Voltage sensitive dye imaging reveals shifting spatiotemporal spread of whisker-induced activity in rat barrel cortex

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Running head: Voltage sensitive dye imaging of barrel cortex.

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ABSTRACT

In rats, navigating through an environment requires continuous information about objects near the head. Sensory information such as object location and surface texture are encoded by spike firing patterns of single neurons within rat barrel cortex. Although there are many studies using single unit electrophysiology, much less is known regarding the spatiotemporal pattern of activity of populations of neurons in barrel cortex to whisker stimulation. To examine cortical response at the population level, we used voltage sensitive dye (VSD) imaging to examine ensemble spatial-temporal dynamics of barrel cortex in response to stimulation of single or two adjacent whiskers in urethane anesthetized rats. Single whisker stimulation produced a post-stimulus fluorescence response peak within 12-16 ms in the barrel corresponding to the stimulated whisker (principal whisker). This fluorescence subsequently propagated throughout the barrel field, spreading anisotropically preferentially along a barrel row. Following paired whisker stimulation, the VSD signal showed sub-linear summation (less than the sum of two single whisker stimulations), consistent with previous electrophysiological and imaging studies. Surprisingly, we observed a spatial shift in the center of activation occurring over a 10-20 ms period with shift magnitudes of 1-3 barrels. This shift occurred predominantly in the posteromedial direction within the barrel field. Our data thus reveal previously unreported spatiotemporal patterns of barrel cortex activation. We suggest that this non-topographic shift is consistent with known functional and anatomical asymmetries in barrel cortex and that it may provide an important insight for understanding barrel field activation during whisking behavior.

KEYWORDS: whisker function, optical imaging, somatosensory, barrel cortex, VSD.
INTRODUCTION

In rodents, the topographic single whisker to single cortical barrel relationship has long been recognized as a principle of cortical functional organization (Woolsey and Van der Loos 1970). Despite this clear one-to-one relationship, neurophysiological studies have revealed that principal whiskers are also modulated by subthreshold influences from distant sites (Moore and Nelson 1998, Ghazanfar and Nicolelis 1999, Veinante and Deschenes 1999, Brecht and Sakmann 2002, Manns et al 2004, Higley and Contreras 2003, 2005). For example, anisotropic suppressive effects of an adjacent whisker on principal whisker neuron response have been demonstrated (Simons and Carvell, 1983, 1989), suggesting that there may be spatial asymmetries in inhibitory interactions across the barrel field (cf. McCasland et al. 1991). In addition to spatial interaction effects, there are also temporal dependencies (Simons 1985; Armstrong-James and Fox, 1987). Displacing whiskers with different interwhisker stimulation times (from 0 to 40 ms) produces either suppression or facilitation of primary whisker responses depending on the interstimulus interval (Simons and Carvell 1983 and 1989, Shimpegi 1999 and 2000, Bruno and Simons 2002). These results suggest the response of cortical barrel field is a result of a complex array of spatial and temporal interactions. While electrophysiological studies have given us tremendous understanding of single neuron behavior in barrel cortex, we lack a good understanding of the spatiotemporal profile of the population response across the whisker barrel field, something which is extremely relevant for understanding cortical activity during whisking behavior in rodents that actively move their whiskers.

To examine the population response of barrel cortex in rats, we have used the voltage sensitive dye (VSD) imaging method to provide high spatial and temporal resolution visualization of cortical activity. VSD imaging measures changes in membrane potential over large populations of neurons primarily in layer 2/3 of cortex (Kleinfeld and Delaney 1996, Jin et
al 2002, Civillico and Contreras 2006, Ferezou et al 2006, Lippert et al 2007) and is thus useful for revealing the time and spread of barrel responses as well as more distant subthreshold modulatory response. Civillico and Contreras (2006) used this method to study barrel fields in mice and found, surprisingly, only suppressive effects in multi-whisker responses. There are several differences between barrel field of the mouse and rat species, including distinctly different arrangements on the level of cytoarchitecture and overall laminar connectivity (Welker and Woolsey, 1974, Simons and Woolsey 1984, Bureau et al 2006). Unlike mice, rats have more developed septal zones (Petersen and Sakmann 2001), regions which are not dominated by a single principal whisker, but respond equally well to 2 or 3 whiskers. This raises the possibility of differences in spatiotemporal activation profiles between these two species. Whether multi-whisker stimulation in the rat leads to facilitation or suppression at the population level remains unclear (although see Kleinfeld and Delaney 1996 for alternating multi-whisker stimulation in rats).

In this study, we examine the cortical response to single and paired whisker stimulation with VSD methods. Our results show that paired whisker stimulation leads to sub-linear summation of responses when compared to single whisker stimulation. In addition, over a 20-25 ms period following whisker stimulation, we find a surprising non-topographic shift in activation over a distance of 1-3 barrels away from the center of the initially stimulated whisker barrel.
MATERIALS AND METHODS

Surgical Procedures: Four adult Long Evans rats 250 to 450 g were used for voltage sensitive dye imaging (VSDI). All procedures were conducted in accordance with National Institutes of Health guidelines and approved by the Vanderbilt University Animal Care and Use Committee. Rats were anesthetized with urethane (1.5 g/kg, 30% aqueous solution, i.p.) and given additional doses as needed to maintain surgical levels of anesthesia. Following initial sedation, rats were given 0.03 mg/kg atropine sulfate by intramuscular injection as well as 1 cc of lactated ringers IP. A tracheotomy was performed and the animal ventilated. A craniotomy was carefully performed over the left hemisphere centered over barrel cortex; with 0.5 mm Mannitol injected IP just prior to removal of the skull as a preventative measure to minimize brain swelling. Dura was then carefully removed. Cortex was covered with 4% agar and a glass coverslip to reduce pulsation and to create an optical window.

Optical Imaging: A CCD camera (NeuroCCD 256, SciMeasure Analytical Systems, Inc. Decatur, GA) was positioned over the craniotomy. Images were collected with a Redshirt Imaging System running CortiPlex software (Redshirt Imaging, Decatur, GA). Initially, intrinsic optical imaging (632 nm illumination) was performed by previously published methods (Cayce et al, 2011) to functionally locate barrel cortex. To localize the barrels, several rostral (condition 1) or caudal whiskers (condition 2) were stimulated and the activation maps were compared to a blank no stimulus condition (condition 3). The imaging run consisted of 20 to 50 trials of each stimulus condition. Trial averaging was used to improve signal to noise.

Subsequently, barrel cortex was stained with voltage sensitive dye RH-1691 or 1692 (Optical Imaging, New York, NY) dissolved in artificial CSF by applying, for 1.5 - 2 hours, dye to cortex exposed by excising the covering agar. After staining, cortex was again covered with 4% agar and a glass coverslip. For VSD imaging, cortical exposure to 632 nm illumination was
gated by an externally controlled shutter (Uniblitz, Vincent Associates, Rochester NY) to minimize light exposure and dye bleaching. To image VSD fluorescence the camera lens was outfitted with a 650 nm high pass filter. The CCD camera acquired frames at either 100 Hz with a 256 by 256-pixel array or 360 Hz with a 64 by 64 pixel array.

All whiskers were trimmed on the contralateral whisker pad except for C2, C3, and C4. Individual piezoceramic actuators (Noliac, Alpharetta, GA) were arranged to contact the whiskers 5 mm from each whisker base. Each piezoceramic stimulator was driven by a 20 ms 75 V square wave pulse (S88 stimulator, Astro-Med, West Warwick RI) calibrated to displace each whisker ~250-300 μm in a rostral direction equal to a peak velocity of around 1700 degrees per second. For each imaging trial an in-house written computer program (LabVIEW, National Instruments Corp, Austin, TX) controlled the temporal sequence of events. The sequence was as follows: (1) each trial began with illumination shutter opening at 100 msec prior to whisker stimulation and illumination of cortex with 632 nm light; (2) the CCD camera started acquiring frames 80 ms prior to whisker stimulation; (3) at 0 ms whiskers were stimulated for 20 ms; (4) at 180 ms the camera stopped collecting frames and the shutter was closed blocking the 632 nm illumination; (5) an 8 s inter-stimulus interval separated trials.

To explore multi-whisker interactions we compared maps generated by four to six conditions. The conditions included 1) C2 stimulation, 2) C3 stimulation, 3) C4 stimulation, 4) simultaneous C2 and C3 stimulation, 5) simultaneous C3 and C4 stimulation, and 6) no whisker stimulation. Each imaging run consisted of 50 to 100 trials of each stimulus condition in random order.

Image Analysis: Multiple steps of image analysis were used to measure the spatial and temporal spread of neural activity (fluorescence) in barrel field cortex. Image analysis consisted of (a) aligning the field of view (FOV) of the image maps with the underlying anatomy, (b)
generating functional maps within the image frames, and (c) selecting a region of interest (ROI) for time course signal analysis.

**Image Alignment with Anatomy and ROI Selection:** To select regions of interest (ROI) for image analysis as well as to link the anatomical with the functional aspects of the images, a cortical blood vessel map of the camera’s field of view (FOV) was acquired with green light (578 nm). The FOV was 4 mm x 4 mm for the collected images (Fig 1A).

After an imaging session, the brain was perfused with 4% paraformaldehyde in PBS and 40 μm frozen sections were cut parallel to the pial surface (tangential plane) and stained for cytochrome oxidase activity (Wong-Riley 1979). The blood vessel image was aligned with the vessel patterns in the most superficial sections to register the FOV with the histology (Fig 1B and the cytochrome oxidase stained barrels in layer IV (Fig 1C). The boundaries of the cytochrome-defined whisker cortical barrels (Fig 1D) were then superimposed over the optical images (Figs 1E and F).

**Image Processing:** To generate maps for visualizing the spread of activity and for making threshold measurements of the area of activation, the raw ΔF/F images (Fig 1F) were spatially filtered using a spatial Gaussian filter with a sigma of 2.00 pixels for the 360 Hz images (64 x 64 pixels) and a sigma of 8.00 pixels for 100 Hz images (256 x 256 pixels) (Fig 1G). With the map of barrel cortex superimposed over the functional recordings, ROI’s were selected with respect to individual barrels. To confirm functional image alignment with the histologically determined barrel field location, ROIs were also outlined using the initial area of activity after whisker stimulation (thresholded above 75-90% of peak activity). The area of initial activity, before spreading across the barrel field, lined up well with the primary barrel of an activated whisker (Fig 1H). To ease visualization of functional activity, images were often scaled to a color map where warm to hot colors (orange, red, white) indicated the level of increased activity and cool
to cold colors (green, blue, black) indicated the level of decreased activity (Fig 1I and J). Image analysis was performed using custom Matlab code (MathWorks Inc, Natick, MA) as well as ImageJ (http://rsbweb.nih.gov/ij/index.html) and FeatureJ (http://www.imagescience.org/meijering/software/featurej/).

**Analysis of Time Course:** ROIs were centered over individual barrels to assess a response of a barrel to whisker stimulation. To examine percent change in signal over time, functional images were derived by calculating for each frame on a pixel-by-pixel basis the percent change in signal. The first few frames, up to 70 ms prior to stimulation, were used as a baseline for \((F_b - F_a)/F_a \times 100\), where \(F_a\) is the pixel fluorescence prior to stimulation and \(F_b\) represents the pixel fluorescence collected at a subsequent time point. Trial averaging was used to improve signal to noise. Figure 2 shows two plots of a \% ΔF time course. Figure 2A illustrates the raw averaged \% ΔF on the y-axis plotted against the frame number (each frame 2.7 ms) on the x-axis for a 100 trials of single whisker stimulation (black trace). Also plotted in Figure 2A are the values collected for 100 blank trials (no stimulus, gray trace). The gradual decrease in VSD amplitude was a consequence of the photobleaching of the VSD and was the same in the blank and stimulus conditions. Figure 2B shows the response profile after subtracting the \% F of the blank (Fig 2A gray trace) from the single whisker stimulus condition (Fig 2A black trace), which removed the decay in fluorescence amplitude and revealed the change in fluorescence (\% ΔF) caused by whisker stimulation. The response amplitude for each stimulus condition was measured from the peak response that typically occurred in a window 10-15 ms after stimulus onset (Fig 2B marked with green line).

**Thresholding Areas of Activation and Measuring Centroid Location:** As size and location of activation can be influenced by thresholding criteria, we examined activation sequences with different threshold criteria. As shown in Figure 3, lower thresholds provide larger apparent
activation zones (e.g. 50% threshold) and higher thresholds reveal smaller activations (e.g. 90% threshold). We based our threshold on two criteria: (1) the latency to initial activation was consistent with electrophysiological studies and (2) the activation size was confined to the whisker barrel size. Based on this, we determined that 75% was the most appropriate threshold level. This criterion was also used in previous studies and agree with the profile of activation spread from single barrels (in which activity in layer 2/3 is initially confined to a single barrel before spreading to the surrounding barrel field) revealed in previous electrophysiological studies (Armstrong-James et al 1992, Petersen and Sakmann 2001, Petersen et al 2003, Ferezou 2006, Armstrong-James 1992). The centroid for each frames’ area of activity was determined by calculating the average of the X and Y coordinates for all the pixels with activations above threshold. In order to evaluate changes in the center of activation X and Y coordinates were determined for the centroid of activation of the first frame of activity above threshold and the last frame of activity above threshold or when the area of activation dropped below the typical area size of a barrel. The coordinates for the centroid of activation for the first frame of activity above threshold were designated as the starting coordinates (SC = Xs,Ys); while coordinates for the centroid of area of activation above threshold for the last frame was designated ending coordinates (EC= Xe,Ye).

To examine whether the centroid measurements were affected by the thresholding procedures we also determined the centroid of activation by fitting, in a program written in LabVIEW (NI), the unfiltered voltage sensitive dye images with a 2D Gaussian function with additional variables for baseline offset and Gaussian orientation. We report only the centroid measurements as determined by the thresholding procedures since we found comparable shifts in centroid activity after reanalyzing all the data (average absolute difference in vector lengths and angles were 0.12 mm and 11.4 degrees, respectively) and highly correlated centroid coordinates (average
R2 = 0.86. Furthermore, to gain an estimate of the variability of the centroid location during an imaging run we generated half-maps where we summed together different halves of the collected trials (even vs. odd trials or first half vs. second half). While we only report findings based on average maps determined from all the trials within a run, we found that the average standard deviation within a run in the centroid location as determined from the xy coordinates from 4 half maps (1 odd, 1 even, 1 first half, 1 second half) was only 0.121 mm (n = 3 experimental days, 15 whisker conditions multiplied by all centroid locations above 75% threshold value).
RESULTS

VSD imaging provided the opportunity to evaluate at the population level spatial and temporal aspects of cortical barrel response to whisker stimulation. In particular, we compared the time course and amplitude of response to single and two whisker stimulation, and the accompanying spatial change of activation center over time.

Spatiotemporal Response to Single Whisker Stimulation

As a first step for evaluating our VSD images we confirmed that the initial activity produced by single whisker stimulation was located over the topographically appropriate barrel in cortex. Previous studies of single whisker responses using VSDI reported that initial activity is confined to a single barrel in layer 2/3 before rapid spreading across cortex (Petersen and Sakmann 2001, Petersen et al 2003, Ferezou et al 2006; cf. Tanifuji et al 1994, Takashima 2001, Kleinfeld and Delaney 1996). Imaging frames captured 11.2 ms after whisker stimulation confirmed that initial activity was confined to a single whisker’s corresponding barrel (Fig 4A-C).

In Figure 4A barrels revealed with CO are outlined in white with the C2 barrel outlined in red. In response to C2 whisker stimulation, the sequence of activation shown in Figure 4E (pseudo color activation maps) and 4F (maps thresholded at 75% overlaid on outlines of barrel field) was observed. Frame 1 (0 ms after whisker stimulation) shows the activity map at the onset of whisker stimulation. At that time the change in fluorescence in the ROI was near zero and comparable to spontaneous activity observed during the prior 80 ms. Significant response to whisker stimulation began at frame 5 (11.2 ms after whisker stimulation), resulting in a rise in fluorescence, localized over the C2 barrel. In subsequent frames 6 and 7 (14 to 16.8 ms after whisker stimulation) activity quickly spread to surrounding barrels. Within a short time, from frame 5 to 6 (2.8 ms), the area of activation increased to more than twice the initial area.

Thresholding images to 75% of peak revealed that peak activation, superimposed over the
barrel field map (Fig. 4F), spread primarily along the stimulated whisker row and appeared to shift slightly towards the D whisker row of barrels before subsiding below threshold by 25 ms after whisker stimulation (Fig. 4F, frame 9). Similar patterns were observed for C3 and C4 whisker stimulation (Fig 4B and C). For all three whiskers, initial activation within the barrels aligned well with the histology (Fig. 4D). The thresholded areas for the three whisker stimulations 1) aligned well with their corresponding barrels, 2) did not overlap with initial activation zones of other whisker stimulations, and 3) aligned in an orderly manner within the barrel row. It is worth noting that when we looked at the thresholded area of initial activation (Fig 4D) with respect to the barrels, the active area was actually smaller (at 75% threshold) than the size of a barrel. [See Movie1]

In a single case with particularly good signal to noise, we subdivided the trials to measure the degree of variability of the sub-barrel activation areas within the first frame of activity. For two runs where the C2 and C3 whiskers were stimulated, we divided our 100 trial dataset into blocks of 10 (10 trials per block) and examined the degree of variability in intra-barrel location for this first frame. We found that the standard deviation of the intra barrel location for C2 was 4.6 pixels on the x axis (~288 μm) and 3.1 pixels on the y axis (~194 μm) and for C3 the deviations were 2.1 pixels on the x axis (~131 μm) and 3.5 on the y axis (~219 μm) (i.e. both less than 300 x 250 μm, while the typical barrel size is approximately 500 x 440 μm or 8 x 7 pixels), indicating that the location of activation was stable within the respective barrels. For both the C2 and C3 barrels the activity was offset from the geometric center and located in the more rostral division of the barrel (see Fig 4D, Red and Blue areas of activity). The presence of this offset indicates possible intra-barrel bias.

Spread of Activity
Anatomical studies report more connections between barrels in a row than within an arc; consistent with this, VSD studies in mice and rats report spread of activity preferentially within a barrel row (Petersen and Sakmann 2003, Armstrong-James 1992, Chapin 1986, Kleinfeld and Delaney 1996). To examine if there was preferential spread of activity to adjacent whisker barrels in our recordings we used several ROIs to measure and compare the peak response amplitude in the four adjacent barrels (caudal, rostral, medial, lateral). Figure 5A shows peak response amplitude of activity averaged across all cases and across C2, C3, and C4 whisker stimulations for five ROIs: primary PW (yellow), rostral adjacent AW (red), caudal AW (blue), medial AW (green), lateral AW (purple). As expected, the peak response amplitude in the PW barrel was significantly larger than other peak fluorescent measurements in adjacent barrels (p<0.0001, Student’s t-test). To confirm earlier reports of preferential spread of activity within a barrel row versus a barrel arc, which is also in agreement with anatomical studies showing more connections within a row, we pooled the peak responses for the rostral and caudal adjacent barrels and the medial and lateral barrels to compare the peak response amplitudes within the barrel row and arc respectively. Figure 5B shows the comparison of peak response amplitudes for the pooled adjacent arc and row barrels. The amplitude of fluorescence change was significantly greater in the stimulated barrel’s adjacent row barrels than adjacent arc barrels, consistent with preferential spread within the barrel row (row versus arc, p<0.05, Student’s t-test).

To rule out the possibility that the decline in activity in adjacent barrels was a consequence of uneven staining we measured peak activation in the C2, C3 and C4 barrels in response to C2, C3, and C4 whisker stimulation. The response of a barrel to the primary whisker was always earlier and larger than responses to secondary whiskers. Thus the response amplitudes of surrounding barrels was not at an upper limit supporting the conclusion
the spatiotemporal pattern of activity in response to stimulating a single whisker was not due to uneven dye staining.

Effects of dual whisker stimulation

Previous electrophysiological studies have reported that, compared to single whisker stimulation, dual whisker stimulation can have either a facilitatory or a suppressive effect depending on the interstimulus interval (Shimegi et al. 1999; Mirabella et al. 2001). To examine the population response to multi-whisker stimulation, we compared the change in fluorescence responses for single and simultaneous dual whisker stimulation. Image sequences (acquired at 360 Hz) from one such experiment that included C2, C3, and paired C2/C3 stimulation are shown in Figure 6. In each sequence, the time of whisker stimulation is highlighted by the frame outlined in bold. Using the peak amplitude response (see Fig 2B), we found the response for paired whisker stimulation under these conditions was significantly sub-linear (p < 0.001, Students t-test). Pooling measurements for all cases (n = 4) gave population responses for the PW, AW, paired PW+AW, and Linear Sum conditions. As quantified in Figure 7, across cases we found: 1) PW responses were significantly greater than AW (p<0.01, Students t-test), 2) paired PW+AW responses where significantly greater than PW (p<0.01, Students t-test), and 3) paired PW+AW responses were significantly smaller than the linear sum of single AW and PW responses (p<0.001, Students t-test), indicating sub-linear summation (Fig 7). Collectively these findings point toward a spatial temporal inhibitory process leading to sub-linear responses to paired stimulation, in agreement with the reported effect of paired stimulation in mouse VSD recordings (Civilico and Contreras 2006).

Shifting Centroid of Activation

As shown in Figure 4, the area of evoked activity showed a distinct pattern of activation; starting with a small focal area of activity (11.2 ms post stimulus) centered over the principal
whisker's barrel, rapidly expanding primarily within a barrel row (12-18 ms), followed by a decline in area of activation (20-25 ms) and return to baseline. Surprisingly, the center of activation appears to shift over time. In Figure 8A, the center of activity (75% threshold) shifted from the C2 barrel (first panel), towards a location roughly between the C2 and C3 (second panel), then towards a location more in the septal area between the C2 and D2 barrels (third panel), then into the D2 barrel itself (fourth and fifth panel). [See Fig 8B and Movie2.] As shown in Figure 8C, overlaying the earliest frame of activity above threshold (11.2ms after stimulus onset) and the final frame of activity above threshold (22.4ms after stimulus onset) revealed a clear spatial shift in the location of activation. The shift in activation was confirmed by plotting time courses of activation at the initial (SC, blue) and final (EC, red) centers of activation above threshold (Fig 8D). Plotting the change in fluorescence (above threshold activity over time) for the two regions of interest revealed two temporally distinct peaks of activation indicative of a clear shift in activation over both space and time. This shift was not simply due to variability of signal. In fact, at 75% threshold, in the first frame, activity was centered on the initial start point (SC ROI) and had not yet reached the end point (EC ROI); in the last two frames, activity had fallen below threshold at the start point but was still above threshold in the end point (Fig 8D). This is consistent with a true shift in center of activation.

We examined whether this shift occurred across all cases and whether it occurred under both single and dual whisker stimulation conditions. If the spread of activity was determined by the intrinsic connectivity of the barrel cortex (i.e. preferential spread within the row) then the expectation would be that the shift in activity over time would be similar for single or paired whisker stimulations. We found that shifts in activation center occurred consistently across cases, and furthermore, that this shift tended to occur in the posteromedial direction towards the more ventral whisker row and more caudal whisker arc. The mean distance of the shift was
1.2mm (n=15) with 100% (15/15) of the shifts moving towards the more ventral whisker row and
80% (12/15) of the shifts moving towards the more caudal whisker arcs. There was no
significant difference in the mean distance of the shift between paired and single stimulations.

Figure 9A illustrates the shift from start point (large colored dot) to the end point (small colored
dot) for 3 single whisker stimulation cases. Following C2 whisker stimulation (A, left, 3 cases),
activation centers shifted towards the D whisker row. Following C3 whisker stimulation (A,
middle, 3 cases), activation centers also shifted to the D whisker row. Following C4 whisker
stimulation (A, right), activation shifted clearly towards the D whisker row in one case and
towards the C3 whisker barrel in two cases. Moreover, such shifts were also observed under
dual whisker stimulation conditions. Six cases are shown in the lower illustrations in Figure 9A.
(Left: 3 cases C2+C3; right: 3 cases C3+C4). A summary of all cases (both single and dual
whisker stimulation conditions) is shown in Figure 9B. All cases are displayed so that the
primary whisker barrel is centered over the C3 barrel. We observed that all activations shifted
towards the more ventral whisker rows (D and E: blue and purple rows), with no shift towards
the more dorsal whisker rows (A and B: orange and red rows). Shifts toward caudal arcs were
also more common than towards anterior arcs. Thus, our 15 cases showed an overall trend for
shift in activation in the posteromedial direction of cortex towards the more ventral whisker rows
and more caudal whisker arcs. Such a directional shift is consistent with known anisotropies in
underlying interbarrel connections (see discussion).
DISCUSSION

Summary

We used VSD imaging to examine cortical activity population responses to single and simultaneous dual whisker deflections in urethane-anesthetized rat barrel cortex. We found that activation to single whisker stimulation originated at the principal whisker barrel and quickly (within 20 ms) spread extensively to surrounding regions. This spread was oriented preferentially along a barrel row. Paired whisker stimulation produced a significant sub-linear summation when compared with stimulation of two adjacent whiskers individually. Surprisingly, in response to both single and dual whisker stimulation, we consistently found a spatial shift of the centroid of activity in the barrel field, one that occurred over 10-20 ms and offset as far as 1-2 barrels as the activity faded. This shift occurred predominantly in the posteromedial direction of barrel cortex.

Spatiotemporal Responses

The VSDI responses measured in barrel cortex to whisker stimulation showed several spatiotemporal characteristics consistent with previous studies. In the temporal structure of the images, detecting responses as early as 11ms after whisker stimulation corresponded with the known timing of excitatory activity reaching layer 2/3 of cortex (Armstrong-James et al 1992). The spatial profile of the VSD images showed initial focal activity roughly the diameter of a barrel column (~400 μm) followed by a rapid horizontal spread preferentially within a whisker row and then to surrounding cortex in agreement with previous studies (Petersen and Sakmann 2001, Kleinfeld and Delaney 1996). Similarly, in previous studies using electrophysiology and VSDI of barrel cortex, it was shown that responses start in layer 4 of barrels, progress vertically directly above the barrel into layer 2/3, and then spread horizontally in layer 2/3 in a “pagoda

We did note in a single case with particularly good signal to noise, the initial area of activation above threshold was confined to an area within the single barrel. Because the signal to noise for the two barrels was exceptionally strong we were able to sub divide the trials to measure the degree of variability of sub-barrel activation area. For both, the center of activation was located rostral from the geometric center of the barrel, consistent with our caudal to rostral direction of whisker stimulation. Thus, based on the data analysis from these two barrels, our results could be consistent with the presence of an intra-barrel somatotopic directional map (Andermann and Moore 2006; Kremer et al 2011; Kerr et al 2007). However, the results from two barrels with exceptional signal to noise are far from conclusive and further studies are needed (Tsytsarev et al 2010 a and b).

The barrel responses reported here with VSDI were monophasic. Other reports using VSDI report a triphasic response (Derdikman et al 2003). This difference was likely due to our stimulus parameters. Derdikman et al (2003) found the profile of barrel response varies with different stimulus intensities and durations. They found a monophasic response for weak stimuli, whereas, stronger stimulation led to a triphasic response. In particular, presentation of a 1 ms deflection evokes a late hyperpolarization; while whisker deflections of longer durations, 10 and 30 ms, does not (Derdikman et al 2003). Thus our finding that the ~250-300 μm whisker deflections for a duration of 20 ms we used induced monophasic responses is consistent with previous studies.

**Paired Stimulation**

The study of cortical responses to paired simultaneously stimulated whiskers was designed to examine how multiple foci of activity were integrated in cortex. Previous studies
have found both facilitatory and suppressive effects. In Shimegi et al. (1999) the facilitatory or suppressive effect is dependent on the timing between whisker stimulation. For example a population of layer 2/3 cells recorded in rats showed facilitation of firing rate with paired whisker stimulations, either simultaneously or with a <20 ms (maximum facilitation at 4 ms) interval between successive whisker stimuli. In the same study Shimegi and colleagues reported suppressive effects when the timing between adjacent whisker stimuli was greater than 20ms. Mirabella et al. 2001 found some slight facilitation to paired whisker stimuli, but primarily a sub-linear summation when they stimulated an increasing number of whiskers, indicative of suppressive effects (Mirabella et al. 2001).

To date, the bulk of studies on simultaneous stimulation of multiple whiskers report suppressive effects (Brumberg et al 1996, Higley and Contreras 2003, 2005, Webber and Stanley 2004, Ego-Stengel et al 2005, Civilico Contreras 2006). What has become clear is that the nature of cortical interactions is crucially dependent on the timing between whisker deflections on a millisecond time scale (Shimegi 1999, Simons and Carvell 1989; see also subcortical effects, Simons 1983, 1985, Simons and Carvell 1989, Contreras 2011). In our study, simultaneous stimulation of whiskers produced sub-linear summation of the VSD signal in barrel cortex. These results are consistent with the bulk of previous studies and parallels a similar VSD study conducted in the mouse barrel field (Civilico and Contreras 2006). Thus, the findings point to a common feature of cortical processing in whisker somatosensory systems of the two rodent species, despite the anatomical differences identified above.

Spatiotemporal shift of activation in barrel cortex

To our knowledge, this is the first report in rodent barrel cortex of substantial shifts in location of the center of activation in response to single whisker stimulation. While there are reports of shifting activations within single barrels (Andermann and Moore 2006), and
asymmetries of inputs to single barrels (Furata et al 2011), these and other studies specifically note that the center of activation remains essentially the same and the spread is isotropic across the row. Some intracellular studies do show a later onset of subthreshold activation from surround vibrissal inputs, from which one could infer a shift (Moore and Nelson 1998). Otherwise, there is one study, using multi-array recordings in layer 5 that illustrates shifts (Figure 6 Ghazanfar and Nicolelis 1999). Outside of this one report, this is the first observation of the center of activation (albeit at a population level) shifting from one barrel to or towards another, and moreover that this shift occurs across barrels with a tendency in the caudal-ventral direction.

Although this finding was unexpected, we propose that such shifts may be consistent with known functional and anatomical asymmetries in rat barrel cortex. In 1989 Simons and Carvell reported functional asymmetries in single unit recordings in the barrel field. They found that the caudally adjacent whisker evoked more inhibition on PW barrel neurons than the rostrally adjacent whisker; furthermore, the ventral adjacent whisker evoked more inhibition than the dorsal whisker (Simons and Carvell 1989). This asymmetry was later confirmed in the responses of inhibitory interneurons (Fast Spiking Units) and the observed gradient of inhibitory effect produced on Regular Spiking Units (Simons 1995). These effects were not evident in subcortical VPM thalamic projection neurons, suggesting the asymmetric gradient of inhibition was established in cortex (Brumberg et al 1996).

Consistent with these functional findings, there are anatomical asymmetries in the projections of cortical axons in the barrel fields. Bernardo et al. (1990a and b) reported intracortical connections are stronger between barrels in the same row than between rows, and, moreover, a large directional bias in connections towards the more anterior barrel row in cortex (corresponding to the more ventral whisker row on the face, closer to the mouth). The
asymmetries in connections coupled with the functional asymmetries in inhibition are further confirmed in early 2-deoxyglucose (2DG) studies in behaving animals where the majority of activation is observed in the barrels of the more ventral and caudal whiskers followed by a clear global gradient of declining activity towards the barrels of the more dorsal and rostral whiskers (McCasland et al 1991).

Our data add an interesting temporal component to these spatial relationships. First, we suggest that the anisotropic connections within barrel rows are likely to underlie our observed preferential spread of VSD signal along, rather than across, rows. We also propose that the temporal posteromedial shift derives from the presence of stronger anatomical connections with anterior than posterior barrel rows. The tendency for activity to move in the posteromedial direction (towards the ventral and caudal whiskers) would further explain the enhanced activity in these barrels found with 2DG studies. Furthermore, the time course of this shift is consistent with delays associated with the interbarrel propagation of activity. Under normal whisking conditions, the summation of spreading EPSPs towards posteromedial direction in cortex (i.e. the ventral and caudal whiskers) would bring the potentials closer to threshold, leading to higher firing rates, which results in higher activity of these barrels with respect to the entire barrel field. Such a functional bias could be important for integrating salient sensory information from the ventral/caudal large vibrissae close to the mouth.

Analysis of the location of the centroid of activation above threshold over time found a preferential shift of the center of activation towards the more ventral whisker rows and more caudal whisker arcs. These findings suggest that the inhibitory component of a barrel response is stronger in the lateral portion of the barrel field, resulting in activity above threshold being sustained longer and spread preferentially into the ventral/caudal whisker barrels. This effect
could be related to the optimal sequence of whisker stimulation (Kleinfeld and Delaney 1996, Ghazanfar and Nicolelis 1999) as well as being based on the intracolumnar connections.

**Updated view of spatiotemporal events in barrel cortex following whisker stimulation**

Our VSDI supports a model with the following sequence of events after whisker stimulation. 1. An initial short latency, high amplitude excitatory response in cortical layers 2/3 as well as layer IV, based largely on thalamocortical projections to layer 4, and limited to the confines of the corresponding barrel of the stimulated vibrissae. 2. Activity quickly spreading to the surrounding barrels allowing for an integration of responses of nearly simultaneous whisker stimulations (< 20 ms) resulting in an enhancement of excitatory amplitudes, especially in layer 2/3. 3. A rapid directional shift of activation in the population determined by timing of whisker contact, influenced by inherent biases in anatomical connection patterns and spatial distribution of inhibitory influences. This directional bias may have behavioral relevance, since it could lead to preferential amplification of sensory stimulation of larger whiskers near the mouth.

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**Reference List:**


**Brumberg JC, Pinto DJ, Simons DJ.** Spatial gradients and inhibitory summation in the rat whisker barrel system. *J Neurophysiol* 76:130-140, 1996.


**Figure Captions:**

**FIG 1. Image alignment and processing.** A: blood vessel map used for image alignment. 4 x 4 mm field of view (FOV). B: camera FOV aligned with surface blood vessels on histological tissue. C: FOV extended to the subsequent sections of cortex stained for cytochrome oxidase revealing the barrel field in layer 4. D: tracing of the barrel field. E: tracing of barrels superimposed on the FOV of the collected images. F: VSD subtraction image (see Fig 2 and methods) acquired 11.2 ms after whisker stimulation. G: image (F) after Gaussian filtering with a sigma value of two pixels. H: image (G) with aligned barrel field superimposed in yellow. Thresholded area of activation at 75% of peak amplitude response is colored in red. I: image (G) with level of activity illustrated with color intensity. J: image (I) with barrel field superimposed in black. For A and D-F top right scale bar = 1 mm. A = anterior, P = posterior, M = medial, L = lateral.

**FIG 2. Time course analysis.** A: plot of VSD emission ($\Delta F$) vs. time averaged for 100 trials of whisker stimulation (black line) and the blank condition (gray line) for a ROI centered over the C2 barrel. Decrease in VSD emission in both is a consequence of the photo bleaching. Time of whisker stimulus for black plot is marked with a red dashed vertical line and time course on the x-axis is aligned with the time of stimulus as 0 ms. B: plot showing the change in VSD signal due to whisker stimulation revealed by subtracting the blank condition (gray line in A) from the stimulus condition (black line in A) and thus removing the contribution of photo bleaching from the signal. Time 0 ms locates the onset of whisker stimulation and is marked on the plot with a red dashed line. The time period used in the measurement of peak response amplitude is indicated by the green bar.
FIG 3. Determining the appropriate threshold level. Areas of activation for C2 whisker response is shown for thresholds set at 50, 70, 75, 80, 85, and 90% of peak response. Notice how increasing the threshold level decreases the measured area of activation. We used 75% threshold to determine the first time point at which activation is evident (latency) because it is reproducibly significant, but yet confined to a single barrel (see text). Scale bar for y axis is $\Delta F = 0.01$. Scale for x-axis is 30 milliseconds.

FIG 4. Cortical response to a single whisker stimulation. A-C: the initial thresholded area and intensity of activation is shown for C2 (A), C3 (B) and C4 (C) whisker stimulation. Individual barrels (white outlines) are overlaid on the FOV blood vessel image. The stimulated whisker barrel is outlined in red. D: outlines of the initial areas of activation for C2 (red), C3 (blue) and C4 (green) whisker stimulation overlaid on the CO histology of the barrel field. Note that the initial areas of activation were confined to the barrel row representing the stimulated whiskers. E: sequence of color intensity images starting at the time of C2 whisker stimulation and ending 25.2 ms later (each frame is 2.7 ms) illustrating the spatiotemporal progression of cortical activation. F: intensity maps (E) after thresholding above 75% of peak activation superimposed over the barrel field (white outlines) and camera FOV. This sequence is produced from our second case, with signal averaged over 100 trials.

FIG 5. Spread of activation greater along rows than arcs. A: top map shows the five regions of interest used to measure for a single whisker response the peak amplitude of the primary (star, yellow) as well as the rostral (red), caudal (blue), medial (green) and lateral (purple)
adjacent barrels. Below are the peak amplitude responses for single whisker stimulations normalized to the largest response and combined across all cases (region of interest, mean, standard error; caudal, 0.87 ,+/− 0.02; rostral, 0.89, +/− 0.02; medial, 0.84,+/− 0.02; lateral, 0.83, +/− 0.03; n = 11. The response for the primary barrel corresponding to the homologous stimulated whisker was significantly larger than the responses in all surrounding adjacent barrels (p<0.0001 Students t-test) B: top map shows the groupings of regions of interest made to compare the peak amplitude of responses for adjacent barrels within the corresponding whisker arc (red) versus the corresponding whisker row (blue). Bar graph below is the normalized peak amplitude responses for the arc versus row for all cases and all conditions (arc, 0.83, +/- 0.02; row, 0.88, +/- 0.02; n=11). The response was significantly larger within the whisker row compared to the whisker arc (p<0.05 Students t-test).

**FIG 6.** Montages of time courses of activation for C2 (left), C3 (middle), and paired C2+C3 (right) whisker activation. Colored bold outline indicates time of whisker stimulation. Images acquired at 360 Hz. Prior to whisker stimulation, cortical reflectance is at baseline levels (primarily blue and green). Following whisker stimulation, barrel specific activation (yellow and red) is first seen at around 11 ms, followed by spread of activity to surrounding regions (20-30 ms), after which there is a return to baseline levels. Bottom shows first three frames of thresholded activation (black bold outline in montages above) superimposed over the vessel map from the imaging field of view with the barrel field outlines in white as in Figure 4F.

**FIG 7.** Sublinear effect of simultaneous paired whisker stimulation. Peak amplitude of responses in a single ROI over the principal whisker barrel to principal whisker (PW), adjacent whisker (AW), paired whisker (AW + PW) and the linear sum of single AW and PW responses.
Responses were normalized to the peak response across conditions and then pooled across all cases. The response to paired whisker stimuli was significantly greater than single PW or AW conditions (Paired vs PW, p<0.01; Paired vs AW, p<0.01, Students t-tests). The linear sum of AW and PW responses was significantly larger than the observed paired responses (Paired vs. Linear Sum, p<0.0001, Students t-test). Bar graph shows condition, mean, S.E.M.; AW, 0.58, +/- 0.06; PW, 0.70, +/- 0.05; Paired, 0.86, +/- 0.04; Linear Sum, 1.28, +/- 0.06, n= 11 whisker pairings.

**FIG 8. Shift in centroid location over time.** A: VSD image sequence shows the area of activation thresholded above 75% of peak response for C2 whisker stimulation overlaid on an outline of the barrel fields. Activation was above threshold for five frames, starting 11.2 ms after whisker stimulation and ending 22.4 ms after stimulation onset. The area of activation above threshold for each frame is magnified below with the centroid of the area of activation for each frame marked with a black dot. Area of activation is colored differently for each frame. B: Trajectory of the centroid shift in location over time. Each centroid spot is colored the same as its' activation area represented in the frames above in A. C: magnified areas of activation for the first and last frames of activity above threshold are overlaid on the barrel field with their respective centroids of activation marked in black. Notice the clear spatial shift in location of the area of activation. D: plot of the change in fluorescence over time for two ROIs shows temporally distinct peaks of activation indicative of a spatial shift in activity. First ROI is over the centroid of activation for the first frame (Starting Coordinates (SC, blue)) and the second ROI is located over the centroid of activation for the last frame of activation (End Coordinates (EC,
Plots are of ΔF over time only for activation thresholded above 75% of peak response (0.034 %).

Fig 9. Coordinates for shift in centroids of activation above threshold for each condition.
A: for each condition (C2: red, C3: blue, C4: green, C2 & C3: purple, C3 & C4: turquoise) across three cases, the locations of the initial coordinates for the centroid of activity above threshold is plotted on a model barrel field (larger circles) while the final coordinates for the centroids of areas of activation above threshold are plotted as smaller circles. B: the trend for shift of activation with respect to whisker rows (left) are shown by collapsing the starting coordinates for all conditions onto the center of the C3 barrel and plotting their respective end coordinates due to shift of the centroid of activation over time. Notice the shift in activity appears to move primarily in the posteromedial direction (towards the more ventral whisker rows and caudal whisker arcs).

Supplemental Material:
VSDMovie1. Movies of imaged fluorescence VSD response to stimulation of D1, D2, and D3 whiskers (acquired at 360 Hz, each frame ~2.7 ms). Field of view: 5 mm. Initial movie segment is raw signal. Colored movie segment is filtered color-coded activity map. Final segment shows thresholded (75%) activation with overlay of D1 (blue circle), D2 (green circle), and D3 (yellow circle) centers.

VSDMovie2. Shifting center of activation. Nine frame movie (2.7 ms/frame) of C2 and C3 whisker stimulation showing shifting center. Circle indicates location of initial centroid (at frame 3). Blue circle shows location of centroid for initial area of activation while black circle shows the
location of the centroid after the shift. Red overlay indicates 75% threshold activation for each frame.
FIGURE 2

A

$\Delta F \%$

-0.20

-0.15

-0.10

-0.05

0.00

-\(55\)

-\(28\)

0

28

56

83

111

139

167

Time (ms)

C2 whisker

Blank

B

$\Delta F \%$

-0.01

0.00

0.01

0.02

0.03

0.04

0.05

-\(55\)

-\(28\)

0

28

56

83

111

139

167

Time (ms)
FIGURE 3

50% 70% 75% 80% 85% 90%

Time (ms)
FIGURE 5

Normalized Peak Fluorescence

A

B

caudal
rostral
primar
medial
lateral
FIGURE 7

Normalized Peak Fluorescence
FIGURE 8

A

B

C

D

Time (ms)

Δ

F %

0.044 0.042 0.04 0.038 0.036 0.034

0 10 20 30 40

SC

EC

11.2 ms  14 ms  16.8 ms  19.6 ms  22.4 ms

A

C D

% EF A

22.4 ms

19.6 ms

16.8 ms

14 ms

11.2 ms

B

C

D

A

C

D

B

FIGURE 8

Time (ms)

Δ

F %

0.044 0.042 0.04 0.038 0.036 0.034

0 10 20 30 40

SC

EC

11.2 ms  14 ms  16.8 ms  19.6 ms  22.4 ms

A

C D

% EF A

22.4 ms

19.6 ms

16.8 ms

14 ms

11.2 ms

B

C

D

A

C

D

B