Diversity of Neural Signals Mediated by Multiple, Burst-Firing Mechanisms in Rat Olfactory Tubercle Neurons

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Chiang E, Strowbridge BW. Diversity of neural signals mediated by multiple, burst-firing mechanisms in rat olfactory tubercle neurons. J Neurophysiol 98: 2716–2728, 2007. First published September 12, 2007; doi:10.1152/jn.00807.2007. Olfactory information is processed by a diverse group of interconnected forebrain regions. Most efforts to define the cellular mechanisms involved in processing olfactory information have been focused on understanding the function of the olfactory bulb, the primary second-order olfactory region, and its principal target, the piriform cortex. However, the olfactory bulb also projects to other targets, including the rarely studied olfactory tubercle, a ventral brain region recently implicated in regulating cocaine-related reward behavior. We used whole cell patch-clamp recordings from rat tubercle slices to define the intrinsic properties of neurons in the dense and multiform cell layers. We find three common firing modes of tubercle neurons: regular-spiking, intermittent-discharging, and bursting. Regular-spiking neurons are typically spiny-dense-cell-layer cells with pyramidal-shaped, dendritic arborizations. Intermittently discharging and bursting neurons comprise the majority of the deeper multiform layer and share a common morphology: multipolar, sparsely spiny cells. Rather than generating all-or-none stereotyped discharges, as observed in many brain areas, bursting cells in the tubercle generate depolarizing plateaus that trigger graded but time-limited discharges. We find two distinct subclasses of bursting cells that respond similarly to step stimuli but differ in the roles transmembrane Ca currents play in their intrinsic behavior. Calcium currents amplify depolarizing inputs and enhance excitability in regenerative bursting cells, whereas the primary action of Ca in nonregenerative bursting tubercle neurons appears to be to decrease excitability by triggering Ca-activated K currents. Nonregenerative bursting cells exhibit a prolonged refractory period after even short discharges suggesting that they may function to detect transient events.

INTRODUCTION

The olfactory bulb is the primary conduit that enables chemo-sensory inputs to reach cortical areas (Shepherd and Greer 1998). However, projections from the bulb target a wide variety of brain areas, including the piriform cortex as well as several poorly understood regions, such as the anterior olfactory cortex, agranular insula cortex and olfactory tubercle (Heimer et al. 1985; Luskin and Price 1983; Shepherd 2004). Even among these “secondary” targets of the olfactory bulb, the olfactory tubercle (the anterior perforated substance in humans) stands out both for how little is known about its function and for the enigmatic structures found there, such as the islands of Calleja and the striatal bridges. Few previous studies have reported intracellular recordings from tubercle neurons in either brain slices or in vivo, and these have focused on neurons in islands of Calleja within the tubercle (Halliwell and Horne 1995, 1998). No previous studies have applied modern patch-clamp methods to the tubercle. Millhouse and Heimer (1984) used Golgi staining techniques to define the major cell types in the tubercle, which include spiny neurons located predominately in the dense cell layer (DCL; Fig. 1A) and sparsely spiny, multipolar neurons located in the deeper multiform layer (MFL). The elemental properties of tubercle neurons (intrinsic physiology, neurotransmitter, synaptic targets) have not been defined, although a subpopulation of tubercle cells, and most granule cells within islands of Calleja, appear to be GABAergic (Gritti et al. 1993). Even the fundamental question of whether the tubercle is primarily an olfactory brain region or is, instead, a component of the limbic system or basal ganglia is unresolved (Haberly and Price 1978; Heimer et al. 1985; Luskin and Price 1983; Millhouse and Heimer 1984). Also unknown is whether the neurons in the tubercle generate distinctive responses when driven with slow, phasic input during sniffing. Previous work in the olfactory bulb (Balu and Strowbridge 2007; Balu et al. 2004) demonstrated that mitral cells express specific ion currents that enable them to phase-lock to sniffing-like periodic inputs with very high temporal precision. It is not known whether tertiary olfactory targets, like the olfactory tubercle, also have intrinsic properties that are linked to the temporal dynamics of olfactory receptor neuron activation.

Although the functional properties of tubercle neurons (and the tubercle itself) are mysterious, new behavioral and neurochemical findings suggest that the tubercle may be a critical brain region that mediates the rewarding effects of cocaine. Recent work that reexamined how lesions of the ventral forebrain affect self-administration of psychomotor stimulants, such as amphetamines and cocaine, points to the tubercle’s significance. Classic work on this question implicated the nucleus accumbens in mediating the stimulant-driven reward because relatively large lesions in this region diminish amphetamine self-administration by rodents (Lyness et al. 1979) and conditioned place-preference (Syrzaki et al. 1982). Direct injections of amphetamine into the nucleus accumbens support self-administration (Hoebel et al. 1983) and conditioned place-preference (Carr and White 1986). Although these results often have been generalized to include cocaine, Ikemoto (2003) found that relatively small lesions of the olfactory tubercle, but not of the nucleus accumbens, disrupt cocaine-induced conditioned place-preference. The same group also found that rats will self-administer cocaine into the olfactory tubercle more...
readily than into the core of nucleus accumbens and that the rewarding aspects of cocaine were abolished by co-infusion of dopamine receptor antagonists into the tubercle (Ikemoto 2003; Ikemoto and Donahue 2005).

Although understanding how the tubercle modulates behavioral reward and the role that dopamine receptors play in this system are critical, long-term questions, these issues cannot be resolved without first defining the intrinsic properties of the major cell types in the tubercle. These functional elements may represent both potential sites of action for cocaine and therapeutic targets to combat addiction. In this study, we define the intrinsic properties of tubercle neurons and find three broad classes of intrinsic responses in these neurons: regular-spiking, intermittently discharging, and bursting. The first two neuronal classes closely resemble similarly named cells in cortical regions. Bursting cells in the tubercle, however, are unusual and can generate graded, time-limited clusters of action potentials that may function to signal sudden changes in synaptic input. We also find two subclasses of bursting cells, both located predominately in the MFL, that differ dramatically in the underlying cellular mechanism responsible for their intrinsic bursts and in their short-term, intrinsic plasticity.

**METHODS**

**Slice preparation and recording**

Olfactory tubercle slices (300 μm thick) were prepared by making coronal slices from anesthetized (ketamine, 140 mg/kg ip) P14–24 Sprague-Dawley rats using a modified Leica (Nussloch, Germany) VT100S vibratome. An artificial cerebrospinal fluid (ACSF) dissec-
tion solution with reduced Ca was used when preparing and storing slices. This solution contained (in mM) 124 NaCl, 2.6 KCl, 1.23 NaH2PO4, 3 MgSO4, 26 NaHCO3, 10 dextrose, and 1 CaCl2, equilibrated with 95% O2-5% CO2, and chilled to 4°C during slicing.

Brain slices were incubated in a 30°C water bath for 30 min and then maintained at room temperature. During experiments, slices were superfused with ACSF that contained (in mM) 124 NaCl, 3 KCl, 1.23 NaH2PO4, 1.2 MgSO4, 26 NaHCO3, 10 dextrose, and 2.5 CaCl2, equilibrated with 95% O2-5% CO2 and warmed to 30°C. Whole cell patch-clamp recordings were made from neurons visualized under IR-DIC optics, using either an Axiostar 1 FS (Carl Zeiss, Thornwood, NY) or BX51WI (Olympus, Center Valley, PA) fixed-stage upright microscope and a Multiclamp 700A or Axopatch 1D amplifier (both from Axon Instruments/Molecular Devices, Sunnyvale, CA).

We avoided recording from granule cells located in or near islands of Calleja in the MFL. Because of this selection bias away from small, granule-like cells, our results may not represent the full extent of variation in intrinsic properties among MFL cells. Patch pipettes (typically 4–6 MOm resistance) contained (in mM) 140 K-methylsulfate, 4 NaCl, 10 HEPES, 0.2 EGTA, 4 MgATP, 0.3 Na3GTP, and 10 phosphocreatine. In some experiments, this internal solution was supplemented with a polar intracellular marker (Alexa594, 100 μM or neurobiotin, 0.5%). All chemicals were obtained from Sigma except for Alexa594 hydrazide (Molecular Probes/Invitrogen, Eugene, OR), neurobiotin (Vector Labs, Burlingame, CA), and TTX (Calbiochem/EMD, San Diego, CA).

Neuronal imaging

Live imaging experiments utilized a custom two-photon microscope based on the Verdi V10 pump laser, Mira 900 Ti-sapphire laser (both from Coherent, Santa Clara, CA) and a high-speed XY galvanometer mirror system (6210; Cambridge Technology, Lexington, MA). Intracellularly loaded fluorescent dyes were excited at 830 nm through a ×60 water-immersion objective (Olympus). Emitted light was detected through an epifluorescent light path that included a 700DCLPXR dichroic mirror, a BG39 emission filter (both from Chroma Technology, Rockingham, VT) and a cooled PMT detector module (H7422P-40; Hamamastu, Bridgewater, NJ). Photomultiplier output was converted into an analog voltage by a high-bandwidth current preamplifier (SR-570; Stanford Research Systems, Sunnyvale, CA). Custom Visual Basic software written by BWS controlled the scanning system and image-analysis functions. Laser beam intensity was controlled electronically through a Pockels cell attenuator (ConOptics, Danbury, CT) and a Uniblitz shutter (Vincent Associates, Rochester, NY). In most experiments, the output of the Mira laser was attenuated by 90–95%.

In some experiments, slices containing labeled neurons were fixed (4% paraformaldehyde) and either viewed with a standard, wide-field epifluorescence microscope (Axioskop 2, Carl Zeiss) after being mounted in Proling (Invitrogen; for Alexa593-filled cells) or processed to visualize neurobiotin labeling using the ABC peroxidase method (Horikawa and Armstrong 1988) and using the Vector Elite kit (Vector Labs) and the NeuronLucida 3D reconstruction system (Microbrightfield, Williston, VT). Neurobiotin-filled cells were used predominately to assess the overall dendritic arborization. The Alexa-filled neurons were used to assess fine morphological details (e.g., dendritic spines), especially when imaged using 2-photon microscopy. Soma area was estimated by measuring the length of orthogonal major and minor axes of the best-fit ellipse. Spine density was estimated by averaging the density at four different dendritic locations in maximal intensity projections of 2-photon Z-stack images (final image formed from the maximal intensity recorded in the image Z stack, determined on a pixel by pixel basis; mean dendritic length examined = 54 μm).

Data acquisition and analysis

Electrophysiological data were recorded and analyzed using custom software written in Visual Basic 6 (Microsoft, Redmond, WA) and Origin 7.5 (OriginLab, Northampton, MA). Current and voltage records were low-pass filtered at 2 kHz and then digitized at 5 kHz, using a 16-bit A/D converter (ITC-18, Instrutech, Port Washington, NY). Input resistance was assessed by measuring the maximal response to hyperpolarizing current steps that caused 5- to 10-mV hyperpolarizations. Action potential properties (amplitude, width, latency-to-peak, spike-AHP, AHP amplitude) were calculated by custom software written in Visual Basic. The AHP latency was calculated from the onset of the action potential to the peak AHP response. Neurons were included in this study if they had overshooting action potentials and input resistances >100 MΩ. The average input resistance across our population of tubercle cells was 263.8 ± 14.5 MΩ. The tendency for some tubercle cells to discharge primarily during the initial phase of long (2 s duration) depolarizing steps was assessed using a spike clustering ratio (number of spikes evoked during the first 500 ms/number of spikes evoked during 1 s to 2,000 ms). This metric would yield a value of 0.25 for tonically discharging neurons and 1.0 for neurons that only fired during the initial 500 ms. The metric was averaged over multiple (mean 5.8 ± 0.2) trials from each neuron. Discharge efficiency (Fig. 6B) was calculated by dividing the total number of action potentials evoked by a 2-s depolarizing step by the step amplitude and is expressed as APs per nA current injected. In Fig. 5, we positioned gray rectangles (III) at visually identified inflections in the membrane potential record to mark the temporal extent of depolarizing plateau potentials. Pharmacological agents were applied by changing the perfusion solution. Voltages presented are not corrected for the liquid junction potential. Unless noted, statistical significance was assessed using the Student’s t-test. Data are presented as means ± SE.

RESULTS

Intrinsic firing patterns of olfactory tubercle neurons

We recorded from 176 rat olfactory tubercle neurons that fit within our selection criteria. Neurons in the dense cell layer (DCL) and multiform layer (MFL) of the rat olfactory tubercle could be classified into three broad groups based on their intrinsic firing responses: regular-spiking (RS), intermittently firing (IF), and bursting. The multiform layer (ML) is sparsely populated with neurons and was not included in this study. Responses to graded depolarizing steps from each cell type are shown in Fig. 1B.

The firing rate in step-evoked discharges in RS neurons (n = 36) initially adapts, then becomes tonic throughout the remainder of the step stimulus. This adaptation pattern is shown in the plot of instantaneous firing-frequency in Fig. 1C, left. Step-evoked discharges in these tubercle neurons closely resemble the firing behavior of RS neocortical pyramidal cells (McCormick et al. 1985) and, therefore were classified as regular-spiking. The vast majority of neurons recorded in the DCL (82.6%) were RS, although this discharge pattern was encountered occasionally in the MFL (12.6% of MFL cells). RS discharge patterns were not dependent on a specific resting membrane potential and were observed following steps from holding potentials of −80 to −60 mV. IF neurons (n = 32) also discharged throughout a 2-s depolarizing step stimulus but did not fire tonically, except for short (~100–300 ms) epochs. Responses in these neurons were dominated by long (>100 ms) pauses, giving rise to their classification name. IF neurons were encountered most frequently in the multiform layer (21.5% of all MFL neurons) and occasionally in the DCL (8.7% of all DCL neurons). IF
neurons had significantly greater coefficients of variation (CV) of the interspike intervals assayed in responses to 2-s step responses (mean CV_{isi} = 0.59 ± 0.09) than did RS neurons (0.18 ± 0.02; P < 0.001; Table 1). Intermittent discharges often are associated with GABAergic interneurons in other brain regions (McCormick et al. 1985), although occasionally this intrinsic behavior occurs in excitatory principal neurons, such as mitral cells in the olfactory bulb (Balu et al. 2004).

In bursting neurons (n = 98), action potential discharges were concentrated largely in the initial 500 ms of 2-s depolarizing steps (bursting cells stopped firing 555 ± 58 mm after step onset; n = 98 cells) and were followed by a pronounced afterhyperpolarization that began during the step response (Fig. 1B, right). Bursting tubercle neurons had a greater initial spike-clustering ratio (0.88 ± 0.02; see methods) than did either RS (0.30 ± 0.01) or IF tubercle cells (0.45 ± 0.05; both significantly less than bursting neurons; P < 0.001; Table 1). Rather than generating all-or-none bursts, tubercle bursting neurons’ discharges were modulated with step amplitude (Fig. 2). Firing within burst discharges was typically intermittent not tonic (mean CV_{isi} = 0.52 ± 0.03; significantly greater than RS cells; P < 0.001; not significantly different from intermitent neurons; P > 0.05; see also Fig. 4A). As described in the following text, functional tests can subdivide this category of tubercle neurons into two classes: regenerative and nonregenerative bursting cells. The cellular mechanisms and functional relevance of discharges in both types of bursting cells are described in the following text. Most bursting neurons were found in the MFL (65.9% of MFL neurons) with a minority found in the DCL (8.7% of DCL neurons). Neurons in the DCL were relatively homogenous with most classified as RS. By contrast, the multiform layer was heterogeneous and included all three intrinsic cell types. A small percentage of tubercle cells (5.7%; 10 of 176) had firing patterns that contained elements of both IF and bursting neurons (intermittent spikes clustered near the beginning of the step that did not trigger a burst AHP) and were excluded from further analysis.

As shown in Table 1, many intrinsic properties (input resistance, membrane time constant, action potential amplitude and width, latency-to-maximum-spike AHP) were similar across the three major cell types in the tubercle. We observed a modest, but statistically significant, difference in resting membrane potential between bursting (−62.2 ± 1.1 mV) and RS cells (−69.5 ± 3.6 mV; P < 0.05). We also noted a slightly depolarized action potential threshold in RS neurons (−34.6 ± 1.9 mV), compared with both IF (−39.4 ± 1.2 mV; P < 0.05) and bursting tubercle neurons (−39.2 ± 0.6 mV; P < 0.05). Spike-evoked AHP responses varied dramatically in tubercle cells, even among neurons in the same category (for example, see Fig. 5, A–C). On average, bursting neurons had significantly smaller spike AHP amplitudes (−7.8 ± 0.6 mV) than did either RS (−11.7 ± 1.6; P < 0.001) or IF cells (−11.7 ± 0.9; P < 0.05). A majority of bursting neurons (80.8%) had a “sag” in response to hyperpolarizing steps. We observed similar membrane potential sags in hyperpolarizing step responses less frequently in both RS (42.9%) and IF (38.9%) cells.

### Morphological correlates of olfactory tubercle neurons

We next sought to define the morphological properties of RS, IF, and bursting tubercle neurons. We visualized 34 tubercle neurons filled with either Alexa594 (n = 26) or neurobiotin (n = 8) through the patch pipette. All visualized RS neurons (n = 11) were spiny (3.0 ± 0.7 spine/10 μm), multipolar neurons (mean number of processes emanating from the soma = 5.8 ± 0.7; Fig. 2). The mean cross-sectional cell body area of DCL neurons was 172 ± 30.7 μm². RS cells located in the DCL (n = 4) had extensive dendritic arborization in both the ML and MFL layers (Fig. 2, A and B). Morphological features of many RS cells (e.g., Fig. 2B) suggest a similarity to superficial neocortical pyramidal cells with an apical dendrite that was oriented perpendicular to the DCL. Electrophysiologically, all visualized DCL neurons were regular-spiking (Fig. 2B, inset).

We recorded from 30 filled MFL neurons, including 10 IF cells and 13 bursting neurons. We found that IF and bursting neurons had generally similar morphologies (Fig. 3) that tended to have both fewer spines (1.0 ± 0.5 and 1.7 ± 0.1 spine/10 μm for IF and bursting, respectively) and fewer primary dendrites (3.3 ± 0.4 and 2.5 ± 0.3) than did RS cells. Grouped together, IF and bursting MFL cells had significantly fewer spines (1.2 ± 0.3; P < 0.05) and fewer primary processes (2.9 ± 0.3; P < 0.001) than did RS tubercle neurons. The cell bodies of IF neurons (mean cross-sectional area =

### Table 1. Intrinsic properties of olfactory tubercle neurons

<table>
<thead>
<tr>
<th></th>
<th>Regular Spiking</th>
<th>Intermittent Firing</th>
<th>Bursting (total)</th>
<th>Bursting Regenerative</th>
<th>Bursting Non-Regen.</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>36</td>
<td>32</td>
<td>98</td>
<td>21</td>
<td>77</td>
</tr>
<tr>
<td>Percentage in DCL</td>
<td>82.6</td>
<td>8.7</td>
<td>8.7</td>
<td>0</td>
<td>8.7</td>
</tr>
<tr>
<td>Percentage in MFL</td>
<td>12.6</td>
<td>21.5</td>
<td>65.9</td>
<td>12.6</td>
<td>53.3</td>
</tr>
<tr>
<td>RMP, mV</td>
<td>−69.5 ± 3.6</td>
<td>−65.1 ± 2.0</td>
<td>−62.2 ± 1.1</td>
<td>−62.9 ± 2.9</td>
<td>−62.1 ± 1.3</td>
</tr>
<tr>
<td>R_{ac}, ΩM</td>
<td>281.4 ± 52.9</td>
<td>250.2 ± 37.7</td>
<td>248.6 ± 15.4</td>
<td>242.1 ± 25.8</td>
<td>251.0 ± 19.1</td>
</tr>
<tr>
<td>tau, ms</td>
<td>32.0 ± 5.3</td>
<td>25.6 ± 2.0</td>
<td>27.0 ± 1.5</td>
<td>31.4 ± 3.0</td>
<td>25.4 ± 1.6</td>
</tr>
<tr>
<td>AP amplitude, mV</td>
<td>61.4 ± 3.0</td>
<td>68.7 ± 2.6</td>
<td>63.4 ± 1.4</td>
<td>60.9 ± 1.8</td>
<td>64.6 ± 1.8</td>
</tr>
<tr>
<td>AP width, ms</td>
<td>2.12 ± 0.04</td>
<td>2.05 ± 0.06</td>
<td>2.13 ± 0.02</td>
<td>2.05 ± 0.06</td>
<td>2.17 ± 0.02</td>
</tr>
<tr>
<td>AP threshold, mV</td>
<td>−34.6 ± 1.9</td>
<td>−39.4 ± 1.2</td>
<td>−39.2 ± 0.6</td>
<td>−38.7 ± 1.3</td>
<td>−39.5 ± 0.7</td>
</tr>
<tr>
<td>AHP amplitude, mV</td>
<td>−11.7 ± 1.6</td>
<td>−11.9 ± 0.9</td>
<td>−7.8 ± 0.6</td>
<td>−7.5 ± 1.6</td>
<td>−8.0 ± 0.5</td>
</tr>
<tr>
<td>AHP peak time, ms</td>
<td>8.7 ± 0.9</td>
<td>9.3 ± 1.3</td>
<td>9.4 ± 0.7</td>
<td>7.3 ± 1.0</td>
<td>10.3 ± 0.8</td>
</tr>
<tr>
<td>Initial clustering</td>
<td>0.30 ± 0.01</td>
<td>0.45 ± 0.05</td>
<td>0.88 ± 0.02</td>
<td>0.79 ± 0.06</td>
<td>0.92 ± 0.02</td>
</tr>
<tr>
<td>Firing CV</td>
<td>0.18 ± 0.02</td>
<td>0.59 ± 0.09</td>
<td>0.52 ± 0.03</td>
<td>0.62 ± 0.08</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>Sag, %</td>
<td>42.9</td>
<td>38.9</td>
<td>80.8</td>
<td>71.4</td>
<td>84.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. DCL, dense cell layer; MFL, multiform layer; RMP, resting membrane potential; AP, action potential; AHP, afterhyperpolarization.  
* and ** Significantly different from regular spiking P < 0.05 (*) and P < 0.001 (**); † and ‡ significantly different from intermittent P < 0.05 (*) and P < 0.001 (**); †† and ‡‡ significantly different from regenerative burst P < 0.05 (*) and P < 0.01 (**).
tical types: spiny, pyramidal-like neurons that discharge in a RS firing pattern and sparsely spiny neurons that show IF and bursting discharge patterns.

Mechanism of intrinsic bursts in tubercle neurons

The absence of all-or-none, stereotyped discharges in tubercle bursting neurons raises the possibility that aspects of the burst response may represent attributes of the stimulus, such as intensity or slope. Although the mean firing frequency within the discharge was correlated with step amplitude in the example shown in Fig. 4A, this relationship existed only through part of the stimulus range. Responses to larger steps deviated from this linear relationship (Fig. 4B; mean $R^2 = 0.69 \pm 0.08; n = 11$ cells). We found a more robust correlation between stimulus amplitude and the first interspike interval (expressed as an instantaneous frequency), in the example shown in Fig. 4B ($R^2 = 0.92$) and in the population of 11 bursting cells tested systematically (mean $R^2 = 0.91 \pm 0.01$). Surprisingly, given the intermittent nature of the firing within the discharge (mean CV$_{ISI} = 0.52 \pm 0.03$) and the relatively weak correlation with mean firing-frequency, we also found a strong correlation between step amplitude and the total number of spikes evoked ($R^2 = 0.97$ in the example shown in Fig. 4B; mean $R^2 = 0.88 \pm 0.02$ for the population of 11 bursting cells tested). These results are summarized in Fig. 4C and suggest that stimulus intensity in bursting cells may be represented by the first interspike interval within the burst response.

Bursting tubercle neurons reliably discharged near the peak of ramp stimuli, such as those shown in Fig. 5A1. Both mean firing-frequency and the instantaneous firing-frequency, determined by the initial two spikes, correlated well with the slope of the ramp stimulus ($R^2 = 0.76 \pm 0.08$ for mean frequency and $0.91 \pm 0.04$ for instantaneous frequency; $n = 5$). As shown in enlargements in Fig. 5A2, the phase relationship between the end of the ramp stimulus and the burst discharge was not constant; steeper ramps triggered bursts near the end of the ramp, whereas discharges began before the peak stimulus intensity in less steep ramps. The ability of bursting neurons to detect both rapidly (steps) and slowly changing stimuli (ramps) suggests a possible role for these neurons in signaling the rate of change in the firing of presynaptic neurons.

It is likely that the graded nature of discharges in bursting ON neurons is caused by a combination of active currents. Rather than being evoked by an underlying Ca spike, discharges in bursting ON neurons often appear on top of a steady, depolarizing plateau potential, such as shown in Fig. 5. As shown in the following text, the graded nature of OT burst discharges is likely due to voltage-dependent modulation in this underlying plateau potential. Burst-generating plateau potentials in the tubercle often continue beyond the last action potential (Fig. 5B, top trace) before they are terminated abruptly by the afterhyperpolarization.

Graded intrinsic bursts in tubercle neurons appear to result from the interactions among at least four active currents: $I_{Na}$, $I_{AHP}$, subthreshold Na current, and low-threshold Ca current. Most (80.8%) tubercle bursting neurons exhibit membrane potential sag during steady hyperpolarizations, presumably reflecting the slow activation of $I_{Na}$ during the step response. The same current can diminish after depolarizing steps, especially during the initial response, due to a transient reduction in

![FIG. 2. Morphology of regular-spiking tubercle neurons. A: neurobiotin reconstruction of a neurobiotin-filled regular-spiking neuron in the dense cell layer (DCL) with a dendritic arborization that was oriented perpendicular to the DCL and entered both the molecular layer (ML) and the multiform layer (MFL). B: 2-photon image of another regular-spiking DCL neuron showing a pyramidal-shaped dendritic arborization and numerous dendritic spines. Image is a composite made from multiple maximum projection Z-stack compressions. Inset: adapting discharge pattern recorded in this neuron to a 2-s depolarizing step. The ventral surface of the brain is at the bottom of both images.](image-url)
input resistance (Maccaferri and McBain 1996). Consistent with this model, we found that bath application of the $I_{\text{H}}$ blocker Cs (4 mM) facilitated discharges in bursting neurons (Fig. 5B; mean number of action potentials evoked in Cs $= 224.7 \pm 61.7\%$ of control; significantly greater than control; $P < 0.05$; $n = 6$ cells). This increase in excitability was paralleled by a reduction in the membrane potential sag in response to hyperpolarizing steps (see inset in Fig. 5B) and was reversible on washout of Cs. In most tubercle bursting cells tested (5 of 6), depolarizing steps still evoked time-limited burst responses in Cs (Fig. 5B, middle trace; number of APs in burst responses $= 164.3 \pm 15.3\%$ of control; $n = 5$ cells with plateau responses in Cs), suggesting that the underlying plateau potentials are modulated, but not mediated, by $I_{\text{H}}$.

In all nonregenerative bursting tubercle neurons tested (6/6), blockade of transmembrane Ca currents by perfusion with a low (0.5 mM) Ca/high (6 mM) Mg extracellular solution converted the burst response into a prolonged discharge that persisted throughout most of the 2-s depolarizing step (number of APs evoked $= 310 \pm 86\%$ of control; significantly greater than control; $P < 0.05$; $n = 6$; Fig. 5C). Low-Ca ACSF also reduced the normally prominent burst AHP response in these cells (see $\uparrow$ in Fig. 5C). Surprisingly, in regenerative bursting cells (5 of 5 cells tested; see Fig. 6G), reduction of Ca currents with low-Ca ACSF had the opposite effect—decreasing excitability and the number of spikes evoked by the step stimulus. Because of this difference in response to low-Ca ACSF, the mechanisms underlying bursting in regenerative and nonregenerative cells will be considered separately.

Tetrodotoxin (TTX) blocked Na-based action potentials and reduced, but did not abolish, the underlying depolarizing plateau potentials in nonregenerative bursting cells (5 of 5 cells tested; Fig. 5D). The amplitude and duration of these TTX-resistant plateau potentials were graded with stimulus amplitude (Fig. 5D, right traces), suggesting that voltage-gated Na current is not required to generate plateau potentials in nonregenerative bursting cells. Although low-Ca extracellular solution increased excitability in nonregenerative bursting cells under control conditions (Fig. 5C), the same treatment reduced excitability and eliminated plateau responses evoked in TTX (Fig. 5E), suggesting that low-threshold Ca channels may contribute to the underlying plateau potential. A role for
low-threshold Ca channels also is suggested by the ability of weak, subthreshold depolarizations to trigger both plateau potentials and AHP responses in control conditions (Fig. 5D, bottom left trace). In these examples, steady-state plateau potentials were triggered by <20 mV depolarizations from rest (to approximately −55 mV), within the range of typical T-type low-threshold Ca channels (Randall and Tsien 1997). The burst AHP response was associated with a transient decrease in input resistance (to 71.2 ± 7.0% of control; n = 4; Fig. 5F) that reversed polarity at −99 mV (Fig. 5F, inset), consistent with the activation of a Ca-activated K current. Presumably, the counterintuitive effect of low-Ca ACSF we find in control conditions (increasing excitability and prolonging discharges) reflects the critical role Ca-activated K currents play in truncating the burst discharge.

Together, our experiments suggest that low-threshold Ca currents underlie the depolarizing plateau response in bursting tubercle neurons and that $I_{\text{AHP}}$ and subthreshold Na current function to enhance this response. Presumably, the inward current caused by low-threshold Ca channels is opposed by K channels, generating the periods of steady-state depolarization that trigger burst discharges. Although the identity of the channels involved in “flattening” the plateau response is not known, the plateau depolarization itself appears to be terminated by a Ca-activated K current.

Regenerative bursting tubercle neurons

In a minority of bursting tubercle neurons (21.4%; 21 of 98), short-duration (25–100 ms) stimuli could trigger regenerative discharges that outlasted the step depolarization (Fig. 6A1). Responses from similar depolarizing stimuli applied to a non-regenerative bursting cell are shown in Fig. 6A2. Both regenerative and nonregenerative generated self-limiting discharges in response to 2-s-duration depolarizing steps (mean duration = 329.7 ± 87.5 and 263.8 ± 40.5 ms, respectively; mean number of spikes = 3.7 ± 0.5 and 3.7 ± 0.3, respectively) and thus were categorized as bursting. Although discharges generated by regenerative and nonregenerative bursting cells were similar, bursts were initiated at significantly longer latencies in regenerative cells (119.9 ± 26.8 vs. 51.2 ± 7.3 ms; $P < 0.01$; Fig. 6B) and required less depolarizing current from the same membrane potential (mean efficiency = 81.1 ± 32 vs. 30.9 ± 4.9 spike/nA; $P < 0.05$). Discharges in nonregenerative bursting cells also had slightly but significantly higher initial clustering ratios (0.92 ± 0.02) than regenerative cells (0.79 ± 0.06; $P < 0.01$; Fig. 6B). All regenerative bursting cells were located in the MFL (12.6% of MFL neurons), whereas nonregenerative bursting cells occurred in both the DCL (8.7% of DCL neurons) and MFL (53.3% of MFL neurons).

Most regenerative bursting cells generated plateau potentials at fixed membrane potentials that were relatively insensitive to step amplitude (Fig. 6, C and D). Depolarizing steps could elicit a stereotyped plateau potential with the latency of the initial action potential graded with stimulus intensity. In regenerative cells that fired doublets, the plateau depolarization often by 10.220.32.247 on October 27, 2016 http://jn.physiology.org/ Downloaded from...
Short-term plasticity of intrinsic responses in tubercle neurons

The large AHP that terminates the burst response in nonregenerative cells also imparts a dramatic, short-term plasticity to burst responses in tubercle neurons. As shown in Fig. 7A1, a single burst could completely inhibit the response to a subsequent, identical depolarizing step evoked 3 s later (n = 12 cells). Bursting neurons appear to recover completely ~10 s after the initial response with partially recovered responses evoked at intermediate intervals. The suppression of burst responses is not caused exclusively by the membrane hyperpolarization associated with the AHP because altering the bias current to match the membrane potential to the same voltage reached during the peak of the AHP response had only a minor effect on the burst discharge (Fig. 7A1, top right trace). This form of two-pulse plasticity was not observed in regenerative bursting cells (Fig. 2A and Fig. 7). Figure 7A3 summarizes the degree of two-pulse inhibition in recordings from 8 regenerative and 12 nonregenerative bursting cells.

The short-term plasticity of the burst response did not depend on the duration of the depolarizing stimulus used to trigger the burst. Discharges evoked by 500-ms steps could completely suppress burst responses to 2-s depolarizing steps (Fig. 7B). Subthreshold depolarizing stimuli partially inhibited subsequent burst responses (Fig. 7B, 2nd trace), suggesting that the mechanism responsible for triggering the short-term plasticity has a low threshold. The suppression of the burst response by a depolarizing prepulse was not absolute. As shown in Fig. 7C, the suppressed burst response could be recovered by increasing the step amplitude. These results argue that relatively weak depolarizations (including subthreshold depolarizations) can activate intrinsic mechanisms that dampen the responsiveness of tubercle neurons by raising their burst-discharge threshold over a period of several seconds.

Two-pulse inhibition in bursting cells dramatically alters neuronal responses to slow, phasic excitatory input. We applied trains of alpha functions (τ = 100 ms, interval = 400 ms) that mimic the normal pattern of glomerular activation in...
the olfactory bulb during sniffing (Charpak et al. 2001; Margrie and Schaefer 2003) and have been used previously to define intrinsic currents that govern mitral cell responses to periodic input (Balu and Strowbridge 2007; Balu et al. 2004; Halabisky and Strowbridge 2003), to the four classes of olfactory tubercle inputs. In support of this hypothesis, we found that low-Ca ACSF reversibly abolished burst responses triggered by near-threshold trains of alpha functions in regenerative cells (Fig. 8B). Responses to similar stimuli in IF cells differed dramatically from bursting cells and were potentiated following the response to the initial cycle (mean number of spikes triggered by sEPSP2 in IF cells = 30.5 ± 1.2 vs. 13.1 ± 2.2 spikes triggered by sEPSP1; *P < 0.001; n = 8). Figure 8C summarizes the modulation of discharges during alpha function trains in bursting, IF, and RS tubercle cells.

**DISCUSSION**

We made three principal conclusions in this study. First, we found three types of firing patterns of neurons located in the dense and multiform cells layers that could be revealed by recording responses to 2-s depolarizing steps from the same membrane potential: RS, ID, and bursting. Most neurons located in the DCL were RS, whereas we found all three firing modes in the MFL. Second, we found a strong correlation between regular discharges and spiny, pyramidal cell-like den-
dritic morphology. By contrast, both bursting and IF neurons appeared to share a common sparsely-spiny, bipolar, or multi-polar dendritic morphology. The related morphology of bursting and IF neurons raises the possibility that these two intrinsic phenotypes may represent variations (or differential modulation) of a single cell type. In support of that hypothesis, we found that burst discharges are neither all-or-none nor regular/tonic but, instead, are intermittent. However, the wide variety of dendritic morphologies we encountered in intracellular fills of MFL neurons precludes an unequivocal answer at this time. Finally, we found two distinct mechanisms that generate burst responses in tubercle neurons. The most common burst discharge, termed nonregenerative, involves both voltage-gated Na and Ca currents, is modulated by \( I_{\text{H}} \), and is terminated by Ca-activated K current. In a minority of cells, a regenerative Ca current amplified weak depolarizing inputs and appeared to generate bursts directly. These different bursting mechanisms could be separated by their sensitivity to blockade of Ca currents and by using tests for short-term intrinsic plasticity.

**Relationship to previous work on the olfactory tubercle**

Previous work using Golgi staining (Millhouse and Heimer 1984) defined two common morphologies of tubercle cells, medium-sized densely spiny cells, and larger, spine-poor neurons. Both cell types occurred in DCL and MFL layers, though most DCL neurons appeared to be densely spiny neurons. Our work suggests that most medium densely spiny tubercle neurons are RS, whereas the spine-poor neurons consist primarily of IF and bursting neurons. Although the transmitter used by either cell type has not yet been directly established using paired recordings, many DCL neurons are immunoreactive for glutamic acid decarboxylase (Gritti et al. 1993), suggesting that they are GABAergic. This transmitter identity is consistent with the morphological parallels between spiny tubercle neuron and medium spiny neurons in the striatum, which are GABAergic, and the dense cholinesterase staining pattern that clearly links the tubercle with structures in the basal ganglia (Heimer et al. 1985). Like spiny tubercle neurons, most striatal medium spiny neurons are RS, though some fire tonically (Venance and Glowinski 2003). Relatively few studies have defined the intrinsic properties of tubercle cells using intracellular recordings. Halliwell and colleagues (Halliwell and Horne 1995, 1998) recorded from granule cells in islands of Calleja and demonstrated gap junction coupling within this neuronal population. The same group also used field recordings in tubercle brain slices to investigate the synaptic circuits activated by extracellular stimulation in the ML and MFL (Owen and Halliwell 2001).

**Multiple mechanisms generate intrinsic bursts in tubercle neurons**

The two types of bursting tubercle neurons we found could be separated by both pharmacological tests (reducing Ca currents using low-Ca ACSF; Figs. 5C and 6G) and by using two-step protocols to test for short-term intrinsic plasticity (Fig. 7A). Preliminary experiments testing the effects of blockers of specific types of Ca channels on the burst response have yielded complex results, suggesting that time-limited bursts in tubercle cells may result from multiple Ca and Ca-activated...
currents. A parallel study is underway in which the primary Ca currents are dissected pharmacologically and the resulting data used to generate a realistic computer model of bursting cells. Our present results suggest that the principal difference between the two bursting cell types relates to the function of Ca currents. In the more-commonly encountered nonregenerative bursting cells, Ca currents play a relatively minor role in generating the burst itself but are critical in terminating the discharge (through Ca-activated K currents). Reducing Ca currents in these cells increases excitability and enables the discharge to continue throughout the stimulus. The time-limited nature of the burst response largely reflects the recruitment of a large-amplitude AHP response mediated by these K channels. Similar counterintuitive effects of Ca currents that function primarily to decrease excitability (by triggering AHP responses) have been reported in other neurons, including hippocampal pyramidal cells (Madison and Nicoll 1982). Because relatively weak depolarizations (to approximately \(-50\) mV; Fig. 5D) appear to trigger AHP responses in tubercle neurons, it is possible that Ca influx through low-threshold Ca currents may trigger the AHP response either directly or by amplifying weak stimuli to activate high-threshold Ca channels. Burst discharges in these neurons appear to be potentiated by subthreshold Na currents, consistent with recent work on bursting in hippocampal neurons (Yue et al. 2005), and are dampened by \(I_{\text{H}}\).

Regenerative bursting neurons resemble the classic bursting phenotype of CA3 pyramidal neurons, including the ability of brief depolarizations to trigger prolonged depolarizing envelopes (Wong and Prince 1981). These depolarizing responses could be truncated by brief hyperpolarizing stimuli in both hippocampal (Wong and Prince 1981) and tubercle neurons (Fig. 6F). Bursts were not observed after Ca currents were reduced with low-Ca ACSF, suggesting that regenerative Ca currents contribute to the burst response. Calcium currents, including low-threshold Ca currents (Fig. 6), play an important role in amplifying inputs and enable regenerative cells to respond to very weak depolarizing stimuli that would not evoke a response in nonregenerative bursting cells. The mechanism of burst termination also differs between the two types of bursting cells with Ca-activated K currents playing a less dominant role in truncating discharges in regenerative cells than in nonregenerative cells. Given the significant differences in the underlying mechanisms, the similarity in the overall burst discharges between regenerative and nonregenerative tubercle cells was surprising and may indicate a common underlying role for low-threshold Ca currents. The difference between regenerative and nonregenerative bursting cells may reflect differences in the density or distribution of these low-threshold Ca currents as well as the contribution of other types of ion channels.

Many regenerative bursting cells also displayed pronounced plateau potentials that were apparent during long pauses between action potentials. This discharge pattern is reminiscent of recently-described “silent plateau” responses in subthalamic neurons (Kass and Mintz 2006) and may reflect one or more stable “up” states generated by active conductances in the different soma-dendritic compartments. Since many “silent plateaus” in both tubercle cells (e.g., Fig. 6D) and subthalamic neurons (Kass and Mintz 2006) appeared to be terminated by...
a single action potential, it is possible that either the rapid depolarization or, more likely, the spike AHP can disrupt the local plateau potential.

**Potential significance of intrinsic properties of tubercle neurons**

RS and IF neurons are found in many diverse brain regions often associated with excitatory principal cells and GABAergic interneurons, respectively, especially in cortical areas (Shepherd 2004). However, this correlation is not universal. In the olfactory system, glutamergic mitral cells in the olfactory bulb discharge intermittently (Balu et al. 2004; Chen and Shepherd 1997). The pyramidal cell-like morphology of spiny RS neurons, as well as their location in the DCL, is suggestive of an excitatory, glutamatergic phenotype, paralleling the pyramidal cells found in the major cell layers of the neighboring piriform cortex (Shepherd 2004), whereas immunocytochemical methods suggest that at least a subpopulation of DCL and MFL neurons are GABAergic (Gritti et al. 1993). Determination of which transmitter is released by the major tubercle cell types will likely require either paired recordings or more detailed co-labeling studies.

Of the three firing modes we identified in tubercle neurons, the graded bursting appears to be the most unusual and interesting. The pronounced short-term intrinsic refractory period we found for MFL nonregenerative bursting cells also was surprising and presents an obvious potential target for modulation by centrifugal afferents (Heimer et al. 1985). Also surprising were the large differences in the pattern of responses to slow phasic (sniffing-like) input among the different classes of tubercle neurons. Nonregenerative bursting cells discharged only in response to the first or second phasic input while the other types of tubercle neurons followed each slow, simulated EPSP (sEPSP). Olfactory bulb mitral cells, by contrast, fail to respond to the first slow sEPSP in a train and then follow subsequent sEPSPs reliably (Balu and Strowbridge 2007; Balu et al. 2004). The results from the present study suggest that different subtypes of tubercle cells likely play very different roles in processing periodic olfactory input. This difference is especially pronounced for IF and bursting cells, which show an opposite modulation in their responses during trains of sniffing-like excitatory input.

Although morphology and intrinsic behavior can be used to define the major cell types in a brain region, a functional understanding of this area also requires a description of the synaptic connectivity between cell types. Unfortunately, the presumptive axon appeared to be severed in most of our intracellular fills. This finding may help explain our difficulty in activating tubercle neurons synaptically using extracellular stimulation in cornal slices. A similar paucity of axonal labeling was reported following Golgi staining (Millhouse and Heimer 1984). Additional work using intracellular fills in different slice orientations will likely be necessary to determine how bursting and IF cells are synaptically interconnected with other tubercle cells. Because MFL tubercle cells appear to integrate both olfactory information, through molecular layer synaptic inputs, and inputs from the basal ganglia and the hippocampal formation, through synapses on dendrites within the MFL (Haberly and Price 1978; Heimer et al. 1987; Luskin and Price 1983), it is appealing to speculate that the these two classes of inputs may selectively activate different plateau potentials in MFL neurons. Using fast Ca imaging and focal stimulation methods (Balu et al. 2007), it should be possible to test whether the different “up states” we record in bursting cells (e.g., Fig. 6, D and E) reflect regenerative currents localized within different dendritic compartments and if these intrinsic responses are regulated by centrifugal modulators such as dopamine.

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**References**


