Functional and structural diversity of external tufted and deep short-axon cells in the main olfactory bulb

Doctoral Theses

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INTRODUCTION

The main olfactory bulb, in most vertebrates, is the most rostral part of the brain, that receives a single source of direct sensory input from olfactory receptor neurons (ORNs) of the olfactory epithelium and sends its output directly to the olfactory cortices. Similar to other sensory systems in the brain, the multilayered architecture of the MOB hosts a wide array of neuronal building blocks which can be categorized into three groups: input, output and intrinsic elements. The sensory input is made up of a sophisticated arrangement of axons originating from the ORNs in the nasal olfactory epithelium, which terminate in synaptic complexes, also known as glomeruli within the glomerular layer. These spherical regions of neuropil are the first processing station of the olfactory pathway where sensory axons form synapses on the apical dendrites of the principal cells (mitral and tufted cells) and on some intrinsic neurons termed juxtaglomerular cells (JGCs). Complex dendro-dendritic and axo-dendritic interactions also occur within the glomeruli between principal cells and JGCs and among JGCs.

It has long been recognized that JGCs are morphologically diverse and are comprised of short-axon, periglomerular and external tufted cells (ETCs). Among the JGCs, ETCs have the largest somata (10-15 μm) and have a single, relatively sparse, fan-shaped apical dendritic tuft that most often arborizes in a single glomerulus. Most ETCs lack secondary, basal dendrites. However, ETCs with basal dendrites have also been described, as have ETCs with apical tufts ramifying in two glomeruli, indicating the morphological heterogeneity of these cells. Juxtaglomerular cells are also diverse based on their active and passive electrical properties and their synaptic connectivity. A few studies have functionally characterized several JGCs and found robust differences between them, but most physiological properties of the ETCs were found to be rather uniform and their functional role remains poorly understood.

The extraordinary diversity of nerve cells was already recognized over a century ago. It is now widely accepted that within most brain regions, the glutamatergic principal cells are rather homogeneous, whereas the GABAergic non-principal cells, or interneurons (INs), form a much more diverse population. Traditionally, the classification of IN types has relied primarily on their axonal and dendritic morphologies. However, recently their intrinsic electrical properties, gene expression profiles and firing behavior in relation to ongoing network activity have all been taken into account. Such combined molecular, morphological and physiological approaches have provided compelling evidence for the astonishing diversity of INs and for the variety of roles they play in neuronal networks. The classical view regarding the connectivity of INs has been that they primarily innervate the principal cells, and to a smaller extent each other. However, additional populations of hippocampal and neocortical INs have been revealed, that are specialized to selectively control the activity of other GABAergic INs, and the presence of such cells has also been suggested to occur in the MOB.

As in many other brain regions, the GABAergic periglomerular and granule cells (GCs) also receive GABAergic inputs, but their source is largely unknown. A recent report has demonstrated that GCs receive $GABA_A$ receptor-mediated inhibition from a subpopulation of inframitral deep short-axon cells (dSACs) called Blanes cells, but it remains to be seen whether other cell types also contribute to the GABAergic control of GCs. Short-axon cells form another, rather diverse GABAergic IN population distinct from the numerically dominant GCs and periglomerular cells. However, very little is

known about their axonal arborizations, their intrinsic electrical properties, their synaptic inputs and their postsynaptic targets.
To understand the cellular and synaptic mechanisms of olfactory information processing, the intrinsic properties and synaptic connectivity of these different types of nerve cells need to be deciphered. The main objective of my work was to identify whether heterogeneity in structural and functional properties as well as synaptic connectivity of certain nerve cell populations in the main olfactory bulb correlate with each other, resulting in well defined subpopulations of cells; or whether these measured features of the cells vary independently, resulting in a single but highly diverse population.

AIMS

First, I aimed to characterize the intrinsic electrical and morphological properties of external tufted cells (ETCs) in the rat main olfactory bulb. Based on a large number of measured physiological and morphological parameters, I asked whether:

1. External tufted cells comprise a *single* population or *multiple subpopulations* exist, and whether

2. the pattern of *dendritic arborization* of ETCs could be *predicted* from their active and passive *electrical properties* and vice versa?

Second, I aimed to provide a combined characterization of deep short-axon cells, by carrying out a detailed analysis of their

3. intrinsic *electrophysiological properties* and *synaptic input*,

4. *molecular content*

5. axo-somato-dendritic *morphologies*

in order to reveal their place in the olfactory bulb network.

METHODS

Acute *in vitro* slice preparation and electrophysiological recordings
For my experiments I used male Wistar rats (20–78 days old), which were anesthetized with ketamine (license number: 2288/003/Főv/2006). After decapitation, the brain was removed and placed into ice-cold artificial cerebro-spinal fluid (ACSF) containing (in mM): 230 sucrose, 2.5 KCl, 25 glucose, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 4 $MgCl_2$, and 0.5 $CaCl_2$. Horizontal or sagittal slices from the olfactory bulb were cut at 300 µm thickness with a Vibratome and were stored in ACSF containing (in mM): 85 NaCl, 75 sucrose, 2.5 KCl, 25 glucose, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 4 $MgCl_2$, and 0.5 $CaCl_2$. After 30 minutes, this medium was gradually or instantly replaced with normal ACSF

containing (in mM): 126 NaCl, 2.5 KCl, 25 glucose, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 2 $MgCl_2$, and 2 $CaCl_2$. All extracellular solutions were bubbled continuously with 95% O_2 and 5% CO_2, resulting in a pH of 7.4. After an additional 30-60 minute period of recovery at 33°C, slices were incubated at room temperature until they were transferred to the recording chamber.

I performed cell-attached and somatic whole-cell current and voltage recordings at 31-36°C. Cells were identified with an infrared differential interference contrast or an oblique illumination technique using an Olympus BX50WI microscope and a 40x water immersion objective. External tufted cell recordings were carried out from somata with a diameter of >10 µm and with a juxtaglomerular location. Deep short-axon cell recordings were carried out from visually identified cells with a somatic diameter of >10 µm located in the infra-mitral layers. For current-clamp experiments, I used a potassium gluconate-based intracellular solution containing (in mM): 120 K-gluconate, 5 KCl, 2 $MgCl_2$, 0.05 EGTA, 10 HEPES, 2 Mg-ATP, 0.4 Mg-GTP, 10 creatinine phosphate, and 5.3-8 biocytin (pH=7.25; osmolarity: 270–290 mOsm). Using intracellular solutions containing 5.3-8 mM biocytin allowed us visualization and *post hoc* anatomical analysis of recorded cells following electrophysiological recordings. Trains of action potentials were evoked by injecting one-second long depolarizing current pulses of different amplitudes. The passive properties were derived from single exponentials fitted to traces of membrane voltage responses to small hyper- and depolarizing current injections. For testing the subthreshold resonance properties of ETCs, two-second long sinusoidal currents of different amplitudes and frequencies were injected into the cells. The peak amplitude of the fast Fourier transform of the voltage responses were then compared to that of a single compartment passive resistance-capacitance model cell, which was generated in Berkeley Madonna 8.0.1. (written by R.I. Macey and G.F. Oster at UC Berkeley, CA).
During paired recordings of dSACs and granule cells the same intracellular solution described above was used for recording from the presynaptic dSACs. Three to 5 ms 2.5-4.0 nA currents were injected to evoke a single spike, which was repeated at either 25 or 50 Hz to evoke pairs of action potentials. To isolate AMPA receptor-mediated excitatory postsynaptic currents (EPSCs), cells were voltage-clamped at -70 mV in the presence of 20 µM SR95531 and 50 µM D-AP5 in the bath solution. To examine spontaneous and action potential-evoked inhibitory postsynaptic currents (ISPCs), I used a mixed potassium gluconate and potassium chloride internal solution containing (in mM): 90 K-gluconate, 40 KCl, 2 $MgCl_2$, 0.05 EGTA, 10 HEPES, 2 Mg-ATP, 0.4 Mg-GTP, 10 creatinine phosphate, and 8 biocytin (pH=7.33; osmolarity: 270–290 mOsm). For recording spontaneous IPSCs, cells were voltage-clamped at -80 mV and recordings were carried out in the continuous presence of 20 µM CNQX and 50 µM D-AP5 in the bath solution. Miniature synaptic events were recorded in the presence of 1 µM tetradotoxin to block voltage-gated sodium channels. During paired recordings, postsynaptic granule cells were recorded with this same mixed potassium gluconate and potassium chloride intracellular solution, they were voltage-clamped at -80 mV, but CNQX and AP5 were not included in the extracellular solution.
To investigate whether deep to superficial GABAergic connections exist in the main olfactory bulb, electrical stimulation of the internal plexiform layer (IPL) was carried out. A bipolar tungsten stimulating electrode was placed in the IPL and brief (350 µs, 0-100 µA) electrical pulses were delivered through an A395 Linear Stimulus Isolator.

Evoked postsynaptic responses were recorded from periglomerular cells using the above described mixed potassium gluconate and potassium chloride intracellular solution in the presence of 5 μM NBQX and 5 μM R-CPP in the bath solution; the cells were clamped at -80 mV. The $GABA_A$ receptor blocker, 20 μM picrotoxin was then washed in.
Recordings were performed with a dual-channel MultiClamp 700A amplifier. Patch pipettes were pulled from thick-walled borosilicate glass capillaries with an inner filament. Data were digitized on-line at 10 or 20 kHz, and analyzed with EVAN 1.3 and with an in-house analysis software (SPIN 1.0.1.) written in Matlab 7.0.

Visualization of the recorded cells
All of the anatomical work, including processing of slices, light and subsequent electron microscopy, *post hoc* reconstruction and anatomical analysis of recorded cells were performed by Dr. Mark D. Eyre Ph.D. (Laboratory of Cellular Neurophysiology, Institute of Experimental Medicine).
After recordings, slices were fixed in a 1.25% glutaraldehyde containing fixative at 4°C. Cells were visualized using an avidin–biotin–horseradish peroxidase complex (ABC) - 3'3-diaminobenzidine tetrahydrochloride (DAB) method.
This protocol extensively labeled the filled cell processes and masked epitopes for post-embedding immunolabeling, so in a number of slices an alternative, silver enhancement method utilizing streptavidin-conjugated 1 nm gold particles was used to visualize the biocytin. In cases when projection neurons were retrogradely labeled by fluorescent microspheres followed by physiological recordings, prior to the ABC-DAB reaction the biocytin was visualized with Alexa488 or Cy5 conjugated to streptavidin and the colocalization of biocytin and the fluorescent microspheres was confirmed using confocal laser scanning microscopy.

***In vivo* injection of fluorescent microspheres**
In vivo injections were also performed by Dr. Mark D. Eyre Ph.D. (Laboratory of Cellular Physiology, Institute of Experimental Medicine). Male Wistar rats (28-42 days old) were anaesthetized with a mixture of Ketamine and Xylazine and were mounted in a stereotaxic apparatus. Solutions of 40 nm diameter fluorescent latex microspheres were pressure injected into the anterior olfactory nucleus, the olfactory tubercle, the piriform cortex and the deep endopiriform nucleus. Three to 14 days after the surgery, acute horizontal slices from the main olfactory bulb were prepared as described above, and fluorescently labeled cells with infra-mitral somatic locations were subject of whole cell recordings.

Immunofluorescent microscopy
Slices were fixed after recordings as above except the glutaraldehyde concentration of the fixative was only 0.05%. Slices were subjected to immunreactions for the $GABA_A$ receptor α1 subunit using an anti-$GABA_A$ receptor α1 subunit primary antibodies (gifts of Prof. J-M. Fritschy and Prof. W. Sieghart) and a mixture of either streptavidin conjugated to Alexa488 and IgG conjugated to Cy3 or streptavidin conjugated to Cy5 and IgG conjugated to Alexa-488. Fluorescent immunolabeling of periglomerular cells for tyrosine hydroxylase and calbindin was carried out using anti-tyrosine hydroxilase and anti-calbindin primary antibodies and a mixture of streptavidin conjugated to Alexa488, IgG conjugated to Cy3 and IgG conjugated to Cy5. Slices were viewed

using an Olympus BX62 microscope equipped with appropriate fluorescence filters and a DP30BW CCD camera or an Olympus FV1000 confocal microscope. Biocytin was subsequently visualized with the ABC-DAB method as described above.

Three-dimensional reconstructions of deep short axon cells
Labeled cells were identified as ETCs or dSACs from their size, the location of their somata and their dendritic morphology. Neurons with no obvious truncations in their dendrites or no obvious truncations in their dendrites and axons, in the case of ETCs or dSACs, respectively, were fully reconstructed with the NeuroLucida system attached to a Zeiss Axioscope 2 microscope using a 100x oil-immersion objective, and were subjected to morphometric measurements.

Electron microscopy and postembedding immunolabeling
Portions of cells were re-embedded and serial sections were cut at 60 nm thickness using an ultramicrotome. Sections were subjected to postembedding immunoreactions for GABA. This was followed by incubation in primary GABA antibodies (GABA9; gift of Prof. P. Somogyi), which was visualized with 10 nm gold-conjugated secondary antibodies. Sections were viewed using a JEOL1011 electron microscope. Digital images were captured with a cooled CCD camera.

Statistical procedures
All data are expressed as mean ± standard deviation. Following Shapiro-Wilk normality test, either paired t-test or sign-test was used to compare data measured at the beginning and at the end of the recording. Parameters that significantly ($p<0.01$) changed during the whole-cell recordings were discarded from my analysis. I utilized Pearson's regression analysis to determine whether the measured physiological parameters depended on the amplitudes of the injected current. Parameters where a significant correlation ($p<0.01$) was found were also discarded from my analysis. Principal component analysis (PCA) and agglomerative clustering methods were carried out. Agglomerative or joining clustering was employed to group data (PCA factors) without any *a priori* assumption concerning the number of clusters. Dissimilarities between cells were measured as Euclidean distances and Ward's amalgamation rule was used to determine cluster linkage. For analysis of ETCs, the number of clusters that were statistically different from one another were determined by calculating the squared Euclidean distance of each cell from the center of each cluster and then compared them. By using Mann-Whitney-U test, we statistically compared the mean distance of the members of cluster 1 and 2 from the center of cluster 1. Then the reverse comparison was carried out. If both of these tests were statistically significant ($p<0.001$), we then concluded that ETCs form two subpopulations. Subsequently, a similar statistical comparison was carried out with 3 clusters, 4 clusters etc. using a Kruskal-Wallis non-parametric test, and if significant, a *post hoc* test (multiple comparisons of mean ranks for all groups) was used to decide whether the groups were different or not.
Following deep short-axon cell (dSAC) recordings, the data were subject to the Shapiro-Wilk normality test, and the Levene and Brown-Forsythe or Hartley F-max tests for homogeneity of variances. To compare averaged spontaneous and miniature synaptic events within subtypes I employed the t-test for dependent samples or the Wilcoxon matched pairs test. At the individual cell level, I carried out comparisons using a non-parametric Kolmogorov-Smirnov test. Either parametric ANOVA or non-parametric

Kruskal-Wallis ANOVA was used to compare data between dSAC subtypes, depending on whether the distribution was normal or not. Where appropriate, data were further assessed by conducting a *post hoc* test (Tukey's Unequal n HSD test or multiple comparisons of mean ranks, respectively). All differences were considered significant if $p<0.05$. Morphological data was also subjected to PCA and agglomerative clustering. The gap statistic method was used to determine the number of clusters. All analyses (including analysis of parameter correlations, PCA and unsupervised clustering) were performed using Statistica 6.0.

RESULTS

I. Correlation of functional and structural characteristics predicts two subpopulations of external tufted cells.

Active and passive electrical properties of external tufted cells

To ensure a representative sampling of the external tufted cell (ETC) population, I randomly selected juxtaglomerular cells with a somatic diameter of >10 µm without any additional selection criterion (e.g. soma shape, number of emerging dendrites etc). All cells were filled with biocytin and were subject to *post hoc* morphological identification. Several cells with somatic diameter of >10 µm were subsequently identified as short-axon cells, which were discarded. First, I assessed the spontaneous spiking activity of the ETCs in cell-attached recording configuration. The recorded cells displayed large heterogeneity based on their spontaneous firing behavior. A large proportion (57%) fired bursts of action potentials (APs; from 0.03 to 8 Hz) some (25%) fired individual APs at frequencies ranging from 0.1 to 38 Hz, and the remaining 18% of the cells were completely silent. This spontaneous activity was also observed during the first minute of whole-cell recordings. When a cell showed spontaneous suprathreshold activity, small hyperpolarizing DC current was injected to keep the membrane potential just below firing threshold. Under such conditions, trains of action potentials were evoked by injecting one-second long depolarizing currents of different amplitudes into the cells.

The firing patterns of ETCs were extremely diverse; qualitative descriptions commonly used to characterize such patterns are regular spiking, irregular spiking with accomodation and burst firing. Eighty eight percent of the cells that showed spontaneous bursting in the cell-attached configuration also displayed bursts of APs upon DC current injections. In 82% of the cells that did not fire bursts spontaneously, somatic current injections evoked regular and irregular firing patterns. Of the silent cells 37.5% fired bursts of APs and the remaining 62.5% were non-bursting following somatic current injections. To quantitatively characterize the spikes of ETCs, I measured and calculated numerous active electrical parameters (e.g. the threshold, peak amplitude of APs, amplitude of spike after-hyperpolarization (AHP), etc.). To characterize the firing patterns of ETCs, I calculated the mean, maximum, minimum and coefficient of variation (CV) of the inter-spike interval (ISI) distributions. These parameters allow distinctions to be made among qualitatively different firing patterns. Dissimilarities in individual spike shape, spike frequency adaptation and AP amplitude accommodation are also reflected by our measurements. The membrane time constant

(τ) and the input resistance (R_{in}) of the cells were also measured and revealed tremendous heterogeneities: τ (5.7 to 63.7 ms, CV of 0.71) and R_{in} (43 to 990 MΩ, CV of 0.6). I also characterized the cells with respect to their intrinsic, subthreshold resonant properties. In one fourth of the cells, no active resonance was found at the frequencies tested, whereas in the rest of the cells, the peak resonant frequency varied from 1 to 10 Hz.

Next, I selected those measured and calculated parameters that did not show any dependence on two important experimental variables, and therefore reliably characterized the cells. In order to ensure that the elapsed time from the establishment of the whole-cell configuration did not affect my parameters, I re-measured them at the end of the whole-cell recording period. All parameters were statistically compared between the beginning (first 3.5±1.2 minutes) and at the end (10±3 minutes later) of the recording periods, and parameters showing significant difference were discarded from my analysis. I also tested whether the measured and calculated parameters depended on the amount of injected current to evoke the spike trains. If regression analysis yielded a significant correlation between a parameter and the amount of current injected, the parameter was also discarded. Following these selection criteria, 12 physiological parameters remained and were used for quantitative characterization of ETCs.

The presence of two subpopulations of external tufted cells based on their physiological properties

To determine whether ETCs form multiple subpopulations based on their active and passive electrical properties or whether a single heterogeneous population exists, I performed PCA and cluster analysis. Because the number of available cells (62 in total) is likely to be insufficient for clustering in a 12-dimensional space, PCA of the parameters was first carried out. The first 4 factors had Eigenvalues of >1 and accounted for >75% of the total variance; therefore they were selected for subsequent cluster analysis. Agglomerative clustering revealed a joining tree with two dominant and several additional sub-branches.

Next we developed a method of objective determination of the number of distinct subpopulations following such clustering. We considered two clusters significantly different in an n-dimensional space if the distance of the members of cluster 1 from the center of cluster 1 was significantly smaller than the distance of the members of cluster 2 from the center of cluster 1, and vice versa. By performing this analysis on ETCs based on the first 4 factors obtained with PCA, I found that two clusters differed significantly, but additional subdivisions (into 3, 4 … clusters) were not supported statistically.

Next, I asked how different the individual active and passive electrical properties between the two subpopulations were. Statistical comparisons revealed that *i*: the threshold of the first AP (cluster 1: -36.1±3.7 mV vs. cluster 2: -33.3±4.9 mV); *ii-iii*: the amplitude of the AHP after the first (4.5±4.0 mV vs. 13.3±4.6 mV) and last (7.2±3.8 mV vs. 14.3±3.7 mV) APs; *iv*: the ratio of the last and first spike width (1.5±0.4 vs. 1.1±0.1); *v-vii*: the mean (77.6±35.2 ms vs. 39.8±18.7 ms), maximum (319.6±140.3 ms vs. 104.1±71.4 ms) and CV (1.6±0.5 vs. 0.6±0.5) of the ISI distributions; and *viii*: the membrane time constants (13.6±6.5 ms vs. 27.8±17.2 ms) are significantly different between the two subpopulations. Qualitatively, the most prominent difference between the two subpopulations was that all members of cluster 1 fired bursts of APs, whereas those of cluster 2 had regular or irregular firing patterns and did not change to burst

firing when hyperpolarized to -60 mV or more negative membrane potentials. However, it is important to note that members of each subpopulation displayed large variability based on several parameters. For example, cells in cluster 1 showed large variability in the degree of the spike amplitude accommodation or the amplitude of the AHP. Cells belonging to cluster 2 displayed very different spike frequency adaptations, amplitude and time course of AHP or τ. The peak resonance of the cells was also significantly different; cells in cluster 1 displayed a higher peak resonant frequency (2.5±2.2 Hz vs. 0.9±0.8 Hz). However, both subpopulations had cells without detectable subthreshold resonance, but cells that showed active resonance had a higher peak frequency in cluster 1.

Quantitative characterization of external tufted cells based on their dendritic arborizations

Following the recordings, biocytin was visualized and the cells were analyzed at the light microscopic level and were judged for completeness. All morphological analysis was performed by Dr Mark D. Eyre Ph.D. Cells with incomplete filling or with truncations in their dendritic tree were discarded from our quantitative morphological analysis. However, if a cell showed signs of obvious truncation (e.g. secondary dendrites), but could be still positively identified as an ETC (e.g. from the soma and apical tuft), it remained in our study for physiological analysis only. The most noticeable difference between ETCs was the presence of secondary, basal dendrites in approximately one third of the cells (14/41 cells).

The branching patterns of the basal dendrites were dissimilar; some cells had a non-extensively branching large diameter basal dendrite, spanning a large distance only in one direction from the soma, whereas in some other cells the basal dendrites extensively branched and symmetrically occupied the external plexiform layer below the cell. Several additional morphological differences were also observed, including the presence of apical dendrites ramifying in two glomeruli, large differences in the extent of arborization of the apical dendritic tufts and in the branching patterns. Cells also had very different axonal arborizations; some emitted a large number of collaterals in the glomerular and external plexiform layers before entering the internal plexiform and granule cell layers, whereas some others crossed through the EPL with little branching. Because we hardly ever found cells without truncation in their axonal arbors, the quantitative characterization of the ETCs was based exclusively on their dendritic morphologies. Forty-one cells with complete filling and no obvious dendritic truncations were reconstructed using the Neurolucida system.

To quantitatively describe differences in the dendritic arborizations among ETCs, we measured and calculated 9 parameters for the apical as well as the basal dendrites. To reduce the number of dimensions within which clustering was carried out, we performed principal component analysis (PCA) of the 18 anatomical parameters. Four factors had Eigenvalues >1, accounting for >73% of the total variance. Agglomerative clustering using the first 4 PCA factor scores for each cell revealed two main branches on the joining tree with several additional sub-branches. Similar statistical analysis to that performed above indicated the presence of two subpopulations of ETCs based on their dendritic arborization patterns. To determine the morphological parameters that are significantly different between the two ETC subpopulations, we performed a between-groups statistical comparison for each variable. Fifteen out of the 18 variables differed significantly ($p<0.05$) between the groups.

These results demonstrate that most of the measured morphological parameters are significantly different between the two ETC subpopulations. However, similarly to the physiological parameters, large within-group variability was observed for most measurements, indicating that ETCs within each subpopulation are highly heterogeneous.

Comparing physiologically and morphologically determined external tufted cell subpopulations

Finally, I asked how the two morphologically determined ETC subpopulations correspond to those derived from the physiological properties. To address this question, I restricted my analysis to a total of 37 ETCs in which all 30 (12 physiological and 18 morphological) parameters were determined.

Principal component analysis indicated 7 factors with Eigenvalues of >1, accounting for 78.5% of the total variance. Agglomerative clustering showed a joining tree with two major branches. The presence of two subpopulations was statistically confirmed. When the position of each individual cell was compared, a perfect match was found between the anatomically determined groups and the groups based on all parameters. This could be the consequence of a large dominance of the anatomical parameters in the first 7 factors derived from PCA. However, a very close agreement between the results of clustering based on all parameters and only the physiological parameters was also observed. Only one cell was clustered into the first group based on its physiological properties, which was clustered to the second group when all parameters were taken into account. The rest of the cells (20) in the first cluster based on the electrophysiological properties alone were also in the first cluster based on all parameters.

Our results demonstrate that a number of electrophysiological (e.g. burst firing) and morphological (e.g. lack of basal dendrites) parameters of ETCs are highly predictive of one another.

II. Novel intra- and extrabulbar GABAergic connections in the main olfactory bulb are provided by distinct subtypes of deep short-axon cells.

The morphological diversity of deep short-axon cells

I carried out patch pipette recordings from the somata of 158 neurons located below the mitral cell layer (MCL) in acute main olfactory bulb slices. Cells were recorded first in cell-attached, and then in whole-cell configurations. Following electrophysiological recordings, the slices were fixed and the biocytin-filled cells were subjected to neurochemical and morphological characterizations.

All of the recorded cells exhibited dendritic and axonal morphologies distinct from both mitral/tufted and granule cells. The location and shape of the somata and the dendritic arbors of these cells were heterogeneous, and examples of all categories of deep short-axon cells (dSACs) described previously were observed. Large spiny Blanes cells (73/158 cells) were the most frequent cell morphology encountered, whereas sparsely spiny large Golgi cells (28/158), medium-sized horizontal cells (42/158) and smaller Cajal cells (15/158) were less common. Due to the advantages offered by intracellular labeling techniques compared to Golgi impregnations, we were able to recover

extensive axonal trees of the recorded cells. Many dSACs had a very extensive axonal arbor, ramifying in distinct layers of the main olfactory bulb.
A substantial number of cells projected their axons across the external plexiform layer (EPL) and ramified broadly in the glomerular layer (GL). The majority of these branches encircled many glomeruli, and only a few collaterals were found in the EPL and granule cell layer (GCL). Their somata were elongated parallel to the MCL, and were often located in the internal plexiform layer (IPL) (32/51 cells). Their dendrites were either sparsely (30/51 cells) or densely (8/51 cells) spiny and predominantly confined to the IPL, corresponding to horizontal cells. The remaining cells (13/51) had an overall appearance of Golgi cells. In contrast, many other dSACs had axonal ramifications predominantly within the EPL, with some collaterals in the IPL and superficial GCL. The axonal arbor was dense, column-like and had a characteristic sharp border between the EPL and GL; axons never entered the GL. The majority (72/79) displayed vertically-polarized somata in the GCL and their stellate or vertically oriented dendrites were usually covered with spines (62/79 cells), giving them the appearance of Blanes cells. However, some cells with similar axonal morphologies possessed smooth dendrites almost devoid of spines, typical of vertical Cajal cells (14/79 cells). Finally, the smallest subset of dSACs had axonal arbors restricted to the GCL. In order to exclude the possibility that the lack of axons in the superficial layers was the consequence of truncation during slice preparation, we discarded all such cells from our analysis. The somata of these cells were often located in the GCL (13/28 cells) and their dendrites were usually sparsely spiny (25/28 cells), having an overall appearance of horizontal or Golgi cells.
Thus, our analysis of the somato-dendritic morphologies was in agreement with the classical Golgi studies, but when the axonal arborizations were also considered, a more complex picture emerged. In order to assess whether dSACs can be objectively divided into well-defined subpopulations based on both their axonal and dendritic morphologies, we reconstructed a subpopulation of these cells in 3D and subjected them to morphometric measurements. Following principal component analysis, agglomerative clustering suggested 3 main subpopulations, which was confirmed using the gap statistic method. Based on the predominant axonal arborizations of the subpopulations in different layers, they were termed as GL-, EPL- and GCL-dSACs. Our results also demonstrate that many morphological parameters significantly differed among the subtypes.

Granule cell layer-dSACs project to higher olfactory areas
When the local axon collaterals of distinct dSAC subtypes were analyzed, we noticed that a branch of the main axon of GCL-dSACs often entered the white matter and projected caudally in the lateral olfactory tract, suggesting that GCL-dSACs might form an extrabulbar projection. In order to address this issue directly, we injected fluorescent microspheres into many higher order olfactory areas, including the anterior olfactory nucleus, the olfactory tubercle, the piriform cortex and the deep endopiriform nucleus.
Three to 14 days after surgery, I prepared acute *in vitro* slices from the main olfactory bulb and fluorescently labeled cells with inframitral somatic locations were subject of whole cell recordings. Following the characterization of their active and passive electrical properties and their synaptic inputs (see below), the presence of microspheres in the biocytin-filled cells was verified followed by full morphological reconstructions. Somata of labeled cells were located in the GCL, had horizontally oriented dendrites,

and the local axon collaterals were also restricted to the inframitral layers. Qualitatively very similar axonal and dendritic arborizations were found in all cells in which sufficient axons and dendrites were recovered. The axo-dendritic morphologies of these retrogradely labeled cells were indistinguishable from the randomly recorded and filled GCL-dSACs, but it remains to be seen whether all GCL-dSACs project outside the main olfactory bulb.

Molecular differences between deep short-axon cells
We then addressed whether molecular differences also exist among dSAC subtypes. Previous immunolabeling studies have indicated that neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), calbindin (CB) and nitric oxide synthase (NOS) are neurochemical markers for dSACs in the inframitral layers. However, because these markers labeled very few cells, we found none of them to be a good candidate as a selective marker of a particular dSAC subtype. In contrast, the $GABA_A$ receptor ($GABA_AR$) α1 subunit selectively labels the somato-dendritic domain of a large population of non-granule cells in the inframitral layers of the main olfactory bulb, making it a potential subtype-selective marker.
Only dSACs are immunopositive for this subunit in the GCL. A subset of the *in vitro* recorded cells (n=50 cells) was processed for immunolabeling for the $GABA_AR$ α1 subunit in conjunction with fluorescent visualization of the biocytin. All EPL-dSACs (n=30 cells) were intensely labeled for the α1 subunit. Eleven out of 15 GL-dSACs were found to be moderately labeled, whereas two were strongly labeled and two appeared to be immunonegative. Finally, the majority (4/5 cells) of GCL-dSACs were apparently immunonegative; only a single cell showed very weak immunoreactivity.
These results clearly demonstrate that molecular differences exist, but future experiments will be needed to identify other dSAC subtype-selective molecular markers.

Deep short-axon cells show distinct intrinsic electrical properties
Given the morphological and molecular heterogeneity of dSACs, I also aimed to reveal potential subtype-specific differences in the electrophysiological properties of the cells. Cell-attached recordings prior to the establishment of the whole-cell configuration demonstrated differences in the spontaneous spiking activity of dSACs in acute *in vitro* slices. Both GL- and GCL-dSACs were found to be spontaneously active, whereas 27 out of 28 EPL-dSACs were silent.
In whole-cell recordings, dSACs exhibited a variety of action potential firing patterns in response to one-second long 100 pA depolarizing current injections. Quantitative analysis of a large number of parameters derived from the spike trains, individual spikes and the passive electrical properties of the cells revealed significant dSAC subtype-specific differences and prominent within-subtype heterogeneity for many parameters.

Excitatory and inhibitory inputs onto deep short-axon cells
In order to gain insights into the potential role of a neuron in a network, not only the morphological, molecular and intrinsic electrical properties should be deciphered, but the synaptic connectivity must be determined as well. To investigate the synaptic inputs of dSACs, I performed *in vitro* voltage-clamp recordings of spontaneous excitatory and inhibitory postsynaptic currents before and during the application of 1 µM tetrodotoxin (TTX). Potential sources of glutamatergic excitation are either the local mitral/tufted

cells or centrifugal inputs from the anterior olfactory nucleus and the piriform cortex. I argued that if TTX reduces the frequency of spontaneous excitatory postsynaptic currents (sEPSCs), then the cells providing the excitatory inputs must be present in the slice and should be spontaneously active.

When examined at the population level, a significant decrease in EPSC frequency was detected in all subtypes without any change in the kinetics of the responses. The amplitude of sEPSCs was significantly reduced in GL- and EPL-, but not in GCL-dSACs. I also noted the prevalence of bursts of sEPSCs in control conditions, which disappeared following TTX application, indicating that the sources of these synaptic currents are likely to be bursting external tufted or some mitral cells. The amplitude, rise and decay kinetics showed no significant differences among the subtypes.

In addition, the identical decay time constants (weighted decay time constant (τ_w) = 1.4 ms for all subtypes) indicate similar postsynaptic AMPA receptors and synaptic glutamate concentration waveforms. In summary, dSACs receive some of their AMPA receptor-mediated excitatory input from spontaneously active mitral/tufted cells. In agreement with this, electron microscopy (EM) analysis revealed that the presynaptic elements establishing asymmetrical synapses on dSACs were axon terminals.

Next, I examined the inhibitory inputs of dSAC subtypes by recording spontaneous inhibitory postsynaptic currents (sIPSCs) using high chloride containing intracellular solutions in the presence of the glutamate receptor antagonists, CNQX and D-AP5. The average frequency of sIPSCs ranged from 3.4 to 7.1 Hz under control conditions, which was significantly reduced following the application of TTX in all dSAC subtypes. Application of TTX did not change the amplitudes of sIPSCs significantly, suggesting a low quantal content (~1) in all dSACs. Comparisons between dSAC subtypes showed no statistically significant differences in mIPSC amplitudes or kinetics. The comparable decay time constants (τ_w=2.3, 2.6 and 2.9 ms for EPL-, GL- and GCL-dSAC, respectively) suggest similar postsynaptic $GABA_A$ receptor subunit compositions and synaptic GABA concentration waveforms. These data demonstrate that all dSAC subtypes receive some of their fast synaptic inhibition from spontaneously active, local GABAergic interneurons. From our current knowledge of the main olfactory bulb microcircuit, these could be either granule or short-axon cells. By using EM analysis, we found that the presynaptic elements of symmetrical synapses onto dSACs were exclusively GABA immunoreactive axon terminals. No evidence of dendro-dendritic synapses, either established by or formed onto dSAC subtypes was observed, suggesting that the majority of the GABAergic inputs of dSACs originate from SACs.

The postsynaptic targets of GABAergic deep short-axon cells are other GABAergic interneurons

Following the identification of the synaptic inputs of dSACs, we carried out EM analysis of the postsynaptic targets of biocytin-filled dSAC axon terminals. In addition to the classification of postsynaptic profiles based on ultrastructural features, their neurotransmitter content was also revealed using postembedding immunogold labeling for GABA. Axon terminals of GL-dSACs (n=3 cells) ramifying in the juxtaglomerular space established symmetrical synapses onto small diameter somata, and large and small diameter dendrites, which all showed ultrastructural features of periglomerular cells (PGCs) and were immunopositive for GABA. Because PGCs form a very diverse cell population, we asked whether GL-dSAC axons selectively innervate a single

subpopulation or indiscriminately synapse on all cell types. Using fluorescent immunolabeling for tyrosine hydroxylase and calbindin, and visualizing biocytin in the intracellularly filled axons, we found that at least these two neurochemically defined PGC subpopulations are among the postsynaptic targets of GL-dSACs. In addition, axon collaterals of one GL-dSAC ramifying in the EPL and a different one arborizing in the GCL were found to synapse exclusively on granule cell (GC) dendrites. Next, the postsynaptic targets of EPL-dSAC (n=4 cells) axons were examined in the EPL, where 38 out of 43 synapses were formed onto GC dendritic shafts and the remaining 5 synapses established contacts onto GC gemmules. The ultrastructural identification of these targets was assisted by the presence of reciprocal dendro-dendritic synapses with mitral/tufted cells and their GABA immunoreactivity. In the GCL, 38 axon terminals from 3 EPL-dSACs were also examined and 37 formed synapses onto GC dendrites. The only exception was a synapse onto the perisomatic region of a horizontally elongated soma in the internal plexiform layer, which was strongly GABA immunopositive and was considered to be a dSAC. Finally, the axons of two GCL-dSACs were examined in the granule cell layer. Out of the 13 synapses analyzed, 11 contacted GC proximal dendrites and 2 contacted GC somata. No evidence of synaptic contacts onto mitral/tufted cells was observed for any dSAC subtype in any layer.

Visualization of intracellular biocytin at the EM level with a diaminobenzidine (DAB) reaction prevents the access of primary antibodies to the aldehyde-fixed neurotransmitter molecules. To overcome this technical drawback, we visualized the intracellular biocytin with silver-enhanced ultrasmall gold particles. For each dSAC subtype, all examined terminals were immunopositive for GABA, as were their postsynaptic target cells.

Functional characterization of the synaptic outputs of deep short-axon cells

To functionally characterize the output of dSACs, I carried out paired whole-cell recordings from presynaptic dSACs and postsynaptic GCs. In 7 pairs, action potentials in the presynaptic dSACs induced short-latency, fast rising, exponentially decaying inward currents in the postsynaptic GCs. The short latency of the responses (1.0 ± 0.4 ms, coefficient of variation (CV) of the latencies: 0.18 ± 0.8, n=7) indicate the monosynaptic nature of the unitary IPSCs (uIPSCs). These results offer functional evidence for our EM predictions that GABAergic interneurons are the postsynaptic targets of dSACs. The specific $GABA_AR$ antagonist SR95531 completely and reversibly blocked uIPSCs in all of the 6 pairs tested, functionally validating our immunohistochemical results that dSACs are GABAergic. The amplitude of the uIPSCs showed large within-cell (CV= 1.5 ± 0.8, n=7) and between-cell (CV= 1.7) variability. Unitary IPSCs with small amplitudes had slow rise times (RT), indicating severe dendritic filtering and distal dendritic locations of the synapses on the postsynaptic GC. When examining the kinetics of uIPSCs, I restricted my analysis to uIPSCs with a 10-90% RT of <0.55 ms. For the 4 pairs meeting this criterion, the 10-90% RT was 0.42 ± 0.1 ms and the weighted decay time constant was 6.6 ± 1.6 ms, which is 2.5-fold slower than that of mIPSCs recorded from dSACs (2.6 ± 0.6 ms). In two pairs, 1 µM zolpidem, a benzodiazepine site agonist, prolonged the decay of uIPSCs.

Next, I investigated the short-term plasticity of the postsynaptic responses. Two action potentials in the presynaptic dSACs were evoked at 50 or 25Hz and their effects on uIPSC amplitudes were analyzed. The short-term plasticity showed large variability;

some responses showed depression, some facilitation and in one pair no plasticity was observed. In this pair, however, changing the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) revealed short-term facilitation and depression, and demonstrated that the plasticity depends on the initial release probability (P_r). Changing the $[Ca^{2+}]_e$ from 1 mM to 3 mM resulted in a 9-fold increase in the success rate (from 0.05 to 0.45), but did not affect the mean postsynaptic response amplitude excluding failures (potency at low P_r=50.9±8.6 pA; high P_r=51.3±11.3 pA), demonstrating the presence of a single functional release site. At this site, the P_r at 2 mM $[Ca^{2+}]_e$ and 2 mM $[Mg^{2+}]_e$ was 0.25 and the quantal size was 51 pA. The variance in the peak current of the successful events was remarkably small (CV~0.2), indicating high postsynaptic receptor occupancy.

To provide functional evidence for the deep to superficial GABAergic connection in the MOB, I carried out whole-cell recordings from PGCs and evoked IPSCs by extracellular stimulation in the internal plexifrom layer (IPL). In the presence of ionotropic glutamate receptor antagonists, extracellular stimulation in the IPL evoked short latency (1.9±0.5 ms) inward currents of variable amplitudes (49.4±34.4 pA**).** The presence of glutamate receptor antagonists in the bath, the short and uniform latencies, and the monotonous rise indicate the monosynaptic nature of these IPSCs. The $GABA_A$ receptor blocker picrotoxin (20 μM) completely and reversibly eliminated the inward currents, providing a functional demonstration of a $GABA_A$ receptor-mediated deep to superficial layer connection in the main olfactory bulb.

SUMMARY OF RESULTS AND CONCLUSIONS

To understand the cellular and synaptic mechanisms of olfactory information processing, the intrinsic properties and synaptic connectivity of the different types of nerve cells in the main olfactory bulb need to be deciphered. The main objective of my work was to identify whether heterogeneity in structural and functional properties as well as synaptic connectivity of certain nerve cell populations in the main olfactory bulb correlate with each other, resulting in well defined subpopulations of cells; or whether these measured features of the cells vary independently, resulting in a single but highly diverse population.

I. First, I determined the active and passive electrical properties of external tufted cells (ETCs) using *in vitro* whole-cell recordings and then we **correlated them to their dendritic arborization patterns.**

1) Principal component followed by cluster analysis revealed two distinct subpopulations of ETCs based on their electrophysiological properties. Eight out of 12 measured physiological parameters exhibited significant difference between the two subpopulations, including the membrane time constant, amplitude of spike after-hyperpolarization, variance in the inter-spike interval distribution and subthreshold resonance.

2) Cluster analysis of the morphological properties of the cells also revealed two subpopulations, the most prominent dissimilarity between the groups being the presence or absence of secondary, basal dendrites.

3) Finally, clustering the cells taking all measured parameters into account also indicated the presence of two subpopulations that mapped in an almost perfect one-to-one fashion to both the physiologically and the morphologically derived groups.

My results demonstrate that a number of functional and structural properties of ETCs are highly predictive of one another. However, cells within each subpopulation exhibited pronounced variability, suggesting a large degree of specialization evolved to fulfill specific functional requirements in olfactory information processing.

A universal feature of neuronal microcircuits is the presence of GABAergic interneurons that control the activity of glutamatergic principal cells and each other.

II. In the second part of my work I used a combined electrophysiological and morphological approach to investigate the rather mysterious deep short-axon cell (dSACs) population of the main olfactory bulb.

1) Deep short-axon cells of the inframitral layers are GABAergic and have extensive and characteristic axonal ramifications in various layers of the bulb, based on which unsupervised cluster analysis revealed 3 distinct subtypes, the glomerular (GL-), external plexiform (EPL-) and granule cell layer- (GCL-) dSACs.

2) Each dSAC subtype exhibited different electrical properties,

3) However, they received similar GABAergic and glutamatergic inputs.

4) The local axon terminals of all dSAC subtypes selectively innervate GABAergic granule and periglomerular cells and evoke $GABA_A$ receptor-mediated inhibitory postsynaptic currents.

5) One subpopulation of dSACs (GL-dSAC) creates a novel intrabulbar projection from deep to superficial layers.

6) Finally, another subpopulation (GCL-dSAC) is labeled by retrogradely-transported fluorescent microspheres injected into higher olfactory areas, constituting a novel projection-cell population of the main olfactory bulb.

My results reveal multiple dSAC subtypes, each specialized to influence main olfactory bulb activity by selectively innervating GABAergic interneurons, and provide direct evidence for the presence of novel intra- and extrabulbar GABAergic projections.

PUBLICATIONS

Publications that form the basis of the doctoral dissertation

Antal M, Eyre M, Finklea B, Nusser Z (2006) External tufted cells in the main olfactory bulb form two distinct subpopulations. Eur J Neurosci 24:1124-1136.

Eyre MD, **Antal M**, Nusser Z (2008) Distinct deep short-axon cell subtypes of the main olfactory bulb provide novel intrabulbar and extrabulbar GABAergic connections. J Neurosci 28:8217-8229.

Other publications

Köllő M, Holderith N, **Antal M**, Nusser Z (2008) Unique clustering of A-type potassium channels on different cell types of the main olfactory bulb. Eur J Neurosci 27:1686-1699.