Patch Clamp Analysis of Gene-Targeted Vomeronasal Neurons Expressing a Defined V1r or V2r Receptor: Ionic Mechanisms Underlying Persistent Firing

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ABSTRACT

Sensory neurons in the mouse vomeronasal organ are comprised of two major groups, apical and basal, which project to different brain regions, express unique sets of receptors, and serve distinct functions. Electrical properties of these two subpopulations, however, have not been systematically characterized. V1rb2-tau-GFP and V2r1b-tau-GFP tagged vomeronasal sensory neurons (VSNs) were selected as prototypical apical or basal VSNs, respectively and their biophysical properties were analyzed in acute slices that minimized cell damage. Basal V2r1b-expressing VSNs had voltage-gated conductances, and especially Na\(^+\) (Nav) and Ca\(^{2+}\) (Cav) currents, that were substantially larger than those observed in apical V1rb2 VSNs, although the resting membrane potential, input resistance and membrane capacitance were similar in both cell types. Of several types of Cav currents, T-type and L-type Cav currents contributed to action potential firing, and both currents alone were capable of generating oscillatory Ca\(^{2+}\) spikes. The L-type Cav current was uniquely coupled to a BK large-conductance K\(^+\) current, and interplay between these channels played a critical role in repolarizing spikes and maintaining persistent firing in VSNs. Larger Nav and Cav conductances, along with a more positive inactivation voltage of the Nav current in the V2r1b VSNs contributed to the larger spike amplitude and higher spike-frequency induced by depolarizing current in these cells compared to V1rb2 VSNs. Basal GFP-negative VSNs and V2r1b VSNs responded to prolonged depolarization with persistent, but adapting discharge that could be relevant in sensory adaptation. Collectively, these results suggest a novel mechanism for regulating and encoding neuronal activity in the accessory olfactory system.

Keywords: VNO, patch clamp, spike-frequency adaptation, L-type Ca\(^{2+}\) current, BK current
INTRODUCTION

The vomeronasal system has long been a focus of study in mammalian sensory physiology. In rodents, the vomeronasal organ (VNO) is responsible for detection and transmission of a wide variety of pheromonal cues that are critically involved in social and reproductive behavior (Brennan and Keverne 2004; Brennan and Zufall 2006; Dulac and Torello 2003; Spehr et al. 2006). In mice, the sensory epithelium of the VNO is segregated into at least two structurally and functionally distinct layers: an apical layer that contains sensory neurons (VSNs) expressing the G-protein $G_{i2}$ and members of the V1r family of vomeronasal receptors, and a basal layer that contains VSNs expressing $G_{o}$ and members of the V2r receptor family (Ryba and Tirindelli 1997; Buck 2000; Halpern and Martinez-Marcos 2003; Mombaerts 2004). These distinct populations of VSNs have been hypothesized to detect and process different classes of chemical signals (Leinders-Zufall et al. 2000, 2004; Kimoto et al. 2005) and are likely to employ different signal transduction mechanisms (Leypold et al. 2002; Kelliher et al. 2006). This dichotomy is maintained in the accessory olfactory bulb (AOB) where apical VSNs project their axons to the rostral part of the AOB whereas basal VSNs innervate the caudal AOB (for reviews see Buck 2000; Dulac and Torello 2003; Mombaerts 2004). While it is generally difficult to predict with certainty which layer of the VNO a given VSN belongs to because of the diffuse boundary between the zones (Martini et al. 2001; Leinders-Zufall et al. 2004), cells are clearly identifiable in gene-targeted strains of mice in which individual VSNs express GFP under the control of a known V1r or V2r receptor.

Over the past decade substantial progress has been made towards understanding of the molecular logic and coding strategies in the mammalian vomeronasal system (Rodriguez et al.}
However, surprisingly little is known about the detailed biophysical properties of individual VSNs and how they transmit olfactory information to the AOB by spiking in response to sensory stimulation (Inamura et al. 1997, 2000; Leinders-Zufall et al. 2000; Holy et al. 2000; Leinders-Zufall et al. 2004). Earlier, dissociated mouse VSNs were used to investigate the major voltage-dependent conductances (Liman and Corey 1996; Fieni et al. 2003), and it was suggested that differences between voltage-dependent conductances in apical and basal VSNs may provide the initial basis for differences in excitability between the two types of VSNs in rodents. Recently, an acute mouse VNO slice preparation was used to measure and model the excitability of basal VSNs due to voltage-dependent Na$^+$ and K$^+$ currents (Shimazaki et al. 2006). Here, we sought to extend that work by characterizing the electrophysiological properties of both basal and apical mouse VSNs using transgenic mice in which VSNs express GFP under the control of either the V1rb2 or V2r1b receptor gene, and by focusing also on the voltage-gated Ca$^{2+}$ (Cav) conductances that are important for spike generation and maintenance of persistent firing in VSNs. We show that coupling between the L-type Cav current and the BK large-conductance K$^+$ conductance repolarizes spikes and controls the persistent firing of the cells, suggesting a novel mechanism for regulating and encoding neuronal activity in the mammalian accessory olfactory system.
METHODS

Slice Preparation

To clearly distinguish between VSNs of the apical or basal layer of the VNO epithelium, we used two mouse strains which harbor a targeted mutation in either the V1rb2 or the V2r1b locus, resulting in cotranslation of tauGFP along with V1rb2 or V2r1b from a bicistronic message (Rodriguez et al. 1999; Del Punta et al. 2002b). Axons from these two distinct neuronal populations project to the anterior and posterior portions of the accessory olfactory bulb, respectively (Rodriguez et al. 1999; Del Punta et al. 2002b). All experiments were performed on 2-6-months-old male or female mice. Original transgenic animals were generated in a mixed (129 x C57BL/6) background and then were backcrossed at least four times to the C57BL/6 background (Rodriguez et al. 1999). The procedures were carried out in accordance with protocols approved by the Universities of Maryland and Saarland. Acute coronal VNO tissue slices were prepared as described previously (Leinders-Zufall et al. 2000, 2004; Lucas et al. 2003). The mice were euthanized by inhalation of carbon dioxide, decapitated and the entire VNO capsule was removed (Leinders-Zufall et al. 2000, 2004). The VNO was dissected in oxygenated, ice-cold modified artificial cerebrospinal fluid (ACSF, 95% O₂, 5% CO₂) containing (in mM): 120 NaCl, 25 NaHCO₃, 5 KCl, 5 BES (N,N-bis[2-hydroxyethyl]-2-aminoethansulphonic acid), 1 MgSO₄, 1 CaCl₂, 10 glucose, osmolarity adjusted to 300 mOsm, pH 7.3. The dissected VNO was embedded in 3% low melting temperature agarose (Sigma) and coronal slices (250 μm) were prepared using a Vibratome (Pelco101, model 1000). After sectioning, slices were transferred to a recording chamber and kept under continuous flow (1-2
ml/min) of oxygenated ACSF or remained on ice in oxygenated medium until needed (for up to 4 hours). All experiments were performed at ambient temperature (20-22 °C).

Electrophysiological Recording

GFP-expressing (GFP+) VSNs were visualized in intact tissue slices with a 40X water immersion objective lens (Olympus) using fluorescent illumination and a GFP filter set attached to the microscope (BX50WI, Olympus). These identified GFP+ neurons were inspected carefully to ensure that their dendrite and axon was not damaged during the slicing procedure. As a control, we also recorded from regular non-fluorescent (GFP-) neurons from the same V2r1b- or V1rb2-tau-GFP mice. Somatic recordings were made using patch pipettes pulled from a standard borosilicate glass (OD 1.5mm, WPI, Sarasota, FL) on a vertical micropipette puller (PP-830, Narishige, Japan) followed by fire polishing. To assess the spontaneous firing properties of the VSNs under non-invasive conditions, we used extracellular loose-patch recording in some experiments. In this case, the pipette solution contained (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, pH 7.4, 300 mOsm. The electrode solution used for current clamp or voltage clamp recording of voltage-dependent K⁺ currents contained (in mM): 140 KCl, 1 EGTA, 10 HEPES, 0.5 GTP Na-salt, 2 ATP Mg-salt, pH 7.1, 290 mOsm. Nav currents were recorded using a CsCl-based electrode solution containing (in mM): 140 CsCl, 1 EGTA, 10 HEPES, 0.5 GTP Na-salt, 2 ATP Mg-salt, pH 7.1, 290 mOsm. After establishing stable whole-cell recordings in the voltage clamp mode (seal resistance ≥ 3 GΩ), we switched to current clamp by using the software-controlled “soft switch” of the EPC-9 patch clamp amplifier (HEKA, Lambrecht, Germany). This was usually done at “zero” holding current, thus enabling us to determine the spontaneous resting potential of the cell. VSNs were stimulated with current injections of
different duration, polarity and amplitude to investigate action potential generation. Signals were
low-pass filtered at 2.9 kHz and acquired at a 10-kHz sampling rate or directly recorded to video
tape. Cav currents were recorded using a bath solution without added Cl− to minimize a possible
contribution from Cl− currents. This solution contained (in mM): 10 Ca(OH)2, 110 NMDG, 140
methanesulfonic acid, 10 TEA-Cl, 10 HEPES, pH 7.4, 290 mOsm. In a subset of the
experiments, extracellular Ca2+ was isotonically replaced with 20 mM Ba2+. The electrode
solution used for recording of Cav currents contained (in mM): 140 Cs-methanesulfonate, 1
EGTA, 0.5 GTP Na-salt, 2 ATP-Mg salt, 10 HEPES, pH 7.1, 290 mOsm. Data were corrected
off-line for a liquid junction potential of +4 mV for Nav and Kv currents, and +11 mV for Cav
currents.

Data analysis

Ionic currents were analyzed using PulseFit 8.54 (HEKA, Lambrecht, Germany) and
IGOR Pro 4.09 software. Voltage-activated currents were leak subtracted. Cells exhibiting leak
currents > 10 pA were not included in this analysis. Cell capacitance (Cm) was monitored using
the automated function of the EPC-9 amplifier. A stable Cm-value over time was an important
criterion for the quality of an experiment. Activation and inactivation curves of ionic currents
were fit by the Boltzmann equation, giving values for the midpoint V1/2 (voltage at half-
activation) and slope k where I/I_{max} = 1/[1+\exp(V_{1/2} - V_m)/k]. These values are listed in Table 1
with the following parameters: slope k (slope^{activ} or slope^{inactiv}) and V_{1/2}^{activ} or V_{1/2}^{inactiv}.
Spike analysis was done off-line using IGOR Pro 4.09 software together with custom written
macros or the Neuromatic 1.86 software package (written by Jason Rothman, available at
http://www.neuromatic.thinkrandom.com). Instantaneous spike frequency was measured as the
reciprocal of the interspike intervals. Curves of the spike frequency as a function of injected depolarizing current were obtained by fitting the Michaelis-Menten equation to the data points, giving values for the maximal frequency $F_{\text{max}}$ and half-effective current $I_{1/2}$ where $F = (F_{\text{max}} * I)/(I + I_{1/2})$. Statistical tests were performed using StatView 5.01 (SAS Institute, North Carolina). The t-test (paired and unpaired) was used for measuring the significance of difference between two distributions. Multiple groups were compared using a one-way or two-way analysis of variance (ANOVA). The Fisher’s least significant difference (LSD) test was used as a post hoc comparison of the ANOVA. If not otherwise stated results are presented as means ± standard error of the mean (SEM).

Chemicals

All chemicals were purchased from Sigma unless otherwise stated. Drugs were prepared as stock solutions in DMSO or distilled water and diluted to the final concentration in ACSF for bath application. Peptide toxins were applied directly via the bath. Mibefradil and nimodipine were used to block T-type and L-type Cav currents, respectively. When used at micromolar concentrations, mibefradil preferentially inhibits T-type over other Cav channels (Randall and Tsien 1997; Martin et al. 2000). We applied this compound for a limited time only to avoid any side effects on other voltage-dependent channels. The peptide toxins $\omega$-Conotoxin MVIIA and MVIIC (both from Tocris, Ellisville, MO) were used to block N-type (Cav2.2) and N/P/Q-type (Cav2.1, 2.2) voltage-dependent Cav channels, respectively. Iberiotoxin (Tocris) and charybdotoxin (Sigma) were used as specific blockers of large-conductance BK Ca$^{2+}$-activated $K^+$ channels. Apamin and the synthetic non-peptide inhibitor UCL1648 (both from Tocris) were
used to assess the role of small-conductance SK Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels. Final DMSO concentrations (≤ 0.1 %, vol/vol) were tested in control solutions and had no effects.

RESULTS

Patch-clamp recording from GFP-expressing VSNs in VNO tissue slices

Individual, fluorescent VSNs were readily identifiable in the acute VNO slices prepared from the genetically-altered mice (Fig. 1). With a combination of fluorescence or infrared differential interference contrast (IR-DIC) illumination (Fig. 1), patch clamp recordings were obtained from such identified VSNs. The electrophysiological properties of V1rb2 \((n = 53)\) and V2r1b \((n = 66)\) neurons were then examined and compared to each other, as well as to some GFP\textsuperscript{−} VSNs \((n = 42)\). For these latter experiments, we chose VSNs that were located deep in the basal layer of the VNO sensory epithelium so that they would most likely represent basal VSNs. All GFP-expressing (GFP\textsuperscript{+}) cells analyzed in this study exhibited an intact cytoarchitecture with a single dendrite projecting toward the lumen of the VNO ending in a distal dendritic knob, and a thin axon projecting from the soma toward the basal lamina (Fig. 1).

Passive membrane properties and spontaneous activity

The electrical properties of V1rb2 and V2r1b cells were examined by whole cell voltage-clamp or current-clamp recording. In some cases, we used extracellular loose-patch recording to measure spontaneous action potential firing rates under less invasive conditions. With KCl
intracellular solution, both types of VSNs exhibited rather negative resting membrane potentials, averaging \(-74.3 \pm 0.6\) mV \((n = 19)\) in V1rb2 and \(-75.3 \pm 0.4\) mV \((n = 43)\) in V2r1b cells. GFP\(^-\) cells had very similar values \((-76.8 \pm 0.9\) mV; \(n = 17)\). Thus, VSN resting potentials in the VNO slice, irrespective of whether or not GFP is overexpressed, are significantly more negative than those reported for freshly dissociated mouse VSNs (Liman and Corey 1996; Fieni et al. 2003). These values are also more negative than a previous report that used acute VNO slices (Shimazaki et al. 2006).

We compared input resistance \((R_i)\) and cell capacitance \((C_m)\) among V1rb2 and V2r1b cells and found no significant differences between the two cell types \((V1rb2: R_i = 1.9 \pm 0.2 \text{ G}\Omega, n = 16; C_m = 6.8 \pm 0.3 \text{ pF}, n = 16; V2r1b: R_i = 1.8 \pm 0.2 \text{ G}\Omega, n = 14; C_m = 6.4 \pm 0.2 \text{ pF}, n = 15), t\text{-test: } P = 0.40 \text{ for } C_m \text{ and } P = 0.59 \text{ for } R_i; \text{ GFP}^+: R_i = 2.0 \pm 0.1 \text{ G}\Omega, n = 10; C_m = 7.1 \pm 0.5 \text{ pF}, n = 18). These values are broadly consistent with previously reported results from mouse VSNs (Liman and Corey 1996; Fieni et al. 2003; Shimazaki et al. 2006). Together, these results indicate that VSNs expressing tau-GFP can be considered healthy by electrophysiological standards and should serve as an excellent model for the investigation of vomeronasal signaling mechanisms.

GFP\(^+\) as well as GFP\(^-\) VSNs in the slice preparation were both spontaneously active at rest. This was observed by using either noninvasive, extracellular loose-patch recordings or whole cell current-clamp recordings (at zero holding current). A total of 39 GFP\(^+\) cells were analyzed with respect to spontaneous firing. The vast majority of these cells \((33/39, 84.6\%)\) showed relatively low spontaneous firing frequencies \((F_s)\) averaging \(0.29 \pm 0.12\) Hz in 12 V1rb2 cells and \(0.26 \pm 0.07\) Hz in 21 V2r1b cells, respectively. However, a subset of these cells \((6/39, 15.4\%)\) encompassing both VSN types exhibited an enhanced rate of resting activity, averaging
3.0 ± 0.7 Hz. This phenotype was correlated with leak currents > 10 pA and elevated resting potentials. In 17 GFP+ VSNs, we found an average resting activity of 0.35 ± 0.07 Hz. None of these cells exhibited an enhanced spontaneous firing rate. Therefore, we limited our study to VSNs exhibiting low resting frequencies.

Voltage-gated Na+ and K+ currents

V1rb2 and V2r1b cells both possessed large, rapidly activating and inactivating voltage-gated Na+ (Nav) inward currents (usually >1 nA) in response to a series of step depolarizations (Fig. 2A). These currents were recorded with Cs+-based intracellular solution to block outward K+ current. Because VSNs also showed robust voltage-gated Ca2+ (Cav) currents (see below), we suspected that a portion of the inward current could be due to currents through Cav channels. Indeed, when we added Cd2+ (100 μM) to the bath solution, peak inward currents were reduced in both cell types, by as much as 4 - 16% (n = 14) (Fig. 2A). The remaining currents were fully abolished by application of TTX (2 μM), indicating that they were due to currents through Nav channels (Fig. 2A). It was previously reported that Nav currents in VSNs of the apical layer are larger than those of basal VSNs and that this phenotype can serve to distinguish between the two cell types (Fieni et al. 2003). By contrast, we found that V2r1b VSNs possessed much larger Nav currents than V1rb2 cells (Fig. 2A). This finding was further substantiated by analyzing current density (peak current normalized to cell capacitance) as a function of voltage in both cell types (Fig. 2B). On average, peak current density was approximately two-fold greater in V2r1b (−274 ± 17 pA/pF, n = 7) vs. V1rb2 cells (−126 ± 16 pA/pF, n = 9; t-test: P < 0.0001). We compared activation and inactivation parameters of Nav currents in both VSN types (Fig. 2C, D). Whereas
activation curves were nearly indistinguishable, there was a significant difference in half-inactivation voltage, with \(-65.7 \pm 2.4\) mV \((n = 14)\) in V1rb2 vs. \(-53.5 \pm 0.8\) mV \((n = 15)\) in V2r1b cells (t-test: \(P < 0.0001\)). Detailed parameters obtained from Boltzmann fits of the activation and inactivation curves of Nav currents are listed in supplemental Table 1.

In the presence of TTX and Cd\(^{2+}\) and with intracellular KCl solution, V1rb2 and V2r1b cells both possessed prominent outwardly rectifying K\(^+\) currents in response to step depolarizations (Fig. 2E). These currents showed delayed rectifier gating properties typical of members of the Kv family of voltage-gated K\(^+\) channels. With Cd\(^{2+}\) in the bath, currents through Ca\(^{2+}\)-activated K\(^+\) channels were absent, due to blockade of Ca\(^{2+}\) entry through Cav channels (see below, Fig. 9). Kv current density in V2r1b cells was slightly larger compared with V1rb2 cells (Fig. 2F). Mean current density (at 60 mV) was \(176.6 \pm 16.1\) pA/pF \((n = 7)\) in V2r1b vs. \(133.7 \pm 9.9\) pA/pF \((n = 8)\) in V1rb2 cells (t-test: \(P < 0.05\)).

**Repetitive firing in VSNs involves spike frequency adaptation**

A characteristic property of both freshly dissociated mouse VSNs (Liman and Corey 1996) and those maintained in VNO slices (Shimazaki et al. 2006) is that injection of only a few picoamperes of inward current leads to repetitive action potential firing. Fig. 3A shows that this specific feature was also observed in GFP\(^+\) VSNs. Under current-clamp, both V1rb2 and V2r1b cells generated repetitive action potentials in response to depolarizing 2-s current steps of 2 to 10 pA (Fig. 3A). Resting membrane potential refers to 0 pA current injection. A plot of mean instantaneous spike frequency as a function of injected current revealed that, in response to the same stimulus, V2r1b cells generated considerably more spikes than V1rb2 cells, at least in the
mid- to high-range of the curves (Fig. 3B). These curves were well-fitted by a Michaelis-Menten
equation, showing saturation above 20 pA with $F_{\text{max}} = 11.2 \pm 0.5 \text{ Hz (} n = 6 \) in V1rb2 and $F_{\text{max}} = 19.1 \pm 1.6 \text{ Hz (} n = 5 \) in V2r1b cells. Larger Nav currents could lead to larger spikes in V2r1b
VSNs. Indeed, we found that V1rb2 cells generated much broader and smaller spikes than
V2r1b. Spike duration (SpD) was measured as width at 50% of the amplitude and spike
amplitude (SpA) as distance between the threshold and the peak (Fig. 3C, D) with the following
results [V1rb2: SpD = 15.8 \pm 0.7 \text{ ms, SpA = 37.9 \pm 2.8 mV (} n = 53 \text{ spikes from 9 cells); V2r1b:
SpD = 11.1 \pm 0.3 \text{ ms, SpA = 64.8 \pm 1.2 mV (} n = 62 \text{ spikes from 10 cells)}]. Injection of
hyperpolarizing currents of -2 to -8 pA revealed the presence of a hyperpolarization-activated
“sag” in both cell types (Fig. 3A) which is typical of the activation of H-currents described in rat
VSNs (Trotier et al. 1998). Both cell types produced rebound spikes during repolarization from
hyperpolarizing current steps (Fig. 3A).

On the basis of anatomical and physiological evidence, chemical cues, once present in the
VNO lumen, might be capable of stimulating VSNs for extended periods of time. For example,
recordings from mitral cells in the accessory olfactory bulbs of behaving mice have revealed
prolonged excitatory responses lasting for tens of seconds (Luo et al. 2003). These unusually
long-lasting responses could well be mediated by prolonged sensory input from VSNs.
Therefore, we investigated firing behavior of V1rb2 and V2r1b cells in response to prolonged,
depolarizing current steps (Fig. 3E-G). Representative recordings to 10-s steps from both cell
types are shown in Fig. 3E. These experiments revealed two unexpected results. First, an
important difference between the two cell types was that V1rb2 cells exhibited a tendency to
oscillate and produce broad spikes with complex waveforms under these conditions, whereas
V2r1b cells were capable of maintaining persistent firing. Second, we observed profound spike
frequency adaptation in both cell types, in contrast to the general view that spike frequency adaptation is absent in VSNs (Liman and Corey 1996). Because of the exceptional regularity of firing in V2r1b cells, we analyzed spike frequency adaptation in these cells in more detail (Fig. 3F). Over the course of a 10-s current step, the frequency declined from 7.4 ± 0.8 Hz to 3.1 ± 0.4 Hz ($n = 6$). Spike frequency-time plots were well-fitted with a monoexponential function yielding an average decay time constant $\tau = 2.4 ± 0.3$ s ($n = 6$). Profound spike frequency adaptation occurred also in GFP–VSNs (Fig. 3G). Here, with 60-s current steps, the frequency declined from 6.7 ± 0.4 Hz to 1.3 ± 0.2 Hz ($n = 5$). With these even longer pulses, spike frequency-time plots were fitted best with two exponentials ($\tau_1 = 2.36 ± 0.96$ s; $\tau_2 = 36.6 ± 14.9$ s; $n = 5$) (Fig. 3G).

Regenerative calcium spikes in VSNs

Given the relatively long action potential durations observed in both types of VSNs and the appearance of oscillatory, complex spikes in at least the V1rb2 cells, we suspected that VSNs are capable of generating calcium spikes that might underlie some of these properties. This was tested by examining voltage responses to depolarizing current steps (10 s, 10 pA) in the presence of TTX (Fig. 4). Remarkably, application of 2 μM TTX, which is sufficient to eliminate all Nav currents in these cells (Fig. 2), did not completely disrupt spike generation. Instead, this treatment both reduced the firing frequency and dramatically broadened individual spikes as compared to control recordings in both V1rb2 and V2r1b cells (Fig. 4A-C). Following TTX treatment, mean instantaneous spike frequency changed from 5.3 ± 0.3 Hz to 3.2 ± 0.2 Hz in V1rb2 ($n = 6$) and from 6.5 ± 0.7 Hz to 3.7 ± 0.4 Hz in V2r1b cells ($n = 9$). At the same time,
spike duration (width at 50% of spike amplitude) increased from 23 ± 5 ms to 91 ± 15 ms in V1rb2 (n = 6) and from 12 ± 1 ms to 44 ± 8 ms in V2r1b cells (n = 9). In both cell types, TTX-resistant spikes showed profound spike frequency adaptation (Fig. 4A).

The properties of these TTX-resistant regenerative spikes were somewhat reminiscent of low-threshold calcium spikes described in a variety of CNS neurons (Golding et al. 1999; Perez-Reyes 2003). When we applied Cd2+ (100 μM) to the bath solution, repetitive spiking was fully abolished, leaving only a single and strongly diminished initial spike followed by tonic membrane depolarization (Fig. 4A, TTX/Cd2+; n = 15). Subsequent experiments using inhibitors of Cav channels established that the TTX-resistant spikes indeed were caused by the activation of T- and L-type Cav channels (see Fig. 7) and thus represent regenerative calcium spikes.

Presence of L-, T- and N/P/Q-types of Cav channels

Given the results of Figs. 3 and 4, we hypothesized that calcium spikes may underlie VSN action potential firing. To investigate this, it was necessary to first gain a detailed understanding of Cav conductances and their pharmacological properties in GFP− and GFP+ neurons. Mouse VSNs express at least two current components related to the activity of Cav channels, a low-voltage-activated T-type current and an L-type current (Liman and Corey 1996; Fieni et al. 2003). In accord with these studies, we consistently found a TTX-resistant inward current (2 μM TTX, Cs+-based intracellular solution) in GFP− VSNs that was activated by voltage steps from -100 to 0 mV and showed relatively slow inactivation kinetics typical of high-voltage-activated Cav currents (Fig. 5A, insert). Even under normal physiological conditions with 1 mM external Ca2+, the average amplitude of this current exceeded -50 pA. Intriguingly
and in contrast to previous work using isolated VSNs (Liman and Corey 1996; Fieni et al. 2003), there was relatively little rundown of this current in the slice preparation over the course of 20 min (Fig. 5A), which enabled pharmacological dissection of its individual components.

Using the same voltage protocol but with 20 mM Ba\(^{2+}\) as the charge carrier, we found that this current was reduced to 71.8 ± 14.9 % of control (n = 4) by application of mibefradil (Mib, 10 μM) which selectively blocked native T-type Cav current over the L-type current in rat retinal bipolar cells (Pan et al. 2001) (Fig. 5B). The remaining current was suppressed to 18.1 ± 2.4 % of control (n = 6) by nimodipine (Nim, 5 μM), indicating that it was mostly due to L-type Cav channels (Helton et al. 2005) (Fig. 5B). Thus, T- and L-type Cav channels appeared to be the main ion channels underlying this current. To confirm this, we applied a voltage protocol that favored selective activation of T-type currents (Fig. 5C). Indeed, under these conditions nimodipine had relatively little effect on Cav currents (89.4 ± 10.0 % of control, n = 9), whereas the addition of mibefradil diminished the currents to 25.5 ± 2.0 % of control (n = 5), leaving only a relatively small residual component. Thus, although not specific in absolute terms, these two blockers nonetheless should be useful in dissecting the roles of T- and L-type channels in intact VSNs. The nimodipine/mibefradil-insensitive current was further suppressed to 21.5 ± 6.1 % of control (n = 5) by ω-Conotoxin MVIIIC (1 μM, CTx), indicating that it was likely mediated by P/Q- and N-types of Cav channels (Catterall et al. 2005) (Fig. 5D). A second peptide toxin, ω-Conotoxin MVIIA which specifically blocks N-type Cav channels, was less potent in suppressing this current (data not shown). Fig. 5D shows that there was also a ω-Conotoxin MVIIIC-insensitive but Cd\(^{2+}\)-blockable, residual component that could be due in part to the activation of R-type Cav channels [but see Lipscombe et al. (2004) on the drug resistance of L-
type Cav1.3]. Thus, individual VSNs are likely to express members of Cav1, Cav2 and Cav3 channel families, with L- and T-type channels as the most prominent ones.

We next compared L- and T-type Cav currents in V1rb2 and V2r1b cells (2 μM TTX, Cs⁺-based intracellular solution; Fig. 6). These experiments used 10 mM Ca²⁺ as the charge carrier. Fig. 6A (control) depicts raw traces of families of currents in response to a series of step depolarizations from -120 mV to voltages between -80 and 40 mV in V1rb2 and V2r1b cells, respectively. To isolate L-type currents, we applied nimodipine (Nim, 5 μM, Fig. 6A) and constructed current density-voltage plots of the nimodipine-sensitive component measured at the end of each voltage step (Fig. 6E). Unexpectedly, these currents activated at relatively negative potentials, between -60 and -50 mV, and reached a maximum between -10 and 0 mV (Fig. 6E). These properties suggest that both L- and T-type Cav channels could underlie low-threshold calcium spikes and contribute to action potential-dependent Ca²⁺ entry in VSNs. The density of L-type currents was significantly larger in V2r1b (-15.2 ± 1.3 pA/pF, n = 7) vs. V1rb2 cells (-8.9 ± 1.9 pA/pF, n = 7; t-test: P < 0.05).

T-type Cav currents were isolated by applying a mixture of nimodipine (5 μM) and ω-Conotoxin MVIIC (Nim/CTx, 1 μM, Fig. 6A) and current density-voltage plots were constructed from peak values of the residual currents (Fig. 6B). These currents activated near -80 mV and reached a maximum between -40 mV to -20 mV. Current density was slightly larger in V2r1b (-6.9 ± 0.1 pA/pF, n = 7) vs. V1rb2 cells (-5.5 ± 0.4 pA/pF, n = 7; t-test: P < 0.05). Notably both the T- and L-type Cav currents had no significant differences in the voltage-dependence of current activation and inactivation between the two cell types (Fig. 6C, D, F, G; Table 1). Thus, both cell types express L- and T-type Cav currents with very similar properties, but L-type currents are much more prominent in V2r1b cells as compared with V1rb2 cells.
Calcium spikes depend on L- and T-type Cav channels in V1r and V2r cells

Having provided evidence for L- and T-type Cav channel subtypes in V1rb2 and V2r1b cells, we next assessed the extent to which these channels were involved in the generation of calcium spikes in each cell type. A single action potential was evoked by a brief depolarizing current step (6 pA, 200 ms) (Fig. 7A). Addition of TTX (2 μM) revealed, in each cell type, the presence of TTX-insensitive spikes characterized by an increased delay and duration as well as a reduced amplitude compared with Na+-dependent action potentials (Fig. 7A, B). When we applied nimodipine (5 μM) these spikes were fully abolished in V1rb2 cells (n = 5). However, Ca\(^{2+}\) spikes in V2r1b cells were still present after this treatment with further increased duration (LSD: \(P < 0.01\)) and diminished spike amplitude (LSD: \(P < 0.025\)). These results provide evidence that, in at least the V2r1b cells, L-type Cav channels are involved in both determining the threshold of Ca\(^{2+}\) spikes as well as contributing to their repolarization phases. Subsequent application of mibefradil (10 μM) fully abolished calcium spikes in V2r1b cells (Fig. 7A, B).

Finally we assessed the role of Cav currents in controlling spike firing when Na\(^+\) channels are not blocked with TTX. Both nimodipine (5 μM) and mibefradil (10 μM) were applied in a mixture (Fig. 7C). Basal GFP VSNs (n = 8) were depolarized with steps of current until a single spike was fired. The inhibitor mixture reduced spike amplitude (control: 65.1 ± 1.9 mV, inhibitors: 52.4 ± 2.7 mV), increased spike width (control: 8.2 ± 0.5 ms, inhibitors: 11.5 ± 1 ms), and also increased the delay of spike firing (control: 115.6 ± 7.4 ms, inhibitors: 164.5 ± 11.8 ms) (Fig. 7D) thus clearly indicating an essential role of the T-type and L-type Cav channels in action potential control.
L-type Cav channels are essential for maintaining persistent spike firing

We next asked whether Cav channels are involved in VSN action potential firing. Fig. 8A shows a representative experiment \((n = 4)\) in which persistent action potential firing was induced by current injection in a GFP- neuron. On the basis of the firing pattern, this cell is clearly identifiable as a basal VSN. We sequentially applied \(\omega\)-Conotoxin MVIIC (1 \(\mu\)M), mibefradil (10 \(\mu\)M) and nimodipine (5 \(\mu\)M) and monitored firing behavior under these conditions. Whereas \(\omega\)-Conotoxin MVIIC and mibefradil had no obvious effect on sustained action potential firing, nimodipine, strikingly, prevented maintained firing leaving only a single action potential in response to the current step. These results suggest that maintained action potential firing was critically dependent on the activity of L-type Cav channels. This was further substantiated in experiments using V1rb2 \((n = 4)\) and V2r1b cells \((n = 5)\), in which application of nimodipine (5 \(\mu\)M) alone gave very similar results and effectively prevented maintained action potential firing in both cell types (Fig. 8B). The only significant difference with the experiment of Fig. 8A was that VSNs fired several action potentials before discharges finally stopped. Since T-type channels were already blocked in the experiment of Fig. 8A (bottom trace), this may indicate that T-type channels play a role in driving action potentials especially at the beginning of the burst sequence, whereas the role of L-type channels becomes more pronounced after a few spikes.

Interplay between L-type Cav and BK channels underlies persistent action potential firing
Block of L-type Cav channels by nifedipine not only affected sustained action potential firing but also often caused action potential broadening and a loss of afterhyperpolarization, together with an increase in the interspike interval. This effect was particularly evident in V1rb2 cells, where the repolarization amplitude became progressively reduced within a few spikes (Fig. 8B V1rb2), but a qualitatively similar effect was also observed in V2rb1 neurons (Fig. 8B V2r1b). Recent evidence indicates that L-type Cav channels can form macromolecular complexes with large conductance Ca\(^{2+}\)-activated K\(^+\) (BK K\(_{Ca}\)) channels, producing Ca\(^{2+}\) nanodomains through which Cav channels activate BK channels with submillisecond kinetics (Berkefeld et al. 2006). We hypothesized that, due to the effect of nimodipine, action potential-induced Ca\(^{2+}\) entry through L-type Cav channels was significantly reduced or eliminated, potentially causing diminished activation of K\(_{Ca}\) channels and, therefore, a loss of repolarization during repetitive action potential firing. If so, selective blockade of K\(_{Ca}\) channels should mimic the effect of nimodipine.

To test this, it was necessary to first gain insight into the properties of K\(_{Ca}\) currents in voltage-clamped V1rb2 and V2r1b cells (Fig. 9A, B). We focused on those K\(^+\) currents that depended on the activity of L-type Cav channels. Families of K\(^+\) currents were activated by voltage steps from –40 mV to 130 mV (Fig. 9A). In both cell types, this resulted in large outward currents that showed partial inactivation (Fig. 9A, control), in contrast to the outward currents that we observed in the presence of Cd\(^{2+}\) (compare Fig. 2E). We found that application of nimodipine (5 μM) caused a substantial reduction in the outward current amplitude (Fig. 9A, nimodipine), whereas application of either ω-Conotoxin MVIIC (1 μM) or mibefradil (10 μM) had very little or no effect (not shown). We plotted the nimodipine-sensitive component of the outward currents (Fig. 9A, subtraction) and constructed current density-voltage plots (Fig. 9B).
These curves showed outward rectification and a characteristic N-shape, typical of $K_{Ca}$ currents. Similar curves were obtained when we removed extracellular Ca$^{2+}$ instead of adding nimodipine, confirming the dependence of these currents on Ca$^{2+}$ entry ($n = 3$; Fig. 9B, *control minus low Ca$^{2+}$*). Thus, it appears that activation of L-type Cav channels is selectively coupled to $K_{Ca}$ currents in these cells. As with Nav and L-type Cav currents, the density of $K_{Ca}$ currents (measured at 50 mV) was nearly two-fold greater in V2r1b (-109 ± 17 pA/pF; $n = 11$) vs. V1rb2 cells (-47 ± 11 pA/pF; $n = 5$) (Fig. 9B).

Are such $K_{Ca}$ currents caused by the activation of BK channels? This was tested in basal GFP$^{-}$ cells by employing subtype-specific blockers of $K_{Ca}$ channels (Fig. 9C). Using the same basic protocol as in Fig. 9A, families of outward K$^{+}$ currents were activated by voltage steps from –40 mV to 130 mV. But instead of nimodipine, we now applied iberiotoxin (1 μM), a highly specific peptide inhibitor of BK$_{Ca}$ channels (Garcia et al. 2001; Wei et al. 2005). Remarkably, current density-voltage plots revealed that the iberiotoxin-sensitive component was nearly indistinguishable from the nimodipine-sensitive one ($n = 4$; Fig. 9C). A very similar effect occurred when we added charybdotoxin (1 μM, $n = 3$), another specific inhibitor of BK channels (Fig. 9C, *control minus Charybdotoxin*). By contrast, application of highly selective inhibitors of small conductance (SK) $K_{Ca}$ channels, UCL1648 (1 μM) or apamin (250 nM), failed to promote this effect (data not shown). Hence, we conclude that activation of L-type Cav channels is specifically coupled to BK channels in these neurons.

These findings enabled us to test directly whether maintained action potential firing in VSNs depends on BK channel activation. To do this, we investigated the effect of iberiotoxin (1 μM) on spike discharges in current-clamped basal GFP$^{-}$ VSNs (Fig. 9D). Strikingly, iberiotoxin prevented maintained spiking in a manner that was nearly indistinguishable from the nimodipine-
induced effect ($n = 4$). For instance, there was a progressive reduction of the afterhyperpolarization, combined with considerable spike broadening during the first few action potentials and increased interspike interval before firing finally stopped (Fig. 9D). In these experiments, the average spike width increased from $7.8 \pm 0.9$ ms (control, C) to $10.2 \pm 1.1$ ms in the presence of iberiotoxin ($n = 4$; paired t-test, $P < 0.01$) (Fig. 9D, inset). No such effect could be induced by the SK channel inhibitors apamin (250 nM) or UCL1648 (1 μM) (not shown). Hence, we conclude that action potential-dependent Ca$^{2+}$ entry mediated by L-type Cav channels that couple specifically to BK channels is essential for maintaining action potential firing in VSNs.

DISCUSSION

A primary goal of this study was to explore whether gene-targeted, identifiable VSNs can serve as a model for investigating signaling mechanisms in the mouse VNO. By selecting V1rb2-tau-GFP and V2r1b-tau-GFP VSNs as prototypical apical or basal VSNs, respectively, we have begun to systematically define the functional properties of sensory neurons located in each of the two primary expression zones of the VNO. Collectively, our results suggest a novel mechanism for regulating and encoding neuronal activity in the mammalian accessory olfactory system that depends specifically on the interplay between L-type Cav channels and BK channels. Potentially, these findings could be important for gaining a deeper understanding of the mechanisms that underlie long-term potentiation and pheromonal learning during social recognition in the accessory olfactory bulb (see below and Kaba and Huang 2005).
Nav current as a fingerprint of the VSN type

We found that Nav currents recorded in apical V1rb2 VSNs were consistently smaller than those in the basal GFP- and V2r1b cells. Nav currents were previously shown to differ in apical vs. basal VSNs using a dissociated cell preparation. However, in that case the Nav currents were found to be smaller in the basal VSNs (Fieni et al. 2003). One reason for this discrepancy could be the truncation of axons during the dissociation procedure, leading to the loss of Nav channels. To avoid this problem, we visually inspected each GFP+ VSN before experimentation to ensure the integrity of the cytoarchitecture. Fieni et al. (2003) correlated dendritic length of VSNs with cell-type specific immunolabeling and found that, on average, apical VSNs have significantly shorter dendrites than basal VSNs. Hence, it has been assumed that apical VSNs express fewer Nav channels than basal VSNs. By contrast, Dean et al. (2004) found no correlation between the size of both the Nav and Kv currents and VSN dendritic length. Both groups used mice of different strains, CD1 (Fieni et al. 2003) vs. BALB/c and CBA mice (Dean et al. 2004). It is unclear whether these differences could be accounted for solely by strain differences. In fact, we recorded from several basal VSNs in slices obtained from CD1 mice and found Nav and Kv current densities similar to those reported above in V2r1b cells (unpublished data). In the intact VNO slice, both apical V1rb2 and basal V2r1b VSNs are located in partially overlapping regions of the VNO neuroepithelium. Therefore, VSNs of both neuronal types exhibit dendrites of variable length. Recording from genetically-labeled VSNs that are maintained in intact epithelial slices provides a means to ensure the morphological integrity of the cells and to unequivocally identify each VSN prior to physiological analysis.
Basal V2r1b neurons are optimized for maintained action potential firing

We asked if larger Nav currents in V2r1b VSNs correlate with more efficient action potential generation. Both the V1rb2 and V2r1b VSNs showed input resistances in the range of several gigaOhms, similar to reported values in VSNs of different species (frog Gjerstad et al. 2003; rat Inamura et al. 1997; garter snake Taniguchi et al. 2000; mouse Liman and Corey 1996; Shimazaki et al. 2006). In both neuronal types, injection of only a few picoamperes of inward current was sufficient to elicit maintained spiking (Fig. 3). However, V2r1b VSNs were capable of firing at higher frequencies than V1rb2 VSNs. Analysis of action potential waveforms showed that V2r1b cells produce faster and larger spikes than V1rb2 VSNs, in accordance with the presence of larger Nav and Cav currents in the V2r1b cells. A finding of importance was that V1rb2 and V2r1b cells differ also in their ability to fire action potentials in response to prolonged, depolarizing current steps. Whereas V1rb2 cells exhibited a tendency to begin to oscillate after a few seconds and produce broad spikes with complex waveforms under these conditions, V2r1b cells were exquisitely capable of maintaining persistent firing for tens of seconds. This remarkable regularity is likely to underlie the prolonged excitatory responses that have been recorded from some mitral cells in the accessory olfactory bulb of behaving mice (Luo et al. 2003). In this respect, it is interesting to note that long-term potentiation at the mitral-to-granule cell synapse in the accessory olfactory bulb, which might underlie pheromonal learning in the context of selective pregnancy termination (the Bruce effect), is effectively triggered by low-frequency, 10-Hz pulses applied for extended periods of time (theta frequency stimulation, Kaba and Huang 2005). Hence mouse VSNs, especially those in the basal zone, are optimized to produce exactly the kind of firing properties that this form of cellular plasticity requires.
Cav currents underlie oscillatory calcium spikes and VSN action potential generation

Sustained Cav currents were a prominent component of the VSN whole-cell current in the slice preparation. Surprisingly, these currents did not show much rundown over time, in contrast to Cav currents in dissociated VSNs (Liman and Corey 1996; Fieni et al. 2003). These remarkably stable Cav currents thus allowed us to assess the role of Cav channel subtypes in VSN action potential firing.

A previously unknown feature of mouse VSNs is their ability to generate slow, regenerative calcium spikes in response to membrane depolarization (Fig. 4). Low-threshold Cav conductances have been described to drive action potentials in CA1 pyramidal neurons (Golding et al. 1999) and to maintain dendritic calcium spike propagation in Purkinje neurons (Cavelier et al. 2002). We, therefore, expected low-threshold T-type Cav currents to mediate calcium spikes in mouse VSNs as well. However, in at least the V1rb2 cells, calcium spikes were fully abolished after inhibition of L-type Cav channels with nimodipine. In the V2r1b cells, block of L-type Cav current did not fully abolish the calcium spikes but further increased their duration and diminished their amplitude (Fig. 7). Here, inhibition of both the T-type and L-type Cav currents was required for complete suppression of calcium spikes. This differential inhibition may be well accounted for by differences in the size of Cav currents in both cell types (Fig. 6). Thus, both types of Cav channels are required for shaping action potential properties in mouse VSNs. This notion was further strengthened by the fact that under normal physiological conditions i.e. with Na\(^+\) channels not inhibited by TTX, a combination of mibefradil and nimodipine was required to affect action potential kinetics (Fig. 7).
T- and L-type currents contributed most to the net Cav conductance in mouse VSNs, with a relatively minor contribution from N/P/Q-type and possibly R-type Cav currents. The pharmacological profile of these Cav currents does not allow us to identify any particular Cav channel isoform with certainty (Catterall et al. 2005). However, a recent study employing immunohistochemical and RT-PCR approaches revealed the presence of multiple Cav channel subunits including the L-type Cav1.2 (alpha1C) channel in mouse VNO (Murakami et al. 2006). A growing body of evidence indicates the presence of multiple isoforms of L- and T-type Cav channels in olfactory sensory neurons as well (Gautam et al. 2007). Both T-type and L-type Cav currents are known to activate fast enough to contribute to the spike firing in a variety of neuronal types (Helton et al. 2005; McCobb and Beam 1991). The role of T-type Cav channels is well established in setting the low-voltage spike threshold in mammalian neurons (Perez-Reyes 2003). Some neuronal L-type Cav channels, especially the Cav1.3 isoform, also activate at relatively low voltages sufficient to drive action potential firing and being involved in action potential-dependent Ca$^{2+}$ entry (Lipscombe et al. 2004). In olfactory sensory neurons, however, it is especially the T-type Cav current that sets the threshold for action potential firing (Kawai et al. 1996). Furthermore, T-type Cav channels are critical for propagation of a Ca$^{2+}$ wave from the distal dendrite to the cell soma (Gautam et al. 2007).

In mouse VSNs, the T-type Cav current activates at around –80 mV (Fig. 6), close to the resting membrane potential of –75 mV, which is slightly more negative than the activation voltage for Nav currents (around –70 mV, Fig. 2). Interestingly, we also found relatively negative activation thresholds for L-type Cav currents (near –60 mV, Fig. 6). Therefore, it appears that both T- and L-type Cav channels are essential for maximal excitability in mouse VSNs. The exact contribution of T- and L-type Cav channel subtypes in VSN spike generation...
and maintained rhythmic firing should be dissected in future experiments by using genetic models (e.g. see Chan et al. 2007), together with more elaborate spike waveform voltage command protocols (e.g. see Bean 2007).

*Interplay between L-type Cav and BK channels is crucial for persistent VSN firing*

A key finding of this communication, specifically in the basal VSNs, is a novel mechanism for regulating persistent neuronal activity in the accessory olfactory system, which depends on specific coupling of L-type Cav channels and BK channels. BK channels were previously shown in mouse brain to co-localize with both Cav1.2 and Cav1.3 L-type channels (Grunnet and Kaufmann 2004) and, more recently, Berkefeld et al. (2006) reported clear evidence that Cav1.2 channels are capable of forming functional macromolecular complexes with BK channels in rat brain. We used established pharmacological tools to specifically assess the role of those Cav channels that might control persistent action potential firing in VSNs. Treatment with mibefradil and ω-Conotoxin MVIIC demonstrated that T-type and N/P/Q-type Cav channels are not critically involved in this regulation. By contrast, application of nimodipine effectively disrupted persistent firing induced by membrane depolarisation (Fig. 8). Thus, an L-type Cav channel, most likely involving Cav1.3, is essential for maintained firing in these VSNs. This effect was reminiscent of the contribution of L-type Cav channels to maintained rhythmic firing in some invertebrate respiratory neurons (Spafford et al. 2006).

Nimodipine - but not mibefradil or ω-Conotoxin MVIIC - inhibited outward K\(^+\) currents to the same extent as treatment with Cd\(^{2+}\) or removal of extracellular Ca\(^{2+}\) ions. These nimodipine-sensitive K\(^+\) currents were caused by the activation of BK channels because they were selectively inhibited by iberiotoxin, a specific peptide blocker binding to the pore-forming
alpha subunit (Slo) of the BK channels (Giangiacomo et al. 1992, Garcia et al. 2001; Wei et al. 2005), but not by specific inhibitors of SK Ca\(^{2+}\)-activated K\(^+\) channels such as UCL1648 and apamin. BK channels are thought to control spike repolarization, firing frequency and spike-frequency adaptation (SFA) in many neurons by giving rise to the fast afterhyperpolarization (AHP) following a spike (Sah and Faber 2002). Similar mechanisms may exist also in mouse VSNs since iberiotoxin effectively disrupted persistent firing and increased the width of the initial spike. However, we also observed another component of the AHP with a substantially slower time course that was always present after termination of depolarizing current injections (see Figs. 3 and 8). This AHP was usually not affected by Cav channel inhibitors such as nimodipine or Cd\(^{2+}\). As discussed by Sah and Faber (2002) there might exist an as yet unidentified ion channel underlying KC\(_{Ca}\)-independent AHPs.

*Spike-frequency adaptation: possible link to sensory adaptation*

Spike-frequency adaptation (SFA) is a fundamental feature of neural dynamics. In the VNO, it was generally thought that SFA is not present in VSNs. However, previous studies had only injected current steps for approximately 2-3 seconds (Liman and Corey 1996). By using prolonged current steps (10 or 60 seconds, respectively), we found that all three sampled VSN populations exhibit profound SFA. Various ionic currents modulating spike generation have been implicated to cause this type of neural adaptation including Kv currents (M-type currents), the cross-talk of Ca\(^{2+}\) currents and intracellular Ca\(^{2+}\) dynamics with KC\(_{Ca}\) channels (AHP-type currents), persistent Nav currents, and slow recovery from inactivation of the fast Nav current (Benda et al. 2003; Miles et al. 2005; Zeng et al. 2005). In our case, it is unlikely that
inactivation of the fast TTX-sensitive Nav channels solely mediates SFA because TTX-treated VSNs that generate pure Ca\(^{2+}\) spikes still exhibited this feature (Fig. 4). Treatment with inhibitors of SK \(K_{Ca}\) channels also did not affect SFA. Furthermore, we can exclude a role of T-type and N/P/Q-type Cav channels since their block had essentially no effect on SFA (Fig. 8A). However, a role of L-type Cav and BK channels could not be tested properly because blockade of both conductances suppressed repetitive firing. In olfactory sensory neurons, SFA is also present and has been proposed to be involved in sensory adaptation (Kawai 2002; Delgado et al. 2003; Narusuye et al. 2003). VSNs have been thought to lack any form of sensory adaptation (Holy et al. 2000). It remains to be seen how SFA affects the encoding of different classes of chemical cues in the VNO.

In summary, our study provides several novel ionic mechanisms for regulating neural activity in the accessory olfactory system that may have been overlooked previously due to potential rundown of Cav conductances in preparations of acutely isolated neurons. We expect that these findings are of direct relevance to future studies aimed at understanding how social cues are encoded in the mammalian vomeronasal system and how social memories are formed.

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REFERENCES


Inamura K, Kashiwayanagi M, and Kurihara K. Inositol-1,4,5-trisphosphate induces


**Lucas P, Ukhanov K, Leinders-Zufall T, and Zufall F.** A diacylglycerol-gated cation channel in vomeronasal neuron dendrites is impaired in TRPC2 mutant mice: mechanism of pheromone


FIGURES AND LEGENDS

FIGURE 1.

A, B: Fluorescence images (pseudocolor) of acute, coronal VNO tissue slices (250 μm thick) showing the spatial location of V1rb2-tau-GFP (A) and V2r1b-tau-GFP VSNs (B) within the sensory epithelium. Each image was reconstructed by merging six individual optical sections obtained by confocal imaging. Each optical section had a thickness of approximately 5 μm. Hence, each image represents the distribution of genetically-labeled cells up to a depth of 30 μm.

C-H: High-power micrographs of VNO slices showing individual V1rb2- (C-E) or V2r1b-expressing cells (F-H). Transmitted light images (C, F) and respective GFP fluorescence images (greyscale; D, G) were merged to show the position of the labeled cells (E, H). All VSNs analyzed in this study exhibited an intact cytoarchitecture including a thick dendrite projecting to the lumen (L) and ending in a knob-like swelling, and a thin axon projecting toward the basal lamina. Patch electrode, P.

FIGURE 2.

Comparison of voltage-gated inward and outward currents in V1rb2- and V2r1b-expressing VSNs. A: Representative families of whole-cell currents to a series of depolarizing voltage steps (as indicated in the figure) recorded from V1rb2- (upper panel) and V2r1b cells (lower panel), respectively. VSNs were exposed successively to extracellular bath solution (control), bath solution containing Cd²⁺ (100 μM), and bath solution containing Cd²⁺ (100 μM) and TTX (2 μM). Voltage-activated K⁺ channels were blocked by using a Cs⁺-based pipette solution. B: Current density-voltage curves were constructed using peak amplitudes of the currents shown in
A (with Cd^{2+}). Plotted data are means ± SEM. In this and all subsequent figures, the symbols (open and closed circles, closed triangles) will denote the three sampled populations of neurons. On average, peak current density was approximately two-fold greater in V2r1b cells (closed circles; n = 7) vs. V1rb2 cells (open circles; n = 8). Data from GFP VSNs (closed triangles; n = 7) are shown for comparison. C: Activation curves of the normalized Nav conductance derived from the data in B were similar in all three VSN populations. D: Steady-state inactivation of the Nav currents was assessed by stepping membrane voltage to different pre-pulse voltages (from –120 mV to -20 mV) and measuring peak current amplitudes during a 10-ms step to –30 mV. The half-inactivation voltage of V1rb2 VSNs was shifted to more negative values than that of V2r1b and GFP VSNs. Data in both sets were fitted by the Boltzmann equation with parameters given in supplemental Table 1. E: Representative families of whole-cell outward currents [in the presence of TTX (2 μM) and Cd^{2+} (100 μM)] to a series of depolarizing voltage steps (as indicated in the figure) recorded from V1rb2- (upper panel) and V2r1b-cells (lower panel), respectively. K^{+}-based pipette solution. F: Current density-voltage plots obtained from families of Kv currents as shown in E. G: Activation curves of the normalized Kv conductance derived from the data in F were indistinguishable in all three neuronal populations.

FIGURE 3.

Firing properties of V1rb2 and V2r1b VSNs. A: V1rb2- and V2r1b-expressing VSNs generated repetitive action potentials in response to 2-s depolarizing current steps of only a few picoamperes. Holding current, 0 pA. With negative current injections a prominent ”sag” was observed in both cell types. Multiple or single rebound spikes occurred upon termination of the hyperpolarizing current steps. B: Mean firing frequency plotted as a function of the injected
depolarizing current. Data were fitted with the Michaelis-Menten equation. C: Representative normalized spikes of V1rb2 and V2r1b-expressing VSNs. The spike width at half spike amplitude was smaller in V2rb1 (grey shaded area) compared to V1rb2 VSNs. D: Histogram showing collected results of spike width and amplitude in V1rb2 and V2r1b neurons. Mean spike duration and amplitude differed significantly in the two types of neurons (t-tests: ***P < 0.0001). E: Representative spike discharges induced by 10-s depolarizing current steps (10 pA). Holding current, 0 pA. V2r1b VSNs are capable of firing persistently under these conditions whereas V1rb2 VSNs generate complex oscillatory waveforms. F: Raster- (left) and instantaneous frequency-time plots (right) of discharges induced by 10-s depolarizing currents steps (10 pA) in six different V2r1b VSNs. Solid lines are single exponential fits to the instantaneous spike frequency decrease from peak at F_max to the minimum at F_min. Spike-frequency adaptation is clearly present in these cells. G: Raster- (left) and instantaneous frequency-time plots (right) from five GFP- cells that were depolarized with 60-s current steps (8 pA). Under these conditions, the time course of spike frequency adaptation was best fit with two exponentials.

FIGURE 4.
Oscillatory Ca^{2+} spikes in VSNs. A: Representative action potential discharges to 10-s depolarizing current steps (10 pA) recorded from V1rb2- (left panel) or V2r1b cells (right panel), respectively. VSNs were exposed successively to extracellular bath solution (control), bath solution containing TTX (2 μM), or bath solution containing TTX (2 μM) and Cd^{2+} (100 μM). TTX-insensitive spikes were abolished by Cd^{2+}. B, C: Histograms showing average spike frequency (B) and spike width (C) under control conditions (black bars) and in the presence of
TTX (white bars) in V1rb2- and V2r1b neurons. The spike frequency was calculated during the first two seconds of the response. TTX-insensitive Ca\(^{2+}\) spikes (white bars) had lower instantaneous frequency and were significantly broader than spikes under control conditions in both types of VSNs (LSD: \(P < 0.01\); \(***P < 0.001\); \(****P < 0.0001\)). Spike width in the presence of TTX was significantly broader in V2r1b neurons compared to V1rb2 neurons (LSD: \(**P < 0.001\)).

FIGURE 5.
VSNs express multiple types of Cav currents. A: High-voltage activated (HVA) Cav currents recorded in 5 GFP\(^{-}\) cells under normal ionic conditions (1 mM Ca\(^{2+}\) in the bath) showed little rundown over time. 200-ms long voltage steps were applied every 20 s and mean current was measured at the end of each step. Peak amplitudes were normalized to control values obtained immediately after establishing the whole-cell configuration at time zero. Inset shows examples of original currents recorded at different time points (a, b, c). B: Pharmacological dissection of individual Cav currents in GFP\(^{-}\) cells using 20 mM Ba\(^{2+}\) as charge carrier. Stepping the membrane voltage from conditioning –100 mV to 0 mV for 500 ms activated a slowly-inactivating inward current (Ctrl, thick line). 10 \(\mu\)M mibefradil (Mib) slightly inhibited this current whereas addition of nimodipine (5 \(\mu\)M, Mib/Nim) almost completely suppressed it. C: A low-voltage activated (LVA) T-type Cav current was observed by stepping a conditioning 300-ms prepulse from –120 mV to –40 mV for 200 ms (Ctrl, thick line). Nimodipine had little effect on this current whereas the addition of mibefradil significantly reduced it within 3 min, leaving only a small residual component. D: The mibefradil/nimodipine-insensitive current (Mib/Nim,
thick line) was blocked by ω-Conotoxin MVIIC (1 μM, CTx), indicating the presence of N/P/Q HVA Cav currents. Addition of Cd^{2+} completely suppressed any remaining current.

FIGURE 6.
Properties of the LVA T-type and HVA L-type Cav currents in V1rb2 and V2r1b VSNs. Recordings were done with 10 mM Ca^{2+} in the bath. A: Families of Cav currents V1rb2 and V2r1b VSNs to a series of voltage steps from −80 mV to +40 mV following a 300-ms conditioning pulse to −120 mV. Treatment with nimodipine (Nim, 5 μM) and a mixture of nimodipine and 1 μM ω-Conotoxin MVIIC (Nim/CTx) was used to isolate L-type and T-type Cav currents, respectively. B: Current density-voltage plots of T-type Cav currents derived from peak amplitudes measured in the presence of nimodipine and ω-Conotoxin MVIIC. V1rb2 VSNs (open circles), V2rb1 cells (closed circles). C: Steady-state inactivation of T-type current was assessed by stepping membrane voltage to different pre-pulse voltages from −120 mV to −10 mV and then measuring peak current amplitude during a 50-ms step to −40 mV. D: Activation curves of the T-type Cav conductance were derived from data in B. Both activation and inactivation curves were fitted with the Boltzmann equation with parameters given in supplemental Table 1. E: Current density-voltage curves of L-type Cav currents were constructed by plotting the nimodipine-sensitive, sustained component measured at the end of each voltage step. F, G: Inactivation and activation curves of L-type Cav currents both neuronal types. Inactivation curves were determined by stepping pre-pulse voltages from −100 mV to +30 mV and measuring plateau current amplitude during a 100-ms step to 0 mV.
FIGURE 7.
Both T-type and L-type Cav currents contribute to action potential firing. A: Single action potentials induced by brief 200-ms steps of depolarizing current (6 pA) recorded from V1rb2- (left panel) and V2r1b-cells (right panel). VSNs were exposed successively to extracellular control solution, control solution containing either 2 µM TTX alone, a mixture of TTX and 5 µM nimodipine (TTX/Nim), or a mixture of TTX, nimodipine and 10 µM mibefradil (TTX/Nim/Mib). Thin lines represent control measurements. Thick lines indicate the waveform under each condition. B: Histograms showing a quantitative analysis of spike delay, width, and amplitude in multiple V1rb2 and V2r1b neurons (numbers indicated above each bar) under the different conditions. C (control, black bar); T (TTX, grey bar), TN (TTX/Nim, white bar), ; TNM (TTX/Nim/Mib). LSD: *P < 0.025; **P < 0.01; ***P < 0.001. C: Effect of a mixture of mibefradil and nimodipine on Na+-dependent action potentials (in the absence of TTX) in GFP- cells (n = 8). D: Treatment with nimodipine and mibefradil significantly changed the action potential delay, width and amplitude (t-test: *P < 0.05; **P < 0.01; ***P < 0.001).

FIGURE 8.
L-type Cav channels are necessary for the maintenance of persistent firing in the VSNs. A: Effect of different Cav channel inhibitors were tested on the firing in basal GFP- VSNs induced by a 10-s step of depolarizing current (10 pA, n = 4). ω-Conotoxin MVIIC (CTx) and mibefradil (CTx/Mib) did not affect prolonged firing, whereas the addition of nimodipine (CTx/Mib/Nim) severely impaired persistent firing. B: Representative recordings of the firing discharge induced by a 10-s step of depolarizing current (10 pA) in V1rb2 (n = 3) and V2r1b (n = 4) VSNs before
and after adding nimodipine to the bath. Nimodipine alone potently prevented persistent firing in both the VSN types leaving only a few initial spikes at the beginning of the current injection.

FIGURE 9.
Specific coupling of large-conductance BK Ca\(^{2+}\)-activated K\(^{+}\) current to L-type Cav channels is critical for persistent firing in the VSNs. A: Depolarizing 500-ms voltage steps from –40 mV to +130 mV were applied to V1rb2 and V2r1b VSNs to measure net outward Kv currents. Recordings were obtained with KCl-based electrode solution without any additional inhibitors. Outward K\(^{+}\) currents in both types of VSNs were substantially reduced after treatment with nimodipine (5 \(\mu\)M) and subtraction yielded families of inactivating outward currents. B: In both V1rb2 and V2r1b VSNs the current density-voltage curves derived from the peak amplitudes of the nimodipine-sensitive component had a characteristic N-shape (control minus Nimodipine). In the experiment shown in the lower graph (control minus low Ca\(^{2+}\)) we lowered the extracellular Ca\(^{2+}\) concentration to 1 \(\mu\)M instead of adding nimodipine. This revealed a component with the same basic properties (\(n = 3\)), demonstrating the Ca\(^{2+}\)-dependence of the N-shaped conductance. C: Similar N-shaped current density-voltage curves were also obtained after treatment with two specific inhibitors of BK channels, iberiotoxin (1 \(\mu\)M, \(n = 4\), control minus Iberiotoxin) and charybdotoxin (1 \(\mu\)M, \(n = 3\), control minus Charybdotoxin). D: Essential role of BK channels in persistent firing. Treatment with iberiotoxin (1 \(\mu\)M) disrupted persistent firing in VSNs in a manner very similar to the effect observed with nimodipine in Fig. 8 (\(n = 4\)). Insert: Analysis of the width of the first spike in response to a 10-s current injection under control conditions (\(C, black bar\)) and after treatment with iberiotoxin (IbTx, gray bar). The BK channel blocker caused
significant spike broadening (t-test: **$P < 0.01$). No significant change occurred with respect to spike delay and amplitude (t-test: $P > 0.2$).
Supplemental Table 1. Parameters of the voltage-dependent currents in mouse vomeronasal neurons, values are means ± SEM (number of cells). The Student’s t-test was used for measuring the significance of difference between V1rb2- and V2r1b-expressing neurons. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

<table>
<thead>
<tr>
<th></th>
<th>GFP-negative</th>
<th>V1rb2</th>
<th>V2r1b</th>
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<tbody>
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<td>Nav current</td>
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<tr>
<td>$I_{\text{max}}$, pA/pF</td>
<td>-258.7±8.9 (7)</td>
<td>-126.4±16.6 (9)</td>
<td>-274.1±17.1 (7) ***</td>
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<td>$V(I_{\text{max}})$, mV</td>
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<td>-25</td>
<td>-25</td>
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<td>2.2±0.7 (7) *</td>
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<td>slope $^{\text{inactiv}}$, mV</td>
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<td>Kv current</td>
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<td>T-type Cav current</td>
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<td>Ca$^{2+}$ activated K$^+$ current</td>
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<td>46.6±10.9 (5)</td>
<td>109.2±17.4 (11)</td>
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Figure A: Comparison of control, Nimodipine, and subtraction for V1rb2.

Figure B: Control - Nimodipine graph showing pA/pF against mV.

Figure C: Control - Iberotoxin and control - Charybdoxin graphs showing pA/pF against mV.

Figure D: Control and Iberotoxin comparison showing spike width and average duration.