Csn induces locomotor activity in embryos expressing TRPV1 in sensory neurons.

(a,b) A representative 24 h.p.f. Tg(islet1:GAL4VP16,4xUAS:TRPV1–RFPT) embryo (lateral view, rostral at left) expresses trpv1–rfpt (magenta) in trigeminal and Rohon–Beard sensory neurons (marked by islet1, green). The boxed region in (a) is shown at higher magnification in (b). Arrows indicate Rohon–Beard neurons that express both trpv1–rfpt and islet1. trpv1–rfpt expression is restricted to islet1–expressing Rohon–Beard neurons, but is only expressed in a subset of these neurons (17/40 islet1–expressing Rohon–Beard neurons express trpv1–rfpt, 3 embryos quantified). Scale bars, 100 μm (a) and 25 μm (b). (c–e) At 2 d.p.f., transgenic embryos (bold colors), but not their WT siblings (faint colors), exhibited a significant increase in locomotor activity (c,d) and in the number of movement bouts (e) in response to 1–10 μM Csn that lasted for up to 2 hours. Mean (c) and mean ± s.e.m. (d,e) are shown. **P<0.01 for transgenic embryos compared to their WT siblings by the Kruskal–Wallis test followed by the Steel–Dwass test to correct for multiple comparisons. 20 embryos were tested for each condition.
Supplementary Figure 2

Increased temperatures induce locomotor activity and neuronal activity in embryos expressing TRPA1 in sensory neurons.

A representative 24 h.p.f. Tg(islet1:GAL4VP16,4xUAS:TRPA1–RFPT) embryo exhibits RFPT fluorescence in trigeminal (arrowhead) and Rohon–Beard sensory neurons (a), as confirmed by trpa1–rfpt (magenta) and islet1 (green) double FISH (b). The boxed region in (b) is shown at higher magnification in (c). Arrows indicate Rohon–Beard neurons that express both trpa1–rfpt and islet1. (28/45 islet1–expressing Rohon–Beard neurons express trpa1–rfpt, 3 embryos quantified). This transgenic line also expresses trpa1–rfpt in some spinal cord interneurons (magenta cells without arrows in [c]). To test the ability of TRPA1 to activate zebrafish sensory neurons, we raised embryos expressing the channel in sensory neurons at or below 26.5°C because the channel is activated at and above 28°C 12. (d–f) Embryos were raised at 26.5°C and tested at 30 h.p.f., at which time they were developmentally similar to 24 h.p.f. embryos raised at 28.5°C. Embryos were acclimated in E3 medium at 22°C several hours before behavioral assays. Slowly raising the temperature using a heating block (approximately 1°C increase/minute) from 22.5°C to 28.5°C failed to produce a robust locomotor response (data not shown). In contrast, when the temperature was rapidly increased by transferring embryos from E3 medium at 22.5°C to E3 medium pre–heated at specific warmer temperatures, locomotor activity was induced by 1°C to 5°C temperature changes (d), consisting of intense locomotor activity lasting up to 15 seconds (e) with a response latency of 0–5 seconds (f). Rapidly increasing the temperature from 25°C to 28°C produced similar results (data not shown). Temperature increases up to 30°C failed to elicit behavioral responses in WT siblings (Supplementary Video 3 and data not shown), consistent with previous observations 14. The intense TRPA1–dependent behavioral response could be induced again in 95% of TRPA1–expressing embryos after returning them to 22°C for as little as 2 minutes. (g–i) Embryos were raised at 22.5°C and tested at 3 d.p.f., at which time they were developmentally similar to 2 d.p.f. embryos raised at 28.5°C, and acclimated in E3 medium at 25.5°C for several hours. Transgenic embryos (red), but not their WT siblings (blue), exhibited an increase in locomotor activity (g,h) and in the number of movement bouts (i) following a change in the water temperature from 25.5°C to 28.5°C (approximately 1 minute ramp time) that lasted for up to 2 hours. Arrow in (g) indicates the time of temperature change. Mean (g) and mean ± s.e.m. (e,f,h,i) are shown. *P<0.05 and **P<0.01 for transgenic embryos compared to their WT siblings by the Wilcoxon rank–sum test. (j–l) Representative images showing that c–fos expression is induced in trpa1–rfpt–expressing Rohon–Beard neurons in transgenic embryos after a change in water temperature from 25°C to 28°C (k), but not in non–transgenic siblings subjected to the temperature change (l) or in transgenic siblings maintained at 25°C (j). Animals were fixed 45 minutes after the temperature change. (m) Mean ± s.e.m. percentage of trpa1–rfpt–expressing Rohon–Beard neurons that express c–fos following a 25°C to 28°C temperature change. n indicates the number of embryos analyzed. At least 11 neurons were analyzed per animal in (m). Scale bars, 100 μm (a,b) and 25 μm (c,j–l).
Supplementary Figure 3

Menthol induces locomotor activity and neuronal activity in embryos expressing TRPM8 in sensory neurons.

A representative 24 h.p.f. WT embryo injected with a plasmid containing the islet1:GAL4VP16,4xUAS:TRPM8–RFPT transgene exhibits...
TRPM8–RFPT fluorescence in a subset of sensory neurons (a), as confirmed by trpm8–rfpt (magenta) and islet1 (green) double FISH (b). The boxed region in (b) is shown at higher magnification in (c). Arrows indicate Rohon–Beard neurons that express both trpm8–rfpt and islet1. (d–f) At 24 h.p.f., transgenic embryos exhibited a dose–dependent locomotor response to menthol, responding to as little as 30 μM (d), with intense locomotor activity lasting up to 9 seconds (e) and a response latency of 0–8 seconds (f). WT embryos did not respond to menthol at any of the concentrations tested (Supplementary Video 4 and data not shown). The behavioral response to menthol could be repeatedly induced in 90% of embryos following drug washout for 2 minutes. Mean (d) and mean ± s.e.m. (e,f) are shown. (g–i) Representative images showing that c–fos expression is induced in trpm8–rfpt–expressing Rohon–Beard neurons in embryos exposed to 100 μM menthol for 45 minutes (h), but not in trpm8–rfpt–expressing Rohon–Beard neurons exposed to the vehicle control (g) or WT neurons exposed to 100 μM menthol (i). Arrows and arrowheads indicate Rohon–Beard sensory neurons that do and do not express c–fos, respectively. islet1 expression was used to identify Rohon–Beard neurons in WT neurons (i). (j) Mean ± s.e.m. percentage of trpm8–rfpt–expressing Rohon–Beard neurons that express c–fos following exposure to 100 μM menthol. n indicates number of embryos analyzed. Average number of neurons analyzed per embryo in (j): TRPM8+ vehicle = 3.3, TRPM8+ 100 μM menthol = 5.2, TRPM8– 100 μM menthol = 8.7. Scale bars, 100 μm (a,b) and 25 μm (c,g–i).
Supplementary Figure 4

Csn induces neuronal activity in embryos expressing TRPV1 in sensory neurons.

(a–e) Representative images showing TRPV1–RFPT+ neurons (a–e) and GCaMP5G fluorescence before (a'–e') and after (a''–e'') addition of Csn. White arrows indicate neurons whose ΔF/F₀ values are shown in panels (a''–e''). Black arrows (a''–e'') indicate Csn addition (t = 0 s) after 10 s of baseline recording. Rohon–Beard neurons were identified in WT embryos by their morphology and location using basal GCaMP5G fluorescence (e). (f) Percentage of TRPV1–RFPT+ Rohon–Beard neurons that showed at least a 50% increase in GCaMP5G fluorescence following exposure to Csn. (g) Average number of calcium transients with at least a 50% increase in ΔF/F₀, per min during 440 s of imaging. Lower values were observed for 10 μM Csn because many neurons exhibited prolonged calcium responses. (h) Average peak ΔF/F₀ value of all calcium transients. (i) Average ΔF/F₀ during the entire Csn application period. (j) Prolonged calcium response was defined as a calcium transient lasting more than 60 s. Panels (g–i) show mean ± s.e.m. for neurons that respond to Csn. *P<0.05 and **P<0.01 by the Kruskal–Wallis test followed by the Steel–Dwass test to correct for multiple comparisons. n indicates number of embryos (f) or neurons (g–j) analyzed. At least 7 cells were analyzed per embryo in (f). Scale bars, 25 μm.

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Supplementary Figure 5

Csn can reversibly and repeatedly stimulate TRPV1–expressing Rohon–Beard sensory neurons.

(a) Images showing TRPV1–RFPT expressing Rohon–Beard neurons and GCaMP5G fluorescence before addition of Csn in a representative Tg(elavl3:GCaMP5G);Tg(hcrt:TRPV1–RFPT) embryo. (b) Change in fluorescence ($\Delta F/F_0$) is plotted for each cell in the field of view for both the first (1–12) and second (1’–12’) round of 1 μM Csn application, with an intervening 15 minute washout of Csn. (c–e) In each trial, Csn was added after 10 s of baseline recording. For 3 embryos tested, 13 neurons responded to 1 μM Csn, and 10 of these neurons responded to a second exposure to 1 μM Csn (77%). A response was defined as at least a 50% increase in $\Delta F/F_0$. Average number of calcium transients/min during 290 s of imaging (c), average peak $\Delta F/F_0$ value for all calcium transients (d) and average $\Delta F/F_0$ during the entire Csn application period (e) are quantified for these 10 neurons. Error bars indicate s.e.m.. Scale bar, 50 μm.

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Supplementary Figure 6

Csn exposure changes the excitability of TRPV1–expressing Rohon–Beard neurons in exposed spinal cord.

(a) Schematic of experimental setup and representative images from a recorded neuron (inset). Whole–cell patch–clamp recordings were made from TRPV1–RFPT+ neurons in the exposed dorsal spinal cord of Tg(islet1:Gal4VP16,4xUAS:TRPV1–RFPT) embryos at 2 d.p.f. Cells were filled with Alexa Fluor 488 hydrazide (AF488) during recordings and imaged afterward (DIC and fluorescence; white arrowhead indicates a TRPV1+ Rohon–Beard neuron filled with AF488 during recording). Csn was applied by diffusion (b) or perfusion (c) at the side of the recording dish. D, dorsal. V, ventral. nc, notochord. (b) Representative trace from a single TRPV1+ Rohon–Beard cell exposed to Csn by diffusion of a small volume (10 μL) of 100 mM solution from the edge of the dish (100 μM final concentration). For all recorded cells (n=7), after a variable diffusion delay, membrane potential began to depolarize. A stimulus that elicited an action potential before the depolarization ramp (red) was unable to elicit an action potential after the ramp (blue). (c) Responses of a single TRPV1+ Rohon–Beard cell to current injections (–25 and +25–400 pA, 200 ms each, 2 s inter–stimulus interval) during perfusion with 100 μM Csn. Responses to a single stimulus train are overlaid. Early in exposure, the cell’s steady–state membrane potential has not begun to depolarize (pre–ramp), and only single action potentials are evoked by supra–threshold stimuli. As the membrane potential begins to depolarize (mid–ramp), multiple action potentials can be elicited by a single stimulus pulse. Once the membrane potential depolarizes even further (post–ramp), the same stimuli fail to drive a single action potential. Inset scale bars, 5 ms.
Supplementary Figure 7

Perfusion of Csn during whole–cell recording from intact embryos is associated with modest Rohon–Beard cell body depolarization but significantly higher average firing rates compared to DMSO vehicle alone.

(a) Mean number of spikes fired per minute (calculated for the entire recording period for each condition) before, during and after perfusion of 10 μM Csn (magenta lines) or DMSO vehicle alone (black lines). Mean firing rates were significantly higher during perfusion with Csn \( (P = 0.0119 \text{ by Wilcoxon rank–sum test}) \). (b) Resting membrane potential of neurons before, during, and after perfusion of either 10 μM Csn (magenta lines) or DMSO vehicle alone (black lines).
Supplementary Figure 8

Csn induces apoptosis in TRPV1–expressing cells exposed to high Csn levels but does not ablate sensory neurons that do not express TRPV1.

(a–d) Representative images showing that TUNEL labeling was only observed in TRPV1+ neurons exposed to 10 μM Csn. Starting at 24 h.p.f., larvae were exposed to Csn for 6 hours and then processed for TUNEL staining. (e) Mean ± s.e.m. percentage of TRPV1+ Rohon–Beard neurons that are TUNEL positive in a 340 μm region of spinal cord that typically contained ~10 Rohon–Beard neurons. Seven embryos were analyzed for each condition. (f,g) Representative images of E(e1b:GAL4VP16)s1102t;Tg(14xUAS:EGFP-Aequorin) embryos incubated in vehicle control or 10 μM Csn starting at 28 h.p.f. EGFP fluorescence is unaffected by Csn treatment. (h) Mean ± s.e.m. EGFP fluorescence intensity for the conditions shown in (f,g). Three embryos were analyzed for each condition. The yellow boxes in (f–f”) indicate the regions of interest used to quantify fluorescence at each time point. Scale bars, 50 μm (a–d) and 100 μm (f,g).
Supplementary Figure 9

TRPV1–mediated activation and ablation of Hcrt neurons affects sleep–wake behaviors.

(a) A representative example of Tg(hcrt:TRPV1–RFPT) and Tg(hcrt:EGFP) co–expression in a 5 d.p.f. larva detected using anti–EGFP (green) and anti–RFPT (magenta) IHC. Images show a maximum intensity projection of a 50 μm confocal z–stack. Scale bars, 20 μm. (b) RFPT–positive and EGFP–positive cell counts in Tg(hcrt:TRPV1–RFPT); Tg(hcrt:EGFP) larvae (n = 8) and control Tg(hcrt:EGFP) larvae (n = 2). TRPV1–RFPT was expressed in 85% of EGFP–positive Hcrt neurons, and EGFP was expressed in 95% of TRPV1–RFPT–positive neurons. Thus, the Tg(hcrt:TRPV1–RFPT) line shows specific and nearly comprehensive expression of the transgene in Hcrt neurons. (c,d) Behavioral phenotypes during activation of Hcrt neurons with 1 μM Csn in Tg(hcrt:TRPV1–RFPT) larvae (n = 44) compared to their WT siblings (n = 44). (e,f) Behavioral phenotypes following ablation of Hcrt neurons with 10 μM Csn in Tg(hcrt:TRPV1–RFPT) larvae (n = 52) compared to their WT siblings (n = 43). Box plots indicate median (solid black line), 25th and 75th percentiles (box) and data range (whiskers). *P<0.05, **P<0.01, ***P<0.001 for transgenic larvae compared to their WT siblings by the Wilcoxon rank–sum test. (g,h) Representative images of QRFP neurons detected by anti–EGFP IHC (green), and c–fos expression detected by FISH (magenta), in Tg(hcrt:TRPV1–RFPT) larval brains after incubation 10 μM Csn for 20 minutes. Sagittal images of a 3 d.p.f. larval brain (g, scale bar, 100 μm) and a higher magnification view of the boxed area (h, scale bar, 20 μm) are oriented with rostral at left. Images are maximum intensity projections of 40 μm confocal z–stacks. A gamma correction was uniformly applied across all images to visualize cells with lower signal.

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Supplementary Figure 10

Csn does not affect WT larval zebrafish behavior.

Locomotor activity (a,b) and sleep (c,d) of WT larvae treated with DMSO vehicle control (black, $n = 40$) or 10 μM Csn (magenta, $n = 40$) are shown. Line plots indicate mean ± s.e.m. Box plots indicate median (solid black line), 25th and 75th percentiles (box) and data range (whiskers). There is no significant difference between vehicle and Csn–treated larvae during any day or night period (p>0.05) by Wilcoxon rank–sum test.
**Supplementary Discussion**

Zebrafish are particularly well suited among vertebrate model organisms for the TRP channel approach. Small molecules are administered via the water and can access neurons deep within the brain. Furthermore, the activation temperature of rattlesnake TRPA1 is within the normal temperature range of zebrafish, yet is high enough that larvae can be raised and assayed at lower temperatures without activating the channel. While TRP channel chemical and thermal agonists lack the millisecond temporal control of optogenetics, such control may not be necessary for behaviors that occur over long time scales. Indeed, TRPA1 is commonly used for long-term neuronal activation in *Drosophila*\(^\text{18}\). Similar technologies that use small molecule agonists of modified G protein–coupled receptors (DREADDs)\(^\text{7}\) or ion channels (PSAMs)\(^\text{8}\) are commonly used in mammals. We tested these reagents by expressing them in Rohon-Beard neurons, but we failed to observe agonist-induced locomotor activity (see Online Methods for details of transgenes tested). Channel expression level, agonist concentration, and tissue depth will determine the number of open TRP channels in a cell, and each cell’s physiological characteristics will determine its response to channel opening, similar to how cellular and illumination properties determine the response to optogenetic tools\(^\text{18}\). Our electrophysiological recordings indicate that activation of TRPV1 can increase neuronal spiking in Rohon–Beard cells, but firing patterns will likely differ in other neuronal classes depending upon their ion channel composition and TRPV1 expression levels. Appropriate Csn concentrations that increase activity without causing ablation will need to be determined empirically for each cell type, as for other tools that manipulate neuronal activity\(^\text{18}\). However, we found that 1 \(\mu M\) Csn is sufficient to robustly stimulate both Rohon-Beard sensory neurons and hypothalamic Hcrt neurons without causing cell ablation, suggesting that this concentration is a good starting point to identify appropriate Csn levels to stimulate other cell types. For ablation studies, we note that 10 \(\mu M\) Csn, which was sufficient to induce robust ablation of Rohon-beard sensory neurons and hypothalamic Hcrt neurons, had no detectable effect on larval health or behavior (Supplementary Fig. 10). This contrasts with nitroreductase\(^\text{17}\), the current method of choice for cell ablation, which often requires the use of metronidazole levels that are somewhat toxic and can affect the behavior of WT animals, although this may not be a problem with recently described mutant nitroreductase transgenes\(^\text{19, 20}\).