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TRPV3 channel activity helps cortical neurons stay active during fever

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eLife Assessment

This is a **valuable** study of the physiological mechanisms promoting network activity during fever in the mouse neocortex. The supporting evidence is **solid**, and has improved with revision, along with increased clarity of presentation.

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Abstract

Fever raises body temperature (T_b) from $\sim 37^\circ\text{C}$ to beyond 38.4°C to combat pathogens. While generally well tolerated below 40°C , in rare cases fever can abnormally elevate neural activity and induce seizures in neurotypical children aged 2–5 years. This study investigates the mechanisms by which neuronal activity is maintained and stabilized during exposure to fever-range temperatures. Recordings of layer (L)4-evoked spiking in L2/3 pyramidal neurons (PNs) of mouse somatosensory cortex revealed four outcomes as temperature increased from 30°C to 36°C and 39°C (fever-range): neurons remained inactive, stayed active, ceased activity, or initiated activity. Roughly equal proportions of neurons ceased or initiated spiking, making the subset of “STAY” PNs, those that remain active across temperatures, crucial for maintaining stable cortical output. STAY PNs were more prevalent at younger postnatal ages. Their firing stability was supported by a distinct ion channel composition, including the thermosensitive channel TRPV3, which enables continued spiking by adjusting depolarization to meet spike threshold. Intracellular blockade of TRPV3, but not TRPV4, significantly reduced the proportion of STAY PNs and suppressed spiking at 39°C . Moreover, in *Trpv3*^{-/-} mice, temperature increases to 39°C reduced both spiking and postsynaptic potential amplitude, and these mice exhibited a delayed seizure onset. Together, these findings suggest that TRPV3 contributes to the preservation of cortical activity during fever.

Introduction

In most mammals, including humans, core body temperature (T_b) is tightly regulated between 36.1 and 37.8°C , with a daily fluctuation of 0.5°C (Campbell I, 2008). Fever or other thermal challenges can impede heat dissipation to significantly increase T_b beyond 38°C (Wang et al., 2014). Because global brain temperature and core body temperature are largely congruent, large increases in temperature affect both regions (Wang et al., 2014). Increases in body temperature up to 40°C are generally well tolerated, but temperatures above 42°C (within the hyperthermia and heat stress range) become harmful, leading to protein denaturation and cell death. The body and brain employ protective mechanisms, such as heat shock proteins, to cope with heat stress. These proteins are rapidly induced or upregulated at noxious temperatures, aiding in the

refolding or degradation of damaged proteins once temperatures return to physiological levels (Miller and Fort, 2018). However, the mechanisms that stabilize and preserve neuronal firing at lower, fever-range temperatures are not well understood.

In rare cases, moderate-grade fevers ($T_b = 38.1$ to 39°C) cause seizures in children aged 6 months to 6 years, with the highest susceptibility between 2 and 5 years (Oakley et al., 2009; Shinnar and O'Dell, 2004). A seizure is defined as a sudden, temporary burst of synchronized, excessive neuronal firing in the brain. These febrile seizures (FS) are almost typically triggered by the thermal component of fever, as infections or metabolic disorders are absent (Shinnar and O'Dell, 2004). Additionally, FS can be induced solely by increased T_b due to ambient heat, such as a warm bath, hot room, or overdressing. Beyond age five, FS rarely occur, suggesting that developmental changes in brain physiology stabilize network excitability during this window of susceptibility (Shinnar and O'Dell, 2004). Identifying the electrophysiological mechanisms underlying this protective shift could provide insight into normal brain maturation and reveal processes whose disruption may increase susceptibility to febrile seizures.

Rodents exhibit a similar age-dependent susceptibility to thermally induced seizures. The second postnatal week is proposed as a developmental window of heightened seizure susceptibility. During this period, fever-range temperatures (38.1 to 39°C), comparable to the range at which FSs occur in children (Holtzman et al., 1981), do not typically induce seizures. Instead, seizures arise only at higher thresholds ($> 42.7^\circ\text{C}$), consistent with hyperthermic rather than febrile seizures. This raises the question: what physiological changes occur during the second postnatal week that prevent seizures from being triggered at fever-range temperatures ($\sim 39^\circ\text{C}$)?

For practical reasons, EEG recordings for the onset of FS in humans are limited. However, EEG traces from rodents with thermally induced seizures show that ictal events often initiate in the hippocampus and propagate to the cortex, with the later spread to the cortex being correlated to the more severe behavioral stages of the seizure i.e. Racine stage > 4 (Dube et al., 2010; Baram et al., 1997). Thus, the cortex represents a critical locus in the seizure phenotype and likely contains mechanisms that may alter neural activity in response to temperature changes. In this manuscript, rather than examining how fever provokes seizures, we focus on identifying the electrophysiological adaptations during postnatal development that stabilize cortical activity at 39°C . Specifically, we compare two developmental stages flanking the proposed window of hyperthermic seizure susceptibility (P12–14): an earlier stage (P7–8) and a later stage (P20–23). This design also provides negative controls, allowing us to determine whether changes observed in the P12–14 window are unique.

Early comparative studies correlating rat and human brain development using neuronal birth dates, myelination patterns, and saltatory growth stages, suggest that 5–7-day-old rats approximate full-term human newborns (Dobbing and Sands, 1973). More recent analyses based on hippocampal and cortical maturation indicate that the second postnatal week (P10–14) corresponds to human infancy (Baram et al., 1997; Bender et al., 2004). In contrast, the third and fourth postnatal weeks align with the human juvenile stage (Baram et al., 1997). Here, using wildtype mice across three postnatal developmental periods—postnatal day (P)7–8 (newborn), P12–14 (infancy), and P20–26 (juvenile)—we investigated the electrophysiological properties, *ex vivo* and *in vivo*, that enable excitatory cortical pyramidal neurons (PNs) neurons in mouse primary somatosensory (S1) cortex to remain active during temperature increases from 30°C (standard in electrophysiology studies) to 36°C (physiological temperature), and then to 39°C (fever-range).

Finally, to begin identifying molecular determinants that may mediate the electrophysiological properties enabling firing stability in cortical PNs, we extended our studies to two members of the thermosensitive, Ca^{2+} -permeable, nonselective cation channels of the transient receptor potential vanilloid (TRPV) family: TRPV3 and TRPV4. These channels are activated within the innocuous warm temperature range (TRPV3: 31 – 39°C ; TRPV4: 27 – 34°C) (Su et al., 2023; Shibasaki et al., 2007), unlike TRPV1 and TRPV2, which respond to noxious heat above 42°C and 52°C , respectively (Kashio and Tominaga, 2022). TRPV3 and TRPV4 share low ($\sim 41\%$) amino acid sequence identity (Smith et al., 2002) and are both expressed in the brain (Xu et al., 2002);

Caterina, 2007 [↗](#); Kanju et al., 2016 [↗](#); Chen et al., 2022 [↗](#)). Given its activation within the 31–39 °C range, we hypothesized that TRPV3 may specifically contribute to neuronal adaptations occurring within fever range (Su et al., 2023 [↗](#); Shibasaki et al., 2007 [↗](#)).

In summary, we aimed to elucidate adaptative neural mechanisms that allow cortical excitatory PNs to maintain stable spiking despite substantial increases in brain temperature into the fever range. Across three postnatal developmental ages, excitatory cortical PNs responded to depolarizing stimuli during temperature increases from 30°C to 39°C, by remaining inactive, continuing to spike, ceasing spiking, or initiating spiking. Nearly equal proportions of neurons ceased or initiated activity, so overall firing stability was maintained largely by “STAY” neurons, which remained active throughout temperature increases. We propose that STAY neurons likely achieve this stability through unique ion channel compositions, including those from TRPV3, which allows for higher depolarization levels through increased excitatory synaptic input relative to inhibitory input.

Results

Body Temperature rises to fever levels during regular activity and exposure to higher ambient temperatures

Contrary to common belief, T_b is not fixed but varies with arousal state, motor activity, and environmental temperature (Kiyatkin EA, 2019; Hankenson et al., 2018 [↗](#); Wang et al., 2014 [↗](#)). Using a sterile IPTT-300 implantable temperature transponder (Bio Medic Data Systems, LLC) with a 0.025-second time constant and 0.1°C accuracy, T_b measurements were recorded every five-minutes over six hours from 2–3-week-old wildtype mice in an open-field arena at room temperature (**Figure 1A** [↗](#)). This analysis revealed a stable median temperature of 36.5°C with a maximum and minimum fluctuation of 0.5°C (**Figure 1A** [↗](#)). However, within-subject analysis showed brief T_b elevations into the fever range (shaded red bars) during increased activity like cage climbing or digging (**Figure 1B–C** [↗](#)). Exposure to infrared light elevates ambient temperatures and induces prolonged T_b elevations into the fever range, with an onset within minutes and a duration of several hours (**Figure 1D** [↗](#)).

Spiking in P12-P14 cortical excitatory pyramidal neurons remains stable as temperature enters the fever range

To investigate how postnatal cortical neurons respond to fever-range temperature, we used whole-cell current clamp electrophysiology to record natural spiking, evoked synaptically, as brain slice temperature gradually rose from 30°C to 36°C to 39°C in PNs (**Figure 2A–B** [↗](#)). Acute S1 cortex slices (350 µm thick) were prepared using standard methods (Antoine et al., 2019 [↗](#)). PNs were visually identified via infrared DIC optics, and physiological verification for regular spiking was done in current clamp. Neuronal activity in PNs was evoked by stimulating in the barrel center of cortical layer (L) 4 using a bipolar electrode (0.2 ms pulses) at a specific stimulation intensity i.e. 1.4X E_0 (**Figure 2A** [↗](#)). E_0 is defined as the minimal intensity evoking an excitatory postsynaptic current (EPSC) during more than 3 of 5 consecutive sweeps with 10 s inter-sweep interval. E_0 was determined in voltage clamp for each recorded cell, prior to recording post-synaptic potentials (PSPs) and spiking in current clamp. L4-evoked PSPs and spiking, were recorded from L2/3 PNs, with resting membrane potential (V_m) set just below spike threshold (−50mV) to simulate in vivo conditions (Yamashita et al., 2013 [↗](#)).

Recordings of synaptically-evoked activity in L2/3 wildtype cortical excitatory PNs from P12–14 mice showed significant effects of temperature elevations from 30°C to 39°C. These changes resulted in an increased depolarization required to reach spike threshold (ST) (defined as the minimal V_m that just elicits spiking) (**Figure 2B–C** [↗](#)). Consistent with previous findings, measurements of input resistance (R_{in}) which reflect the extent to which membrane channels are open, showed significant reductions across all recorded neurons with increasing temperature (**Figure 2D** [↗](#)). Similar temperature-induced effects on R_{in} have been reported in L2/3 PNs in the

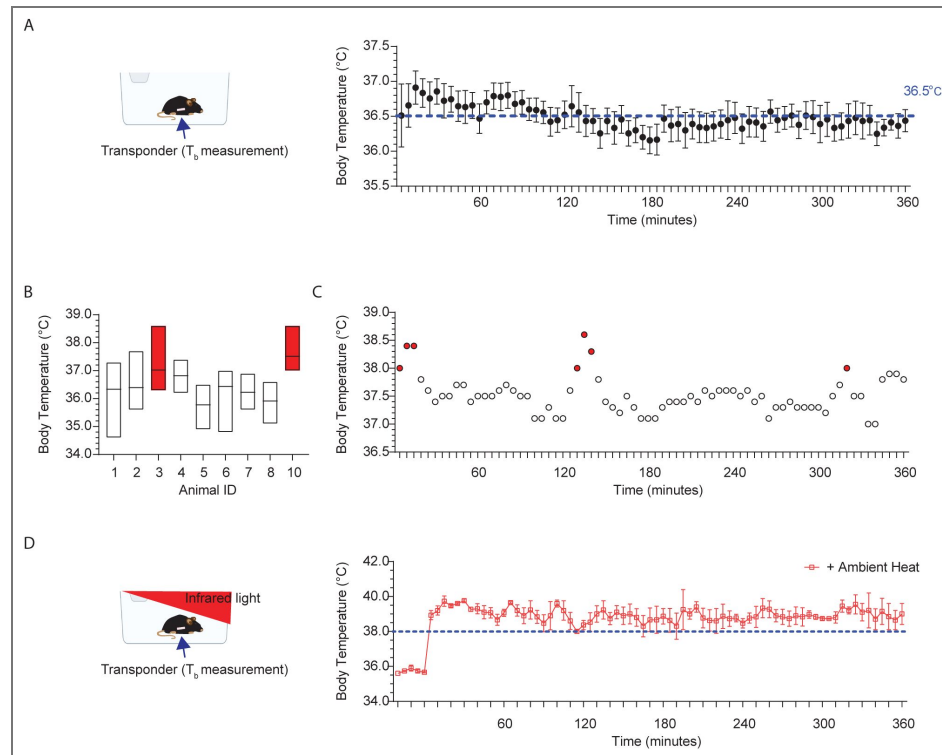


Figure 1. Body Temperature rises to fever levels during regular activity and upon exposure to higher ambient temperatures.

A. Left: Setup for recording mouse body temperature (T_b) at room temperature using an implanted transponder for non-invasive measurement. Right: Average T_b readings over 6 hours at 5-minute intervals for 10 mice. T_b typically hovers around 36.5 $^{\circ}\text{C}$ during the day. **B.** T_b may briefly elevate into fever range (shaded red bars) during regular daily activity. Median with upper and lower limits indicated. **C.** T_b readings over 6 hours, at 5-minute intervals for animal 3 in [Figure 1B](#). Shaded red dots indicate time points when T_b enters the fever range. **D.** Left: Setup for T_b recordings during infrared light exposure. Right: T_b elevates into the fever range (>38 $^{\circ}\text{C}$) for extended durations, lasting minutes to hours. Average T_b readings over 6 hours at 5-minute intervals for 10 mice.

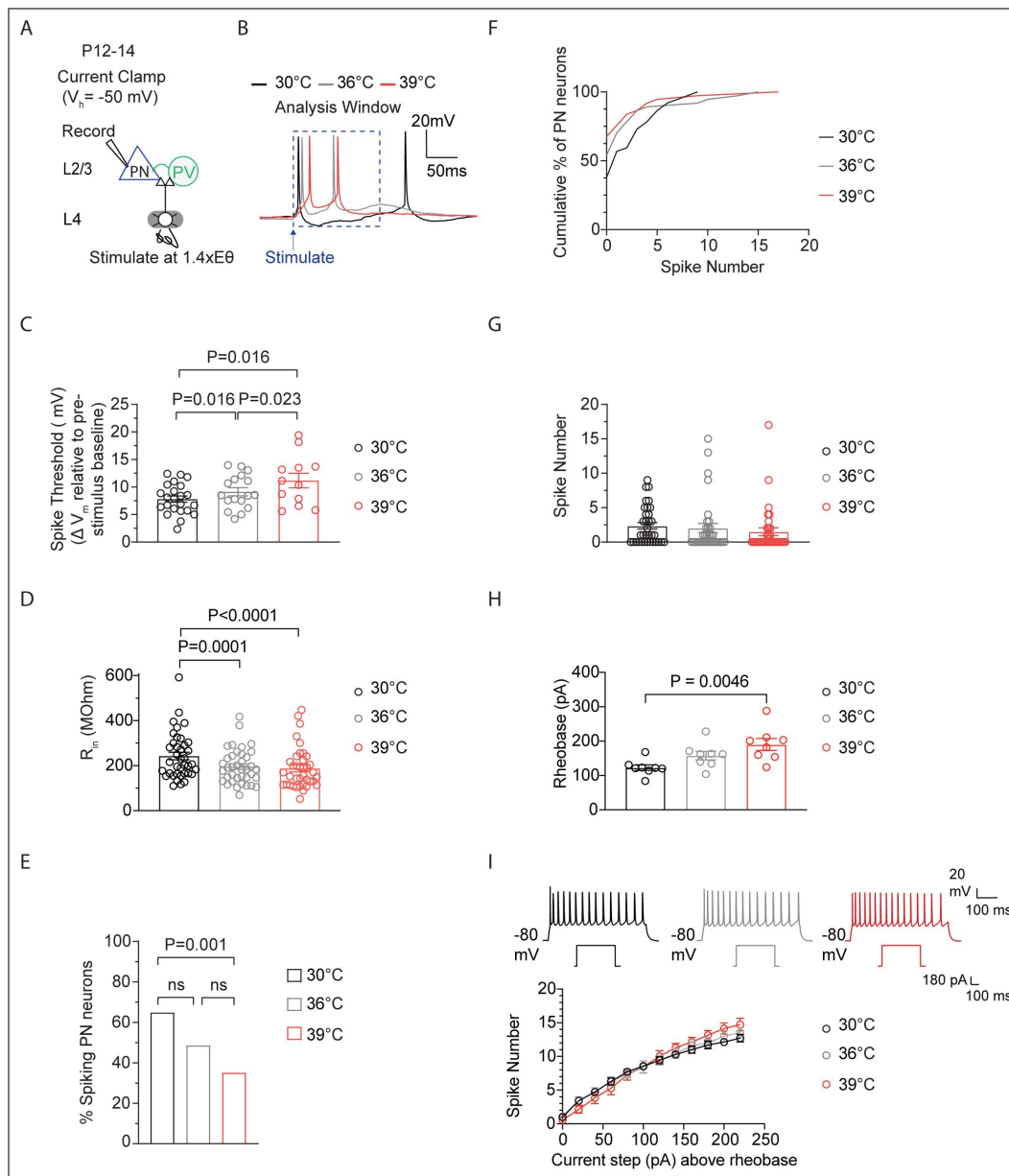


Figure 2. Spiking in P12-14 cortical excitatory pyramidal neurons remains stable as temperature enters the fever range.

A. Setup for recording L4-evoked postsynaptic potential and spiking in a cortical excitatory pyramidal neuron (PN) at just-subthreshold V_m at 30°C, 36°C and 39°C in mouse primary somatosensory (S1) cortex. **B.** Example traces of L4-evoked spikes in L2/3 PN during consecutive recordings at 30°C (black), 36°C (gray), and 39°C (red). **C.** Depolarization required to reach spike threshold (ST) in L2/3 PNs during temperature elevations from 30°C to 39°C. **D.** Same as (C) for input resistance (R_{in}). **E.** Same as (C) for the percentage of spiking L2/3 PNs. **F.** Same as in (C) for the spiking distribution. **G.** Same as in (C) for the number of spikes. **H.** Rheobase in L2/3 PNs during temperature elevations from 30°C to 39°C. 8 PNs recorded from 3 animals. **I.** Quantification of evoked spiking for F-I curves in L2/3 PNs during temperature elevations from 30°C to 39°C. Each recorded cell was exposed to the full temperature range (30 to 39°C). 8 PNs recorded from 3 animals. In **C**, **D** and **G**, each data point represents an individual cell. In **C-F**, 37 PNs recorded from 14 animals, with each cell exposed to temperature elevations from 30°C to 39°C. Data in **C**, **D**, and **G** are shown as mean $\pm$ SEM. Statistical significance was assessed as follows: **C**, **D**, **G**: one-way repeated-measures ANOVA with Tukey's post-hoc test ($\alpha = 0.05$); **E**: two-tailed binomial test; **F**: Kolmogorov-Smirnov (K-S) test; **H**: two-way repeated-measures ANOVA with Tukey's post-hoc test ($\alpha = 0.05$).

rat visual cortex (Hardingham and Larkman, 1997) and in hippocampal CA1 and CA3 PNs from P13-P16 mice (Kim and Connors, 2012). The depolarized ST and reduced R_{in} in PNs would likely decrease cell excitability and spiking. Indeed, the percentage of spiking cells decreased during temperature elevations from 30°C to 39°C (Figure 2E). However, contrary to expectations, average spiking remained remarkably stable (Figure 2F-G). We also examined the temperature-induced effects on L2/3 PNs in the absence of excitatory and inhibitory synaptic inputs. Intrinsic spiking was measured in the presence of synaptic blockers (100 μ M APV, 10 μ M NBQX, and 3 μ M gabazine). PNs were held at -80 mV, followed by positive current injection (500 ms) steps to determine rheobase (the minimum current injection needed to produce one spike). At 39°C, rheobase was increased compared to 30°C (Figure 2H). Current was injected up to 250 pA above rheobase at 30°C, 36°C, and 39°C, using a new brain slice for each recording. Although rheobase was decreased at 39°C compared to 30°C, we found that the mean number of spikes produced did not differ across current steps (Figure 2I).

Spiking in P7-8 cortical excitatory pyramidal neurons decreases as temperature enters the fever range

This stability in average spiking at 39°C, despite the loss of some previously active neurons, suggests the presence of temperature-adaptive changes in PNs that help to maintain normal circuit activity levels. These changes may either initiate firing in previously non-spiking neurons or either maintain or elevate spiking levels in currently active cells. Interestingly, in P7-8 mice, temperature elevations from 30 °C to 39 °C produced effects on ST and R_{in} similar to those observed in P12-14 mice, with ST increasing and R_{in} decreasing (Figure 3A-D). However, in P7-8 PNs, during temperature elevations from 36 to 39°C, ST did not increase, and R_{in} decreased (Figure 3C-D). Additionally, the percentage of spiking PNs decreased during temperature elevations from 30°C to 39°C (Figure 3E). In contrast to P12-14 PNs, the average spiking levels in P7-8 PNs significantly decreased during these temperature elevations (Figure 3F-G). This suggests that temperature-adaptive changes, possibly involving R_{in} and ST, which help maintain average spiking levels at fever temperatures despite the loss of some previously active neurons, may be underdeveloped or absent at this earlier age.

Increased depolarization at higher spike thresholds helps maintain stable spiking activity in cortical pyramidal neurons during temperature elevations

The temperature-induced increases in ST at P12-14, would make it harder for a cell to spike, as larger depolarization levels would be required to maintain spiking (Figure 4A). Therefore, we hypothesized that in cells with increased ST that remain spiking, a temperature-adaptive change might involve a proportional increase in depolarization. To determine if larger depolarizations were associated with higher (less negative) STs, we analyzed the relationship between peak postsynaptic potential (PSP), which indicates maximum depolarization, and ST (Figure 4B).

Correlation analysis revealed a strong positive correlation between peak PSP and spike threshold at P12-14, which was absent at P7-8 and P20-23 (Figure 4C). This correlation was specific to these parameters, as it did not occur for comparisons involving input resistance, an intrinsic property which is affected by temperature changes (Figure 4D-E).

One mechanism that could promote enhanced depolarization is a reduction in the inhibitory PSP (IPSP) (Figure 4F). In addition to the early component of the PSP (the peak), where excitation is maximal, we also quantified the late component, where inhibition levels are greatest and thus largely represents the IPSP (Bhatia et al., 2019; Antoine et al., 2019) (Figure 4B). This analysis revealed that the late PSP (LPSP) was significantly reduced with temperature elevations from 30°C to 39°C (Figure 4G). Further analysis indicated that there was no correlation between the ST and LPSP at any age (P7-8: $r=0.17$, $P=0.41$, XY pairs=25; P12-14: $r=0.24$, $P=0.31$, XY pairs=19; P20-23: $r=0.33$, $P=0.28$, XY pairs=13 Pearson's test). However, a significant, moderate relationship occurred between the magnitude of the LPSP and the PSP threshold (Figure 4H). Thus, PNs with

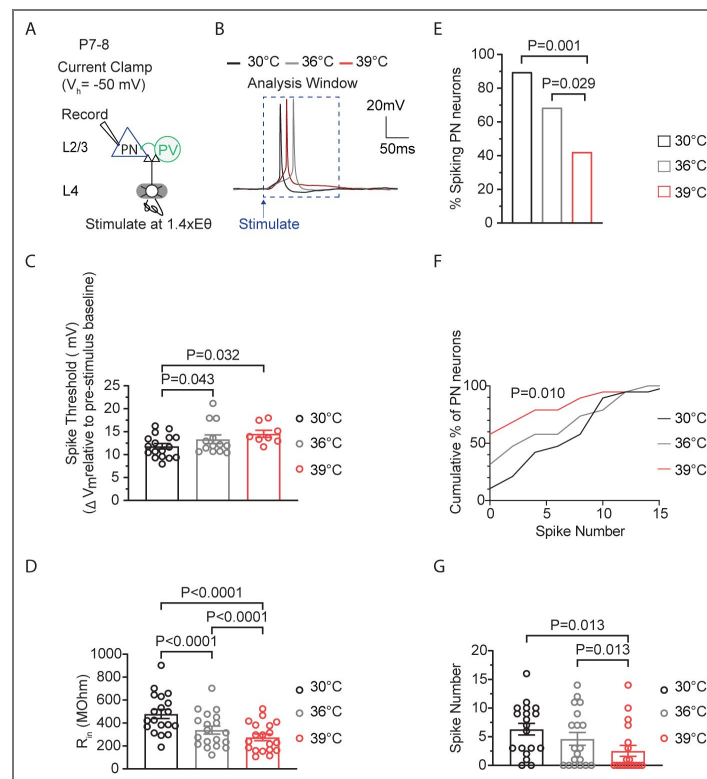


Figure 3. Spiking in P7-8 cortical excitatory pyramidal neurons decreases as temperature enters the fever range.

A. Setup for recording L4-evoked postsynaptic potential and spiking in an excitatory pyramidal neuron (PN) at just-subthreshold V_m at 30°C, 36°C and 39°C in mouse primary somatosensory (S1) cortex. **B.** Example traces of L4-evoked spikes in L2/3 PN during consecutive recordings at 30°C (black), 36°C (gray), and 39°C (red). **C.** Depolarization required to reach spike threshold (ST) in L2/3 PNs during temperature elevations from 30°C to 39°C. **D.** Same as (C) for input resistance (R_{in}). **E.** Same as (C) for the percentage of spiking L2/3 PNs. **F.** Same as in (C) for the spiking distribution. **G.** Same as in (C) for the number of spikes. In **C**, **D** and **G**, each data point represents an individual cell. In **C-G**, 19 PNs recorded from 5 animals, with each cell exposed to temperature elevations from 30°C to 39°C. Data in **C**, **D**, and **G** are shown as mean $\pm$ SEM. Statistical significance was assessed as follows: **C**, **D**, **G**: one-way repeated-measures ANOVA with Tukey's post-hoc test ($\alpha = 0.05$); **E** – two-tailed binomial test; **F** – Kolmogorov-Smirnov test.

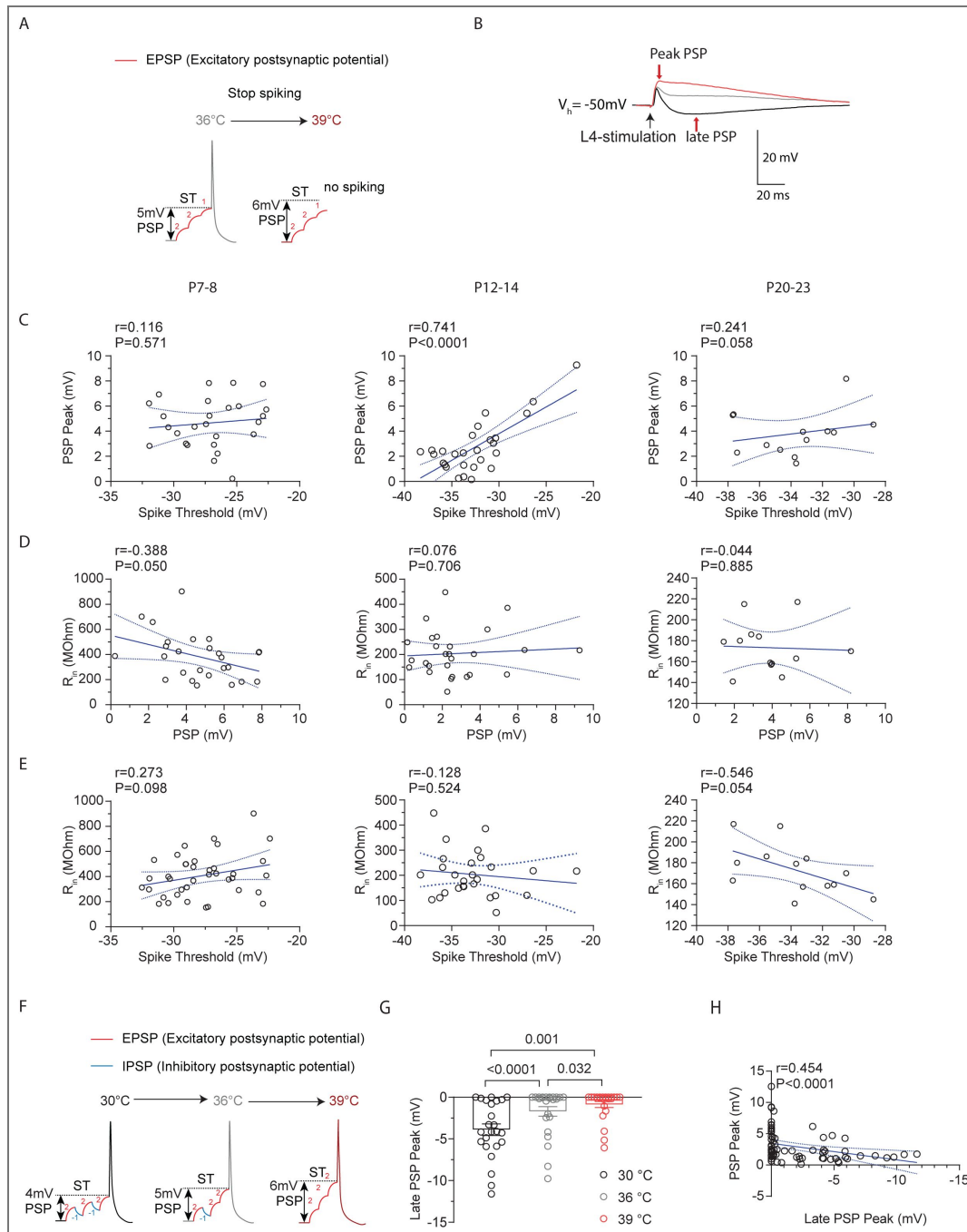


Figure 4. Increased depolarization at higher spike thresholds helps maintain stable spiking activity in cortical pyramidal neurons during temperature elevations.

A. Illustration demonstrating how temperature-induced changes in spike threshold make spiking more challenging for a neuron, requiring larger levels of depolarization to sustain spiking. **B.** Example traces of L4-evoked postsynaptic potentials (PSPs) in L2/3 PN during consecutive recordings at 30°C (black), 36°C (gray), and 39°C (red). **C.** Correlation of PSP peak versus spike threshold (ST). r = Pearson correlation coefficient with Deming linear regression. **D.** Correlation of input resistance versus ST. r = Pearson correlation coefficient with simple linear regression. **E.** Same as D but for input resistance versus PSP. **F.** Illustration showing how temperature-induced loss in inhibition (blue IPSP) could lead to larger levels of depolarization. IPSPs typically bring the membrane potential away from the spike threshold. **G.** The late PSP peak in L2/3 PNs during temperature elevations from 30°C to 39°C. Statistical significance was evaluated on log-transformed data using one-way repeated measures ANOVA with Tukey's test, with significance at $\alpha=0.05$. **H.** Same as D but for PSP versus Late PSP Peak. r = Spearman correlation coefficient with simple linear regression.

increased STs and larger PSPs were part of a circuit where synaptic inhibition was reduced across all or most PNs, regardless of whether STs were elevated. These results suggest that at P12-14, ST is sensitive to temperature increases and spiking is maintained by larger depolarization levels in these neurons.

Cortical excitatory neurons that remain active at febrile temperatures exhibit increased spiking rates

At a later age (P24-P26), we applied external heat to elevate normal T_b (36°C) into the fever range and used *in vivo* high-density, extracellular electrophysiological recordings to capture single-unit activity in the S1 cortex of ketamine/xylazine anesthetized wildtype mice (**Figure 5A**). Unlike *in vitro* whole-cell patch-clamp recordings, extracellular recordings allow analysis only from cells that spike throughout the recording period during temperature elevations from 36°C (baseline) to fever-range. This is largely due to the inability of spike sorting algorithms to reliably track neurons that stop firing during the recording.

Color maps (with yellow and red indicating spiking activity) illustrate multiunit activity obtained in the left and right cortices during baseline temperature (left), warming to fever T_b (middle), and cooling back to 36°C (recovery upon heat removal) are shown in **Figure 5B**. **Figure 5C** presents example traces of spiking activity recorded from three cortical positions (marked by black, gray, and white asterisks on the right side of panels in **Figure 5B**). Following manual curation, single units ($n = 633$) were classified as putative inhibitory interneurons or excitatory pyramidal neurons (PNs) based on their spike waveform duration (Barthó et al., 2004; see Methods). The spike duration was calculated as the time difference between the trough and the subsequent waveform peak of the mean filtered (300 – 6000 Hz bandpassed) spike waveform. Durations of extracellularly recorded spikes showed a bimodal distribution (Hartigan's dip test; $p < 0.001$) characteristic of the neocortex with shorter durations corresponding to putative interneurons (narrow spikes) and longer durations to putative principal cells (wide spikes). Next, k-means clustering was used to separate the single units into these two groups, which resulted in 140 interneurons (spike duration < 0.6 ms) and 493 principal cells (spike duration > 0.6 ms), corresponding to a typical 22% - 78% (interneuron – principal) cell ratio. **Figure 5D** displays representative single units isolated via spike sorting, with unit 3 representing a putative interneuron.

Finally, the firing rates of neurons were computed separately during the three distinguished periods (baseline, thermal fever (39°C) and recovery back to 36°C). To decrease the effect of transient changes (e.g., tissue recovery during the baseline period or the short-term effect of heating during the thermal fever period), for each 45-minute-long period, we used only the last 25-minutes to calculate the mean firing rates of neurons. The normalized average firing rate for each animal ($n = 5$) was calculated for these putative inhibitory interneurons and excitatory PNs across the recording session. We found that *in vivo*, putative excitatory PNs that remained active increased their spike rates at fever temperatures (**Figure 5E**). The normalized firing rates of five representative units are shown in **Figure 5F** during the baseline, warming (red shaded area), and recovery periods. Additionally, we observed that putative inhibitory interneurons that remained active also increased their spike rates at fever temperatures (**Figure 5E**).

Consistent with these *in vivo* findings, *ex vivo* recordings at a similar age (P20-23) showed that 11.1% of recorded excitatory PNs ($n = 27$ cells) spiked at both 36°C and 39°C (**Figure 5G**). However, similar to the *in vivo* condition, neurons that remained spiking at 36°C and 39°C increased their spike/firing rate (**Figure 5H**). Along with the 11.1% of recorded excitatory PNs that stayed spiking during temperature increases from 36°C to 39°C, roughly equal proportions of neurons stopped spiking and started spiking (**Figure 5G**). Thus, neurons that maintain spiking at fever temperatures play a crucial role in determining the overall activity levels of the circuit. Additionally, we found that the proportion of PNs that remain spiking from 36°C to 39°C is higher at earlier ages (P12-14) compared to later ages (P20-23) (**Figure 5I**).

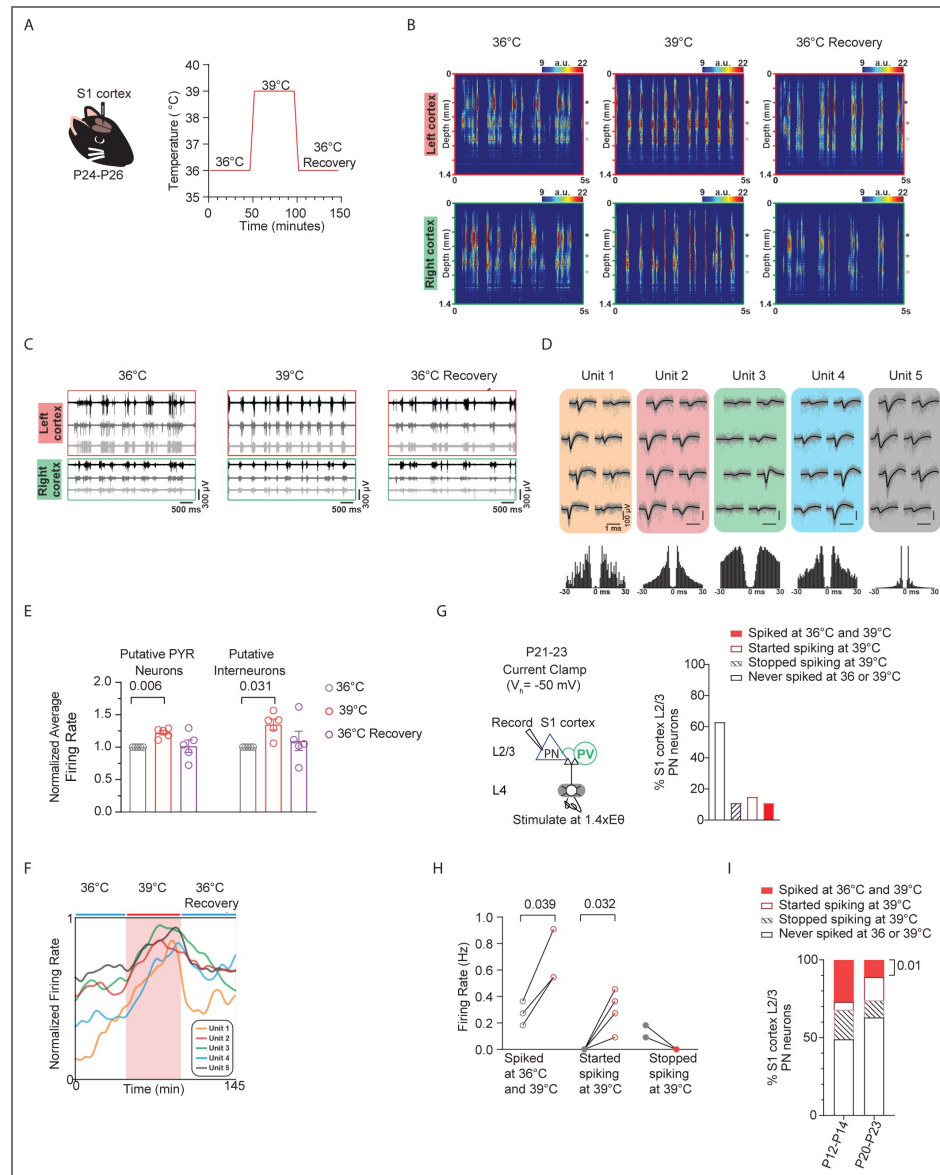


Figure 5. Cortical excitatory neurons that remain active at febrile temperatures exhibit increased spiking rates.

A. Setup for *in vivo* recording of spiking in mouse S1 cortex during temperature increases from 36°C to 39°C and cooling back to 36°C. **B.** Color maps (with yellow and red indicating spiking activity) illustrate multiunit activity obtained in the left and right cortices during baseline temperature (left), warming to fever T_b (middle), and cooling back to 36°C (recovery upon heat removal). **C.** Example traces of spiking activity recorded from three cortical positions (marked by black, gray, and white asterisks on the right side of panels in B). **D.** Mean spike waveforms (black), single spikes (gray) and autocorrelograms (bottom) of single units isolated, with unit 3 representing a putative interneuron. **E.** Normalized firing rates of putative excitatory PNs and interneurons obtained from recordings in A. **F.** The normalized firing rates of five representative units during the baseline, warming (red shaded area), and recovery periods. **F** presents the same data, with color coding consistent with the units in D. **G.** Left: Setup for *ex vivo* recording of L4-evoked postsynaptic potentials and spiking in L2/3 cortical PN at just-subthreshold V_m at 30°C, 36°C and 39°C in mouse S1 cortex. Right: Percent distribution for recorded PNs that never spiked, stopped spiking, stayed spiking, or started spiking during temperature increases from 36°C to 39°C. **H.** L2/3 PN spiking activity in neurons that stayed spiking (left), started spiking (middle), and stopped spiking (right) upon temperature increases from 36°C to 39°C. **I.** Activity distribution for *ex vivo* recorded neurons that never spiked, and those that stopped spiking, stayed spiking, or started spiking upon temperature increases from 36°C to 39°C. P-value = 0.01 indicates a significant increase in the fraction of neurons that stayed spiking during temperature increases from 36°C to 39°C (solid red bar). In **E** and **H**, each data point represents an individual animal and cell, respectively. Data were collected from 5 animals in **B-F**, 11 animals in **G-H**, and 9–14 animals in **I**. Statistical significance was assessed using one-way repeated-measures ANOVA with Tukey’s post-hoc test (**E**), paired two-tailed t-test (**H**), and binomial test (**I**), with significance set at $\alpha = 0.05$.

Excitatory pyramidal neurons that remain spiking with temperature elevations into fever range exhibit unique intrinsic properties

As neurons that remain spiking with temperature elevations may be crucial in setting overall cortical circuit activity levels, we sought to uncover additional information about the intrinsic characteristics of cortical PNs at P12-14 that facilitate their activity at 36°C to 39°C. We refer to neurons that retained spiking at all three temperatures (30°C, 36°C, and 39°C) as STAY neurons, while those cells that stopped spiking upon temperature transitions from 30°C to 36°C or 36°C to 39°C are denoted as STOP neurons. Firstly, in STOP and STAY neurons, ST increased during temperature elevations from 30°C to 39°C (**Figure 6A**). However, in STOP PNs, the increases in ST were greater than in those neurons that continued spiking (**Figure 6A**). The ST of neurons that continued spiking at 39°C was similar in magnitude to that of neurons that stopped spiking at 36°C.

There was an interaction of temperature and whether the PNs were STOP or STAY cells in the PSP ($P=0.048$, two-way ANOVA, mixed-effects model), with a trend toward larger PSPs in the STAY PNs (**Figure 6B**). STAY cells showed a strong positive correlation between PSP and ST (**Figure 6C-D**). At 30°C, STAY neurons had larger LPSPs (**Figure 6E**). However, inhibition levels in these PNs were unique in that inhibition levels significantly decreased with temperature elevations (**Figure 6E**). Moreover, we found a significant moderate inverse correlation between ST and LPSP in the STAY cells that remain spiking at 39°C ($r=-0.57$, $P=0.046$, XY pairs=13, Spearman). Thus, in PNs that stay spiking, the excitation and inhibition levels are matched to facilitate greater depolarization and maintain spiking activity. Temperature-induced alterations in spike height and spike afterhyperpolarization (AHP) were not different between STAY and STOP cells, while R_{in} was largely insensitive to temperature changes in STAY PNs but not in STOP PNs (**Figure 6F-H**).

Temperature elevations in the fever range increase TRPV3 currents in cortical pyramidal neurons

Next, we sought to identify molecular determinants that might underlie the properties of STAY cells. We focused on the transient receptor potential vanilloid 1-4 (TRPV1-4) channels, a family of thermosensitive, Ca^{2+} -permeable, nonselective cation channels. Of these, only TRPV3 and TRPV4 are activated within the innocuous warm temperature range (31-39°C), whereas TRPV1 and TRPV2 respond to noxious heat above 42°C and 52°C, respectively (Kashio and Tominaga, 2022). TRPV3, in particular, has a reported temperature threshold for activation between 31–39°C, with Smith et al., 2002 noting activation at 39°C.

We examined TRPV3 protein expression by immunohistochemical staining at P7, P14, and P21 (**Figure 7A-B**). Staining specificity was confirmed at all ages by co-incubating the TRPV3 antibody with a TRPV3 blocking peptide, with an example shown for a P7 brain section (**Figure 7A**, right panel). TRPV3 expression was detected in S1 cortex at all ages, with strong expression in L2/3 at P14 but not at P7 or P21 (**Figure 7A**). Outside S1 cortex, TRPV3 was also present in the hippocampus, thalamus, and striatum (**Figure 7A-B**). Co-immunostaining for TRPV4 revealed several brain regions where TRPV4 (red color) and TRPV3 (green color) were non-overlapping (**Figure 7B**, top-left panel). In the S1 cortex at P14, TRPV3 was detected in L4 barrels and showed a patchy distribution in L2/3, with some patches displaying more intense staining (**Figure 7B**, bottom-right panel). This pattern was not observed for TRPV4 (**Figure 7B**, bottom-left panel).

Using whole-cell voltage clamp, we recorded TRPV3 currents at different voltages in cortical excitatory PNs with bath application of camphor (5 mM), a TRPV3 agonist (Mogrich et al., 2005) (**Figure 8A**). We observed a significant increase in TRPV3 currents at 39°C (**Figure 8B-C**, two-way repeated measures ANOVA, with Fisher's LCD test). We also measured the net TRPV3 currents by comparing TRPV3 current densities recorded in the absence and presence of forsythoside B, a natural TRPV3 inhibitor found in the plant *Lamiophlomis rotata* with an IC₅₀ of 6.7 $\mu\text{mol/L}$ and no

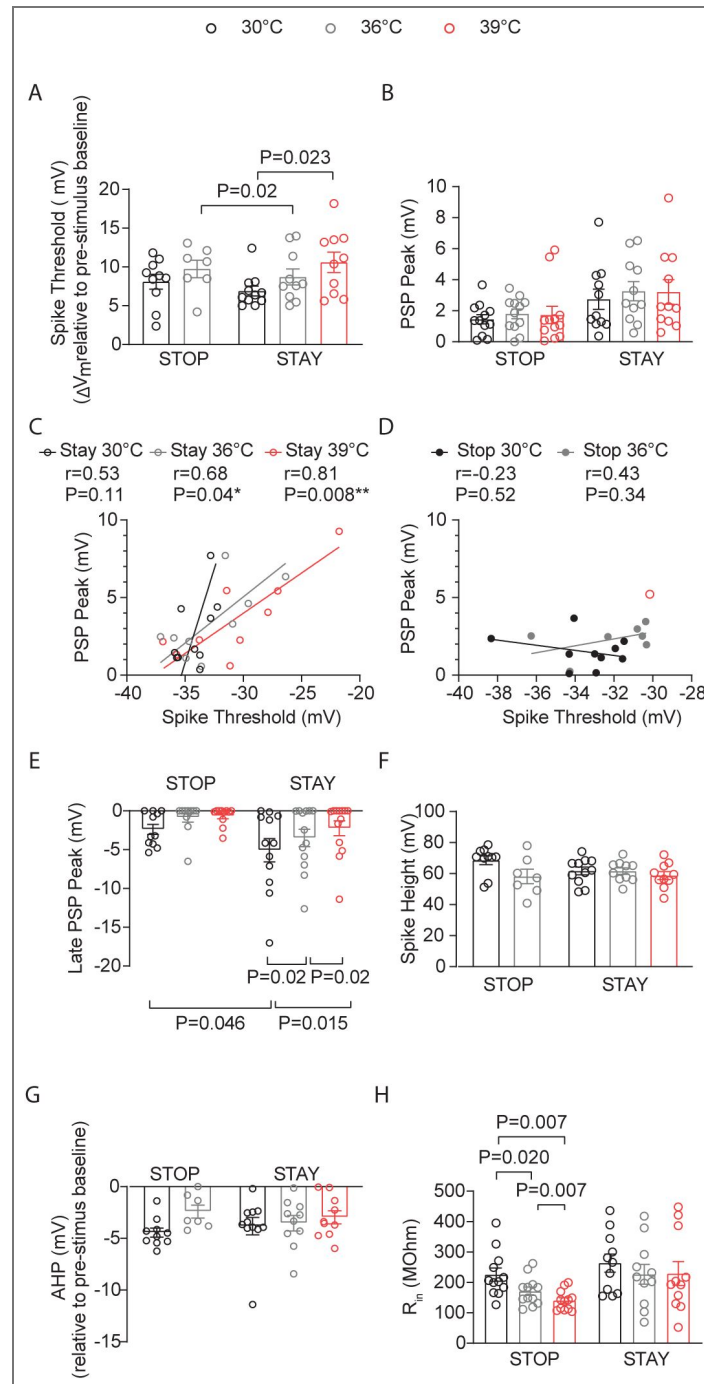


Figure 6. Excitatory pyramidal neurons that remain spiking with temperature elevations into fever range exhibit unique intrinsic properties.

Neurons that spiked at all temperatures (30°C, 36°C, and 39°C) are STAY PNs, while those that stopped spiking at 36°C or 39°C are STOP neurons **A**. Depolarization required to reach spike threshold (ST) in STOP and STAY L2/3 PNs during temperature elevations from 30°C to 39°C. **B**. Same as (A) for L4-evoked postsynaptic potentials (PSPs) **C**. Correlation of PSP peak versus ST in STAY L2/3 PNs at 30°C, 36°C, and 39°C. r = Pearson correlation coefficient with Deming linear regression. **D**. Same as (C) for STOP cells. **E**. Same as (A) for the L4-evoked late PSP peak. **F**. Same as (A) for spike height. **G**. Same as (A) for spike afterhyperpolarization (AHP). **H**. Same as (A) for input resistance. Each data point in **A–H** represents an individual cell. Data were collected from 14 animals. Mean $\pm$ SEM is shown in **A–B** and **E–H**. Statistical significance was assessed using one- or two-way repeated-measures ANOVA with Tukey's or Sidak post-hoc test (**A–B**, **E–H**; $\alpha = 0.05$). In **C–D**, correlations were evaluated using Pearson's r with Deming linear regression.

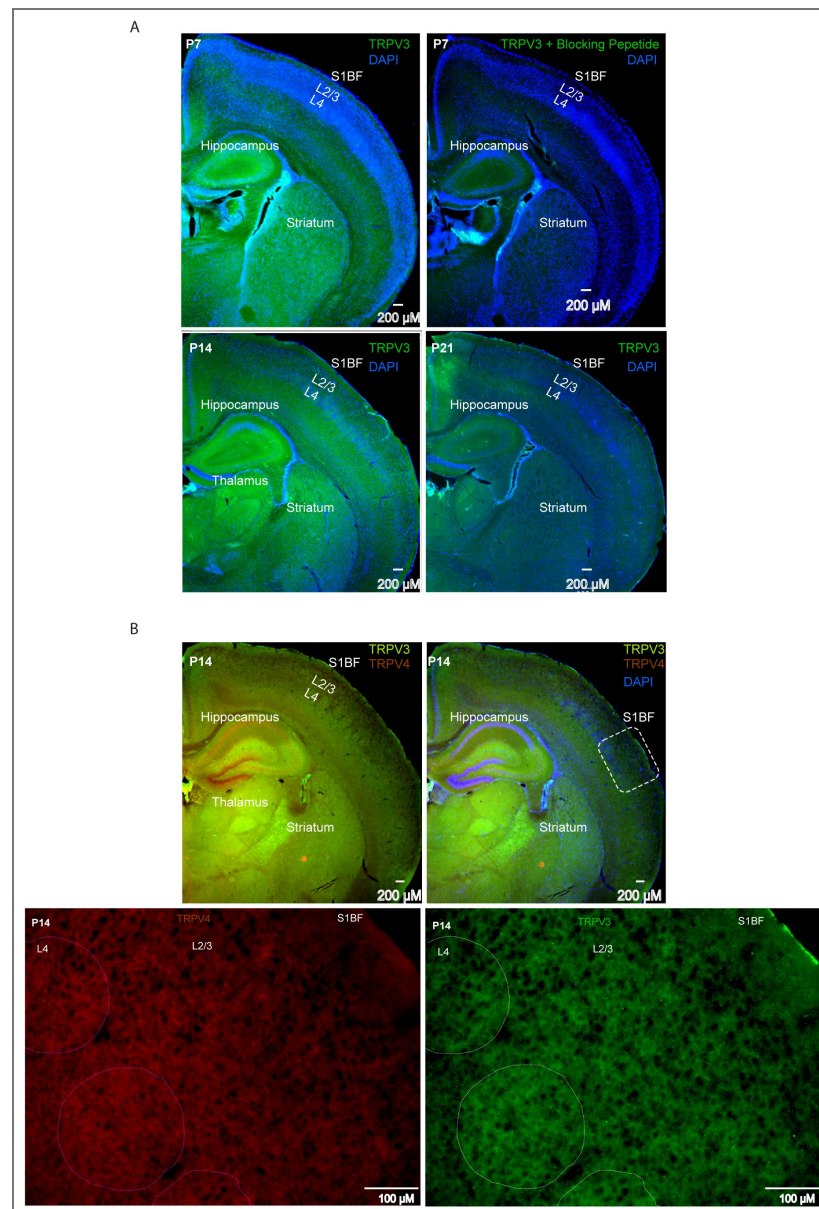


Figure 7. TRPV3 protein expression by immunohistochemistry at postnatal days 7, 14, and 21.

A. TRPV3 immunostaining (green) in mouse primary somatosensory (S1) cortex, striatum, thalamus, and hippocampus, with DAPI counterstain (blue). Top row left: Postnatal day (P)7; bottom row left: P14; bottom row right: P21. Top row right: Sections incubated with TRPV3 antibody plus TRPV3 blocking peptide in S1 cortex, striatum, thalamus, and hippocampus (green) with DAPI counterstain (blue). Remaining TRPV3 staining is shown in green with DAPI in blue. **B.** TRPV3 and TRPV4 immunostaining in S1 cortex, striatum, thalamus, and hippocampus at P14. Top row left: TRPV3 (green) and TRPV4 (brick red). Top row right: TRPV3 (green) and TRPV4 (brick red) with DAPI counterstain (blue). Bottom row left: TRPV4 immunostaining in S1BF (inset from top row left) at 10x. Bottom row right: TRPV3 immunostaining in S1BF (inset from top row left) at 10x.

obvious inhibitory effects on the other TRPV channels like TRPV1 or 4 (Zhang et al., 2019). Consistent with Smith et al., 2002, who noted TRPV3 activation at 39°C, we also observed a net positive TRPV3 current at 39°C (Figure 8D-G).

Inhibiting TRPV3, but not TRPV4 channels, significantly reduced the population of STAY pyramidal neurons and spiking levels at fever temperature

To investigate the potential role of TRPV3 and TRPV4 in regulating STAY PN phenotype in the S1 cortex during temperature increases from 30–39°C, we applied the following blockers: forsythoside B (50 μM) or RN1734 (10 μM, TRPV4 blocker) intracellularly in our recordings of L4-evoked spiking at 1.4X E0 in L2/3 PNs (Figure 9A). RN1734 is a highly selective antagonist for TRPV4 with an IC₅₀ of 5.9 μmol/L and minimal to negligible affinity for the other TRPV channels (Vincent et al., 2009). These blockers phenocopy electrophysiological effects observed in *Trpv4*^{-/-} KO and *Trpv3*^{-/-} KO mice (Chen et al., 2022). For instance, *Trpv3*^{-/-} KO mice exhibit a hyperpolarizing effect on the resting membrane potential (RMP) in striatal MSNs and L2 stellate neurons in the entorhinal cortex (Chen et al., 2022). This hyperpolarization reflects reduced TRPV3 activity, which normally promotes cation influx (Shibasaki et al., 2007; Chen et al., 2022). Although increases in temperature alone can depolarize the RMP, consistent with prior reports in *Trpv3*^{-/-} mice, our recordings of L4-evoked spiking in L2/3 PNs of S1 cortex showed a similar trend toward RMP hyperpolarization with intracellular TRPV3 block via forsythoside at 30 °C and 36 °C, and a significant enhancement at 39°C. Specifically, in the absence of block (n = 13 cells) versus with TRPV3 blocker (n = 15 cells), the RMP values were: 30 °C: -75.1 ± 1.1 vs. -77.1 ± 1.0 mV (P = 0.273); 36 °C: -73.5 ± 1.2 vs. -76.3 ± 1.1 mV (P = 0.273); 39 °C: -71.3 ± 1.0 vs. -74.4 ± 1.1 mV (P = 0.048); two-way repeated-measures ANOVA with Fisher's LSD test.

Furthermore, we found that intracellular blockade of TRPV3 caused a significant reduction in the percentage of STAY neurons (No block: 27% (37 recorded cells), TRPV3 block: 8% (n=26 recorded cells), TRPV4 block: 17% (n=24 recorded cells), two-tailed binomial test) (Figure 9B). Consistent with the STAY cells being largely responsible for maintaining stable average spiking during temperature elevations, spiking was reduced in PNs with intracellular TRPV3 blockade during the temperature transitions from 30°C to 39°C (Figure 9C). TRPV3 block did not occlude the increases in the depolarization required to reach ST (TRPV3 blocker: 30°C: 7.15 ± 0.57, 36°C: 8.39 ± 0.26, 39°C: 10.0 ± 0.94, 30°C vs 39°C: P=0.007, One-way repeated measures ANOVA, mixed-effects model with Tukey's test). However, it did abolish the temperature induced increases in the PSP (TRPV3 blocker: 30°C: 2.37 ± 0.36, 36°C: 2.45 ± 0.38, 39°C: 2.22 ± 0.25; No blocker: 30°C: 2.47 ± 0.32, 36°C: 2.90 ± 0.32, 39°C: 3.32 ± 0.52, 30 vs 39°C:

P=0.030, One-way repeated measures ANOVA, mixed-effects model with Dunnett's test).

Consistently, TRPV3 block abolished the positive correlation between ST and PSP (No block: XY pairs=27, TRPV3 blocker: XY pairs=18, Pearson's test) (Figure 9D).

Furthermore, in keeping with the correlation between the magnitude of the LPSP and the PSP threshold, we found that TRPV3 blockade also prevented the temperature induced decreases in the LPSP (Figure 9E). Moreover, these reductions in spiking with TRPV3 blockade were not dependent on temperature-induced decreases in R_{in}, as changes to PNs were similar in the TRPV3 block and no-block conditions (Figure 9F). Altogether, these results suggest that TRPV3 expression or its functional effects—such as mediating Ca²⁺ or other cation (Na⁺, K⁺) influx to promote increased depolarization with spike threshold increases—may define the subset of STAY neurons. Given this role, the loss of TRPV3 is expected to promote temperature-induced hypoactivity in cortical circuits rather than exacerbate seizure activity.

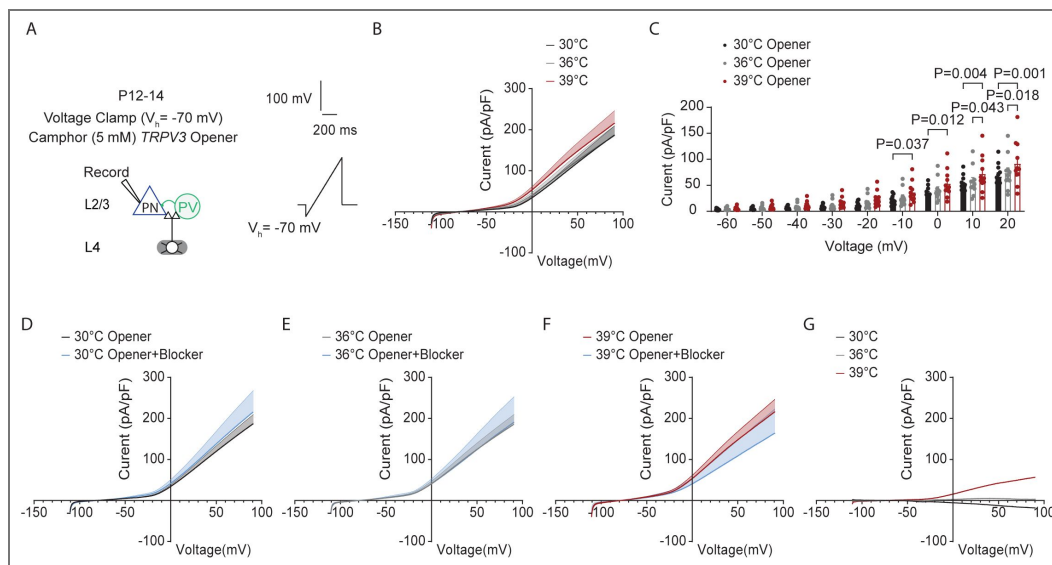


Figure 8. Temperature elevations in fever range increase TRPV3 currents in cortical pyramidal neurons.

A. Setup for recording whole-cell TRPV3 currents at 30°C (black), 36 °C (grey) and 39°C (red). in cortical excitatory pyramidal neurons (PNs) with bath application of camphor (5mM), a *TRPV3* agonist. **B.** Current density-voltage (I-V) relationship of TRPV3 currents at 30°C (black), 36 °C (grey) and 39°C (red) in WT mice: 11 cells from 4 mice. **C.** Scatter dot plots of the current density-voltage measurements. **D.** Current density-voltage (I-V) relationship of TRPV3 currents at 30°C (black) in the presence of camphor (5mM), a TRPV3 agonist, or camphor (5mM) + TRPV3 blocker (Forsythoside B, 50 μM) (blue). **E.** Same as (**D**) but for 36°C. **F.** Same as (**D**) but for 39°C. **G.** Current density-voltage (I-V) plot showing the net TRPV3 current (opener – (opener + blocker) condition). In B-F, statistical significance was assessed using a two-way repeated-measures ANOVA with Tukey's or Sidak post-hoc test ($\alpha = 0.05$)

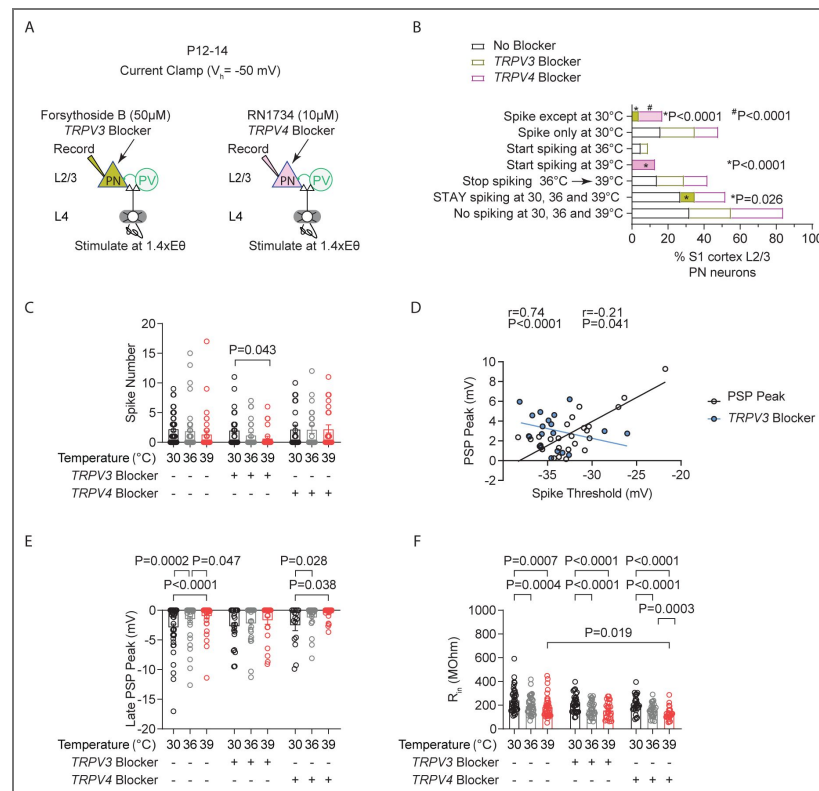


Figure 9. Inhibiting TRPV3, but not TRPV4 channels, significantly reduced the population of STAY pyramidal neurons and spiking levels at fever temperature.

A. Setup for recording L4-evoked postsynaptic potential and spiking in an excitatory cortical pyramidal neuron (PN) with an intracellular blocker of TRPV3 channels (Forsythoside B, 50 μ M) (left) or TRPV4 channels (RN1734, 10 μ M) (right) at just-subthreshold V_m at 30°C, 36°C, and 39°C in mouse S1 cortex. **B.** Percentages of cell types obtained from experiment in A. **C.** Evoked spikes in L2/3 cortical PNs during temperature elevations to 30°C, 36°C, and 39°C under three conditions: no blockers, TRPV3 blocker (Forsythoside B, 50 μ M), or TRPV4 blocker (RN1734, 10 μ M). **D.** Correlation between PSP peak and spike threshold (ST). r = Pearson correlation coefficient with Deming linear regression. **E.** Same as (C) for the L4-evoked late PSP peak. **F.** Same as (C) for input resistance (R_{in}). Each data point in **C–F** represents an individual cell. Data were collected from 26 cells in 7 animals for the TRPV3 blocker, 24 cells in 6 animals for the TRPV4 blocker, and 37 cells in 14 animals for the no-block condition. Mean $\pm$ SEM is shown in **C, E, and F**. Statistical significance was assessed using one- or two-way repeated-measures ANOVA with Tukey's or Sidak post-hoc test ($\alpha = 0.05$). In **D**, correlations were evaluated using Pearson's r with Deming linear regression.

TRPV3 knockout mice exhibit reduced spiking activity at febrile temperatures and delayed seizure onset

Recordings of L4-evoked PSPs and spiking in L2/3 cortical PNs from wildtype (WT) and homozygous *Trpv3* knockout (*Trpv3*^{-/-}) (Moussaieff A et al., 2008) mice revealed similar degrees of temperature sensitivity in ST and R_{in} . Febrile temperatures increased ST and decreased R_{in} (Figure 10A-C). Consistent with findings that TRPV3 blockade using 50μM forsythoside B reduces spiking in cortical L2/3 PNs, we observed significantly reduced spiking in *Trpv3*^{-/-} mice as well (Figure 10D). Analysis of postsynaptic potentials in these neurons, showed that, in WT mice, PSP amplitude increased with temperature elevation into the febrile range, whereas this temperature-dependent depolarization was absent in *Trpv3*^{-/-} mice (Figure 10E). This reduction in spiking is consistent with the idea that, during increases in STs, concurrent increases in PSP amplitude help sustain neuronal spiking. These findings support the conclusion that TRPV3 contributes to enhanced depolarization and excitability at febrile temperatures.

We next examined the effects of reduced *Trpv3* expression on hyperthermia-induced seizures in WT (*Trpv3*^{+/+}), heterozygous (*Trpv3*^{+/-}), and homozygous knockout (*Trpv3*^{-/-}) P12 pups. As described in Figure 1D, mice were exposed to infrared light to raise their core T_b into the range of hyperthermia (>40°C; Figure 10F). To enable wireless, non-invasive T_b recordings, all mice were implanted with temperature transponders. Baseline T_b measurements prior to infrared exposure revealed no significant differences across genotypes (*Trpv3*^{+/+}: 33.7 ± 0.44°C, *Trpv3*^{+/-}: 33.7 ± 0.33°C, *Trpv3*^{-/-}: 32.8 ± 0.10°C; One-way ANOVA with Tukey's multiple comparison test, $F(2,11) = 2.41$, $P = 0.136$). As T_b increased during infrared exposure, P12 mice typically transitioned from rest to active walking and exploratory behavior. The T_b at which mice became ambulatory did not significantly differ across genotypes (*Trpv3*^{+/+}: 37.5 ± 0.55°C, *Trpv3*^{+/-}: 36.3 ± 0.43°C, *Trpv3*^{-/-}: 36.6 ± 0.34°C; One-way ANOVA with Tukey's multiple comparison test, $F(2,11) = 1.34$, $P = 0.301$).

As T_b reached 41–42°C, mice exhibited loss of postural control (LPC), defined as collapse and failure to maintain upright posture. LPC is a key behavioral marker of cortical and motor circuit dysfunction. The T_b at which LPC occurred was significantly lower in *Trpv3*^{-/-} mice compared to wildtype controls (Figure 10G). LPC was followed by the rapid onset of generalized seizures. While the T_b at seizure onset was not significantly different among genotypes (Figure 10H), the time to seizure onset was significantly prolonged in both *Trpv3*^{+/-} and *Trpv3*^{-/-} mice. Specifically, *Trpv3*^{+/-} mice exhibited a 49.4% increase, and *Trpv3*^{-/-} mice a 91% increase in latency to seizure compared to WT mice (Figure 10I). Importantly, the rate of T_b increase leading to seizure did not differ across genotypes (*Trpv3*^{+/+}: 0.011 ± 0.003°C/s, *Trpv3*^{+/-}: 0.013 ± 0.002°C/s, *Trpv3*^{-/-}: 0.012 ± 0.001°C/s; One-way ANOVA with Tukey's multiple comparison test, $F(2,11) = 0.1055$, $P = 0.9008$), suggesting the delayed seizure onset was not due to slower heating. Together, these findings indicate that reduced TRPV3 function increases resistance to seizure initiation and/or propagation under febrile conditions, likely by decreasing neuronal excitability.

Discussion

In this study, we aimed to elucidate adaptive neural mechanisms that enable excitatory PNs to remain active despite substantial increases in brain temperature into the fever range. Our electrophysiological assessments across three postnatal ages, P7-8, P12-14 and P20-23 showed that excitatory PNs responded to temperature increases from 30°C to 39°C by either remaining inactive, staying active, ceasing activity, or initiating activity. Roughly equal portions of neurons ceased or initiated activity, so firing stability was largely maintained by those that remained active, i.e., STAY neurons. The overall proportion of PNs that remain spiking from 36°C to 39°C was higher at P12-14 compared to P20-23. This increased proportion at earlier ages could potentially reflect the increased risk for febrile seizure occurrence at earlier postnatal ages.

STAY neurons likely possess a unique composition of ion channels that enable them to exhibit temperature sensitivity in spike threshold but not in input resistance, and adjust their depolarization levels to match the ST value. Our initial characterization focused on *Trpv3* and *Trpv4*, two members of a family of thermosensitive transient receptor potential vanilloid channels

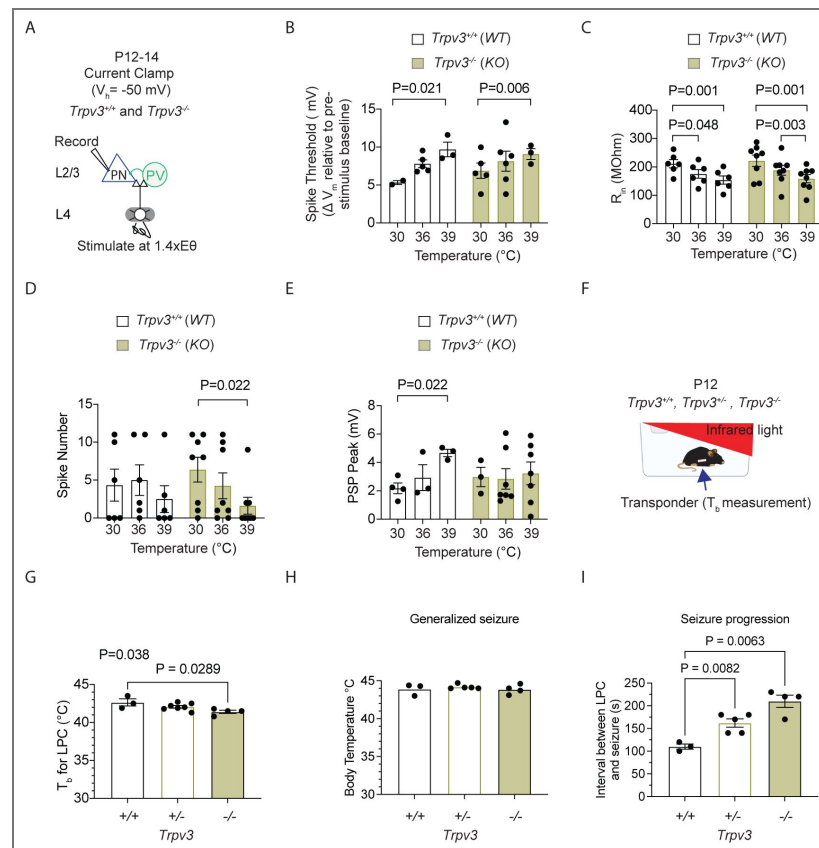


Figure 10. TRPV3 knockout mice exhibit reduced spiking activity at febrile temperatures and delayed seizure onset.

A. Setup for recording L4-evoked postsynaptic potentials and spiking in excitatory cortical pyramidal neurons (PNs) at just-subthreshold V_m at 30 °C, 36 °C, and 39 °C in mouse S1 cortex of wildtype ($Trpv3^{+/+}$) and $Trpv3$ knockout ($Trpv3^{-/-}$) mice. **B.** Depolarization required to reach spike threshold (ST) in $Trpv3^{+/+}$ and $Trpv3^{-/-}$ mice at 30 °C, 36 °C, and 39 °C. **C.** Same as (B) but for input resistance (R_{in}). **D.** Same as (B) but for number of spikes. **E.** Same as (B) but for postsynaptic potential (PSP). **F.** Setup for recording mouse body temperature (T_b) at room temperature and during fever-range and higher, using an implanted transponder for non-invasive measurement, with exposure to infrared light. **G.** Time to loss of postural control (LPC), defined as collapse and failure to maintain upright posture, in wildtype ($Trpv3^{+/+}$), heterozygous ($Trpv3^{+/-}$) and $Trpv3$ knockout ($Trpv3^{-/-}$) mice. **H.** Same as in (G) but showing T_b at seizure onset. **I.** Same as in (G) but for the time from LPC to seizure onset. Each data point in **B–E** represents an individual cell (3 animals per genotype). Statistical significance was assessed using two-way repeated-measures ANOVA with Tukey's or Sidak post-hoc test ($\alpha = 0.05$). Each data point in **G–I** represents an individual animal. Statistical significance was assessed using one-way repeated-measures ANOVA with Tukey's or Sidak post-hoc test ($\alpha = 0.05$).

that are expressed in the brain and activated between 30–39°C, the temperature range of our study. Intracellular blockade of TRPV3, but not TRPV4, significantly decreased the population of STAY PNs and prevented stability in spiking activity during temperature increases into fever range.

TRPV3 is highly expressed in various tissues, including epithelial cells of the skin and oropharynx, the tongue, testis, dorsal root ganglion, trigeminal ganglion, spinal cord and brain (Moussaieff A et al., 2008 and Xu et al., 2002). It plays a crucial role in skin and hair generation as well as in the perception of pain and warmth on the skin. However, its role in the central nervous system remains to be further elucidated. In the brain, TRPV3 is expressed in several neuronal types, including cortical and striatal neurons (Xu et al., 2002; Chen et al., 2022).

TRPV3 has a reported temperature threshold for activation between 31–39°C, with Smith et al., 2002 noting activation at 39°C. Consistent with this, we observed the strongest physiological effects in cortical PNs at 39°C. At this temperature, intracellular blockade of TRPV3 reduced average spiking levels and temperature-induced increases in the PSP. Additionally, a potential early postnatal temperature-dependent activity mechanism in PNs, where levels of excitatory and inhibitory input are adjusted to match the cell's ST, was abolished with TRPV3 blockade. Altogether, these results suggest that TRPV3 blockade reduces net activity in sensory cortical circuits by decreasing activity in STAY cells.

Although, TRPV3 channels are cation-nonselective, they exhibit high permeability to Ca^{2+} ($\text{Ca}^{2+} \gg \text{Na}^+ \approx \text{K}^+ \approx \text{Cs}^+$) with permeability ratios (relative to Na^+) of 12.1, 0.9, 0.9, 0.9 (Xu et al., 2002). Opening of TRPV3 channels activates a nonselective cationic conductance and elevates membrane depolarization, which can increase the likelihood of generating action potentials. Indeed, our observations of a loss of the temperature-induced increases in the PSP with TRPV3 blockade are consistent with a reduction in membrane depolarization. In S1 cortical circuits at P12–14, STAY PNs appear to rely on a temperature-dependent activity mechanism, where depolarization levels (mediated by higher excitatory input and lower inhibitory input) are scaled to match the cell's ST. Thus, an inability to increase PSPs with temperature elevations prevents PNs from reaching ST, so they cease spiking. See summary **Figure 11**.

Previous research in *Trpv3*^{-/-} KO mice revealed a similar phenotype of reduced activity with diminished TRPV3 function (Chen et al., 2022). Whole-cell current-clamp recordings from striatal MSNs and L2 stellate neurons in the entorhinal cortex at 36.5–37.5°C showed that *Trpv3*^{-/-} cells exhibited reduced intrinsic excitability, as evidenced by fewer spikes in response to 400-ms current steps, a hyperpolarized RMP, and a decrease in mEPSC frequency (Chen et al., 2022). These electrophysiological effects were also replicated using pharmacological inhibition of TRPV3 with 50 $\mu\text{mol/L}$ forsythoside B (Chen et al., 2022). Additionally, consistent with the idea that ischemic injury upregulates TRPV3 expression, leading to intracellular Ca^{2+} overload and progressive cell death, inhibition of TRPV3, rather than N-methyl-D-aspartate (NMDA) receptors, protected against progressive cell death after stroke (Chen et al., 2022).

As STAY PNs and neurons that initiate spiking at fever temperature (“START” cells) are active at fever temperature, future research will focus on the molecular characterization of these cells in the cortex and elsewhere in the brain at P12–14 and other ages. Interestingly, neurons that initiate spiking at fever temperature are more prevalent at P20–23 than at the earlier ages examined. TRPV4 block caused a subset of PNs to initiate spiking at 39°C, suggesting that low TRPV4 expression could define the population of START cortical PNs, at least at P12–14.

Molecular characterization of STAY and START cell types may provide additional insights into their function within normal brain circuits. In response to specific experiences, selective subsets of neurons, known as “engrams,” may become active to encode specific memories or behavioral responses (Lee et al., 2021). Alternatively, neurons that become active could initiate responses to specific stimuli, such as an increase in brain temperature. In this latter scenario, these neurons may be part of the brain's homeostatic response to temperature increases, while STAY neurons may play a critical role in facilitating ongoing (cognitive) functions during a fever. Future research into the molecular characterization of these cell types could offer opportunities to target and

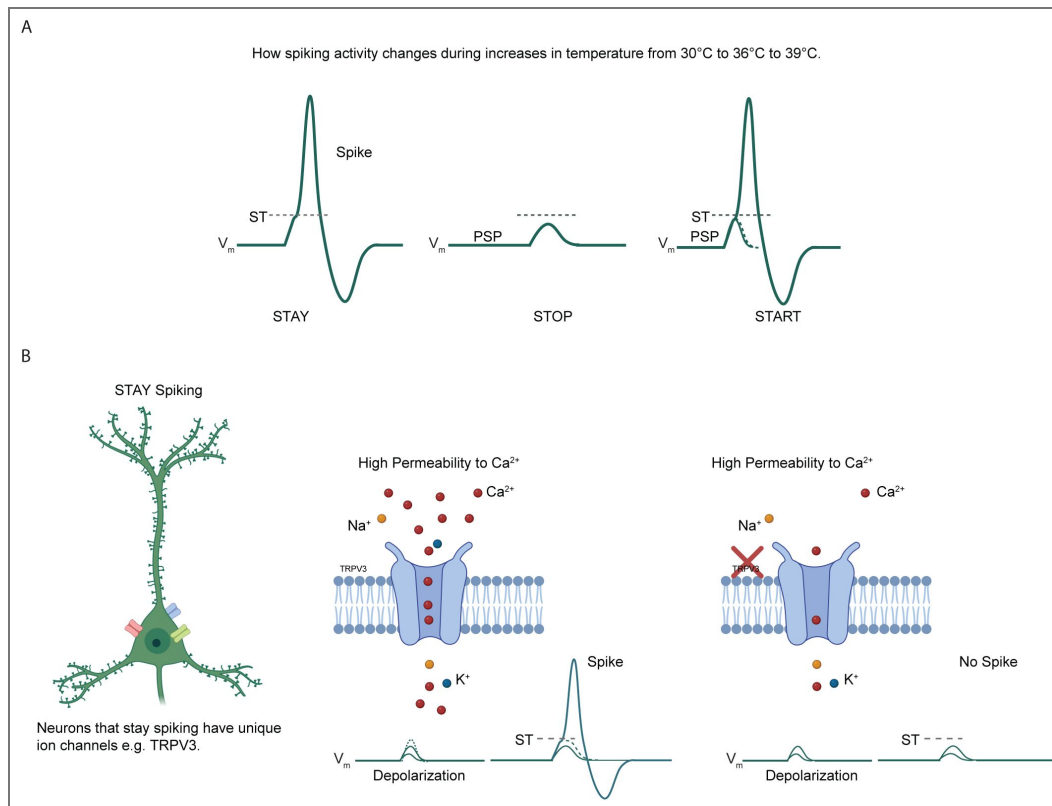


Figure 11. Summary model of how STAY neurons achieve firing stability through unique ion channel compositions, including TRPV3.

A. In cortical L2/3 pyramidal neurons (PNs) with synaptically evoked spiking, gradual increases in brain slice temperature from 30 °C to 36 °C to 39 °C result in four possible outcomes: neurons remain inactive, continue spiking (STAY), stop spiking, or initiate spiking. To spike, PNs must reach spike threshold (ST), defined as the minimal V_m that elicits an action potential, which requires sufficient depolarization via the postsynaptic potential (PSP). STAY neurons consistently reach ST and continue spiking. Neurons that stop spiking fall below the level of depolarization required to reach ST, while neurons that initiate spiking achieve sufficient depolarization to newly reach ST. **B.** STAY neurons may contain unique ion channels, such as TRPV3. TRPV3 channels are highly permeable to Ca^{2+} ions, and Ca^{2+} influx contributes to PN depolarization. The presence of TRPV3 facilitates greater ion entry, enabling depolarization sufficient to reach ST and sustain spiking, whereas its absence reduces depolarization to levels insufficient for spiking.

modulate their activity in a brain region-specific manner, potentially advancing treatments for fever-associated seizures or epilepsies, such as Dravet Syndrome (a genetic epilepsy typically initiating in early infancy) and febrile infection-related epilepsy syndrome (FIRES).

Materials and Methods

Animals

All experimental procedures were performed in accordance with the National Institutes of Health guidelines for care and use of laboratory animals and the EC Council Directive of September 22, 2010 (2010/63/EU). All experiments were approved by the Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism (NIAAA) (Protocol # LIN-MA-1) and the Animal Care Committee of the HUN-REN Research Centre for Natural Sciences (RCNS) and by the National Food Chain Safety Office of Hungary (license number: PE/EA/1004- 5/2021). Mice were housed under standard conditions with ad libitum access to food and water, under a 12-h light/dark cycle. The following mouse strains were obtained from The Jackson Laboratory (JAX): C57BL/6J (JAX 000664), FVB/N mice (JAX 004828), and Trpv3 (JAX 010773) (Moussaieff A et al., 2008; Moqrich et al., 2005 [↗](#)). The day of birth was denoted as postnatal day (P)1. Both male and female mice were used for body temperature (T_b) recordings and electrophysiological experiments.

Body Temperature Measurements

All mice used for body temperature (T_b) recordings were implanted with sterile IPTT-300 Implantable Programmable Temperature transponders (Bio Medic Data Systems, LLC) at 2 weeks of age. The injection site was pre-cleaned with a betadine solution, and the sterile transponders were injected subcutaneously using a pre-loaded 12-gauge syringe into animals anesthetized with 4-5% isoflurane. The injection site was on the left side, approximately 11 mm from the base of the hip, with the animal in the prone position. Post-implantation, the mice were able to move, eat, and drink autonomously, with no adverse phenotypes noted. T_b recording commenced at least 5 days after injection. Prior to recording, mice were brought into the procedure room and allowed to habituate for 1 hour. T_b was recorded every 5 minutes for 6 hours using an IPTT-300 Implantable Programmable Temperature reader (DAS-8027) in a 37L x 16W x 13H cm cage. T_b was recorded in the presence (**Figure 1D** [↗](#)) or absence (**Figure 1A-C** [↗](#)) of infrared light. Infrared light exposure was provided via a 250-W temperature-controlled infrared lamp (catalog #50320, Stoelting), set to 32.5-33.5 °C. The lamp was positioned ~15 cm above the cage, and the ambient temperature was monitored every 5 minutes (**Figure 1D** [↗](#)).

Slice Preparation

Using standard methods, acute primary somatosensory cortex (S1) slices (350 μ m thick) from P7-8, P12-14, or P20-23 mice were cut in the "across-row" plane, oriented 35° toward coronal from midsagittal (Antoine et al., 2019 [↗](#)). The cutting solution contained (in mM): 85 NaCl, 75 sucrose, 25 D-(+)-glucose, 4 MgSO₄, 2.5 KCl, 1.25 NaH₂PO₄, 0.5 ascorbic acid, 25 NaHCO₃, 0.5 CaCl₂. Once cut, slices were transferred to a submerged-style holding chamber containing standard Ringer's solution (in mM: 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 1 NaH₂PO₄, 26.2 NaHCO₃, 11 D-(+)-glucose and 2.5 CaCl₂) and then incubated at 32°C for 30 minutes. Both solutions were at neural pH (i.e., 7.3), 300 mOsm, and saturated with 95% O₂ and 5% CO₂. Slices were kept at room temperature for at least 30 minutes before being transferred to a submerged recording chamber.

In Vitro Physiology

Whole-cell current-clamp recordings were made from pyramidal neurons (PNs) visually identified via infrared DIC optics. Physiological verification for regular spiking was done in current clamp. Recordings were made using 3-6 M Ω micropipettes containing (in mM): 116 K gluconate, 20 HEPES, 6 KCl, 2 NaCl, 0.5 EGTA, 4MgATP, 0.3 NaGTP, 10 Na phosphocreatine, with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Where applicable, forsythoside B (50 μ M, TRPV3

blocker, Millipore Sigma Cat #: PHL83313) or RN1734 (10 μ M, *TRPV4* blocker, Tocris Bioscience Cat #: 3746/25) was added to the internal solution. Camphor (MedChem, Cat. No: HY-N0808) was added to the bath solution for experiments in **Figure 8**.

Signals were filtered (2-6 kHz) and digitized (10-20 kHz). Perfusate temperature for all in vitro experiments was regulated by a PC-connected Peltier heater (SM-4600, Scientifica, UK) and temperature controller (Linlab2, Scientifica, UK), with a high-accuracy, low-noise temperature control system, containing both a built-in temperature sensor and bath sensor for accurate feedback. ACSF flow rates of $\sim$ 3 ml/min were used to facilitate efficient heating/cooling.

To determine the effect of temperature on an activated cortical network, layer 4-evoked spiking was quantified during perfusate temperature increases from 30°C to 36°C, and then to fever range ($\sim$ 39°C) while recording membrane potential. The slicing plane facilitated clear identification of whisker barrel columns, and neuronal activity was evoked by stimulating the barrel center using a bipolar electrode (0.2 ms pulses) at a stimulation of 1.4 E₀. E₀ is defined as the minimal intensity that evoked a consistent excitatory postsynaptic current (EPSC) during more than 3 of 5 consecutive sweeps with 10 s ISI (Antoine et al., 2019). E₀ was determined in voltage clamp for each recorded cell prior to recording PSPs and spiking in current clamp. A new brain slice was used for each recording. L4-evoked feedforward post synaptic potentials (PSPs) and spiking were recorded in single L2/3 PNs from a pre-stimulus baseline membrane potential (V_m) of -50 mV. This V_m , just below spike threshold, was selected to mimic *in vivo* conditions, as PNs *in vivo* can reside within this V_m range during whisker exploration of objects or surfaces (Yamashita et al., 2013). Evoked spikes were analyzed over a 150-ms interval, starting 2-3 ms post-stimulus, for 11 sweeps at 10 s inter-sweep interval (ISI). Spike threshold was defined as the membrane potential (V_m) at which the second derivative of V_m was >5 SDs above the pre-stimulus period. Intrinsic spiking excitability was measured in the presence of glutamate and GABA-A receptor blockers (in μ M: 100 APV, 10 NBQX, 3 gabazine). F-I curves were obtained from PNs held at -80 mV by applying incremental current injections relative to this V_m .

Animal Surgery and *In vivo* Electrophysiological Recordings

In vivo experiments were done under general anesthesia where five mice (age, P24-26; body weight, 7-10 g; both genders) received an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Regular doses of the ketamine/xylazine cocktail were given intramuscularly to maintain the depth of anesthesia during the experimental sessions. Up until the thermal fever protocol started, the T_b of the animals was kept at 36°C using a homeothermic heating pad connected to a temperature controller (Supertech, Pécs, Hungary). To measure the internal body temperature, a Type T thermocouple microprobe (MT-29/5; Physitemp Instruments, Clifton, NJ, USA) was placed in the rectum of the animal. The microprobe has a shaft diameter of 330 μ m, a time constant of 0.025 second and 0.1°C accuracy.

To perform high-density extracellular electrophysiological recordings, mice were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA), then two circular craniotomies ($\sim$ 1.5 mm in diameter) were made with a dental drill above the left and right barrel cortices. The craniotomies were centered at the following stereotaxic coordinates: anterior-posterior (AP): -1.0 mm; medial-lateral (ML): 3.5 mm (with respect to the bregma; Paxinos and Franklin 2001). Two commercially available Neuropixels 1.0 silicon probes (imec, Leuven, Belgium; Jun et al., 2017) mounted on two motorized stereotaxic micromanipulators (Neurostar, Tubingen, Germany) were implanted into the brain, one into the left and the other into the right barrel cortex, to a depth of 1.5 mm. An insertion speed of 2 μ m/s was used to decrease the mechanical trauma caused by the probe insertion (Fiáth et al. 2019).

In order for the probe tracks to be perpendicular to cortical layers, the probes were inserted at an angle of 20 degrees from vertical. The dura mater in the craniotomy was left intact, except when it was too thick and thus the silicon probe could not pierce through this layer (which was indicated by significant probe buckling; $n = 3$ insertions). In these cases a 36 gauge, slightly bent needle was used to carefully cut the dura above the targeted cortical area. After the probe reached its final insertion depth, to allow the brain tissue to settle, we waited at least 10 minutes before

electrophysiological recording was started. Spiking activity of cortical neurons (action potential band, 300–10,000 Hz) was recorded on 384 channels (768 channels in total for the two probes), with a sampling rate of 30 kHz/channel and with a gain of 500 (yielding a resolution of 2.34 μ V per bit). Data were acquired using the SpikeGLX open-source software (<http://billkarsh.github.io/SpikeGLX/>). A common stainless steel wire inserted into the neck muscle of the animal served as the external reference and ground electrode. To avoid the dehydration of the cortex, the skull and the craniotomy was kept moist during the whole experiment using body temperature, sterile physiological saline solution and Gelaspon. Before starting the experimental protocol, manual whisker stimulation (by repetitively touching the whiskers of the animal with a cotton swab) was used to verify the recording position. In all cases ($n = 10$ probe insertions), we detected strong whisker-evoked neuronal activity.

Thermal Fever Protocol during Electrophysiological Recordings

First, we recorded cortical activity for 45 minutes at $\sim 36^{\circ}\text{C}$ body temperature ("baseline" period), then the body temperature of the animal was elevated with the aid of the heating pad and a power bank with warming capability which was placed next to the mouse. The body temperature was increased from 36°C to 39°C in about 5 minutes ($\sim 0.01^{\circ}\text{C/s}$). The elevated body temperature was maintained for 45 minutes ("thermal fever" period). Next, we turned off the heating until the T_b reached physiological temperatures (36°C ; ~ 5 min; $\sim 0.01^{\circ}\text{C/s}$). Cortical activity was recorded for another 45 minutes at 36°C body temperature ("recovery" period).

Continuous recordings with a total duration of 145 minutes were obtained for each mouse.

Spike Sorting and Data Analysis

To extract cortical single-unit activity, spike sorting was performed with Kilosort2 (<https://github.com/MouseLand/Kilosort>; Pachitariu et al., 2016a, 2016b) using the default parameter set (available in the StandardConfig.m file). Channels containing activity acquired by recording sites located outside the cortex were removed before spike sorting (usually ~ 130 channels/probe recorded from the barrel cortex). The list of single unit clusters generated by Kilosort2 was visually inspected to remove units considered as noise (e.g., units with abnormal spike waveform shapes) or multi-unit activity (e.g., clusters with a contaminated refractory period). Manual curation of the Kilosort2 results was done with the Phy Python library, which provides a graphical user interface for interactive visualization of high-density data and supply operations for merging, splitting and marking of clusters (<https://github.com/cortex-lab/phy>; Rossant et al., 2016). In this dataset, we aimed to keep only those single units which had at least 900 spikes ($> \sim 0.1$ Hz firing rate), a clear refractory period, a consistent waveform shape and whose spikes were present throughout the 145-minute-long recording.

Following manual curation, based on their spike waveform duration, the selected single units ($n = 633$) were separated into putative inhibitory interneurons and excitatory principal cells (Barthó et al., 2004). The spike duration was calculated as the time difference between the trough and the subsequent waveform peak of the mean filtered (300 – 6000 Hz bandpassed) spike waveform. Durations of extracellularly recorded spikes showed a bimodal distribution (Hartigan's dip test; $p < 0.001$) characteristic of the neocortex with shorter durations corresponding to putative interneurons (narrow spikes) and longer durations to putative principal cells (wide spikes). Next, k-means clustering was used to separate the single units into these two groups, which resulted in 140 interneurons (spike duration < 0.6 ms) and 493 principal cells (spike duration > 0.6 ms), corresponding to a typical 22% - 78% (interneuron – principal) cell ratio. Finally, the firing rates of neurons were computed separately during the three distinguished periods (baseline, thermal fever and recovery). To decrease the effect of transient changes (e.g., tissue recovery during the baseline period or the short-term effect of heating during the thermal fever period), for each 45-minute-long period, we used only the last 25-minutes to calculate the mean firing rates of neurons.

Immunofluorescence staining

Mice were anesthetized and transcardially perfused using standard methods. Brains were post-fixed overnight in 4% PFA at 4 °C with shaking, rinsed in 1X PBS, and cryoprotected in 30% sucrose. They were embedded in optimal cutting temperature (OCT) compound, frozen on dry ice, and stored at –80 °C until sectioning. Tissue was cut at 30 µm on a cryostat, mounted onto slides, and sections were permeabilized in 0.3% Triton X-100 (in 1X PBS) for 10 min at room temperature. Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ (in 1X PBS) at room temperature. Slides were blocked in 10% normal goat serum for 1 h at room temperature, then incubated overnight at 4 °C with anti-TRPV3-Biotin antibody (#ACC-033-B), TRPV3 blocking peptide (#BLP-CC033), and/or anti-TRPV4 antibody(#ACC-034) (Alomone Labs, Israel). For antibody–peptide controls, the anti-TRPV3-Biotin antibody was pre-incubated with its blocking peptide at a 1:2.5 ratio, using 1 mg/ml of each. The following day, sections were incubated for 1 h at room temperature with fluorescently labeled secondary antibodies (streptavidin-conjugated or unconjugated). Nuclei were counterstained with DAPI Fluoromount-G (Electron Microscopy Sciences, USA). Finally, sections were mounted onto slides, cover-slipped, and stored at 4 °C until imaging. Images were acquired using a Keyence BZ-X810 microscope (BZ-X800 Viewer, Version 1.1.1.8) with BZ-X filter sets: DAPI (Ex 360/40 nm; OP-87762), GFP (Ex 470/40 nm; OP-87763), and TRITC (Ex 545/25 nm; OP-87764).

Statistics

Statistical analyses were conducted using Prism 9.0 (GraphPad). For experiments, at least 3 mice from a minimum of 2 separate litters were used, with littermates typically included across the age groups P7-8, P12-14, and P20-23. Non-Gaussian data were either log-transformed for parametric testing or subjected to nonparametric tests, as specified in the results and figure legends. Significance was consistently reported at $\alpha = 0.05$. Details on the statistical tests used are provided in the figure captions.

Data availability

All behavioral, electrophysiological and immunohistochemical data will be deposited and made publicly available.

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Peer reviews

Reviewer #1 (Public review):

The paper by Chen et al describes the role of neuronal thermo-TRPV3 channels in the firing of cortical neurons at fever temperature range. The authors began by demonstrating that exposure to infrared light increasing ambient temperature causes body temperature rise to fever level above 38{degree sign}C. Subsequently, they showed that at the fever temperature of 39{degree sign}C, the increased spike threshold (ST) increased in both populations (P12-14 and P7-8) of cortical excitatory pyramidal neurons (PNs). However, the spike number only decreased in P7-8 PNs, while it remained stable in P12-14 PNs at 39{degree sign}C. In addition, the fever temperature also reduced the late peak postsynaptic potential (PSP) in P12-14 PNs. The authors further characterized the firing properties of cortical P12-14 PNs, identifying two types: STAY PNs that retained spiking at 30{degree sign}C, 36{degree sign}C and 39{degree sign}C, and STOP PNs that stopped spiking upon temperature change. They further extended their analysis and characterization to striatal medium spiny neurons (MSNs) and found that STAY MSNs and PNs shared same ST temperature sensitivity. Using small molecule tools, they further identified that thermo-TRPV3 currents in cortical PNs increased in response to temperature elevation, but not TRPV4 currents. The authors concluded that during fever, neuronal firing stability is largely maintained by sensory STAY PNs and MSNs that express functional TRPV3 channels. Overall, this study is well designed and executed with substantial controls, some interesting findings and quality of data.

Comments on revisions:

My previous concerns have been addressed in this revised manuscript.

<https://doi.org/10.7554/eLife.102412.2.sa3>

Reviewer #2 (Public review):

Summary:

The authors studied the excitability of layer 2/3 pyramidal neurons in response to layer four stimulation at temperatures ranging from 30 to 39{degree sign}C in P7-8, P12-P14, and P22-

P24 animals. They also measure brain temperature and spiking in vivo in response to externally applied heat. Some pyramidal neurons continue to fire action potentials in response to stimulation at 39°C and are referred to as "stay neurons." Stay neurons have unique properties, aided by the expression of the TRPV3 channel.

Strengths:

The authors focused on layer 2/3 neuronal excitability at three developmental stages: during the window of susceptibility to febrile seizures, before the window opens, and after it closes.

Electrophysiological experiments are rigorously performed and carefully interpreted.

The cellular electrophysiology is further confirmed. The authors compared the seizure susceptibility of TRPV3 knockout, heterozygous, and wild-type mice. EEG recording would have strengthened the study, but they are challenging in this age group.

Finally, the authors studied TRPV3 expression with immunohistochemistry.

<https://doi.org/10.7554/eLife.102412.2.sa2>

Reviewer #3 (Public review):

Summary:

This important study combines in vitro and in vivo recording to determine how the firing of cortical and striatal neurons changes during a fever range temperature rise (37-40 °C). The authors found that certain neurons will start, stop, or maintain firing during these body temperature changes. The authors further suggested that the TRPV3 channel plays a role in maintaining cortical activity during fever.

Strengths:

The topic of how the firing pattern of neurons changes during fever is unique and interesting. The authors carefully used in vitro electrophysiology assays to study this interesting topic.

Weaknesses:

(1) In vivo recording is a strength of this study. However, data from in vivo recording is only shown in Fig 5A,B. This reviewer suggests the authors further expand on the analysis of the in vivo Neuropixels recording. For example, to show single spike waveforms and raster plots to provide more information on the recording. The authors can also separate the recording based on brain regions (cortex vs striatum) using the depth of the probe as a landmark to study the specific firing of cortical neurons and striatal neurons. It is also possible to use published parameters to separate the recording based on spike waveform to identify regular principal neurons vs fast-spiking interneurons. Since the authors studied E/I balance in brain slices, it would be very interesting to see whether the "E/I balance" based on the firing of excitatory neurons vs fast-spiking interneurons might be changed or not in the in vivo condition.

(2) The author should propose a potential mechanism for how TRPV3 helps to maintain cortical activity during fever. Would calcium influx-mediated change of membrane potential be the possible reason? Making a summary figure to put all the findings into perspective and propose a possible mechanism would also be appreciated.

(3) The author studied P7-8, P12-14, and P20-26 mice. How do these ages correspond to the human ages? it would be nice to provide a comparison to help the reader understand the context better.

Comments on revisions:

In this revised version, the authors nicely addressed my critiques. I have no more comments to make.

<https://doi.org/10.7554/eLife.102412.2.sa1>

Author response:

The following is the authors' response to the original reviews

Public Reviews:

Reviewer #1 (Public review):

The paper by Chen et al describes the role of neuronal thermo-TRPV3 channels in the firing of cortical neurons at a fever temperature range. The authors began by demonstrating that exposure to infrared light increasing ambient temperature causes body temperature to rise to a fever level above 38{degree sign}C. Subsequently, they showed that at the fever temperature of 39{degree sign}C, the spike threshold (ST) increased in both populations (P12-14 and P7-8) of cortical excitatory pyramidal neurons (PNs). However, the spike number only decreased in P7-8 PNs, while it remained stable in P12-14 PNs at 39 degrees centigrade. In addition, the fever temperature also reduced the late peak postsynaptic potential (PSP) in P12-14 PNs. The authors further characterized the firing properties of cortical P12-14 PNs, identifying two types: STAY PNs that retained spiking at 30{degree sign}C, 36{degree sign}C, and 39{degree sign}C, and STOP PNs that stopped spiking upon temperature change. They further extended their analysis and characterization to striatal medium spiny neurons (MSNs) and found that STAY MSNs and PNs shared the same ST temperature sensitivity. Using small molecule tools, they further identified that thermo-TRPV3 currents in cortical PNs increased in response to temperature elevation, but not TRPV4 currents. The authors concluded that during fever, neuronal firing stability is largely maintained by sensory STAY PNs and MSNs that express functional TRPV3 channels. Overall, this study is well designed and executed with substantial controls, some interesting findings, and quality of data. Here are some specific comments:

(1) Could the authors discuss, or is there any evidence of, changes in TRPV3 expression levels in the brain during the postnatal 1-4 week age range in mice?

This is an excellent question. To our knowledge, no published studies have documented changes in TRPV3 expression in the mouse brain during the first to fourth postnatal weeks. Research on TRPV3 expression has primarily relied on RT-PCR analysis of RNA from dissociated adult brain tissue (Jang et al., 2012; Kumar et al., 2018), largely due to the limited availability of effective antibodies for brain sections at the time. Furthermore, the Allen Brain Atlas does not provide data on TRPV3 expression in the developing or postnatal brain. To address this gap, we performed immunohistochemistry to examine TRPV3 expression at P7,

P14, and P21 (Figure 7). To confirm specificity, the TRPV3 antibody was co-incubated with a TRPV3 blocker (Figure 7A, top row, right panel). While immunohistochemistry is semiquantitative, we observed a trend toward increased TRPV3 expression in the cortex, striatum, hippocampus, and thalamus from P7 to P14.

(2) Are there any differential differences in TRPV3 expression patterns that could explain the different firing properties in response to fever temperature between the STAY- and STOP neurons?

This is another excellent question, and we plan to explore it in the future by developing reporter mice for TRPV3 expression and viral tools that leverage endogenous TRPV3 promoters to drive a fluorescent protein, enabling monitoring of cells with native TRPV3 expression. To our knowledge, such tools do not currently exist. Creating them will be challenging, as it requires identifying promoters that accurately reflect endogenous TRPV3 expression.

We have not yet quantified TRPV3 expression in STOP and STAY neurons. However, our analysis of evoked spiking at 30, 36, and 39 °C suggests that TRPV3 may mark a population of cortical pyramidal neurons that tend to remain active (“STAY”) as temperatures increase. While we have not directly compared TRPV3 expression between STAY and STOP neurons at feverrange temperatures, intracellular blockade of TRPV3 with fentanyl (50 μM) significantly reduced the proportion of STAY neurons (Figure 9B). Consistently, spiking was also significantly reduced in *Trpv3*^{-/-} mice (Figure 10D).

In our immunohistochemical analysis, TRPV3 was detected in L4 barrels and in L2/3, where we observed a patchy distribution with some regions showing more intense staining (Figure 7B). It is possible that cells with higher TRPV3 levels correspond to STAY neurons, while those with lower levels correspond to STOP neurons. As we develop tools to monitor activity based on endogenous TRPV3 levels, we anticipate gaining deeper insight into this relationship.

(3) TRPV3 and TRPV4 can co-assemble to form heterotetrameric channels with distinct functional properties. Do STOP neurons exhibit any firing behaviors that could be attributed to the variable TRPV3/4 assembly ratio?

There is some evidence that TRPV3 and TRPV4 proteins can physically associate in HEK293 cells and native skin tissues (Hu et al., 2022). TRPV3 and TRPV4 are both expressed in the cortex (Kumar et al., 2018), but it remains unclear whether they are co-expressed and coassembled to form heteromeric channels in cortical excitatory pyramidal neurons. Examination of the I-V curve from HEK cells co-expressing TRPV3/4 heteromeric channels shows enhanced current at negative membrane potentials (Hu et al., 2022).

Currently, we cannot characterize cells as STOP or STAY and measure TRPV3 or TRPV4 currents simultaneously, as this would require different experimental setups and internal solutions. Additionally, the protocol involves a sequence of recordings at 30, 36, and 39°C, followed by cooling back to 30°C and re-heating to each temperature. Cells undergoing such a protocol will likely not survive till the end.

In our recordings of TRPV3 currents, which likely include both STOP and STAY cells, we do not observe a significant current at negative voltages, suggesting that TRPV3/4 heteromeric channels may either be absent or underrepresented, at least at a 1:1 ratio. However, the possibility that TRPV3/4 heteromeric channels could define the STOP cell population is intriguing and plausible.

(4) In Figure 7, have the authors observed an increase of TRPV3 currents in MSNs in response to temperature elevation?

We have not recorded TRPV3 currents in MSNs in response to elevated temperatures. Please note that the handling editor gave us the option to remove these data from the paper, and we elected to do so to develop them as a separate manuscript.

(5) Is there any evidence of a relationship between TRPV3 expression levels in D2+ MSNs and degeneration of dopamine-producing neurons?

This is an interesting question, though it falls outside our current research focus in the lab. A PubMed search yields no results connecting the terms TRPV3, MSNs, and degeneration.

However, gain-of-function mutations in TRPV4 channel activity have been implicated in motor neuron degeneration (Sullivan et al., 2024) and axon degeneration (Woolums et al., 2020). Similarly, TRPV1 activation has been linked to developmental axon degeneration (Johnstone et al., 2019), while TRPV3 blockade has shown neuroprotective effects in models of cerebral ischemia/reperfusion injury in mice (Chen et al., 2022).

The link between TRPV activation and cell degeneration, however, may not be straightforward. For instance, TRPV1 loss has been shown to accelerate stress-induced degradation of axonal transport from retinal ganglion cells to the superior colliculus and to cause degeneration of axons in the optic nerve (Ward et al., 2014). Meanwhile, TRPV1 activation by capsaicin preserves the survival and function of nigrostriatal dopamine neurons in the MPTP mouse model of Parkinson's disease (Chung et al., 2017).

(6) Does fever range temperature alter the expressions of other neuronal Kv channels known to regulate the firing threshold?

This is an active line of investigation in our lab. The results of ongoing experiments will provide further insight into this question.

Reviewer #2 (Public review):

Summary:

The authors study the excitability of layer 2/3 pyramidal neurons in response to layer four stimulation at temperatures ranging from 30 to 39 Celsius in P7-8, P12-P14, and P22-P24 animals. They also measure brain temperature and spiking in vivo in response to externally applied heat. Some pyramidal neurons continue to fire action potentials in response to stimulation at 39 C and are called stay neurons. Stay neurons have unique properties aided by TRPV3 channel expression.

Strengths:

The authors use various techniques and assemble large amounts of data.

Weaknesses:

(1) No hyperthermia-induced seizures were recorded in the study.

The goal of this manuscript is to uncover age-related physiological changes that enable the brain to maintain function at fever-range temperatures, typically 38–40°C. Febrile seizures in humans are also typically induced within this temperature range. Given this context, we initially did not examine hyperthermia-induced seizures. However, as requested, we assessed the effects of reduced *Trpv3* expression on hyperthermia-induced seizures in WT(*Trpv3*^{+/+}), heterozygous (*Trpv3*^{+/-}), and homozygous knockout (*Trpv3*^{-/-}) P12 pups. Please see figure 10.

While *T_b* at seizure onset and the rate of *T_b* increase leading to seizure were not significantly different among genotypes, the time to seizure from the point of loss of postural control (LPC), defined as collapse and failure to maintain upright posture, was significantly longer in *Trpv3*^{+/-} and *Trpv3*^{-/-} mice. Together, these results indicate that reduced TRPV3 function enhances resistance to seizure initiation and/or propagation under febrile conditions, likely by decreasing neuronal depolarization and excitability.

(2) Febrile seizures in humans are age-specific, extending from 6 months to 6 years. While translating to rodents is challenging, according to published literature (see Baram), rodents aged P11-16 experience seizures upon exposure to hyperthermia. The rationale for publishing data on P7-8 and P22-24 animals, which are outside this age window, must be clearly explained to address a potential weakness in the study.

As requested, we have added an explanation in the “Introduction” for our rationale in including age ranges that flank the period of susceptibility to hyperthermia-induced seizures (see lines 80–100). In summary, we emphasize that this design provides negative controls, allowing us to determine whether the changes observed in the P12–14 window are specific to this developmental period.

(3) Authors evoked responses from layer 4 and recorded postsynaptic potentials, which then caused action potentials in layer 2/3 neurons in the current clamp. The postsynaptic potentials are exquisitely temperature-sensitive, as the authors demonstrate in Figures 3 B and 7D. Note markedly altered decay of synaptic potentials with rising temperature in these traces. The altered decays will likely change the activation and inactivation of voltage-gated ion channels, adjusting the action potential threshold.

The activation and inactivation of voltage-gated ion channels can modulate action potential threshold. Indeed, we have identified channels that contribute to the temperature-induced increase in spike threshold, including BK channels and *Scn2a*. However, Figure 4B represents a cell with no inhibition at 39°C, and thus the observed loss of the late postsynaptic potential (PSP). This primarily contributes to the prolonged decay of the synaptic potentials. By contrast, cells in which inhibition is retained, when exposed to the same thermal protocol, do not exhibit such extended decay.

(4) The data weakly supports the claim that the E-I balance is unchanged at higher temperatures. Synaptic transmission is exquisitely temperature-sensitive due to the many proteins and enzymes involved. A comprehensive analysis of spontaneous synaptic current amplitude, decay, and frequency is crucial to fully understand the effects of temperature on synaptic transmission.

We did not intend to imply that E-I balance is generally unchanged at higher temperatures. Our statements specifically referred to observations in experiments conducted during the P20–26 age range in cortical pyramidal neurons. We are conducting a parallel line of investigation examining the differential susceptibility of E-I balance across age and temperature, and we have observed age- and temperature-dependent effects. Recognizing that our earlier wording may have been misleading, we have removed this statement from the manuscript.

(5) It is unclear how the temperature sensitivity of medium spiny neurons is relevant to febrile seizures. Furthermore, the most relevant neurons are hippocampal neurons since the best evidence from human and rodent studies is that febrile seizures involve the hippocampus.

Thank you for the opportunity to provide clarification. The goal of this manuscript is to uncover age-related physiological changes that enable the brain to maintain stable, non-excessive neuronal firing at fever-range temperatures (typically 38–40°C). We hypothesize that these changes are a normal part of brain development, potentially explaining why most children do not experience febrile seizures. By understanding these mechanisms, we may identify points in the process that are susceptible to dysfunction, due to genetic mutations, developmental delays, or environmental factors, which could provide insight into the rare cases when seizures occur between 2–5 years of age.

Our aim was not to establish a link between medium spiny neuron (MSN) function and febrile seizures. MSNs were included in this study as a mechanistic comparison because they represent a non-pyramidal, non-excitatory neuronal subtype, allowing us to assess whether the physiological changes observed in L2/3 excitatory pyramidal neurons are unique to these cells. Please note that the handling editor gave us the option to remove these data from the manuscript, and we chose to do so, developing these findings into a separate manuscript.

(6) TRPV3 data would be convincing if the knockout animals did not have febrile seizures.

We find that approximately equal numbers of excitatory neurons either start or stop firing at fever-range temperatures (typically 38–40 °C). Neurons that continue to fire (“STAY” cells), thus play a key role in maintaining stable, non-excessive network activity. While future studies will examine the mechanisms driving some neurons to initiate spiking, our findings suggest that a reduction in the number of STAY cells could influence more subtle aspects of seizure dynamics, such as time to onset, by decreasing overall network excitability. We assessed the effects of reduced *Trpv3* expression on hyperthermia-induced seizures in WT(*Trpv3*^{+/+}), heterozygous (*Trpv3*^{+/-}), and homozygous knockout (*Trpv3*^{-/-}) P12 pups. As you stated, these mice have hyperthermic seizures, however, we noted that the time to seizure from the point of loss of postural control (LPC), defined as collapse and failure to maintain upright posture, was significantly longer in *Trpv3*^{+/-} and *Trpv3*^{-/-} mice. Normally, seizures happen shortly after this point, but notably, *Trpv3*^{-/-} mice took twice as long to reach seizure onset compared with wildtype mice. In an epileptic patient, this increased time may be sufficient for a caretaker to move the patient to a safer location, reducing the risk of injury during the seizure.

Consistent with findings that TRPV3 blockade using 50 μM forsythoside B reduces spiking in cortical L2/3 pyramidal neurons, we observed significantly reduced spiking in *Trpv3*^{-/-} mice as well (Figure 10D). Analysis of postsynaptic potentials in these neurons showed that, in WT mice, PSP amplitude increased with temperature elevation into the febrile range, whereas this temperature-dependent depolarization was absent in *Trpv3*^{-/-} mice (Figure 10E). Together, these results indicate that reduced TRPV3 function enhances resistance to seizure initiation and/or propagation under febrile conditions, likely by decreasing neuronal depolarization and excitability.

Reviewer #3 (Public review):

Summary:

This important study combines in vitro and in vivo recording to determine how the firing of cortical and striatal neurons changes during a fever range temperature rise (37-40 oC). The authors found that certain neurons will start, stop, or maintain firing during these body temperature changes. The authors further suggested that the TRPV3 channel plays a role in maintaining cortical activity during fever.

Strengths:

The topic of how the firing pattern of neurons changes during fever is unique and interesting. The authors carefully used in vitro electrophysiology assays to study this interesting topic.

Weaknesses:

(1) In vivo recording is a strength of this study. However, data from in vivo recording is only shown in Figures 5A,B. This reviewer suggests the authors further expand on the analysis of the in vivo Neuropixels recording. For example, to show single spike waveforms and raster plots to provide more information on the recording. The authors can also separate the recording based on brain regions (cortex vs striatum) using the depth of the probe as a landmark to study the specific firing of cortical neurons and striatal neurons. It is also possible to use published parameters to separate the recording based on spike waveform to identify regular principal neurons vs fast-spiking interneurons. Since the authors studied E/I balance in brain slices, it would be very

interesting to see whether the "E/I balance" based on the firing of excitatory neurons vs fast-spiking interneurons might be changed or not in the in vivo condition.

As requested, we have included additional analyses and figures related to the in vivo recording experiments in Figure 5. Specifically, we added examples of multiunit and single-spike waveforms, as well as autocorrelation histograms (ACHs). ACHs were used because raster plots of individual single units would not be very informative given the long recording period. Additionally, Figure 5F was also aimed to replace raster plots as it helps to track changes in the firing rate of a single neurons over time.

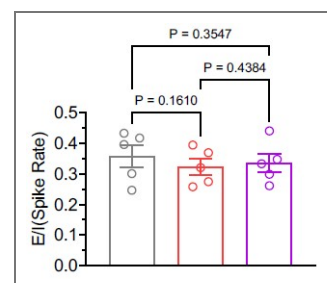
Additionally, all recordings were conducted in the cortex at a depth of ~1 mm from the surface, and no recordings were performed in the striatum. Based on the reviewing editor's suggestions, we decided to remove the striatal data from the manuscript and develop this aspect of the project for a separate publication.

Lastly, we used published parameters to classify recordings based on spike waveform into putative regular principal neurons and interneurons. To clarify this point, we have now included descriptions that were previously listed only in the "Methods" section into the "Results" section as well.

The paragraph below from the methods section describes this procedure.

"Following manual curation, based on their spike waveform duration, the selected single units (n= 633) were separated into putative inhibitory interneurons and excitatory principal cells (Barthóet al., 2004). The spike duration was calculated as the time difference between the trough and the subsequent waveform peak of the mean filtered (300 – 6000 Hz bandpassed) spike waveform. Durations of extracellularly recorded spikes showed a bimodal distribution (Hartigan's dip test; $p < 0.001$) characteristic of the neocortex with shorter durations corresponding to putative interneurons (narrow spikes) and longer durations to putative principal cells (wide spikes). Next, k-means clustering was used to separate the single units into these two groups, which resulted in 140 interneurons (spike duration < 0.6 ms) and 493 principal cells (spike duration > 0.6 ms), corresponding to a typical 22% - 78% (interneuron – principal) cell ratio".

As suggested, we calculated the E/I balance using the average firing rates of excitatory and inhibitory neurons in the in vivo condition. Our analysis revealed that the E/I balance remained unchanged (see Author response image 1). Nonetheless, following the option provided by the reviewing editor, we have chosen to remove the statement referencing E/I balance from the manuscript.



Author response image 1.

(2) The author should propose a potential mechanism for how TRPV3 helps to maintain cortical activity during fever. Would calcium influx-mediated change of membrane potential be the possible reason? Making a summary figure to put all the findings into perspective and propose a possible mechanism would also be appreciated.

Thank you for your helpful suggestion. In response, we have included a summary figure (Figure 11) illustrating the hypothesis described in the Discussion section. We agree with your assessment that *Trpv3* most likely contributes to maintaining cortical activity during fever by promoting calcium influx and depolarizing the membrane potential.

(3) *The author studied P7-8, P12-14, and P20-26 mice. How do these ages correspond to the human ages? it would be nice to provide a comparison to help the reader understand the context better.*

Ideally, the mouse to human age comparison should depend on the specific process being studied. Per your suggestion, we have added additional references in the Introduction (Dobbing and Sands, 1973; Baram et al., 1997; Bender et al., 2004) to help readers better understand the correspondence between mouse and human ages.

Recommendations for the authors:

Reviewer #2 (Recommendations for the authors):

(3) *Perform I-F curves to study the intrinsic properties of layer 2/3 neurons without the confound of evoked responses.*

We performed F-I curve analyses (Figures 2H–I), as suggested by Reviewer 2, to study intrinsic properties of L2/3 neurons without evoked responses. Although rheobase increased at 39 °C compared to 30 °C, consistent with findings such as depolarized spike threshold and reduced input resistance, the mean number of spikes across current steps did not differ.

Reviewer #3 (Recommendations for the authors):

Some statistical descriptions are not clearly stated. For example, what statistical methods were used in Fig 2E? The effect size in Fig 2D seems to be quite small. The authors are advised to consider "nested analysis" to further increase the rigor of the analysis. Does each dot mean one neuron? Some of the data points might not be totally independent. The author should carefully check all figures to make sure the stats methods are provided for each panel.

We apologize for not including statistical details in Figure 2E. We have now added this information and verified that statistical descriptions are provided in all figure legends. In Figure 2D, each dot represents a cell, with measurements taken from the same cell at 30°C, 36°C, and 39°C. Given this design, the appropriate test is a one-way repeated-measures ANOVA.

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