Supporting Information

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SI Materials and Methods

Preparation of Solutions. The medium for culturing ventral respiratory column (VRC) neurons, Eagle's minimal essential medium (MEM) + supplements, was MEM (Invitrogen) supplemented with 10% (vol/vol) FBS (HyClone), penicillin (100 U/mL), streptomycin (100 μg/mL), 1× Glutamax (Invitrogen), 10 mM Hepes, and 0.4% (wt/vol) glucose. The medium was adjusted to pH 7.4 with NaOH and then was filtered through a 0.22-μm filter under sterile conditions. Before use, the medium was stored at 4 °C. It was warmed to 37 °C in a tissue-culture incubator just before use.

We used artificial cerebrospinal fluid (aCSF) for tissue preparations (brain slices and microdissection) and calcium-imaging experiments. It consisted of the following (in mM): 118 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1 NaH_2PO_4, and 30 D-glucose. For use in tissue preparations, we bubbled carbogen (95% O_2 and 5% CO_2) through the aCSF to oxygenate the solution and to adjust its pH to 7.4. For use in calcium-imaging experiments, aCSF was adjusted to pH 7.4 with HCl and then was stored at 4 °C until used in experiments at room temperature.

All stock solutions of pharmacological agents were stored at −20 °C in small-volume aliquots to avoid repetitive freezing and thawing. All working concentrations were obtained by diluting stock solutions into aCSF. The following stocks were kept in aqueous solutions, typically either physiological saline or water: 2 mM histamine dihydrochloride (Acros Organics), 1 mM ATP disodium salt trihydrate (Sigma-Aldrich), 1 M acetylcholine chloride (Sigma-Aldrich), 200 μM substance P (Peptides International), 1 mM bradykinin, 20 mM norepinephrine, 3 M glutamate, 1 M atropine. α-Conotoxin ArIB[V11L;V16D] was not stored in solution. PNU-120596 stocks were 100 mM in DMSO. Fura-2-acetoxymethyl ester (Fura-2-AM; Invitrogen) stocks were 1 mM in DMSO, distributed into single-use aliquots and stored at −20 °C.

Mouse Cell Preparations and Culture. Preparation and culture of mouse dorsal-root ganglion (DRG) cells was reported previously in detail (1, 2). The following methods apply to preparation and culture of mouse VRC cells. All procedures in this study comply with the rules and regulations in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (3) and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Utah Health Sciences Center. WT C57BL/6 mice at postnatal day (P) 7-8 were anesthetized by rapid hypothermia on ice before rapid decapitation.

We prepared a homemade dissecting plate by pouring silicone elastamer (Sylgard) into a 100-mm-wide tissue-culture dish and allowed the silicone elastamer to harden. The head was pinned to the Sylgard plate, and the skin and the connective tissues were removed. The skull was cut along the sutures with fine scissors to separate the interparietal region of the skull and expose the superior colliculus, inferior colliculus, and cerebellum. Ice-cold aCSF was applied to keep the tissue moist and cold. A one-sided razor blade was used to make a deep cut along the interface between the inferior colliculus and cerebellum, and the cerebellum was removed to isolate the brainstem. The brainstem and upper cervical spinal cord were isolated in ice-cold aCSF bubbled with carbogen. The brainstem was glued to a slant agar block using cyanoacrylate with its rostral end up and its dorsal side attached to an agar block, which was mounted and secured on a specimen tray. The agar block was cut at a slant forming a 110° angle between the agar block surface and the horizontal specimen tray (a 20° angle from vertical). The vibratome blade was set at a 20° angle below horizontal so that it sliced at a right angle to the surface of the agar block. Slicing at this angle enabled us to observe the projections from the pre-Bötzinger complex (preBötC) to the XII nucleus and from the XII nucleus to the rootlets.

We sectioned the brainstem serially in a rostral-to-caudal direction. The first slice was cut at a thickness of 200 μm from the top (rostral end). From this point onwards, slices were made to observe facial nerves, which were the first landmark. The slice width varied with preparations; usually a slice of 400–500 μm from the top had to be removed to observe facial nerves. These facial nerves were observed over a thickness of 250–300 μm. Thus, a slice of 300 μm was made from the point of observing facial nerve. The next landmark was the opening of ventricle at the interface of the brainstem and the agar block. A slice of ∼300–400 μm was cut to reveal the opening of the ventricle. From this point, 200-μm slices were cut until the ventricle closed. The last three slices before the complete ventricle closure were taken and were designated "rostral," "medial," and "caudal," respectively. To ensure that we were cutting at the level of the preBötC, we calculated the percentage of class B cells responsive to substance P (a marker for preBötC) within dissociated cell cultures obtained from each slice: As expected, the 200-μm-thick medial slice, on average, had the highest percentage of substance P-responsive cells (8.6%) when compared with the rostral (7.3%) and caudal (4.1%) slices ($n = 7$ preparations). This finding suggested that the medial slice, on average, was near the center of the preBötC. In these slices, a faint hypoglossal nerve was observed, and the nucleus ambiguus sometimes was observed in the rostral slice.

The medial slice was placed in ice-cold HBSS, where the segments that contained the VRC and preBötC were microdissected and then transferred with a large-diameter fire-polished Pasteur pipette to a 15-mL conical tube. The total volume was adjusted to 900 μL with HBSS. Then 100 μL of 2.5% (wt/vol) trypsin was added [for a working concentration of 0.25% (wt/vol) trypsin)], and the tube was incubated at 37 °C in a water bath for 3–4 min. After incubation, the intact tissue segments were washed three times with 4 mL MEM + supplements. Each wash was performed by adding 4 mL MEM + supplements, allowing microdissected segments to settle to the bottom of the tube, and removing as much medium as possible with a fire-polished Pasteur pipette while avoiding the accidental removal of any tissue. After the final wash, the tissue segments were resuspended in 1.5 mL MEM + supplements. The VRC suspension was triturated 5–10 times (or until there was no resistance) through a series of fire-polished pipettes, where each successive pipette had a smaller tip diameter. The solution became cloudy as individual cells dissociated from the slice fragments.

The cell suspension was centrifuged at 50 × *g* for 10 min. After centrifugation of the cell suspension, the supernatant was removed by aspiration, leaving behind the volume of medium required to plate dissociated VRC neurons, typically into six wells (130 μL total). Neurons were resuspended in the remaining medium by gentle trituration with a 100-μL disposable plastic pipette tip. In several of the inner wells of a 24-well plate, 20 μL of the cell suspension was placed in the center of a silicone ring (3 mm i.d.) that was attached to the floor of each well as described previously (2). The wells at the edge of the plate were half-filled with sterile water to humidify the culture. Each plate was placed in the 37 °C incubator for 45–60 min to allow cells to settle and adhere. Then 1 mL MEM + supplements solution was added very gently at the edge of each well to avoid dislocating

any loosely adherent cells within the silicone ring. The plates were placed in a 37 °C, 5% CO_2 tissue-culture incubator, and the cultures were used for imaging after 18–36 h.

Calcium Imaging. We have described the calcium-imaging methods in detail previously (1, 2). Briefly, the cells were loaded with Fura-2-AM in their growth medium for 1 h at 37° C, followed by 30 min at room temperature; then the medium containing Fura-2-AM was replaced with aCSF at room temperature for calcium imaging. Changes in cytosolic calcium concentration, $[Ca^{2+}]_i$, were monitored over time by standard ratiometric calcium-imaging methods, i.e., the ratio of fluorescence intensities at 510 nm obtained from intermittent (typically once every 2 s) excitation by 340-nm and 380-nm light (labeled as "340/380 nm" in the *y*-axis of calcium-imaging figures and described as "340/380-nm ratio" in the text). Upward or downward deflection of a calcium-imaging trace represents an increase or decrease in $[Ca^{2+}]_i$, respectively. In all figures, arrows indicate a 15-s application of the specified compound or other perturbation. Horizontal bars indicate when other compounds were present in the bath solution.

Slice Electrophysiology. Electrophysiological experiments were performed in accordance with the protocols approved by the IACUC of the Seattle Children's Research Institute. Transverse medullary brainstem slices were taken from male and female P7–10 mice with a C57BL/6 background as described previously (4). Briefly, animals were anesthetized with 4% isoflurane and were decapitated rapidly, and the brainstem was isolated in ice-cold aCSF equilibrated with carbogen (95% O_2 and 5% CO_2), pH 7.4, 305–312 mOSM. The brainstem was glued to an agar block on the mounting plate of an Electron Microscopy Sciences vibratome. The rostral face of the experimental slice was ∼530 μm caudal to the opening of the fourth ventricle. From this landmark, a 550- to 580-μm slice that contained the preBötC was cut. The slice was transferred to a recording chamber with circulating aCSF (flow rate 10–15 mL/min, total circulating volume 100 mL) and was allowed to equilibrate to experimental temperature (30–33 °C). The population rhythm was stimulated by elevating the extracellular potassium concentration from 3 to 8 mM over the course of 30 min.

Extracellular recordings were obtained with glass suction electrodes (tip resistance <1 MOhm) filled with aCSF placed on the slice surface over the ventral respiratory column containing the preBötC. The recorded signal was sampled at 1.67 kHz, amplified 10,000×, filtered (low pass, 1.5 kHz; high pass, 250 Hz), rectified, and integrated using an electronic filter. Blind whole-cell patch-clamp recordings were obtained in current clamp configuration using a Molecular Devices Multiclamp 700B amplifier sampling at 20 kHz. Patch electrodes were pulled (P-97 Flaming/Brown micropipette puller; Sutter Instrument Co.) from borosilicate glass with a resistance of 6–12 MΩ. The electrodes were filled with a saline solution containing (in mM): 140 K-gluconic acid, 1 $CaCl_2$, 10 EGTA, 2 $MgCl_2$, 4 Na_2ATP, 10 Hepes (pH = 7.4). Both extracellular and intracellular recordings were obtained with Clampex 10.0 data acquisition module (Molecular Devices). When an intracellular recording was established, the inspiratory neuron was isolated pharmacologically from fast synaptic transmission by applying the mixture of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione, 10 μM 4-(3-phosphonopropyl)piperazine-2-carboxylic acid, 50 μM picrotoxin, and 1 μM strychnine to inhibit AMPA glutamate receptors, NMDA glutamate receptors, $GABA_A$ receptors, and glycinergic receptors, respectively. Once the inspiratory neuron was isolated, the agonists substance P (1 μM), bradykinin (10 μM), and histamine (10 μM) were applied sequentially to the circulating medium at 10-min intervals. Recordings were stored on a computer for post hoc analysis. All drugs were obtained from Sigma Aldrich or Tocris Bioscience.

Statistical Data Analysis. The values shown under the heading "Average response to 100 mM $[K^+]_o$ ± SD" in Table 2 were calculated using all the individual responses to 100 mM $[K^+]_o$ of class A, B, and C cells from five independent experimental trials, with the SD calculated from all the individual responses. The quantification of the response shown in Table 2 is the change in the magnitude of the 340/380-nm ratio elicited by 100 mM $[K^+]_o$, as described above and as depicted in the *y*-axis of traces from Fig. 1. Larger numbers indicate a greater response or greater relative change in $[Ca^{2+}]_i$ elicited by 100 mM $[K^+]_o$. The *P* value reported in *Results* for the difference in the average response to 100 mM $[K^+]_o$ between class A and B cells was derived as follows: For each independent experimental trial (each trial included more than 100 cells), a sample mean for the response to 100 mM $[K^+]_o$ was calculated separately for class A and B cells. Then the sample means for class A and B cells from independent experimental trials were compared using Student *t* test (two-tailed, paired samples).

For the cluster analysis summarized in Table S1, 1,586 cells were scored for their responses to each of eight stimuli. Responses were scored as 0 = no response or as 1 = response. A response to 100 mM $[K^+]_o$ was defined as a maximum peak height ≥ 0.1 on the *y*-axis scale of the calcium-imaging experiments (340/380-nm ratio, as described above). A response to all other stimuli was defined as a maximum peak height ≥0.05 on the *y*-axis scale of the calcium-imaging experiments (340/380-nm ratio). The binary cell-response data were clustered using the pam function (5) in the cluster library of R (6). The manhattan distance function was used to define dissimilarities between pairs of cells which correspond to the number of scored differences between any two cell profiles. Five hundred bootstraps were used to establish the robustness of the medoids (prototype response profiles, as shown in Table S1) selected by the pam function. For each bootstrap trial, a random set of 1,586 cell-response profiles was selected with replacement and used for input into the pam function. The amount of variation explained by clustering was calculated as 1 − (diss)/n, where diss is the total dissimilarity remaining in the clusters and *n* is the total number of cell scores.

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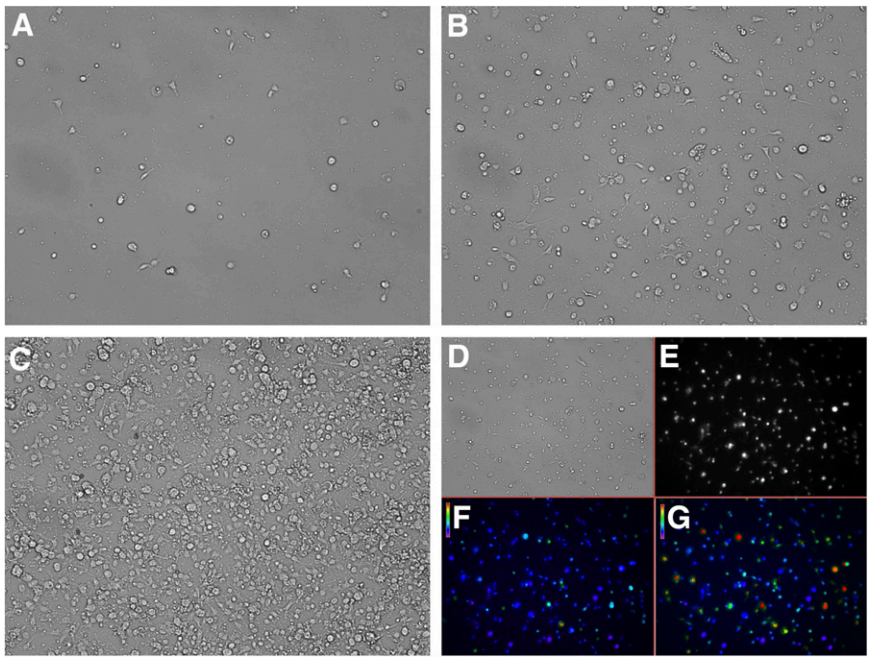


Fig. S1. Bright-field (*A*–*D*) and fluorescence (*E*–*G*) images of dissociated VRC cells in culture. Shown are cell cultures of inadequate, optimal, or excessive density for calcium-imaging experiments. (*A*) Inadequate density, 400 cells/mm^2. (*B*) Optimal density, 800 cells/mm^2. (*C*) Excessive density, 2,000 cells/mm^2. (*D*–*G*) All panels show the same field of view. (*D*) Bright-field image of dissociated VRC cells. (*E*) Fluorescence image of the cells loaded with Fura-2-AM dye (380-nm excitation and 510-nm emission). (*F*) Pseudocolored ratiometric image of cells at rest. The ratiometric image is a relative measure of $[Ca^{2+}]_i$ and is obtained from the ratio of fluorescence intensities obtained at 510-nm emission by alternate exciting by 340-nm and 380-nm light. (*G*) Pseudocolored ratiometric image of cells immediately following a stimulus, demonstrating that only a subset of the cells responded to the stimulus with an increase in $[Ca^{2+}]_i$. Color scale (vertical bars) in *F* and *G* indicates relative $[Ca^{2+}]_i$, where purple/blue represent low levels of resting $[Ca^{2+}]_i$ and yellow/red represent high levels of $[Ca^{2+}]_i$.

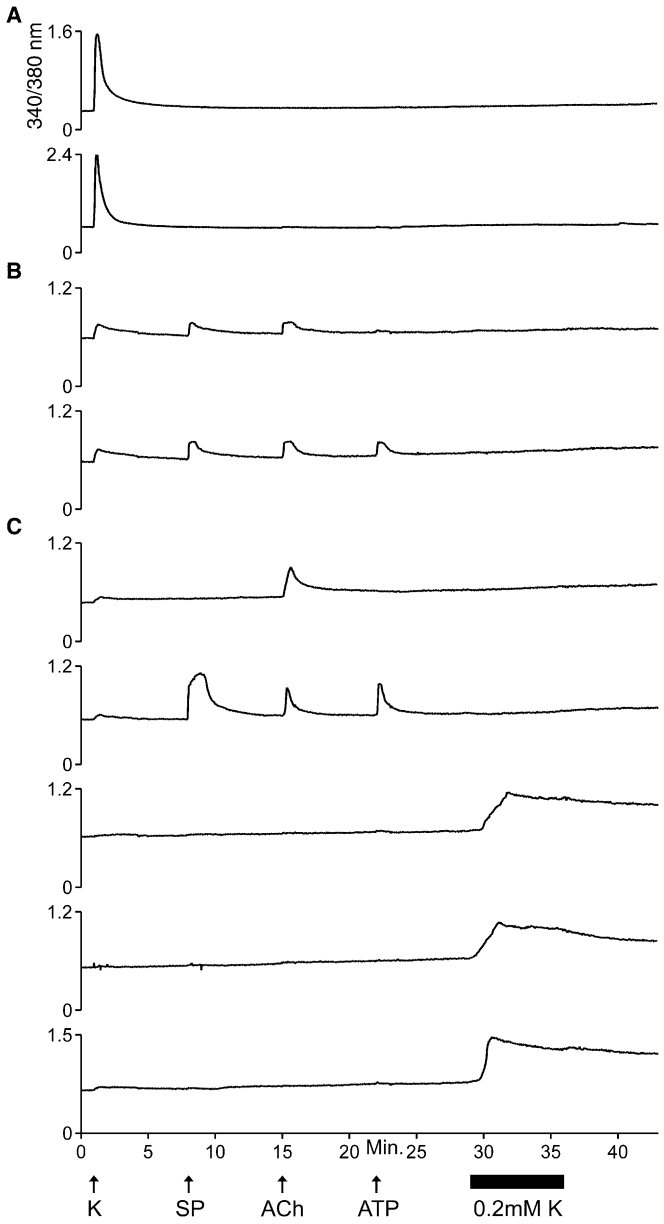
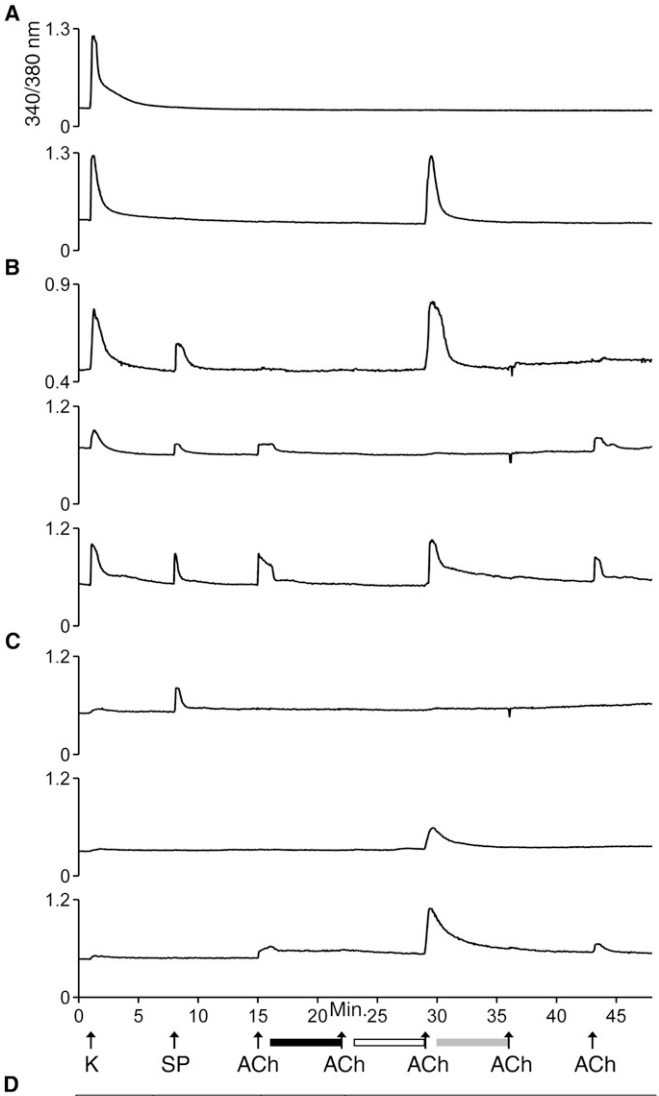


Fig. S2. Examples of calcium-imaging traces, presented as in Fig. 1, demonstrating responses to 0.2 mM $[K^+]_o$ in VRC class C cells and the lack of responses to 0.2 mM $[K^+]_o$ in class A and B cells. (*A*) Two examples are shown of class A cells that did not respond to 0.2 mM $[K^+]_o$ with an increase in $[Ca^{2+}]_i$. (*B*) Two examples are shown of class B cells that did not respond to 0.2 mM $[K^+]_o$. (*C*) The upper two traces are examples of class C cells that did not respond to 0.2 mM $[K^+]_o$ with an increase in $[Ca^{2+}]_i$. The lower three traces are examples of class C cells that did respond to 0.2 mM $[K^+]_o$ with an increase in $[Ca^{2+}]_i$.



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			% of ACh-responsive cells in each cell class		
Cell Class	Avg Resp. to 100 mM $[K^+]_o$	% of Total Cells	α7 nAChRs Only	mAChRs Only	Mix of α7 nAChRs and mAChRs
A	0.8	8%	66%	0%	0%
B	0.2	21%	19%	24%	49%
C	0.0	71%	4%	1%	3%

Fig. S3. Examples of calcium-imaging traces, presented as in Fig. 1, from experiments investigating acetylcholine (ACh) receptors (AChRs) in VRC cells. The black horizontal bar below the *x*-axis indicates the presence of 10 μM atropine in the bath, which blocked the response to the second application of 1 mM ACh. The open horizontal bar below the *x*-axis indicates the presence of both 10 μM atropine and 5 μM PNU-120596 (PNU) in the bath. Many cells responded to ACh only after application of PNU and in the continued presence of atropine. The gray horizontal bar below the *x*-axis indicates the presence in the bath of atropine, PNU, and 200 nM α-conotoxin ArIB[V11L;V16D], a highly selective blocker of α7 nicotinic acetylcholine receptors (nAChRs), which blocked the ACh responses elicited by PNU. After washout of these pharmacological agents, the final application of ACh at minute 43 demonstrated that muscarinic acetylcholine receptors (mAChRs) responded again (see ACh application at minute 15), but α7 nAChRs no longer responded in the absence of PNU. (*A*) Examples of traces from class A cells. (*B*) Examples of traces from class B cells. (*C*) Examples of traces from class C cells. (*D*) Compilation of data for class A, class B, and class C cells. This dataset was compiled for 2,267 cells from seven independent experimental trials, using cells prepared separately from three different mice.

Table S1. Cellular subclasses defined by cluster analysis

Prototype response profile	Fraction of cells per cluster that responded to each stimulus: K	SP	ACh	ATP	NE	H	Br	Glu	Cell count	No. of subclusters	Mean K^+ response	K^+ response SEM	Color code
10000001	1.00	0.01	0.00	0.00	0.00	0.00	0.02	1.00	177	3	0.76	0.03	
10000000	1.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	47	2	0.58	0.05	
Total									224				
Mean											0.72		
10111101	1.00	0.00	0.99	1.00	1.00	1.00	0.00	1.00	104	2	0.35	0.02	1.00
10111111	1.00	0.00	0.93	1.00	1.00	1.00	1.00	1.00	58	2	0.34	0.02	0.90–0.99
10110101	1.00	0.08	0.92	1.00	0.00	1.00	0.00	1.00	38	3	0.28	0.03	0.80–0.89
10101101	1.00	0.06	0.94	0.00	1.00	1.00	0.00	1.00	36	3	0.29	0.03	0.50–0.79
10110001	0.85	0.09	0.91	1.00	0.00	0.00	0.00	1.00	33	4	0.21	0.03	0.21–0.49
10101111	1.00	0.00	0.97	0.00	1.00	0.97	1.00	1.00	30	3	0.27	0.03	0.11–0.20
10111100	0.96	0.00	1.00	1.00	1.00	1.00	0.15	0.00	26	3	0.34	0.04	0.01–0.10
10110000	1.00	0.09	0.82	1.00	0.00	0.00	0.00	0.00	22	3	0.24	0.04	0.00
10111001	1.00	0.00	1.00	0.80	1.00	0.00	0.05	0.85	20	4	0.37	0.04	
10100101	1.00	0.00	0.94	0.00	0.00	1.00	0.00	1.00	17	2	0.19	0.03	
10100001	1.00	0.06	1.00	0.00	0.00	0.00	0.00	1.00	17	2	0.38	0.08	
10101110	1.00	0.06	1.00	0.00	1.00	0.94	1.00	0.00	16	3	0.32	0.05	
10100111	0.93	0.00	0.86	0.00	0.00	0.71	1.00	1.00	14	4	0.35	0.08	
10100110	0.71	0.07	1.00	0.00	0.00	1.00	1.00	0.00	14	3	0.14	0.03	
11111101	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00	13	1	0.40	0.05	
10100000	0.92	0.00	1.00	0.00	0.08	0.00	0.23	0.00	13	4	0.25	0.04	
10110100	1.00	0.00	1.00	1.00	0.00	1.00	0.00	0.00	12	1	0.25	0.04	
10110111	0.92	0.00	1.00	1.00	0.00	1.00	1.00	0.58	12	3	0.27	0.04	
11111111	0.91	1.00	0.91	0.82	1.00	1.00	1.00	0.82	11	4	0.27	0.04	
10011010	1.00	0.10	0.00	0.90	1.00	0.00	0.70	0.30	10	5	0.19	0.05	
10101100	1.00	0.10	1.00	0.00	1.00	1.00	0.00	0.00	10	2	0.30	0.06	
10100100	1.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	6	1	0.18	0.04	
10111010	1.00	0.20	1.00	1.00	1.00	0.00	1.00	0.00	5	2	0.16	0.06	
10010010	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.20	5	2	0.13	0.02	
Total									542				
Mean											0.30		
00000000	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	723	2	0.03	0.00	
00010000	0.00	0.14	0.00	1.00	0.00	0.00	0.00	0.00	21	2	0.03	0.00	
00000100	0.00	0.00	0.15	0.08	0.08	1.00	0.08	0.00	13	5	0.04	0.01	
00000010	0.06	0.00	0.00	0.13	0.13	0.00	1.00	0.13	16	5	0.05	0.01	
00110000	0.00	0.15	1.00	1.00	0.00	0.00	0.00	0.00	13	2	0.05	0.01	
00110010	0.22	0.00	1.00	1.00	0.11	0.11	1.00	0.00	9	4	0.07	0.02	
00101110	0.00	0.00	0.89	0.11	1.00	1.00	1.00	0.11	9	4	0.05	0.00	
00011001	0.17	0.00	0.00	1.00	0.83	0.00	0.33	1.00	6	4	0.05	0.01	
00000001	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	6	1	0.06	0.01	
00011110	0.00	0.00	0.00	1.00	1.00	0.75	0.75	0.00	4	3	0.04	0.01	
Total									820				
Mean											0.03		

Prototype response profile is the defining response pattern of each cluster to the eight stimuli (in order: K, SP, ACh, ATP, NE, H, Br, Glu), where 1 = response and 0 = no response. Under the heading of Fraction of cells per cluster that responded to each stimulus, each value different from 0 or 1 indicates that the response profiles of some cells in the cluster did not match the cluster perfectly. This cluster analysis, which produced 36 clusters, assumes an error rate in scoring of ~1%. Colors indicate the fraction of cells that responded to a particular stimulus; the color code is given in the far right column of the table. For this cluster analysis, we discarded 173 cells scored for the prior data analysis summarized in Table 2 (mostly from class C), because responses from those cells could not be scored accurately by automated methods. ACh, acetylcholine; Br, bradykinin; Glu, glutamate; H, histamine; K, $[K^+]_o$; NE, norepinephrine; SP, substance P.