Revisiting the role of spike afterhyperpolarization and spike threshold in motoneuron current-frequency gain

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In this study, we explore the inner workings of the input current-firing frequency mechanism in spinal motoneurons of the adult cat in the primary firing range. In this range, the relationship between steady input current levels and firing rate is often characterized as the “F-I gain.” The basic theory behind this F-I gain is based on the summation of spike afterhyperpolarizations and has stood for four decades (Baldissera and Gustafsson 1971; MacGregor and Sharpless 1973). The theory essentially describes a negative feedback loop containing both summation and firing rate, wherein the accumulation of the afterhyperpolarization current acts to delay the arrival of the next spike and thereby lower F-I gain. The inputs to that negative feedback loop consist of the input current itself but also a stereotyped afterhyperpolarization and a constant spike threshold. Thus this theory explicitly makes two key assumptions: 1) afterhyperpolarizations are stereotypic responses to spiking, and 2) spike threshold is constant. Of course, these assumptions are not literally true, as both are known to vary. For example, the afterhyperpolarizations can be altered pharmacologically, and these changes do affect F-I gain (Miles et al. 2007). Likewise, spike threshold variability has also been observed (Pinter et al. 1983). However, the key aspect of those assumptions is not constancy per se but rather, independence from the feedback loop. That is, the assumptions are best articulated as afterhyperpolarization and spike threshold being independent of firing rate. Thus we are particularly interested to know whether there is covariance between these measures and firing rate as input is changed, since this would imply that they are part of the feedback loop and therefore not independent.

We present experimental data that the “stereotyped afterhyperpolarization” assumption appears to hold that afterhyperpolarization is an input to, but not an integral part of, the summation-firing rate control loop. Furthermore, observations indicate that the reason for the afterpolarization being out of the loop is that it is surprisingly insensitive to spike properties. In contrast, we observe that the “constant threshold” assumption cannot be supported, in that observations indicate that the spike threshold covaries with firing rate as input level is changed. Thus we conclude that the spike threshold is “in the loop” in a manner not predicted by the original algebraic summation theory.

The observed linkage between spike threshold and F-I gain requires an extension to the original theory, which we propose as follows: in brief, we contend that the curvature (that is, the second derivative), with voltage activation of persistent sodium currents, forms a spike threshold that shifts with increasing summation of afterhyperpolarizations (due to accumulating potassium conductance). Thus spike threshold both affects firing rate and is affected by summation, making spike threshold an integral component of the F-I gain control mechanism. Indeed, our experimental observations show that this curvature does correlate with F-I gain.

Direct incorporation of persistent inward currents and spike threshold dynamics into the F-I gain mechanism may explain several lines of prior experimental observation. For example, Riluzole, a persistent sodium channel blocker, reduces F-I gain (Kuo et al. 2006). Also, spike threshold variability during fictive locomotion causes changes to firing rates (Fedirchuk and Dai 2004). Additionally, the connection between “subprimary” range firing and spike threshold (Manuel et al. 2009), as well as recent dynamic clamp data (Gabrielaitis et al. 2011), may be due to the proposed linkage. Consequently, we discuss the ramifications of this extended theory to these observations, as well as its potential effects in other neurons and properties.

METHODS

All experiments (n = 9) were performed in accordance with procedures approved by The Georgia Institute of Technology Animal Care Committee and are described below.
Preparation

Data were obtained from nine cats (average weight, ~2.5 kg), using the standard procedures for our lab (Jones and Lee 2006). Briefly, initial surgical preparations were done under deep gaseous anesthesia. In the left hindlimb, the nerves to medial gastrocnemius (MG) and lateral gastrocnemius–soleus (LGS) muscles were carefully isolated and left in continuity. The surgically exposed areas of the hindlimb were covered with a pool of mineral oil, which was formed within the pulled-up skin flaps. Animals were converted to pentobarbital anesthesia with an initial dose of 25–30 mg/kg i.v., diluted 1:9 with saline. The gaseous anesthesia was then discontinued, and the animal was allowed to breathe room air. The preparation was paralyzed with Flaxedil (10 mg initial dose, supplemented at regular intervals with additional doses of 1–2 mg) and artificially respired. Anesthesia level was maintained by supplemental doses of pentobarbital (5 mg/kg) and monitored via changes in blood pressure and respiratory rate. Radiant heat was used to maintain hindlimb and core temperatures within physiological limits. At the end of the experiment, the animals were killed with a lethal dose (100 mgl/kg i.v.) of pentobarbital.

Intracellular recording

Intracellular recordings of motoneurons, antidromically activated by stimulation of the MG and LGS nerves, were obtained in the lumbar cord with sharp microelectrodes, and microelectrode tips were broken back under microscopic observation and control. Because of the large currents required for successful single electrode voltage clamp protocols, the electrodes were filled with a solution combining potassium citrate (1.5 M) and potassium chloride (1.5 M). All currents were read out with Flaxedil (10 mg initial dose, supplemented at regular intervals with additional doses of 1–2 mg) and artificially respired. Anesthesia level was maintained by supplemental doses of pentobarbital (5 mg/kg) and monitored via changes in blood pressure and withdraw reflexes (evaluated in periods where the preparation was allowed to recover from paralysis). In addition, a bilateral pneumothorax was done to enhance intracellular recording stability. End tidal CO2 was monitored and kept within acceptable limits by adjusting respiration rate and volume. Radiant heat was used to maintain hindlimb and core temperatures within physiological limits. At the end of the experiment, the animals were killed with a lethal dose (100 mgl/kg i.v.) of pentobarbital.

Assessment protocols. Two protocols were used to capture firing and current-related properties (Fig. 1). Firing properties were assessed by application of a slowly ascending (5 s) injected current ramp during discontinuous current clamp (Fig. 1A). Ramp magnitudes varied between 20 and 50 nA, averaging ~30 nA. Current-related properties were assessed by application of a slowly ascending (5 s) voltage ramp during voltage clamp (Fig. 1B). Ramp magnitudes were 30–40 mV. Superimposed on the voltage ramp command was a small amplitude (0.25 mV) sinusoid with a frequency of 125 Hz, with which the admittance at 125 Hz could be determined as a function of the voltage swept by the ramp (see below and Mitchell and Lee 2011 for details of this protocol).

Data analysis

Data acceptance criteria. The main acceptance criteria were that the amplitude of the antidromic spike exceeded 70 mV, and the resting membrane potential did not vary by more than ±5 mV during the course of the data collection.

Firing-related measures. The firing properties were assessed by tracking spike threshold, spike peak overshoot, interspike interval (ISI) minimum, and the timing of all of these measures over the duration of the rising phase of the injected current ramp protocol. The exact definitions for these assessments, as well as metrics derived from these data, are described below.

Spike threshold was determined by the location of the first peak of the second derivative of the membrane voltage, just preceding a spike. This method tends to pick threshold just at or slightly prior to the abrupt upswing. It results in a much more stable measure of voltage threshold than rate of rise criteria (e.g., 50 mV/ms), since by definition, voltage is highly variable at this point. Figure 2 shows examples of the threshold chosen (see Sekerli et al. 2004 for details of spike threshold evaluation). Spike peak overshoot was determined by a second order curve regression of the five data points surrounding the peak data point (i.e., two points before and two after). The spike peak overshoot was then the peak of the regression curve. The purpose of curve fitting the spike peak was to minimize measurement jitter associated with the timing of data acquisition vs. the timing of the spike peak. ISI minimum was determined by finding the recorded minimum between consecutive spike thresholds. Instantaneous firing rate is the reciprocal of the time between consecutive spike peaks.

Note that “AHP” is often used to denote afterhyperpolarizations. However, AHP was originally defined as the afterhyperpolarization following a singly elicited spike, whereas ISI was used to designate afterhyperpolarizations elicited during rhythmic firing. Although this distinction is often not necessary, it is important in this context, so we will use AHP and ISI and use “afterhyperpolarization” to refer collectively to AHP/ISI.

Fig. 1. Example current and voltage ramp protocols. A: current ramp during current clamp. Example ramp command is 20 nA over 5 s (top trace) and produces repetitive firing (bottom trace). B: voltage ramp during voltage clamp. Example ramp command is 30 mV over 5 s (top trace) and produces a current (bottom trace).
A second layer of derived metrics was based on relating all of the above to input level (Fig. 3). All of these metrics are defined as the slope of the base metric vs. input and are denoted with “gain”. Thus the slope of the relationship between firing rate and input is the F-I gain, and the slope of voltage threshold with respect to input is the “threshold-I gain” and so on. As has long been observed, the F-I gain is not constant with respect to input (e.g., Bennett et al. 2001). Likewise, the various slopes measured here are no different. Consequently, all slopes were assessed over the primary firing range only. The primary range was determined by manual, visual inspection of all of the above relationships but with emphasis on the firing rate-to-input relationship. The first one or two ISIs often have a notably steeper F-I relationship. Subsequently, there is a relatively linear region, followed at some point by an increase in the F-I slope. The primary range was therefore manually defined as starting at the first spike of the linear region and ending at the last spike of that region. Attention was paid to the other relationships to ensure that no abrupt changes in their slopes occurred during this range. In these few cases, truncating the primary range a few spikes sooner resolved the issue without a significant effect on the F-I gain measure.

Current-related measures. Current-voltage (I-V) properties were assessed over the duration of the rising phase of the voltage ramp protocol. The exact definitions for these assessments, as well as metrics derived from these data, are described below.

As in our previous work, we assess the effective negative conductance associated with persistent sodium by assessing admittance during our voltage ramp command (Fig. 4) (Lee and Heckman 2001; Mitchell and Lee 2011). Based on our prior analysis (Mitchell and Lee 2011), we use 125 Hz as a reasonable a priori estimate of the optimal sinusoid frequency for assessing the fast inward currents responsible for the instability and use the method for extracting constant frequency information, which was described therein. Briefly, the “Fast I-V” relationship was obtained by low-pass filtering the voltage clamp ramp data. The fast inward current was extracted by converting the real portion of the response to the sinusoid into an effective conductance (Fig. 4A) and integrating the result to produce a current (Fig. 4B), which represents only fast, active conductances that can participate in spike initiation and the leak. Thus the activation of slow currents, such as that produced by calcium-dependent potassium channels, was excluded. As we have shown previously, this Fast I-V curve exhibits an instability (i.e., a negative slope region), as described by Koch et al. (1995). This negative slope region in motoneurons is capable of sustaining repetitive firing, and the onset of this region corresponds to spike threshold (Lee and Heckman 2001).

Based on the generated Fast I-V curve, several metrics were defined to characterize the effective negative conductance. The curves were assessed at three points: onset of the zero slope, slope at onset + 4 mV, and the point of the most negative slope. In our prior work, we have shown that the zero-slope point (VOnset) is related to spike threshold; thus we refer to this point at the Fast I-V threshold. We use the onset + 4 mV point as the measure of the negative slope, GFast. We assess curvature, dGFast/dV, across all three points. Finally, whole cell input (or leak) conductance was assessed from the slope of the voltage-current relationship regressed over a 5-mV range, just below the resting membrane voltage (i.e., the point of zero current).
The goal of the work presented here is to gain a greater understanding of how F-I gain in motoneurons is regulated. We start with an experimental examination of the afterhyperpolarization following spikes (Fig. 5). The original theory assumes that the afterhyperpolarization is constant. Thus we would predict that any measured changes to the afterhyperpolarization would not covary with firing rate as input level changed. The most direct and independent measure of changes to afterhyperpolarization during rhythmic firing is changes to ISI minimum voltage (i.e., the bottom of the ISI). As predicted, the slope of the ISI minimum voltage appears to have no relation whatsoever with F-I gain (Fig. 5A; \( n = 71; r = 0.05; P > 0.5 \)). Taken together, with the prior experimental evidence, we contend that these data show that the afterhyperpolarization is an input to firing but not actually part of any loop regulating firing rate (see DISCUSSION).

In contrast to the lack of relation among spiking, ISIs, and F-I gain, spike threshold appears to be linked to F-I gain (Fig. 6). As has been observed previously, spike threshold often increases with increasing firing rate/input level. We show explicitly here that this increase negatively correlates with F-I gain itself (\( n = 71; r = -0.42; P = 0.002 \)). Thus the original theory assumption regarding spike threshold is called into question. However, it is difficult to overturn such a long-standing assumption variation of these measures, due to changes in firing rate/input level, result in a significant correlation. A clear example of this is the lack of a correlation between the changes in the afterhyperpolarization and changes in spike overshoot as input level is increased (Fig. 5C; \( n = 71; r = 0.05; P > 0.5 \)).

Fig. 5. Input current-frequency modulation (F-I) gain vs. afterhyperpolarization-related measures. A: F-I gain vs. ISI minimum voltage gain. F-I gain appears to be independent of ISI minimum voltage. B: F-I gain vs. ISI current/conductance gain. Gain (Gn) × ISI minimum voltage is the key calculation to convert to both current and conductance. Again, F-I gain appears to be independent. C: ISI minimum voltage gain vs. spike overshoot gain. The apparent independence of ISI minimum on spike overshoot may explain the observations in A and B.
without some explanation. Thus we now turn our attention to
expanding the original theory.

We have previously examined spike threshold in relation to
fast inward currents, assessed during I-V relationships (Lee
and Heckman 2001; Mitchell and Lee 2011). We contend that
this spike threshold-generating mechanism also affects F-I gain
(Fig. 7). Specifically, we can see that the voltage at the onset of
the negative slope correlates with spike threshold (Fig. 7A; n =
57; r = 0.79; P < 0.0001). Additionally, F-I gain is correlated
significantly with GFast (Fig. 7B; n = 58; r = 0.51; P <
0.0001), as well as dGFast/dV (Fig. 7C; n = 61; r = −0.39;
P = 0.002). Notably, it is not correlated with the inward
current itself (Fig. 7D). These observations are collectively
used to develop an expanded theory of F-I gain (see DISCUSS-
ION).

DISCUSSION

In the work presented here, we observe that F-I gain
negatively correlates with changes in spike threshold. That
is, cells with thresholds that have a greater tendency to
depolarize with increasing firing rate/input level also tend to
have lower F-I gains. This observation undermines one of
the two key assumptions in the original F-I gain theory
(Baldissera and Gustafsson 1971; MacGregor and Sharpless
1973). Furthermore, our observations indicate that persistent
sodium currents, in conjunction with input conductance,
play a crucial role in spike threshold and F-I gain. However,
we also observe that the second assumption—namely, that
spike afterhyperpolarizations are stereotyped and so are not
affected by firing rate/input level—is supportable, likely
because the afterhyperpolarizations are not sensitive to
spike properties. The ramifications of these observations are
multifaceted and are discussed below.

First off, these findings require that our understanding of
how motoneurons and possibly how all neurons generate a
F-I gain be revised. The central question is: How can the
onset of a negative slope in the I-V relationship mechanis-
tically affect F-I gain? The curvature of the Fast I-V rela-
tionship from a positive into a negative slope indicates the
presence of a dynamic instability in membrane voltage,
which reveals itself as a spike, the threshold of which
corresponds to the VOnset in the Fast I-V curve. Triggering
an afterhyperpolarization effectively adds conductance to
the cell and therefore, alters the Fast I-V relationship. Given
the curve from positive to negative slope, adding conduc-
tance will shift the VOnset in a depolarizing direction. Thus
we suggest that spike threshold traverses a path that mirrors
the afterhyperpolarization (Fig. 8A). This suggestion ap-
pears to be in conflict with experimental observations of
spike threshold during the afterhyperpolarization (Powers
and Binder 1996). However, these observations were made
by injecting current pulses during the afterhyperpolarization

Fig. 6. F-I gain vs. spike threshold gain. In contrast to the lack of relation
between ISI-related measures and F-I gain, spike threshold gain is correlated
(n = 71; r = −0.42; P = 0.002).

REVISITING F-I GAIN MODULATION
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With the above threshold shifting during the afterhyperpolarization in view, it is easy to see how spike threshold could shift with increasing firing rate. Higher input levels bring membrane voltage to threshold sooner in the afterhyperpolarization when the threshold is higher but also later than it would have been if threshold had been constant (Fig. 8B). Thus spike threshold both affects and is affected by firing rate and is therefore in the F-I gain control loop.

To summarize how these ideas and our experimental observations affect the F-I gain theory, we have illustrated the concepts diagrammatically (Fig. 9). The original theory simply included the outer loop, which incorporated only summation and firing rate. Based on the instability introduced by the curvature in the Fast I-V relation, we have introduced an inner loop, capable of relating either or both spike threshold and afterhyperpolarization to firing rate. Our observations indicate that the insensitivity of the afterhyperpolarization to firing rate effectively breaks that loop pathway in motoneurons. However, our observations support the loop pathway that includes spike threshold.

It is worth noting that the spike threshold loop has a net negative sign of effect. Thus it is a negative feedback loop, just like the outer original theory loop was. This extension to the theory also implies that the shifting spike threshold helps to reduce overall gain and to stabilize firing, making the cell input-output relationship more robust across a larger input range. In contrast, if the afterhyperpolarization loop were not suppressed, it would have a positive net sign of effect. Thus it would reduce robustness and operating range, which may explain why it is suppressed.

It is also worth noting that this theory directly explains experimental results, indicating that drugs like Riluzole, which selectively block persistent sodium currents, reduce F-I gain (Kuo et al. 2006).

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GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: R.H.L. conception and design of research; R.H.L. performed experiments; R.H.L. analyzed data; R.H.L. and C.S.M. interpreted results of experiments; R.H.L. prepared figures; R.H.L. and C.S.M. drafted manuscript; R.H.L. and C.S.M. edited and revised manuscript; R.H.L. approved final version of manuscript.

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