Interglomerular Center-Surround Inhibition Shapes Odorant-Evoked Input to the Mouse Olfactory Bulb In Vivo

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Mouse olfactory receptor proteins have relatively broad odorant tuning profiles, so single odorants typically activate a substantial subset of glomeruli in the main olfactory bulb, resulting in stereotyped odorant- and concentration-dependent glomerular input maps. One of the functions of the olfactory bulb may be to reduce the extent of this rather widespread activation before transmitting the information to higher olfactory centers. Two circuits have been studied in vitro that could perform center-surround inhibition in the olfactory bulb: one acting through the classical reciprocal synapses between granule cells and the lateral dendrites of mitral cells and the dendrites of granule cells. One unanswered question from these in vitro measurements was how these circuits would affect the response to odorants in vivo. We made measurements of the odorant-evoked increase in calcium concentration in the olfactory receptor neuron terminals in the anesthetized mouse to evaluate the role of presynaptic inhibition in reshaping the input to the olfactory bulb. We compared the glomerular responses in 2- to 4-wk-old mice before and after suppressing presynaptic inhibition onto the receptor neuron terminals with the GABA\(_B\) antagonist, CGP46381. We find that the input maps are modified by an apparent center-surround inhibition: strongly activated glomeruli appear to suppress the release from receptor neurons terminating in surrounding glomeruli. This form of lateral inhibition has the effect of increasing the contrast of the sensory input map.

INTRODUCTION

Olfactory receptor neurons project axons to specific glomeruli of the olfactory bulb so that all the sensory input detected by a single type of olfactory receptor protein converges onto one or two glomeruli in each bulb (Ressler et al. 1994; Treloar et al. 2002). An odorant typically binds to many receptor proteins with different affinities and thus at moderate concentrations can activate a substantial subset of all glomeruli (Meister and Bonhoeffer 2001; Rubin and Katz 1999; Wachowiak and Cohen 2001). One of the functions of the olfactory bulb may be to reduce the extent of this rather widespread activation before transmitting the information to higher olfactory centers (Laurent 1999; Perez-Orive et al. 2002).

Considerable evidence shows that the mitral cell output activity differs in several dimensions from the olfactory sensory neuron input (e.g., Kay and Laurent 1999). Two circuits have been studied in vitro that could perform center-surround inhibition in the olfactory bulb: one acting through the classical reciprocal synapses between granule cells and the lateral dendrites of mitral cells (Rallis et al. 1966) and the other between glomeruli (Aungst et al. 2003). Both these circuits can directly modify mitral cell output, the former by acting onto their basal dendrites, the latter by acting on the tuft of the apical dendrite inside the glomerulus. The later circuit could, however, also presynaptically inhibit neurotransmitter release from the olfactory receptor axon terminals.

From experiments in vitro it is known that synaptic transmission from olfactory receptor neurons can be modulated by presynaptic GABA\(_B\) and dopamine D\(_2\) receptors (Duchamp-Viret et al. 2002; Ennis et al. 2001; Nickell et al. 1994; Wachowiak and Cohen 1999; Wachowiak et al. 2005). In vertebrates, the activation of these receptors suppresses synaptic vesicle release by reducing the amount of calcium that enters the axon terminal as a result of an action potential (Wachowiak and Cohen 1999; Wachowiak et al. 2005). The component of presynaptic inhibition controlled by GABA could result from spillover within a glomerulus from GABAergic periglomerular neurons activated either directly by the olfactory receptor neurons or indirectly by activity in interglomerular superficial short axon cells (Aungst et al. 2003) or the mitral/tufted cells (Murphy et al. 2005).

We studied the effects of blocking presynaptic inhibition in vivo to determine its role in shaping the response to odor stimulation. The spatio-temporal structure of the response to odorants in vivo will be much more complex than the input resulting from electric shocks to the olfactory nerve layer in a slice preparation. In addition, the milieu of the presynaptic terminals might differ substantially in the tonic concentration of neurotransmitters and neuromodulators between in vitro and in vivo preparations. Finally, some of the three-dimensional structure of the olfactory bulb is lost and feedback from higher brain centers is removed in the in vitro preparation.

Here we present evidence that interglomerular center-surround inhibition shapes the odorant-evoked input in vivo through GABAergic presynaptic inhibition at olfactory receptor axon terminals. We used a local drug perfusion protocol to compare the maps of odorant-evoked input to the mouse olfactory bulb under control conditions and after blocking the presynaptic GABA\(_B\) receptors with the antagonist CGP46381. We found that the GABA\(_B\) antagonist qualitatively changes the input map in a manner consistent with blockage of interglo-
merular center-surround inhibition acting presynaptically on the afferent axon terminals.

**METHODS**

Experiments were performed on 24 female C57/BL6 mice that were between 14 and 30 days of age at the time of recording; effects of CGP46381 were not reliably detected in older animals—the penetration of drugs through the dura and brain tissue of older animals may be restricted. Olfactory receptor neurons were loaded with calcium green-1 dextran as described previously (Wachowiak and Cohen 2001). During surgery and recording, animals were anesthetized with pentobarbital sodium. Bone over one dorsal olfactory bulb was removed, but the dura matter was left intact. The exposed part of the brain was kept immersed in Ringer solution and covered with a coverslip. CGP46381 was bath-applied by replacing this solution. In control measurements, the solution was replaced at the intervals used for the drug protocol. Before addition, the replacement solution was heated to 34°C to approximate the temperature of the exposed portion of the olfactory bulb. A double tracheotomy was performed to allow controlled flow of air through the nasal cavity; the animals breathed freely through the lower canula. All procedures were approved by the Yale University and the Marine Biological Laboratory animal care committees.

**Olfactometer**

To minimize the possibility of any mechanical response to the introduction of odorant into the airflow, we made several modifications, shown in Fig. 1, to the odorant delivery and respiration apparatus described earlier (Lam et al. 2000). The new olfactometer (Fig. 1A) was connected so that the saturated odorant vapor, when introduced into the airflow, replaced the equivalent volume of diluent air. This insured that the rate, humidity, and direction of airflow over the nares stayed constant throughout the measurement. The artificial respiration system (Fig. 1B) was connected so that the open end of the valve that performed artificial sniffing was taking air in through a partially open tap, which was adjusted so that its resistance to air flow matches the resistance of the nasal passages. This allowed a more precise metering of the flow rate through the nose. An electronic pressure sensor (Honeywell ASCX01DN) connected at the output of the rostrally directed tracheal canula enabled early detection of tracheal collapse.

The concentration of the odorants is given in percentage of saturated vapor. We selected odorants that activate the visually accessible part of the olfactory bulb. The odorants and their final concentrations in the 24 preparations were: 2-hexanone (1%, n = 11; 2%, n = 1), isoamyl-acetate (0.4%, n = 1; 1%, n = 2; 1.3%, n = 1; 2%, n = 3), and benzaldehyde (0.4%, n = 1; 2%, n = 3). In this study, we did not attempt to measure the dependence of map alteration on odorant concentration.

**Optical recording**

The dorsal olfactory bulb was imaged through Wild 10 × 0.4 NA or 7 × 0.2 NA objectives mounted onto an antique Leitz Ortholux II upright microscope; the actual magnifications of the two lenses were ×15 and ×10, respectively. Excitation light from a 150-W xenon arc lamp was passed through a 480/50-nm band-pass filter and reflected by a 515-nm dichroic mirror. The fluorescence above 530 nm was recorded with a NeuroCCD-SM or NeuroCCD-SM256 camera using NeuroPlex software (RedShirtImaging, Fairfield, CT).

**Data analysis**

Activated glomeruli were identified manually in each preparation based on the spatial pattern of stimulus-evoked activation (between 17 and 42 per preparation). Small shifts in the position of the preparation relative to the camera were corrected for in software by comparing the spatial patterns of the resting fluorescence intensity at every recording.

The blue trace in the top section of Fig. 2A shows the time-course of the calcium green-1 fluorescence response from a single glomerulus to a 2-s presentation of the odorant 2-hexanone at 1% of saturation. The black trace shows the same data temporally filtered with a low-pass Gaussian filter (f = 1.2 Hz); the two black bars below the trace indicate the frames used for the subtractions to make the maps of activity shown in Figs. 2B and 4. The prestimulus period of every recording was fit with an exponential decay curve; the extension of this fit into the stimulus period was subtracted from the signal to give the signal amplitude (Fig. 2A). In the data presented here, only the first 2 s after stimulus onset were used in quantifying the response (Fig. 2A, gray area) to minimize contributions from any intrinsic signal. Similar results were obtained if we used the peak or the values at 0.5, 1, 1.5, or 2 s after stimulus onset (data not shown).

Each vertical tic mark in the bottom section of Fig. 2A shows a trial like that shown in the top section. To reduce the noise, four such trials were averaged for the data in Figs. 4–7.

The data in Fig. 3C come from 14 preparations where there was a control period with at least three sets of four trials before the drug application and two sets of four trials in the presence of the GABA B antagonist CGP46381. The last set of four trials before the application of the drug was taken as the baseline data, and the last four trials in the presence of CGP46381 were taken as the data in the presence of the drug.

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**FIG. 1.** Schematic diagram of the olfactometer (A) and respiration apparatus (B) used to make the measurements. A: odorant vapor displaces an equivalent volume of diluent air when introduced into the airflow, thus minimizing the possibility of mechanical artifacts. B: valve is adjusted to present an equal resistance to airflow as the nasal passages of the preparation, allowing a more accurate measurement of the flow rate. An electronic pressure sensor, whose output is recorded by the data acquisition system, enables early detection of tracheal collapse.
For the plots in Fig. 6, A and B, we used the glomerulus and the surround dimensions shown in Fig. 2B. All of the pixels within the delineated surround region were used to measure the cumulative response amplitude of the surround, not just the pixels belonging to previously identified glomeruli; otherwise the manual glomerulus identification process would introduce a bias into the measurement by excluding some glomeruli that were less activated (and potentially more affected by any center-surround circuit). Similar results were obtained with 50-μm-wide annular surrounds starting at 50, 100, 150, and 200 μm from the center of the glomerulus (data not shown).

Normalization of glomerular amplitudes for Figs. 3–5 was performed by dividing by the largest amplitude in each recording. The lognormal distribution function fit to the data in Fig. 3B is defined as

\[ y = \frac{A}{\sqrt{2\pi}wx} \exp\left(-\ln(x/x_0)^2/2w^2\right) \]

where \( x \) is the independent variable, \( x_0 \) is the center, and \( w \) is the width.

**FIG. 6.** Recording of presynaptic population signals from olfactory afferents entering the mouse olfactory bulb. A: protocols for the drug perfusion and optical recordings and for quantifying the signal observed as a change in the level of calcium green-1 dextran fluorescence. Two-second odorant stimuli were given not less than 1 min apart to minimize the possibility of habituation. The last 4 recordings in the presence of a drug in the bath (drug) were averaged and compared with the average of the 4 recordings just before drug application (baseline). Inset: prestimulus period of every recording was used to estimate the time-courses of dye bleaching and filter setting (red trace) on the time progression of fluorescence; gray area was used as the measure of response amplitude; blue: unfiltered trace recorded at 31.25 frames/s; black: temporally filtered trace used for making map images (low-pass Gaussian, \( f_0 = 1.2 \) Hz). B: map of the fluorescence increment observed in a dorsal olfactory bulb in response to 1% 2-hexanone. A substantial subset of the dorsal glomeruli is activated by the odorant. Glomeruli were identified manually in each preparation. Traces from pixels that lie within the distances outlined by circles in the image were averaged to measure the response of a glomerulus and of its surround.
We used a fluorescent calcium indicator dye, loaded into the olfactory receptor neurons through the nasal epithelium (Friedrich and Korsching 1997; Wachowiak and Cohen 2001), to monitor the level of presynaptic activity in the receptor nerve terminals in the olfactory bulb glomeruli. Figure 2B shows a grayscale map of odorant-induced activity; individual glomeruli are readily distinguishable.

Response stability

To quantify the stability of our preparations, we determined the signal amplitudes (Fig. 2A) of 367 glomeruli recorded from 14 animals through periods of undisturbed repeated measurements (Fig. 2A, control period). The results of these control measurements are shown in Fig. 3A. Figure 3B shows a histogram (points) of the relative change of amplitudes of 367 glomeruli between two measurements (each consisting of 4 averaged trials) made 20 min apart under unchanged conditions. This distribution agrees well with a lognormal distribution function shown in red. Consistent with the result shown in Fig. 3A, the center of the distribution lies slightly below 1, indicating a slow decline of the average amplitude over time, which could be caused by a combination of dye bleaching, photodynamic damage, and run-down of the preparation. From the fitted distribution function, we conclude that amplitudes of <5% of all glomeruli are expected to fluctuate upward by chance by >21% (the region to the right of the vertical line; Fig. 3B) over the course of the drug protocol. Because some sources of noise are expected to have effects that are correlated between glomeruli (e.g., fluctuations in odorant delivery will tend to affect all glomeruli in a similar manner; Fig. 6 in Wachowiak and Cohen 2001), we used this distribution as a conservative way to estimate the significance of any drug-induced change in the amplitude of an ensemble of glomeruli.

Effect of GABA<sub>B</sub> antagonists on signal amplitude

The brain-penetrant, competitive GABA<sub>B</sub> antagonist CGP46381 caused a long-lasting increase in the average amplitude of the presynaptic calcium signal when bath-applied at millimolar concentration (Fig. 3C). This effect was dependent on the age of the animal; it was usually present in juvenile mice (14–29 days old) and usually absent in mature mice (>30 days old). We only used the data recorded from animals younger than 30 days. Furthermore, we did not analyze the experiments

RESULTS

We used a fluorescent calcium indicator dye, loaded into the olfactory receptor neurons through the nasal epithelium...
where the increase of signal amplitude over the course of the protocol was <21% above the baseline (the last set of 4 trials before the application of the drug) amplitude; the probability of the baseline signal fluctuating past this cut-off as a result of noise was measured to be <5% as described above. Nine experiments passed these criteria.

**Effect of GABA_B antagonists on the input map**

Odorant-evoked input maps are altered when GABA_B receptors are blocked. Figure 4 shows examples of the alteration from two different preparations. The color scale has been adjusted between the baseline and drug-perfused recordings to compensate for the increase in calcium signal amplitude after the addition of CGP46381 in the glomeruli with the largest signals. In both preparations, there is a more prominent activation of many weakly activated areas after presynaptic inhibition is blocked, as would be expected if strongly activated glomeruli normally suppressed the weakly activated ones. In the following, we present two different measurements quantifying this observation.

**REDUCTION OF MAP CONTRAST.** First, in Fig. 5, we plot cumulative distributions of normalized glomerular signal amplitudes. Figure 5B shows that there is a shift to the right after perfusion with CGP46381 (solid line) compared with the baseline distribution (dashed line; a total of 251 glomeruli in 9 animals; \( P < 0.001 \), Kolmogorov-Smirnov test). This is a manifestation of the reduction of input map contrast. Figure 5A shows that no such shift occurred when we compared two measurements from the control dataset where no drug was applied (a total of 367 glomeruli in 14 animals; \( P > 0.7 \)).

**CENTER–SURROUND ANALYSIS.** Next we measured the effect of the GABA_B antagonist on the glomerular signal and the surround signal (Fig. 2B) as a function of the relative activation of the glomerulus and the surround. In Fig. 6B, we plot the ratio of relative change of the amplitudes of glomeruli and their surrounds against the ratio of the amplitude of a glomerulus and its surround before the drug was applied. When presented in this manner, the glomeruli will be distributed along a horizontal line at \( y = 1 \) if their amplitudes did not change but also if all the amplitudes were scaled by a constant. The noise in the measurement is shown by the analogous plot (Fig. 6A) from a control data set, where we only replaced the vehicle. Figure 6B shows that the GABA_B antagonist produced a marked correlation between the two quantities that would not be present if the drug had a proportional effect on all glomeruli. To quantify the level of correlation, we fit the function \( y = 1 + a \log(x/b) \) to these data (red lines in the figure): the slope of the best fit is \( a = 0.53 \pm 0.04 (P < 0.0001) \) compared with \( a = 0.07 \pm 0.03 \) for the control data (optimal value ± SD). These results were not significantly affected by excluding the data from glomeruli whose identity was questionable (e.g., the diffuse spot in the bottom right of Fig. 2B). Errors in the calculation of \( \Delta F/F \) resulting from the fact that we did not subtract the background fluorescence (before staining) and blurring of the signals from scattering or out-of-focus light would both tend to reduce the slope seen in Fig. 6B. The slope of the line in Fig. 6B indicates that for glomerular signals that are eight times larger than the average surround, CGP46381 caused an increase in the surround that was approximately twice as large as the increase in the glomerulus. As another control, after perfusion of a D_2 receptor agonist we observed an overall reduction in signal amplitude that was not accompanied by a significant correlation when the data were plotted as in Fig. 6 (data not shown). We conclude that strongly activated glomeruli are relatively less suppressed by GABAergic presynaptic inhibition than their surround.

One concern was the possibility that the correlation shown in Fig. 6B, as well as the reduction of contrast in Fig. 4 and the shift in the cumulative distribution in Fig. 5B, are a consequence of a simple nonlinearity in the effect of the drug on the population calcium signal. For example, if strongly activated glomeruli are operating near the saturation of the calcium signal, it is conceivable that the drug could simply have a greater effect on weakly activated glomeruli, thereby changing quality of the input map but not actually resulting from lateral inhibition. To test for this possibility, in Fig. 7 we plotted the relative change of amplitude of a glomerulus caused by perfusion of CGP46381 against its normalized baseline amplitude, without regard for the surround. This distribution does, indeed, show that weakly activated glomeruli are relatively more affected by the GABA_B antagonist, an effect that could be caused either by saturation or by blocking lateral inhibition. To distinguish the two possibilities, we compared the surrounds of
only modestly activated glomeruli that show similar baseline activation but undergo a different change after drug perfusion. The noise in the data necessitates the comparison of several glomeruli, so we divided all the glomeruli near the middle of the observed dynamic range into two groups along the median (outlined in red and blue in Fig. 7). This band was chosen with the following rationale: strongly activated glomeruli were not used because any saturation effects would show here first; weakly activated glomeruli were not used because noise dominates in this region; the band was extended from a normalized amplitude of 0.5 to lower values until enough glomeruli were included to reach a conclusion that was statistically significant. The glomeruli so selected that were more affected by the GABA_B antagonist did, in fact, have more strongly activated, nonlinearity in effect of the drug. R = A(glomerulus)/A(surround); values are means ± SE; 251 glomeruli from 9 animals.

**D I S C U S S I O N**

In this study, we investigated the role of presynaptic inhibition of neurotransmitter release from receptor neuron axon terminals in shaping the map of input from the nose to the olfactory bulb in an in vivo mouse preparation. Presynaptic modulation at receptor neuron axon terminals through GABA_B and dopamine D_2 receptors has been well established in in vitro preparations (Aroniadou-Anderjaska et al. 2000; Ennis et al. 2001; Duchamp-Viret et al. 2002; Nickell et al. 1994; Wachowiak and Cohen 1999; Wachowiak et al. 2005). A question left unanswered by these studies was what role, if any, do these presynaptic modulatory mechanisms play in shaping the input elicited by odorants in vivo. The results we present here provide the first evidence that presynaptic inhibition modifies the receptor neuron input evoked in vivo by odorant presentation to the nose.

The roles suggested for presynaptic inhibition of release from olfactory afferents were thought to be local to a glomerulus: the relative suppression of weak signals through tonic activation, the relative enhancement of sensitivity to novel odors (Aroniadou-Anderjaska et al. 2000), and the increase of the dynamic range of glomerular response to different concentrations of an odorant (Wachowiak et al. 2002). More recent evidence points to the possibility of disynaptic inhibitory communication between glomeruli through superficial short axon cells with mean axon lengths of 300–400 μm, four to five glomeruli (Aungst et al. 2003), as well as dendrodendritic activation of GABAergic periglomerular interneurons by mitral/tufted neurons (Murphy et al. 2005), where the release of GABA inhibits further glutamate release from afferent terminals. In addition, the mitral/tufted neurons form a widespread network through the classical dendro-dendritic synapses with granule cells (Rall et al. 1966). Because of the relatively long space constant of the mitral cell apical dendrite (Djurisic et al. 2004), membrane potential changes can reliably spread from the soma back to the glomerular tuft. Thus there are a number of candidate pathways for inhibiting afferent input as a result of activity that is not local to a glomerulus.

Our results indicate that one component of the modification of the presynaptic calcium signal is a relative suppression of the surround of strongly activated glomeruli, which increases the contrast of the input map. The apparent absence of axoaxonic synapses in glomeruli (Bonino et al. 1999; Shepherd and Greer 1998) suggests that this effect is a result of spillover within a glomerulus of GABA released from periglomerular neurons that are activated by one or more interglomerular circuits. Thus the presynaptic terminal responds to the average level of GABA within a glomerulus at a time scale of spillover, an order of magnitude slower than direct synaptic communication.

Several pieces of evidence suggest that the map change we observed is not an artifact of nonlinearity of the effect of the GABA_B antagonist on the amplitude of the calcium signal. First, if we select a subset of glomeruli on the basis of their modest level of activation to minimize the possibility of encountering saturation, and without regard for their location in the map, the glomeruli that are nearer strongly activated regions behave differently under the antagonist than the ones that are further away (Fig. 7). Such an effect cannot be produced by a local, intraglomerular circuit. For instance, while tonic activation of GABA_B receptors is likely to introduce a nonlinearity into the response to an exogenous GABA_B receptor antagonist, the manifestation of this kind of nonlinearity will be the same regardless of how distant a glomerulus is from a strongly activated one. In addition, the proximity effect cannot be artificially produced by cross-talk between glomeruli that mixes their signals, such as the scattering of light and the limited spatial resolution of the measurement apparatus, as any indiscriminate mixing will dilute the observed difference.

Second, recent reports point to a nearly linear dependence of the probability of release from olfactory receptor neuron terminals on the extracellular calcium concentration (Murphy et al. 2004; Wachowiak et al. 2005) and to a nearly linear dependence of release failure probability at a central synapse.
on the calcium signal reported by an indicator of affinity similar to the one we used (Koester and Johnston 2005). These results do not favor a large nonlinearity between the response of our indicator and neurotransmitter release. Because network-mediated center-surround inhibition is expected to create results like those shown in Figs. 4–6, we conclude that interglomerular communication is the most likely explanation for the map modification. The modification of the presynaptic map is not the only effect of CGP46381; the amplitude of the calcium signal is increased (Fig. 3C) implying that presynaptic inhibition has other important functions in addition to altering the map.

From recent experiments using synaptopHluorin loaded presynaptic terminals, McGann et al. (2005a) concluded that “inhibitory connections between glomeruli mediated . . . lateral inhibition between olfactory sensory neuron inputs in slices but did not do so in response to odorant stimulation in vivo”. Surprisingly, some data that they present (McGann et al. 2005b; Fig. 8A of McGann, et al. 2005a) show clear examples of relatively increased responses by weakly responding glomeruli after blocking presynaptic inhibition.

The relative contribution of the different interglomerular circuits to the modification of the presynaptic input map are unknown. In the future this question might be addressed by recording the maps of the activity of specific juxtaglomerular neuron types through the use of genetically targeted indicators of activity in concert with drugs that selectively inactivate the particular circuits. Similar measurements from the output neurons (mitral/tufted cells) would provide a measure of the sum of all the interglomerular effects generated in the olfactory bulb.

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