The *Drosophila* Gr28bD product is a non-specific cation channel that can be used as a novel thermogenetic tool

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Extrinsic control of single neurons and neuronal populations is a powerful approach for understanding how neural circuits function. Adding new thermogenetic tools to existing optogenetic and other forms of intervention will increase the complexity of questions that can be addressed. A good candidate for developing new thermogenetic tools is the *Drosophila* gustatory receptor family, which has been implicated in high-temperature avoidance behavior. We examined the five members of the Gr28b gene cluster for temperature-dependent properties via three approaches: biophysical characterization in *Xenopus* oocytes, functional calcium imaging in *Drosophila* motor neurons, and behavioral assays in adult *Drosophila*. Our results show that Gr28bD expression in *Xenopus* oocytes produces a non-specific cationic current that is activated by elevated temperatures. This current is non-inactivating and non-voltage dependent. When expressed in *Drosophila* motor neurons, Gr28bD can be used to change the firing pattern of individual cells in a temperature-dependent fashion. Finally, we show that pan-neuronal or motor neuron expression of Gr28bD can be used to alter fruit fly behavior with elevated temperatures. Together, these results validate the potential of the Gr28bD gene as a founding member of a new class of thermogenetic tools.
We examined here the temperature response of the five members of the Gr28b family (Gr28bA through Gr28bE) via three approaches: biophysical characterization in Xenopus laevis oocytes, functional calcium imaging in Drosophila motor neurons, and behavioral assays in adult Drosophila. Heterologous expression systems (e.g., oocytes or mammalian cell lines) are ideally suited for biophysical analysis, as any current can be better isolated and studied. Despite successful characterization of related gustatory and olfactory receptors in various artificial expression systems,14,15 previous attempts to heterologously express Gr28b proteins were reported ineffective. However, we were able to establish a quick and robust expression system to study the Grs in Xenopus oocytes, and found that Gr28bD is the only member of the Gr28b family that generates a temperature-dependent current. When expressed in Drosophila motor neurons, Gr28bD can alter the firing patterns of individual cells as a function of temperature. In behaving flies, neuronal expression of Gr28bD leads to temperature-dependent changes in behavior. Altogether, these results validate the potential of the Gr28bD gene as a founding member of a new class of thermogenetic tools.

Results

Biophysical properties of GR28bD in Xenopus oocytes. We report here the first successful expression of Gr28bD in Xenopus laevis oocytes, using established procedures (see Methods). We examined the temperature dependence, ionic selectivity, and basic kinetic properties of Gr28bD, using the two-electrode voltage-clamp (TEVC) technique to record oocyte currents under different conditions of temperature, membrane potential, and ionic composition of the extracellular solution. The other members of the Gr28b family were also tested, but no currents were observed.

When subjected to temperature steps (Fig. 1A, bottom panel), oocytes injected with Gr28bD cRNA exhibit large, temperature-sensitive currents (Fig. 1A, middle panel). In contrast, water-injected oocytes exhibit only a tiny amount of background current, with no significant voltage dependence (Fig. 1A, top panel). As can be observed from the example in Fig. 1A, our temperature application system cannot change temperature rapidly, which makes it difficult to estimate the kinetics of the Gr28bD current in response to a change in temperature. Cross-correlation analysis indicates that the current lags behind the temperature by approximately five seconds. Whether this lag is an experimental artifact or genuine channel kinetics remains to be determined through more precise temperature application experiments. For thermogenetic applications, it is very important to know whether the temperature-induced current can be maintained over long durations. From the response to temperature steps (Fig. 1A), we determined that the Gr28bD currents do not inactivate significantly over a 100-second time course. Moreover, the currents return to their initial value upon cooling.

The properties of a temperature-dependent current can be summarized by three quantities: the threshold of activation, the steepness of the response, and the saturation. To examine these properties, we subjected the oocytes to slow temperature ramps (approximately 2.5 °C/60 s), which should allow the channel to reach a state of quasi-equilibrium with respect to slow activation or inactivation kinetics, if any, and would also minimize temperature distribution artifacts within the recording chamber. The temperature was limited to a range of 10–40 °C to avoid damage to the oocyte and to maintain reliable recording conditions. An example of the current elicited by a temperature ramp is shown in Fig. 1B. In this case, the cross-correlation analysis indicates a lag of less than one second. Furthermore, the on-ramp and the off-ramp produce approximately symmetrical responses, altogether confirming that channels are at quasi-equilibrium at these slow ramp rates. The logarithm of the normalized current versus temperature is shown in Fig. 1C. Inspection of the current response reveals a threshold of activation of approximately 14 °C. Above this temperature, the logarithm of the current increases linearly with a slope that corresponds to a Q10 of 9.05 ± 0.88, until 20 °C, where Q10 changes to 5.20 ± 0.95 (n = 12). These results were obtained with an extracellular solution (ND96) commonly used for oocyte recordings, containing 1.8 mM Ca2+.

However, oocytes are known to express endogenous calcium-activated chloride currents,16 which could contaminate our measurements, should the Gr channel have a significant calcium conductance. We tested this possibility using a nominally calcium-free ND96 recording solution17 and found Q10 values of 9.9 ± 1.3 and 5.6 ± 1.2 (n = 6), below and above 20 °C, respectively. These values are not statistically different from those reported above, obtained with 1.8 mM Ca2+ (unpaired t-test, p-value > 0.12).

The Gr28bD currents do not exhibit any detectable voltage dependence, at either low (10 °C) or high (35 °C) temperature, as illustrated in Fig. 1D and E. In control oocytes, the endogenous currents are very small across a broad voltage range (−100 mV to +30 mV), with no discernable voltage dependence. This implies that the currents recorded from Gr28bD-injected oocytes are generated by Gr28bD, with very little contamination from endogenous currents across a wide range of temperatures and membrane potentials.

Under the ND96 extracellular solution, containing (in mM) 96 NaCl, 2 KCl, and 1.8 CaCl2, the Gr28bD currents are inward at −60 mV (see Fig. 1A), with a measured reversal potential of −12 ± 0.7 mV (Fig. 1E). This value suggests that Gr28bD would act as an excitatory agent in thermogenetic applications. Nevertheless, to be able to predict the response of the channel and the nature of its effect in other biological preparations and solutions, it is necessary to determine its ionic selectivity. Under the ND96 solution and assuming 7 mM Na+, 100 mM K+, and 0.1 mM Ca2+ intracellular concentrations,16 the predicted Nernst potentials would be approximately +69 mV for Na+, −104 mV for K+, and +24 mV for Ca2+, at 35 °C. The measured reversal potential of −12 mV under ND96 does not match any of these values, indicating that the channel is conducting more than one type of ion. Considering these measured and predicted values, the relative K+ conductance must be at least 35%, as would be calculated assuming zero relative Na+ conductance, or as much as 47%, assuming zero relative Ca2+ conductance.

To further investigate the relative conductance for Na+ ions, we recorded Gr28bD currents under various Na+ concentrations, with Na+ ions accordingly replaced by NMDG+, which is known not to permeate through Na+ or cation non-selective channels.18 The relative conductance for an ion can be calculated from the ratio between the observed shift in the reversal potential of the current and the predicted shift in the Nernst potential for that ion.
ion. As illustrated in Fig. 1E, when the extracellular Na\(^+\) concentration is reduced from 96 to 48 mM, the measured reversal potential changes from \(-12.0 \pm 0.7\) to \(-23.7 \pm 0.2\) mV, while the predicted Na\(^+\) Nernst potential changes from \(+69\) to \(+51\) mV, corresponding to a 65% relative Na\(^+\) conductance. When the extracellular Na\(^+\) is reduced to 2 mM, the measured reversal potential changes to \(-59.0 \pm 0.9\) mV, corresponding to an estimated 45% relative Na\(^+\) conductance. These results average to a 55% relative Na\(^+\) conductance.

Considering this and the above estimates for the K\(^+\) conductance (35% to 47%), the partial Ca\(^2+\) conductance should be less than 10%. We also tested low-calcium extracellular solutions, one nominally Ca\(^2+\)-free and one with 0.6 mM Ca\(^2+\), and found only a small negative shift in the reversal potential of less than 1 mV. This result is consistent with a Ca\(^2+\) conductance of less than 4%. Additional experiments in which Cl\(^-\) was replaced with gluconate (C\(_6\)H\(_{11}\)O\(_7\)^-) did not alter the reversal potential, suggesting that the Gr28bD current is neither carried by Cl\(^-\) ions nor contaminated by endogenous chloride currents (data not shown).

Altogether, these results clearly indicate the non-selective cationic nature of the current, similar to that of several TRP channels\(^{99}\). Although further experiments are necessary to obtain more precise values, most of the Gr28bD conductance appears to be due to Na\(^+\) (55%) and K\(^+\) (35–47%), with any potential remainder due to Ca\(^2+\) ions. In contrast to Gr28bD, expression of the four other Gr28b isoforms (A, B, C, and E) did not produce any measurable current, temperature-sensitive or not, above the endogenous level. Moreover, co-expression of all possible binary combinations of these isoforms did not elicit any temperature-dependent current (data not shown). However, the possibility of functional Gr28b heteromers cannot be ruled out, as more than two isoforms may be needed to form a functional protein.
Modulation of neuronal activity by Gr28bD. To use a thermogenetic tool, one must have a good way to calibrate and monitor its effects on neuronal activity. As shown by our biophysical experiments and by a previous study, Gr28bD generates a temperature-sensitive non-selective cation current in both *Xenopus* oocytes and *Drosophila* neurons, and elicits strong proboscis extension when expressed in sweet-responsive fruit fly chemosensors. According to these results, Gr28bD should, in principle, generate excitatory inward currents in target neurons. However, enough current must be generated relative to the battery of endogenous currents normally expressed by a cell to modulate cellular activity. Electrophysiology is potentially the most precise measuring method, but it is not suitable for large-scale monitoring of neuronal populations. Therefore, we used imaging techniques to test Gr28bD in adult *Drosophila*, in combination with a genetically-encoded calcium indicator (GCaMP6f), to determine if it produces a robust, temperature-dependent effect that can be easily identified, analyzed, and interpreted with common imaging and data analysis techniques. We also tested control flies expressing only GCaMP6f.

For these tests, we focused on the motor neurons in the lateral abdominal neuromeres, located in the ventral nerve cord (VNC). As illustrated in Fig. 2A, these neurons are a good target for calcium imaging experiments, as they are easy to identify and consistently exhibit robust firing activity. Moreover, these neurons are conveniently located in the same plane and exhibit calcium fluorescence with comparable intensity and good signal-to-noise ratio, altogether making it easy to image multiple cells at the same time. Additionally, motor neurons are generally larger than other neuronal types, making them a good test system to determine whether expression of Gr28bD can generate enough current to modulate cellular activity.

The thermosensitive effect of Gr28bD on neuronal activity was tested with temperature steps alternating between 19 and 28 °C. We recorded from 21 GCaMP6f control cells in five flies and 20 Gr28bD + GCaMP6f cells in six Gr28bD-expressing flies. Under repetitive application of temperature steps, calcium fluorescence activity lasted for 73.6 ± 4.5 minutes in control flies and 49.7 ± 7.4 minutes in Gr28bD-expressing flies. One would anticipate that changing temperature by approximately 10 °C would have an effect on neuronal activity, even in control flies. Would changes be different between control and Gr28bD-expressing flies? Indeed, as illustrated in Fig. 2B, control flies (top panel) and Gr28bD-expressing flies (middle and bottom panels) respond differently to temperatures steps. The most notable distinction is in how the average fluorescence intensity changes between low and high temperature (Fig. 2C). In control flies, the average fluorescence level is attenuated by high temperature (ΔF/F = −0.1524 ± 0.1358), but it is slightly increased in Gr28bD-expressing flies (ΔF/F = 0.03656 ± 0.10) (unpaired t-test; p-value < 0.00001).

Although the temperature-induced change in average fluorescence in Gr28bD-expressing neurons is small (∼3.7%) it should be regarded as relative to the control group. Accordingly, the Gr28bD-specific effect is an overall increase in fluorescence of almost 20%. The average calcium fluorescence is proportional to the overall neuronal activity, independent, to some degree, of the specific firing pattern (e.g., tonic spiking vs. bursting). Therefore, the observed changes in average fluorescence elicited by high temperature can be interpreted as a slow-down of neuronal activity in control flies versus an increase in activity in Gr28bD-expressing flies.

GCaMP6f is in principle capable of reporting individual action potentials. However, without simultaneous electrical recordings, it is difficult to determine whether the individual calcium fluorescence events detected in our data correspond to single action potentials or to more complex patterns of activity (e.g., episodes of high-frequency firing). Thus, we used the continuous wavelet-transform (CWT) with a Morlet wavelet to examine the spectral content of the calcium fluorescence data. As illustrated by the example recordings in Fig. 2B, there are significant differences between control (top panel) and Gr28bD-expressing flies (middle and bottom panels).

In control neurons, the spectral content of fluorescence data changes relatively little with temperature, with respect to both frequency and amplitude. These neurons are active at both low and high temperatures, as demonstrated by the multiple spectral bands that are visible in the 1–4 Hz frequency range, some of which potentially correspond to the repetition rate of action potentials. The strongest of these bands (1–2 Hz) becomes slightly more intense and more regular at high temperature. In contrast, some of the Gr28bD-expressing neurons exhibit similar tonic activity at 19 °C (n = 8), while others are in a more quiescent state (n = 12). The onset of high temperature triggers a remarkable increase in the amplitude of high-frequency oscillations, lasting as much as 100 seconds. The effects of temperature were reversible in both control and Gr28bD-expressing flies. After each four-minute exposure to 28 °C, the cells resumed their normal activity when temperature was returned to 19 °C, throughout the lifetime of the preparation.

Activation of Gr28bD alters behavior. Our imaging experiments demonstrate the ability of Gr28bD to induce temperature-dependent effects in the firing activity of individual neurons. However, we also see temperature effects in control flies. To determine how these specific and non-specific effects scale up to the level of the entire organism, we investigated the behavioral response of flies to different temperatures in the 24–40 °C range. Gr28bD and each of the other four different transgenes in the Gr28b family were individually tested with pan-neuronal overexpression using the nSyb-GAL4 driver. An initial screening exposed flies to temperatures ranging from 24–40 °C in two-degree steps (Fig. 3A). Most of the flies overexpressing Gr28bD became incapacitated at 34 °C, in contrast to flies overexpressing the Gr28ba, C and E transgenes, which did not incapacitate. A temperature response was also observed for Gr28bB flies, but only at the upper temperature limit of our experimental paradigm (40 °C), which prevented further investigation. We also tested the effect of a more restricted overexpression of Gr28bd in motor neurons only, using the OK6-GAL4 driver. In this case, we found that most of the flies were incapacitated at 36 °C (Fig. 3A). In the remainder of the Results section all references to overexpression should be read as “pan-neuronal” overexpression. To obtain a better resolution of the Gr28bD-induced effect, flies from control and experimental genotypes were subjected to temperatures in the 32–40 °C range in one-degree steps. Flies overexpressing Gr28bD became incapacitated between 33 and 36 °C, with most of the flies incapacitated at 34 °C (Fig. 3B). In contrast, no genetic control flies were incapacitated.
Figure 2. Temperature-dependent modulation of cellular activity by Gr28bD. (A) Left panel: dorsal view of adult Drosophila dissected to expose ventral nerve cord motor neurons to saline bath. Right panel: composite image of epifluorescence (green on black) and two-photon 3D reconstruction (gray scale) of abdominal neuromere neurons expressing GCaMP6f. The green trace is an example of single-cell activity, as obtained via epifluorescence from the indicated region of interest. (B) Single-cell fluorescence signals (blue traces) obtained in response to a temperature step (red traces), analyzed by continuous wavelet-transform (spectrogram). Top panel: representative data from control flies expressing only GCaMP6f in motor neurons. Middle and bottom panels: representative data sets illustrating two types of temperature responses in flies expressing GCaMP6f + Gr28bD in motor neurons. (C) Change in average fluorescence between low and high temperature. ΔF/F was calculated as (F_H − F_L)/F_L, where F_L and F_H are the average fluorescence intensities (in arbitrary units) for a given cell, at low and high temperature. Data points are mean ± SDV (n = 21 cells from five flies for GCaMP6f, and n = 20 cells from six flies for GCaMP6f + Gr28bD). The blue lines represent mean ± SDV for each data set. ΔF/F = −0.1524 ± 0.1358 in control flies and 0.03656 ± 0.10 in GR28bD-expressing flies (unpaired t-test; p-value < 0.00001).
Next, we tested whether the time to incapacitation and the time to recovery from incapacitation are affected by the exposure temperature. We examined these two quantities by exposing flies overexpressing Gr28bD to a sudden increase in temperature for 90 seconds (Fig. 3C). All flies exposed to 36, 38, and 40 °C became incapacitated during the 90 second exposure, with more than 50% reaching incapacitation within 10 seconds (Fig. 3D).

At 34 °C, only ≈65% of the exposed flies became incapacitated and the time to incapacitation was significantly longer, suggesting that 34 °C is a threshold value for temperature-sensitive behavior. In contrast, none of the flies recovered faster when subjected to lower temperature. In A, B, and D, statistical difference between control and experimental genotypes was determined with a Kaplan-Meier test, p < 0.000001 = ****. In each experiment, 60–80 flies of each genotype were tested.
genetic control flies were incapacitated within the 90 seconds exposures at any tested temperature, with the exception of the UAS-Gr28bD heterozygous flies. About 10% of these flies became incapacitated at 40 °C, potentially due to a leaky expression of the UAS-Gr28bD transgene. The time to recovery for flies overexpressing Gr28bD was more graded than the time to incapacitation (Fig. 3E). Flies exposed to high temperatures took an exponentially longer time to recover than flies exposed to low temperatures. From 34, 36, 38, and 40 °C, the 50% recovery time was 20, 162, 383, and 760 seconds, respectively. Moreover, at 40 °C, the recovery was not complete within the 1080 seconds of the recovery phase of the experiment with 22% of the flies remaining incapacitated.

Discussion

The ability to control neuronal activity is the most direct and powerful approach for understanding how neural circuits control behavior. An ideal suite of tools for extrinsic neuronal control would include activating and inactivating methods that can target multiple cell types and neuronal populations in a behaving animal. The development of thermogenetic tools as a complement to optogenetics and other approaches is a promising step toward multi-modal control of neuronal activity. The Drosophila gustatory receptor gene family, which has been largely unexplored with respect to temperature responsiveness, provides an opportunity to identify novel thermogenetic tools.

We show here that one member of the Gr28b family, Gr28bD, generates a temperature-dependent cation current when expressed in Xenopus oocytes. This current does not require any explicit co-expression of additional factors, suggesting that Gr28bD forms a previously uncharacterized temperature-sensitive ion channel (Fig. 1). Moreover, we show that overexpression of Gr28bD in motor neurons can be used to drive temperature-dependent neuronal activity (Fig. 2). The expression system is sufficient to drive activity in these relatively large neurons, suggesting that the Gr28bD product can be used in multiple cellular contexts. Finally, we show that temperature activation of Gr28bD overexpressed in neurons can have profound effect on behavior (Fig. 3). Expression of Gr28bD in all neurons gives rise to temperature-dependent paralysis, with the time to incapacitation and the recovery time both related to exposure temperatures. We also demonstrate that flies with Gr28bD expression restricted to motor neurons display a similar temperature-dependent paralysis, but shifted by approximately +2 °C. This small shift could be explained by a difference in Gr28bD expression levels, as caused by different strengths of the two GAL4 drivers, and/or by differences between the neuronal types overexpressing Gr28bD. According to these results, the Gr28bD gene product is useful as a thermogenetic tool and is a good starting point for designing new molecular tools for extrinsic control of neuronal activity.

Our first attempts to demonstrate that behavior can be altered with Gr28bD activation showed that the gene product has a sharp temperature effect, with nearly all flies reaching paralysis within a narrow temperature window (33–36 °C). This temperature range for Gr28bD is about 4 °C higher than that required to activate and paralyze TRPA1-expressing flies (data not shown). Interestingly, the time required for recovery from paralysis was temperature dependent, with flies exposed to higher temperatures taking longer to recover from paralysis. The difference in recovery at different temperatures could be a function of the Gr28bD product or of the neural systems that need to recover from extrinsic activation. However, the temperature-sensitive currents recorded in Xenopus oocytes expressing Gr28bD follow the change in temperature equally rapidly (Fig. 1A), within seconds, between any two temperatures. This suggests that the temperature-dependent slow recovery rates observed in the behavioral experiments are the result of neuronal properties, rather than Gr28bD temperature sensitivity and kinetics.

Further experiments are necessary to more precisely determine the time response of the Gr28bD to changes in temperature. With the estimates we have so far (seconds), it is possible that Gr28bD kinetics are slow compared to fly behaviors such as the escape response or the tuning of wing movements in flight. Nevertheless, the response kinetics are compatible with the testing of other behaviors, such as phototaxis, negative gravitaxis, and conditioning, which are typically measured in the seconds range.

While Gr28bD has clear temperature sensitive effects that can be corroborated at all tested levels (oocytes, individual neurons, and whole organism), the other members of the Gr28b family have no observable effect (Gr28bA and C) or are less consistent across experimental paradigms (Gr28bB and E). Expression of Gr28bB in Xenopus oocytes does not show a temperature-dependent current (data not shown). However, pan-neuronal expression of Gr28bB gives rise to temperature-dependent paralysis at the highest probed temperature of 40 °C (Fig. 3A). One possible explanation is that Gr28bB requires higher temperatures to activate and generate current, temperatures which could not be reached in the Xenopus experiments. Alternatively, there may be insufficient expression of Gr28bB in Xenopus oocytes, causing a lack of detectable current. Heterologous expression systems that can tolerate higher temperatures may be able to address this issue. Nevertheless, the observed temperature-dependent paralysis is an encouraging result, suggesting that the Gr28bB gene product may also generate a temperature responsive current. Finally, there is evidence from a previous study that Gr28bE has temperature response properties when expressed in the hot-cells of the Drosophila antenna. In our experiments, expression in Xenopus oocytes and in all Drosophila neurons does not show any detectable temperature-dependent effect of Gr28bE. There may be factors in the hot-cells that provide the environment for Gr28bE temperature responsiveness that are lacking in other neurons and in Xenopus oocytes.

To summarize, we show that Gr28bD, a member of the Drosophila gustatory receptor family, can be used as a thermogenetic tool. Gr28bD produces a temperature-dependent current in heterologous cells, is able to activate multiple cell types in the fly, and can be used to interrogate neural circuit function in imaging and behavioral studies. Further testing of the gustatory receptor family may identify new members with temperature sensitive properties that may aid in the development of designer thermogenetic tools. To be useful in multiple model systems and cellular contexts, these proteins could be further engineered with respect to their temperature sensitivity (activation threshold and Q10), ionic selectivity, regulation by other physical and chemical factors, and cellular localization.
Methods
All reagents were purchased from Sigma-Aldrich, USA (St. Louis, MO).

**Gr28b constructs and oocyte expression.** The Gr28b genes were cloned in pcDNA3.1+/- (Invitrogen) (GenScript). Complementary RNA (cRNA) was synthesized using T7 polymerase (mMessage mMACHINE kit, Ambion, USA), after linearizing the DNA with Xmal. All Gr28b constructs were expressed in Xenopus laevis oocytes, and studied 1–3 days after cRNA injection. The oocytes were incubated at 15 °C in an ND96 solution containing (in mM): 96 NaCl, 2 KCl, 5 HEPES, 1 MgCl₂, 1.8 CaCl₂, 7.5 NaHCO₃, 1.5 CaCl₂, 4 MgCl₂, with an osmolarity between 270–275 mOsm. The saline was continuously bubbled with 95% O₂/5% CO₂ to a pH of 7.3. Perfusate temperature was regulated with a bipolar temperature controller (CL-100, Harvard Apparatus, USA) and a dual in-line heater/cooler (SC-20, Warner Instruments, USA). Q₁₀ values were calculated from slow temperature ramp experiments, using the equation

\[ Q_{10} = \left( \frac{T_2}{T_1} \right)^{\text{slope}} \]

where \( Q_{10} \) is the temperature coefficient, \( T_1 \) and \( T_2 \) are the temperatures in °C, and \( s \) is the slope of the linear fit to the logarithmic plot of the normalized current versus temperature. The equilibrium potentials were calculated using the Nernst equation:

\[ E_X = \frac{RT}{zF} \ln \left( \frac{[X]^e}{[X]^i} \right) \]

where \( E_X \) is the equilibrium potential for a given ion X, \([X]^e\) and \([X]^i\) are the extracellular and intracellular concentrations of X, respectively. \( R \), \( T \), and \( F \) are the usual quantities: the gas constant, the temperature, the valence of the ion, and Faraday’s constant, respectively. The data were analyzed with Clampfit 10 (Molecular Devices, USA) and Origin 2017 (OriginLab, USA).

**Calcium fluorescence imaging and data analysis.** Flies, 5–11 days old, were dissected as previously described. To eliminate descending thermotaxis signals to the ventral nerve cord, the head of the fly was removed before imaging. The dissected preparations were perfused in external saline containing (in mM): 103 NaCl, 3 KCl, 5 N-tris(hydroxymethyl) methyl-2-aminoethane-sulfonic acid, 8 trehalose, 10 glucose, 26 NaHCO₃, 1 NaHPO₄, 1.5 CaCl₂, 4 MgCl₂, with an osmolarity between 270–275 mOsm. The saline was continuously bubbled with 95% O₂/5% CO₂ to a pH of 7.3. Perfuse temperature was regulated with a bipolar temperature controller (CL-100, Harvard Apparatus, USA), a SC-20 dual in-line heater/cooler (Warner Instruments, USA), and a LCS-1 liquid cooling system (Warner Instruments, USA). A custom imaging stage was designed in SketchUp (sketchup.com) and 3D printed with PLA using an Ultimaker Original+ (Ultimaker, Netherlands). Lateral motor neurons in the abdominal neuromeres were imaged on a customized upright microscope (Scientifica, UK) equipped with epifluorescence and two-photon imaging. For wide-field illumination, CoolLED p100 LED sources (CoolLED Ltd., UK), GFP filters (Chroma, USA), and a 40X water-immersion objective (Olympus LUMPLFLN40XW) were used. Images were acquired using a Hamamatsu SC81000 Flash 4.0 V2 camera (Hamamatsu Photonics KK, Japan), typically with a 25 ms exposure time. For two-photon imaging, we used a Ti:Sapphire MaiTai HP laser (Maimi Microscopy, USA), typically with a 25 ms exposure time. A custom imaging stage was designed in SketchUp and 3D printed with PLA and Extrudr Original+. The movement of each fly was measured at each temperature step for 90 seconds. Six or seven trials were conducted for each experimental genotype, with 10–12 flies in each trial. A fly was considered incapacitated if it was inactive for at least 45 s within a 90 s window. To determine the time-to-incapacitation and the time-to-recovery, flies were allowed to acclimate at 24 °C for 90 s prior to a temperature jump to 34–40 °C for 90 s, and then allowed a recovery time of 15 minutes.
The movement of flies was recorded for the entire duration of the test. Observations from incapacitation assays were analyzed with the Statistica 8.0 software (StatSoft, USA), using Kaplan–Meier survival analysis with p-values of less than 0.05 considered significant.

Data availability. The datasets generated during the current study are available from the corresponding authors on reasonable request.

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Acknowledgements
We thank Paul Garrity for generously providing fly stocks. The Bloomington Drosophila Stock Center also provided fly stocks. We also thank Marco Navarro for help with the experiments and Dr. Andrew McClellan for help with the data analysis. The project was supported by NSF Grant 1535790.

Author Contributions
Performed experiments: A.M., A.S., B.B., M.K., M.A., A.R., J.L., and B.Z. Data analysis: A.M., A.S., B.B., K.M., M.A., A.R., B.Z., J.L., M.M., and T.Z. Wrote the paper: A.M., A.S., B.B., L.M., M.M., and T.Z., with input from all authors.

Additional Information
Competing Interests: The authors declare that they have no competing interests.
