TRP channel mediated neuronal activation and ablation in freely behaving zebrafish

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The zebrafish (Danio rerio) is a useful vertebrate model system in which to study neural circuits and behavior, but tools to modulate neurons in freely behaving animals are limited. As poikilotherms that live in water, zebrafish are amenable to thermal and pharmacological perturbations. We exploit these properties by using transient receptor potential (TRP) channels to activate or ablate specific neuronal populations using the chemical and thermal agonists of heterologously expressed TRPV1, TRPM8 and TRPA1.

Attempts to decipher neuronal mechanisms that regulate vertebrate behaviors are limited by the difficulty of manipulating mammalian neuronal circuits and the complexity of mammalian brains. The larval zebrafish has emerged as a model that overcomes these challenges due to its transparency, lack of a mature blood-brain barrier and amenability to chemical and genetic perturbations. Anatomical and molecular analyses have shown that zebrafish and mammalian brains have similar features, and neuronal circuits that regulate certain zebrafish behaviors are likely to be conserved in mammals. However, use of zebrafish is limited by a paucity of validated tools to manipulate neuronal activity. Optogenetic tools that stimulate or inhibit neurons in response to light have been used to study restrained zebrafish larvae. However, the light stimulus elicits a behavioral response in unrestrained larvae, which is problematic for many behavioral assays. The confounding effects of light can be avoided using tools that modulate neuronal activity in the presence of specific small molecules or at specific temperatures, but these technologies have not been applied to zebrafish.

To develop these tools for use in freely swimming larval zebrafish, we tested three heterologous TRP channels that are activated by specific small molecules or temperatures. We used rat TRPV1, which is activated by capsaicin (Csn), rat TRPM8, which is activated by menthol, and rattlesnake TRPA1, which is activated at and above 28 °C (ref. 12). Importantly, the zebrafish TRPV1 ortholog is insensitive to Csn, zebrafish lack a TRPM8 ortholog, and the two zebrafish TRPA1 paralogs are not thermosensitive.

We used the islet-1 sensory neuron enhancer to express TRP channels in trigeminal and Rohon–Beard sensory neurons (Fig. 1 and Supplementary Figs. 1–3) and assayed for behavioral responses to channel agonists. Wild-type (WT) embryos did not respond to Csn (Supplementary Fig. 1c and Supplementary Video 1), and TRPV1+ embryos exhibited little locomotor activity when treated with vehicle or 0.1 μM Csn (Fig. 1b,c). However, exposure to 0.3 μM or more Csn induced intense locomotor activity in transgenic embryos (Fig. 1b,c and Supplementary Video 1), consistent with the activation of sensory neurons, and similar to the phenotype induced by channelrhodopsin-2 (ChR2). At 1 μM, Csn induced a response in 100% of embryos (Fig. 1b) consisting of 45 s of intense locomotor activity (Fig. 1c) and up to 2 h of less intense activity (Supplementary Fig. 1c–e and Supplementary Video 2). These results suggest that Csn can activate TRPV1+ neurons, and thus affect behavior, over long time periods. Following a 5-min washout, reaplication of Csn elicited a similar behavioral response in 95% of embryos, indicating that the effect can be repeatedly induced. Similar responses were observed using increased temperature to activate TRPA1 and menthol to activate TRPM8 (Supplementary Figs. 2 and 3 and Supplementary Videos 3 and 4). In contrast, we did not observe agonist-induced effects on locomotor activity in larvae that expressed designer receptors exclusively activated by designer drugs (DREADD) or pharmacologically selective actuator modules (PSAM) in Rohon–Beard sensory neurons.

To more directly test whether TRPV1+ sensory neurons are activated by Csn, we assayed neuronal activity using c-fos fluorescence in situ hybridization (FISH) and Tg(elavl3:GCaMP5G) embryos, which express GCaMP5G in most postmitotic neurons. We observed a dose-dependent increase in the number of c-fos–positive TRPV1+ neurons (Fig. 1f,h), but no c-fos expression in controls (Fig. 1e,g,h). Consistent with these results, Csn induced a dose-dependent increase in GCaMP5G fluorescence in TRPV1+ neurons (Supplementary Fig. 4). Exposure to 1 or 3 μM Csn induced transient GCaMP5G signals in 21% and 62% of TRPV1+ neurons, respectively, with larger and longer effects at 3 μM Csn. At 10 μM Csn, 88% of TRPV1+ neurons showed large and prolonged increases in fluorescence, which may indicate deleterious effects, and which were sustained until Csn was washed out. We did not observe changes in GCaMP5G fluorescence in controls.

To determine whether TRPV1–induced calcium signaling is reversible, we exposed TRPV1–expressing embryos to 1 μM Csn, washed out the Csn, and then reapplied 1 μM Csn. Most cells that responded to the first application showed a similar response to the second application (Supplementary Fig. 5), indicating that Csn can reversibly and repeatedly stimulate TRPV1+ neurons. We observed similar c-fos responses when activating TRPA1 or TRPM8 (Supplementary Figs. 2 and 3).

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To confirm that the observed calcium transients reflected Csn-evoked neuronal activity, we performed whole-cell patch clamp recordings from TRPV1+ Rohon-Beard neurons in 2 days post-fertilization (d.p.f.) embryos. Recordings from exposed spinal cord confirmed that Csn depolarized the membrane potential and transiently increased excitability of TRPV1+ neurons (Supplementary Fig. 6) but not TRPV1− neurons (data not shown). However, Csn did not evoke action potentials, possibly because the sensory endings of Rohon-Beard neurons were damaged during surgery. We therefore performed recordings from intact spinal cord (Fig. i). We perfused 10 μM Csn into the recording chamber, with the Csn concentration increasing gradually over several minutes as fluid was exchanged. Before Csn exposure and during perfusion with vehicle, some Rohon-Beard neurons fired action potentials, probably stimulated by small movements of the microelectrode in the skin. In response to Csn (n = 6) but not DMSO (n = 3), TRPV1+ neurons fired a prolonged burst of action potentials that began within 1−2 min of the beginning of exposure (when Csn concentration was 1−5 μM) and tapered off several minutes. Both the peak firing rate (Fig. ii) and the average number of spikes (Supplementary Fig. 7a) were higher during Csn exposure. The resting membrane potential of the cell body depolarized only marginally (Supplementary Fig. 7b), indicating that Csn likely initiated action potentials by depolarizing the sensory endings. Thus, Csn increases neuronal activity in TRPV1+ neurons.

We next tested the effect of prolonged activation of TRPV1 (Fig. 2). Using TRPV1-RFPT fluorescence as a proxy for cell number, we found that exposure to 1 μM Csn for 24 h did not affect the number of TRPV1+ neurons (Fig. 2b,d). However, these neurons were
reduced by treatment with 10 µM Csn for 10 h, and were essentially absent after 24 h (Fig. 2c,d). Consistent with these observations, TRPV1+ cells were TUNEL+ when treated with 10 µM, but not 1 µM, Csn (Supplementary Fig. 8a–e). Neurons expressing EGFP alone were unaffected by exposure to 10 µM Csn for 24 h (Supplementary Fig. 8f–h). TRPV1+ embryos exposed to 10 µM Csn for up to 48 h developed normally and exhibited normal locomotor activity. We conclude that strong and prolonged activation of TRPV1 results in apoptosis, allowing for targeted and inducible neuronal ablation. We did not observe neuronal ablation following prolonged activation of TRPA1 or TRPM8, likely because these channels induced weaker neuronal activity (Supplementary Figs. 2 and 3) that was insufficient to induce excitotoxicity.

To determine whether TRP channels can be used to activate neurons deep within the brain, we expressed TRPV1 in hypothalamic neurons that express the neuropeptide hypocretin (Hcrt) (Supplementary Fig. 9). We found that 1 µM and 10 µM Csn induced c-fos expression in 95% of Hcrt neurons (Fig. 3a,b,e,f,i), whereas little or no c-fos expression was observed in neighboring neurons that express the neuropeptide QRFP15 (Fig. 3i and Supplementary Fig. 9g,h) or in Hcrt neurons of TRPV1− siblings (Fig. 3c,d,g,h,i). Similar to sensory neurons, high Csn levels ablated TRPV1+ Hcrt neurons. At 10 µM Csn, over 50% of Hcrt neurons in Tg(hcrt:EGFP);Tg(hcrt:TRPV1-RFPT) larvae were absent after as little as 1 h of exposure (Fig. 2c–g,q). This likely underestimates the number of ablated cells because of the persistence of EGFP, because immunohistochemical (IHC) detection of Hcrt showed loss of 80% of Hcrt neurons after treatment with 10 µM Csn for 24 h (Fig. 2h,q). Exposure to 10 µM Csn for 24 h had no effect on Hcrt neurons in Tg(hcrt:EGFP) larvae or on QRFP neurons in Tg(qrfp:EGFP);Tg(hcrt:TRPV1-RFPT) larvae (Fig. 2k,l,q). Treating Tg(hcrt:EGFP);Tg(hcrt:TRPV1-RFPT) larvae with 1 µM Csn, which induces c-fos expression, for 24 h did not affect Hcrt cell number (Fig. 2m,q). TRPV1 is thus a single tool with two applications: neuronal activation or ablation at low or high Csn levels, respectively.

Hcrt overexpression promotes locomotor activity and inhibits sleep in larval zebrafish16. Consistent with this result, activating Hcrt neurons using 1 µM Csn resulted in increased locomotor activity, decreased sleep, hyperactivity and fewer sleep bouts (Fig. 3m and Supplementary Fig. 9c,d). Conversely, ablatin Hcrt neurons using 10 µM Csn resulted in decreased locomotor activity, increased sleep and more daytime sleep bouts (Fig. 3n–q and Supplementary Fig. 9c,f), similar to ablation of zebrafish Hcrt neurons using nitroreductase17 and to narcolepsy, a disorder caused by reduced Hcrt neurons. Exposure to 10 µM Csn for over 48 h had no effect on the health or behavior of WT larvae (Supplementary Fig. 10). Thus, TRPV1 can be used to manipulate neurons and affect behavior in freely behaving larvae.

TRP channels offer several advantages over alternative technologies (see Supplementary Discussion for comparison to similar tools). First, they can be used in freely behaving larvae without disrupting light-dependent behaviors. Second, because they do not require a light stimulus, they are compatible with bioluminescent and fluorescent neuronal activity reporters. Third, TRP channels conduct 1,000-fold more current than ChR2 (ref. 18), and thus can drive neuronal activity at lower expression levels. Fourth, our results suggest that cell ablation using TRPV1 may be faster and more robust than using nitroreductase17, although enhanced versions of the latter were recently reported19,20. Fifth, the ability of TRPV1 to activate or ablate neurons enables efficient screens for neurons that affect behavior. However, Csn concentrations that increase activity without causing ablation must be determined empirically for each cell type, as for other tools that manipulate neuronal activity18. Sixth, because

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**Figure 2 | Csn dose-dependent ablation of TRPV1-expressing neurons.** (a–c) Representative images of Tg(islet1:GAL4VP16,UAS:TRPV1-RFPT) embryos incubated for the indicated times in vehicle (a), 1 µM Csn (b) or 10 µM Csn (c) starting at 28 h.p.f. (d) Mean ± s.e.m. RFPT fluorescence intensity for the conditions shown in a–c. (e–q) Representative images of Hcrt neurons, detected by anti-EGFP IHC in Tg(hcrt:TRPV1-RFPT); Tg(hcrt:EGFP) (e–g,m,n) or Tg(hcrt:EGFP) (f–k,o,p) larvae. Hcrt protein was detected by IHC in Tg(hcrt:TRPV1-RFPT) (h) or WT (i) siblings. Dorsal views of 5 d.p.f. larval brains with rostral at left are shown. 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 in e–p. (q) Mean ± s.e.m. number of cells for the conditions shown in e–p. Six animals were analyzed for each condition (d,q). *P < 0.05 and **P < 0.01 by the Kruskal-Wallis test followed by the Steel-Dwass test to correct for multiple comparisons (d) and the Wilcoxon rank-sum test (q). Scale bars, 100 µm (a–a″) and 20 µm (l,p).
**BRIEF COMMUNICATIONS**

Figure 3 | TRPV1-mediated activation and ablation of Hcrt neurons affects sleep-wake behaviors. (a–h) Representative images of Hcrt neurons (anti-EGFP IHC, green) and c-fos expression (ISH, magenta) in TRPV1+ (a,b,e,f) and TRPV1– (c,d,g,h) larvae after incubation in 1 μM (a–d) or 10 μM (e–h) Csn for 20 min. Sagittal images of 3 d.p.f. larval brains (a,c,e,g; scale bars, 100 μm) and magnified views of the boxed areas (b,d,f,h; scale bars, 20 μm) are oriented with rostral at left. Images are maximum intensity projections of 40 μm z-stacks. A gamma correction was uniformly applied across all images to visualize cells with lower signal. (i) Mean ± s.e.m. fraction of c-fos–positive cells for a–h and Supplementary Figure 9g,h. See Online Methods for number of samples. (j–m) Behavioral phenotypes following ablation of Hcrt neurons with 10 μM Csn in Tg(hcrt:TRPV1-RFPT) larvae (n = 44) compared to WT siblings (n = 44). (n–q) Behavioral phenotypes comparing ablation of Hcrt neurons with 10 μM Csn in Tg(hcrt:TRPV1-RFPT) larvae (n = 52) compared to WT siblings (n = 43). Behavioral recording began on night 5 and ended on night 7 of development. Line plots indicate mean ± s.e.m. 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.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

D.A.P. conceived of and supervised the project. S.C. and C.N.C. designed, performed, and analyzed all experiments, except physiology experiments, which were designed, performed and analyzed by K.L.M. and J.R.F. All authors collaborated to write the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Ethics statement. All experiments were performed using zebrafish (Danio rerio) larvae aged from 24 h.p.f. to 7 d.p.f. in accordance with the California Institute of Technology and Cornell University Institutional Animal Care and Use Committee guidelines.

Transgenic zebrafish. We fused rat TRPV1 (NM_031982), containing the E600K mutation that increases sensitivity to Csn by over 10-fold21, rat TRPM8 (NM_134371), and Crotalus atrox (rattlesnake) TRPA1 (GU562967), to TagRFPT22 at their C termini. For expression in trigeminal and Rohon-Beard sensory neurons, we cloned the 4 kb islet1 sensory neuron specific enhancer23 upstream of the GAL4VP16 transcriptional activator24, followed by 4xUAS: E1b minimal promoter–TRP channel–RFPT. We used the 1 kb zebrafish hctr promoter23 to express TRPV1-RFPT in Hcrt neurons. Each open reading frame was followed by an SV40 polyA sequence and each cassette was flanked by ISce1 meganuclease sites and Tol2 transposase arms. We generated the Tg(hctr:TRPV1-RFPT)ct824 transgenic line using the Tol2 transposase method26. We generated the Tg(islet1:GAL4VP16,4xUAS:TRPV1-RFPT)ct825 and Tg(islet1:GAL4VP16,4xUAS:TRP channel–RFPT)ct826 transgenic lines using the ISce1 method27. TRPM8 experiments used transient injection of an islet1:GAL4VP16,4xUAS:TRPM8-RFPT transgene. See Supplementary Data files for plasmid sequences. The Tg(elavl3:GCaMP5G)at598 (ref. 28), Et(e1b:GAL4VP16)s1102t (ref. 29), Tg(14xUAS:EGFP-Aequorin)a127 (ref. 30), Tg(−2.0Trp. Hcrt:EGFP)zfl1Tg18 and Tg(qrtp:EGFP)ct820 (ref. 15) transgenic lines have been described.

In situ hybridization and immunohistochemistry. We fixed zebrafish samples in 4% paraformaldehyde (PFA) in PBS for 12–16 h at room temperature. We performed double fluorescence in situ hybridization (FISH) using digoxigenin (DIG)- and 2,4-dinitrophenol (DNP)-labeled riboprobes and the TSA Plus DIG and DNP System (NEL747A001KT, PerkinElmer). We amplified an 818 bp template for the c-fos (also known as fosab, GenBank ID AL929435) probe from a larval zebrafish cDNA library using the primers 5′-CAGCTCCACCAAGTGAAGA-3′ and 5′-TGCAAACAATTCGCAAGTTC-3′. We amplified a 735 bp template for the rfp probe from a TRPV1-RFPT plasmid using the primers 5′-ATGGTGTCTAAGGGCGAAGA-3′ and 5′-ATGCTGGTCCTAAGGGCGAAGA-3′ and 5′-TTACTTGTACAGCTCGTCCATG-3′. We amplified an 818 bp template for the Y115FL141F-RFPT probe from a larval zebrafish cDNA library using the primers 5′-CATCTCCACCAAGTGAAGA-3′ and 5′-TGCAAACAATTCGCAAGTTC-3′. We amplified a 735 bp template for the rfp probe from a TRPV1-RFPT plasmid using the primers 5′-ATGGTGTCTAAGGGCGAAGA-3′ and 5′-ATGCTGGTCCTAAGGGCGAAGA-3′ and 5′-TTACTTGTACAGCTCGTCCATG-3′. We generated the islet-1 probe as described23 and performed immunohistochemistry as described16. Primary antibodies were chicken anti-GFP (1:500, GFP-1020, Aves), rabbit anti-rTBP (1:200, EVN-AB233-C100, Evrogen) and rabbit anti-orexin A (1:500, AB3704, Millipore). We used Alexa 488- and 568-conjugated secondary antibodies (1:500, Invitrogen). We mounted samples in 50% glycerol in PBS, and imaged them using a Zeiss LSM 780 laser-scanning confocal microscope with 488 nm and 561 nm lasers and 10×, 25× and 40× objectives. Numbers of samples analyzed in hctr:TRPV1-RFPT cell activation experiment (Fig. 3a–k): 10 μM Csn, TRPV1+, hctr: GFP (n = 5); 10 μM Csn, TRPV1−, hctr:GFP (n = 5); 1 μM Csn, TRPV1+, hctr:GFP (n = 5); 1 μM Csn, TRPV1−, hctr:GFP (n = 2); 10 μM Csn, TRPV1+, qrtp:GFP (n = 5). In Figures 2e–p and 3a–h, because we report the presence of labeled cells, and not the intensity of labeling within cells, we uniformly applied a gamma correction across all images to visualize cells with lower signal.

Small-molecule treatment. We prepared frozen stock solutions of 100 mM Csn (M2028, Sigma) and 500 mM Menthol (M2772, Sigma) by dissolving the compounds in dimethyl sulfoxide (DMSO, 4948-02, Macron Chemicals). We prepared working concentrations just before embryo treatment by diluting the stock solutions into E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, pH 7.4). All treatments contained a final concentration of 0.05% DMSO (hctr experiments) or 0.2% DMSO (islet1 experiments).

Temperature-dependent activation of TRPA1. We raised Tg(islet1:Gal4VP16, UAS:TRPA1-RFPT) larvae in E3 medium at 22.5 °C or 26.5 °C to avoid activating the TRPA1 channel. We transferred embryos to E3 medium at temperatures up to 28.5 °C to activate the channel.

PSAM and DREADD experiments. DREADD. We transiently injected the following transgenes into WT embryos: Tg(islet1:Gal4VP16,4xUAS:hM3Dq-RFPT) and Tg(islet1:Gal4VP16,4xUAS:rM3Ds-RFPT). At 24 h.p.f., we immerseed RFPT+ embryos in 1 mg/ml, 500 μg/ml, 50 μg/ml, 25 μg/ml and 10 μg/ml clozapine-N-oxide but did not observe any behavioral responses. We also tested larvae transiently injected with Tg(islet1:Gal4VP16,4xUAS:hM3Dq-RFPT) at 4 d.p.f. in 1 mg/ml and 300 μg/ml CNO. We did not observe any difference in behavior compared to mock injected WT larvae. See Supplementary Data files for plasmid sequences.

PSAM. We transiently injected the following transgenes into WT embryos: Tg(islet1:Gal4VP16,4xUAS:5HT3 HC Y115FL141F-2A-RFPT), Tg(islet1:Gal4VP16,4xUAS:5HT3 HC Y115FL141F-RFPT), Tg(islet1:Gal4VP16,4xUAS:5HT3 HC Y115FL141F-2A-RFPT). At 24 h.p.f., we immerseed injected RFPT+ embryos in 400 μM, 300 μM, 200 μM, 100 μM, 40 μM and 20 μM S89 (a gift from S. Sternson, Janelia Farm) but did not observe any behavioral responses. See Supplementary Data files for plasmid sequences.

TRPV1-mediated cell ablation. For TRPV1-mediated Rohon-Beard cell ablation, we dechorionated 28 h.p.f. embryos and treated them with 1 μM or 10 μM Csn for 24 h. We imaged embryos just before Csn addition, and at 10 and 24 h after Csn addition. At each time point, each embryo was anesthetized with 0.03% tricaine and oriented on its side. We acquired fluorescent images using a dsRed filter on a fluorescent stereomicroscope (M250c, Leica Microsystems Inc.) and a color CCD camera (DFC310FX, Leica Microsystems Inc.). We quantified fluorescence intensity using ImageJ in a 200 μm × 1.6 mm (28 h.p.f.) or 100 μm × 2 mm (38 h.p.f. and 52 h.p.f.) region of interest that encompassed the dorsal spinal cord and most Rohon-Beard sensory neurons. We calculated fluorescence intensity by subtracting background fluorescence from the measured fluorescence.

For the TUNEL assay, we treated dechorionated 24 h.p.f. embryos with vehicle, 1 μM or 10 μM Csn for 6 h then fixed them in 4% PFA in PBS overnight at 4 °C. Fixed embryos were dehydrated using methanol and stored at ~20 °C. Embryos were rehydrated using decreasing concentrations of methanol in PBST (PBS + 0.1% Tween), treated with 10 μg/ml Proteinase K for 8 min, and fixed in 4% PFA in PBS for 20 min. Embryos were treated with
0.1% sodium citrate in PBST, then incubated with 90 µl labeling solution and 10 µl enzyme solution using the In situ Cell Death Detection Kit, Fluorescein (11684795910, Roche) for one hour at 37 °C. Embryos were washed three times with PBST and imaged using a Zeiss 780 confocal microscope with 25× water objective. The bright field overlay in Supplementary Figure 8a–d shows the position of Rohon-Beard neurons in the spinal cord.

**Calcium imaging.** Embryos at 2 d.p.f. were paralyzed with 1 mg/ml α-bungarotoxin (2133, Fisher Scientific) dissolved in E3 medium, mounted on their side in 0.8% low melting point agarose in a 35 × 10 mm culture dish, and covered with 3 mL E3 medium. We imaged Tg(elav3:GCaMP5G) fluorescence using a Zeiss 780 confocal microscope with a 20× water immersion objective. Samples were excited with a 488 nm laser and emitted light was collected through a 493–569 nm filter. We acquired images at 1 frame per second for 10 s before and 440 s after Csn administration. Frame acquisition time was approximately 900 ms. We added 1 mL of Csn at 4 µM, 12 µM, or 40 µM to the side of the culture dish for a final concentration of 1 µM, 3 µM, or 10 µM, respectively. We measured fluorescence intensity by drawing regions of interest around Rohon-Beard neurons using ImageJ. We calculated change in fluorescence (∆F/F0) as the fluorescence minus the initial fluorescence (defined as the mean fluorescence for the first 10 images) divided by initial fluorescence. To test whether TRPV1-induced calcium signaling is reversible, we performed calcium imaging as described above, except images were acquired for 10 s before and 290 s after adding 1 mL of 4 µM Csn to the side of the dish (final concentration = 1 µM). We then washed embryos three times for 5 min with E3 solution to remove Csn. The same embryos were then treated with Csn and imaged as described above.

**Physiology.** We performed whole-cell patch clamp recordings on 2 d.p.f. Tg(islet1:Gal4VP16,4xUAS:TRPV1-RFPT) embryos. We paralyzed embryos in α-bungarotoxin (Biotoxins, Inc.; 1 mg/mL in purified water or 10% Hank’s solution) and transferred them to a Sylgard-coated, glass-bottom specimen dish. Embryos were secured to the dish with custom etched tungsten pins through the notochord and covered with extracellular solution. For recordings from intact spinal cord, we immediately covered larvae with extracellular recording solution (in mM: 134 NaCl, 2.9 KCl, 1.2 MgCl2, 10 HEPES, 10 glucose, 2.1 CaCl2; adjusted to pH 7.8 with NaOH) and transferred them to the recording apparatus with no further dissection. For recordings from surgically dissected spinal cord, we first submerged larvae in 0.1% collagenase (Sigma) dissolved in extracellular recording solution. We removed the skin overlying the dorsal tail musculature with a sharp tungsten pin; after 10–15 min in collagenase, the blunt edge of the probe was used to gently abrade away the loosened muscle tissue to expose the spinal cord. We washed embryos 3× with fresh extracellular recording solution to remove collagenase and debris, and then transferred them to the electrophysiological recording apparatus. Cells were visualized with an Olympus BX51WI inverted microscope equipped with infrared DIC optics, epifluorescence, and a 40× immersion objective, and were viewed with a CCD camera controlled by QCapture Pro 6.0 software (QImaging). We selected cells with moderate TRPV1-RFPT expression for recording; each cell was filled with fluorescent dye during recording (from the micropipette; see below) and imaged after recording to confirm neuron identity. We performed all experiments at room temperature.

We pulled micropipettes for whole-cell recording from thin-walled filamented capillary glass (A-M Systems) on a Flaming-Brown puller (Sutter Instruments), to a tip diameter of ~3–5 µm and resistance of 8–15 MΩ. We backfilled micropipettes with intracellular recording solution (in mM: 125 K-glucoclate, 2 MgCl2, 10 HEPES, 10 EGTA, 4 Na2-ATP; adjusted to pH 7.2 with KOH) containing 0.01% Alexa Fluor 488 hydrazide fluorescent dye (Life Technologies). Micropipettes were advanced into the spinal cord using a motorized micro manipulator (MP-225, Sutter Instruments), under positive tip pressure (60 mm Hg) maintained by a pneumatic transducer (DPM-1B, Bio-Tek Instruments). Once the tip was near the soma of the Rohon-Beard neuron, positive pressure was released to obtain a gigahm seal to the cell membrane. A holding voltage of ~65 mV was applied, and a sharp suction pulse ruptured the cell membrane to initiate whole-cell recording. We performed all recordings in current-clamp mode, where standard corrections were made for bridge balance and pipette capacitance.

Electrophysiological data were acquired with a MultiClamp 700A amplifier (Molecular Devices) and a Digidata 1322A digitizer (Molecular Devices), recorded with Clampex 8.2 software (Molecular Devices), and analyzed offline with Clampfit (Molecular Devices) and Matlab (Mathworks). Electrical signals from Rohon-Beard neurons were filtered at 30 kHz and digitized at 100 kHz (Rf = 500 MΩ). Access resistance was monitored periodically during recording and was typically less than 80 MΩ; in cases where access resistance did increase over time, gentle suction was used to unplug the tip and bring the access resistance back down to an acceptable value. All recorded neurons had stable resting membrane potentials between ~40 and ~70 mV. Since we were concerned primarily with changes in membrane potential and excitability (rather than absolute values), we have reported values as recorded without applying a correction for the junction potential, estimated to be 18.5 mV at 23 °C.

We detected action potentials using a thresholding function, implemented in Clampex (Molecular Devices). We defined spike times by the peak of each action potential and converted to raster plots. We calculated average firing rate per unit time as the number of spikes fired over the course of an entire trial, divided by the length of that trial in minutes. To calculate peak firing rates, we convolved spike trains (1 ms bins) with a Gaussian kernel (sigma = 5 s), and determined the maximum value. We performed statistical analyses using Matlab (Mathworks). We used single depolarizing current pulses (50–300 pA) to coarsely determine the firing threshold of each neuron before Csn exposure. We used hyperpolarizing current pulses (10–40 pA) to determine the input resistance of neurons before and after Csn exposure (input resistance = steady-state change in membrane voltage divided by amplitude of applied current step; calculated as a linear fit to data points). We used repeated sets of depolarizing current pulses (25–400 mV, 200 ms each, 2s inter-stimulus interval) to probe changes in excitability during the depolarizing ramp response to Csn.

We applied Csn to the embryos in two ways. In perfusion experiments (used to collect data shown in Fig. 1i and Supplementary Figs. 6c and 7), Csn was pre-dissolved in...
extracellular recording solution (10 or 100 µM) and released into the dish (~1 mL/30 s) with simultaneous suction at the other side, such that the dish solution was replaced by the Csn solution over time. To estimate the dynamics of fluid exchange in the recording chamber during perfusion, we collected images as Fast Green dye (10 µM; Sigma) was perfused into the recording chamber, initially filled with water. As the concentrated dye mixed with the chamber solution, the mean luminance of the chamber images decreased over time. We collected images at 20 s intervals for 10 min, then used the time course of decreasing mean luminance to estimate how long it took for the dye concentration in the chamber to reach 10% and near 100% of the maximum dye concentration. Based on our observations, we estimate that the concentration of Csn in the chamber reached 10% of maximum within the first minute of perfusion and would reach near 100% of maximum only after 10 min of perfusion. For a subset of wash-out experiments, we replaced the Csn solution over time with fresh extracellular solution, and we repeated membrane potential, spiking threshold, and input resistance measurements following several minutes of perfusion with the wash-out solution. In diffusion experiments (Supplementary Fig. 6b), we released concentrated Csn (100 mM in DMSO) through a broken-off recording pipette positioned at the edge of the recording dish (final concentration of 100 µM in extracellular recording solution).

**Locomotor activity behavioral assay.** We raised larval zebrafish on a 14 h:10 h light:dark cycle at 28.5 °C (or 22 °C for Tg(islet1: GAL4VP16,4xUAS:TRPA1-RFPT) larvae) with lights on at 9 a.m. and off at 11 p.m. We placed individual larvae into each well of a 96-well plate (7701-1651, Whatman) containing 650 µL E3 embryo medium. In most experiments, we sealed plates with an optical adhesive film (4311971, Applied Biosystems) to prevent evaporation. This introduces air bubbles in some wells, which occludes tracking of larvae, and thus we excluded these wells from analysis. In islet1 experiments, we transferred 2 d.p.f. larvae into E3 medium containing Csn or DMSO vehicle control immediately before the start of behavioral recording at 12 p.m. In hcrt experiments, we transferred 5 d.p.f. larvae into E3 medium containing Csn 1 h before the start of behavioral recording at 11 p.m. We monitored locomotor activity using an automated videotracking system (Viewpoint Life Sciences) with a Dinion one-third inch monochrome camera (Dragonfly 2, Point Grey) fitted with a variable-focus megapixel lens (M5018-MP, Computar) and infrared filter. We recorded the movement of each larva at 15 Hz, with an integration time of 1 s for islet1 experiments and 1 min for hcrt experiments, using the quantization mode. The 96-well plate and camera were housed inside a custom-modified Zebrabox (Viewpoint Life Sciences) that was continuously illuminated with infrared lights and illuminated with white lights from 9 a.m. to 11 p.m. The 96-well plate was housed in a recirculating water chamber to maintain a constant temperature of 28.5 °C (or 25.5 °C for Tg(islet1: GAL4VP16,4xUAS:TRPA1-RFPT) larvae). The parameters for detection were: detection threshold, 15; burst, 29; freeze, 3. We processed data using custom PERL and Matlab (Mathworks) scripts. We performed statistical tests using JMP (SAS) and Matlab (Mathworks). In figures showing behavioral data, activity refers to the average amount of locomotor activity of all animals of a particular genotype or condition during the indicated time interval. It was previously shown that zebrafish larvae that are inactive for one or more minutes exhibit an increased arousal threshold, indicating that one or more minutes of inactivity is a sleep-like state

Statistical analyses. For c-fos FISH, GCAMP5G, cell ablation and behavioral data, we used the Wilcoxon rank-sum test for pairwise comparisons, and the Kruskal-Wallis test followed by the Steel-Dwass test for analyses involving multiple comparisons. To compare peak and average firing rates recorded during perfusion of DMSO vehicle alone and 10 µM Csn, we performed one-tailed Wilcoxon rank sum tests to test the hypothesis that firing rates were significantly higher with Csn than with DMSO vehicle. For experiments in involving quantification, samples were genotyped following analysis, and thus were scored in a blind fashion.

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