Supporting Information

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SI Materials and Methods

Experiments were conducted with an observation solution (also called "bath solution") that consisted of 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM sodium citrate, 10 mM Hepes, and 10 mM glucose, pH 7.4. Working concentrations of pharmacological agents (i.e., challenge compounds; see Table S1) were made by diluting concentrated stock solutions in observation solution. Stock solutions were as follows: 10.2 M allyl isothiocyanate (AITC) oil (Sigma-Aldrich); 1 mM ATP disodium salt trihydrate (Sigma-Aldrich) in observation solution; 10 μM α-Dendrotoxin (α-Dtx) (Alomone Labs) in observation solution; 10 μM κM-conopeptide RIIIJ (κM-RIIIJ) in observation solution; 640 mM (100 mg/mL) (1R, 2S, 5R)-(-)-menthol (Sigma-Aldrich) in ethanol; 100 μM μO-conopeptide MrVIB (μO-MrVIB) in observation solution + 0.1 mg/mL BSA and 0.01% DMSO; 2 mM nicardipine hydrochloride (Sigma-Aldrich) in DMSO; 10 μM SNX-482 (Peptides International) in observation solution + 0.1 mg/mL BSA; 1 mM TTX in distilled water; 30 mM veratridine in DMSO; 50 μM ω-conopeptide GVIA, and ω-conopeptide MVIIC (ω-MVIIC) in observation solution + 0.2 mg/mL BSA. Elevated extracellular potassium concentrations were achieved by reducing the concentration of NaCl in the observation solution by the corresponding increase in concentration of KCl, to keep osmolarity constant.

All experimental trials began with an ~15-s application of a high concentration of extracellular KCl ($[K^+]_o$) to depolarize the neurons. Nonneuronal cells and nonviable neurons that did not respond to high $[K^+]_o$ were excluded from further analysis. In all figures, arrows indicate the ~15-s application of high $[K^+]_o$ or other challenge compounds described below (or bath solution); after each application the free compound or high $[K^+]_o$ was washed out of the well with room-temperature bath solution. As shown in the figures, each experimental trial included at least one replacement of bath solution with identical bath solution at room temperature, which served as a negative control to ensure that an increase in the cytoplasmic calcium concentration ($[Ca^{2+}]_i$) was not elicited merely by exchanging solutions. In addition to the application of high $[K^+]_o$ to depolarize neurons, changes in $[Ca^{2+}]_i$ were elicited by two types of experimental protocols: receptor-agonist challenges (RA challenges) and membrane-potential challenges (MP challenges).

An RA-challenge compound elicits an increase in $[Ca^{2+}]_i$ through activation of a calcium-permeable ionotropic receptor or through activation of a metabotropic receptor (G protein-coupled receptor, GPCR) that stimulates release of calcium from the endoplasmic reticulum. We also used physiological challenges, i.e., cold bath solutions, which were a type of RA challenge, because cold temperature is the natural agonist of TRPM8 channels (also activated by menthol). Except when cold bath solution was applied, as indicated in the figures, experiments were conducted at room temperature (~24 °C).

An MP-challenge compound alters $[Ca^{2+}]_i$ by changing membrane potential through agonist or antagonist activity on particular voltage-gated ion channels. MP-challenge compounds were used in combination with various depolarizing stimuli, such as high $[K^+]_o$ or RA challenges, to determine whether the MP-challenge compounds amplified or blocked the $[Ca^{2+}]_i$ signal elicited by such depolarizing stimuli. MP-challenge compounds were allowed to equilibrate with their molecular targets over time, as indicated by horizontal bars in the figures, typically between applications of identical depolarizing stimuli. In all figures, horizontal bars indicate the duration of time (>15 s) that a compound (or cold bath solution) was present in the well.

In the presence of an MP-challenge compound, the change in $[Ca^{2+}]_i$ elicited by a depolarizing stimulus was evaluated in terms of peak height, which is a measurement from the $[Ca^{2+}]_i$ baseline (i.e., relative resting $[Ca^{2+}]_i$) to the top of the elicited peak (i.e., relative maximal $[Ca^{2+}]_i$ achieved by the depolarizing stimulus). For each cell (i.e., trace), the peak height after application of MP-challenge compounds was compared with the average peak height of controls obtained before application of MP-challenge compounds. The result for each cell then was expressed as a percent response (i.e., percent of the control value). The results from each experimental trial were averaged to obtain a sample mean for M+A− and M+A+ neurons. Experimental trials were replicated to obtain an average percent response ± SEM.

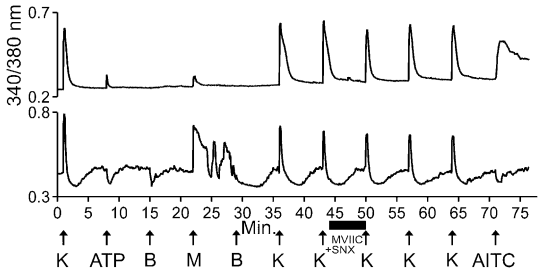

Fig. S1. The combination of an N- and P/Q-type Ca-channel blocker (ω-MVIIC) with an R-type Ca-channel blocker (SNX-482) did not inhibit the KCl-elicited calcium signal in M+A− neurons (*Lower*) but attenuated the signal in M+A+ neurons (*Upper*). The experimental protocol shown in Fig. 2 was used to obtain these traces, with the exception of the compounds used to block the KCl-elicited calcium signals. (*Upper*) The KCl-elicited calcium signal in this M+A+ neuron was attenuated modestly by the combination of ω-MVIIC and SNX-482. (*Lower*) The KCl-elicited calcium signal in this M+A− neuron was not blocked by the combination of ω-MVIIC and SNX-482.

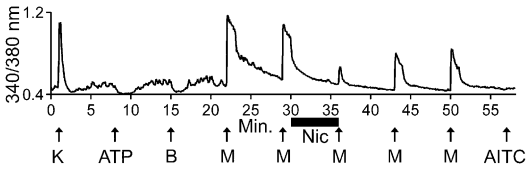

Fig. S2. Nicardipine blocked responses to menthol in M+A− neurons. After application of nicardipine, the average menthol-elicited response as a percentage of control peak height was 34 ± 7% (n = 3 trials, 8 cells total). The reversibility was moderately slow (as shown), similar to the reversibility observed when blocking KCl-elicited responses in M+A− neurons (Fig. 2).

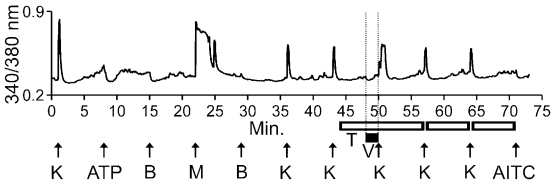

Fig. S3. Although KCl-elicited responses were blocked (on average) in the presence of both TTX and veratridine in M+A− neurons (Fig. 3*B* and Table 2), in a few rare instances, M+A− neurons exhibited a reversible peak broadening. The experimental protocol shown in Fig. 3*B* was used to obtain this trace. T, TTX; V, veratridine.

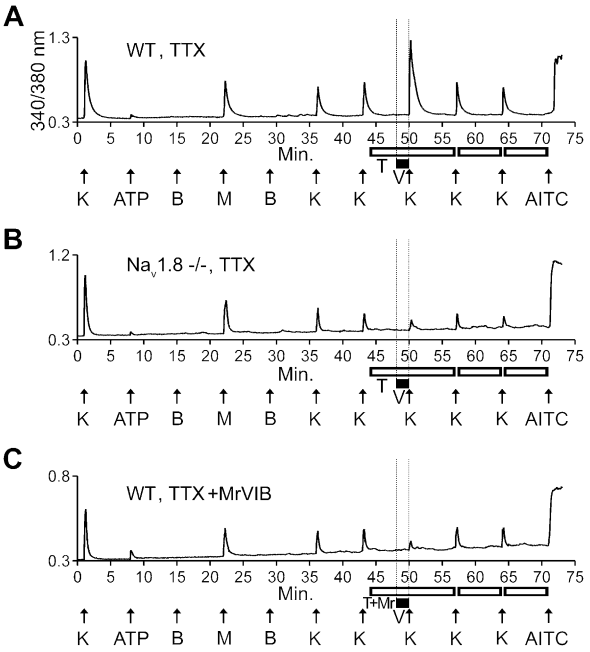

Fig. S4. In the presence of TTX and veratridine, KCl-elicited responses were amplified in M+A+ neurons in WT mice (*A*) but were blocked in NaV1.8$^{-/-}$ mice (*B*). In WT mice, the KCl-elicited responses also were blocked when both TTX and μO-MrVIB were coapplied with veratridine (*C*). The vertical dashed lines are for clarity only, to indicate when veratridine was present in the well. The experimental protocol shown in Fig. 3*B* was used to obtain these traces, with the exception that μO-MrVIB also was used in C. The letter "K" is an abbreviation for 25 mM KCl at minute 1, and then 15 mM KCl at subsequent time points in *A*–*C*. The use of 15 mM KCl made evident the amplification or blocking of the KCl-elicited response. These data are summarized in Table 2. (*A*) The upper trace in Fig. 3*B*, shown here for comparison with traces in *B* and *C*. Using neurons from WT mice, in the presence of TTX and veratridine, KCl-elicited responses were amplified in M+A+ neurons. (*B*) In contrast to *A*, using neurons from Na$_V$1.8$^{-/-}$ mice, KCl-elicited responses were blocked in M+A+ neurons in the presence of TTX and veratridine. (*C*) Also in contrast to *A*, using neurons from WT mice, KCl-elicited responses were blocked in M+A+ neurons in the presence of TTX, μO-MrVIB and veratridine.

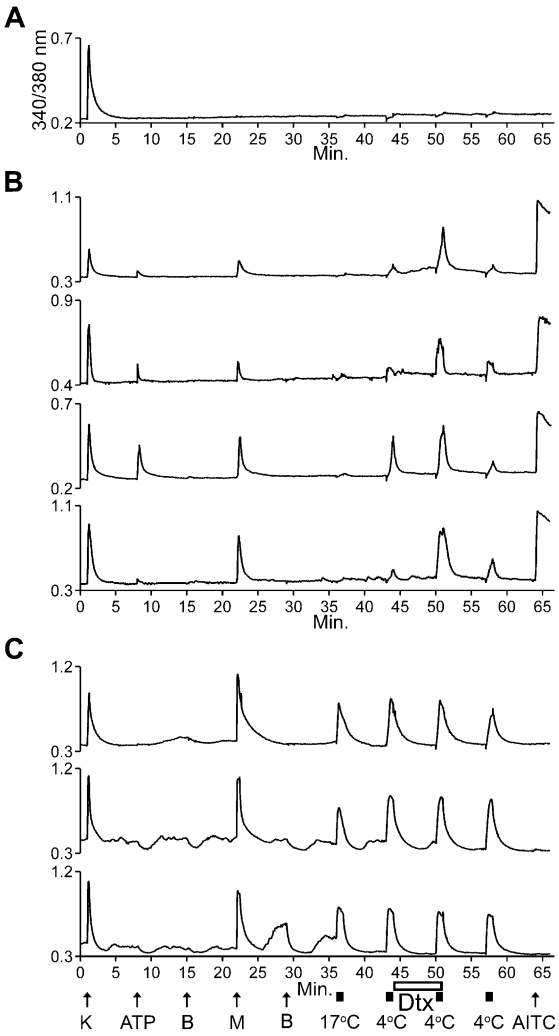

Fig. S5. α-Dtx substantially amplified the response to cold temperature in M+A+ neurons but not in M+A− neurons. Note that each application of 4 °C bath solution (designated by the small, black bar) was for 1 min. Application of α-Dtx is designated by the open horizontal bar. (*A*) A trace from a control neuron that did not respond to menthol, ATP, AITC, or to cold temperatures. The neuron responded to KCl depolarization only. The slight downward inflection that is observed each time the 4 °C bath solution was applied to the well suggests a decrease in $[Ca^{2+}]_i$, rather than an increase. (*B*) Four traces from different M+A+ neurons that responded to 4 °C bath solution with an increase in $[Ca^{2+}]_i$. Their responses to 4 °C bath solution were amplified by α-Dtx. After application of α-Dtx, the average cold-elicited response as a percentage of control peak height was 226 ± 28% for M+A+ neurons (n = 4 trials, 42 cells total). (*C*) Three traces from different M+A− neurons that responded to the 17 °C bath solution and the 4 °C bath solution with an increase in $[Ca^{2+}]_i$. Their responses to 4 °C bath solution were not amplified by α-Dtx. After application of α-Dtx, the average cold-elicited response for M+A− neurons as a percentage of control peak height was 96 ± 1% (n = 4 trials, 9 cells total).

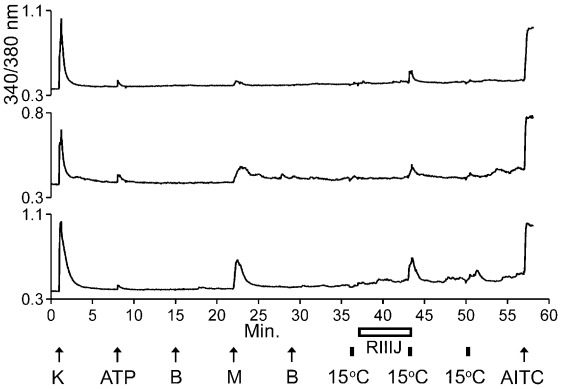

Fig. S6. M+A+ neurons, which ordinarily do not respond to 15 °C, will respond to 15 °C in the presence of κM-RIIIJ. Note that each application of 15 °C bath solution (designated by small, black bars) was for 30 s. Application of κM-RIIIJ is designated by the open horizontal bar.

Table S1. Abbreviations of compound names and controls, with the working concentrations of compounds used in all figures (except as otherwise indicated in figure legends)

Compound	Abbreviation	Working concentration	Function	Molecular target
RA-challenge compounds				
Allyl Isothiocyanate (mustard oil)	AITC	100 μM	Agonist	TRPA1 channel
ATP	ATP	10–20 μM	Agonist	P2X or P2Y receptors
Menthol	M	250–500 μM	Agonist	TRPM8 channel
MP-challenge compounds				
K channels*				
α-Dendrotoxin	Dtx	200 nM	Antagonist	K_V1.1, 1.2, 1.6
κM-Conopeptide RIIIJ	RIIIJ	1 μM	Antagonist	K_V1.2
Ca channels				
ω-Conopeptide GVIA	GVIA	1 μM	Antagonist	Ca_V2.2 (*N*-type)
ω-Conopeptide MVIIC	MVIIC	10 μM	Antagonist	Ca_V2.1 (P/Q-type) Ca_V2.2 (*N*-type)
Nicardipine	Nic	4 μM	Antagonist	Ca_V1 (L-type)
SNX-482	SNX	300 nM	Antagonist	Ca_V2.3 (R-type)
Na channels				
Tetrodotoxin (TTX)	T	1 μM	Antagonist	TTX-S Na channels
Tetrodotoxin (TTX) +	T+Mr	1 μM	Antagonist	TTX-S Na channels +
μO-Conopeptide MrVIB		1 μM	Antagonist	Na_V1.8 (TTX-R)
Veratridine	V	30 μM	Agonist	Na channels
Controls				
KCl	K	25 mM	+ Control (depolarize)	Ca channels
Bath replacement at room temperature, ~24 °C	B	NA	− Control	None

Some of the abbreviations in the table, which are used in all figures, are shortened more than the abbreviations used in the text, because of space constraints in the figures. NA, not applicable.

*Properties of the large and functionally diverse voltage-gated K channel family have been reviewed comprehensively (1, 2).

1. Gutman GA, et al.; International Union of Pharmacology (2003) International Union of Pharmacology. XLI. Compendium of voltage-gated ion channels: Potassium channels. *Pharmacol Rev* 55:583–586.
2. Gutman GA, et al. (2005) International Union of Pharmacology. LIII. Nomenclature and molecular relationships of voltage-gated potassium channels. *Pharmacol Rev* 57:473–508.