The superficial tufted and mitral cell output neurons of the mouse olfactory bulb have a dual role in insulin sensing

Running Title: Insulin sensing superficial tufted cells

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SUMMARY STATEMENT

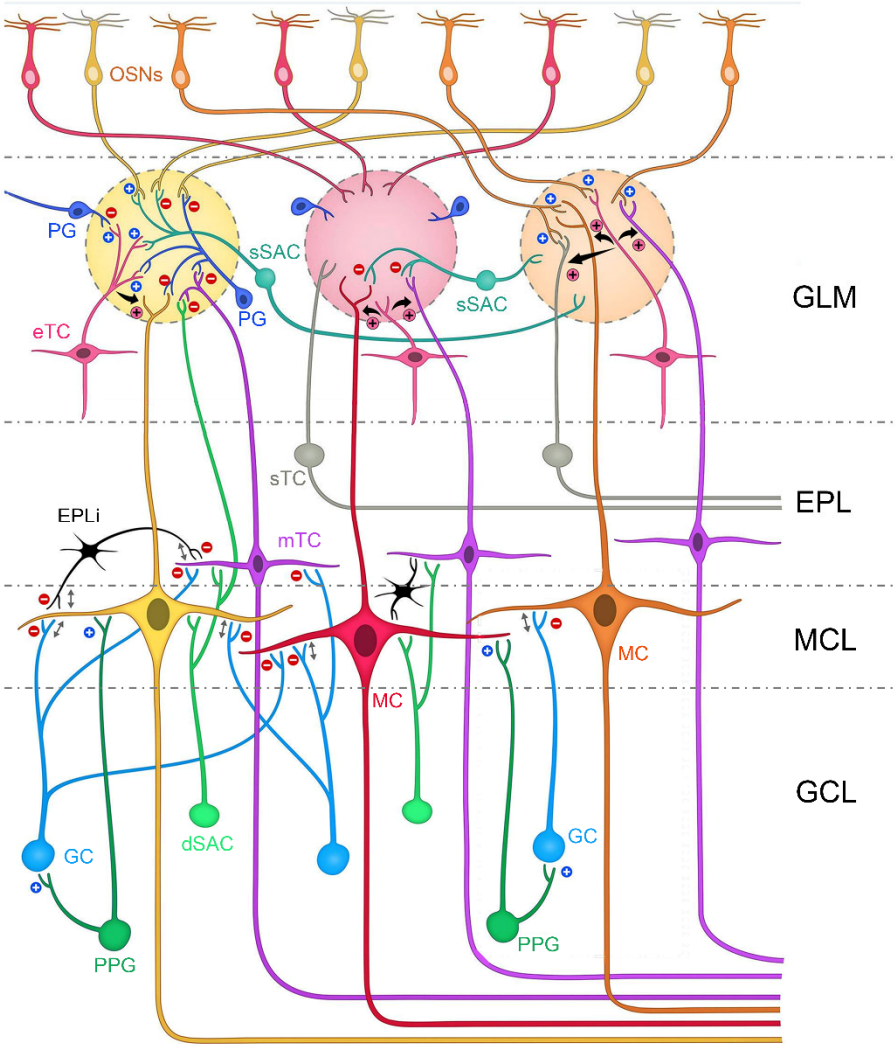
Superficial tufted cells, as output neurons of the olfactory bulb, were electrophysiologically studied to be insulin sensitive. Brain insulin signaling represents a manner in which olfactory and metabolic circuitry are intertwined.

ABSTRACT

The olfactory bulb (OB) contains multiple, parallel projection neurons to relay the nature of a stimulus. In a mouse *ex vivo* slice preparation, we used patch-clamp electrophysiology to measure intrinsic properties, excitability, action potential (AP) shape, voltage-activated conductances, and neuromodulation in the newly-categorized superficial tufted cells (sTCs) compared with those of mitral cells (MCs). We propose that a marked difference in voltage-dependent current represents distinct ion channel populations that affect the kinetics of action potentials, and evokes an increase in sTC firing frequency, albeit both types of projection neurons having similar AP spiking activity. Triple-colored immunofluorescence and RNA scope were used to detect co-localization of the Kv1.3 ion channel and the insulin receptor in sTCs, with ~73% of sTCs expressing both. The sTCs were modulated by bath application of insulin – increasing AP firing frequency by 97%, attributable to an 8% decrease in the intraburst interval, and a reduction of the latency to first spike by 37%. We conclude that there may be a range of neuromodulators of sTCs that may alter excitability and fine-tune olfactory information processing or metabolic balance.

INTRODUCTION

Sensory systems typically carry multiple types of parallel output, or projection neurons, to relay differing specificity with regards to the nature of the stimulus. The olfactory bulb (OB) processes electrically-encoded odor information, and relays it to overlapping, but not identical, cortical regions via two types of primary projection neurons – namely mitral (MCs) and tufted cells (TCs) (Price and Powell, 1970; Vaaga and Westbrook, 2016). Albeit both receiving synaptic connections from the olfactory sensory neurons (OSNs) at defined and evolutionarily-conserved integration units called glomeruli, less has been studied concerning TCs in terms of biophysical properties and sources of modulation (Liu and Shipley, 2008; Burton and Urban, 2014; Jones et al., 2020). We and other investigators typically recorded from both populations of neurons, referring to them collectively as M/TCs (Fadool and Levitan, 1998; Tucker and Fadool, 2002; Youngstrom and Strowbridge, 2015; Liu et al., 2016). In model organisms, such as drosophila and zebrafish, MCs and TCs are not readily distinguishable from one another (Nagayama et al., 2014). The neurolaminae of mice are significantly more distinct, allowing the complexity of the olfactory circuity to be more easily assessed.



86 **Fig. 1**. **Categorization and interactions of the principal projection neurons of the olfactory**
87 **bulb.** The olfactory bulb neurolamina is divided across the glomerular layer (GLM), external
88 plexiform layer (EPL), the mitral cell layer (MCL), and the innermost granule cell layer (GCL). Also
89 shown is the olfactory mucosa containing the olfactory sensory neurons (OSNs). This schematic
90 highlights the four classes of output or projection neurons, namely the external tufted cells (eTCs,
91 pink), the superficial tufted cells (sTCs, gray), the middle tufted cells (mTCs, purple), and the mitral
92 cells (MCs, red). The soma of the eTCs are found within the GLM. The sTC soma reside in the
93 superficial one-third of the EPL. The deep two-thirds of the EPL contain both the mTCs and
94 misplaced MCs. The MCL houses the eponymous MCs. Blue plus signs (+) represent excitatory
95 synapses, red minus signs (-) represent inhibitory synapses, and pink plus signs (+) represent
96 excitatory dendro-dendritic interactions originating from external tufted cells (eTCs). Reciprocal
97 synapses are denoted by a double arrow. PG = periglomerular cell, sSAC = superficial short axon
98 cell, EPLi = interneuron of the external plexiform layer, GCL = granule cell layer, GC = granule cell,
99 PPG = preproglucagon neuron, dSAC = deep short axon cell. *Modified from Huang* et al. *2021.*

100

101 Like many budding areas of research, the standardization of categorical terminology is not
102 considered until there is enough information to classify. Jones et al. 2020 aptly stated that the
103 *"nomenclature around TC subtypes has been somewhat confusing in the field* (Jones et al.,
104 2020)." Both early works and more recent reviews describe 4 distinct classes of projection
105 neurons – external TC, internal TC, middle TC, and MC (Pinching and Powell, 1971a,b;
106 Mori, 1987; Shepherd, 2004; Hayar et al., 2004; Mori and Sakano, 2022). A more recent
107 work proposed to alternatively reclassify output neurons based upon size and location into
108 three types: putative MCs (pMC; >20 um diameter and located in the MCL), EPL located TC
109 (lower EPL TC; <20 um diameter), and putative TC (pTC; <20 um diameter located in the
110 MCL) (Gadiwalla et al., 2025). Herein, our study uses the naming convention originating
111 from Cajal and then reestablished by Jones et al. (2020), based upon the position of the TC
112 soma within its respective neurolamina – namely, external tufted cells (eTCs), middle tufted
113 cells (mTCs), and superficial tufted cells (sTCs) (**Fig. 1**). Of the three subclasses of TCs, the
114 eTC are the best studied. The eTCs are a select population of juxtaglomerular cells that
115 demonstrate rhythmic and spontaneous action potential bursting (Hayar et al., 2004a,b, 2005;
116 Antal et al., 2006; Zhou et al., 2006; Ma and Lowe, 2007; Liu and Shipley, 2008). The eTC
117 are known to receive monosynaptic input from olfactory sensory neurons (OSNs) and
118 establish glutaminergic synaptic connections to short-axon cells and periglomerular cells
119 within the bulb (Hayar et al., 2004a,b, 2005), and their activation can provide feedforward
120 excitation of MC (De Saint Jan et al. 2009). eTCs actually process sensory input before it
121 reaches the sTCs and MCs (De Saint Jan et al., 2009; Najac et al., 2011; Gire et al., 2012;
122 Igarashi et al., 2012; Vaaga and Westbrook, 2016). The eTCs receive the greatest signal from
123 activated OSNs, and stimulate neighboring sTCs, mTCs, and MCs via glutamatergic dendro-
124 dendritic interactions (De Saint Jan *et al*., 2009; Najac *et al*., 2011; Jones *et al*., 2020). This
125 feed-forward excitation from eTCs provides "biphasic input" to other M/TCs that may also
126 receive direct monosynaptic input from a specific OSN family member (Najac *et al*., 2011;
127 Vaaga and Westbrook, 2016; Jones *et al*., 2020).
128
129 Because the eTC are well characterized, and the mTCs are less accessible for patch-clamp
130 recordings, we decided to focus our study on a lesser known type of TC, the sTC, which are
131 expressed superficially in the external plexiform layer (EPL)(Jones et al., 2020; Chen et al.,

132 2024). mTC soma are found between the MCL and the inner (deep) two-thirds of the external
133 plexiform layer (EPL), whereas the soma of superficial tufted cells (sTCs) are located in the
134 outer (superficial) one-third of the EPL. Like MCs, sTCs and mTCs receive input from
135 olfactory sensory neurons (OSNs) at defined glomeruli, and pass sensory information to
136 various subcortical areas. The sTCs and mTCs, however, project, to distinctly different brain
137 regions than MCs; even if a M/TC pair projects to the same subcortical region, it will project
138 to different parts of it (Nagayama et al., 2010, 2014; Igarashi et al., 2012). Excellent
139 foundational work has been done to characterize the innate biophysical differences between
140 TCs and MCs (Burton and Urban, 2014), but the categorization of the assessed cells preceded
141 the boundaries set by the current naming methods (Jones et al., 2020). As such, no previous
142 report has characterized the innate biophysical differences between MCs and sTCs, nor
143 investigated the function of sTCs to sense metabolically-relevant molecules or
144 neuromodulators.
145
146 In this study, we compare the biophysical properties of MCs and sTCs in *ex vivo* brain slices
147 from male and female mice. Using current- and voltage-clamp recordings, we found that
148 action potential (AP) properties and voltage-activated currents differed between sTCs and
149 MCs. To better understand the complementary function of sTCs to MCs, we examined the
150 response of sTCs to a known modulator and a vestibule blocker of Kv1.3 – a predominant ion
151 channel carrying 60-80% of the outward current in MCs (Fadool and Levitan, 1998; Colley et
152 al., 2004). Our results directly show that sTCs are more excitable than MCs. This is correlated
153 with differing voltage-activated currents, and a differing AP shape. We further find that sTCs
154 are modulated by the presence of insulin, suggesting a role for sTCs in metabolic circuitry
155 (Fadool et al., 2004; Palouzier-Paulignan et al., 2012; Aimé et al., 2014). Our study
156 demonstrates that the Kv1.3 ion channel is necessary for the insulin-sensing property of sTCs,
157 as it is in MCs (Fadool et al., 2004, 2011; Tucker et al., 2013).
158

159 ## RESULTS

160 ### Superficial tufted cells have greater input resistance and slightly lower voltage-
161 ### activated current at hyperpolarizing potentials than that of mitral cells

162 Superficial tufted cells (sTCs) have greater EPSC amplitude when stimulated by direct
163 olfactory sensory neuron activation over that of mitral cells (MCs) (Jones et al., 2020), albeit
164 both being the predominant output neurons of the OB. Less has been studied to reveal the
165 nature of intrinsic properties and voltage-activated conductances in sTCs. We used ex vivo
166 slice electrophysiology in the voltage-clamp configuration, therefore, to study intrinsic
167 properties as well as outward currents, which are largely driven by the Kv1.3 channel in MCs
168 (Fadool *et al*., 1997; Fadool and Levitan, 1998). The resting membrane potential (RMP) was
169 not significantly different between MCs and sTCs (MCs = -55.8 $\pm$ 6.3 mV (20) vs. sTCs = -
170 58.0 $\pm$ 6.5 mV (22); Student's *t*-test, p = 0.2906).
171
172 Macroscopic currents were acquired in response to a series of 400 ms duration (P_d) voltage
173 steps that increased in increments of 20 mV from -100 mV to +40 mV. The resulting current-
174 voltage (IV) relationship was plotted in **Fig. 2A** using the peak transient current value for each
175 step normalized to the +40 mV step. It was noted that the amplitude of the outward current at
176 hyperpolarizing potentials (a window between -60 to -20 mV) was significantly greater in
177 MCs vs. that of TCs (**Fig. 2B**). A two-way repeated measure, mixed factor analysis of
178 variance (2-w RM mixed ANOVA) was applied using cell type and voltage as factors. There
179 was a main effect of cell type (F (1, 22) = 9.430; p = **0.0056) as well as a voltage x cell type
180 interaction (F (7, 154) = 5.542; p <0.0001). A Sidak's post-hoc test showed that the evoked
181 current was significantly greater for MCs at the -60 mV (p = 0.0202), -40 mV (p = 0.0049)
182 and -20 mV (p = 0.0311) voltage steps. This difference only existed in this hyperpolarized
183 window and was not observed once highly depolarized at the +40 mV step (**Fig. 2C**, MCs =
184 5180 $\pm$ 2540 pA (11) vs. sTCs = 3746 $\pm$ 1030 pA (13), Mann-Whitney U, p = 0.2066). We
185 next measured the kinetics of inactivation and deactivation for the voltage-activated currents
186 at a hyperpolarized (V_c = -40 mV) and depolarized (V_c = +40 mV) range, respectively. The
187 kinetics of inactivation (τ_{Inact}) did not differ between cell types (**Fig. 2D, Left**, MCs = 71.2 $\pm$
188 19.0 ms (10) vs. sTCs = 57.0 $\pm$ 28.6 ms (13), Student's *t*-test, p = 0.1894) at V_c = -40 mV,
189 however the kinetics of deactivation (τ_{Deact}) was significantly faster for sTCs at this command
190 voltage (**Fig. 2D, Right**, MCs = 2.19 $\pm$ 0.67 ms (11) vs. sTCs = 1.47 $\pm$ 0.61 ms (13), Mann-
191 Whitney U, *p = 0.0107). Neither time constant differed between cell types at a V_c = +40 mV
192 step (**Fig. 2E**, τ_{Inact}: MCs = 75.6 $\pm$ 30.9 ms (11) vs. sTCs = 158 $\pm$ 140 ms (13), Mann-Whitney

U, $p = 0.3031$; τ_{Deact}: MCs = 1.14 ± 0.41 ms (10) vs. sTCs = 1.12 ± 0.52 ms (13), Student's *t*-test, $p = 0.9043$). Overall, although sTCs tend to have higher input resistance than MCs, the outward voltage-activated currents of sTCs are less responsive to changes in voltage.

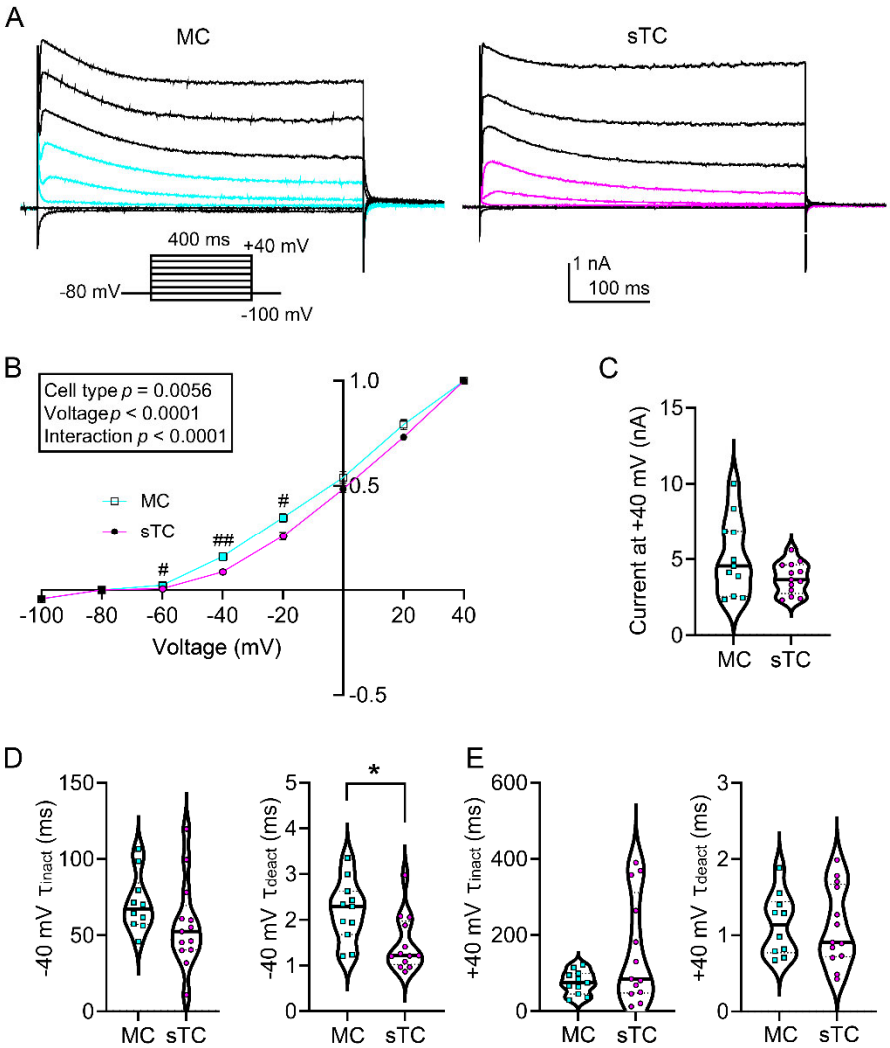


Fig. 2. Outward voltage-activated current properties of mitral cells and superficial tufted cells. ***A,*** Family of voltage-activated currents, V_h = -80 mV where V_c was stepped in 20 mV increments from -100 mV to +40 mV using a P_d = 400 ms. Colored sweeps correspond to significantly different colored data points in (***B***). ***B,*** Normalized IV plots (2-w mixed RM ANOVA, cell type ***p = 0.0056, Sidak's post-hoc test, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; voltage x cell type interaction ****$p < 0.0001$). ***C,*** Violin plot of non-normalized peak transient voltage-activated current at +40 mV, Mann-Whitney U. Violin plot of the *Tau* of inactivation (*left*) and deactivation (*right*) at **(D)** Vc = -40 mV and **(E)** +40 mV, respectively, Mann-Whitney U (*$p = 0.0107$). MC = mitral cell population sampled, sTC = superficial tufted cell population sampled.

205 **Kv1.2 and Kv1.3 expression is similar between superficial tufted and mitral**
206 **cells**

207 The drug MgTx is a selective blocker of Kv1.2 and Kv1.3 ion channels when applied to the
208 extracellular membrane at 1 nM concentration (Garcia-Calvo et al., 1993; Garcia et al., 1997;
209 Bartok et al., 2014). It has a slow k_{on} of 10 min and an even slower k_{off} of 2 h (Garcia-Calvo et
210 al., 1993; Nikouee et al., 2012; Chen and Chung, 2014), therefore, we did not attempt a
211 washout of the vestibule blocker during our patch recording. After collecting baseline voltage-
212 clamp recordings on MCs for 15 min, 1 nM MgTx was added to the bath to wash over the
213 slice for 15 min. Another set of voltage-clamp recordings was then collected (**Fig. 3A**). The
214 same repeated-measure paradigm was then applied to a separate group of sTCs (**Fig. 3B**).
215 MgTx reduced voltage-activated currents in both MCs and sTCs in a voltage-dependent
216 manner (**Fig. 3C).** Here, a 2-w mixed RM ANOVA was applied using drug and voltage as
217 factors. For the MC recordings, there was a main effect of drug ($F(1, 9) = 43.62$, ****$p <$
218 0.0001) as well as a voltage x drug interaction ($F(7, 63) = 43.86$, ****$p < 0.0001$). A Sidak's
219 post-hoc test demonstrated significant differences at the -20 mv ($^{\#\#\#}p = 0.0008$) voltage step
220 and all further depolarizing steps ($^{\#\#\#\#}p < 0.0001$). For the sTC recordings, there was a main
221 effect of drug ($F(1, 12) = 25.51$, ***$p = 0.0003$) as well as a voltage x drug interaction ($F(7,$
222 $60) = 19.87$, ****$p < 0.0001$). A Sidak's post-hoc test demonstrated significant differences at
223 -30 mv and above ($^{\#\#\#\#}p < 0.0001$). Using a 3-way mixed-effects analysis (3-w mixed RM
224 ANOVA), we found no difference in MgTx-mediated effects on voltage dependence between
225 MCs and sTCs (**Fig. 3C, Bottom**, voltage x cell type x drug interaction $F(7, 123) = 0.7633$, p
226 $= 0.6190$).
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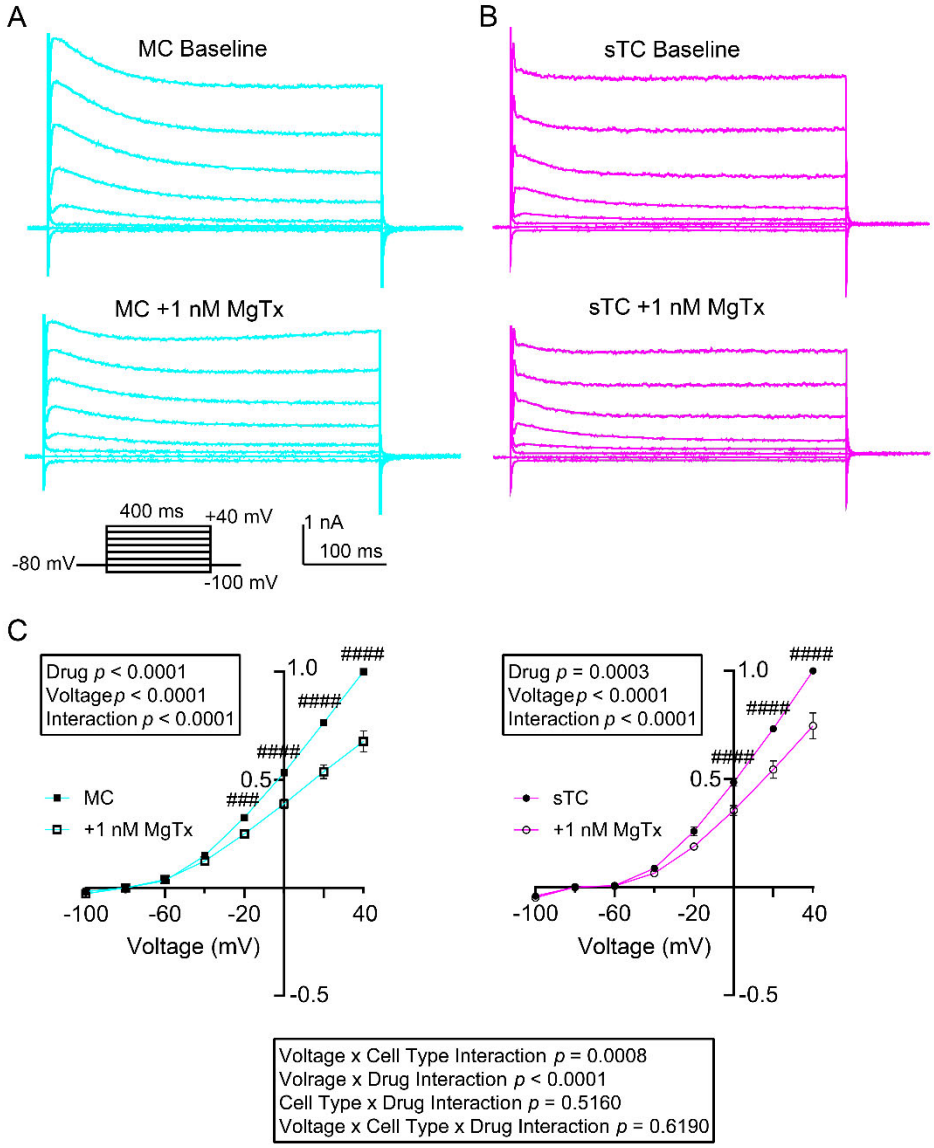

Fig. 3. Sensitivity of mitral and superficial tufted cells to a selective blocker of Kv channels. ***A,*** Family of voltage-activated currents recorded as in Figure 3 prior (***Top*,** Baseline) and following (***Bottom*,** +1 nM MgTx) 15-min bath application of margatoxin (MgTx) in a representative ***A,*** mitral cell (MC) and ***B,*** superficial tufted cell (sTC). ***C,*** Normalized IV plots prior (solid symbols) and following (open symbols) bath application of MgTx in a population of MCs (***Left,*** 2-w mixed RM ANOVA, drug F (1, 9) = 43.62, ****$p < 0.0001$, with a Sidak's post hoc test, $^{\#\#\#}p = 0.0008$, $^{\#\#\#\#}p < 0.0001$; voltage x drug interaction F (7, 63) = 43.86, ****$p < 0.0001$) and a population of sTCs (***Right,*** 2-w mixed RM ANOVA, drug ***$p = 0.0003$, Sidak's post-hoc test, $^{\#\#\#\#}p < 0.0001$; voltage x drug interaction ****$p < 0.0001$). ***C, Bottom*** **–** 3-way mixed RM ANOVA result comparing the two IV plots in ***C, Left-Right*** (3-w mixed RM ANOVA, voltage x cell type interaction F (7, 123) = 3.879, ***$p = 0.0008$; voltage x drug interaction F (7, 123) = 57.42, ****$p < 0.0001$; cell type x drug interaction F (1, 123) = 0.4244, $p = 0.5160$; voltage x cell type x drug interaction F (7, 123) = 0.7633, $p = 0.6190$).

248 **Superficial tufted cells are more excitable than mitral cells**

249 We next wanted to compare evoked AP firing frequency between MCs and sTCs as a metric
250 for cellular excitability. After obtaining the whole-cell configuration, cells were current-
251 clamped using a V_h of -60 mV (near RMP) for comparison of excitability across cell types.
252 Each MC or sTC was stimulated with a family of injected current steps that increased in 25
253 pA increments from -50 pA to 175 pA (**Fig. 4A**) to determine the threshold for first firing. On
254 average, the current needed to evoke APs (rheobase) was lower in sTCs than in MCs (**Fig. 4B**,
255 MCs = 69.3 $\pm$ 44.9 pA (22) vs. sTCs = 34.2 $\pm$ 24.1 pA (30), Mann-Whitney U, $^{**}p = 0.0021$).
256 Both cell types increased AP firing frequency in response to current step, but sTCs had a
257 significantly greater frequency than that of MCs (**Fig. 4C**). Here, we applied a 2-w mixed RM
258 ANOVA using cell type and current as factors. As anticipated, there was a main effect for
259 injected current ($F(7, 263) = 71.39$, $^{****}p < 0.0001$). There was also a main effect for cell
260 type ($F(1, 51) = 11.92$, $^{**}p = 0.0011$) and a current x cell type interaction ($F(7, 263) = 3.656$,
261 $^{***}p = 0.0009$). A Sidak's post-hoc test indicated a significant difference in current
262 dependence at the current injection window between 25 and 75 pA (25 pA - $^{\$\$}p = 0.0077$, 50
263 pA - $^{\$\$}p = 0.0017$, and 75 pA - $^{\$\$\$}p = 0.0001$). The increased AP firing frequency in response
264 to injected current was likely attributed to a change in interspike interval (ISI). As shown in
265 **Fig. 4D**, the ISI was shorter in sTCs compared to that of MCs. A 2-w mixed RM ANOVA
266 using cell type and injected current as factors demonstrated a main (negative) effect for
267 injected current ($F(1.643, 38.07) = 33.74$). There was also a main effect for cell type ($F(1,$
268 $49) = 37.53$, $^{****}p < 0.0001$) and a current x cell type interaction ($F(6, 139) = 5.873$, $^{****}p$
269 < 0.0001). A Sidak's post-hoc test indicated a significant difference between cell types at the
270 current injection window between 100 and 150 pA (100 pA - $^{\#}p = 0.0101$, 125 pA - $^{\#\#}p$
271 $= 0.0022$, and 150 pA - $^{\#}p = 0.0294$).
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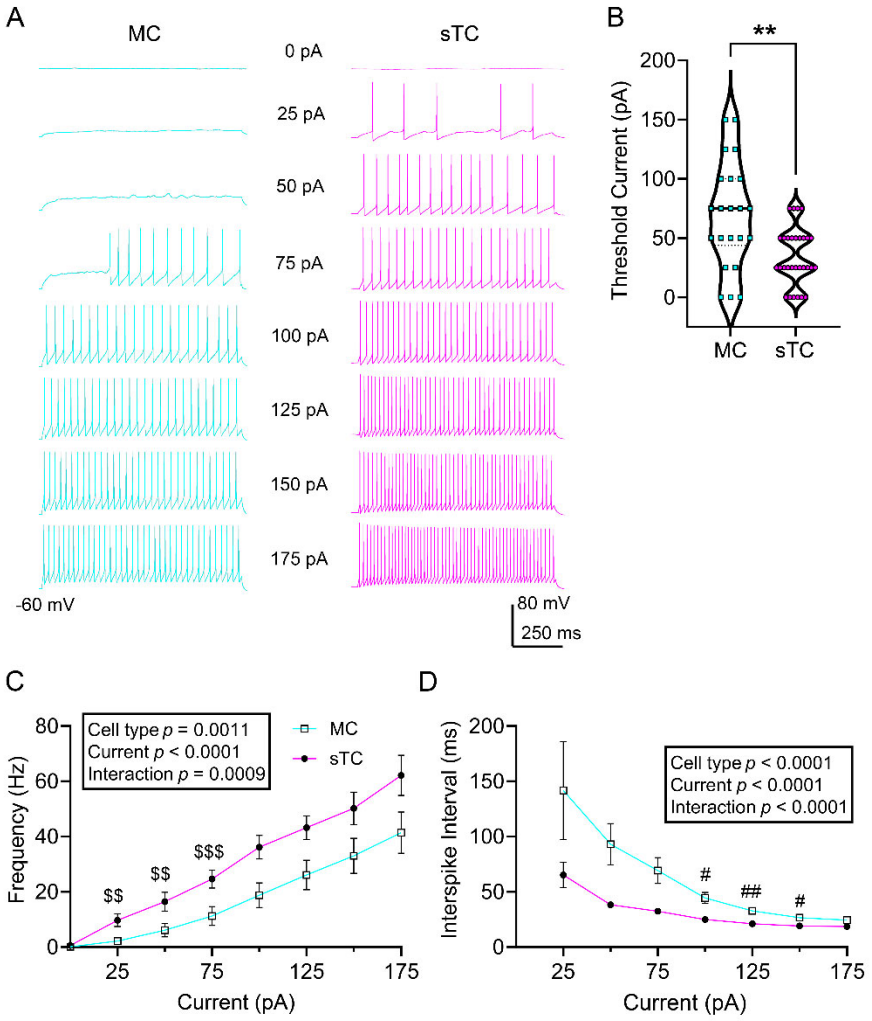

Fig. 4. Spike firing properties compared between mitral cells (MC) and superficial tufted cells (sTC). ***A*,** Representative action potential trains evoked by various current steps as indicated, recorded from a MC (***Left***) vs. sTC (***Right***). ***B*,** Violin plot comparing the current needed to evoke action potentials (rheobase), Mann-Whitney U, $^{**}p = 0.0021$. Line plots for ***C*,** mean spike frequency across current step and ***D*,** mean interspike interval (ISI). Line plot data were analyzed with a ***(C)*** 2-w mixed RM ANOVA, cell type $^{**}p = 0.0011$; current x cell type interaction $^{***}p = 0.0009$, Sidak's post-hoc test, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, ***(D)*** 2-w mixed RM ANOVA, cell type $^{****}p < 0.0001$; current x cell type interaction $^{****}p < 0.0001$, Sidak's post-hoc test, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

Because potassium channel currents can greatly influence the characteristics of APs (**Fig. 2-4**), we wanted to determine any differences in AP shape or kinetics that could accompany these findings. Many AP parameters require a clearly defined baseline in order to be assessed accurately, and the parameters are dependent on current injection magnitude. The metrics of

291 'maximum decay slope' and 'time to achieve maximum decay slope' are not dependent upon
292 correct identification of the recording baseline, and we therefore examined these metrics
293 simultaneously across all current injection steps. sTCs achieved steeper maximum decay
294 slopes than MCs, which also differed in a current-dependent manner (**Fig. 5A, C**). We applied
295 a 2-w mixed RM ANOVA using cell type and current step as factors. There was a main effect
296 of cell type (F (1, 50) = 5.914, $^{*}p = 0.0186$) and a current x cell type interaction (F (6, 127) =
297 5.562, $^{****}p < 0.0001$); a Sidak's post-hoc test indicated that cell-type significant differences
298 occurred at current steps 50 pA - $p^{\#\#\#} = 0.0006$, 75 pA - $^{\#\#\#\#}p < 0.0001$, and 100 pA - $^{\#\#}p =$
299 0.0074. APs recorded in sTCs also exhibited faster times to achieve maximum decay slope
300 than those in MCs, independent of current injection step (**Fig. 5B, C**). We applied a 2-w
301 mixed RM ANOVA using cell type and current step as factors. There was no effect of current
302 step (F (0.1425, 3.016) = 9.085, $p = 0.0524$), but there was a main effect of cell type F (1, 50)
303 = 4.088, $^{*}p = 0.0486$. A Sidak's post-hoc test, demonstrated that sTCs were faster at all
304 current injections at and above 75 pA (75 pA - $^{\#\#\#}p = 0.0002$, 100 pA - $^{\#\#}p = 0.0031$, 125 pA -
305 $^{\#}p = 0.0300$, 150 pA - $^{\#}p = 0.0274$).
306
307 Due to differences in excitability between MCs and sTCs (**Fig. 4**), the current injection step of
308 75 pA had the largest sample size for the comparison of AP characteristics that are dependent
309 upon verifiable baseline. Overall, we found that the APs of sTCs have greater peak
310 magnitude, greater antipeak (after-hyperpolarization) amplitude, faster rise kinetics, and faster
311 decay kinetics than MCs (**Table 1**). Mean values, sample sizes, *p* values, and statistical tests
312 are reported within each column.
313

	Peak Amplitude (mV)	Time to Peak Amplitude (ms)	Antipeak Amplitude (mV)	Time to Antipeak (ms)	Half Width (FWHM) (ms)
Mitral Cells	51.1 ± 10.0	5.83 ± 0.26	-12.1 ± 2.4	11.4 ± 0.7	1.78 ± 0.36
N	11	11	11	11	11
Superficial Tufted Cells	60.8 ± 7.0	5.72 ± 0.09	-15.1 ± 2.5	9.20 ± 0.90	1.32 ± 0.16
N	11	11	11	11	11
p-value	**0.0150**	ns (0.1970)	**0.0079**	**< 0.0001**	**< 0.0001**
Test used	†	†	⊤	†	⊤

	Max Rise Slope (mV/ms)	Time to Max Rise Slope (ms)	Max Decay Slope (mV/ms)	Time to Max Decay Slope (ms)	Rise Time to Half Amplitude (ms)
Mitral Cells	88.4 ± 35.2	5.11 ± 0.17	-44.3 ± 13.2	0.866 ± 0.167	5.09 ± 0.11
n	15	11	15	15	11
Superficial Tufted Cells	117.0 ± 44.0	5.06 ± 0.17	-79.5 ± 20.4	0.612 ± 0.105	5.11 ± 0.05
n	20	11	20	20	11
p-value	**0.0493**	ns (0.8338)	**< 0.0001**	**< 0.0001**	ns (0.7969)
Test used	†	⊤	‡	†	⊤

	Decay Time to Half Amplitude (ms)	Rise Slope 10% to 90% (mV/ms)	Rise Time 10% to 90% (ms)	Decay Slope 90% to 10% (mV/ms)	Decay Time 90% to 10% (ms)
Mitral Cells	6.87 ± 0.38	58.8 ± 24.1	1.04 ± 0.82	-31.3 ± 8.2	1.41 ± 0.21
n	11	11	11	11	11
Superficial Tufted Cells	6.43 ± 0.16	74.1 ± 18.3	0.784 ± 0.151	-56.7 ± 11.2	0.911 ± 0.107
n	11	11	11	11	11
p-value	**0.0021**	ns (0.1087)	ns (0.3192)	**< 0.0001**	**< 0.0001**
Test used	†	‡	†	‡	†

314 **Table 1.** Action potential shape compared between mitral cells (MCs) vs. superficial tufted cells
315 (sTCs). Recordings were made while holding cells near rest (V_h = -60 mV) and injecting 75 pA of
316 current. Values are mean ± standard deviation. ns = not significant, †= Student's *t*-test, ‡= *t*-test with
317 Welch's correction, ₸= Mann-Whitney U.

318

319 **Superficial tufted cells do not show a higher propensity to bursting activity**
320 **than mitral cells**

321 Previous reports demonstrate that MCs are less likely to exhibit trains of AP activity than TCs
322 in general (Liu and Shipley, 2008; Burton and Urban, 2014; Jones *et al*., 2020). As such, we
323 wanted to determine if this was also true of sTCs by applying further refined spike analysis.
324 After determining the supra-threshold current injection step, we injected cells at that threshold
325 using a P_d of 5000 ms and a T_s = 30 s for 10 total sweeps (**Fig. 5D**). We defined a "train" as
326 having 3 or more APs within a 100 ms period (Balu et al., 2004), and only directly compared
327 cells receiving identical current injection magnitude. sTCs did not exhibit more AP trains
328 within their respective recording periods (**Fig. 5E,** MCs = 28.7 ± 21.1 (7) AP trains vs. sTCs =
329 25.1 ± 21.3 AP trains (7), Student's *t*-test, p = 0.7582), nor did the trains of sTCs contain more
330 AP spikes (**Fig. 5F,** MCs = 10.8 ± 11.9 spikes/train (6) vs. sTCs = 11.3 ± 7.2 spikes/train (6),
331 Student's *t*-test, p = 0.2796). sTCs did not exhibit differing pause duration between trains
332 (**Fig. 5G,** MCs = 950 ± 890 ms (6) vs. sTCs = 1660 ± 1720 ms (6), Mann-Whitney U, p =
333 0.3939), nor a differing latency period to first spike (**Fig. 5H,** MCs = 327 ± 249 ms (6) vs.
334 sTCs = 535 ± 404 ms (7), Mann-Whitney U, p = 0.1375). To assess whether sTCs showed a
335 higher propensity to be a 'bursting cell' (as opposed to a regularly-firing cell), we assessed the
336 firing activity from all current-clamp recordings to determine the categorical type of each MC
337 and sTC. A cell was categorized as a "bursting cell" if any of its sweeps contained at least two
338 bursts separated by a pause duration of > 100 ms. sTCs did not show a statistically different
339 preference toward bursting or regularly firing than that of MCs (**Fig. 5I**, MCs = 28.6% (21),
340 sTCs = 40% (30), Chi-squared test, p = 0.40061, X^2 = 0.7065).

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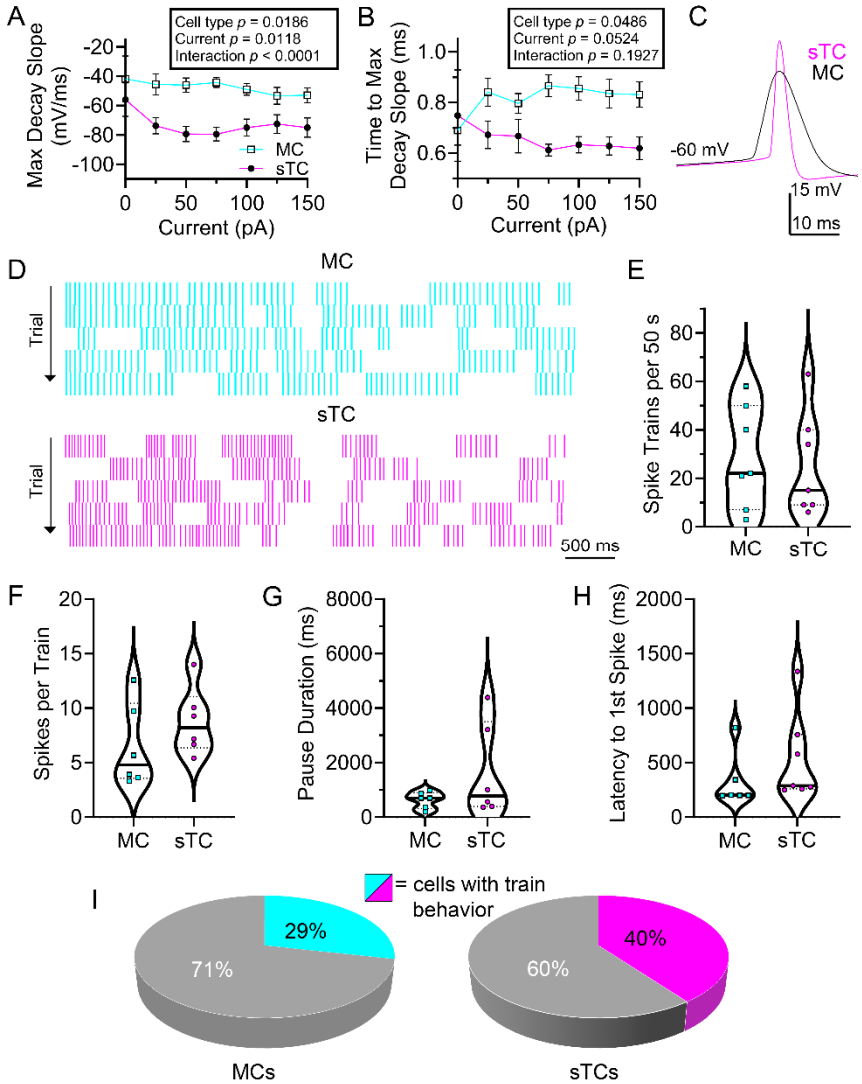

Fig. 5. Action potential shape and spike train activity compared between mitral cells (MC) and superficial tufted cells (sTC). Line plot for ***A,*** maximum decay slope (max decay slope) and ***B,*** time to achieve maximum decay slope from peak magnitude (time to max decay slope) across current step. Line plot data were analyzed with **(A),** 2-w mixed RM ANOVA, cell type *p = 0.0186, Sidak's post-hoc test, ##p < 0.01, ###p < 0.001; current x cell type interaction ****p < 0.0001, **(B),** 2-w mixed RM ANOVA, cell type *p = 0.0486, Sidak's post-hoc test, #p < 0.05, ##p < 0.01, ###p < 0.001. ***C,*** Representative pair of action potentials recorded in response to a 75 pA current injection held near rest (Vh = -60 mV). ***D,*** Representative raster plot for a cell stimulated with a 50-pA current injection using a P_d = 5 s for each cell type, respectively. Successive trials are stacked vertically below. Violin plots computed across a 50 s recording period (10, 5s sweeps) compare ***E,*** mean number of bursts, ***F,*** mean spikes per train, ***G,*** mean pause duration between trains, and ***E,*** mean latency to first spike. ***E-F,*** were analyzed using a Student's *t*-test, ***G-H*** were analyzed using Mann-Whitney U; $p > 0.05$. ***I,*** Relative proportion of cells eliciting spike train behavior among sampled MCs (***Left***) and sTCs (***Right***), respectively (Chi-squared test, p = .40061, $X^2 = 0.7065$, n = 51).

359 **Co-localization of Kv1.3 channel and the insulin receptor in superficial tufted**
360 **cells**
361
362 MCs are known be modulated by insulin, and Kv1.3 is necessary for this modulation (Fadool
363 and Levitan, 1998; Fadool et al., 2000, 2011; Tucker et al., 2010; Kuczewski et al., 2014). We
364 found sTCs to be modulated by margatoxin (**Figure 3**), suggesting expression of Kv1.3. We
365 therefore performed multiplex fluorescent *in situ* hybridization (RNAscope) to determine co-
366 expression of Kv1.3 and the insulin receptor (IR) in sTCs, similar to what we have observed
367 for MCs (Marks and Fadool, 2007; Colley et al., 2009; Marks et al., 2009; Thiebaud et al.,
368 2016). Using a transgenic GFP label for M/TCs under control of the *Tbx21* promoter, we
369 performed RNAscope targeting *Kv1.3*, *GFP*, and *Insr* on six coronal sections sampled 512 µm
370 apart along the antero-posterior axis (AP axis), in both male and female mice. We observed
371 co-localization of the channel and the receptor markedly distributed with the expression of the
372 *Tbx21* promoter by imaging at low power across the entire olfactory bulb (**Fig 6A-D**). We
373 then moved to higher magnification in the EPL to visualize the sTC specifically, and the
374 colocalization of the channel and receptor with the *Tbx21* reporter expression (**Fig. 6E-H**). A
375 2-way comparison revealed a main effect of AP axis on Tbx21+ cell count (**Fig. 6I**, **p* =
376 0.0118) with no interactive effect of axis on sex or laminar layer ($p = 0.3020$; M MCL, M
377 EPL, M GLM, F MCL, F EPL, F GLM as columns variables; M = male, F = female). We then
378 examined the percentage of Tbx21+ cells co-labeled with both *Kv1.3* and *Insr* along the
379 antero-posterior axis, and found no main effect of AP axis (**Fig. 6J,** $p = 0.5736$) or interactive
380 effects on sex or laminar layer ($p = 0.7359$). Without regard for antero-posterior position or
381 sex (since these were not found to be factors for co-labeling), we assessed the percentage of
382 Tbx21+ cells that express both *Kv1.3* and *Insr*. We find triple-labeling of 82.1 $\pm$ 4.3 % in the
383 MCL, 73.4 $\pm$ 8.2 % in the EPL, and 76.2 $\pm$ 6.8 % in the GLM. ANOVA comparisons across
384 neurolaminae revealed a subtle difference in the percentage of all Tbx21+ cells expressing
385 *Kv1.3* alone (**Fig. 6K**, **p* = 0.0159) and expressing both *Kv1.3* and *Insr* (**Fig. 6M**, **p* =
386 0.0373). We further performed immunolabeling for Kv1.3 and Insr proteins in tandem with
387 Tbx21 reporter expression. As predicted based upon previous reports (Marks *et al*., 2009;
388 Colley *et al*., 2009), co-labeling for both IR and Kv1.3 was observed in the soma and near the
389 axon initial segment of most MCs imaged (**Fig. S1**).

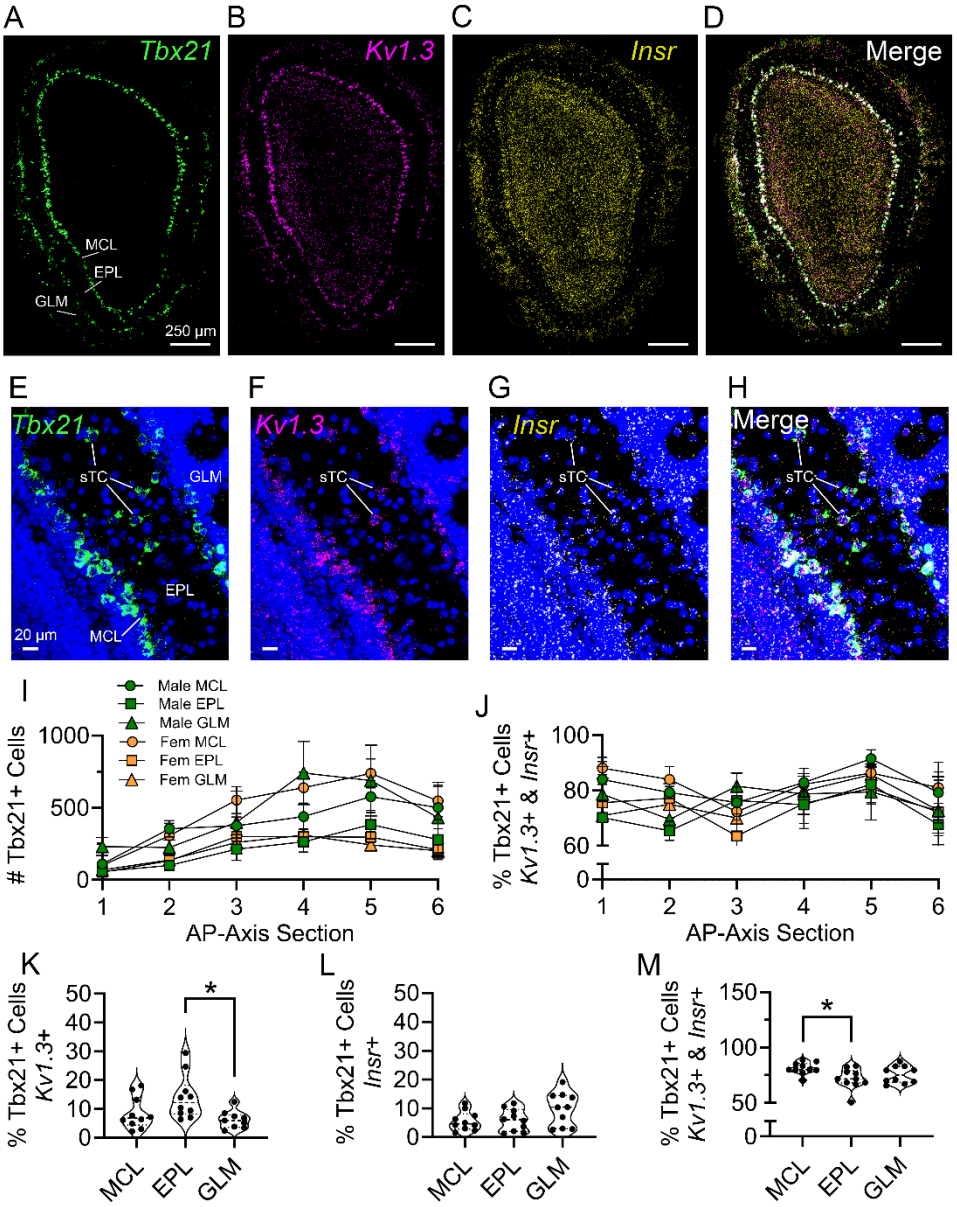

Fig. 6. Colocalization of M/TCs with the Kv1.3 channel (*Kv1.3*) and insulin receptor (*Insr*) using RNAscope. Photomicrographs demonstrating neurolaminar distribution of Tbx21 reporter expression (green, ***A***), *Kv1.3* expression (magenta, ***B***), and *Insr* expression (yellow, ***C***), separately and merged (white, ***D***). ***E–H***: Same as ***A–D***, but to demonstrate localization to sTCs with the inclusion of a DAPI nuclear stain. ***I:*** Coronal sections were sampled along the anter-posterior axis (AP axis) at ~512 µm intervals and assessed for total number of M/TCs (Tbx21+ cells) and for percentage of M/TCs expressing both *Kv1.3* and *Insr* (***J***). Each data point represents the average from 5 separate mice, separated by laminar layer and sex. ***K–M:*** Percentage of M/TCs (Tbx21+ cells) expressing *Kv1.3* alone (***K***), *Insr* alone (***L***) or both (***M***). Statistical comparisons for ***K–M*** are Kruskal-Wallis (ANOVA) with Dunn's multiple comparisons test. Each data point represents the average of all six antero-posterior sections from each mouse, one data point per mouse per neurolaminar layer, respectively. $^{*}p < 0.05$, GLM = glomerular layer, EPL = external plexiform layer, GCL = granule cell layer.

402 **Superficial tufted cells are modulated by insulin via the Kv1.3 ion channel**

403 To further explore the complementary physiological role of sTCs, we wanted to determine if
404 their excitability could be modulated by insulin—knowing that MCs are modulated by 0.1 μm
405 insulin (Fadool *et al*., 2000, 2011). After sampling for supra-threshold current, synaptic
406 blockers were added to the bath solution. Evoked APs were established using the threshold
407 current with a P_d = 5000 ms and T_s = 30 s for a 5-min paradigm (10 recordings). A
408 representative set of baseline recordings and an associated raster plot is shown in **Fig. 7A,**
409 prior (left) and subsequent (right) to bath application of insulin for 15 min.
410
411 Insulin significantly reduced the average latency to first spike by ~38% (**Fig. 7B**, sTC pre =
412 1220 $\pm$ 723 ms (11) vs. sTC post = 649 $\pm$ 403 ms (11), paired *t*-test, **p* = 0.0132). On average,
413 insulin also increased the AP frequency by ~98% (**Fig. 7C**, sTC pre = 4.76 $\pm$ 1.76 Hz (11) vs.
414 sTC post = 8.49 $\pm$ 4.98 Hz (11), paired *t*-test, **p* = 0.0381), and decreased the average
415 intraburst interval by ~8% (i.e., the pause between APs within a particular burst) (**Fig. 7D**,
416 sTC pre = 65.1 $\pm$ 13.6 ms (11) vs. sTC post = 60.3 $\pm$ 14.9 ms (11), paired *t*-test, **p* = 0.0136).
417 All statistical tests were performed on non-normalized data, however, plots were generated as
418 normalized paired values to visualize individual cell changes across the sampled cells.
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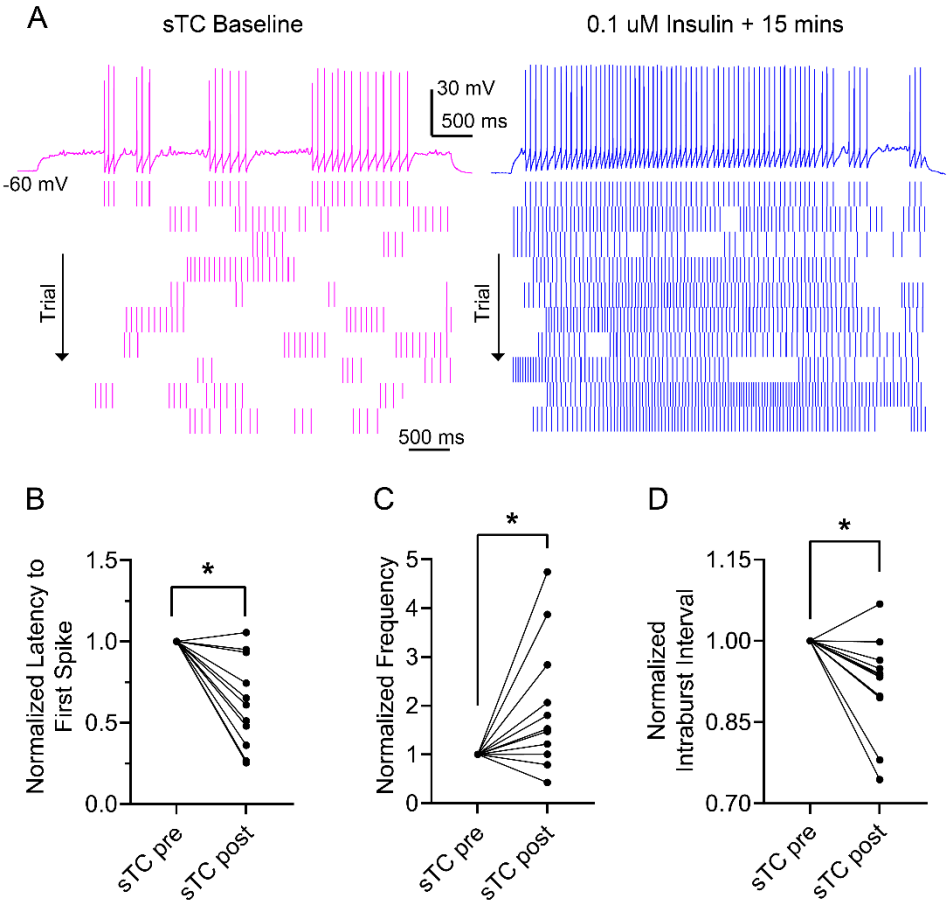

Fig. 7. Bath application of insulin increases spike firing frequency, and shortens latency to first spike and intraburst interval in superficial tufted cells (sTCs). ***A,*** Representative spike trains (***Top***) that were elicited in response to 25 pA current injection while holding the cell near rest (V_h = -60 mV) prior (***Left,*** Baseline) and subsequent (***Right,*** Insulin) to hormone stimulation using P_d = 5 s. (***Bottom***) Representative associated raster plot. Line graph comparing ***B,*** latency to first spike, ***C,*** spike firing frequency, and ***D,*** intraburst interval, prior (pre) and following (post) insulin stimulation in the same cell. Data were analyzed with a paired *t*-test using non-normalized data ***(B)*** **p* = 0.0132, ***(C)*** **p* = 0.0381, and ***(D)*** **p* = 0.0136.

The modulation of MCs by insulin has been shown to depend on the Kv1.3 ion channel using either pharmacological block or targeted gene-deletion (Fadool *et al*., 2000, 2011; Colley *et al*., 2004). Since previous reports demonstrate Kv1.3 expression in sTCs (Kues and Wunder, 1992; Fadool *et al*., 2000; Colley *et al*., 2007), we wanted to determine if insulin modulation of sTCs was also Kv1.3-dependent. Using transgenic mice lacking the *Kv1.3* gene (Kv1.3-/-, see methods) we first compared the AP bursting characteristics between the sTCs of GFP mice (Kv1.3 +/+), and the sTCs of Kv1.3 -/- mice. In accordance with the findings in Kv1.3 -

/- MCs (Fadool *et al*., 2004, 2011; Tucker *et al*., 2010; Thiebaud *et al*., 2016), we found that Kv1.3 -/- sTCs tend to burst more often, and have shorter bursts with faster intraburst frequency. Kv1.3 -/- sTCs also have shorter latency to first spike and faster overall evoked AP frequency than Kv1.3 +/+ sTCs (**Table 2**). Mean values, sample sizes, *p* values, and statistical tests are reported within each column.

	Bursts per 50 s (n)	Spikes per Burst (n)	Burst Duration (ms)	Intraburst Interval (ms)	Intraburst Frequency (Hz)
Kv1.3 +/+ sTCs	24.8 ± 10.0	7.6 ± 3.1	414.6 ± 209.5	65.1 ± 12.9	17.0 ± 5.6
n	11	11	11	11	11
Kv1.3 -/- sTCs	55.0 ± 9.6	6.0 ± 1.4	215.0 ± 82.4	45.6 ± 11.7	26.1 ± 8.7
n	4	4	4	4	4
p-value	**0.0003**	ns (0.2362)	**0.0305**	**0.0291**	**0.0048**
Test used	†	‡	‡	†	₸

	Pause Duration (ms)	Latency to First Spike (ms)	AP Frequency (Hz)	Event Frequency (Hz)	% Bursting Cells
Kv1.3 +/+ sTCs	729.8 ± 354.1	1221.6 ± 689.4	4.8 ± 1.7	7.6 ± 2.6	
n	11	11	11	11	
Kv1.3 -/- sTCs	655.3 ± 210.4	419.5 ± 298.7	7.1 ± 2.2	7.6 ± 2.5	
n	4	5	5	5	
p-value	ns (0.7183)	**0.0090**	**0.0475**	ns (0.7427)	
Test used	†	‡	†	₸	

Table 2. Spike bursting properties in superficial tufted cells (sTCs) compared between wildtype (WT) and Kv1.3 -/- mice. Recordings were made while holding cells near rest (V_h = -60 mV) and injecting 75 pA of current. Data were computed across a 50 s recording period (10, 5s sweeps). Values are mean ± standard deviation. ns = not significant, †= Student's *t*-test, ‡= *t*-test with Welch's correction, ₸= Mann-Whitney U

457

458 Using Kv1.3 -/- mice, we next repeated the previous insulin experiment shown **Fig. 7**. A
459 representative set of baseline recordings and associated raster plot for sTCs evoked APs is
460 shown in **Fig. 8A,** prior (left) and subsequent (right) to bath application of insulin for 15 min
461 as recorded in the Kv1.3-/- mice. Unlike in recordings acquired from Kv1.3 +/+ mice, insulin
462 did not alter latency to first spike (**Fig. 8B**, Kv1.3-/- sTC pre = 420 $\pm$ 334 ms (5) vs. Kv1.3-/-
463 sTC post = 390 $\pm$ 232 ms (5), paired *t*-test, $p = 0.5694$). Insulin also did not affect AP firing
464 frequency (**Fig. 8C**, Kv1.3-/- sTC pre = 7.09 $\pm$ 2.48 Hz (5) vs. Kv1.3-/- sTC post = 7.13 $\pm$
465 2.93 Hz (5), paired *t*-test, $p = 0.9300$, n = 5), nor affect the intraburst interval (**Fig. 8D***,* Kv1.3-
466 /- sTC pre = 45.6 $\pm$ 13.6 ms (4) vs. Kv1.3-/- sTC post = 52.7 $\pm$ 17.8 ms (4), paired *t*-test, $p =$
467 0.1990, n = 4) in Kv1.3-/- mice. The same 5 cells were used for all pre/post comparisons, but
468 one cell did not fire quickly enough for burst analysis (see methods).

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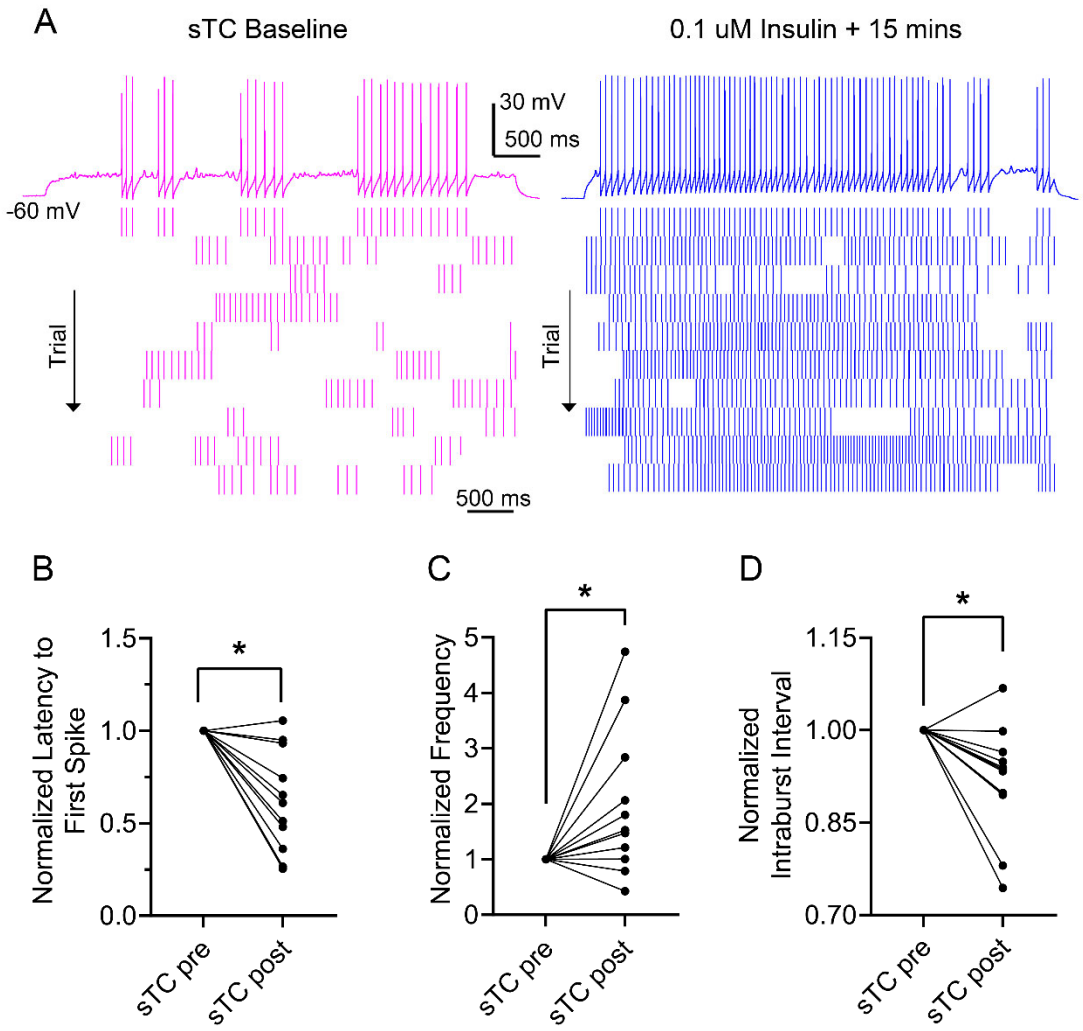

485 **Fig. 8.** Insulin fails to modulate spike firing frequency in superficial tufted cells of Kv1.3-/- mice. ***A,***
486 Representative spike trains (***Top***) and associated raster plot (***Bottom***) recorded as in *Figure 9*. Line
487 graphs Line graph comparing ***B,*** latency to first spike, ***C,*** spike firing frequency, and ***D,*** intraburst
488 interval, prior (pre) and following (post) insulin stimulation in the same cell. Data were analyzed with
489 a paired *t*-test using non-normalized data, $p > 0.05$.

490

491 ## Discussion

492 We have demonstrated that the two major output neurons in the olfactory bulb have different
493 biophysical properties, despite both neurons expressing IR kinase and exhibiting modulation
494 by insulin. sTCs are more excitable, as evidenced by the lower threshold needed to evoke an
495 AP, a decreased ISI, and a greater AP firing frequency particularly at lower current injection.
496 We found that the APs of sTCs have greater peak amplitude, greater anti-peak amplitude,

497 faster rise kinetics, and faster decay kinetics than those of MCs – while at the same time, the
498 incidence of a train of AP activity was similar between the two output neurons. While there
499 are differences in the voltage dependence of voltage-activated outward currents between MCs
500 and sTCs, both are blocked by the Kv1.3 vestibule blocker, MgTx. We determined that
501 voltage-activated outward currents in sTCs are modulated by insulin, and that the Kv1.3 ion
502 channel is necessary for this modulation.
503
504 Burton and Urban (Burton and Urban, 2014) first compared mitral cell biophysical properties
505 with that of what they called "tufted cells", which were projection neurons whose cell bodies
506 resided in the superficial 50% of the EPL. Prior to their publication, physiologists often
507 clustered mitral and tufted cells together as a homogenous neuronal population (Fadool and
508 Levitan, 1998; Tucker and Fadool, 2002; Youngstrom and Strowbridge, 2015; Liu et al.,
509 2016) due to their similar function as output neurons. Using new nomenclature described by
510 Jones et al. (Jones et al., 2020), we now know that TCs are comprised of a variety of sub-
511 classes that are functionally distinct with differential sensitivity to inhibitory/excitatory
512 circuits to putatively sharpen tuning specificity for odorants during sensory processing (Zhou
513 et al., 2023). The tufted cells first measured by Burton and Urban (2014) likely consisted of a
514 mixed population of sTCs and mTCs. Nonetheless, some of our data measured in strictly
515 sTCs, align well with what they reported for "tufted cells" in general. Burton and Urban found
516 that their TCs had a higher instantaneous frequency (peak instantaneous rate) that differed
517 with current injection magnitude than that of MCs, and that the AP shape of their TCs
518 exhibited a narrower half width (FWHM), a steeper decay slope, and faster time to anti-peak
519 (after-hyperpolarization). Our comparative measurements were quite similar. Thus, we
520 similarly report that sTC have greater intrinsic excitability than that of MCs. Unlike this
521 earlier study, however, we found that the AP shape in sTCs compared with that of MCs, has a
522 greater peak amplitude, steeper maximum rise slope, a greater anti-peak amplitude, and the
523 minimum current necessary to evoke a first AP (threshold) is lower.
524
525 Burton and Urban further found that their TCs were more likely to elicit spike trains (stutter)
526 compared with that of MCs, which they characterized based upon the increased co-efficient of
527 variation of the ISI observed in the TCs. We did not observe a greater overall firing

528 irregularity in our recorded sTCs, and in fact, we found the opposite - our recorded MCs had a
529 greater ISI variance at lower levels of current injection than that of sTC. Also inconsistent
530 with our findings; sTCs and MCs did not have significantly different AP train properties
531 (spikes per train, pause duration, or latency to first spike), nor did sTCs have a statistically
532 higher propensity to elicit a spike train. The likely inclusion of both sTCs and mTCs in their
533 study could account for these differential findings. In fact, Antral et al. (2006) also reports in
534 their study of eTC, that they observed two subclassifications, nonbursting (contained lateral
535 dendrites) and bursting (lacked lateral dendrites), that were found to be both morphologically
536 and functional diverse. We believe that we have complemented the findings of Burton and
537 Urban, expanding upon their comparisons to more specifically characterize selectively the
538 sTCs and not multiple subtypes clustered together.
539
540 Differences in ion channel expression could underlie the greater excitability of sTCs
541 compared to that of MCs. We found voltage-activated currents in the window of -60 to -20
542 mV to be greater in MCs than those of sTCs. It is well known that *shaker* family members
543 have a voltage-activation above -50 to -40 mV (Zagotta and Aldrich, 1990), therefore Kv1.2
544 and Kv1.3 likely are not highly responsible for the difference in voltage-activated currents
545 between the two cell types. This is corroborated by the fact that both cell types exhibited a
546 similar pharmacological response to MgTx and both are shown to be similarly modulated by
547 bath application of insulin, previously demonstrated to be the result of Kv1.3 being used as a
548 substrate for tyrosine phosphorylation in the OB in a use- or activity-dependent manner
549 (Fadool et al., 2000, 2011). We also measured a select slowing of the deactivation kinetics in
550 MCs at the sampled -40 mV hyperpolarizing step that might selectively lower MC excitability
551 vs. that in sTCs. A comparison of MC activity in Kv1.3-/- mice vs. pharmacological block of
552 the channel in the current study, may reveal differences in underlying ion channel expression
553 between sTCs and MCs. The threshold for activation of voltage-activated outward currents in
554 the MCs of Kv1.3-/- mice were previously found to be shifted +20 mV in the positive
555 direction compared to wild-type MCs (Fadool et al., 2004), but these Kv1.3-/- MCs have an
556 upregulation of a *slack* sodium-activated potassium (K_{Na}) channel (Lu et al., 2010), an
557 increase in calcium channel expression (Fadool et al., 2004), and may have increased chloride
558 currents (Koni et al., 2003). Therefore, the threshold for activation of voltage-activated

559 currents in sTCs and Kv1.3-/- MCs is both higher than that of wildtype MCs, but the precise
560 nature of any differences in channel expression (i.e., perhaps sTCs express higher levels of
561 K_{Na} channels than MCs) is not known. This opens the door to a larger area of research that is
562 beyond the scope of this report.
563
564 sTCs are modulated by the presence of insulin, and our data demonstrate that this modulation
565 depends upon the Kv1.3 ion channel. Insulin's primary role in the periphery is to mobilize
566 glucose from the bloodstream following a meal; however, insulin is known to have many roles
567 as a signaling molecule in the central nervous system (CNS). The levels of insulin in the CNS
568 do not mirror the levels of insulin in the periphery, and the highest concentration of insulin
569 receptors and binding affinity to insulin (outside the pancreas) is found in the OB (Baskin et
570 al., 1983; Hill et al., 1986; Gupta et al., 1992; Schwartz et al., 1992; Myers Jr. and White,
571 1993; White, 1997; Wickelgren, 1998; Banks et al., 1999; Fadool et al., 2000; Aimé et al.,
572 2012; Palouzier-Paulignan et al., 2012; Fadool and Kolling, 2020). MCs have an interesting
573 co-localization of IR kinase and Kv1.3 with adaptor proteins that functions to cluster signaling
574 transduction cascades in close proximity (Marks and Fadool, 2007; Marks et al., 2009). In the
575 brain, insulin resistance is implicated in the progression of neurodegeneration and dementia
576 (Kuljiš and Salković-Petrišić, 2011; Arnold et al., 2018; Ferreira et al., 2018; Hong et al.,
577 2021; Femminella et al., 2021). Our report elevates the role of Kv1.3 as a target for the
578 investigation of olfactory function, and, by extent, the complexities of insulin signaling in the
579 OB. Though not annotated directly, previous histological reports suggested expression of
580 Kv1.3 in sTCs. These include Figure 1E from Kues 1992 (Kues and Wunder, 1992), Figure 7
581 from Fadool 2000 (Fadool et al., 2000), and Figure 9 from Colley 2007 (Colley et al., 2007).
582 At this time, we do not know of any reports that previously suggested expression of insulin
583 receptors on sTCs. MCs are known to be modulated by insulin in a similar manner to our sTC
584 findings (Fadool et al., 2000, 2011; Das et al., 2005; Tucker et al., 2010; Aimé et al., 2012;
585 Kuczewski et al., 2014), and this modulation in MCs is known to occur via phosphorylation of
586 Kv1.3 (Bowlby et al., 1997; Fadool and Levitan, 1998; Fadool et al., 2000, 2011).
587
588 While we know that IR kinase is also co-localized with glucagon-like 1 peptide receptors in
589 MCs (Thiebaud et al., 2016) and have discovered an array of neuromodulators that alter Kv1.3

590 channel function and general excitability of these output neurons (Cook and Fadool, 2002;
591 Tucker and Fadool, 2002; Colley et al., 2009; Fadool et al., 2011; Mast and Fadool, 2012;
592 Tucker et al., 2013; Huang et al., 2017; Thiebaud et al., 2019), the possibility of
593 neuromodulation of sTCs remains highly likely and greatly unexplored. Herein, we have
594 distinguished these two output neurons in terms of biophysical function, but there may be a
595 range of neuromodulators that can affect and fine tune odor information coding or regulation
596 of metabolic state (Thiebaud et al., 2016, 2019; Kolling et al., 2022). Moreover, we have
597 recently enhanced total excitability of the output neurons (MCs and all classes of TCs) using
598 cell selective CRISPR genome editing of Kv1.3 to support a new function of the olfactory
599 system – metabolic regulation and energy homeostasis (Kolling et al., 2022). Thus, there is
600 much to discover concerning both the division of olfactory function between MCs and TCs
601 (odor context, valence, vs. odor identity), as well as the combined parallel function of
602 olfactory output neurons in regulating whole-body metabolism.
603

604 # MATERIALS AND METHODS

605 ## Ethical approval

606 All animal procedures were reviewed and approved by Florida State University (FSU)
607 Laboratory Animal Resources (Protocol 202000045) that abided by the American Veterinary
608 Medical Association (AVMA) and the National Institutes of Health (NIH). In preparation for
609 OB slice electrophysiology, mice were anesthetized with isoflurane (Aerrane; Baxter,
610 Deerfield, IL, USA) using the Institutional Animal Care and Use Committee (IACUC)-
611 approved drop method and then were killed by decapitation (AVMA Guidelines on
612 Euthanasia, June 2007). In preparation for histology, mice were anaesthetized with a mixture
613 of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) prior to
614 intracardial perfusion with 4% paraformaldehyde and euthanasia by decapitation (AVMA
615 Guidelines on Euthanasia, June 2007). Our work was guided by the animal ethics checklist
616 reported by Grundy, 2015 (Grundy, 2015).
617

618 ## Solutions and reagents

619 Salts and sugars, unless otherwise noted, were obtained from Thermo Fisher Scientific
620 (Waltham, MA, USA) or Sigma-Aldrich Chemical (St. Louis, MO, USA). Artificial cerebral

621 spinal fluid (aCSF) contained (in mm): 119 NaCl, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 2.5 KCl, 1.3
622 $MgCl_2$, 2.5 $CaCl_2$ and 22 d-glucose (pH 7.3; 300–310 mOsm). Sucrose-modified aCSF was
623 used for vibratome sectioning and recovery, and contained (in mm): 83 NaCl, 26.2 $NaHCO_3$,
624 1 NaH_2PO_4, 3.3 $MgCl_2$, 0.5 $CaCl_2$, 72 sucrose and 22 d-glucose (pH 7.3; 300-310 mOsm) (Jan
625 and Westbrook, 2007). Slice intracellular (pipette) solution contained (in mm): 135 potassium
626 gluconate, 10 KCl, 10 HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid), 10
627 $MgCl_2$, 0.4 NaGTP and 2 NaATP (pH 7.3; 280–285 mOsm). PBS contained the following (in
628 mm): 136.9 NaCl, 2.7 KCl, 10.1 Na_2HPO_4, and 1.8 KH_2PO_4 (pH 7.4). Paraformaldehyde
629 (PFA) for tissue fixation consisted of 4% PFA (Eastman Kodak, Rochester, NY, USA) in
630 PBS. Blocking buffer and incubation buffer consisted of 2% bovine serum albumin fraction V
631 (BSA) (Invitrogen, Waltham, MA, USA) and 0.2% Tween-20 (Fisher, Hampton, NH, USA)
632 in 1X PBS (Salameh et al., 2017).
633
634 The following synaptic blockers and channel vestibule blocker were prepared as concentrated
635 stock solutions in aCSF, stored at -20ºC until day of use, and then diluted to the following
636 working concentrations: 10 µM gabazine (catalog #104104-50-9, MilliporeSigma, Burlington,
637 MA, USA), 5 µM 2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline (NBQX, catalog
638 #ab120045, Abcam, Waltham, MA, USA), 25 µM (2R)-amino-5-phosphonovaleric acid (AP5,
639 catalog #ab120003, Abcam), 1 nM margatoxin (MgTx, catalog # M8278, MilliporeSigma),
640 0.1 uM insulin (Fadool et al., 2011*a*) (catalog #I2643, MilliporeSigma), and 100 nM
641 tetrodotoxin (TTX, catalog # 120055, Abcam). Prior to use, MgTx and insulin were
642 additionally mixed with 0.05% bovine serum albumin (BSA fraction V, catalog # BSAV-RO,
643 Sigma-Aldrich) to prevent loss of peptide to tubing and plasticware while recording.
644

645 **Animals**

646 All mice were housed at the FSU vivarium on a standard 12 h/12 h light/dark cycle and in
647 accordance with institutional requirements for animal care. Mice were individually housed in
648 conventional-style rodent cages containing separate food and water that could be obtained *ad*
649 *libitum*. Mice were maintained on chow that was comprised of 13.5% kcal fat, 59.1% kcal
650 carbohydrate, and 28.05% kcal protein (Catalog #5001, Purina Company, St. Louis, MO,
651 USA).

652

653 B6:CBA-Tg(Tbx21-cre)1Dlc/J mice with cre recombinase expression limited to mitral and
654 tufted cells (catalog #024507, Jackson Labs, Bar Harbor, ME, USA;
655 RRID:IMSR_JAX:024507) were crossed with B6J.129(B6N)-Gt(ROSA)26Sor$^{tm1(CAG\text{-}cas9^{\wedge},\text{-}}$
656 $^{EGFP)Fezh}$/J mice (catalog #026175, Jackson Labs; RRID:IMSR_JAX:026175) to generate
657 progeny mice with bicistronic spCas9/EGFP expression limited to the mitral and tufted cells.
658 The EGFP signal, along with anticipated physiological parameters, was used to identify mitral
659 vs. tufted cells (Chen and Shepherd, 1997; Marks *et al*., 2009). A total of 76 Tbx21-cre x
660 Cas9/EGFP male and female mice were used in our experiments for electrophysiology and
661 RNAscope (referred to as simply 'GFP mice' throughout) and ranged from 19 to 28 postnatal
662 days (PD) in age.

663

664 Kv1.3-/- mice were crossed with Thy1-YFP mice to generate progeny mice with
665 fluorescently-labeled OB neurons. Kv1.3-/- mice (RRID:IMSR_JAX:027392) were produced
666 previously by excision of the N-terminal one-third of the *Kv1.3* gene (Xu et al., 2003; Koni et
667 al., 2003) and were a generous gift from Drs. Leonard Kaczmarek and Richard Flavel (Yale
668 University, New Haven, CT, USA). Thy1-YFP mice (RRID:IMSR_JAX:014130) were a gift
669 from Dr. Gouping Feng (MIT Broad Institute, Boston, MA, USA) (Feng et al., 2000). A total
670 of 5 Kv1.3-/- x YFP male and female progeny mice were used for electrophysiology (referred
671 to as simply 'Kv1.3-/- mice' throughout) and ranged from PD19-28 in age.

672

673 **Olfactory bulb slice recordings**

674 OB slices for electrophysiological recordings were acquired from GFP or Kv1.3-/- mice as
675 described previously (Fadool et al., 2011; Mast and Fadool, 2012; Huang et al., 2017;
676 Thiebaud et al., 2019; Kolling et al., 2022). Briefly, mice were anesthetized by inhalation of
677 isoflurane, quickly decapitated, and the OBs were rapidly exposed by removing the dorsal and
678 lateral portions of the skull (Nickell et al., 1996). The OBs (while still attached to the
679 forebrain) were quickly removed, glued to a sectioning block with Superglue (Lowe's Home
680 Improvement, Tallahassee, FL, USA) and submerged in oxygenated (95% O_2/5%CO_2), ice-
681 cold, sucrose-modified artificial cerebral spinal fluid (aCSF) to prepare the tissue for
682 sectioning. Coronal sections (300 μm) were cut in oxygenated, ice-cold, sucrose-modified

683 aCSF using a Series 1000 Vibratome (Leica, Wetzlar, Germany; RRID:SCR_016495). The
684 OB sections were allowed to recover in an interface chamber (Krimer and Goldman-Rakic,
685 1997) with oxygenated, sucrose-modified aCSF at 33° C for 15 min and then were maintained
686 at room temperature (rt) in oxygenated normal aCSF until needed (Fadool *et al*., 2011). OB
687 slices were recorded in a continuously-perfused (Ismatec; 1-2 ml/min), submerged-slice
688 recording chamber (RC-26, Warner Instruments, Hamden, CT, USA) with aCSF at rt.
689
690 OB slices were visualized at 10x and 40x using an Axioskop 2FS Plus Microscope (Carl Zeiss
691 Microimaging, Thornwood, NY, USA) equipped with fluorescent excitation and infrared
692 detection capabilities (CCD100; Dage-MTI, Michigan City, IN, USA). Electrodes were
693 fabricated from borosilicate glass (#1405002; Hilgenberg GmbH, Malsfeld, Germany) to a
694 diameter of ~2 μm to yield pipette resistances ranging from 4 to 8 MΩ. Positive pressure was
695 retained when navigating through the OB laminae until a high resistance seal (1.5–10 GΩ)
696 was obtained on a positionally-identified MC or sTC in the slice (Fadool et al., 2011). The
697 morphology and biophysical properties of the neurons were used to distinguish MCs and sTCs
698 (Nickell et al., 1996; Schoppa and Westbrook, 2001; Fadool et al., 2004; Fadool and Kolling,
699 2020). MCs were selected only if > 50% of their cell body resided within the MCL. sTCs
700 were selected only if their cell body resided in the outer one-third of the EPL, and were not
701 selected if their cell body partially resided within the GLM. In addition, GFP mice provided
702 fluorescent markers that served as secondary confirmation of cell identity, and were readily
703 distinguishable. The cell-attached configuration was established by applying gentle suction to
704 the lumen of the pipette while monitoring resistance. Whole-cell access was obtained by
705 applying a 100 ms, 1 mV pulse across the membrane while applying sharp suction to the
706 lumen of the pipette. Membrane voltage and current properties were generated using
707 pCLAMP, version 9 or 10, in conjunction with a Multiclamp 700B amplifier (Axon
708 Instruments; RRID:SCR_011323/Molecular Devices; RRID:SCR_018455). The analog signal
709 was filtered at 3 kHz and minimally digitally sampled every 100 μs with a Digidata 1440A
710 digitizer (Axon Instruments, Molecular Devices; RRID:SCR_021038). The pipette
711 capacitance was electrically compensated through the capacitance neutralization circuit of the
712 Multiclamp 700B amplifier. Resting membrane potentials were corrected for a calculated -7
713 mV junction potential offset. Membrane capacitance was acquired from the membrane test

714 function of Clampex 10.7 (Axon Instruments, RRID:SCR_011323). Input resistance was
715 calculated using Ohm's law by injecting a -50 pA current and measuring the induced voltage
716 deflection. For clarity across disciplines, this report uses the electrophysiological terminology
717 that is used by the pCLAMP software suite. Where applicable, other common names are
718 included in parentheses.
719
720 After establishing a whole-cell configuration, cells were first sampled for adequate resting
721 potential (less than -50 mV), proper access resistance (less than 40 MΩ), and adequate input
722 resistance (greater than 150 MΩ) prior to initiating either a series of current- or voltage-clamp
723 recordings. For current-clamp recordings, cells were held at -60 mV (near rest; V_h) between
724 pulses to compare data across cells. Peri-threshold current levels were determined by
725 incrementally injecting a family of current pulses of 1000 millisecond (ms) duration (P_d).
726 Current was injected every 30 seconds (s) and was stepped in 25 pA increments from -50 pA
727 to 175 pA to obtain action potential (AP) characteristics and current-dependence data.
728 Following the determination of spike threshold, cells were stimulated with 10 super-threshold
729 sweeps using a P_d of 5000 ms (typically ranging from 15 to 150 pA) every 30 s (T_s) to acquire
730 spike frequency and latency data. Current-clamp data were acquired in the presence of
731 synaptic blockers and voltage-clamp data were acquired in the presence of 20 μM TTX to
732 eliminate unwanted AP spiking (see Solutions).
733
734 AP characteristics, latency to first spike, spike event frequency, interspike interval, intratrain
735 interval, and spike train length were measured using Clampfit software as described
736 previously (Balu et al., 2004; Fadool et al., 2011; Mast and Fadool, 2012). A train was defined
737 as three or more consecutive spikes within a period of 100 ms or less, as established by Balu
738 et al. (Balu et al., 2004). M/TC firing is intrinsically intermittent and is characterized by
739 variable train characteristics. As such, classical means of computing spike timing variability,
740 such as peri-stimulus time histograms, were less suitable for the behavior of these neurons.
741 Therefore, alternative means of spike analysis were applied as described previously (Balu et
742 al., 2004; Fadool et al., 2011; Thiebaud et al., 2016; Kolling et al., 2022). For voltage-clamp
743 recordings, cells were held at -80 mV (V_h), then stepped from -100 mV to +40 mV (V_c) in
744 +20 mV increments for a duration of 400 ms (P_d) with a stimulation interval (T_s) of 45 s.

745

746 **RNA scope and quantification**

747 Five male and five female GFP mice (10 mice total) were anesthetized with ketamine/xylazine
748 and fixed via transcardial perfusion of PBS followed by 4% PFA. Brains were cryoprotected
749 in 30% sucrose before being embedded in OCT and flash frozen. Mouse brains were
750 cryosectioned coronally at 16 µm, and sections were collected 1:10. Sections were mounted
751 on histobond glass slides, and RNAscope was performed according to the ACDbio
752 manufacturer's protocol using commercially available probes for *GFP, Kv1.3* and *Insr*. The
753 sequence of the mouse *GFP* transgene was used to verify the selection of GFP RNAscope
754 probe. Images were acquired using an Olympus VS200 slidescanner at 20X, and analysis was
755 performed using QuPath software (v 0.5). The 'cell detection' function was first used to
756 identify Tbx21+ cells, then object classifiers were created for *Kv1.3* and *Insr* using a threshold
757 for mean fluorescent intensity within each cell. Validation of this workflow was performed
758 manually, once per neurolaminar layer (MCL, EPL, and GLM).

759

760 **Experimental design and statistical analyses**

761 Before performing any statistical comparisons, data were first analyzed with the Dixon's Q
762 test to identify any outliers. Data were then checked for normal distribution and homogeneity
763 of variance using the Fmax test. Analysis of data collected did not identify any outliers.
764 Where collected data violated homogeneity of variance (failed the Fmax test), the selection of
765 applied statistics was changed to the nonparametric equivalent. Paired comparisons (innate
766 properties, action potential kinetics, bursting metrics) were analyzed using Student's *t* test or
767 Mann-Whitney U at the 95% confidence level ($\alpha < 0.05$). There were no sex-specific
768 differences in the measured parameters, so measurements from both sexes were combined. IV
769 plots, frequency and interspike interval (ISI) were analyzed using a mixed two-way RM
770 ANOVA with current and voltage as factors (IV) or current and cell type as factors (frequency
771 and ISI); at the 95% confidence level ($\alpha < 0.05$). Modulation of voltage-activated current
772 between cell types was analyzed using a mixed three-way RM ANOVA with voltage, cell
773 type, and drug as factors; at the 95% confidence level ($\alpha < 0.05$). Insulin modulation data
774 were analyzed using within-cell comparisons by applying a paired *t* test at the 95% confidence
775 level ($\alpha < 0.05$). Categorical AP spike train type analysis was performed using a Chi-Square

test, where the expected proportion of sTCs eliciting spike trains was set as the observed proportion of MCs eliciting spike trains; 95% confidence level ($\alpha < 0.05$). For the two-way RM ANOVA tests, the Sidak method for multiple comparison testing was used as the post-hoc analysis to make mean-wise comparisons between cell types or between drug treatments. All reported values in the text represent the mean ± SD. Figures largely report individual data points using violin graphs and represent number of cells as noted in the figure legends. Sample sizes for the line graphs are reported in the figure legends. Where means are reported in the figures, the error is reported as the SEM. Individual *p* values are reported for each experiment within the corresponding graph with the individual F statistic described in the results section. Statistical tests were performed using GraphPad Prism 9 (GraphPad Software) while the graphs and figures were produced using a combination of Origin v8 (OriginLab Corporation; RRID:SCR_014212), Adobe Photoshop CS4 (Adobe; RRID:SCR_014199), and GraphPad Prism 9 (GraphPad Software; RRID:SCR_002798).

All electrophysiological data were analyzed using pClamp, version 10 (Clampfit 10.7; Molecular Devices; RRID:SCR_011323) and Igor Pro, version 6.12A (WaveMetrics Inc.; RRID:SCR_000325) with the plug-in NeuroMatic, version 2 (written by Jason Rothman; http://www.neuromatic.thinkrandom.com; RRID:SCR_004186). Exact *p* values, statistical tests, and parameters are given in the results text/figures for each data set. Specific variables required for independent replication (sample size, number of animals, etc.) and full statistical reporting can be found in the methods and results text/figures for each respective experiment and the supplemental statistical table (Supplemental Table 1).

Figure Legends

Fig. 1. **Categorization and interactions of the principal projection neurons of the olfactory bulb.** The olfactory bulb neurolamina is divided across the glomerular layer (GLM), external plexiform layer (EPL), the mitral cell layer (MCL), and the innermost granule cell layer (GCL). Also shown is the olfactory mucosa containing the olfactory sensory neurons (OSNs). This schematic highlights the four classes of output or projection neurons, namely the external tufted cells (eTCs, pink), the superficial tufted cells (sTCs, gray), the middle tufted cells (mTCs, purple), and the mitral cells (MCs, red). The soma of the eTCs are found within the GLM. The sTC soma reside in the superficial one-third of the EPL. The deep two-thirds of the EPL contain both the mTCs and misplaced MCs. The MCL houses the eponymous MCs. Blue plus signs (+) represent excitatory synapses, red minus signs (-) represent inhibitory synapses, and pink plus signs (+) represent excitatory dendro-dendritic interactions originating from external tufted cells (eTCs). Reciprocal synapses are denoted by a double arrow. PG = periglomerular cell, sSAC = superficial short axon cell, EPLi = interneuron of the external plexiform layer, GCL = granule cell layer, GC = granule cell, PPG = preproglucagon neuron, dSAC = deep short axon cell. *Modified from Huang* et al. *2021.*

Fig. 2. Outward voltage-activated current properties of mitral cells and superficial tufted cells. ***A,*** Family of voltage-activated currents, V_h = -80 mV where V_c was stepped in 20 mV increments from -100 mV to +40 mV using a P_d = 400 ms. Colored sweeps correspond to significantly different colored data points in (***B***). ***B,*** Normalized IV plots (2-w mixed RM ANOVA, cell type ***p = 0.0056, Sidak's post-hoc test, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; voltage x cell type interaction ****$p < 0.0001$). ***C,*** Violin plot of non-normalized peak transient voltage-activated current at +40 mV, Mann-Whitney U. Violin plot of the *Tau* of inactivation (*left*) and deactivation (*right*) at **(D)** Vc = -40 mV and **(E)** +40 mV, respectively, Mann-Whitney U (*p = 0.0107). MC = mitral cell population sampled, sTC = superficial tufted cell population sampled.

Fig. 3. Sensitivity of mitral and superficial tufted cells to a selective blocker of Kv channels. ***A,*** Family of voltage-activated currents recorded as in Figure 3 prior (***Top*,** Baseline) and following (***Bottom*,** +1 nM MgTx) 15-min bath application of margatoxin (MgTx) in a representative ***A,*** mitral cell (MC) and ***B,*** superficial tufted cell (sTC). ***C,*** Normalized IV plots prior (solid symbols) and following (open symbols) bath application of MgTx in a population of MCs (***Left,*** 2-w mixed RM ANOVA, drug F (1, 9) = 43.62, ****$p < 0.0001$, with a Sidak's post hoc test, $^{\#\#\#}p = 0.0008$, $^{\#\#\#\#}p < 0.0001$; voltage x drug interaction F (7, 63) = 43.86, ****$p < 0.0001$) and a population of sTCs (***Right,*** 2-w mixed RM ANOVA, drug ***p = 0.0003, Sidak's post-hoc test, $^{\#\#\#\#}p < 0.0001$; voltage x drug interaction ****$p < 0.0001$). ***C, Bottom –*** 3-way mixed RM ANOVA result comparing the two IV plots in ***C, Left-Right*** (3-w mixed RM ANOVA, voltage x cell type interaction F (7, 123) = 3.879, ***p = 0.0008; voltage x drug interaction F (7, 123) = 57.42, ****$p < 0.0001$; cell type x drug interaction F (1, 123) = 0.4244, p = 0.5160; voltage x cell type x drug interaction F (7, 123) = 0.7633, p = 0.6190).

Fig. 4. Spike firing properties compared between mitral cells (MC) and superficial tufted cells (sTC). ***A*,** Representative action potential trains evoked by various current steps as indicated, recorded from a MC (***Left***) vs. sTC (***Right***). ***B,*** Violin plot comparing the current needed to evoke action potentials (rheobase), Mann-Whitney U, **p = 0.0021. Line plots for ***C,*** mean spike frequency across current step and ***D,*** mean interspike interval (ISI). Line plot data were analyzed with a ***(C)*** 2-w mixed RM ANOVA, cell type **p = 0.0011; current x cell type interaction ***p = 0.0009, Sidak's post-hoc test,

842 $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, ***(D)*** 2-w mixed RM ANOVA, cell type ****$p < 0.0001$; current x cell type
843 interaction ****$p < 0.0001$, Sidak's post-hoc test, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

844 **Fig. 5.** Action potential shape and spike train activity compared between mitral cells (MC) and
845 superficial tufted cells (sTC). Line plot for ***A,*** maximum decay slope (max decay slope) and ***B,*** time
846 to achieve maximum decay slope from peak magnitude (time to max decay slope) across current step.
847 Line plot data were analyzed with **(A),** 2-w mixed RM ANOVA, cell type *p = 0.0186, Sidak's post-
848 hoc test, ##p < 0.01, ###p < 0.001; current x cell type interaction ****p < 0.0001, **(B),** 2-w mixed
849 RM ANOVA, cell type *p = 0.0486, Sidak's post-hoc test, #p < 0.05, ##p < 0.01, ###p < 0.001. ***C,***
850 Representative pair of action potentials recorded in response to a 75 pA current injection held near
851 rest (Vh = -60 mV). ***D,*** Representative raster plot for a cell stimulated with a 50-pA current injection
852 using a P_d = 5 s for each cell type, respectively. Successive trials are stacked vertically below. Violin
853 plots computed across a 50 s recording period (10, 5s sweeps) compare ***E,*** mean number of bursts, ***F,***
854 mean spikes per train, ***G,*** mean pause duration between trains, and ***E,*** mean latency to first spike. ***E-F,***
855 were analyzed using a Student's *t*-test, ***G-H*** were analyzed using Mann-Whitney U; $p > 0.05$. ***I,***
856 Relative proportion of cells eliciting spike train behavior among sampled MCs (***Left***) and sTCs
857 (***Right***), respectively (Chi-squared test, p = .40061, $X^2 = 0.7065$, n = 51).

858 **Fig. 6.** Colocalization of M/TCs with the Kv1.3 channel (*Kv1.3*) and insulin receptor (*Insr*) using
859 RNAscope. Photomicrographs demonstrating neurolaminar distribution of Tbx21 reporter expression
860 (green, ***A***), *Kv1.3* expression (magenta, ***B***), and *Insr* expression (yellow, ***C***), separately and merged
861 (white, ***D***). ***E–H***: Same as ***A–D***, but to demonstrate localization to sTCs with the inclusion of a DAPI
862 nuclear stain. ***I:*** Coronal sections were sampled along the anter-posterior axis (AP axis) at ~512 µm
863 intervals and assessed for total number of M/TCs (Tbx21+ cells) and for percentage of M/TCs
864 expressing both *Kv1.3* and *Insr* (***J***). Each data point represents the average from 5 separate mice,
865 separated by laminar layer and sex. ***K–M:*** Percentage of M/TCs (Tbx21+ cells) expressing *Kv1.3*
866 alone (***K***), *Insr* alone (***L***) or both (***M***). Statistical comparisons for ***K–M*** are Kruskal-Wallis (ANOVA)
867 with Dunn's multiple comparisons test. Each data point represents the average of all six antero-
868 posterior sections from each mouse, one data point per mouse per neurolaminar layer, respectively. $^{*}p$
869 < 0.05, GLM = glomerular layer, EPL = external plexiform layer, GCL = granule cell layer.

870 **Fig. 7.** Bath application of insulin increases spike firing frequency, and shortens latency to first spike
871 and intraburst interval in superficial tufted cells (sTCs). ***A,*** Representative spike trains (***Top***) that
872 were elicited in response to 25 pA current injection while holding the cell near rest (V_h = -60 mV)
873 prior (***Left,*** Baseline) and subsequent (***Right,*** Insulin) to hormone stimulation using P_d = 5 s.
874 (***Bottom***) Representative associated raster plot. Line graph comparing ***B,*** latency to first spike, ***C,***
875 spike firing frequency, and ***D,*** intraburst interval, prior (pre) and following (post) insulin stimulation
876 in the same cell. Data were analyzed with a paired *t*-test using non-normalized data ***(B)*** $*p = 0.0132$,
877 ***(C)*** $*p = 0.0381$, and ***(D)*** $*p = 0.0136$.

878 **Fig. 8.** Insulin fails to modulate spike firing frequency in superficial tufted cells of Kv1.3-/- mice. ***A,***
879 Representative spike trains (***Top***) and associated raster plot (***Bottom***) recorded as in *Figure 9*. Line
880 graphs Line graph comparing ***B,*** latency to first spike, ***C,*** spike firing frequency, and ***D,*** intraburst
881 interval, prior (pre) and following (post) insulin stimulation in the same cell. Data were analyzed with
882 a paired *t*-test using non-normalized data, $p > 0.05$.

883 **Table 1.** Action potential shape compared between mitral cells (MCs) vs. superficial tufted cells
884 (sTCs). Recordings were made while holding cells near rest (V_h = -60 mV) and injecting 75 pA of

current. Values are mean ± standard deviation. ns = not significant, †= Student's *t*-test, ‡= *t*-test with Welch's correction, ⊤= Mann-Whitney U.

Table 2. Spike bursting properties in superficial tufted cells (sTCs) compared between wildtype (WT) and Kv1.3 -/- mice. Recordings were made while holding cells near rest (V_h = -60 mV) and injecting 75 pA of current. Data were computed across a 50 s recording period (10, 5s sweeps). Values are mean ± standard deviation. ns = not significant, †= Student's *t*-test, ‡= *t*-test with Welch's correction, ⊤= Mann-Whitney U

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COMPETING INTERESTS

The authors have no interests to declare, scientific or financial.

AUTHOR CONTRIBUTIONS

All authors critically evaluated the work for important intellectual content. All authors acquired, analyzed, or interpreted data in the study. As such, we confirm that all approved the final version of the manuscript; agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; and all persons designated as authors qualify for authorship, and all who qualify for authorship are listed. In using the CRediT (Contributor Roles Taxonomy) guidelines, L.K. and D.F. were responsible for project conceptualization, data curation, formal analysis, funding acquisition, methodology, validation, visualization, and writing the original draft, reviewing, and editing. L.K. was responsible for electrophysiological recordings. C.M. and D.F. were responsible for project administration and supervision. L.K., C.M. and D.F. were responsible for the provision of resources.

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DATA AVAILABILITY

All data referenced in this report are summarized in the primary figures contained in the publication. All summarized electrophysiological recordings data are stored in the Fadool Laboratory and are available upon request.

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Supplemental figures

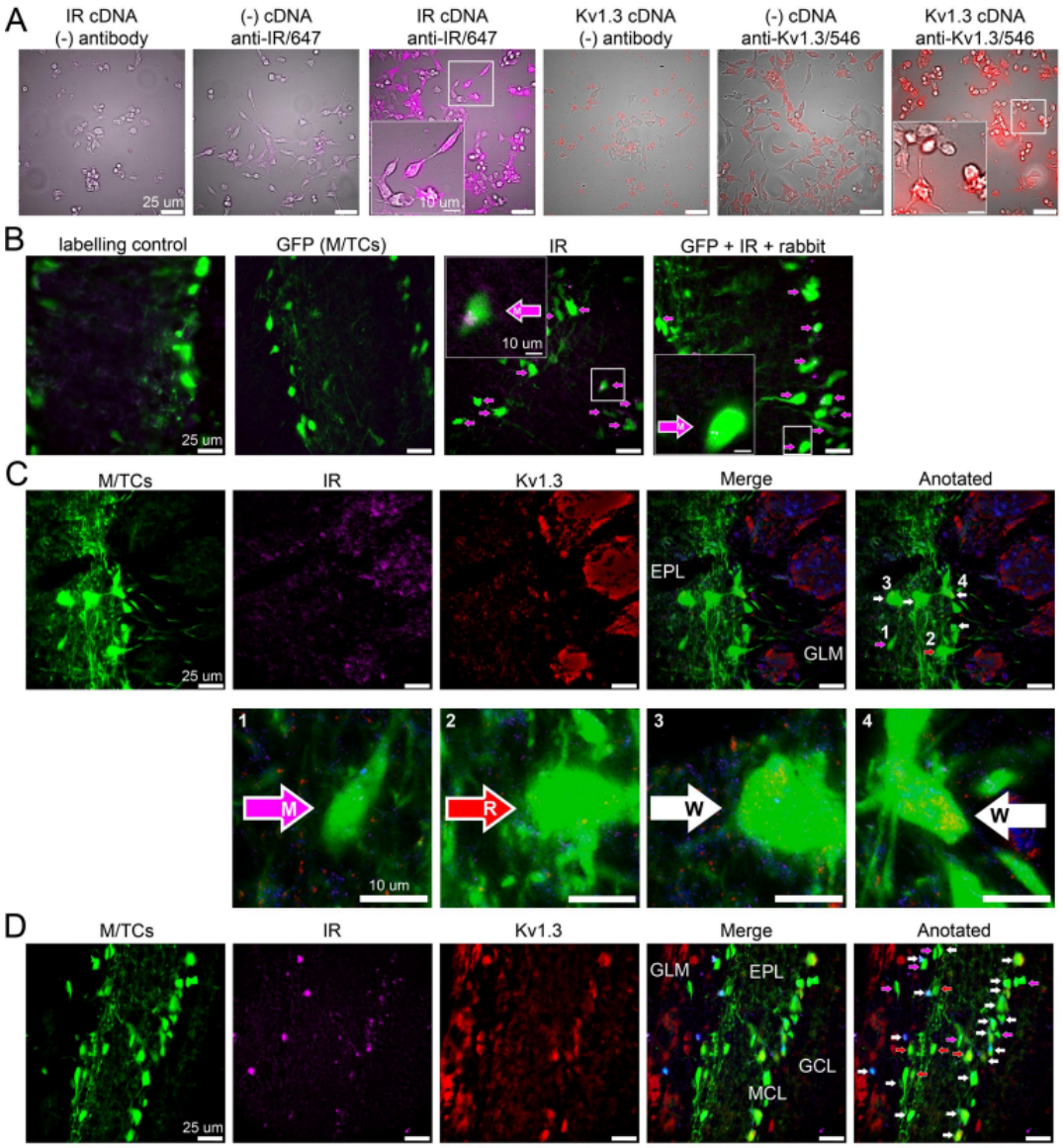

Fig. S1. Colocalization of the insulin receptor (InsR) and Kv1.3 channel (Kv1.3) in superficial tufted cells (sTCs) and mitral cells (MCs). Photomicrographs demonstrating antisera control experiments in ***A,*** HEK 293 cells (*in vitro* specificity controls) and ***B,*** coronal mouse olfactory

1164 bulb sections acquired from GFP mice (*ex vivo* controls where transgenic GFP labels M/TCs
1165 under control of the Tbx21 promoter; green). ***A,*** Cells were transiently transfected with cDNA
1166 encoding the InsR receptor, the Kv1.3 channel, or were mock transfected, and then were
1167 incubated with primary antisera or the omission of primary antisera (***panels 1-6***). ***B,*** Olfactory
1168 bulb sections were incubated with (***panel 1***) omission of primary and secondary antisera,
1169 (***panel 2***) antisera against GFP, (***panel 3***) antiserum against GFP + InsR, or (***panel 4***)
1170 antiserum against GFP + InsR + anti-rabbit. ***C-D,*** Photomicrographs demonstrating labeling
1171 in coronal mouse olfactory bulb sections acquired from GFP mice using both InsR and Kv1.3
1172 antiserum. ***C,*** Separate channels were imaged for endogenous GFP+ M/TCs (***panel 1***), InsR
1173 (***panel 2***), Kv1.3 (***panel 3***), Merge (overlayed green + magenta + red, respectively, yields
1174 cyan; ***panel 4***), and Annotated (see arrow code below, ***panel 5***). ***D,*** Same as in (C), but for
1175 tufted cells and glomeruli (***panel 1***), InsR (***panel 2***), Kv1.3 (***panel 3***), Merge (glomeruli
1176 overlayed magenta + red yields violet; ***panel 4***), and Annotated (***panel 5***).
1177
1178 **Supplemental methods**
1179
1180 **cDNA constructs and antisera**
1181 Insulin receptor (IR) cDNA was a gift from Dr. R. Roth, Stanford University, in the pECE
1182 vector. The entire IR gene was removed using the unique restriction sites Sal I and Xba I, and
1183 ligated into pcDNA3 between the Xho I and Xba I sites in the multiple cloning region under
1184 control of the cytomegalovirus (CMV) promoter (Fadool *et al*., 2000). Rat Kv1.3 (rKv1.3)
1185 was subcloned into the multiple cloning region of $pcDNA_3$ (now available only as 3.1;
1186 RRID:Addgene_23252; originally 3.0 Invitrogen, Carlsbad, CA) at the unique HindIII
1187 restriction site, under control of the CMV promoter (Holmes et al., 1996).
1188
1189 Monoclonal antibodies targeting the beta subunit of the IR or green fluorescent protein were
1190 purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-IR antibody was
1191 purchased pre-conjugated to Alexa Fluor 647 (Anti-INSR catalog # sc-57342 AF647;
1192 RRID:AB_784102); validated by Wu and associates (Wu et al., 2019). Anti-IR antibody was
1193 generated against the C-terminus of the beta subunit of human insulin receptor protein. Anti-
1194 GFP antibody was purchased pre-conjugated to Alexa Fluor 488 (catalog # sc-9996 AF488;

1195 RRID:AB_627695); validated by Kwon and associates (Kwon et al., 2020). Anti-GFP was
1196 generated against amino acids 1-238, representing full length GFP. FSU120, a rabbit
1197 polyclonal antiserum, was generated against the 46-amino acid sequence 478-
1198 MVIEEGGMNHSAFPQTPFKTGNSTATCTTNNNPNDCVNIKKIFTDV-523, representing
1199 the unique coding region of the Kv1.3 channel between transmembrane domain 6 and the
1200 carboxy terminal (Fadool *et al*., 2000). The purified peptide was produced by Genmed
1201 Synthesis (San Francisco, CA, USA), the antiserum was produced and then affinity purified
1202 by Cocalico Biologicals (Reamstown, PA, USA). We have validated this epitope in several
1203 previous studies (Fadool *et al*., 2000, 2004; Colley *et al*., 2007; Marks and Fadool, 2007; Biju
1204 *et al*., 2008; Marks *et al*., 2009) and this latest regeneration of the antisera, specifically (Velez
1205 *et al*., 2016; Kolling *et al*., 2022). Alexa Fluor 546 (Invitrogen, catalog #A-11035;
1206 RRID:AB_2534077) was used to label the FSU120 antibody for imaging.
1207

1208 **Culturing, immunocytochemistry, and immunohistochemistry**

1209 Human embryonic kidney 293 (HEK 293; RRID:CVCL_0045) cells were used that are not
1210 listed as a commonly-misidentified cell line at the International Cell Line Authentication
1211 Committee Registry (ICLACR). Our HEK 293 cells were a generous gift from Dr. Robert
1212 Margolskee (Monell Chemical Senses Center, Philadelphia, PA, USA) and have been
1213 authenticated for cross-contamination and misidentification by profiling the cellular genome.
1214 Cell culture practices at Florida State University have followed "Guidance on Good Cell
1215 Culture Practices" (Coecke et al., 2005). Our most recent cryopreserved batch of HEK 293
1216 cells was prepared in 2016, and each aliquot is used between passages 4 and 25. HEK 293
1217 cells were grown in supplemented Modified Eagle Medium composed of minimal essential
1218 media (MEM; 12360; GIBCO, Life Technologies/ Invitrogen, Durham, NC) plus 2%
1219 penicillin/streptomycin (15140; GIBCO, Invitrogen) and 10% fetal bovine serum (16000;
1220 GIBCO, Invitrogen). Before transient transfection, cells were grown to 90% confluency (7
1221 days), dissociated with trypsin-EDTA (Sigma-Aldrich) and mechanical trituration, diluted in
1222 MEM to a concentration of 600 cells/mL, and replated on Corning dishes (catalog no. 25000;
1223 Thermo Fisher Scientific). To accommodate imaging on a glass substrate, two, 12 mm circle
1224 glass coverslips (Catalog #12-545-90, Thermo Fisher Scientific) were placed flat inside each
1225 35 mm dish. cDNA was introduced into HEK 293 cells with Lipofectamine 3000 transfection

1226 reagent (Invitrogen catalog #L3000015) in OptiMEM serum-reduced media
1227 (Invitrogen/GIBCO) 3-5 days after passage as previously described (Cook and Fadool, 2002).
1228 Briefly, cells were transfected for 4 hours (h) with 1.0 □g of each cDNA construct per 35-mm
1229 dish. Plasmid DNA with no coding insert (control vector) served as the control to equalize
1230 total ug of cDNA added to each dish/slip. Thirty-six hours after transfection, cover slips were
1231 placed "cells up" on glass slides and fixed in 4% PFA for 20 min at rt. Cells were rinsed in
1232 two 5-min changes of 1X PBS before proceeding with immunocytochemistry. Cells were first
1233 blocked in blocking buffer for 30 min followed by three 5-min changes of 1X PBS. Antibody
1234 labeling for GFP and IR was performed using a 1:50 dilution in incubation buffer for 60 min
1235 at rt. Labeling for Kv1.3 was performed using a 1:50 dilution in incubation buffer for 90 min
1236 at rt, followed by secondary labeling with a species-specific antibody at a 1:500 dilution in
1237 incubation buffer for 45 min at rt.
1238
1239 Immunohistochemistry (IHC) was performed in the same manner as immunocytochemistry.
1240 GFP mice were anesthetized and then terminated by cardiac perfusion using 4% PFA as
1241 previously described (Thiebaud et al., 2014). The mice were decapitated, and skulls
1242 decalcified to flash freeze the brain in protective medium (Biju *et al*., 2008). Coronal sections
1243 at 14-µm thickness (Model 1850UV, Leica Biosystems) were prepared for slide-mounting.
1244 Labeling control consisted of blocking and incubation in buffer without antibodies. No-
1245 primary (omission of FSU120 primary antisera) and singly-labeled controls were performed to
1246 determine the specificity of the anti-rabbit secondary antibody with pre-conjugated antibodies.
1247 Slides were cover-slipped with Fluoromount-G™ (Invitrogen) before imaging. Imaging was
1248 performed on a Leica confocal microscope (THUNDER Imager, Wetzlar, Germany;
1249 RRID:SCR_023794) using a 40X oil-immersion lens, and appropriate laser lines for each
1250 reporter (475 nm, 551 nm, 642 nm). Post-hoc analyses were performed using Photoshop CS4
1251 (Adobe, San Jose, CA, USA; RRID:SCR_014199). The minimum and maximum thresholds
1252 for each color channel were adjusted uniformly for all images before using the "difference"
1253 feature to align multidimensional images in Photoshop. This method allowed for transient
1254 removal of specific channels in order to more clearly determine co-localization of reporters.
1255