Olfactory Nerve Stimulation-Induced Calcium Signaling in the Mitral Cell Distal Dendritic Tuft

Q. Yuan¹ and T. Knöpfel¹

¹Laboratory for Neuronal Circuit Dynamics, Brain Science Institute, RIKEN, Saitama, Japan

Submitted 13 September 2005; accepted in final form 26 November 2005

Yuan, Q. and T. Knöpfel. Olfactory nerve stimulation-induced calcium signaling in the mitral cell distal dendritic tuft. J Neurophysiol 95: 2417–2426, 2006. First published November 30, 2005; doi:10.1152/jn.00964.2005. Olfactory receptor neuron axons form the olfactory nerve (ON) and project to the glomerular layer of the olfactory bulb, where they form excitatory synapses with terminal arborizations of the mitral cell (MC) tufted primary dendrite. Clusters of MC dendritic tufts define olfactory glomeruli, where they involve in complex synaptic interactions. The computational function of these cellular interactions is not clear. We used patch-clamp electrophysiology combined with whole field or two-photon Ca²⁺ imaging to study ON stimulation-induced Ca²⁺ signaling at the level of individual terminal branches of the MC primary dendrite in mice. ON-evoked subthreshold excitatory postsynaptic potentials induced Ca²⁺ transients in the MC tuft dendrites that were spatially inhomogeneous, exhibiting discrete “hot spots.” In contrast, Ca²⁺ transients induced by backpropagating action potentials occurred throughout the dendritic tuft, being larger in the thin terminal dendrites than in the base of the tuft. Single ON stimulation-induced Ca²⁺ transients were depressed by the NMDA receptor antagonist d-aminophosphonovaleric acid (D-APV), increased with increasing stimulation intensity, and typically showed a prolonged rising phase. The synthetically induced Ca²⁺ signals reflect, at least in part, dendrodendritic interactions that support intraglomerular coupling of MCs and generation of an output that is common to all MCs associated with one glomerulus.

INTRODUCTION

Mitral cells (MCs) are the principal output neurons of the olfactory bulb and represent the first stage of central odor processing. The distally tufted primary dendrites of rodent MCs outline complex structures termed olfactory glomeruli. A given glomerulus is innervated by axons of olfactory receptor neurons (ORNs), each of which expresses the same odor receptor protein (Vassar et al. 1994). The convergence of ORN axons onto topographically defined glomeruli creates a spatial map of the odor’s molecular features in the glomerular layer of the olfactory bulb (Mombaerts et al. 1996; Vassar et al. 1994; Wachowiak and Cohen 2001).

The glomeruli of rodent MCs exhibit complex synaptic interactions between each other and with periglomerular cells (Isaacson 1999; Schoppa and Westbrook 2001, 2002; Urban and Sakmann 2002). Because a given glomerulus receives inputs essentially from the same type of ORNs, glomerular processing of odor information may primarily serve to average over ORN inputs (approximately 11,000 in rodents; Shepherd et al. 2004), and to produce a uniform signal carried by each of the approximately 25 (Allison 1953) MCs that are associated with a given glomerulus. Averaging of ORN inputs increases sensitivity far beyond the sensitivity of isolated ORNs (Van Drongelen et al. 1978), while coupling and synchronization of MCs likely also increase fidelity of the output signal (Isaacson 1999; Schoppa and Westbrook 2001 2002). In this concept, the MC firing rate from approximately 1,800 glomeruli in the mouse olfactory bulb (Royer et al. 1988) represent the combinatorial odor code. Interglomerular signaling may, in addition, enhance odor discrimination (Aungst et al. 2003). Glomerular interactions likely also determine the exact temporal structure of the oscillatory synchronization between MCs, which may be important for entraining network oscillations within the olfactory bulb (Adrian 1950; Zochowski and Cohen 2005) and for enhancing the signal-to-noise ratio when the MC activity is encoded in the olfactory cortex (Friedrich et al. 2004; Laurent et al. 2001). Conceptually, MC firing may represent independent rate and temporal coding (Huxter et al. 2003).

The plausibility of any of these concepts is constrained by the detailed features of glomerular signal processing. Direct electrophysiological measurement of the activity of terminal dendritic branches in the tuft is not feasible because these structures are too small to tolerate intracellular electrode recordings. A powerful method to access subcellular signaling is provided by combining somatic patch clamp and dendritic Ca²⁺ imaging techniques. Ca²⁺ signals in the glomerular tuft of the primary dendrite of the MC have been used to access backpropagating action potentials (bAPs), presynaptic inputs, and postsynaptic signaling (Debarbieux et al. 2003; Ma and Lowe 2004; Urban and Castro 2005). There is controversial experimental evidence about some characteristics of these Ca²⁺ signals (Debarbieux et al. 2003; Ma and Lowe 2004; Urban and Castro 2005), and little information is available as to the mechanism associated with the intercellular Ca²⁺ concentration ([Ca²⁺]₀) increase of bAP and local, subthreshold excitatory postsynaptic potentials (EPSPs). The bAP-induced Ca²⁺ signals of MC dendritic tufts are likely mediated by voltage-gated Ca²⁺ channels including N- and P/Q-type, but not dihydropyridine-sensitive L-type channels (Isaacson and Strowbridge 1998; Yuan et al. 2004). In addition, olfactory nerve (ON) synapses that release glutamate might mediate Ca²⁺ signals via N-methyl-D-aspartate (NMDA) receptor or mGluR1 (Aroniadou-Anderjaska et al. 1999; Carlson et al. 2000; Ennis et al. 1996; Friedman and Strowbridge 2000; Hayar et al. 2004; Heinbockel et al. 2004; Isaacson 1999; Trombley and Westbrook 1990).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: T. Knöpfel, Laboratory for Neuronal Circuit Dynamics, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-shi, Saitama, 351-0198, Japan. (E-mail: tknopfel@brain.riken.jp).

www.jn.org 0022-3077/06 $8.00 Copyright © 2006 The American Physiological Society
In this work, we studied subthreshold synaptic stimulation and bAP-induced signals using combined patch clamp and Ca\(^{2+}\) imaging techniques. We characterized Ca\(^{2+}\) transients induced by bAPs in the primary dendrite of the MC and used these signals as a reference for synaptically induced signals. We found that focal ON stimulation induced patch-like Ca\(^{2+}\) signals that were NMDA receptor dependent and exhibited a prolonged rising phase that, at least in part, reflect dendrodendritic interactions (Isaacson 1999). Our results are consistent with the concept that intraglomerular signaling supports coupling of MCs and generation of an output that is common to all MCs associated with one glomerulus.

**Methods**

**Slice preparation and electrophysiology**

Horizontal slices (350 μm) of olfactory bulbs were obtained from 18- to 26-day-old ICR mice of both sexes. Slices were cut by a Vibratome (VT 1000S, Leica), recovered at 32°C for the 1st hour and afterwards at room temperature (23–25°C). After more than 1 hour of recovery, slices were transferred into a recording chamber and perfused with artificial cerebrospinal fluid (ACSF) containing (in mM) 118 NaCl, 25 NaHCO\(_3\), 1 NaHPO\(_4\), 3 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), and 10 glucose, equilibrated with 95% O\(_2\)-5% CO\(_2\). Experiments were performed at either room temperature or 32°C. For electrophysiological and fluorescence recordings, slices were placed in an immersion-type perfusion chamber mounted on the stage of an upright microscope (Leica, DMLEFS or Nikon, E600FN) and visualized using Nikon 63X water immersion lenses (NA = 0.9). The procedures had approval from the animal ethical committee of the RIKEN Brain Science Institute.

Patch-clamp recordings were carried out in whole cell configuration. Glass pipettes (3.5–5 MΩ resistance) were pulled from borosilicate glass using a two-stage vertical puller (Narishige, Tokyo). Pipettes contained (in mM) 0.2 Oregon green BAPTA-1, 125 K-glucuronate, 2 MgCl\(_2\), 0.025 CaCl\(_2\), 10 HEPES, 1 EGTA, 2 Na,ATP, and 0.5 Na,GTP, pH 7.25. In one set of experiments, the Na\(^{+}\) channel blocker QX-314 (10 mM) was included in the intracellular solution.

Whole cell patch-clamp recordings were made from the somata of MCs using an Axon Instrument Axonpatch 200B (Axon Instruments, Sunnyvale, CA). Under differential interference contrast visual guide, MCs were identified and selected by their location in the MC layer, size and shape of the somata (around 25 μm diameter), and the distinct single primary dendrite that project into a glomerulus. Cells that had resting membrane potential more negative than −45 mV at zero holding current and without correction for junction potentials were selected for recording. Holding current of ≤300 pA was used to hold the membrane potential at rest between −60 and −65 mV. Back-propagating APs were generated by current injection (0.7–1.2 nA, 2 ms) through the patch pipette (AP amplitude 128.1 ± 2.1 mV, overshoot 65.8 ± 2.1 mV; mean ± SE, n = 15 cells). Size and shape of bAPs remained stable throughout the experiments.

The EPSPs were elicited by extracellular stimulation of the olfactory nerve using either a glass pipette (0.8–1 MΩ) containing normal ACSF solution or a miniature bipolar concentric electrode (tip diameter 25 μm). Model MCE-100; Rhodes Medical Instruments, Woodland Hills, CA). The electrode was positioned, under visual control, on a small bundle of presynaptic axons just outside the imaged glomerulus. Both types of electrodes produced identical results (in particular, focal Ca\(^{2+}\) signals were seen with both types of electrodes).

Drugs applied in the bath were made from aqueous stock solutions. For recording of bAP-induced Ca\(^{2+}\) signals in the absence of synaptic transmission, the ACSF contained 50 μM D-aminophosphonovaleric acid (D-APV, an NMDA receptor antagonist), 40 μM 2,3-dixo6-nitro-1,2,3,4-tetrahydrobenzoflavinoxaline-7-sulfonamide (NBQX), and 40 μM bicuculline. For recording of EPSPs in the absence of inhibition, the ACSF contained 40 μM bicuculline and, in a series of experiments, also contained 2–4 μM NBQX (to prevent synaptically driven oscillatory activity). To test the contribution of NMDA and mGluRs in generating local Ca\(^{2+}\) signals, D-APV (100 μM) and methyl-4-carboxyphenylglycine (MCPG, 1 mM, a group I and II mGluR antagonist) were used through bath application. All drugs were purchased from Tocris Cookson (Bristol, United Kingdom).

**Loading of olfactory nerve axons with Calcium Green-1 dextran**

For Ca\(^{2+}\) imaging in olfactory nerve axons, mice (16 to 18 days old) were anesthetized by pentobarbital sodium (50 mg/kg, s.c.). Olfactory receptor neurons in nasal cavities were exposed to 2% isoflurane in 95% O\(_2\)-5% CO\(_2\) and, in a series of experiments, also contained 2–6 μM bicuculline. For recording of bAP-induced Ca\(^{2+}\) signals in the absence of synaptic transmission, the ACSF contained 40 μM bicuculline and, in a series of experiments, also contained 2–4 μM NBQX (to prevent synaptically driven oscillatory activity). To test the contribution of NMDA and mGluRs in generating local Ca\(^{2+}\) signals, D-APV (100 μM) and methyl-4-carboxyphenylglycine (MCPG, 1 mM, a group I and II mGluR antagonist) were used through bath application. All drugs were purchased from Tocris Cookson (Bristol, United Kingdom).

Fluorescence of Oregon green BAPTA-1 or Calcium Green-1 dextran was excited by a two-photon laser (810 nm, Verdi, Coherent, Palo Alto, CA) using a multifocal scanner (frame rate 7 Hz) (Straub et al. 2000) or by whole field epifluorescence images (488 nm; frame rate 20 Hz), with light provided by a monochromator (Polychrome IV, TILL photonics, Gräfelfing) and detected by a cooled CCD camera (PCO Sensicam, PCO imaging, Kelheim) with spatial resolution of 0.226 μm/pixel (×63 objective, 520x680 pixels, 2x2 binning) under the control of ImagePro software (Media Cybernetics, Silver Spring, MD). Optical filters for whole field epifluorescence consisted of a dichroic beam splitter (DCLP 505 LP; Chroma Technology, Brattleboro, VT) and an emission filter (535 ± 25 nm). Fluorescence from intracellularly loaded dye equilibrated throughout the cell within 30–40 min of commencing whole cell configuration. Changes of [Ca\(^{2+}\)] were expressed as relative fluorescence changes (ΔF/F, where F is the baseline fluorescence before a stimulus and ΔF is the evoked change in fluorescence). ΔF/F was measured in areas of interest in the shaft and in the terminal tuft of the MC primary dendrite. Fluorescence values were determined by subtracting pixel values averaged over regions outside the stained cell from each pixel of the series of images (i.e., fluorescence values are “background subtracted”). The ΔF/F images were spatially low-pass filtered (Gaussian kernel with half-width of 0.5–5 μm). Ca\(^{2+}\) maps represent the average of two to five series of images (trials) unless stated otherwise. For recordings under two-photon excitation, Ca\(^{2+}\) images from different layers of the tufts were added to yield projection images. Responsive areas within the dendritic tuft were identified as “hot spots” if their peak amplitude exceeded the response averaged over the whole tuft (“global tuft response”) by a factor of 2. The border of these hot spots was defined by the half-maximal value of the hot spot amplitude relative to the global tuft response. Color-coded maps of ΔF/F were obtained using custom-made macros in ImagePro Plus. Color-coded images show the maps of the peak Ca\(^{2+}\) transient if not otherwise stated.

**Results**

**Ca\(^{2+}\) signals induced by bAPs in the MC primary dendrite glomerular tuft**

MCs were loaded with the fluorescent Ca\(^{2+}\) indicator Oregon green BAPTA-1 (200 μM) from the patch-pipette attached to the soma. Brief intrasomatic depolarizing pulses (2 ms, 0.7–1.2 nA) were used to evoke single bAPs, and related Ca\(^{2+}\) transients were recorded from the MC soma.
transients were imaged from the glomerular tuft of the primary dendrite (Fig. 1).

Under whole field fluorescence excitation, somatically triggered APs robustly produced a rise in $[\text{Ca}^{2+}]_i$, in dendritic shafts and tuft terminals, indicating that these APs equally invaded all of the terminal branches in the tuft (Fig. 1, A–C). The relatively homogeneous distribution of the Ca$^{2+}$ signal was not due to the saturation of the Ca$^{2+}$ indicator because increasing numbers of bAPs raised the peak amplitude of the Ca$^{2+}$ transient in the dendritic tuft near-linearly ($n = 3$, Fig. 1D–E). The rise in $[\text{Ca}^{2+}]_i$ was clearly larger in thinner terminal branches of the tuft as compared with the more proximal dendritic regions (Fig. 1, B and C). This clear gradient of increasing Ca$^{2+}$ transients from the primary dendrite toward the fine tuft branches was seen in all cells examined ($n = 12$).

We measured Ca$^{2+}$ transients in different portions of dendritic tufts (including dendritic shaft, first order branch and second order branch) in $n = 7$ cells with clear morphological details. We found significantly larger Ca$^{2+}$ transients with higher order dendritic compartments (peak amplitude $[\Delta F/F]$: dendritic shaft: $6.5 \pm 0.7\%$, first order branch: $10.2 \pm 0.8\%$; second order branch: $12.9 \pm 0.8\%$, mean $\pm$ SE, $P < 0.0002$, One-way analysis of variance [ANOVA], post hoc Tukey test). Because the diameter of dendrites decreases toward more distal dendritic branches of the MC primary tuft (Kasowski et al. 1999), the simplest explanation for the observed gradient is the difference in surface-to-volume ratio; larger surface-to-volume ratio of thinner dendrites would be expected to result in a larger increase in $[\text{Ca}^{2+}]_i$.

An alternative explanation for the gradient could be that auto-excitation of tuft branches or inhibitory mechanisms at more proximal sites are involved (Aroniadou-Anderjaska et al. 1999; Carlson et al. 2000; Chen et al. 2000; Didier et al. 2001; Isaacson 1999; Lowe 2002; Salin et al. 2001). These mechanisms may produce inhomogeneities in the bAP-Ca$^{2+}$ maps that cannot be resolved by whole field fluorescence excitation (Debarbieux et al. 2003).

To clarify this point, we performed an additional series of measurements using two-photon laser scanning excitation to improve spatial resolution. The two-photon system employed uses full frame integration; therefore it gives an accurate measure of the Ca$^{2+}$ signal over the integration period (100 ms) (Straub et al. 2000). The advantage of two-photon excitation, as employed here, lies in the high spatial resolution, and the complementary whole field fluorescence recording allowed a high temporal resolution. In the experiments employing

![Image](https://example.com/image1.png)

**FIG. 1.** Backpropagating action potentials (bAP) induce Ca$^{2+}$ transients throughout the dendritic tuft. A–E: bAP induced Ca$^{2+}$ transients under whole-field fluorescence excitation. A: fluorescence image of a mitral cell (MC) dendritic tuft filled with Oregon green BAPTA-1 via a somatic recording pipette. B: color-coded map of the peak Ca$^{2+}$ transient induced by a single bAP evoked by short current pulse (2 ms, 0.7–1.2 nA) injected into the cell body. Blue to red indicates relative fluorescence change $\Delta F/F$ from 0 to 19%. C: time-courses of Ca$^{2+}$ transients in four regions of interest (indicated by numbers in A). Note that the bAP invaded all parts of the dendritic tree, being larger in fine terminal branches. D: Ca$^{2+}$ transients induced by 1, 2 and 4 bAPs. E: peak amplitudes of Ca$^{2+}$ transients normalized to that induced by one bAP ($n = 3$ cells). Dotted line indicates linear relationship. F–J: bAP induced Ca$^{2+}$ transients under two-photon laser excitation. F: fluorescence image of a MC dendritic tuft. G: color-coded map of the peak Ca$^{2+}$ transient induced by a single bAP. Blue to red indicates relative fluorescence change $\Delta F/F$ from 0 to 8%. H: selected areas of interest outlining shaft (1, blue), first order branches (2 and 3, green), and second order branches (4, 5, and 6, red). I: voltage traces of bAP and Ca$^{2+}$ transients from areas of interest in C. bAP shape is shown as inset. J: peak amplitudes of Ca$^{2+}$ transients measured in different regions as the example in C. Averaged values from four cells. Note that largest Ca$^{2+}$ transients were observed in higher order terminal branches of the dendritic tuft. *, $P < 0.01$. Black squares in C, D, and I indicate time of current injection. Bars in A and F indicate 50 μm.
two-photon excitation, both excitatory and inhibitory synaptic transmissions were blocked by 40 μM NBQX, 50 μM t-APV, and 40 μM bicuculline. The results obtained under these conditions were consistent with those obtained with whole field imaging in control ACSF: bAPs invaded all parts of the MC dendritic tuft, and with synaptic transmission blocked, Ca\(^{2+}\) transients still increased in amplitude with the decrease in dendritic diameter (Fig. 1, F–J). We therefore measured and compared Ca\(^{2+}\) transients from different locations. Data from \(n = 4\) cells were pooled in Fig. 1J. The peak amplitude \(\Delta F/F\) values were 4.2 ± 1.3% at the base of the dendritic tuft; 6.4 ± 1% at first order branch, and 12 ± 1% at second order branch (mean ± SE; \(P = 0.0005\), one-way ANOVA, post hoc Tukey test). We concluded that larger Ca\(^{2+}\) transients in more distal branches were based on differences in the surface-to-volume ratio and were not due to auto-excitation or dendrodendritic interactions.

**Ca\(^{2+}\) signals induced by activation of ON synapses**

The subthreshold EPSPs were evoked by stimulating bundles of ON axons that innervate the glomerular tuft of the dye-filled MC. Optical and electrical recordings of the evoked EPSP were interleaved with recordings of bAPs under either whole field illumination (Fig. 2) or two-photon excitation (Fig. 3). Synaptic stimulation induced Ca\(^{2+}\) maps that were distinct in several respects from those induced by bAP. First, Ca\(^{2+}\) transients associated with subthreshold EPSPs were pronounced in the tuft, whereas changes in the primary dendrite proximal to the tuft were either small or absent (Fig. 2, B–E). Second, synaptically induced Ca\(^{2+}\) transients were clearly localized to one or several hot spots (Fig. 2, C and D; \(n = 12\)). The localization of Ca\(^{2+}\) hot spots was robust throughout single trials, with only small variation in peak amplitudes (Fig. 2, C1–C3).

Two-photon microscopy revealed that these hot spots correspond spatially to short portions of terminal dendritic branches (Fig. 3). To account for possible errors in recorded Ca\(^{2+}\) maps that may have resulted from a biased normalization of the fluorescence signals to resting fluorescence levels, we also normalized the data by dividing the EPSP-Ca\(^{2+}\) of the fluorescence signals to resting fluorescence levels, we concluded that larger Ca\(^{2+}\) transients in more distal branches were based on differences in the surface-to-volume ratio and were not due to auto-excitation or dendrodendritic interactions.

**FIG. 2. Olfactory nerve (ON) stimulation-induced localized Ca\(^{2+}\) transients.** A: fluorescence image using whole field fluorescence excitation of a dendritic tuft with areas of interest (shaft, blue; local tuft area, red). Yellow rectangle indicates area shown in D and E. B: bAPs and EPSPs recorded with the somatic pipette (bottom traces) and Ca\(^{2+}\) transients (top traces) measured from the two areas of interest indicated in A. Time of bAP is indicated by black square; time of synaptic stimulation is indicated by black arrow. C: maps (C1–C3) of synthetically induced Ca\(^{2+}\) transients in three sequential trials. Note reproducibility of pattern of yellow/red hot spots. D: average of maps shown in C1–C3. E: color-coded map of bAP-induced Ca\(^{2+}\) elevation. Synaptic recordings were interleaved with bAP recordings, with a 2-min interval. Blue to red indicates \(\Delta F/F\) from 0 to 5% (C1–C3) and 0 to 15% F; bar in A indicates 50 μm.

at hot spot), 6.53 ± 1.55% (bAP over the whole tuft; \(P > 0.08\), compared with bAP at hot spot), 5.14 ± 0.60% (EPSP at hot spot), and 1.35 ± 0.22% (EPSP over the whole tuft; \(P < 0.0005\), compared with EPSP at hot spot). The mean ratio between the EPSP-induced signals over the hot spots and the signals measured over the whole tuft was 4.8 ± 1.2 (SE) and is significantly larger than the corresponding value for the bAPs (1.2 ± 0.1, mean ± SE; \(P < 0.02\), paired \(t\)-test).

To better understand the input-output relationship between ON axons and MC terminal tufts, we estimated the spread of activated fibers in our slice preparation by imaging Ca\(^{2+}\) signals in the presynaptic terminals of ON axons. Ca\(^{2+}\) staining in the ON axons was achieved by nose injection of Calcium Green-1 dextran 2–5 days before slice physiology and imaging (\(n = 5\)). Figure 3 shows examples of presynaptic Ca\(^{2+}\) signals that were obtained by the same protocol used to image postsynaptic responses. These examples indicate activation of a subset of dye-loaded ON fibers with relatively low stimulation intensity (Fig. 4, A2 and B1), and under higher stimulation intensity, a more widespread activation of ON fibers with subregions that exhibit larger than average signals (Fig. 4, A3 and B2). The subset of activated ON fibers depended on the positioning of the stimulation electrode, as illustrated by the extreme case, in
which the stimulation electrode was placed onto the lower border of a glomerulus (Fig. 4B3). The presynaptic Ca\textsuperscript{2+}/H\textsubscript{11001} signals were graded with stimulation intensities (Fig. 4, A2–B2) and thus represented compound responses, as does the ON-EPSP. This result suggests that postsynaptic hot spots may be recruited at locations where the density of activated synapses is high.

Localized Ca\textsuperscript{2+} signals induced by activation of ON synapses are mediated by NMDA receptors

The localized Ca\textsuperscript{2+} changes in the MC dendritic tuft could be due to Ca\textsuperscript{2+} influx through NMDA receptors or voltage-gated Ca\textsuperscript{2+} channels, or may reflect the release of Ca\textsuperscript{2+} from internal stores following activation of group I mGluRs. To test the involvement of NMDA receptors, we compared EPSP-induced Ca\textsuperscript{2+} transients under control conditions and after bath application of 100\,\mu M of the NMDA receptor antagonist D-APV. D-APV did not affect the initial fast phase of the ON-EPSP that is mediated by AMPA receptors but did block the slow EPSP component (n = 11, Fig. 5A, Carlson et al. 2000; Ennis et al. 1996; Trombley and Westbrook 1990). Single bAPs that consistently generated larger Ca\textsuperscript{2+} signals than subthreshold EPSPs (Fig. 2) did not induce any detectable D-APV-sensitive potential (Fig. 5A3).

The ON-EPSP-associated Ca\textsuperscript{2+} signal, however, was either completely blocked or greatly attenuated by D-APV (n = 8, Fig. 5B and 6A and C). To take into account an increased dye concentration with subsequent increase in Ca\textsuperscript{2+} buffering and decrease in fluorescence transients (Ma and Lowe 2004) during

FIG. 3. Synaptically induced local Ca\textsuperscript{2+} transients imaged with two-photon laser scanning microscopy. A: fluorescence image of a MC dendritic tuft. Yellow rectangle indicates area shown in D-G. B: bAPs (bottom) and associated Ca\textsuperscript{2+} transients (top), from regions of interest outlined in D. Time of bAP is indicated by black square. C: ON-EPSPs (lower traces) and associated Ca\textsuperscript{2+} transients (top traces) from regions of interest outlined in D. Time of synaptic stimulation is indicated by black arrow. D: regions of interest. E–F: maps of Ca\textsuperscript{2+} transients induced by bAP (E) and ON stimulation (F). G: ratio of fluorescence during synaptically and bAP-induced Ca\textsuperscript{2+} transients. Bar in A indicates 50\,\mu m.

FIG. 4. ON stimulation induced Ca\textsuperscript{2+} transients in ON axon terminals. A1: fluorescence image of dye-loaded ON, outline of two glomeruli (dotted circles), and position of the stimulation electrode (white lines) in the ON layer (ONL). A2–A3: color-coded maps of Ca\textsuperscript{2+} transients evoked at low (30\,\mu A, A2) and higher (60\,\mu A, A3) stimulation intensities. Note that higher stimulation intensity recruited more nerve fibers (yellow arrow indicated ON branch that was recruited only at higher stimulation intensity). B1–B3: example maps of ON Ca\textsuperscript{2+} transients at different stimulation intensities (60\,\mu A, B1 and 90\,\mu A, B2) and after moving the stimulation electrode to the deeper portion of the glomerulus (B3; stimulation intensity 90\,\mu A).
the time period required to wash in D-APV, we interleaved recordings of EPSPs with recordings of bAPs. The peak amplitude and the decay time of bAP-induced Ca\textsuperscript{2+} transients were only slightly reduced and prolonged after wash in D-APV (Fig. 5C and 6A). The bAP-induced Ca\textsuperscript{2+} signals slowed even further after D-APV was washed out, whereas the EPSP-induced Ca\textsuperscript{2+} signals recovered, at least partially (n = 3, Fig. 5B). Comparison of the resulting synaptically induced signals before and after wash in D-APV (3.78 ± 0.374%, mean ± SE before D-APV, vs. 0.77 ± 0.194% after D-APV; P < 0.0005, n = 8, paired t-test) with bAP-induced signals before and after wash in D-APV (13.03 ± 1.66% before D-APV, vs. 11.04 ± 2.20% after D-APV; P > 0.13, n = 8, paired t-test) confirmed that the drug specifically reduced ON-EPSP-related Ca\textsuperscript{2+} signals (Fig. 6A). To take into account a decrease over time in the measured Ca\textsuperscript{2+} transients, we compared the ratio of synaptically induced signals after and before D-APV (0.21 ± 0.04, mean ± SE) to that of bAP-induced signals (0.72 ± 0.07; P < 0.0001, t-test; Fig. 6C).

MCs of the main olfactory bulb express high levels of the group I mGluR1 (Heinbockel et al. 2004; Sahara et al. 2001; Shigemoto et al. 1992; Van den Pol 1995), and probably also low levels of mGluR5 (Sahara et al. 2001). These receptors can mediate a release of Ca\textsuperscript{2+} from internal stores (Coutinho and

**FIG. 5.** Synaptically induced Ca\textsuperscript{2+} transients are mediated by NMDA receptors. A1–A3: ON-EPSPs and bAPs induced before, during D-APV application, and after D-APV washout. A1: representative EPSP. A2: average EPSP over nine cells. Note that D-APV blocked the late component of the EPSP but did not affect EPSP peak amplitude. A3: membrane voltage during induction of bAP (Arrow indicates truncated AP that is followed by AP afterhyperpolarization; average over 9 cells). Note that the membrane potential following the bAP was not affected by the blockage of the NMDA receptor. B: synaptically induced Ca\textsuperscript{2+} transients before, during D-APV application, and after D-APV washout. Color-coded maps of Ca\textsuperscript{2+} show hot spots (yellow arrows) before and after application of D-APV. Bottom panel shows time course of Ca\textsuperscript{2+} transients (region of interest indicated by yellow rectangle in C). Note that D-APV application abolished the local Ca\textsuperscript{2+} increase induced by synaptic stimulation of the ON. Blue to red indicates ΔF/F from 0 to 3%. C: bAP-induced Ca\textsuperscript{2+} transients before, during D-APV application, and after D-APV washout. Color-coded map (top) and time course of Ca\textsuperscript{2+} transients (bottom) induced by bAPs. Blue to red indicates ΔF/F from 0 to 12%. Bar indicates 50 μm. Black arrows and squares indicate time of synaptic stimulation and bAP induction, respectively.
Ca^{2+} signals induced by activation of ON synapses are graded

The ON stimulation-induced local Ca^{2+} signals may represent all-or-none “Ca^{2+} spikes” (Urban and Castro 2005) or may be graded with recruitment of increasing numbers of ORN axons. To investigate this issue, we determined the minimal ON stimulation intensity needed to evoke an AP in the patch-clamped MC and then measured the responses evoked by fractions of this reference intensity. Compound EPSP amplitude varied with stimulation intensity, as did the evoked Ca^{2+} signals (Fig. 6). The peak amplitude of the EPSP and the initial portion of the slower EPSP component (EPSP area, between 50 and 200 ms after ON stimulation) varied in near-linear relation to stimulation intensity (Fig. 7C). However, larger minimal stimulation intensities are required to evoke the Ca^{2+} transients and the later NMDA receptor-mediated EPSP component (EPSP area, between 200 and 500 ms after ON stimulation; Fig. 7C, n = 5 cells). To test the effect of larger stimulation intensities without AP firing in the recorded cell, we performed the next set of experiments with pipettes containing the Na+ channel blocker QX-314 (10 mM). Higher stimulation intensities induced large stimulus-locked EPSPs and a fast rise in Ca^{2+}, followed by unsynchronized synaptic events and a further increase in Ca^{2+} (Fig. 8, n = 3 cells). A prolonged or delayed rise in Ca^{2+} and unsynchronized synaptic events were also seen in cells with standard pipettes (see Fig. 7B) and were efficiently depressed by D-APV (see Fig. 5, A and B).

DISCUSSION

Our results showed that ON stimulation, subthreshold for spike initiation in the recorded MC, induced localized Ca^{2+} responses that required the activation of NMDA receptors in the MC dendritic glomerular tuft. Ca^{2+} transients evoked by bAPs spread throughout the dendritic tuft, with amplitudes that increased with the decrease of the diameters of the terminal dendrites. In contrast to bAP-associated Ca^{2+} transients, synaptically induced Ca^{2+} transients exhibited clear hot spots, were longer lasting, and were associated with prolonged electrical activity.

Ca^{2+} signals induced by bAP in the glomerular tuft

Ca^{2+} signals evoked by bAPs in the MC glomerular dendritic tuft have attracted much attention because terminal dendritic branches in the tuft behave both as postsynaptic structures that receive olfactory receptor cell terminals and as presynaptic structures that release transmitter (Bischofberger and Jonas 1997; Murphy et al. 2005; Rall et al. 1966). It has been shown previously by indirect measurements using Ca^{2+} imaging (Charpak et al. 2001; Debarbieux et al. 2003), as well as by direct measurement using voltage imaging (Djurisic et al. 2006), and Jonas 1997; Murphy et al. 2005; Rall et al. 1966).
that bAPs propagate in a nondecremental manner into the dendritic tuft of MCs. Our results are in agreement with previous reports showing that single bAPs trigger significant Ca\(^{2+}\)/H\(^{11001}\) transients in the distal branches of the dendritic tuft. We did not, however, find failures of bAPs in triggering Ca\(^{2+}\)/H\(^{11001}\) signals in selected dendritic tuft branches as reported in earlier in vivo measurements (Debarbieux et al. 2003). This discrepancy might be due to differences in intraglomerular inhibitory processes between in vitro, slice preparation, and in vivo recording from an anesthetized animal. Also in contrast to one earlier report showing that the amplitude of Ca\(^{2+}\)/H\(^{11001}\) transients did not depend on the diameter of the dendrite (Debarbieux et al. 2003), our current results clearly demonstrated Ca\(^{2+}\)/H\(^{11001}\) signals that were consistently larger in thinner dendrites, in agreement with concurrent observations made in the accessory olfactory bulb (Ma and Lowe 2004; Urban and Castro 2005). The reason for this discrepancy between the in vitro and in vivo data is not clear. Our results also demonstrated that bAP-induced Ca\(^{2+}\)/H\(^{11001}\) transients were not influenced by autoexcitation (Figs. 1 and 5).

FIG. 7. Synaptic EPSPs and Ca\(^{2+}\) transients increase with increasing stimulation intensities. A: fluorescence image of a MC dendritic tuft (top, left) and color-coded Ca\(^{2+}\)/H\(^{11001}\) maps representing Ca\(^{2+}\)-transients induced by synaptic stimulations with increasing intensities, as indicated. The time of imaging is indicated by the black rectangle in (B) left top panel. B: Ca\(^{2+}\) transients and synaptic EPSPs induced by synaptic stimulation at different intensities. Top panels show Ca\(^{2+}\) transients obtained from two regions of interest as indicated in the fluorescence image in A. The transients shown in the left and the right were obtained from the regions outlined in yellow and green colors, respectively. Bottom panels show sub- and suprathreshold EPSPs induced by synaptic stimulation at different intensities at the time scale of the Ca\(^{2+}\) transients (left) and at faster time scale (right). C: plots showing the relationship between ON stimulation intensity (fraction of intensity threshold for induction of an AP) and EPSP components or Ca\(^{2+}\) transients. Note that the peak amplitude of the EPSP (top left) and the early EPSP component (EPSP area, between 50 to 200 ms after ON stimulation, top right) varied close to linearly with stimulation intensity whereas larger minimal stimulation intensities are required to evoke the late EPSP component (EPSP area, between 200 to 500 ms after ON stimulation, bottom left) and the Ca\(^{2+}\) transients (bottom right). Early and late NMDA components are indicated by black rectangles in B left bottom.

FIG. 8. Synaptic EPSPs and Ca\(^{2+}\) transients recorded with pipettes containing QX-314. A: fluorescence image of a MC dendritic tuft (top, left) and color-coded Ca\(^{2+}\)/H\(^{11001}\) maps representing ON stimulation-induced Ca\(^{2+}\) transients. B: Ca\(^{2+}\) transients and synaptic responses induced by ON stimulation at different intensities in the region of interest indicated in A. Note prolonged rising phase of Ca\(^{2+}\) transient (arrow). Inset illustrates an enlarged view of a voltage trace (100 μA) with asynchronous synaptic events.

2004), that bAPs propagate in a nondecremental manner into the dendritic tuft of MCs. Our results are in agreement with previous reports showing that single bAPs trigger significant Ca\(^{2+}\) transients in the distal branches of the dendritic tuft. We did not, however, find failures of bAPs in triggering Ca\(^{2+}\) signals in selected dendritic tuft branches as reported in earlier in vivo measurements (Debarbieux et al. 2003). This discrepancy might be due to differences in intraglomerular inhibitory processes between in vitro, slice preparation, and in vivo recording from an anesthetized animal. Also in contrast to one earlier report showing that the amplitude of Ca\(^{2+}\) transients did not depend on the diameter of the dendrite (Debarbieux et al. 2003), our current results clearly demonstrated Ca\(^{2+}\) signals that were consistently larger in thinner dendrites, in agreement with concurrent observations made in the accessory olfactory bulb (Ma and Lowe 2004; Urban and Castro 2005). The reason for this discrepancy between the in vitro and in vivo data is not clear. Our results also demonstrated that bAP-induced Ca\(^{2+}\) transients were not influenced by autoexcitation (Figs. 1 and 5C) or GABAergic local inhibition (Fig. 1). We also found no direct evidence for NMDA receptor-dependent autoexcitation...
in the voltage trace following a single bAP (Aroniadou-Anderjaska et al. 1999; Isaacson 1999; Salin et al. 2001).

\[ \text{Ca}^{2+} \text{ signals induced by ON-EPSPs in the glomerular tuft} \]

In contrast to the widespread \( \text{Ca}^{2+} \) signals induced by bAPs, dendritic \( \text{Ca}^{2+} \) signals following subthreshold ON stimulation were pronounced in the tuft, whereas changes in the primary dendrite proximal to the tuft were either small or absent. Also, these synaptically induced \( \text{Ca}^{2+} \) transients were clearly localized to one or several hot spots.

The EPSP-associated dendritic \( \text{Ca}^{2+} \) transients could theoretically be caused by mobilization of \( \text{Ca}^{2+} \) from internal stores. Our experiments excluded the role of mGlurS because the mGlur antagonist MCPG did not significantly affect the \( \text{Ca}^{2+} \) transient associated with the ON-EPSP. The mGlur antagonist had also only a minor effect on the ON-EPSP itself, consistent with a previous report (De Saint and Westbrook 2005). The \( \text{Ca}^{2+} \) signals were, however, either blocked completely or greatly attenuated by the NMDA receptor antagonist D-APV. Another potential entry pathway is via voltage-gated \( \text{Ca}^{2+} \) channels that are activated by the NMDA receptor-mediated depolarization. Because the peak amplitude of the ON stimulation-induced EPSP was not dependent on NMDA receptors, it is unlikely that voltage-gated \( \text{Ca}^{2+} \) channels are the major source for the NMDA receptor-dependent \( \text{Ca}^{2+} \) transients. The source of the D-APV-insensitive residual component of the \( \text{Ca}^{2+} \) signal following ON stimulation (approximately 25% of the control response), however, has not been clarified. It is, therefore possible that a part of the \( \text{Ca}^{2+} \) signal is mediated by low-threshold voltage-gated \( \text{Ca}^{2+} \) current, as suggested by previous experiments of Charpak et al. 2001. However, on the basis of indirect evidence, these authors concluded that \( \text{Ca}^{2+} \) signals are completely independent of NMDA receptor activation, which is markedly different from our results.

NMDA receptors may be activated directly by glutamate released from the ON, and by glutamate released from MC dendrites following an initial activation of dendritic glutamate release by the ON input. Our data provide evidence for additional dendritically released glutamate. Thus \( \text{Ca}^{2+} \) signals and slower synaptic components increase supralinearly with stimulation intensity, while the EPSP peak amplitudes (which is a measure of recruited ON fibers) increase more linearly with stimulation intensity (Fig. 7C). Moreover, ON stimulation was often followed by barrages of unsynchronized synaptic events and a prolonged rising phase of the \( \text{Ca}^{2+} \) transient. Indeed, the slow NMDA receptor-dependent component has previously been attributed to recurrent, intraglomerular dendrodendritic interactions (Carlson et al. 2000).

The most likely scenario for glomerular integration of ORN inputs is that dendrodendritic mutual excitation of dendrites of neighboring MCs can become self-supportive after an initial ON input. The hot spots in our \( \text{Ca}^{2+} \) maps would then reflect regenerative glutamate release in chemical glomerular compartments, as proposed by Schoppa and Westbrook (2001). The mechanisms for termination of this regenerative mutual excitation of dendritic tufts is unclear but may involve depletion of releasable dendritic glutamate, \( \text{Ca}^{2+} \) activated outward currents, or GABAergic inhibition. It should be noted that natural stimulation most likely would activate ORN axon terminals that are more homogenously distributed over the glomeruli than was the case under our experimental focal stimulation conditions. It is therefore likely that the local hot spots of slow NMDA receptor-dependent process studied here will fuse to a more homogenous pattern under natural conditions. In the absence of hot spots, the dendrodendritic interactions that are reflected by the hot spots seen with focal ON activation will, however, still contribute to the coupling of MCs. This scenario is in agreement with previous proposals that glutamate released from MC dendrites is regenerative and contributes to synchronization of glomerular responses to ON activity (Christie et al. 2005; Isaacson 1999; Schoppa and Westbrook 2001).

**Functional implications**

The elaborated MC dendritic tufts and multifaceted dendrodendritic interactions are suggestive of specific dendritic information processing (as opposed to simply relaying ON inputs) in olfactory glomeruli. All signaling mechanisms that are known to occur at the level of the olfactory glomeruli can be attributed to one specific computational outcome, namely, to increase sensitivity for the specific class of ORNs that provide input to that given glomerulus. Increase in sensitivity is achieved by sampling of and averaging over all ORN inputs (Van Drongelen et al. 1978). This process requires coupling of MCs so that the average ORN inputs can be represented by the population of MCs that are associated with one given glomerulus. In addition to sampling and averaging, dendrodendritic interaction can boost the ON inputs by regenerative mutual excitation. In this picture, inhibitory mechanism is required to dampen sensitivity for strong ON inputs. Faster interaction such as gap-junction coupling (Christie et al. 2005) may synchronize spike timing of MCs. This synchronization of spiking may further enhance sensitivity via stochastic resonance, or may provide information that is encoded by OB network oscillations or the MC target cells in the olfactory cortex.

**ACKNOWLEDGMENTS**

We thank H. Mutoh for help with \( \text{Ca}^{2+} \) imaging in the olfactory nerve and technical support. We also thank all members of the Knöpfel lab for many helpful discussions and encouragement and J. Yokoyama for editorial help.

**REFERENCES**


