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Point/Counterpoint: Heat-induced membrane depolarization of hypothalamic neurons: a putative/an unlikely mechanism of central thermosensitivity

POINT: HEAT-INDUCED MEMBRANE DEPOLARIZATION OF HYPOTHALAMIC NEURONS: A PUTATIVE MECHANISM OF CENTRAL THERMOSENSITIVITY

HEATING AND COOLING VARIOUS sites of the preoptic area and anterior hypothalamus (PO/AH) of mammals with fine thermodes reflexively evoke thermoregulatory responses, indicating that many central thermostats regulate temperature (T) of the brain (16). In PO/AH, heating- and cooling-activated neurons have been recorded in vivo (5, 14) and in vitro (1, 2, 6–8, 10, 12, 20). This suggests that some PO/AH neurons have primary T receptors similar to peripheral T receptors, and that these PO/AH neurons themselves act as the central thermostats (9–11). However, it has been controversial whether primary PO/AH neurons show heating-induced depolarization (6, 12) or not (1, 2, 20). Here, we state that primary PO/AH neurons show heating-induced depolarization based on our study (6) and thermo transient receptor potential (TRP) channels (4, 13, 18, 19).

As reported by Hori et al. (7) and Kelso and Boulant (8), T-sensitive neurons in PO/AH slices have been classified into two types. One is primary T-sensitive neurons, which retain thermal sensitivity after synaptic blockade. The other is secondary T-sensitive neurons whose T sensitivities disappear after synaptic blockade. Therefore, we analyzed the ionic basis of primary heating-activated neurons visualized in PO/AH slices with the patch-clamp method (6, 12).

To inhibit evoked release of synaptic vesicles, solution of Ca^{2+}-free/high Mg^{2+} was prepared from normal Krebs solution by replacing Ca^{2+} with Mg^{2+} ions (7, 8). However, this cannot eliminate miniature release of synaptic vesicles (17) that strongly affect postsynaptic neurons in the brain. In the brain, the main excitatory transmitter is glutamate affecting non-*N*-methyl-D-aspartate (non-NMDA) receptors and the main inhibitory transmitter is GABA affecting GABA$_A$ receptors. Therefore, blockers (CNQX) for non-NMDA receptors and blockers (bicuculline methiodide) for GABA$_A$ receptors were added to the above Ca^{2+}-free/high-Mg^{2+} solution. This was finally used as a test solution for synaptic blockade. In this solution, evoked or miniature postsynaptic currents disappeared in postsynaptic neurons (17), indicating that this procedure enabled synaptic blockade.

Secondary T-Sensitive Neurons Do Not Show Heating-Induced Depolarization

In this study, T sensitivity was tested at 32–40°C (6). In normal Krebs solution, we easily found heating-sensitive neurons. In whole cell current-clamp recordings, these neurons evoked action potentials (impulses) spontaneously with regular intervals. When heated, frequency of action potentials increased substantially, but heating-induced depolarization from resting potential did not appear. Thus, these neurons behaved as if they were heating-sensitive neurons without depolarization. However, when normal Krebs solution was replaced by

the test solution for synaptic blockade, all of these neurons stopped impulse generation. Therefore, we identified them as secondary T-sensitive neurons, which received inputs from presynaptic T-sensitive cells in the same slice.

These secondary T-sensitive neurons may correspond to the T-sensitive neurons that Boulant and his colleagues have repeatedly recorded in rat PO/AH slices in normal Krebs solution without synaptic blockade. Their heating-activated neurons have not shown membrane depolarization (1, 2, 20).

Primary T-Sensitive Neurons Show Heating-Induced Depolarization

Throughout the following experiments, we recorded neurons in the test solution for synaptic blockade (6). With loose-seal cell-attached patch recordings of impulses, we extracellularly searched for primary T-sensitive neurons. Under synaptic blockade, the proportion of primary T-sensitive neurons was very low. When found, we made whole cell configuration with a new patch pipette. In current-clamp recordings (Fig. 1*A*), when T increased from 32 to 40°C, membrane potential started to depolarize from resting potential at threshold T (35.5 ±
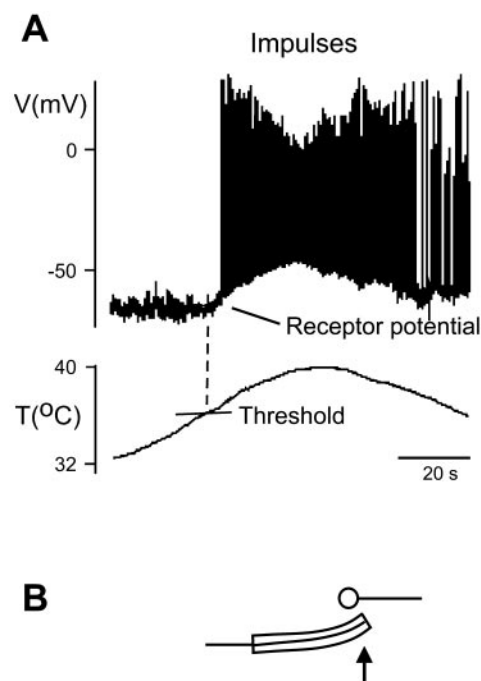
Fig. 1. Whole cell current-clamp recording of intracellular membrane potential of a primary heating-activated neuron in rat anterior hypothermus (PO/AH) slices. *A*: when temperature (T) increased above threshold T (bar), heating-induced depolarization (receptor potential) occurred from resting potential, which generated repetitive impulses as the trigger to activate target cells. V, voltage. (Modified from Fig. 1 of Ref. 6). *B*: symbol of this neuron as a heating-activated comparator expressed by a bimetal switch that makes output when T is above threshold T. When temperature increases, a top of this bimetal moves upward as indicated by the arrow.

2.1°C, $n = 9$). The heating-induced depolarization (receptor potential) increased with T. When the depolarization increased above threshold potential of excitation, these neurons generated impulses repetitively. The discharge frequency increased with depolarization. Thus, primary T-sensitive neurons showed heating-induced depolarization, which evoked impulses repetitively. We have proposed that this neuron acts as a comparator that compares T with its threshold T, as expressed by a symbol of a bimetal switch (Fig. 1*B*).

In voltage-clamp (−67 mV) recordings, heating above threshold (35.8 ± 1.8°C, $n = 16$) evoked inward current without desensitization. *I-V* curves demonstrated that the heating-activated whole cell conductance showed nonselective cation channel properties. This indicated that heating would cause depolarization to 0 mV from resting potential in a current-clamp mode.

These results implied that there were a few primary T-sensitive neurons in a slice, which sent extensive synaptic outputs to secondary T-sensitive neurons in the same slice.

If there are efferent paths from primary heating-activated neurons in PO/AH to heat-loss effectors in vivo, these comparator neurons act as the central thermostats (Fig. 1*B*) to regulate the brain T against heat with coolers (9, 11). This assumption is reasonable, because local heating of PO/AH evokes various heat-loss responses (16).

There may be afferent paths from peripheral heating-activated receptors to primary heating-activated neurons in PO/AH. Then, primary PO/AH neurons play two roles. One is to work as the central thermostats against heat. The other is to work as relays (interneurons) on a path from peripheral thermostats (receptors) (9, 11) to heat-loss effectors.

Recently-cloned T receptors (4, 13, 18, 19) exist in the TRP-nonselective cation channel family and are expressed in peripheral sensory neurons. TRPM8 (melastatin) channels are receptors, causing depolarization to 0 mV when T is below threshold (~28°C). TRPV1 (vanilloid), TRPV2, TRPV3, and TRPV4 are receptors, also causing depolarization to 0 mV when T is above their respective thresholds. These thermo TRP channels may be comparators with different active ranges, working based on phase transition (15).

Active T ranges (near-normal core T) of TRPV3 (19) and TRPV4 (4) are similar to those of primary PO/AH neurons. TRPV3 (3) and TRPV4 (4) have been reported to be present in the brain. Therefore, TRPV3 (3) or TRPV4 (4) might be expressed in primary PO/AH neurons. However, we must test this hypothesis by future studies. From these results, we conclude that heating-induced depolarization of primary PO/AH neurons is a putative mechanism of central thermosensitivity.

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COUNTERPOINT: HEAT-INDUCED MEMBRANE DEPOLARIZATION OF HYPOTHALAMIC NEURONS: AN UNLIKELY MECHANISM OF CENTRAL THERMOSENSITIVITY

THE HYPOTHALAMIC PREOPTIC region contains thermosensitive neurons that are important in the regulation of body temperature. Mechanisms for preoptic neuronal thermosensitivity remain controversial, and some laboratories continue to believe that the primary mechanism is a heat-induced depolarization of the resting membrane potential. It is my contention that this is not true. As explained below, my reasons are based on the following lines of evidence. First, preoptic neuronal warm sensitivity is due primarily to the effects of temperature on brief depolarizing prepotentials or pacemaker potentials that determine the intervals between successive action potentials. Second, there is no correlation between firing rate thermosensitivity and the thermosensitivity of resting membrane potentials or resting currents. Third, in some laboratories, experimentally-induced conditions (especially the use of injected

Fig. 1. Effect of temperature on the activity of preoptic temperature-insensitive (*A* and *C*) and warm-sensitive (*B* and *D*) neurons recorded intracellularly in rat hypothalamic tissue slices. *A* and *B*: 1-s records of action potentials and depolarizing prepotentials at three different temperatures. *C* and *D*: superimposed records at different temperatures showing individual action potentials and subsequent depolarizing prepotentials and action potentials. Both types of neurons display depolarizing prepotentials and warm-induced decreases in action potential amplitudes. In warm-sensitive neurons (*B* and *D*), however, increasing temperature causes an increase in the rate of rise in the depolarizing prepotential, with no appreciable change in the average membrane potential. There is no change in the average membrane potential at the midpoint of each interspike interval (*D*). Because of the warm-induced increase in the prepotential's rate of depolarization, threshold is reached sooner, interspike interval shortens, and firing rate increases. (Reproduced with permission from Ref. 8.)

holding currents and traumatized neurons) can artificially produce neurons having heat-induced membrane depolarization.

As illustrated in Fig. 1, electrophysiological studies have identified two predominant types of preoptic neurons in both intact animals and hypothalamic tissue slices. The majority of preoptic neurons are classified as "temperature insensitive" (Fig. 1*A*) and show little or no change in their firing rates during a change in preoptic temperature. In contrast, about 20% of preoptic neurons are classified as "warm sensitive" (Fig. 1*B*) because their firing rates increase significantly with an increase in temperature (4).

When comparing warm-sensitive and temperature-insensitive neurons, it should be noted that different laboratories employ different criteria for neuronal thermosensitivity, which is defined by the linear regression of the action potential firing rate (i.e., impulses per second) plotted as a function of hypothalamic temperature. Studies by Kobayashi and colleagues (11, 13, 14) have used 0.5 impulses·s^{-1}·°C^{-1} as the criterion to distinguish between temperature-insensitive and warm-sensitive neurons. For many years, however, our laboratory has used 0.8 impulses·s^{-1}·°C^{-1} as the minimal criterion for warm sensitivity (2, 3). Both physiological and morphological neuronal differences provide compelling reasons to use this later criterion. If the 0.8 impulses·s^{-1}·°C^{-1} criterion is used, most preoptic warm-sensitive neurons (unlike temperature insensitive neurons) can be shown to receive afferent information from skin and spinal thermoreceptors (5). This indicates that the warm-sensitive neurons are capable of integrating central and peripheral thermal information, suggesting a functional role in thermoregulatory responses. Similarly, there are morphological differences between neurons having thermosensitivities greater than 0.8 impulses·s^{-1}·°C^{-1} compared with neurons having lesser thermosensitivities (9). Preoptic temperature-insensitive neurons do not receive peripheral thermal information and often orient their dendrites parallel to the midline third ventricle. In contrast, warm-sensitive neurons tend to orient their dendrites perpendicular to the midline third ventricle, presumably to receive synaptic input from peripheral thermoreceptor pathways that ascend both medially and laterally through the hypothalamus (9). Again, this suggests that that warm-sensitive neurons may integrate central and peripheral thermal information to serve a functional role in thermoregulation.

There continues to be debate over the mechanisms of preoptic neuronal thermosensitivity. Kobayashi and colleagues (11, 14) and Kiyohara et al. (12) suggest that neuronal warm sensitivity is due to heat-induced, inward, cationic currents causing a slow depolarization of a neuron's resting membrane potential. Our own studies, however, indicate that temperature has very little influence on resting potentials, and the small thermal effects on resting membrane potentials are essentially identical for both warm-sensitive and temperature-insensitive neurons (6, 7, 18). Therefore, heat-induced depolarization cannot be the explanation for warm sensitivity.

Warm Sensitivity Is Due To Depolarizing Prepotentials

Our studies indicate that the basis of neuronal thermosensitivity lies in the brief, transient currents that determine the timing between one action potential and the next. As illustrated in Fig. 1, *C* and *D*, the primary distinction between tempera-
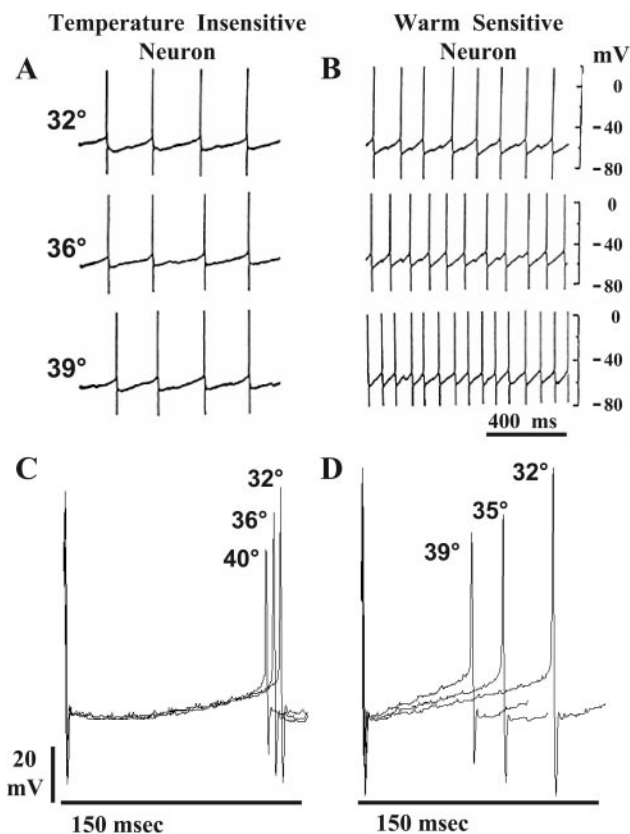
ture-sensitive and -insensitive neurons is the effect of temperature on the pacemaker potential or depolarizing prepotential. These depolarizing prepotentials are not dependent on synaptic inputs and occur in both warm-sensitive neurons and temperature-insensitive neurons. In warm-sensitive neurons, however, temperature strongly affects the prepotential's rate of depolarization. Figure 1D shows that warming increases the depolarizing prepotential's rate of rise. This allows threshold to be reached sooner, causing an increase in firing rate. As illustrated in Fig. 1, it is this prepotential (not the resting membrane potential) that is the primary mechanism of warm sensitivity.

Although various inward cationic currents contribute to the depolarizing prepotential, one study emphasizes the importance of a transient, outward hyperpolarizing K^+ current, i.e., the A-type potassium current (I_A) (8). Unlike temperature-insensitive neurons, warm-sensitive neurons display a gradual decrease in their net ionic conductance as the prepotential depolarizes toward threshold. This suggests that, in warm-sensitive neurons, much of the depolarizing prepotential is due to a gradually decreasing outward potassium current. The I_A is important because temperature strongly affects its inactivation phase (8, 15, 16). Immediately following an action potential, when the membrane begins to depolarize, I_A activates and the outward K^+ current briefly contributes to membrane hyperpolarization. After a short time, however, this outward K^+ current

inactivates, which allows the membrane to depolarize. Warming increases the rate of I_A inactivation, and in turn, this allows the prepotential to depolarize at a faster rate, as shown for 39°C in Fig. 1D. Also shown in Fig. 1D, is that it is important to note that at all three temperatures, the average membrane potential (i.e., the voltage halfway between each interspike interval) remains unchanged. Therefore, the neuron's overall resting membrane potential is not significantly affected by temperature.

No Correlation Between Firing Rate And Membrane Potential Thermosensitivities

Perhaps the best evidence that neuronal thermosensitivity is not due to heat-induced depolarization comes from a recent study comparing the thermosensitivities of resting membrane potentials and resting currents in all types of preoptic neurons (18). As examples, the effects of temperature on firing rate and resting membrane potential are shown in Fig. 2A for a warm-sensitive neuron (1.35 impulses·s^{-1}·°C^{-1}) and in Fig. 2B for a temperature-insensitive neuron (0.14 impulses·s^{-1}·°C^{-1}). Even though these two neurons have widely differing firing rate thermosensitivities, their membrane potential thermosensitivites are identical (i.e., 0.47 mV/°C). For all 117 neurons recorded in this study, Fig. 2C plots the resting membrane potential thermosensitivity as a function of each neuron's firing rate thermosensitivity. This includes 19 warm-sensitive neu-

Fig. 2. Effect of temperature on the firing rate and membrane potential of different types of preoptic neurons. A: warm-sensitive neuron with firing rate thermosensitivity of 1.35 impulses·s^{-1}·°C^{-1} and membrane potential thermosensitivity of 0.47 mV/°C. Warm-sensitive neurons have firing rate thermosensitivities ≥0.8 impulses·s^{-1}·°C^{-1}. B: temperature-insensitive neuron with firing rate thermosensitivity of 0.14 impulses·s^{-1}·°C^{-1} and membrane potential thermosensitivity of 0.47 mV/°C. A and B, left: time records of firing rate (FR) and averaged membrane potential (MP) during changes in tissue slice temperature (°C). Averaged membrane potential was recorded by filtering out rapid changes in membrane potentials, including action potentials. Middle: firing rate is plotted as a function of tissue temperature. Right: membrane potential is plotted as a function of tissue temperature. m, Regression coefficient of FR and MP plots. Temperature had similar effects on the membrane potential thermosensitivity of both neurons. C: relationship between firing rate thermosensitivity and membrane potential thermosensitivity for 117 neurons recorded in rat hypothalamic tissue slices. This includes 15 silent neurons (plotted, left) that did not have spontaneous activity. For the remaining 102 spontaneously firing neurons, the extremely low regression coefficient (m) and correlation coefficient (r) indicate that there is no correlation between neuronal firing rate thermosensitivity and membrane potential thermosensitivity. Vertical dashed line indicates the criterion for neuronal warm-sensitivity, 0.8 impulses·s^{-1}·°C^{-1} (Reproduced with permission from Ref. 18.)
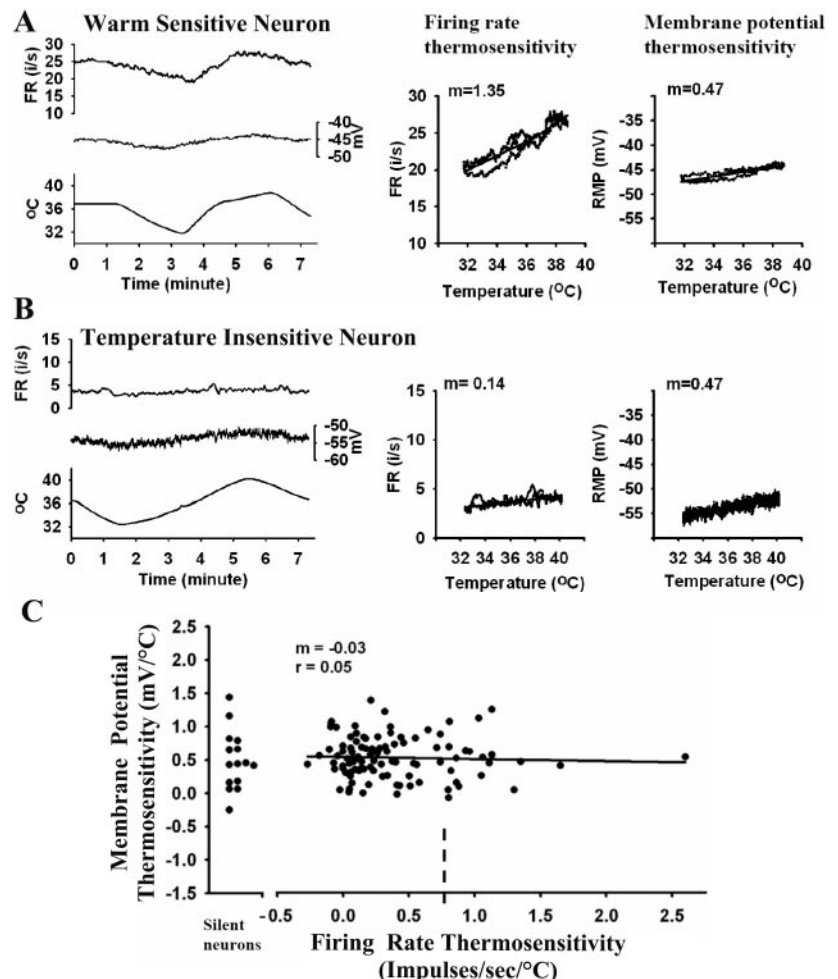
rons, 83 temperature-insensitive neurons, and 15 silent neurons. As indicated by the low regression and correlation coefficients in Fig. 2C, there is no correlation between resting membrane thermosensitivity and firing rate thermosensitivity (i.e., $r = 0.05$). This same study also conducted -92 mV voltage-clamp recordings on these neurons. This was done to maximize inward cationic currents, because other studies suggest that neuronal warm sensitivity is due to persistent, thermally-dependent Na^+ or Ca^{2+} currents (1, 10, 12, 17). Once again, when all of these neuronal current thermosensitivities are plotted as a function of firing rate thermosensitivities, there is no correlation (i.e., $r = 0.01$). These are strong reasons to reject the hypothesis that neuronal warm sensitivity is due to heat-induced depolarization.

Experimentally-Induced Artifact

Why then do some studies report that certain preoptic neurons display heat-induced depolarization? Our previous publications list several possible reasons stemming from experimentally-induced conditions (7, 8, 18). These include grounding problems where the temperature of the indifferent electrode is allowed to change. Also, in some studies (11, 14), the stability of the recording is suspect, particularly when the records are very short (i.e., <2 min) or when the tissue is maintained for long periods at hypothermic temperatures, with brief periods of rapid warming lasting only a minute. Other studies (12) use mechanically- and enzymatically-dissociated neurons from neonatal animals; and because of this, neuronal trauma should be considered a reason for abnormal membrane responses to temperature (reviewed in Ref. 18). Usually, the instability of neuronal recordings is apparent from the amplitude and shape of recorded action potentials and the absence of after-hyperpolarizing potentials following each action potential.

Another problem concerns sample sizes where comparisions are made between a small number of temperature-sensitive and -insensitive neurons. Consider, for example, the data shown in Fig. 2C which plots membrane potential thermosensitivity as a function of firing rate thermosensitivity for 117 preoptic neurons. Because of the variability in membrane potential thermosensitivity, this plot shows that there is no correlation between heat-induced membrane depolarization and firing rate thermosensitivity. On the other hand, within this sample it would certainly be possible to find some warm-sensitive neurons with relatively high membrane potential thermosensitivities and some temperature-insensitive neurons with low membrane potential thermosensitivities.

Finally, an example of experimentally induced artifact comes in studies in which temperature is changed while a neuron is injected with a negative holding current to maintain the membrane potential at a hyperpolarized level. All neurons show thermally induced changes in their input resistance, where warming decreases resistance and cooling increases resistance. By simply applying Ohm's law ($V = IR$), we see that the recorded voltage (V) is equal to the membrane resistance (R) times the current (I). If a study applies constant negative current injection to artificially "hold" a neuron at a hyperpolarized membrane potential, then a warm-induced decrease in resistance will decrease this "experimentally-imposed" hyperpolarization. In other words, during warming the neuron will appear to depolarize, but this is totally artificial.

Our earlier study (7) has shown that if no holding current is applied, temperature has little or no effect on a preoptic neuron's membrane potential. On the other hand, the application of a hyperpolarizing holding current causes a dramatic increase in the same neuron's membrane potential thermosensitivity. These conditions appear to be similar to intracellular recordings conducted by Kobayashi and colleagues (11, 14) in which heat-induced depolarization was observed in some preoptic neurons. One of these studies reported that a hyperpolarizing current was constantly injected to hold the membrane potential at -68 mV (14). Whether negative currents are injected intentionally or unintentionally, these conditions should not be considered physiological and could easily lead to mistaken conclusions regarding the effect of temperature on membrane potentials.

In conclusion, our studies of preoptic neurons find no correlation between the temperature sensitivity of resting membrane potentials and firing rate thermosensitivity. These studies do not support the hypothesis that neuronal warm sensitivity is due to heat-induced membrane depolarization. Instead, the mechanisms for warm sensitivity reside in the brief ionic currents of the prepotential that determines the timing between successive action potentials.

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KOBAYASHI REPLY

Boulant and his colleagues have reported that heating does not elicit depolarization in heating-sensitive and heating-insensitive neurons in anterior hypothalamus (PO/AH) slices (see their Fig. 2*C*, above). This indicates that both neurons (maybe interneurons) do not have channels for heating-induced depolarization, such as transient receptor potential (TRP). If their view is true, their pacemaker prepotential should retain after synaptic blockade. Thus, to explain neuronal thermosensitivity, they must clarify the presence of heating-activated channels to induce prepotential under synaptic blockade.

We analyzed the ionic basis of primary heating-activated neurons under synaptic blockade. In voltage-clamp recordings, heating activated whole cell conductance with nonselective cation channel properties, similar to those of TRP. This conductance would cause heating-induced depolarization to generate impulses (see our Fig. 1). We conclude that heating-induced depolarization explains thermosensitivity of primary heating-activated neurons in PO/AH.

BOULANT REPLY

In refuting Dr. Kobayashi's comments above, I refer the reader to the section *Experimentally-Induced Artifact* in my COUNTERPOINT. This lists conditions producing spurious recordings and is applicable to Dr. Kobayashi's recordings. I would ask the reader to compare the recordings in our two papers. In my paper, Fig. 1 shows action potentials followed by pronounced after-hyperpolarizing potentials (AHPs). In Fig. 1*B*, one can clearly distinguish intrinsic pacemaker activity from occasional postsynaptic potentials (all of which are inhibitory IPSPs). It should be obvious that the neuron's thermosensitivity is due primarily to thermal effects on the rise time of pacemaker potentials. Fig. 2 shows 7-min records of firing rate and membrane potential during temperature changes. Note that temperature is maintained near 37°C, it is then slowly decreased and increased, and finally, it is returned back to 37°C. When firing rate and membrane potential are plotted as a function of temperature, there is little hysteresis. In contrast, Dr. Kobayashi's Fig. 1 shows a 100-s record that does not allow firing rate to be determined. There are no AHPs. The record starts near 32°C, and the tissue is rapidly warmed to 40°C. During the subsequent cooling, the record ends abruptly before the cycle is completed, possibly because of the erratic activity during the last 20 s (as indicated by fluctuating membrane potentials and widely varying action potential amplitudes). Also, during the first 30 s of the record (before any spiking activity) there are large, 10-mV fluctuations in the resting membrane potential. This is odd, because synaptic activity had been eliminated with calcium-free media containing glutamate and GABA antagonists. Dr. Kobayashi's figure is modified from a short paper in *Neuroscience Letters* (his Ref. 6), which contains potential problems. It appears that the tissue was maintained at hypothermic temperatures (32–34°C) with only brief, rapid warming to test for thermosensitivity. These conditions alter neuronal responses to temperature and should not be considered normal. Also, in the Kobayashi figure, note that membrane potential is not in synchrony with temperature. Accordingly, a plot of membrane potential vs. temperature would show considerable hysteresis. In Dr. Kobayashi's paper (his Ref. 6), however, membrane potential was only plotted for increasing (not decreasing) temperature, and thus, the hysteresis was not shown. This is unfortunate since the paper's main point was to show "thermostat" neurons having discrete threshold temperatures. Finally, whether intentionally or unintentionally, it appears that in Dr. Kobayashi's studies, neurons were recorded during hyperpolarizing current injections that "hold" the membrane potential near −67 mV. This explains the lack of AHPs; but more importantly, as described in my COUNTERPOINT, this explains why Dr. Kobayashi's neurons show pronounced depolarization during warming. Again, these are artificial conditions and should not be considered physiological.