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Imaging Odor Coding and Synaptic Plasticity in the Mammalian Brain with a Genetically-Encoded Probe

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Abstract—**We have used the genetically-encoded fluorescent exocytosis indicator synaptopHluorin (spH), expressed selectively in mouse olfactory receptor neurons, to image odor representations at the input to the olfactory bulb. The olfactory bulb is a powerful system for in vivo fluorescence imaging because its inputs are segregated into receptor-specific functional units (glomeruli) that are optically accessible and receive massively convergent input from sensory neurons. In a line of transgenic mice expressing spH under the control of a receptor neuron-specific promoter (OMP), odorant-evoked patterns of receptor neuron input to ~ 10% of the olfactory bulb can be imaged with excellent spatial resolution and sensitivity during single brief odorant presentations. Odor representations are similar across mice and can be imaged repeatedly in the same animal for months. In olfactory bulb slices from OP-spH mice, shock-evoked spH signals are rapid and linear reporters of transmitter release, although control for changes in extracellular pH is critical for proper interpretation of the spH signals. These features have allowed us to characterize the functional organization and mechanisms of presynaptic modulation of transmitter release at the first olfactory synapse. The capacity for long-term chronic imaging permits the direct visualization of the function regeneration and remapping of input to the olfactory bulb after lesions of the nasal epithelium.**

Keywords—**Neural imaging and sensing; Neural Imaging; Neural Injury**

I. Introduction

The rodent olfactory system provides a powerful model for studying the neural coding of sensory stimuli because its circuitry is relatively well understood and experimentally accessible using genetic and optical imaging techniques. Air enters the nose through the naris and passes into the nasal cavity, which is lined by the olfactory epithelium. The epithelium contains approximately 2 million olfactory receptor neurons (ORNs), which extend their cilia into the mucosa where they are exposed to odorants. Each individual ORN expresses a single type of receptor, selected from a family of approximately 1000 receptor genes. These different receptor types are scattered throughout the epithelium. Each ORN projects from the nasal epithelium back to the olfactory bulb by way of the olfactory nerve. Axons from the nerve distribute over the surface of the bulb and then converge into distinctive spherical bundles of neuropil known as glomeruli. Remarkably, the ORN axons segregate within the bulb such that each glomerulus receives input only from ORNs that express a single, specific receptor. In addition to incoming ORN axons, glomeruli contain the apical dendritic tufts of mitral and tufted cells, which are the principal output neurons of the olfactory bulb, and the axons and dendrites of periglomerular cells, which are interneurons that may span glomeruli. The mitral cells project their axons to various higher brain centers, including the olfactory cortex and the amygdala. Because each glomerulus corresponds to a specific population of ORNs that each express the same receptor, this imaging technique permits the simultaneous measurement of the activity of many different molecularly-defined subpopulations of ORNs. The olfactory glomerulus is thus the functional unit of early olfactory coding.

In collaboration with Peter Mombaerts' laboratory, we recently established a mouse model that permits direct observation of ORN output in the glomeruli of the olfactory bulb in vivo through the ORN-specific expression of synaptopHluorin (spH), a genetically-encoded fluorescent indicator of neurotransmitter release [1]. The dorsal bulb can be easily imaged during odorant presentation in vivo, permitting real-time imaging of odor-evoked input from the nose to the olfactory bulb. The olfactory bulb is particularly advantageous for in vivo fluorescence imaging because olfactory bulb glomeruli are discrete, relatively large (~100 μm) anatomical structures located superficially; because receptor neuron input to each glomerulus is massively convergent and activated near-synchronously by odors; and because a receptor neuron-specific promoter (OMP) is known which drives high levels of spH protein expression. Olfactory bulb slice preparations from these mice can also be used to more precisely investigate bulbar circuitry and pharmacology [2, 3]. Here, we present and discuss several recent uses of these mice to investigate how odor information is represented and processed, both under normal conditions and after experience or injury to the olfactory system.

II. Methodology

In vivo imaging was performed in pentobarbital-anesthetized mice, as described previously [1]. Briefly, the olfactory bulbs were imaged through thinned bone using an Olympus BX51WI microscope and epifluorescence condenser, with 4x (0.28 NA), 10x (0.3 NA), or 20x (0.95 NA) Olympus objectives. Illumination was provided by a 150W Xenon arc lamp (Opti-Quip, New York) attenuated with a 25% ND filter. Optical signals are recorded using a back-illuminated CCD camera (NeuroCCD, SM-256, RedShirtImaging, Fairfield, CT) at 256x256 pixel resolution

and a frame rate of 7 Hz. Data acquisition was performed with Neuroplex software (RedShirtImaging, Fairfield, CT).

Odorants were diluted from saturated vapor in cleaned, desiccated air using a mass flow controller (Aalborg, Orangeburg, NY). Nitrogen was used as the vapor carrier to avoid oxidation. Separate lines were used for each odorant to avoid cross-contamination. The olfactometer delivers square pulses at a flow rate of 300 - 500 ml/min. In most acutely-imaged mice a double tracheotomy was performed and odorants presented using a 3 Hz artificial sniff protocol [1], which standardizes nasal airflow across subjects and also permits time-locking across trials for multi-trial averaging.

Slices of olfactory bulb were prepared as described in [3]. Briefly, mice were deeply anesthetized with halothane and decapitated, and the olfactory bulbs rapidly removed under ice-cold aCSF (contents in mM: NaCl 124, $NaHCO_3$ 26, Glucose 10, KCl 3, $CaCl_2$ 2, $MgSO_4$ 1.3, NaH_2PO_4 1.25). 400 µm thick horizontal slices were cut on a rotary slicer and transferred to an incubation chamber containing oxygenated aCSF at room temperature. Slices were then transferred to a standard imaging chamber where they were continuously perfused with oxygenated aCSF at 30-32°. Slices were imaged at 125-1000 Hz with an 80x80 pixel resolution camera (Red Shirt Imaging). Glomerular responses were evoked by 20-500 µA electrical stimuli from a concentric bipolar stimulating electrode in the olfactory nerve layer.

III. Results

A. Visualization of stimulus-evoked transmitter release from ORNs using synaptopHluorin

The synaptopHluorin construct contains a pH-sensitive green fluorescent protein (GFP) whose fluorescence is quenched at acidic pH [4]. Prior to transmitter release the GFP is sequestered in the acidic lumen of a synaptic vesicle. When transmitter is released by exocytosis, the GFP is exposed to the more neutral pH of the extracellular space and abruptly increases its fluorescence. In OMP-spH mice, the gene encoding the synaptopHluorin construct has been inserted into the genome under the control of the promoter for olfactory marker protein (OMP), which is heavily and selectively expressed in ORNs [1].

Figure 1A shows the resting fluorescence of the olfactory bulb of an anesthetized spH mouse, imaged through thinned bone. Individual fluorescent glomeruli are clearly discernable through the bone. The imaged area is approximately 10% of the total surface of the olfactory bulb. Figure 1B illustrates the large change in fluorescence in individual glomeruli evoked by the presentation of the odorant 2-hexanone to the nose (data are from a single trial and have not been filtered). Figure 1C shows the time course of the fluorescence change in two adjacent glomeruli displayed in Figure 1B, one of which (number 1) responded to 2-hexanone and one of which (number 2) did not. Note that the fluorescence increases continuously throughout the odor presentation due to the relatively slow recycling of presynaptic vesicles.

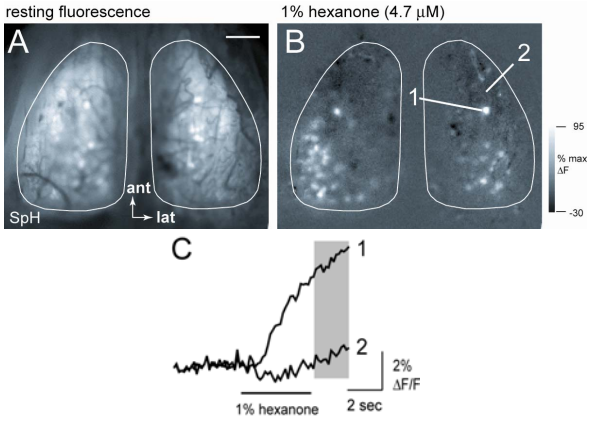

Figure 1. Typical odor-evoked fluorescence increases in the spH mouse *in vivo*. A) Baseline image of glomerular fluorescence prior to odor onset. Scale bar indicates 500 µm. B) Difference map showing change in fluorescence before and after odor presentation. C) Traces show the change in fluorescence over time for glomeruli shown in B. Fluorescence increases gradually throughout the odor presentation, reflecting the accumulation of spH in the membrane of the ORN terminals. Shaded box indicates the post-stimulus window used to create B.

The spatial pattern of spH signal evoked by an odor is very consistent across trials and relatively stereotyped across subjects. For example, the pattern of activity in Fig. 1B is typical of that evoked by hexanone, roughly symmetrical across the left and right bulbs with clusters of active glomeruli in the posterolateral part of the dorsal bulb. Note the distinctive location of the glomerulus labeled 1 in Fig. 1B and its symmetry across bulbs—this is an example of an "identified glomerulus" that can be recognized across subjects by its response to hexanone. Additional identified glomeruli can be routinely recognized in the responses to other odors. The ability to pick out specific glomeruli that correspond across bulbs and across preparations is useful in the quantitative comparison of responses across experimental conditions.

In olfactory bulb slices from OMP-spH mice, electrical stimulation of ORN axon bundles evokes spH signals in the glomeruli they innervate (Fig. 2A). These shock-evoked spH signals are rapid and linear reporters of transmitter release, and correspond very well to similarly evoked activity recorded from postsynaptic neurons [2]. They also reflect the rapid fluctuations in extracellular pH that are produced by synchronized transmitter release from many converging axons [3]. This pH change consists of an initial acidification of the synaptic cleft, resulting in a brief quenching of spH fluorescence (Fig. 2A, arrow and B), followed by a slower alkalinization of the cleft mediated by the transmitter reuptake process (Fig. 2C). Control of extrasynaptic pH changes by increasing buffering capacity

eliminates these pH artifacts, yielding the simple and rapid stimulus-evoked fluorescence increase that purely reflects vesicle fusion (Fig. 2D). Even without correcting for pH artifacts, slice experiments have proven to be a powerful complement to in vivo imaging because they permit precise temporal control over the input to the bulb and effective pharmacological access to the preparation [2, 3].

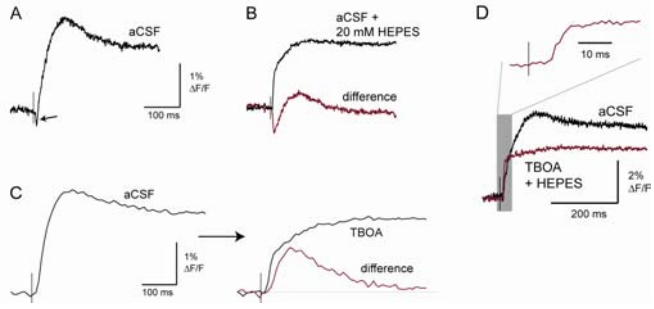

Figure 2. SpH signals imaged from ORN terminals in vitro. A) Trace of ON-evoked spH signal from a glomerulus at 1 kHz frame rate reveals complex signals kinetics. B) Black trace shows the spH signal from the same glomerulus as in A after switching to aCSF containing 20 mM HEPES. The initial fluorescence decrease is eliminated and the rise time is faster. The difference between the traces in A and B (red trace) reveals the change in the evoked signal produced by additional pH buffering. C) The slower alkalization is mediated by glutamate transporters. Left trace shows ON-evoked spH signal imaged from a different glomerulus in control aCSF. Right trace shows response in 50 μM TBOA (a glutamate transporter inhibitor, black) and difference between control and TBOA traces (red). D) ON-evoked spH signals show a rapid and simple fluorescence increase in 20 mM HEPES-buffered aCSF and TBOA (red) without the complex kinetics seen in control aCSF (black). Inset (above) shows the evoked signal in HEPES and TBOA on an expanded time scale.

B. Chronic imaging from spH mice

One major advantage of imaging with a genetically-encoded indicator like spH is the stability of the indicator across time. We have found it possible to image odor maps over at least 3 months in OMP-spH mice by "installing" a chronic imaging window in the skull overlying the dorsal bulb, along with a headscrew allowing precise repositioning of the preparation in repeated sessions. The window, which consists of cyanoacrylate placed over thinned bone, remains optically clear for a period of 3-4 weeks, after which the regrowing bone can be thinned again and cyanoacrylate reapplied, allowing for continuous chronic imaging. Fig. 3 shows an example of spH odor response maps imaged through such a window in two sessions 78 days (11 weeks) apart. As revealed in the maps of fluorescence change (Fig. 3, middle and right panels), the spatial pattern of odorant-evoked transmitter release is stable across this long period of time, and individual glomeruli can be clearly recognized from session to session (arrows).

The capacity for chronic imaging permits a within-subjects design for experiments on long-term plasticity of odorant representations. One area of particular interest is the regeneration of ORN projections to the olfactory bulb following lesion of the nasal epithelium. A remarkable and unique feature of the olfactory system is its ability to recover from even a massive loss of its primary sensory neurons. In the natural world, ORNs may die due to exposure to environmental toxins, infections of the nasal lining, or head trauma that severs all ORN axons passing from the nasal cavity to the olfactory bulb through the cribiform plate. In humans who experience olfactory loss due to any of these causes, olfactory function can recover, but is often associated with dysosmias.

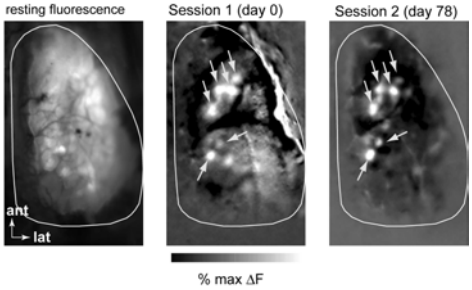

Figure 3. Long-term, chronic imaging with spH. Gray-scale spH response maps evoked by methyl valerate imaged through a chronic optical window in two sessions 78 days apart. The session 1 response map was from only a single trial and so had a smaller signal-to-noise ratio than the session 2 map. Nonetheless individual glomeruli are easily identified across sessions.

The epithelium has the general ability to restore the neuronal population of ORNs across the repertoire of receptors, and these regenerated cells then send their axons to the olfactory bulb. However, there is recent anatomical evidence that the precise targeting of ORN axons to their cognate glomeruli is not restored after wholesale neuronal lesion. Instead, ORN axons extend into the general region of the appropriate target glomerulus but typically fail to converge onto the one or two glomeruli normally seen in unlesioned animals. Over time, this mistargeting may correct itself to its pre-lesion state through axonal pruning, or it may settle into a new organization. Little is known of the functional changes that result from the initial mistargeting, and the processes that underlie the remapping of the initial projections are largely unknown. OMP-spH mice provide the means to repeatedly visualize the functional mapping of ORN input to the bulb, and to do so for a variety of olfactory receptor populations simultaneously through the use of different odorants. By imaging from these mice chronically, it is possible to map the pre-lesion functional organization of input to the bulb, unilaterally lesion the epithelium, and then watch the regeneration process unfold within a single mouse.

C. Investigating intrabulbar information processing

OMP-spH mice provide a convenient and powerful means to image the spatial maps of odorant-evoked input to the olfactory bulb, but provide little direct information about the processing of that information by bulbar circuitry. Recent work from our laboratory has used pharmacological

techniques *in vivo* and in slices to demonstrate that the input to the bulb is modulated by a feedback inhibition that presynaptically inhibits transmitter release from ORNs [2, 3]. This input modulation was shown to be organized on an *intra*glomerular level, rather than an *inter*glomerular level, suggesting that its purpose is to modulate the input from each subpopulation of ORNs independently [3]. However, this approach can only reveal glomerular processing in so far as it affects the presynaptic terminal.

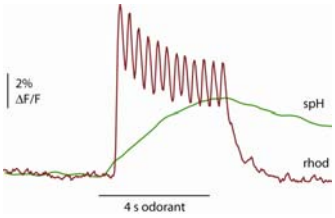

Figure 4. SpH and calcium (rhod) signal from a single glomerulus evoked by a 4 s presentation of 2% (s.v.) methyl valerate. Oscillations in the calcium signal reflect the respiration cycle.

To better investigate information processing in the olfactory bulb, we have developed a new technique that permits the visualization of both the input to the bulb and the postsynaptic responses this input elicits in bulbar neurons. The red fluorescent, calcium sensitive dye Rhod-2 is injected into the olfactory bulbs of an OMP-spH mouse. The dye is initially in AM ester form, which is membrane permeable and nearly non-fluorescent. Once taken up by neurons, the dye is de-esterified, thus producing a population of postsynaptic neurons that are labeled with a red calcium-sensitive indicator that can be optically separated from the green spH indicator. As shown in Fig. 4, odorant presentations result in a rapid, transient calcium signal from the activated postsynaptic neurons that parallels the time course of the spH signal imaged from the same glomerulus. The phasic changes in the rhod signal correspond to the breathing cycle of the mouse, which is usually too fast to be observed using spH alone. The postsynaptic origin of this signal can be confirmed by the application of glutamate receptor antagonists to block the input from ORNs, a manipulation that nearly eliminates the postsynaptic odor-evoked calcium signals. This combination of imaging techniques is a potentially powerful means for studying the processing of olfactory information in the bulb because it essentially visualizes the transformation of the odorant representation by intrabulbar circuitry. This technique may preferentially label mitral cells, which directly receive synaptic input from the ORNs and are the primary output of the olfactory bulb. If so, the comparison of the spH and calcium signals give a direct measure of the input-output function of the olfactory bulb.

IV. Discussion

OMP-spH mice provide a powerful new technique for studying ORN function in particular and sensory representations in general. Unlike previous mapping techniques like radiolabeled 2-deoxyglucose imaging or immunohistochemical detection of c-fos expression, spH imaging provides a direct metric of neural activity in a specific class of cells with sufficient resolution to discriminate individual glomeruli, and thus receptor-specific populations of ORNs. A panel of many different odors can also be tested sequentially, allowing the comparison of responses to different odors for each glomerulus within a single animal. Unlike unit recordings, spH imaging permits the measurement of activity within many glomeruli simultaneously. Unlike other fluorescent activity indicators, spH is expressed constantly and universally in ORNs, despite their constant turnover, thus permitting long-term functional imaging (see 1). The capacity for long-term imaging permits powerful within-subjects experimental designs, such as observing the regeneration and remapping of ORN inputs to the bulb after lesion of the olfactory epithelium.

The use of OMP-spH mice does impose important technical limitations. First, the time course of the spH signal is quite slow (reflecting the slow process of vesicle reuptake and reacidification after transmitter release), which makes it unsuitable for the study of olfactory input dynamics. Second, because the spH gene takes the place of the OMP gene, homozygous OMP-spH mice are OMP-null. The function of OMP in olfactory processing is poorly understood, making the significance of this confound unknown. In our experiments we routinely compare data collected from homozygous OMP-spH mice with that collected from heterozygous OMP-spH mice, which express one OMP allele and one spH allele, thus partially controlling for the reduction in OMP expression. Finally, the spH signal reflects both exocytosis and transient changes in the pH of the synaptic cleft. The relative contribution of these signals can be disentangled in slice experiments, where they are likely to be most significant, but are difficult assess in vivo.

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