The wiring of Grueneberg ganglion axons is dependent on neuropilin 1

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SUMMARY
The Grueneberg ganglion is a specialized olfactory sensor. In mice, its activation induces freezing behavior. The topographical map corresponding to the central projections of its sensory axons is poorly defined, as well as the guidance molecules involved in its establishment. We took a transgenic approach to label exclusively Grueneberg sensory neurons and their axonal projections. We observed that a stereotyped convergence map in a series of coalescent neuropil-rich structures is already present at birth. These structures are part of a peculiar and complex neuronal circuit, composed of a chain of glomeruli organized in a necklace pattern that entirely surrounds the trunk of the olfactory bulb. We found that the necklace chain is composed of two different sets of glomeruli: one exclusively innervated by Grueneberg ganglion neurons, the other by axonal inputs from the main olfactory neuroepithelium. Combining the transgenic Grueneberg reporter mouse with a conditional null genetic approach, we then show that the axonal wiring of Grueneberg neurons is dependent on neuropilin 1 expression. Neuropilin 1-deficient Grueneberg axonal projections lose their strict and characteristic avoidance of vomeronasal glomeruli, glomeruli that are innervated by secondary neurons expressing the repulsive guidance cue and main neuropilin 1 ligand Sema3a. Taken together, our observations represent a first step in the understanding of the circuitry and the coding strategy used by the Grueneberg system.

KEY WORDS: Olfactory system, Axon guidance, Neuropilin 1, Mouse, GC-G, GC-D

INTRODUCTION
Multiple and specialized sensory systems have been selected during the evolution of vertebrates. Among these sensors, olfaction represents a major tool for many species. In most mammals, the olfactory system is divided into at least three physically and functionally separate sensory structures. These consist of the main olfactory neuroepithelium, the vomeronasal organ and the Grueneberg ganglion. This last structure is located at the tip of the nose, in the vestibule of the anterior nasal cavity, and is composed of a few hundred sensory neurons (Gruneberg, 1973).

The function of the Grueneberg ganglion is apparently multimodal, as it is responsive to both thermo- and chemostimuli (Mamasuew et al., 2008; Chao et al., 2010; Schmid et al., 2010; Mamasuew et al., 2011). The structure also responds to volatile molecules produced by stressed mice, an activation that leads to a freezing behavior in the recipient animals (Brechbuhl et al., 2008).

Sensory information is spatially encoded in the brain of vertebrates. This is also true for olfactory information, which is relayed to the brain through sensory axonal projections that coalesce into anatomical structures called glomeruli. Axonal projection maps in the olfactory bulb are characteristic of each type of olfactory sensory neuron. The axonal projections of the Grueneberg ganglion have only recently been associated with the olfactory system (Fleischer et al., 2006a; Fuss et al., 2005; Koos and Fraser, 2005; Roppolo et al., 2006; Storan and Key, 2006). They converge in the olfactory bulb and are part of a group of glomeruli called the necklace glomeruli (Shinoda et al., 1989). These constitute a very peculiar and still poorly defined set of glomeruli, which are linked with each other in a chain-like narrow band that runs between the main and the accessory olfactory bulbs and that surrounds the entire trunk of the olfactory bulb (Fig. 1A). This necklace-shaped group of glomeruli is also innervated by sensory neurons located in the main olfactory system, which detect a variety of semiochemicals (Hu et al., 2007; Leinders-Zufall et al., 2007; Munger et al., 2010). Whether specific necklace glomeruli are co-innervated by Grueneberg and main olfactory projections is unknown.

The establishment of an olfactory topographic map in the bulb results from the integration by axonal fibers of various guidance cues. The immense majority of studies have focused their attention on the molecular signals allowing sensory neurons to navigate toward the main or the accessory olfactory bulbs. As a result, guidance cues responsible for the axonal projection pattern of Grueneberg axons are still unknown.

Here, we took a genetic approach to first identify the projection pattern of the Grueneberg ganglion, and found that Grueneberg ganglion axons form homogeneously innervated glomeruli among other necklace structures. It provided a unique tool for identifying potential cues involved in Grueneberg axonal wiring. We thus evaluated the role played by a guidance molecule expressed by Grueneberg sensory neurons, neuropilin 1, in the elaboration of the Grueneberg projection map in the olfactory bulb. We found that the expression of neuropilin 1 by Grueneberg axons prevents them from innervating an area restricted to the axonal projections of another chemosensor, the vomeronasal organ.

MATERIALS AND METHODS
Animals
Mice were housed and handled in accordance with the guidelines and regulations of the institution and of the state of Geneva. Nrpl1<Sup>B<Sub>ox/fox</Sup> animals were purchased from The Jackson Laboratory.
Generation of the BAC transgenic mice
A zeocin-puromycin cassette flanked by flippase recognition target (FRT) sites was inserted after an IRES-Cre-IRES-eGFP cassette. The resulting construct was inserted by homologous recombination after the stop codon of GC-G in BAC RP23-345B13 using methods described previously (Lee et al., 2001). After the removal of the zeocin-puromycin cassette by flippase in EL250 cells, the resulting bacterial artificial chromosome (BAC) was used for pronuclear injections. Eight transgenic lines were obtained, of which seven lines expressed the transgene in the Grueneberg ganglion. Line 52, which exhibited robust GFP expression in the Grueneberg ganglion, was used for further analysis.

Whole-mount analyses
After dissection of animals, whole-mount images of the Grueneberg ganglion or the olfactory bulb were taken with a Zeiss SteREO Lumar V12. z-stack images were projected to a single image by an extended focus function of Axio Vision (Carl Zeiss).

Immunohistochemistry
Tissues were fixed with 4% paraformaldehyde (PFA) in PBS for 3 hours (to 16 hours) at 4°C, followed by 25% sucrose in PBS overnight, and were embedded in optimal cutting temperature compound (OCT). Cryostat sections (10-20 μm) were mounted on Superfrost + slides. Sections were preincubated with PBS containing 0.3-0.5% Triton X-100 and 5% fetal calf serum (FCS) for 30 minutes at room temperature, then incubated with primary antibodies diluted in PBS with 0.3% Triton X-100 and 3% FCS overnight at 4°C. The following primary antibodies were used: goat anti-carbonic anhydrase II (CAII) (1:800, Santa Cruz Biotechnology), rabbit anti-GFP (1:800, Abcam), chicken anti-GFP (1:800, Abcam), rabbit anti-PDE2 (1:800, FabGennix), goat anti-OMP (1:400-800, Wako Laboratory Chemicals), rabbit anti-V2R2 (1:200) (Martini et al., 2001), goat anti-Npl1 (1:100-800, R&D Systems), rabbit anti-synaptotagmin (1:200, Sigma-Aldrich), rabbit anti-PGC-G (1:200, FabGennix), and goat anti-human semaphorin 3A (1:20, R&D Systems). Slides were washed three times with PBS and incubated with secondary antibodies for 2 hours at room temperature. The following secondary antibodies were used: donkey Cy3-conjugated anti-rabbit (1:800, Jackson Laboratory), donkey Alexa 555-conjugated anti-goat (1:800, Invitrogen), chicken Alexa 488-conjugated anti-rabbit (1:800, Invitrogen), goat FITC-conjugated anti-chicken (1:800, Abcam), donkey Alexa 488-conjugated anti-goat (1:800, Invitrogen), donkey Alexa 680-conjugated anti-rabbit (1:400, Invitrogen). Fluorescent images were taken with a confocal microscope (Zeiss 510 Meta). All sections were counterstained with DAPI (1 μg/ml).

Whole-mount immunohistochemistry
Olfactory bulbs were fixed with 4% PFA in PBS for 2 hours and washed with PBS for 20 minutes. Bulbs were dehydrated by graded methanol/PBS series (25%, 50%, 100% methanol, 10 minutes for each step). Bulbs were frozen at −80°C for 30 minutes and thawed at room temperature for 10 minutes. This procedure was repeated three times. Samples were rehydrated in 50%, 25% methanol/PBS series, washed with PBS for 20 minutes and preincubated with PBS containing 0.5% Triton X-100 and 5% FCS for 30 minutes at room temperature. This was followed by an incubation with primary antibodies diluted in PBS with 0.5% Triton X-100 and 5% FCS for 72 hours at 4°C. Primary antibodies were goat anti-carbonic anhydrase II (CAII) (1:1000, Santa Cruz Biotechnology), rabbit anti-GFP (1:1000, Abcam). After washing with PBS for 1 hour three times, bulbs were incubated overnight with secondary antibodies donkey Alexa 555-conjugated anti-goat (1:1000, Invitrogen) or chicken Alexa 488-conjugated anti-rabbit (1:1000, Invitrogen). Fluorescent images were taken with a Zeiss SteREO Lumar V12. z-stack images were projected to a single image by an extended focus function of Axio Vision (Carl Zeiss).

In situ hybridization
In situ hybridizations were performed as described previously (Roppolo et al., 2007). Briefly, tissues were embedded in OCT and 14 μm cryostat sections were mounted on Superfrost Plus slides. Slides were fixed for 20 minutes with 4% PFA, and hybridized overnight at 65°C in the following buffer: 1 X salt buffer [10 mM NaCl, 5 mM NaH2PO4, 5 mM Na2HPO4, 5 mM EDTA (pH 7.5)], 50% formamide, 10% dextran sulfate, 1 μg/μl tRNA, 1 × Denhardt’s, with 20 ng/μl cRNA probes. Following hybridizations, slides were washed twice at 65°C and once at room temperature with 1 X SSC, 50% formamide and 0.1% Tween20. Slides were preincubated in 1 × MABT, 2% blocking reagent (Roche) for 20 minutes followed by 1 hour incubation with alkaline phosphatase-anti-digoxigenin antibody (1:500, Roche) and peroxidase-anti-fluorescein antibody (1:200, Roche). Peroxidase activity was detected by washing slides three times with TNT [Tris 150 mM, NaCl 150 mM, Tween20 0.05% (pH 7.5)], incubation for 30 minutes with a biotinyl-tyramide solution (PerkinElmer), three washes with TNT and incubation for 30 minutes with streptavidin-Alexa 488 (Invitrogen). Alkaline phosphatase activity was detected by incubating slides with Fast Red substrate (DAKO) for 30 minutes. Fluorescein- and digoxigenin-labeled RNA probes were prepared using a DIG RNA Labeling Kit (Roche) following the manufacturer’s instructions. The entire coding sequence of GFP was used as probe. The Sema3a 3’UTR probe spanned nucleotides 122 to 1110 relative to the stop TGA. The sequences described in (Fleischer et al., 2007) were used for TAAR6 and TAAR7 probes.

Dil labeling
Dil (7 μl of a 250 μg/ml solution, diluted in PBS from a 5 mg/ml Dil solution dissolved in ethanol) was applied to one of the nostrils. Dil was inhaled spontaneously. Thirty minutes later, the same amount of Dil solution was applied to the other nostril. Animals were killed 2-3 days after Dil administration, and the fluorescence of the caudal olfactory bulb was analyzed by confocal microscopy (Zeiss 510 Meta).

RESULTS
A transgene that labels Grueneberg sensory neurons
In order to visualize and discriminate the minute population of Grueneberg ganglion neurons and their axonal projections in the olfactory bulb, we took a transgenic approach. It involved the insertion of a modified BAC into the mouse genome. This transgene contained the guanylyl cyclase G gene, a gene that is transcribed by Grueneberg neurons (Fleischer et al., 2009; Liu et al., 2009; Chao et al., 2010) as early as postnatal day (P) 0 (supplementary material Fig. S1E). Among olfactory sensory neurons, only those pertaining to the Grueneberg ganglion are known to express this cyclase. A double polycistrionic cassette driving the expression of the GFP marker and of the Cre recombinase was added to the cyclase gene (GCG-Cre-GFP transgene; Fig. 1B). Eight GCG-Cre-GFP founders were obtained. Seven GCG-Cre-GFP lines expressed the transgene at different levels in the Grueneberg ganglion. A line with robust expression in Grueneberg neurons was selected for further analysis (Fig. 1C).

An exclusive labeling of V2R2-expressing neurons
The molecular receptors involved in the recognition of Grueneberg stimuli are still elusive. However, a few potential chemoreceptors, which include the vomeronasal panreceptor V2R2 (alternatively known as V2R83), have been identified in Grueneberg neurons (Fleischer et al., 2006b). V2R2 transcripts are found in virtually all Grueneberg neurons in young mice and in adults. The GCG-Cre-GFP transgene labeled olfactory marker protein (OMP) (supplementary material Fig. S1A-D) and V2R2-expressing Grueneberg neurons in P7 mice (supplementary material Fig. S1F-H). A few non-V2R2-expressing neurons were found to express trace amine-associated receptors (TAARs) by in situ hybridization (Fleischer et al., 2007). These were not labeled by the transgene (supplementary material Fig. S1J). The number of these TAAR
positive cells was extremely low, and close to zero in adults. We thus observed that for the transgenic line selected, the large majority if not all of the Grueneberg sensory neurons were GFP positive, whereas the fluorophore was absent from other olfactory sensory populations (Fig. 2D; data not shown). This provided a precise and unique tool to follow the Grueneberg axonal projections, from the ganglion somata to the corresponding glomeruli.

Homogeneous and early fasciculation of Grueneberg axons
An indirect way to evaluate the molecular proximity between olfactory subpopulations is to analyze their putative physical association during axonal migration. Thus, in the vomeronasal system, V1R- and V2R-expressing axons fasciculate together in bundles while running along main olfactory axonal bundles, with which they do not intermingle. This is also true between subpopulations of main olfactory sensory neurons, which segregate early during axonal fasciculation (Imai et al., 2009). We examined Grueneberg fibers from GCG-Cre-GFP mice to evaluate their possible joining and intermingling with other fibers. When leaving the Grueneberg ‘grape-like’ ganglion, axons coalesced into a single or a few bundles. At the tip of the nose, the bundle had a diameter of about 20 µm and was already dense, in the sense that it appeared almost exclusively composed of Grueneberg fibers (Fig. 2A-C). It then entered the area covered by the main olfactory epithelium, where it ran under the neuroepithelium (Fig. 2D). It was found associated with main olfactory bundles, but did not appear to intermingle with them. Scale bars: 100 µm in C, left panel; 20 µm in C, right panel.

Grueneberg ganglion glomeruli are present at birth
The olfactory system is not mature at birth. This is true for both the main olfactory and the vomeronasal systems. The sensory part of the latter first contacts the outside world only a few days after birth. To evaluate the developmental state of the Grueneberg system neonatally, we looked for the potential presence of Grueneberg ganglion fibers and glomeruli in the olfactory bulb in neonates. We analyzed six P0 GCG-Cre-GFP mice, and found, in all of them, OMP-positive Grueneberg ganglion fibers that had coalesced in the necklace glomeruli (Fig. 3A-D). These glomeruli were positive for the presynaptic marker synaptotagmin (Fig. 3E), in contrast to most olfactory glomeruli at this stage, suggesting an early maturation of the Grueneberg circuitry. This early presence of Grueneberg glomeruli was not detected in previous reports (Koos and Fraser, 2005), probably because of the use of less sensitive, non-genetic
GFP-expressing Grueneberg glomeruli (G) can already be observed around the rostral part of the accessory olfactory bulb, and defined mouse. At birth, Grueneberg fibers are organized in a necklace shape divided in two (or sometimes more) smaller bundles, one aiming at the dorsal part of the necklace, the other at the ventral part, strictly avoiding the accessory olfactory bulb. A ventral view of the olfactory bulb of a P0 GCG-Cre-GFP transgenic mouse. Scale bars: 200 μm in A; 50 μm in B,E.

The topographical map of Grueneberg ganglion glomeruli
We then aimed at a global analysis of the topographical map formed by Grueneberg glomeruli in the olfactory bulb. Taking advantage of the very superficial innervation of the bulb by Grueneberg axons, we analyzed whole-mount preparations of olfactory bulbs from GCG-Cre-GFP transgenic mice. We observed dorsal, medial, ventral and lateral views of the bulbs of female P7 mice (n=3) (Fig. 4A-D). After leaving the cribriform plate, the Grueneberg bundle followed the medial side of the olfactory bulb (Fig. 4B). Before reaching the necklace glomeruli, the bundle divided in two (or sometimes more) smaller bundles, one aiming at the dorsal part of the necklace, the other at the ventral part, strictly avoiding the accessory olfactory bulb. A ventral view of the bulb shows that fibers pertaining to the two bundles possibly rejoin each other at the very bottom of the bulb (Fig. 4C). No stray fibers were observed.

The lack of fixed structures for most views did not allow an easy and precise comparison between individuals. We thus decided to restrict our precise analysis of the topographical map to the glomeruli that were visible from the dorsal view of the bulb, a view that benefited from the fixed position of the accessory olfactory bulb. We compiled the dorsal glomerular pattern of six animals (supplementary material Fig. S2A). Grueneberg glomeruli were disposed along the entire length of the necklace glomeruli, and did not exhibit any specific organization, or at least no zones in which glomeruli would be more likely to form. We then evaluated a possible evolution of the pattern with age, and analyzed mice at weaning (P21), and adult animals (Fig. 4E-G). We drew maps similar to the ones generated for P7 mice (supplementary material Fig. S2B-D), and observed, again, no specific zone of convergence. The number of glomeruli was stable from one week to adulthood: at P7 (9.9±1.6), at P21 (9.7±1.8) and at P60 (10.8±1.5) (Fig. 4H). We did not observe differences in number of glomeruli or projection patterns between genders (supplementary material Fig. S2C,D).

These data show that Grueneberg axons project around the entire olfactory trunk, unlike previous reports that only found innervation of the dorsal part of the necklace by Grueneberg fibers (Fuss et al., 2005; Roppolo et al., 2006). Again, similar to our identification of Grueneberg glomeruli in newborns (which was not observed by others), it probably reflects poorly sensitive tracing methods that were used in the past.

GC-G and GC-D axons project to different necklace glomeruli
The necklace glomeruli are a composite group of neuropil-rich structures. They are both innervated by Grueneberg ganglion axons (which express the cyclase GC-G) and by main olfactory neurons (which express the cyclase GC-D). These two sensory populations not only share their peculiar necklace-shaped axonal projections and the expression of a guanylyl cyclase, but they also express elements of a cGMP second messenger pathway that includes the cGMP-stimulated phosphodiesterase PDE2 and the nucleotide-gated channel CNGA3 (Juilfs et al., 1997; Meyer et al., 2000; Mamasuew et al., 2008; Fleischer et al., 2009; Liu et al., 2009). This naturally suggests potential parallels in terms of signal processing or function between these sensory populations. Owing to these intriguing common characteristics, and to the co-convergence of similar but not identical olfactory fibers in specific necklace glomeruli (Cockerham et al., 2009), we investigated the potential crosstalk (i.e. co-convergence) between GC-D and Grueneberg axonal projections (Fig. 5A). In order to discriminate between these two types of glomeruli in the necklace structure, we took advantage of a peculiar characteristic of Grueneberg neurons: in live mice, Grueneberg sensory neurons do not incorporate DiI when the dye is introduced into the nostrils, possibly owing to their lack of direct contact with the outside world. By contrast, main olfactory sensory neurons, including GC-D-positive cells, incorporate the dye, which is readily transported toward the bulb. We took this approach to label olfactory sensory neurons from P5-P7 transgenic mice (n=9). We thus obtained mice with green and red fluorescent Grueneberg ganglion and GC-D/main olfactory neuron axonal fibers, respectively. No co-innervation between the two populations was observed (Fig. 5B,C). We further confirmed the separate nature of the Grueneberg and main olfactory glomeruli by immunohistochemistry with the specific GC-D and necklace markers carbonic anhydrase II (CAII) and PDE2, respectively (Fig. 5D,E). Thus, Grueneberg ganglion neurons and GC-D sensory neurons, although both targeting the necklace glomeruli, do not wire, nor converge together, suggesting a parallel processing of information.
Grueneberg ganglion axons project dorsally relative to GC-D axons

The narrow band occupied by the necklace glomeruli appears to be composed of glomeruli randomly organized along its width. We took a close look at this question and examined dorsal views of the olfactory bulb. We recorded the position of Grueneberg ganglion and GC-D glomeruli in seven bulbs of 3-week-old animals. Both Grueneberg ganglion axons and glomeruli were found rostralized relative to those of GC-D-expressing neurons on the dorsomedial part of the necklace (Fig. 6A-D). This topographic exclusion was not observed or was unclear in other parts of the necklace (supplementary material Fig. S3A-C). This observation suggests that the guidance cues that direct the projection map of Grueneberg ganglion and GC-D axons are not identical.

Neuropilin 1-dependent wiring of Grueneberg ganglion axons

In order to investigate the factors involved in the peculiar organization of Grueneberg ganglion axons, we evaluated Grueneberg sensory neurons for potential expression of neuropilin 1, a guidance molecule that was shown to be involved in the wiring of specific olfactory populations, in particular in pre-target axon sorting (Imai et al., 2009). We found expression of neuropilin 1 in Grueneberg axons and glomeruli in neonates (Fig. 7A,B). In Grueneberg glomeruli of older animals (3 weeks old), the presence of the protein was no more, or barely detectable in Grueneberg glomeruli (data not shown). We did not detect neuropilin 1 expression in the accessory olfactory bulb (Fig. 7B; data not shown).

In the main olfactory system, the level of neuropilin 1 expression is heterogeneous and is dependent on the odorant receptor expressed (supplementary material Fig. S4A-C). This heterogeneous expression is crucial in the axonal guidance process of these sensory neurons. We assessed whether a similar heterogeneous neuropilin 1 expression was present in Grueneberg axonal fibers, and found homogeneous levels of expression between Grueneberg axons (data not shown).

We then explored the expression pattern of the main neuropilin 1 ligand, Sema3a, in the vicinity of the target of Grueneberg axonal projections, i.e. around the necklace glomeruli. We made two observations at the time Grueneberg glomeruli are formed, between embryonic day (E) 19 and P5. First, mitral cells that innervate glomeruli of the main olfactory and accessory olfactory bulbs transcribe Sema3a (Fig. 7C-E). These transcripts were not present in cells located directly beneath Grueneberg glomeruli (Fig. 7C-E), thus creating a narrow semicircular band devoid of Sema3a transcripts that correlates with the position of the rostral necklace glomeruli. Second, vomeronasal sensory neurons transcribe Sema3a at P0 (Fig. 7F), a transcription reflected by its corresponding protein in vomeronasal axonal fibers in the accessory olfactory bulb (Fig. 7G). The distribution of the Sema3a protein in the AOB is thus broader than the in situ data suggest.

To evaluate the potential role played by neuropilin 1 in Grueneberg axonal wiring, we first generated a mouse bearing a neuropilin 1 null allele (Nrp1del), by crossing a line bearing a neuropilin floxed allele (Nrp1flox) to a strain expressing the Cre recombinase constitutively. We then took advantage of the GCG-Cre-GFP transgenic line we produced, which in addition to the GFP fluorophore also drives the expression of the Cre recombinase. We generated Nrp1del/flox;GCG-Cre-GFP and Nrp1flox/flox;GCG-Cre-GFP animals, which were missing neuropilin 1 exclusively in Grueneberg ganglion neurons. The reason for using compound Nrp1 heterozygotes was to facilitate the Cre-mediated generation of two null alleles. We first evaluated potential defects in axonal bundle formation of Nrp1del/flox;GCG-Cre-GFP and Nrp1flox/flox;GCG-Cre-GFP mice. We found no alterations in their compactness or potential intermingling with non-like fibers (supplementary material Fig. S4D,E). We then analyzed, in whole mounts, the corresponding Grueneberg axonal projections. As a control, we evaluated the projections of Nrp1+/-;GCG-Cre-GFP and of Nrp1flox/flox;GCG-Cre-GFP mice. We could not distinguish them from those of Nrp1+/+;GCG-Cre-GFP mice (Fig. 8A,B; data not shown). By contrast, all Nrp1del/flox;GCG-Cre-GFP animals analyzed exhibited a striking alteration of the Grueneberg projections (n=6). The formation of Grueneberg glomeruli and their exclusion from GC-G glomeruli was not affected (supplementary material Fig. S4A-C), but a large number of Grueneberg axonal...
Our results thus support a key role played by neuropilin 1, in the avoidance of Grueneberg neurons of the accessory olfactory bulb.

**DISCUSSION**

By combining a transgenic approach with imaging techniques, we performed an analysis of the axonal wiring of the Grueneberg ganglion, one of the rodent olfactory subsystems. We show that the necklace glomeruli are composed of homogeneous units, innervated by either one of two peculiar sensory populations: the GC-D-expressing neurons from the main olfactory system and those from Grueneberg ganglion. Further, taking advantage of our transgenic tool associated with conditional null alleles, we then show that the expression by Grueneberg sensory neurons of a specific guidance molecule, neuropilin 1, is involved in the establishment of this wiring diagram.

**A specific circuitry for Grueneberg axons**

We first took a look at the way Grueneberg ganglion axonal projections leave the anterior nasal vestibule. We observed an early fasciculation of Grueneberg fibers into one or two axonal bundles. This fasciculation was not affected by the later joining of axonal bundles from the main olfactory epithelium, and remained homogeneous until the fibers reached the caudal part of the olfactory bulb. This early segregation between groups of axonal fibers before reaching their target is consistent with reports that show in the main olfactory system an early sorting of axons pertaining to different olfactory sensory neuron subpopulations (Imai et al., 2009). Grueneberg axons are thus likely to be recognized by main olfactory bundles as a non-like group of olfactory fibers. After crossing the cribriform plate and running along the medial olfactory bulb, Grueneberg axons project to a unique chain of glomeruli, the necklace glomeruli, and surround the entire trunk of the olfactory bulb. The peculiar morphological characteristics of the Grueneberg ganglion sensors are thus reflected at the level of the Grueneberg circuitry, at least up to the olfactory bulb.

The role played by the Grueneberg system is still unclear and is apparently multimodal. A few observations may provide insights into this question. The first observation is linked with the maturation of the Grueneberg system, which we found unusual, as Grueneberg synapse-containing glomeruli are already present in newborn animals. This early maturation of axonal wiring is not the rule for olfactory sensors. Many glomeruli from the main olfactory system are indeed formed during and after the perinatal period. Vomeronasal glomeruli also appear late, probably reflecting the postnatal maturation of this system (in mice, the duct that connects the vomeronasal organ with the outside world opens after the first week of life). The apparent maturity of the Grueneberg system at birth is thus potentially functionally meaningful. It would be consistent with the known activators of the Grueneberg ganglion: namely ‘fear’-induced compounds and cold temperature, two stimuli that could trigger stress, and perception of which could be beneficial to animals of all ages, including newborns. Possibly linked to the latter stimulus, cold temperatures, which induce c-fos transcription in the Grueneberg ganglion of neonates (Mamasuew et al., 2008), trigger ultrasonic vocalizations that are attractive stimuli to the lactating mother. A second observation that may provide insight into the role played by the Grueneberg system may reside in its wiring pattern. The innervation of the bulb by Grueneberg fibers is reminiscent of olfactory subsystems processing socially relevant chemical information. We found about ten Grueneberg...

![Image](https://example.com/image.png)
Neuropilin 1 and Grueneberg axonal projections

How the olfactory maps in the bulb are built is still unclear. The adequate targeting of a functionally homogeneous olfactory sensory axonal population (i.e. expressing a given olfactory chemoreceptor) first requires a capacity to reach a defined area in the olfactory bulb, followed by an ability to coalesce. A few molecules have been shown to play crucial roles in both gliomerulus formation, and in the topographical position of these glomeruli (Cho et al., 2007; Sakano, 2010). In the main olfactory system, in a given sensory neuron, expression of several of these molecules is dependent on the olfactory receptor gene transcribed. It was proposed that each olfactory receptor defines a specific level of cAMP in the olfactory sensory neuron, which in turn drives the expression of a specific set of homophilic adhesion/repulsion and axon guidance molecules, such as krrels, ephrins, and neuropilin 1 (Serizawa et al., 2006; Cho et al., 2007; Imai et al., 2006; Imai et al., 2009; Sakano, 2010; Takeuchi et al., 2010). These guidance receptors can exert a direct effect on the sensory neurons that express them, or can affect the targeting of other neurons.

Most of the studies aimed at understanding olfactory guidance mechanisms targeted the main and the vomeronasal olfactory systems. Thus, the axonal guidance signals that are involved in the establishment of the narrow chain of necklace glomeruli are almost entirely unknown today.

As for the rest of the olfactory map, the formation of the necklace glomeruli surely relies on distinct sets of guidance cues, involved in their general targeting and their ability to coalesce. The very peculiar trajectory of necklace glomeruli around the olfactory bulb suggests either a strong attractive signal present on this path, or concomitant repulsive signals from the structures flanking the glomerular chain (i.e. from the main olfactory and the accessory olfactory bulbs, respectively) to maintain it sandwiched in between. A report suggests that the repulsive hypothesis is correct, at least for the avoidance of structures located rostrally relative to the necklace: a deficiency in expression of neuropilin 2 alters the projection map formed by GC-D-expressing neurons in the necklace glomeruli in mice (Walz et al., 2007). In these null animals, some of the GC-D axonal projections miswire and end their course earlier, in the glomerular layer of the main olfactory bulb. Neuropilin 2 plays thus a role in preventing GC-D axons from arborizing too early, and in innervating rostral areas of the main olfactory bulb. Our observations point to a signal that plays a reverse but complementary role, that is to prevent the overshooting of the Grueneberg axonal projections past the necklace. We found that at a time Grueneberg sensory axons navigate toward the bulb, they express the neuropilin 1 protein. The genetic deletion of its corresponding gene, when restricted to Grueneberg neurons, allows Grueneberg axons to cross the area occupied by the accessory olfactory bulb, an area that is usually entirely devoid of Grueneberg fibers. The overshooting control is thus at least partly played by neuropilin 1. That is the first step in axonal guidance, the general positioning, and not the second step that affects coalescence, which is apparently independent of this protein (as we did not observe co-convergence of Grueneberg axons lacking neuropilin 1 and GC-G axons).

It is tempting to speculate that our miswiring observation results from the lack of response by Grueneberg axons to a repulsive neuropilin 1 agonist such as Sema3a in the accessory olfactory bulb. We in fact observed an expression pattern of this repulsive protein (as we did not observe co-convergence of Grueneberg axons lacking neuropilin 1 and GC-G axons).

Necklace glomeruli per bulb, apparently connected together. This type of wiring is unlike the canonical projection pattern observed in the main olfactory system, but is reminiscent of the one found in the vomeronasal system, where the numerous like glomeruli are often linked together, and is very similar to the one corresponding to GC-D-expressing neurons. Knowing that both vomeronasal and GC-D neurons are specialized in interpreting innate and/or socially relevant signals (Halpern and Martinez-Marcos, 2003; Munger et al., 2010), it naturally suggests that Grueneberg neurons play a role in inter-individual interactions. Making parallels between these different systems should, however, be tempered. Necklace glomeruli are unusual in their innervation: they receive heterogeneous sensory input from main olfactory neurons. They integrate axonal projections from GC-D-expressing neurons, and from another, still elusive population expressing OMP (Cockerham et al., 2009). This very unusual stimulus coding strategy (that is for the sensory part of the olfactory system), which involves multiple and functionally distinct sensory neuron subtypes, led to the suggestion that Grueneberg glomeruli could also share this heterogeneous innervation pattern. We report here homogeneously innervated Grueneberg glomeruli. There is thus no direct crosstalk between Grueneberg axonal projections and other types of olfactory sensory projections, at least until the first projection relay. Thus, the Grueneberg system, although probably involved in innate and/or social interactions like GC-D neurons, possibly plays a different role.

Fig. 6. Dorsomedial Grueneberg ganglion axons project rostrally relative to GC-D axons. (A-C) Topographical position of Grueneberg (green) and GC-D (red) glomeruli on a dorsal view of the necklace glomeruli from the left bulb of 3-week-old GCG-Cre-GFP transgenic mice. GC-D fibers were immunolabeled in red with an anti-CAII antibody and Grueneberg axons were immunolabeled in green with an anti-GFP antibody. (D) The glomerular position of Grueneberg (green) and GC-D (red) glomeruli corresponding to a dorsal view of the necklace glomeruli of seven bulbs from GCG-Cre-GFP mice is shown. Scale bar: 500 μm in A.
specific place. What remains to be found (something that is also lacking for the other olfactory subsystems), is a comprehensive understanding of all players involved. These are undoubtedly specific although partially overlapping for each olfactory subpopulation, as their expression leads to highly stereotyped and specific projection patterns.
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Competing interests statement
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Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.077008/-/DC1

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