG) is a primary olfactory subsystem bilaterally located in the far-anterior nasal vestibule of many mammals. Its ~1,000 constituent neurons are believed to serve multimodal functions in the detection of low ambient temperatures and alarm and urinary pheromones. *In vivo* studies have supported the specialization of mouse GG function to these various sensory modalities at different ages of the animal. Here, we examined behavioral manipulations that induced nuclear accumulation of c-Fos protein in the GG. As c-Fos immunoreactivity (c-Fos-IR) in the nucleus is an established marker of neuronal activation in the primary olfactory system, these experiments should provide information about the function of the GG. In mice younger than weaning age (<p20), manipulations that involved parental separation induced nuclear c-Fos-IR in GG neurons. A greater number of GG neurons were active in mice older than p5. GG responses were independent of the presence of conspecifics but dependent on the expression of the cGMP-gated cationic channel CNGA3. Several aspects of the experiments and results are inconsistent with known modalities of GG function; thus, GG neurons may have additional sensory functions during weaning.

KEYWORDS

c-Fos, nuclear accumulation, immunohistochemistry, maternal separation, weaning, stress

INTRODUCTION

In mice and many other mammals, the detection and discrimination of odors and pheromones is initiated by collections of primary olfactory neurons at several anatomically-distinct locations in the nasal cavity. At its far-anterior end, where the septum is cartilaginous, there are ~1,000 clustered neurons in the mouse that are present throughout life and that project axons forming 8-12 glomeruli in the necklace-like domain of the olfactory bulb (Fleischer et al. 2006a; Fuss et al. 2005; Grüneberg 1973; Koos and Fraser 2005; Roppolo et al. 2006; Storan and Key 2006). On the basis of their expression of olfactory marker protein (OMP), a pheromone receptor and trace-amine associated receptors at birth (Fleischer et al. 2006b; Fleischer et al. 2007), and key components of a cGMP signaling pathway that can mediate odor transduction (Fleischer et al. 2009; Liu et al. 2009), the neurons of this so-called Grueneberg ganglion (GG) have been proposed to have a primary olfactory function.

GG neurons appear to function in multiple sensory contexts. Firstly, i*n vivo* studies measuring the transcription of the c-Fos immediate-early gene in mice have demonstrated that these neurons respond to low ambient temperature primarily at neonatal ages, when mice exhibit poor auto-regulation of body temperature (Mamasuew et al. 2008). These *in vivo* neuronal responses depended on the cGMP-gated cationic channel CNGA3 (Mamasuew et al. 2010). *In vitro* studies support the function of GG neurons as finely tuned temperature sensors. GG neurons exhibited increases in cytosolic [$Ca^{2+}$] to cold. These increases were independent of changes in intracellular $Ca^{2+}$ and the presence of CNGA3, but dependent on fast-inactivating $Na^{+}$ conductance (Schmid et al. 2010). Secondly, GG neurons are also proposed to detect an alarm pheromone. They exhibited cytosolic [$Ca^{2+}$] bursts when exposed to an unknown water-soluble compound collected during the asphyxiation of mice. This compound induced freezing behavioral responses in adult mice (Brechbuhl et al. 2008). Thirdly, GG neurons respond to exposure to 2,5-dimethylpyrazine (Mamasuew et al. 2011a), a known rodent pheromone (Ma et al. 1998), with increases in spontaneous firing rate (Chapter 4). Certain other odorants and pheromones, such as 2-heptanone, are capable of decreasing the spontaneous firing rates in the neurons (Chapter 4).

We have examined patterns of activity in mouse GG neurons by measuring their activity-dependent nuclear accumulation of c-Fos protein. This assay has previously been used to analyze neuronal activity in olfactory neurons of the vomeronasal organ (VNO) (Kimoto and Touhara 2005). We found that parental separation reliably induced nuclear c-Fos immunoreactivity (c-Fos-IR) in GG neurons. In contrast to previous reports (Mamasuew et al. 2008), the activity levels were highest in adolescent mice and were significantly lower in neonates. GG neurons seem primed for activity during adolescent ages and may serve specialized functions during weaning. The results are discussed with attention to a recent report (Chapter 4) suggesting the modulation of GG activity by blood-borne stress hormones.

## MATERIALS AND METHODS

### *Animal Care*

Mice were maintained according to Caltech-approved protocol. Mice were housed in 6000 $cm^3$ plastic cages, provided with food (LabDiet 5001), and attached to an automated water delivery system equipped with quick disconnect manifold mounted drinking valves (A-160). Cages were assembled onto racks and were provided HEPA-filtered air (Enviro-gard). Ambient room temperature was kept constant at 22˚ C.

Once per week, mice were moved from soiled cages to clean cages, as part of normal maintenance procedure. Once per month, sullied water valves were replaced with clean ones. We found that experiments performed within 12 h of a regularly scheduled cage change would yield unreliable results; hence, we avoided this scenario in the experiments discussed below.

Animals were housed with an equal-phase 24 h light-dark cycle. We began and finished all experiments in the range between 1600 and 1900 h, fully within the light cycle. At these times, there was no interference from the quotidian activities of animal facility staff or other researchers. No other mice were handled by the investigators before or during the experiments on the days of the experiments. Control groups and experimental groups were formed from littermates. All mice used were of the

BL/6/129 background, except for *Cnga3*$^{-/-}$ and *Cnga3*$^{+/-}$ mice, which were of the RHJ/LeJ (JR 5415) background. *Cnga3*-mutant mice were described and characterized previously (Chapter 4).

*Transport Stimulus*

This stimulus produced the c-Fos staining patterns shown in Fig. 1. The cage containing the mouse dam, sire, and litter was moved to a sterile hood within the animal facility. Offspring were separated from parental mice and placed into an enclosed 1,800 $cm^3$ cardboard box. The cardboard box (containing mice) was then carried by the investigators out of the animal facility to a dissection station in a separate building. Mice were left in the cardboard box in the dissection station, without food or water, for 5-150 min prior to euthanasia by $CO_2$. Collected tissue was processed for immunohistochemistry.

*Cage Change Stimulus*

Sterilized cages containing clean woodchip bedding, food, and water were prepared. Parents and offspring of a single litter were moved to a sterile hood within the animal housing room. Offspring were separated and moved to the clean cage as a group (2-5 per group). The clean cage (containing the new mice) was brought to a different cubicle on the original housing rack. Mice were euthanized by $CO_2$ at a sterile dissection station within the housing room. Mice of a given experiment were euthanized as a group 5-150 min after the cage change. The dissection of nasal vestibules took ~3 min per mouse. Tissue was processed for immunohistochemistry.

In some experiments, the clean cage containing newly weaned mice was transported to a dissection station in a separate building (Fig. 3A). These mice were euthanized by $CO_2$ and processed for immunohistochemistry at this distally-located station.

The effects of human contact on GG activity (Fig. 3A) were evaluated by moving the cage (parents and offspring) to a sterile hood. Offspring mice were picked up and dropped back down into the original cage. The cage was returned to its original cubicle on the housing rack. Tissue was collected 75-120 min after these manipulations.

To evaluate the necessity of conspecific animals (Fig. 3D), clean cages were prepared in a sterile hood. Unweaned mice were moved into these clean cages such that some cages housed >2 mice and other cages housed only 1 mouse. Cages were returned to separate cubicles on the original housing rack. Mice were sacrificed after 75-120 min.

*Weaning Stimulus*

To discriminate between the contributions of parental separation and environmental novelty (*i.e.*, a clean cage) (Fig. 3A), mouse litters with the dam and sire were placed in a sterile hood. The dam and sire were moved to a clean cage. The cage containing the offspring was returned to its original cubicle on the animal housing rack. After 75-120 min, the offspring mice were sacrificed.

*Ex vivo culture*

p10 mice were transported using the described "Transport Stimulus" procedure. The animals were euthanized by cervical dislocation and decapitated. Heads were skinned. Excess bone and tissue were removed to expose the brain, olfactory bulbs, and nasopalatine duct. Remaining tissue was placed into excess pre-warmed (37˚C), pre-equilibrated (5% $CO_2$) Neurobasal-A media (Invitrogen), supplemented with 1X B-27 (Invitrogen), 200 mM L-glutamine, and 50 U/mL penicillin-streptomycin. Tissue was gently shaken at 37˚C for 2-6 h. Nasal vestibules were removed and fixed in cold 4% paraformaldehyde overnight and processed for immunohistochemistry.

*Immunohistochemistry*

Immunohistochemistry was performed as previously described (Liu et al. 2009), but with an extended incubation time (~60 h) of anti-c-Fos antibody to optimize nuclear labeling. Briefly, nasal vestibules were dissected from mice and fixed overnight in cold 4% paraformaldehyde prepared in phosphate buffered saline (PBS). After cryoprotection in buffered 30% sucrose (w/v), vestibules were embedded in Tissue-Tek O.C.T. (Sakura) and sectioned to 14 µm thickness on a freezing microtome at

-23˚C. Tissue was adhered to slides. For fluorescence staining, slides were sequentially incubated with rabbit anti-c-Fos primary antibody (EMD Chemicals, 1:10,000, cat# PC38), biotinylated goat anti-rabbit secondary antibody (Vector Labs, 3 µg/mL), and tertiary streptavidin-Alexa Fluor 555 label (Invitrogen, 2 µg/mL), with 4 changes of PBS between each incubation. PBS-buffered 0.3% Triton-X was used to promote infiltration during antibody incubations. Final PBS washes included 0.2 µM Topro-3 (Invitrogen) as a nuclear stain.

For diaminobenzidine (DAB)-based revelation of staining on thin sections, slides with adherent tissue was exposed to buffered 0.3% (v/v) hydrogen peroxide solution for 10 min prior to incubation with the primary antibody. A tertiary label of peroxidase-streptavidin (2.5 µg/mL, MP Bio) was used following the incubation of biotinylated secondary antibodies. Labeling was revealed with a Ni-enhanced DAB kit (Thermo Scientific) with a development time of 5-12 minutes in the dark. Thin sections of the VNO were processed in the same manner.

In whole-mount stainings, the superficial epithelium of the nasal vestibule (containing the GG) was peeled apart from the septum in fixed specimens. For DAB staining, the tissue was exposed to buffered 0.3% (v/v) hydrogen peroxide solution for 30 min prior to incubation with the primary antibody. After staining, tissue was adhered to a slide, in preparation for microscopic examination.

*Imaging*

For fluorescent imaging, slides were mounted with Fluoro-Gel (Electron Microscopy Sciences). Imaging was performed on an LSM 510 upright confocal microscope (Zeiss) with excitation laser wavelengths of 488 nm, 543 nm, and 633 nm. Optical sections of various thicknesses could be generated by varying the pinhole diameter. With a 40X/0.75 Plan-NeoFluar (Zeiss) objective, we obtained optical slices of ~4 µm thickness by using a pinhole diameter of 260 µm.

For brightfield imaging, slides were mounted with polyvinylpyrrolidone (PVP) mounting medium, containing: 2.5 g PVP 40K, 0.5 g PVP 360K, 1 mL glycerol, 50 mg n-propyl gallate, 1 crystal

thymol, 25 mL Tris buffer (pH 7.4). Brightfield images of DAB deposition were obtained on a Zeiss Axiophot compound microscope.

*Data Analysis*

The degree of neuronal activation in the GG organ was assessed by counting the number of GG nuclei that exhibited c-Fos accumulation in DAB-developed nasal vestibule sections. GG neurons could be recognized by their larger size, their clustering, and their faint cytosolic labeling by the antibody. Using a hand-tally counter, we performed the counting during analysis of tissue by light microscopy. Repeated counting of the same sample yielded scores within 10%. Data from several different experiments were pooled. Effect sizes were measured by comparing the nuclei counts for the experimental group versus littermate controls in the same experiment. The mean control effects across different experiments were then normalized to 1 to account for variability in baseline activity. The normalized values correspond to a dimensionless "activity index." Error bars indicate standard error. Statistical significance between groups was evaluated using the two-sample t-test with the assumption of unequal variances and a significance level of 0.05. Data analysis was performed in MATLAB 7.4.0 with custom-written routines. Images were prepared in ImageJ (NIH).

RESULTS

We found, fortuitously, that transport of unweaned mice from a housing facility to a separate euthanasia/dissection facility was sufficient to induce the nuclear c-Fos-IR in GG neurons. This was a robust stimulus resulting in >10 active neurons per animal in 25/25 mice younger than p20. The intensely stained GG nuclei were readily discerned in thin sections (Fig. 1A) and whole-mount preparations (Fig. 1B) of the mouse nasal vestibule. Fluorescent stainings with OMP-GFP mice (Potter et al. 2001) revealed a colocalization of c-Fos protein, nuclear dye, and OMP in thin sections from the anterior nasal vestibule in transported mice, confirming the c-Fos-IR to be in GG nuclei. No GG nuclear c-Fos-IR was evident in mice sacrificed within 5 min of transport and whose noses were immediately fixed (Fig. 1C). However,

the GG neurons of animals sacrificed in the range between 45 and 150 min after transport unambiguously exhibited nuclear c-Fos-IR (Fig. 1D). Transport was sufficient to elicit activity, because nuclear c-Fos-IR was still observed in GG neurons of mice that were sacrificed immediately after transport, but whose noses were cultured *ex vivo* for 2 h prior to fixation and processing (Fig. 1E). After 6 h of *ex vivo* culture, c-Fos protein in GG neurons was diffusely distributed throughout the cell (Fig. 1F), similar to transported animals sacrificed after 5 min.

Transport is a heterogeneous stimulus, with components such as temperature changes, stress, and new external chemosensory, audible, and tactile cues that would be expected to trigger activity in sensory systems. We sought to narrow the GG stimulus. To this end, we tested the effect of a cage change on GG activity ("cage change stimulus"). In these experiments, unweaned mouse pups, as a group, were moved from the company of the dam, sire, and littermates to a clean cage, located in proximity to the original cage. Mice older than p13 (but younger than p20) immediately exhibited exploratory locomotor activity in the new cage, consistent with previous observations in adult mice (Drenan et al. 2008). These pups were sacrificed 60 to 90 min after movement. At the time of sacrifice, the mice were usually found huddled together.

The cage change stimulus induced nuclear c-Fos-IR in GG neurons in 53/58 (91%) mouse pups. Fig. 2A shows the number of c-Fos-IR GG nuclei per animal in relation to the age of the animal. On average, a higher number of GG neurons were activated in animals in the age range of p6 to p21 (176 ± 18 nuclei, n=42), compared to animals in the age range of p0 to p4 (46 ± 8 nuclei, n=16, $p < 0.001$) (Fig. 2B). The highest number of observed c-Fos+ nuclei was 531 in a p13 mouse. Mice that were sacrificed at the start of the experiment exhibited almost no nuclear c-Fos-IR in the GG (5 ± 1 nuclei, n=33). A 10 min exposure to the clean cage, with subsequent return to the dam and sire, was sufficient to induce nuclear c-Fos-IR in the GG neurons of p20 mouse pups (349 ± 53 nuclei, n=3).

We found that modifications to the cage change stimulus could increase or decrease the number of active GG neurons. In p3-p7 mice, transport of the clean cage to a separate dissection station resulted in a 64 ± 10% increase (n=5, p=0.0016) in the number of active GG neurons (Fig. 3A, '1+2+3+4'). In

p16-p17 mice, we simulated weaning, in the absence of a cage change, by removing the parental mice from the cage 90 min prior to sacrifice. This was sufficient to induce nuclear c-Fos-IR in the GG of 8/9 (89%) pups, but at a significantly lower level (22 ± 5% of the activity level in cage-changed mice, $p < 0.001$) (Fig. 3A, '1+2'). These results have several implications. First, our manipulations induced nuclear c-Fos-IR in only a subset of the total number of GG neurons that are capable of responding. Second, the magnitude of the organ-level response in the GG is graded. Third, parental separation alone is sufficient to trigger activity in the GG.

We tested whether c-Fos nuclear accumulation in the GG could be due to pheromonal communication. In particular, weaning may induce new pheromonal secretion events that are detected by conspecifics. In p12 mice, the cage change stimulus did not result in nuclear c-Fos-IR in the neurons of the vomeronasal organ (VNO) (Fig. 3B-C), though the GG organs exhibited nuclear c-Fos-IR (n=3). We next examined whether GG activity required the presence of conspecifics. In these experiments, p15 mice were individually weaned to clean cages. We found no significant differences in the number of GG neurons exhibiting nuclear c-Fos-IR between mice weaned individually or as a group ($p=0.61$, n=3 per group) (Fig. 3D). Thus, the observed GG responses were not due to pheromonal exchange.

It has been suggested that odor transduction in the GG relies in cGMP signaling components (Fleischer et al. 2009; Liu et al. 2009). GG neurons express candidate odor receptors in the form of membrane-bound guanylate cyclases and the cGMP-gated cationic channel CNGA3 on their cilia (Liu et al. 2009). The cilia represent putative sites of primary odor transduction in olfactory sensory neurons (Firestein 2001). We examined the role of CNGA3 in GG activity by measuring nuclear c-Fos-IR in GG neurons of *Cnga3*$^{-/-}$ mice. In these experiments, a mixed litter of p16 *Cnga3*$^{-/-}$ and *Cnga3*$^{+/-}$ mice was randomly divided into groups no larger than 4 and weaned into clean cages. In *Cnga3*$^{-/-}$ mice subjected to this protocol, the mean number of active neurons was 24 ± 5 (n=8), a statistically-significant decrease compared to their subjected *Cnga3*$^{+/-}$ siblings (91 ± 6 nuclei, n=5, $p < 0.001$) (Fig. 3E). These results suggest that the behavioral manipulations applied to the mice in this study evoked CNGA3-dependent responses in the GG.

Though a robust stimulus, weaning was not essential for nuclear c-Fos-IR in GG neurons. Though light handling of mouse pups, absent of weaning, generally did not induce nuclear c-Fos-IR, extensive handling could produce clearly stained nuclei (data not shown). In weanlings and adults (1-3 months old), transferring the mice to a clean cage induced GG nuclear c-Fos-IR in 3/5 (60%) mice. Overall, the responses in adults were not as robust; the average number of c-Fos+ nuclei in responding animals was 78 ± 27 (unhandled adult mice had an average score of 8 ± 2 active neurons per animal, n=10).

## DISCUSSION

We have demonstrated that parental separation is sufficient to induce nuclear c-Fos-IR, an established marker of neuronal activity, in the GG. The manipulations performed in this study evoked activity in a fraction (at most ~50%) of the ~1,000 GG neurons (Koos and Fraser 2005) in an animal. Weaning of mouse pups to a clean cage ("cage change stimulus") constituted a robust stimulus of GG neurons. GG responses to the cage change stimulus were more pronounced in p6-p20 mice. The induced nuclear c-Fos-IR in this age range depended on the expression of CNGA3, a cGMP-gated cationic channel. It is likely that the c-Fos protein translocation events monitored in this study correspond to cGMP-initiated electrical transduction in the GG.

Previous *in vivo* studies have shown that low ambient temperatures (Mamasuew et al. 2008) and exposure to isoforms of DMP (Mamasuew et al. 2011a) induce CNGA3-dependent (Mamasuew et al. 2011b; Mamasuew et al. 2010) transcription of the c-Fos gene. However, these previously-observed effects were most pronounced at neonatal ages (p0-p7). In this study, nuclear c-Fos-IR was reduced at neonatal ages. Stronger responses were observed at adolescent ages (>p6), when mice are better able to regulate their own body temperatures. In our cage change stimulus, the ambient temperature was constant at 22˚C; thus, at most the weaned mice experienced a temperature change of 37˚C to 22˚C. Temperature changes in this range do not alter activity in GG neurons in mice ~p14 and older (Mamasuew et al. 2008; Schmid et al. 2010). Thus, the neuronal activity inducing nuclear c-Fos-IR in the GG is unlikely to be

solely attributable to ambient temperature changes. The effects we have analyzed in this study differ from those examined in earlier studies.

GG neurons have been proposed to detect alarm pheromones (Brechbuhl et al. 2008). Because the chemical identities of the alarm pheromones remain elusive, we have not been able to verify their collection and effects in our own experiments. Nevertheless, we tested whether the GG c-Fos protein translocations seen in this study required the presence of other animals. By definition, this is a necessary condition for pheromonal communication. We found that conspecifics were not required for the cage change-induced GG activity and that removal of conspecifics had no effects on the number of activated GG neurons. It is therefore unlikely that the GG activity we observed is due to detection of an alarm pheromone.

What are possible sensory contexts that account for the GG activity patterns observed in this study? Weaned mice all experienced a loss of parental odors and cues that are indicative of an inhabited cage, such as feces and urine. GG neurons may directly respond to the absence of habitation cues or pheromones, especially those that indicate a parental presence. In electrophysiological studies, GG neurons are excited by the removal of odorants (Chapter 4). However, this mechanism cannot solely account for graded organ-level encodings of GG activity in mice that underwent post-weaning manipulations of incrementing complexity (Fig. 3A).

We favor a model in which GG neurons sample cues that relate to an internal physiological state that is induced by weaning. This physiological state has different gradations of intensity, whose levels determine the concentrations or frequency of cues. At a low intensity, such as that elicited by parental separation alone, the physiological state induced only small changes in the baseline activities of GG neurons. As a result, only a few neurons exceeded the detection threshold of our c-Fos protein assay. At a high intensity, such as that elicited by transporting newly weaned mouse pups, this physiological state induces large changes in the activities of GG neurons and incurs nuclear c-Fos-IR throughout the ganglion. Of note, the results described here are consistent with previous findings of c-Fos transcription,

stemming from maternal separation, in the GG. Interestingly, those c-Fos signals were still present with naris occlusion (Mamasuew et al. 2008).

We conjecture that the relevant physiological state induced by the weaning of pups is stress. In guinea pigs, experimental manipulations similar to those performed here induce graded levels of glucocorticoids in the bloodstream (Hennessy and Moorman 1989). Cortisol can directly reduce the spontaneous firing rate of GG neurons (Chapter 4); as c-Fos assays usually report excitatory responses, the recovery period after hormone exposure may underlie the c-Fos responses seen in this study. We do not know if the nuclear c-Fos-IR observed in this study might stem from direct interactions between GG neurons and cortisol, or if serum cortisol might simply indicate the presence of other chemicals that are detected by the GG. Nevertheless, a prediction of this model is that any experimental manipulation that induces extreme stress in an animal should alter GG activity. Consistent with this prediction, we observed GG nuclear c-Fos-IR with some experimental manipulations that did not include weaning. Exposures of mice to alarm pheromones or cold may similarly induce secondary stress states that affect GG neurons.

GG neurons appear most primed for activity in adolescent mice. In mammals, this stage of life is characterized by the natural separation of offspring from their parents. While the caudal glomeruli near the necklace-like glomeruli in the olfactory bulb exhibit increased metabolic activity during suckling (Greer et al. 1982), this role has not yet been substantiated for the GG. Our results show that GG neurons participate in suckling's juxtaposed and necessary condition, weaning. This is a critical time in a mammal's life, in which the ability to recognize and adjust to new situations and environments is a matter of life or death. The special readiness and deployment of GG neurons at this stage of life suggests a critical role for this unusual primary olfactory subsystem in a mammal's adaptation to a life on its own.

ACKNOWLEDGMENTS

We thank B. Chang for kindly providing *Cnga3*$^{-/-}$ mice. We thank J. Gutierrez, S.M.M. Alaniz, and A.R. Douglas for animal care and husbandry. This project was supported by grants from the U.S. National Institutes of Health and the U.S. National Science Foundation.

CONTRIBUTIONS

C.Y.L., D.S.K., and S.E.F. planned experiments. C.Y.L. and D.S.K. performed experiments. C.Y.L. wrote the paper.

REFERENCES

**Brechbuhl J, Klaey M, and Broillet MC**. Grueneberg ganglion cells mediate alarm pheromone detection in mice. *Science* 321: 1092-1095, 2008.

**Drenan RM, Grady SR, Whiteaker P, McClure-Begley T, McKinney S, Miwa JM, Bupp S, Heintz N, McIntosh JM, Bencherif M, Marks MJ, and Lester HA**. In vivo activation of midbrain dopamine neurons via sensitized, high-affinity alpha 6 nicotinic acetylcholine receptors. *Neuron* 60: 123-136, 2008.

**Firestein S**. How the olfactory system makes sense of scents. *Nature* 413: 211-218, 2001.

**Fleischer J, Hass N, Schwarzenbacher K, Besser S, and 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* 125: 337-349, 2006a.

**Fleischer J, Mamasuew K, and Breer H**. Expression of cGMP signaling elements in the Grueneberg ganglion. *Histochem Cell Biol* 131: 75-88, 2009.

**Fleischer J, Schwarzenbacher K, Besser S, Hass N, and Breer H**. Olfactory receptors and signalling elements in the Grueneberg ganglion. *J Neurochem* 98: 543-554, 2006b.

**Fleischer J, Schwarzenbacher K, and Breer H**. Expression of trace amine-associated receptors in the Grueneberg ganglion. *Chem Senses* 32: 623-631, 2007.

**Fuss SH, Omura M, and Mombaerts P**. The Grueneberg ganglion of the mouse projects axons to glomeruli in the olfactory bulb. *Eur J Neurosci* 22: 2649-2654, 2005.

**Greer CA, Stewart WB, Teicher MH, and Shepherd GM**. Functional development of the olfactory bulb and a unique glomerular complex in the neonatal rat. *J Neurosci* 2: 1744-1759, 1982.

**Grüneberg H**. A ganglion probably belonging to the N. terminalis system in the nasal mucosa of the mouse. *Z Anat Entwicklungsgesch* 140: 39-52, 1973.

**Hennessy MB, and Moorman L**. Factors influencing cortisol and behavioral responses to maternal separation in guinea pigs. *Behav Neurosci* 103: 378-385, 1989.

**Kimoto H, and Touhara K**. Induction of c-Fos expression in mouse vomeronasal neurons by sex-specific non-volatile pheromone(s). *Chem Senses* 30 Suppl 1: i146-147, 2005.

**Koos DS, and Fraser SE**. The Grueneberg ganglion projects to the olfactory bulb. *Neuroreport* 16: 1929-1932, 2005.

**Liu CY, Fraser SE, and Koos DS**. Grueneberg ganglion olfactory subsystem employs a cGMP signaling pathway. *J Comp Neurol* 516: 36-48, 2009.

**Ma W, Miao Z, and Novotny MV**. Role of the adrenal gland and adrenal-mediated chemosignals in suppression of estrus in the house mouse: the lee-boot effect revisited. *Biol Reprod* 59: 1317-1320, 1998.

**Mamasuew K, Breer H, and Fleischer J**. Grueneberg ganglion neurons respond to cool ambient temperatures. *Eur J Neurosci* 28: 1775-1785, 2008.

**Mamasuew K, Hofmann N, Breer H, and Fleischer J**. Grueneberg ganglion neurons are activated by a defined set of odorants. *Chem Senses* 36: 271-282, 2011a.

**Mamasuew K, Hofmann N, Kretzschmann V, Biel M, Yang RB, Breer H, and Fleischer J**. Chemo- and thermosensory responsiveness of Grueneberg ganglion neurons relies on cyclic guanosine monophosphate signaling elements. *Neurosignals* 19: 198-209, 2011b.

**Mamasuew K, Michalakis S, Breer H, Biel M, and Fleischer J**. The cyclic nucleotide-gated ion channel CNGA3 contributes to coolness-induced responses of Grueneberg ganglion neurons. *Cell Mol Life Sci* 67: 1859-1869, 2010.

**Potter SM, Zheng C, Koos DS, Feinstein P, Fraser SE, and Mombaerts P**. Structure and emergence of specific olfactory glomeruli in the mouse. *J Neurosci* 21: 9713-9723, 2001.

**Roppolo D, Ribaud V, Jungo VP, Luscher C, and Rodriguez I**. Projection of the Gruneberg ganglion to the mouse olfactory bulb. *Eur J Neurosci* 23: 2887-2894, 2006.

**Schmid A, Pyrski M, Biel M, Leinders-Zufall T, and Zufall F**. Grueneberg ganglion neurons are finely tuned cold sensors. *J Neurosci* 30: 7563-7568, 2010.

**Storan MJ, and Key B**. Septal organ of Gruneberg is part of the olfactory system. *J Comp Neurol* 494: 834-844, 2006.

FIGURE LEGENDS

Figure 1: Transport induces nuclear c-Fos-IR in mouse pups. A) Thin section of the mouse nasal vestibule stained with c-Fos antibody shows nuclear accumulation of c-Fos protein (black) in GG neurons. B) Whole-mount view of the c-Fos-stained nasal vestibule demonstrates labeled clusters of GG nuclei. Arrows in panels A and B point to clusters of GG neurons. C-D) Transported mice were sacrificed at C) 5 min or D) 90 min and stained for c-Fos. Shown are fluorescent photomicrographs of the nasal vestibule. GG cells have green fluorescence in OMP-GFP mice. Many but not all GG neurons show nuclear c-Fos-IR at 90 min (D). E-F) *Ex vivo* culture of nasal vestibules from mice sacrificed immediately after transport. E) Noses were processed after 2 h of culturing. F) Noses processed after 6 h of culturing no longer exhibited GG nuclear c-Fos-IR (compare E and F). Arrows point to clusters of GG cells. Scale bars: A) 60 μm, B) 100 μm, C-D) 30 μm, E-F) 60 μm.

Figure 2: Weaning mouse pups to a clean age constitutes a robust stimulus of the GG. A) Plot of the number of GG nuclei demonstrating c-Fos-IR per animal as a function of the age of the pup. The 'no change' data points represent c-Fos-IR in un-manipulated mice. B) Mice older than p6 had significantly ($p < 0.001$) higher numbers of c-Fos-IR nuclei in the GG.

Figure 3: Experimental permutations of weaning alter nuclear c-Fos-IR in the GG. All effects shown are statistically significant ($p < 0.05$) unless otherwise indicated by 'ns.' Activity index was calculated by normalizing effect sizes of experimental groups to the control effect of the cage change stimulus in littermates. Normalized values from different experiments were pooled together. A) Activity index as a function of different forms of weaning. '0': untouched mice. '1': touched/handled mice. '2': weaned mice (separated from dam and sire). '3': exposure to clean cage. '4': transport. For example, '1+2+3' indicates the mice were handled, separated from the dam and sire, and moved to a clean cage. '1+2+3+4' indicates that the weaned pups in the clean were transported to a separate dissection station. B-C) The cage change stimulus did not alter activity in the VNO. Shown are photomicrographs c-Fos-stained thin sections of the

VNO of B) untouched mice and C) mice weaned to a clean cage. D) The absence of conspecific animals did not change GG nuclear c-Fos-IR under the cage change stimulus. E) *Cnga3*$^{-/-}$ mice had significantly reduced numbers of active GG neurons under the cage change stimulus. Scale bars: 80 μm.

FIGURES

Figure 1

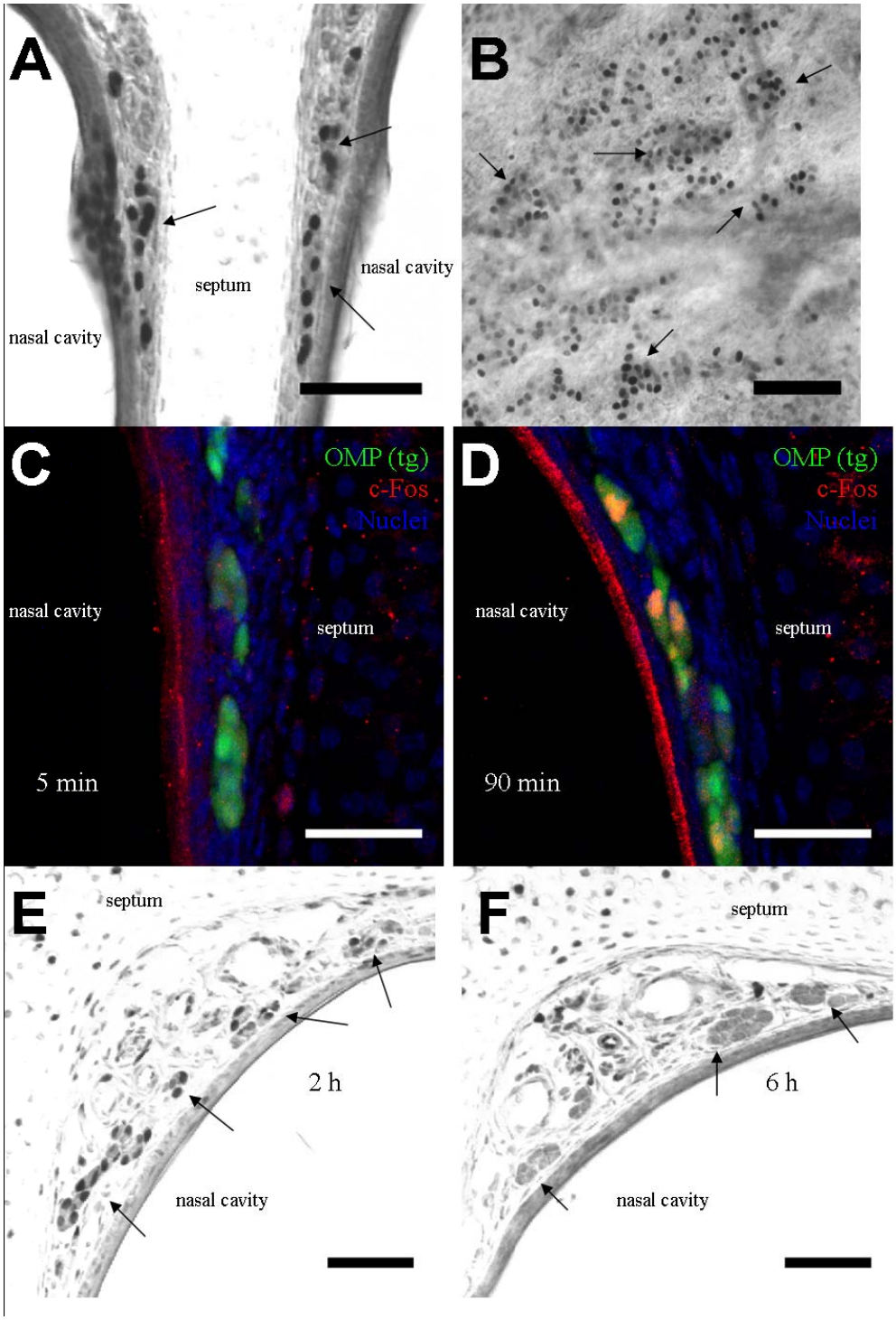


Figure 2

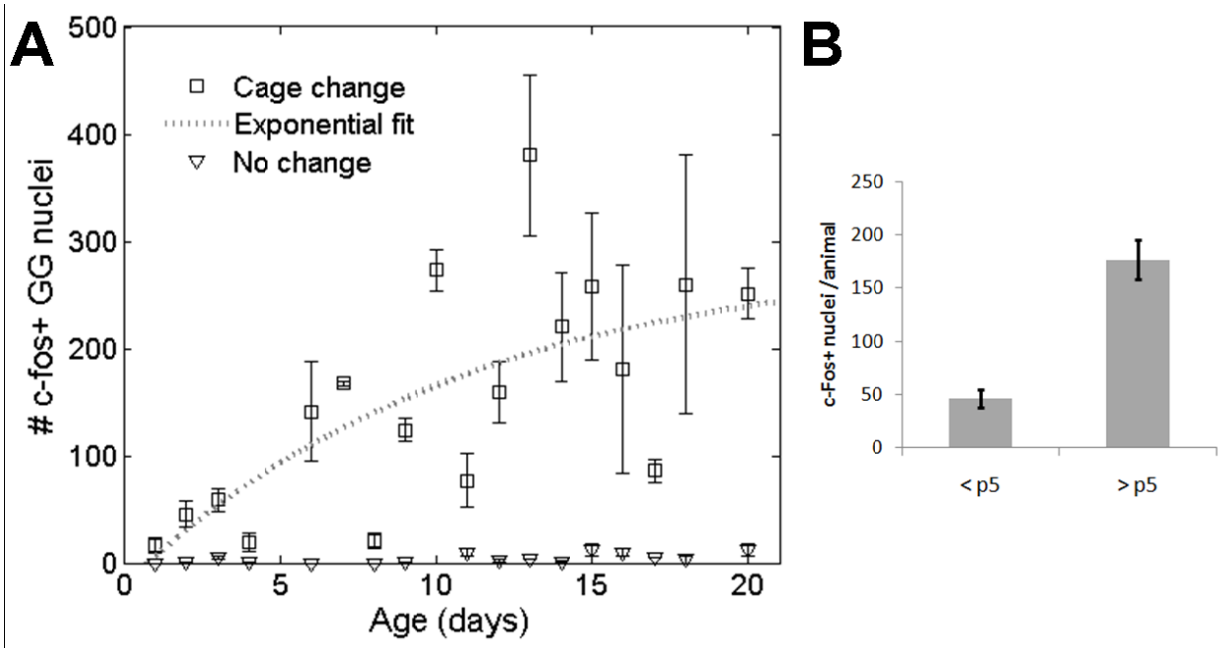
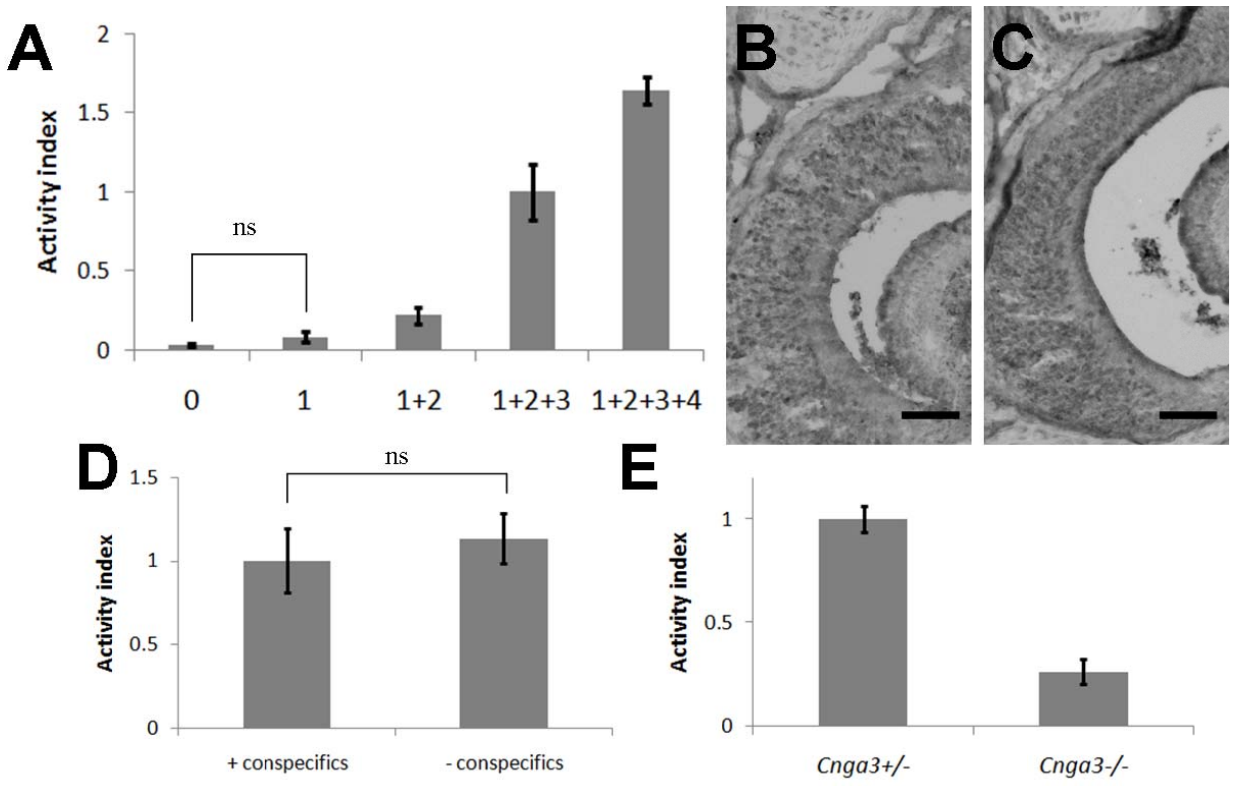

A
# c-fos+ GG nuclei
Cage change
Exponential fit
No change
Age (days)
B
c-Fos+ nuclei /animal
< p5
> p5

Figure 3


A
Activity index
2
1.5
1
0.5
0
ns
0
1
1+2
1+2+3
1+2+3+4
B
C
D
Activity index
1.5
1
0.5
0
ns
+ conspecifics
- conspecifics
E
Activity index
1
0.5
0
Cnga3+/-
Cnga3-/-