Kappe neurons, a novel population of olfactory sensory neurons

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Perception of olfactory stimuli is mediated by distinct populations of olfactory sensory neurons, each with a characteristic set of morphological as well as functional parameters. Beyond two large populations of ciliated and microvillous neurons, a third population, crypt neurons, has been identified in teleost and cartilaginous fishes. We report here a novel, fourth olfactory sensory neuron population in zebrafish, which we named kappe neurons for their characteristic shape. Kappe neurons are identified by their Gα-like immunoreactivity, and show a distinct spatial distribution within the olfactory epithelium, similar to, but significantly different from that of crypt neurons. Furthermore, kappe neurons project to a single identified target glomerulus within the olfactory bulb, mdg5 of the mediodorsal cluster, whereas crypt neurons are known to project exclusively to the mdg2 glomerulus. Kappe neurons are negative for established markers of ciliated, microvillous and crypt neurons, but appear to have microvilli. Kappe neurons constitute the fourth type of olfactory sensory neurons reported in teleost fishes and their existence suggests that encoding of olfactory stimuli may require a higher complexity than hitherto assumed already in the peripheral olfactory system.

Results

A homogenous population of olfactory sensory neurons with characteristic shape and spatial position is labeled by Gα antibody. Gα-positive neurons have been described as a morphologically heterogenous
population including cells with the globose shape typical of crypt neurons. We suspected that at least part of this heterogeneity might be due to different sectioning angles of the labelled cells. Therefore we engaged in analysis of distributions for different cell shape and position parameters, as opposed to focusing on single cell properties. In our experience the former approach is much more powerful, and allows to distinguish homogenous from heterogenous cell populations with high sensitivity and accuracy.

We report here that Go-ir labels a sparse population of pear- or bottle-shaped cells with a characteristic cap of intense Go-ir at the apical end of the cells (Fig. 1a, b, c). We have used the ratio of horizontal to vertical diameter of these cells as measure of their shape $c_f$. A value of 1 would correspond to a perfectly circular shape, with decreasing values pointing to increasingly elongated shapes. We find a median value of 0.66 for Go-ir-positive neurons, distinctly lower than that of crypt neurons and see below, but also much larger than that of ciliated neurons and see below. The distribution of diameter ratios is narrow (Fig. 1f), consistent with a homogenous population. Importantly, the empirical cumulative distribution function (ECDF) of the diameter ratio is a single sigmoid curve (Fig. 1g), indicative of a homogenous population (a mix of different populations would result in either a step or quasi-linear function, $c_f$).

Beyond a characteristic shape, Go-ir-positive neurons also have a very conspicuous position within the olfactory epithelium. It is known that such restricted spatial distributions are characteristic parameters for particular subpopulations, e.g. olfactory sensory neurons expressing a particular receptor. Across a lamella, Go-ir-positive neurons tend to lie very apical, close to the lumen and far away from the basal lamina. We quantified this parameter as relative height ($h_{rel} = h_{center}/$thickness of sensory layer; 0, basal; 1, apical). The histogram shows a rather narrow peak and the ECDF exhibits a sigmoidal shape, indicative of a homogenous population (Fig. 1f, g).

Within a lamella, the distance of Go-ir-positive cells to the median raphe, the center of the epithelium, is generally rather small, compared to the full extent of the sensory epithelium. We quantified this coordinate as relative radius ($r_{rel} = r_{center}/$length of the lamella; 0, innermost; 1, outermost, $c_f$). The histogram of radial distance values shows a steep and narrow peak, and the corresponding ECDF exhibits a sigmoidal shape consistent with a homogenous population (Fig. 1f, g). Within the epithelium, a third axis is defined by the series of horizontal sections. Here, the majority of Go-ir-positive cells were concentrated in a few sections close to the opening of the cup-shaped epithelium, and far away from the basal region containing the olfactory nerve bundles (Fig. 1f, g).

Taken together, we have quantified four different morphological and spatial parameters for Go-ir-positive cells. All four distributions are consistent with the presence of a single, homogenous cell population. Go-ir-positive cells are also labeled by zns2-immunoostaining, a general marker for sensory neurons, and in high magnification initial axon segments are visible, suggesting that Go-ir-positive cells are indeed neurons (Fig. 1a).

Furthermore, whole mount immunohistochemistry of the olfactory bulb using Go antibody results in the labeling of a single, bilateral symmetric glomerulus (Fig. 1d, e), confirming that these cells are sensory neurons that convey information to the brain. Comparison with zns2-immunoostaining, which labels the entire glomerular pattern, allows to identify the Go-ir-positive glomerulus as mdg5, consistent with a previous report. Between 200 to 500 Go-ir-positive neurons are present in a single olfactory epithelium, which is well within the range expected to innervate a single glomerulus, $c_f$.

Go-ir-positive neurons are different from ciliated neurons. The apical laminar position of Go-ir-positive neurons is roughly similar to that of crypt neurons, even though their shape is generally somewhat more slender than that of crypt neurons $c_f$. However, cells with morphology similar to that of crypt neurons have been reported in the Go-ir-positive cell population. Therefore we have used the Go antibody in parallel with an established crypt neuron marker, S100-ir, to examine a potential overlap between these two markers. We report here that Go-ir and S100-ir label mutually exclusive cell populations (Fig. 2a–c, f). Also, as shown above, Go-ir-positive neuron terminals in the olfactory bulb innervate a different glomerulus, mdg5, compared to mdg2, the crypt neuron glomerulus.

Furthermore, we have examined the shape and spatial distribution of Go-ir-positive and crypt neurons identified by a second marker, TrkA-ir, in alternating sections to obtain a stringent comparison of the properties of both cell populations. We find subtle, but highly significant (p < 10$^{-4}$) differences in relative height and relative radius between the two populations (Fig. 2e, g). Go-ir-positive neurons are even more apically situated within the lamella than TrkA-ir-positive neurons (maximal difference between the distributions 25%), and they are found closer to the median raphe than crypt neurons (maximal difference between the distributions 22%). Within the entire olfactory epithelium, Go-ir-positive neurons are found in more apical sections closer to the opening of the cup-shaped olfactory organ, compared to TrkA-ir-positive neurons (Fig. 2h).

Finally, we have investigated whether Go-ir-positive neurons might belong to either microvillous or ciliated neuron populations.

Go-ir-positive neurons are different from ciliated and microvillous neurons. Ciliated neurons in teleosts specifically express the olfactory marker protein (OMP), and a transgenic line is available, in which the OMP promoter faithfully drives expression of a red fluorescent protein (RFP), Tg(OMP:lynRFP). We performed Go immunoostaining with transgenic epithelia, and report here that almost all Go-ir-positive neurons (98%) are negative for RFP (Fig. 3a, f). This suggests that Go-ir-positive neurons do not belong to the population of ciliated neurons.

Moreover, a comparison of cell shape and preferred laminar position within the lamella shows highly significant differences between Go-ir-positive and OMP-positive neurons. Ciliated neurons tend to have a very slender shape, and rather basal cell bodies, whereas Go-ir-positive neurons are mostly pear-shaped and are found at extreme apical positions within the lamella, even more apical than crypt neurons (Fig. 3e, g, h). Only 1–2% overlap are observed between Go-ir and OMP distributions (Fig. 3h).

Next, we investigated, whether Go-ir-positive neurons might express the microvillous neuronal marker TRPC2. First, we employed a transgenic line, which largely reproduces the endogenous TRPC2 pattern, Tg(TRPC2:gap-Venus). We report that Go-ir-positive cells were negative for Venus fluorescence (Fig. 3b, f). Secondly, we also performed direct double labeling in wildtype zebrafish, detecting Go-ir by immunostaining and TRPC2 by in situ hybridization. Again, almost all Go-ir-positive cells (>98%) were negative for TRPC2 signals (Fig. 3c, f), suggesting that Go-ir-positive neurons are different from microvillous neurons.

Furthermore, a comparison of cell shape and preferred laminar position within the lamella shows highly significant differences between Go-ir-positive and TRPC2-positive neurons (p < 10$^{-4}$). Microvillous neurons are somewhat slender in shape, and their cell bodies are preferentially located more basal than crypt neuron somata, whereas Go-ir-positive neurons are mostly pear-shaped and are found at extreme apical positions within the lamella, more apical than crypt neurons (Fig. 3c, e, g, h).

Finally we have examined a potential overlap of Go-ir-positive neurons with the calretinin-positive population of olfactory sensory neurons. Calretinin appears to label subpopulations of ciliated and
Figure 1 | Gα-like immunoreactivity reveals a distinct population of sparse, pear-shaped sensory neurons in zebrafish olfactory epithelium. (a) Gα-ir (green) is seen in a sparse population of pear-shaped cells in horizontal sections of the olfactory epithelium (short-fixed), using DAPI as counter-stain (blue); r, radial distance. Top right inset at higher magnification shows a Gα-ir-positive cell (green), co-labeled with zns2 (red), and visible nucleus (DAPI, blue). Bottom left inset at higher magnification shows a Gα-ir-positive cell with initial axon segment (ax) and cap (cp). (b) At higher magnification the apical position of Gα-ir-positive cells (green) is clearly visible. øv, vertical cell diameter; øh, horizontal cell diameter; h, laminar height; dotted half-circle, the apical dendritic part of Gα-ir-positive olfactory sensory neurons resembles a cap. (c) Nine Gα-ir-positive cells show the typical range of morphologies for these neurons. (d) Whole mount of adult zebrafish olfactory bulb double-labeled with anti- Gα and anti-zns2 antibodies, dorsal view. Zns2 labels all glomeruli, whereas Gα-ir labels a single medial glomerulus (yellow). The olfactory nerves were cut at the entrance to the olfactory bulb before staining. (e) Horizontal vibrotome cross-section (100 μm) reveals the extremely dorsal position of the Gα-immunoreactive glomerulus in each olfactory bulb. A single, thick axon bundle is seen entering the glomerulus. (f,g) One shape parameter and three spatial parameters were quantified for the Gα-ir-positive cell population, and shown as histogram (f) and empirical cumulative distribution function, ECDF (g). From left to right: ratio of horizontal to vertical diameter, laminar height (normalized to maximal height), radial distance (normalized to maximal radius), and number of cells per 10 μm horizontal cross section of the olfactory epithelium; x axis units and labels are valid for both (f) and (g). Scale bars correspond to 50 μm (a), 5 μm (b), and 100 μm (d, e).
Again we observe no co-localisation for Gα-ir and calretinin (<1%, Fig. 3d, f), confirming the results for ciliated and microvillous neuron markers (OMP and TRPC2, respectively). We note that both cell shape and laminar height distribution of calretinin-positive neurons are identical to the respective distributions of OMP-positive neurons, which itself show nearly no overlap with those of TRPC2-positive neurons (Fig. 3e, g, h). These results are consistent with calretinin-positive neurons being ciliated neurons, cf.10. In summary, Gα-ir-positive neurons exhibit a conspicuous distinct shape and preferred laminar position, significantly different from the morphology and location observed for the three known populations of olfactory sensory neurons. Moreover, molecular markers for ciliated, microvillous and crypt neurons are absent in Gα-ir-positive neurons. We conclude that Gα-ir-positive neurons do
not belong to the three known populations, but constitute a fourth type of olfactory sensory neuron. Due to their conspicuous ‘cap’ (German: Kappe) we suggest to name this novel population kappe neurons.

Kappe neurons are tubulin-negative and actin-positive. We performed immunostaining with anti-tubulin and anti-actin antibodies together with Gα antibody to further characterize kappe neurons. We report that tubulin staining mostly does not overlap with Gα-ir.
Rare cases of overlap may be due to technical reasons, since tubulin-positive cilia of ciliated neurons are densely packed in the apical layer, cf. (Fig. 4a). Since tubulin is an essential component of cilia\textsuperscript{17} we conclude that kappe neurons do not possess cilia. Microvilli, on the other hand, require actin as essential component\textsuperscript{18}. We observe nearly complete co-labeling of Go-ir and actin (Fig. 4e), with the actin antibody consistently labeling a small apical spot within the cap of kappe neurons (Fig. 4b). Although immuno-EM studies will be required to draw a firm conclusion, these results suggest that kappe neurons may possess microvilli.

**Discussion**

Three different types of olfactory sensory neurons are known in the vertebrate sense of smell, ciliated, microvillous and crypt neurons\textsuperscript{4}. Here we report the presence of a fourth type of olfactory sensory neurons, kappe neurons, identified by the presence of G\textsubscript{a}-ir\textsuperscript{19}, which do not express the molecular markers commonly accepted as defining ciliated, microvillous, and crypt neurons. We used a population-based quantitative approach to characterize kappe neurons, and show them to be highly significantly different in shape and spatial location from each of the three previously known populations of olfactory sensory neurons. Kappe neurons are a rare cell population with a few hundred cells per olfactory organ, consistent with the expression of only one or very few olfactory receptor genes in this type of sensory neurons. Thus, it is conceivable that additional such populations of olfactory sensory neurons might exist, cf.\textsuperscript{19,20}, but they would presumably only come into view after identification of a molecular marker specific for such a population.

It is not clear, whether G\textsubscript{a}-ir labels the same type of kappe neurons in other teleost fish species. Different patterns of G\textsubscript{a} immunoreactivity have been reported for different fish species, both for sparse neuron populations described as crypt-like neurons\textsuperscript{21–23} and for frequent neuron populations of undefined\textsuperscript{19} or microvillous\textsuperscript{21,24} phenotype. It is conceivable that in the absence of knowledge about kappe neurons, some may have been mistaken as crypt neurons in those earlier studies, since kappe neurons are more similar to crypt neurons than to the other two populations, ciliated and microvillous neurons. In any case, the observed species differences preclude the use of G\textsubscript{a}-ir as a defining criterion of kappe neurons in other fish species.

Kappe neurons project to a single glomerulus in the mediodorsal cluster, mdg5 (this manuscript, see also\textsuperscript{19}). With the identification of kappe neurons two of the six glomeruli in this cluster have been shown to be innervated by distinct populations of olfactory neurons, mdg5 by kappe neurons and mdg2 by crypt neurons. It will be interesting to see, whether this observation will be generalizable to the remaining four glomeruli in this cluster. Indeed, all six mediadorsal glomeruli are negative for ciliated and microvillous markers\textsuperscript{7} in the double transgenic line also used here.

The presence of G\textsubscript{a}-like immunoreactivity in kappe neurons could suggest G\textsubscript{a} as a possible signal transduction molecule for these neurons. However, the subcellular distribution of G\textsubscript{a}-ir in dendrite, cytoplasm, axon and axon terminals is unexpectedly broad. Additionally, in situ hybridization with G\textsubscript{a} shows a large and broadly distributed cell population\textsuperscript{19}, inconsistent with the small and spatially restricted population of G\textsubscript{a}-positive neurons. Thus, we cannot exclude that G\textsubscript{a}-ir in kappe neurons might be caused by a cross-reacting antigen, reminiscent of the situation for standard molecular markers of crypt neurons, S100-ir\textsuperscript{19} and TrkA-ir\textsuperscript{19}.

Kappe neurons feature a dot of intense actin signal somewhat recessed on their apical cap, suggesting the presence of microvilli in these neurons. However, in all other aspects investigated (shape, location, molecular markers), kappe neurons are highly significantly different from microvillous neurons, and in particular they do not express TRPC2, the accepted molecular marker and signal transduction molecule of microvillous neurons.

The functional role of kappe neurons is not known so far, but their sheer existence shows an astonishing complexity of odor representation already in the periphery of the olfactory system.

**Methods**

**Antibodies, tissue and animal handling.** Primary antibodies used were anti-S100 antibody (rabbit IgG; 1: 500; catalog no. Z0311, Dako), anti- G\textsubscript{a} (K-20) antibody (rabbit IgG; 1: 50; sc-387, Santa Cruz Biotechnology), anti- TrkA (763) antibody (rabbit IgG; 1: 100; sc-118, Santa Cruz Biotechnology), anti-zns2 (monoclonal mouse IgG1; 1: 200; Swant (Bellinzona, Switzerland), anti-tubulin (mouse monoclonal antibody IgG, 1: 300; G712, Promega) and beta-actin (mouse monoclonal antibody; A5411; 1: 300; Sigma). Secondary antibodies used were donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (A21206, Invitrogen), goat anti-rabbit IgG conjugated to Alexa Fluor 594 (A11012, Invitrogen) and goat anti-mouse conjugated to Alexa Fluor 594 (A11005, Invitrogen).

Adult wild type zebrafish (Ab/Tü strain, 8–12 months old) were maintained at 28°C on 14-hour light/dark cycle. Adult fish were sacrificed by decapitation during anesthesia with MS-222 (ethyl 3-aminobenzoate, Sigma). Those experiments

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**Figure 4** | Kappe neurons are tubulin-negative and actin-positive. Double labeling of G\textsubscript{a}-ir-positive cells with anti-tubulin or anti-actin antibody is analysed in horizontal cryostat sections of olfactory epithelia. (a) Double fluorescent labeling of G\textsubscript{a}-ir (green) and tubulin (red) shows absence of co-localization; scale bar 10 pm. The insets represent magnified images of single neurons taken at 100× magnification, 0.1 pm optical sections. (b) Double fluorescent labeling of G\textsubscript{a}-ir (green) and actin (red) shows co-localization: G\textsubscript{a}-ir positive neurons exhibit highly localized actin staining at the apical surface of their cell bodies, the expected position for microvilli. Scale bar 10 pm. The insets show single neurons, images taken at 100× magnification, 0.1 pm optical sections. (c) Quantification of co-label for G\textsubscript{a}-ir and actin or tubulin, respectively, shows over 90% co-label (yellow) for actin, but less than 10% co-label for tubulin. The small number of G\textsubscript{a}-ir/tubulin co-labeled cells is likely to result from the dense packing of cells, dendrites, cilia and microvilli, at the limit of light-microscopic resolution. (d) Schematic representation of four types of olfactory sensory neurons with their laminar position. Ciliated neurons (orange) have round somata and slender dendrites that terminate in bundles of cilia on the epithelial surface. They constitute the most basal layer of olfactory sensory neuron. Microvillous neurons (blue) have bundles of microvilli on their apical surface. Crypt neurons (red) are globular-shaped and carry both microvilli and cilia on their apical surface. They are located more apical than microvillous neurons. G\textsubscript{a}-ir-positive kappe neurons (green) are pear-shaped with an apical appendage resembling a cap (German: Kappe). Have no cilia, and are located even more apical than crypt neurons. Kappe neurons (green) constitute a novel olfactory sensory neuron population.
were approved by the governmental animal care and use office (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany, Protocol No. 8.87–51.05.20.10.217) and were in accordance with the German Animal Welfare Act as well as with the General Administrative Directive for the Execution of the Protection of Animals Act. Tissues were embedded in 5% low melting agarose and sectioned by vibration (Pelco 101) or embedded in TissueTek O.C.T. compound (Sakura) and sectioned by cryostat (Leica CM1900) at −20 °C. Fluorescence was analysed using a wide field fluorescence microscope (Keyence BZ-9000) for sections and whole mounts. Transgenic zebrafish lines for ciliated neurons, Tg(OMP:lyn-mRFP-S), and microvillous neurons, Tg(TRPC2:gap-Venus), were used in this study.

Whole mount olfactory bulb immunohistochemistry. The dorsal cranium was removed, exposed brains were fixed by immersion in 4% paraformaldehyde (PFA, pH 7.4) in phosphate-buffered saline (PBS, pH 7.5) overnight at 4°C. Tissues were dissected out, keeping their connection to the telencephalon intact. Staining was thereafter removed, exposed brains were fixed by immersion in 4% paraformaldehyde (PFA, pH 7.4) in phosphate-buffered saline (PBS, pH 7.5) overnight at 4°C. Transgenic zebrafish lines for ciliated neurons, Tg(OMP:lyn-mRFP-S), and microvillous neurons, Tg(TRPC2:gap-Venus), were used in this study.

Immunohistochemistry on cryosections. Heads were either pre-incubated before dissection in cold freshly prepared 4% PFA in PBS for 5 min (pre-fixed tissue) or dissected directly (fresh-frozen tissue). We found that a short fixation step of 5 min does not affect the sensitivity of the Tg(100) antibody for crypt neurons. Tg(OMP:lyn-mRFP-S) or Tg(TRPC2:gap-Venus) sections were both incubated single or in combination at 4°C for 20 to 25 days on a vertical rotator (5 sec/round), followed by several washes over a period of 3 hours at room temperature. Subsequently, the olfactory bulbs were incubated with secondary antibodies for 7 days at 4°C, followed by several washes at room temperature. Tissue was cleared as described5. Both primary and secondary antibodies were used at a final dilution of 1:100 in blocking reagent. For detailed examination 100 μm vibratome sections were analysed.

 Quantification and statistical evaluation. Spatial coordinates were measured in arbitrary units and normalized. Horizontal cell diameter was determined as maximal cell width, i.e. parallel to the basal lamina, and vertical diameter was determined as maximal distance between the central position and the border of the epithelial section. Finally, the cardinal number of sections served as z axis coordinate. One hundred to several hundred cells were measured for each marker and spatial coordinate. Thus the range of values is between 0 (most basal) and 1 (most apical). Radial distance was measured from the apical pole. In the axis of maximal cell width, i.e. parallel to the basal lamina, and vertical distance was determined as maximal cell length perpendicular to the basal lamina (soma and dendrite, if any), see Fig. 1b. For laminar height in the olfactory epithelium the distance between center of the cell soma and basal border of the epithelial layer (see Fig. 1b) was normalized to the distance between basal and apical border of the epithelial layer at the position of the cell to be measured. The thus range of values is between 0 (most basal) and 1 (most apical). Radial distance was measured from the apex of the lamellae ‘curve’, i.e. closest to the median raphe, to the cell soma center (see Fig. 1b), and normalized to the distance between the central position and the border of the epithelial section. Finally, the cardinal number of sections served as z axis coordinate. One hundred to several hundred cells were measured for each marker and spatial coordinate.

Distributions are depicted as histograms or as the corresponding empirical cumulative distribution function (ECDF) of the unbounded distributions2,5,8,22. To estimate whether two spatial or shape distributions were significantly different, we performed Kolmogorov–Smirnov tests on the unbounded distributions as described22. This test is particularly suitable for continuous distributions and makes no assumptions about the nature of the distributions investigated, which is essential because the skewness of the observed distributions shows that these are not Gaussian.

Due to the sensitive nature of the test on large distributions (n > 100), we selected P < 0.01 as cutoff criterion for significant difference. Results of the Kolmogorov–Smirnov test were confirmed by permutation analysis22 without exception.

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Author contributions
The experiments were designed by S.I.K. and G.A., and performed by G.A., V.S., D.K. and Y.O. Illustrations were drafted by V.Z., G.A. and S.I.K. Data analysis was done by S.I.K., S.B. and G.A. S.I.K. wrote the paper.

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