# Sub-optimal Discontinuous Current-Clamp switching rates lead to deceptive mouse neuronal firing

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**Abstract** Intracellular recordings using sharp microelectrodes often rely on a technique called Discontinuous Current-Clamp to accurately record the membrane potential while injecting current through the same microelectrode. It is well known that a poor choice of DCC switching rate can lead to under- or over-estimation of the cell potential, however, its effect on the cell firing is rarely discussed. Here, we show that sub-optimal switching rates lead to an overestimation of the cell excitability. We performed intracellular recordings of mouse spinal motoneurons, and recorded their firing in response to pulses and ramps of current in bridge and DCC mode at various switching rates. We demonstrate that using an incorrect (too low) DCC frequency lead not only to an overestimation of the cell conductance, but also, paradoxically, to an artificial overestimation the firing of these cells: neurons fire at lower current, and at higher frequencies than at higher DCC rates, or than the same neuron recorded in Bridge mode. These effects are dependent on the membrane time constant of the recorded cell, and special care needs to be taken in large cells with very short time constants. Our work highlights the importance of choosing an appropriate DCC switching rate to obtain not only accurate membrane potential readings, but also accurate representation of the firing of the cell.

## Keywords

Techniques in Neuroscience, intracellular recording, sharp electrodes, neuronal excitability, firing frequency

# 1 Introduction

Neurons, by virtue of their plasma membrane and the numerous ion channels that can be found therein, behave to a first approximation like RC circuits. Consequently, a stationary electrical (ionic) current flowing through the membrane causes a change of voltage proportional to the resistance of the cell. This is Ohm's law: $V = I \times R$, where V is sometimes called voltage drop or IR drop. When performing intracellular recordings with microelectrodes, or whole cell recordings using patch electrodes, electrophysiologists can pass current through their electrode ("current clamp") to change the membrane potential of the cell and thereby study its excitability. However, the electrode itself, because of its very small tip, acts as an additional RC circuit, and therefore also experiences an IR drop when current is applied. In these conditions, it is essential to be able to separate the physiological response of the cell from a change of voltage caused by the resistance of the very electrode used to perform the recording. Two main techniques have been developed over the years to overcome this problem. The first one, the so-called "bridge" mode, consists (broadly speaking) in subtracting the voltage drop caused by the current injection through a variable resistor set to a value close to the estimated electrode resistance from the voltage measured by the electrode. This technique works well if the resistance of the electrode can be assumed to be constant over a large range of current intensity. Unfortunately, that is often not the case, particularly with small intracellular microelectrodes, which can exhibit strong non-linearities. A second technique was invented in the early 1970s, which consists in injecting current and measuring the potential at separate times, hence the name "discontinuous current clamp" (DCC) (Brennecke and Lindemann, 1971). Instead of injecting a continuous current, the amplifier will alternate at a high frequency between injecting a pulse of current (scaled appropriately so as to conserve the same charge transfer) for a very short duration (classically 1/3 of the DCC period), while no current is injected for the

remainder of the DCC period. The membrane potential is sampled at the end of the period, when no current is injected through the microelectrode. If the time constant of the electrode is fast enough compared to the time constant of the membrane, then the IR drop through the electrode has had time to vanish when the potential is sampled, while the IR drop through the membrane would have barely decayed. In theory, these two recording modes (bridge and DCC) should yield the same values of membrane potential, as long as they are used in the proper conditions. One important aspect parameter is the DCC switching rate, which needs to be high enough so that the membrane time constant can smooth out the short pulses of current, but not so high as to prevent the IR drop through the electrode to vanish before the end of the sampling period. An incorrectly set DCC rate should, in theory, only lead to under- or over-estimating the membrane potential. Yet, a recent study (Jensen et al., 2020) illustrates that the firing behaviour of a spinal motoneuron in response to a triangular ramp of current can change drastically depending on the DCC switching rate set by the experimenters, suggesting that the choice of the DCC switching rate is a critical parameter to take into consideration not only in order to obtain accurate readings of the membrane potential, but also when studying the firing rates of the cell. In this paper, We demonstrate that using an incorrect (too low) DCC frequency lead not only to an overestimation of the cell conductance, but also, paradoxically, to an artificial overestimation the firing of these cells: neurons fire at lower current, and at higher frequencies than at higher DCC rates, or than the same neuron recorded in Bridge mode.

# 2 Results

## 2.1 Case study

Let us start by observing the effect of changing the DCC rate on the response of a motoneuron to a triangular ramp of current. When recorded with a DCC rate of

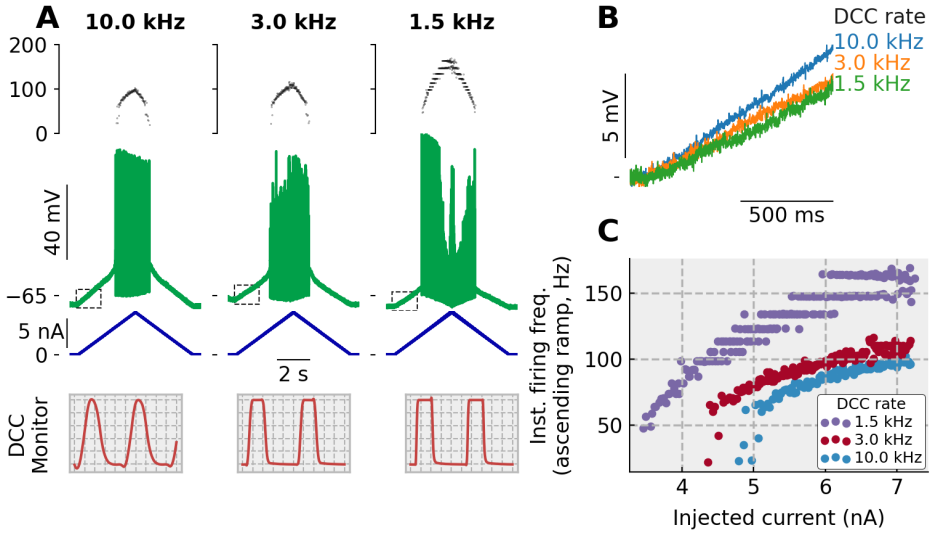


**Figure 1. Typical example showing how DCC rates alter the response of a motoneuron to a stationary input. A.** Response of a triceps Surae motoneuron ($R_{in}$=3.8 MΩ; $\tau_m$=3.3 ms) to a slow (1 nA/s) triangular ramp of current, recorded in DCC mode with switching rates 10, 3 and 1.5 kHz. Bottom traces: injected current. Middle traces: voltage response. Top traces: instantaneous firing frequency. The boxes on the bottom represent the monitoring traces used to check the settling of the electrode, recorded at the top of the ramp. Time bases from left to right: 20 µs, 67 µs, and 133 µs per division. **B.** Expansions of the regions delimited with the dashed box in A. C. F-I curves showing the instantaneous firing frequency plotted against the injected current at the time of each spike.

10 kHz, the motoneuron depolarized progressively until it started to fire repetitively (Figure 1A). The initial firing was irregular and accelerated very steeply over the first few spikes. Then, the firing became more regular and increased approximately linearly with the injected current (Figure 1C). This is the classical response of mouse spinal motoneurons to this kind of current injected: a brief sub-primary range, followed by a linear primary range (Manuel et al., 2009; Iglesias et al., 2011). When recorded with a DCC rate of 3 kHz, the response was similar, but quantitative differences were visible. First, the rate of rise of the membrane potential before the onset of firing was slower than at 10 kHz (Figure 1B). Since the current increases linearly, the rate of rise of the membrane potential is directly proportional to the resistance of the cell. At 3 kHz, the cell thus appears to have a higher conductance than at 10 kHz. Paradoxically, even though the conductance was seemingly increased, the cell started firing at a lower current intensity, and at higher frequencies than at

10 kHz (Figure 1C). These effects were even more pronounced at lower DCC rates. At 1.5 kHz, the apparent conductance was larger than at 10 and 3 kHz (lower rate of rise of the potential, Figure 1B) and the firing started even sooner (Figure 1C). In addition, at 1.5 kHz, the firing frequency increased very steeply with the injected current and reached much higher values (> 100 Hz) than with higher DCC rates. Moreover, when the firing frequency increased beyond 100 Hz, the firing acquired a very distinctive step-like pattern, where the firing frequency had a tendency to oscillate back and forth between two discrete values (Figure 1C).

## 2.2 DCC switching rate affects the apparent cell conductance

The first effect outlined above, namely the increase in cell conductance at low DCC rate, is fairly straightforward to explain. By design, in DCC mode, the amplifier injects a short pulse of current, then stops the injection to allow the voltage drop through the electrode to vanish before the membrane potential is sampled. However, during that time of no current injection, the membrane potential will also decay. The technique only works if the electrode time constant (adjusted to be as fast as possible using the capacitance compensation circuit of the amplifier) is much faster than the membrane time constant. In these conditions, the DCC frequency can be set high enough that the membrane potential has barely decayed by the time the voltage is sampled, and the membrane potential recorded in DCC mode is very close to the membrane potential that would be recorded with a perfectly balanced bridge (Figure 2B). If the DCC rate is too low, however, then the membrane potential has time to decay in between the end of the current pulse and the sampling time (Figure 2A). Consequently, the membrane potential recorded in DCC mode is lower than it would be when recorded in bridge mode. Since the current intensity applied by the amplifier is the same in all cases, an underestimation of the membrane potential produces an apparent increase of the cell conductance at low DCC rates. Conversely, if the DCC rate is too high, then the IR drop through the electrode does

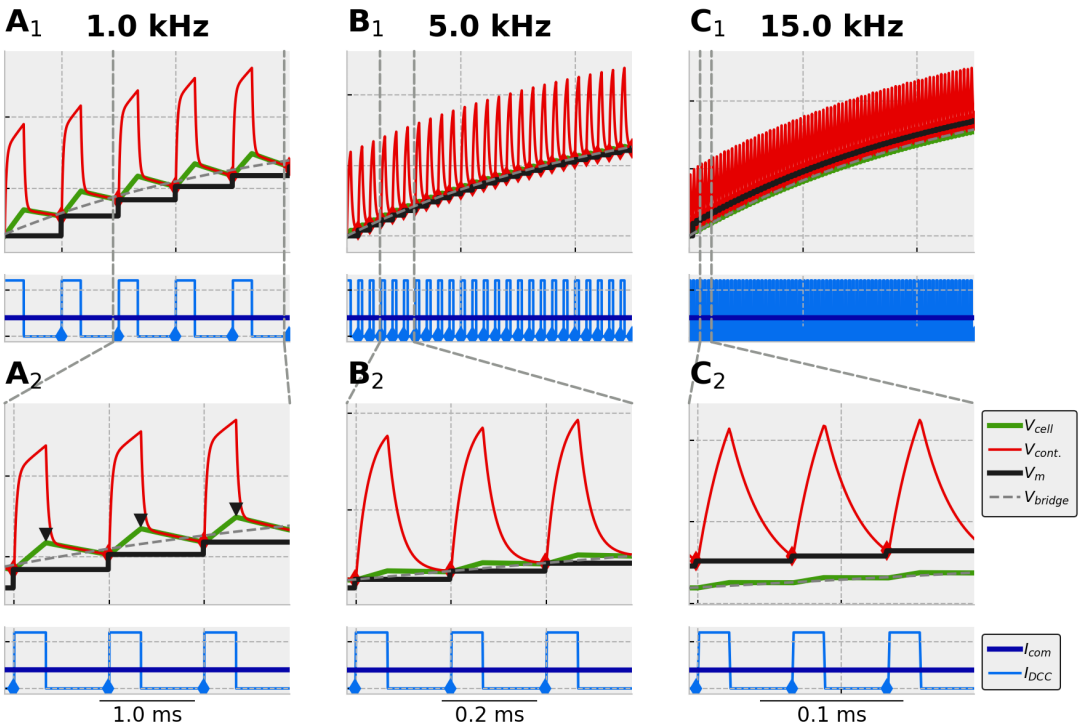


**Figure 2. Effect of DCC rate on the recording of the membrane potential.** Numerical simulations showing the response of a neuron to a stationary current. Top panels: voltage traces. $V_{bridge}$: response of the cells to the continuous current as would be observed in an ideal situation where the electrode resistance was perfectly compensated for by the bridge circuit. $V_{cont.}$: continuous voltage recorded at the tip of the electrode that includes the voltage drop through the electrode and the cell membrane. $V_m$: output of the amplifier, which is the value of $V_{cont.}$ sampled at the end of each DCC period (diamonds) and stored in a sample-and-hold circuit. $V_{cell}$: calculated membrane potential excluding the contribution of the electrode resistance. Bottom panels: current traces. $I_{com}$: stationary current that the experimenter is imposing to the cell. $I_{DCC}$: actual current injected in the cell. That current is 3× the amplitude of $I_{com}$, but injected for only 1/3 of the time. The simulated cell had a resistance of 5 MΩ and a time constant of 5 ms. The electrode had a resistance of 1 MΩ and an effective time constant 200× faster than the membrane time constant. **A.** recording with a DCC rate of 1 kHz (5 cycles per time constant). The arrowheads point to sharp oscillations in the membrane potential. **B.** recording with a DCC rate of 5 kHz (25 cycles per time constant). **C.** recording with a DCC rate of 15 kHz (75 cycles per time constant).

not have time to vanish by the time the potential is sampled (Figure 2C). Therefore the value of the membrane potential of the cell is contaminated by a fraction of the IR drop through the electrode, leading to an overestimate of the potential, and thus an apparent decrease in cell conductance (Figure 2C).

## 2.3 Low DCC switching rates can drive firing

The effect of the DCC on the F-I curves is more subtle. Although the amount of charge transferred to the cell is the same in DCC and in Bridge, the frequency content of the input is not the same. By chopping the current injection in short pulses, the DCC introduces harmonics of the DCC frequency in the input signal (Brette and Destexhe, 2012). This is particularly problematic at low DCC rates, where the membrane potential has time to increase during the pulse injection and then has time to decay substantially in between each current injection, creating oscillations in the membrane potential (Figure 2B, arrowheads). Note that although these oscillations are present in the membrane potential, they are hidden to the experimenter by the DCC sample-and-hold circuit, which samples the potential at the end of the DCC period and holds the amplifier output constant at that value until the next sampling time. A recording with a low DCC frequency becomes equivalent to injecting a series of short pulses of current. This kind of stimulus is highly efficient to trigger motoneuron firing, much more than a continuous current injection (Delestrée et al., 2014; Martínez-Silva et al., 2018). Figure 3A shows the response of a motoneuron to the same 200 ms-long 4 nA pulse of current, recorded in DCC mode with a rate of 1.5 kHz and 8 kHz. With a DCC at 8 kHz, this pulse of current was not able to reach firing threshold (Figure 3A1). At lower DCC rate, however, although the amount of current injected is the same, the motoneuron responded with a strong repetitive discharge (Figure 3B1). Interestingly, the voltage threshold for the first spike was below the steady-stage potential reached with a DCC of 8 kHz (compare dashed lines in Figure 3B2 and A2), suggesting that the appearance of firing at 1.5 kHz was not due to a larger depolarisation (in fact, the depolarisation is smaller, see above), but rather due to the strong sensibility of the cell to transient currents and oscillations in their membrane potential. In the extreme case, a low DCC rate can turn a motoneuron that was not able to fire repetitively in response to a stationary

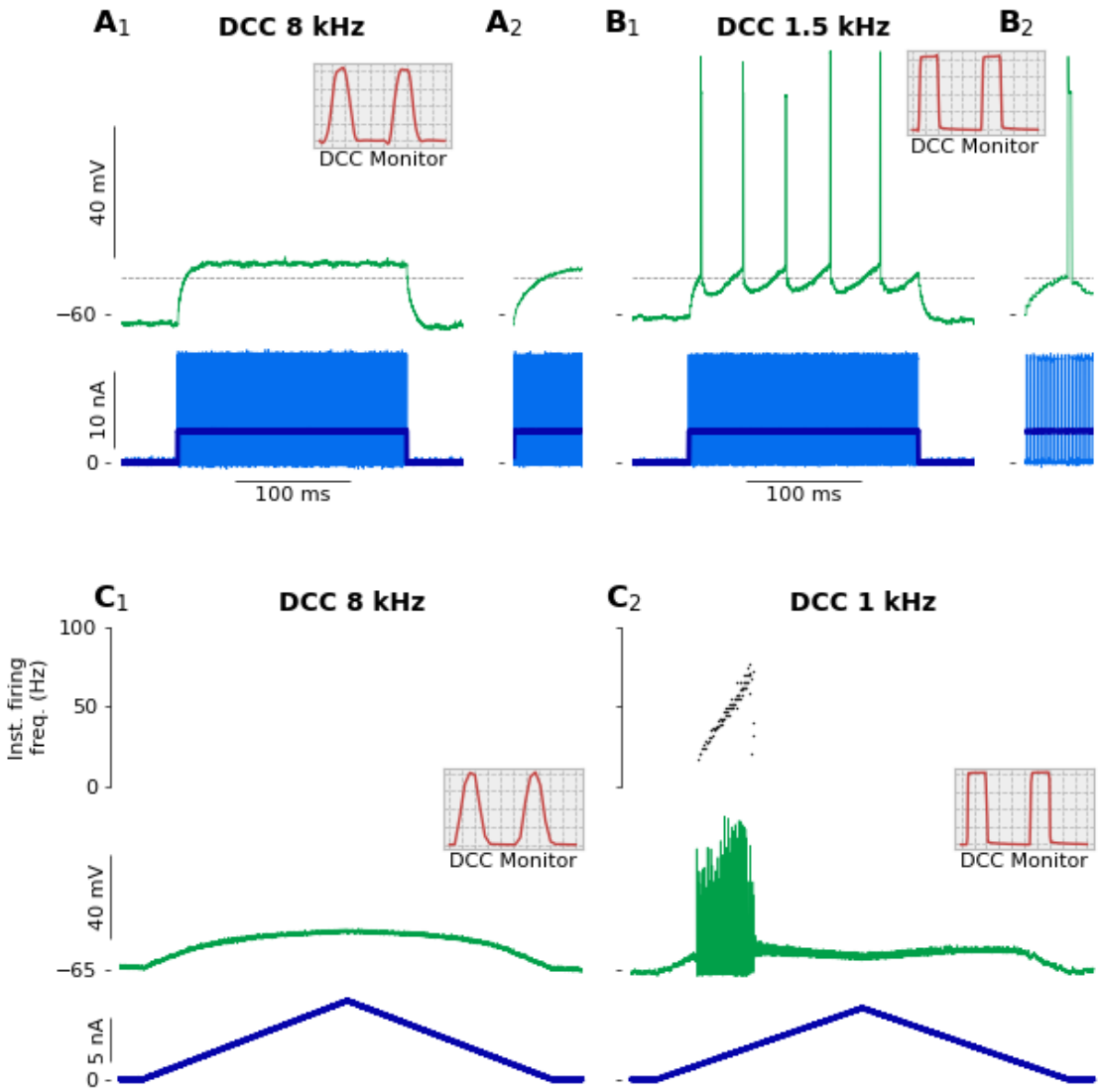


**Figure 3. Spurious firing elicited by low DCC rates. A–B.** Recording from a Triceps Surae motoneuron ($R_{in}$=3.5 MΩ; $\tau_m$=4.9 ms) following the injection of a 200 ms-long 4 nA pulse of current. A. Response recorded with a DCC rate of 8 kHz. The inset in A2 is a zoom over the first 15 ms following the onset of the pulse. B. Response recorded with a DCC rate of 1.5 kHz. The inset in B2 is a zoom over the first 15 ms following the onset of the pulse. The horizontal dashed line represents the voltage threshold measured at the foot of the first spike of the response in B. The grey boxes in A and B represent the monitoring traces used to check the settling of the electrode, recorded at the top of the ramp. Time bases from left to right: 25 μs (C1), and 133 μs (C2) per division. **C.** Response of a Triceps Surae motoneuron ($R_{in}$=5.0 MΩ; $\tau_m$=4.7 ms) to the injection of a triangular ramp of current (1 nA/s) with a DCC rate of 8 kHz (C1) or 1 kHz (C2). The bottom trace is the injected current, the middle trace is the membrane potential and the top graph is the instantaneous firing frequency. The inserts represent the monitoring traces used to check the settling of the electrode, recorded at the top of the ramp. Time bases from left to right: 25 μs (C1), and 200 μs (C2) per division.

input (Figure 3C1) into a motoneuron that elicits a bout of repetitive firing to the same ramp (Figure 3C2). Observation of the DCC monitoring trace on the oscilloscope (inserts in Figure 3C) confirms that the inability to fire repetitively was not due to the electrode becoming blocked. The IR drop through the electrode had fully vanished by the end of the DCC period, and the membrane potential was therefore accurately measured. With a DCC at 1 kHz, however, the DCC period was so long that not only the IR drop through the electrode had time to settle to zero, the membrane potential also rose and decayed during each DCC period. The net effect is a series of large amplitude membrane potential oscillations (which are hidden by the sample-and-hold circuit of the amplifier), superimposed to the slow depolarization of the quasi-stationary ramp. The spiking observed in these conditions is caused by this mixed dynamic and stationary input, rather than a response to the stationary input alone.

As hinted above (Figure 1), this effect can profoundly affect the shape of the frequency-current relationship of a motoneuron in response to a triangular ramp of current, but which F-I curve is the most physiological? Figure 4 illustrates the response of a motoneuron to a series of ramps of current recorded in DCC mode at various frequencies. Because this motoneuron had a fairly low rheobase and did not require a lot of current to fire, we were able to record the response in Bridge mode as well. That response is free of artefacts due to DCC switching, and we will therefore use it as the control firing for this cell. As can be seen, the response recorded in DCC mode at 8 kHz is almost indistinguishable from the one recorded in Bridge mode (Figure 4B). However, when the DCC frequency is too low, the curve is shifted to the left (lower recruitment current), the slope is steeper, and a distinctive “step” pattern appears on the instantaneous frequency.

This effect is seen consistently across motoneurons, but, because the minimum DCC switching rate is dependent on the membrane time constant of the cell, the F-I curves start to diverge at different switching rates. Figure 5A–D shows how the current in-

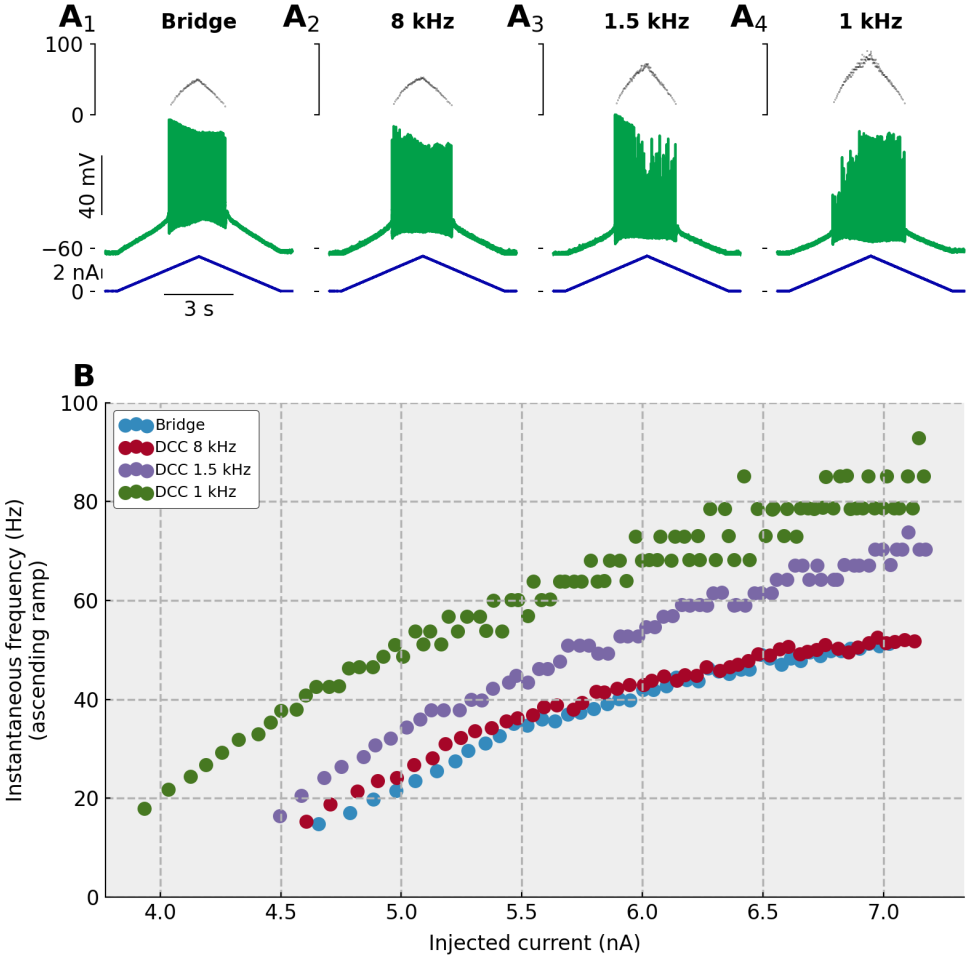


**Figure 4. Effect of DCC switching rate on the response to a ramp of current. A.** Same motoneuron as Figure 3A–B, injected with a triangular ramp of current (2 nA/s) and recorded either in Bridge mode (A1), or in DCC mode at various switching rates (A2: 8 kHz, A3: 1.5 kHz, A4:1 kHz). **B.** The instantaneous firing frequency on the ascending ramp is plotted against the current intensity.

tensity required to start firing (onset current), the current intensity when the cell stopped firing (offset current), the slope of the ascending phase of the F-I relationship (F-I gain), and the voltage threshold measured on the first spike of the ramp vary with DCC frequency. It is clearly apparent that values measured at low DCC rates are usually very different than the ones measured at higher rates, and that the values tend to converge to a stable value when the DCC rate is increased past

a critical point. Moreover, in the one motoneuron where we were able to record the response in Bridge mode (Figure 4), the values measured with the highest DCC rates agree well with the values recorded in bridge mode (green diamond), with the exception of the voltage threshold, which cannot be measured accurately in bridge mode since one cannot completely get rid of the IR drop through the electrode in this mode. Nevertheless, the plateau value is reached for different DCC rates in each motoneuron. We therefore normalized the DCC rate in each cell by the membrane time constant (number of DCC periods per time constant), as well as the measured value to the value estimated at the highest DCC rate (Figure 5E–F). These curves clearly show that all measurements converge towards the same value as the DCC switching rate is increased. However, these curves demonstrate that the rate of 10 cycles per time constant recommended in the Axoclamp manual is not high enough. Rates of at least 15–20 cycles per time constant are necessary to get good estimates of the value of most of these measurements.

## 2.4 Low DCC rate drive firing at discrete intervals

As shown above, using a low DCC switching rate not only leads to cells firing at lower current, but also at higher frequencies. For instance, in the cell exemplified in Figure 6, lowering the DCC rate from 8 to 3 kHz led to both a leftward and upward shift of the F-I curve. Reducing it further to 1 kHz led to the appearance of marked "plateaus" in the instantaneous firing frequency. These plateaus appear at subharmonics of the DCC rate (fundamental frequency), suggesting that they result from the sharp transient currents generated by the DCC mode and the subsequent oscillations in membrane potential phased-locked to the DCC frequency. The cell no longer fires at its natural interspike interval, but instead is driven to fire on the crest of one of these oscillations when the after-hyperpolarization from the preceding spike has relaxed sufficiently for the membrane potential to come close to the voltage threshold. The plateaus are characteristic of recordings with low DCC rates. They are never

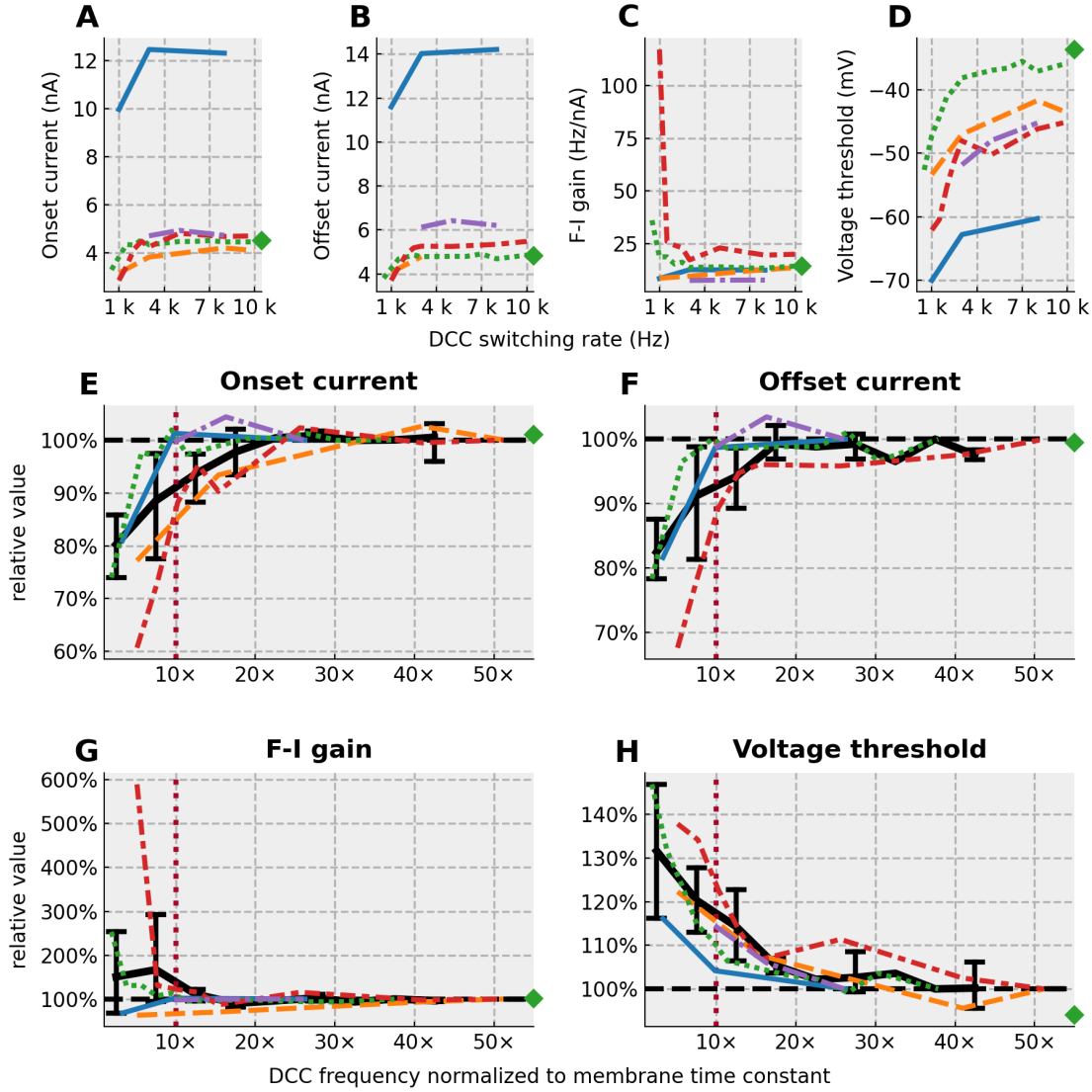


**Figure 5. Relationship between parameters measured on the F-I curves and the DCC frequency used during the recording.** In all panels, each line represents one motoneuron. The green dotted lines correspond to the motoneuron shown in Figure 4, and the parameters extracted from the trace recorded in bridge mode are represented by a green diamond on the right side of each plot. Onset current: current required to elicit the first spike on the ascending ramp. Offset current: current at which the firing stops on the descending ramp. F-I gain: slope of the F-I relationship measured on the ascending part of the ramp. Voltage threshold: voltage measured at the foot of the first spike elicited on the ascending ramp. **A–D.** Absolute value of each of the parameters plotted against the DCC rate used during the recording. **E–F.** Value of each of the parameters normalized to the value measured at the highest DCC rate achieved in each motoneuron (dashed horizontal line) plotted against the DCC rate normalized by the time constant of each motoneuron. The thick black trace represents the average values across motoneurons with error bars representing average±SD. The vertical dotted line shows the recommended minimal DCC rate of 10 cycles per membrane time constant.

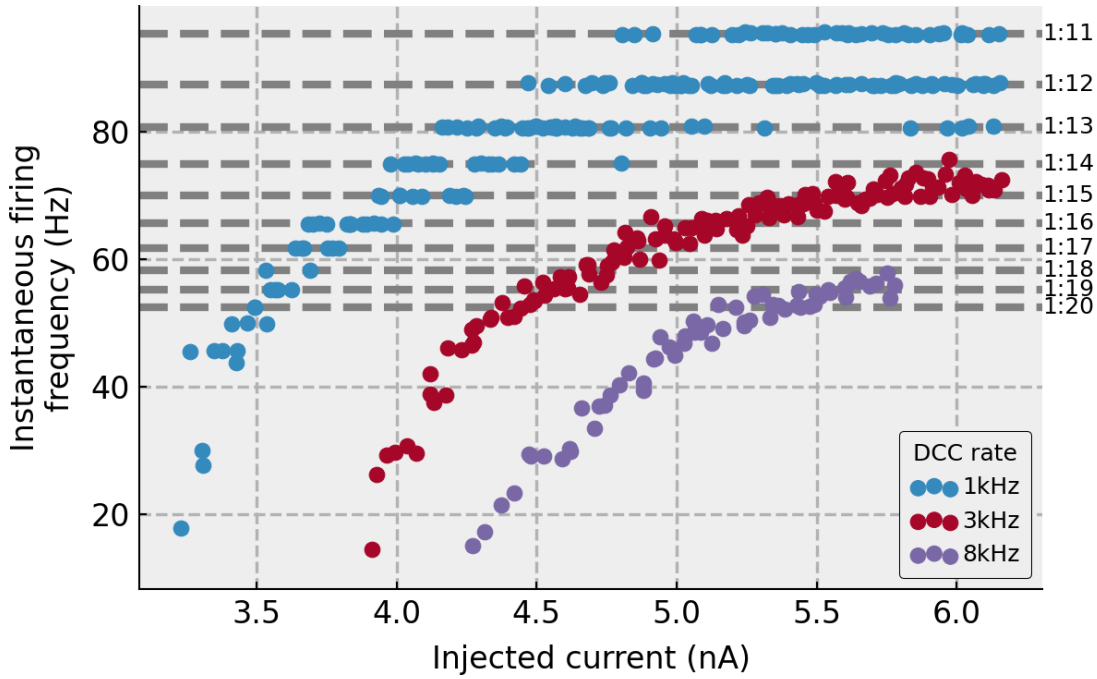


**Figure 6. Example of a stepwise pattern at low DCC rate.** F-I curves from a Triceps Surae motoneuron ($R_{in}$=2.0 MΩ; $\tau_m$=3.8 ms), injected with a triangular ramp of current (1 nA/s) and recorded in DCC mode at three different DCC switching rates. At low DCC rates, a clear stepwise pattern is apparent, which corresponds to multiples of the switching rate (1050 Hz in this instance): 95.5 Hz (or once every 11th current injection), 87.5 Hz (1:12), 80.8 Hz (1:13), 75.0 Hz (1:14), 70.0 Hz (1:15), 65.6 Hz (1:16), etc.

seen at high rates, even when the firing frequency reaches high levels (Figure 7). In such a subharmonic resonator, the distance between subharmonics decreases as the order of the subharmonics increases; i.e. the distance between the first subharmonics $f_1 = f_0/2$ and the second $f_2 = f_0/3$ is $\Delta f_{1-2} = f_0/6$ while the distance between the second and third ($f_3 = f_0/4$) subharmonics is $\Delta f_{2-3} = f_0/12$. Consequently, on the ascending phase of the ramp of current, the firing frequency increases progressively and is attracted to successive subharmonics of the fundamental frequency (the DCC switching rate), with increasing "leaps" between each of these steps (Figure 6). This phase-locking effect could induce an apparent increase in the slope in the F-I relationship, particularly when the firing frequency reaches high values. This phenomenon is illustrated Figure 7 where a motoneuron was stimulated with a high-amplitude (13 nA), fast ramp of current (5 nA/s), expressly for the purpose of

pushing the motoneuron to high firing frequencies. At DCC rates 5 and 8 kHz, the resulting F-I curves are almost indistinguishable, with a first range of current where the frequency was increasing steeply, followed by a region where the frequency increased at a smaller rate (∼7 Hz/nA, dashed grey line). When recorded with a DCC rate of 3 kHz, the F-I curve was shifted upward to higher firing frequencies. The slope of the initial linear phase was slightly higher (∼10 Hz/nA, black dashed line) than with higher DCC rates. When the firing reached frequencies above 175 Hz, a clear step-wise pattern became apparent (once again at multiples of the 3 kHz DCC rate: 150.0 Hz, or 1:20; 157.9 Hz, or 1:19; 166.7 Hz, or 1:18; 176.5 Hz, or 1:17; 187.5 Hz, or 1:16; 200.0 Hz, or 1:15; 214.3 Hz, or 1:14; 230.8 Hz, or 1:13; and 250.0 Hz, or 1:12). While the firing is driven at these discrete interspike intervals, the F-I curve becomes steeper (∼20 Hz/nA), creating the illusion of a "secondary range", even though this change of slope is not present in the data recorded with higher DCC rates for the same current intensities.

# 3 Discussion

This paper describes the effect of an incorrectly set DCC rate on the firing properties of spinal motoneurons. Although a low DCC rate lead to an underestimation of the membrane potential, and therefore an apparent increase in cell conductance, we show that, paradoxically, it has the potential to artificially drive the cells to fire a lower currents and higher frequency, as well as to profoundly alter the shape of the F-I relationship.

Although there are no theoretical upper limits to the DCC cycling rate, in practice, one is limited by the time constant of the electrode and the capacitance neutralization circuit of the amplifier. Indeed, if the voltage drop through the micro-electrode has not relaxed to zero at the sampling time, the membrane potential recorded is contaminated by an unknown fraction of the electrode resistance. More importantly,

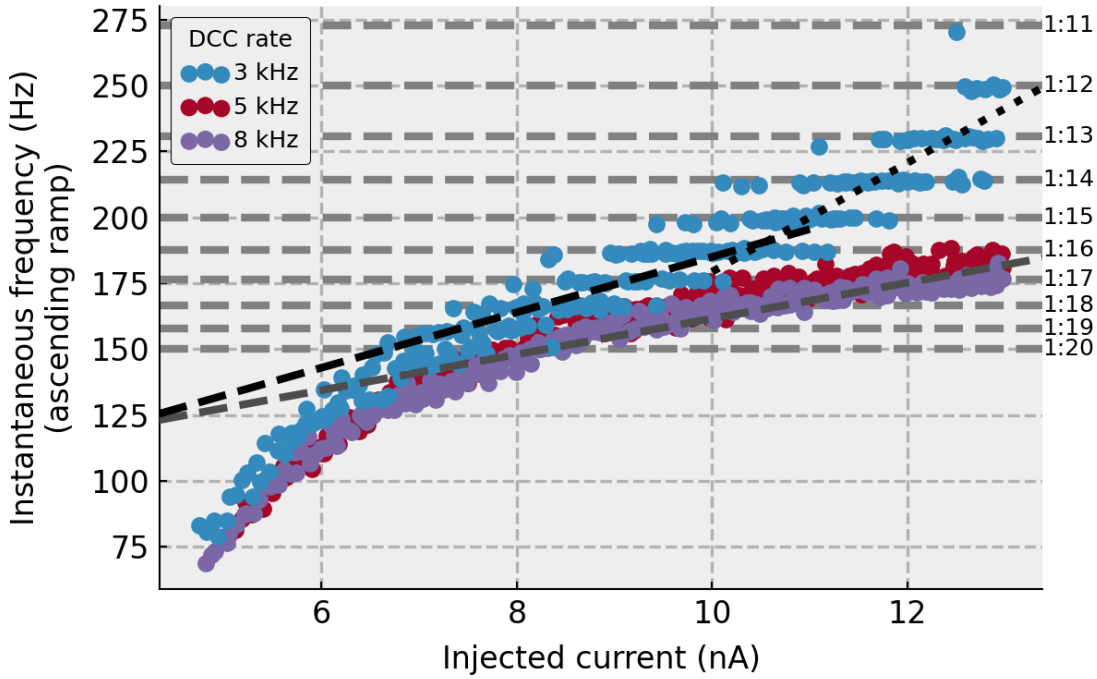


**Figure 7. Apparent change of slope in the F-I curve associated with discrete firing intervals.** F-I curves from a Triceps Surae motoneuron ($R_{in}$=4.1 MΩ; $\tau_m$=3.3 ms). A fast triangular ramp of current (amplitude 13 nA, 5 nA/s) was injected to drive the firing at high frequency. The instantaneous firing frequency is plotted against the ascending ramp current intensity. Grey dashed line: slope of the F-I curve recorded at 8 kHz measured in the second half of the curve. Black dashed line: slope of the F-I curve recorded at 3 kHz, measured over the range 7–10 nA. Black dotted line: slope of the F-I curve recorded at 3 kHz, measured over the range 11–13 nA.

there is a lower limit to the DCC rate. For instance, the Axoclamp manual states that the rate must be such that "that there are ten or more cycles per membrane time constant. This enables the membrane capacitance to smooth the membrane voltage response to the current pulses" (Axon Instruments, 2003). There must be a theoretical "optimal" DCC rate in between those two extremes, but finding that optimal frequency is difficult in practice (Brette and Destexhe, 2012). Instead, most electrophysiologists observe the continuous electrode potential on an oscilloscope synchronized to the DCC sampling clock. The goal is to adjust the electrode capacitance compensation circuit and the DCC switching rate to reach the highest DCC rate possible while ensuring that the response shown on the oscilloscope appears flat, that is to say, that the contribution of the electrode resistance to the recorded

potential has become negligible.

While it is fairly straightforward to check that the DCC rate is not too high based on the settling time of the electrode observed on the monitoring oscilloscope, it is difficult to know whether the DCC rate is fast enough to not distort the firing of the cell. Our experiments show that the minimum value of ten cycles per time constant commonly recommended is too conservative. We show that DCC rates of at least 15 cycles per time constant are required to produce measurements that match the ones obtained in bridge mode. More importantly, above 15 cycles per time constants, the measurements become largely insensitive to the exact DCC rates, and therefore small differences in DCC rates between cells should not impact their respective firing behavior.

Compared to the Bridge mode, the DCC transforms the input signal from a continuous variation in current intensity to a discontinuous situation, where the current can only be injected as short square pulses. This difference is almost negligible when the DCC rate is high enough for the membrane potential to barely move during the current injection and the subsequent inter-pulse interval. However, at lower DCC frequencies, the membrane potential will start to oscillate substantially (Figure 2). It should be noted that, although these oscillations are present across the membrane of the recorded cell, they are hidden from the experimenter since the output of the amplifier is held constant at the level of the previous sampled value during that time (thick black line in Figure 2A). When considering slow ramps of current, like in the present study, decreasing the DCC rate from a high frequency to a lower frequency therefore amounts to transitioning from a situation where the membrane potential is increasing slowly, to a situation where sharp voltage transients are superimposed to a slow depolarisation. These oscillations are particularly efficient at triggering action potentials. Indeed, it has been shown in many neuronal types that the faster the rate of rise of the membrane potential, the more reliably a spike will be generated (Mainen and Sejnowski, 1995; Azouz and Gray, 2000; Agrawal et al., 2001;

Kuo et al., 2006). This high dynamic sensitivity explains why motoneurons recorded with a low DCC rate fire at lower current despite reaching lower membrane potentials. In addition, it also accounts for the fact that, at low DCC rate, firing becomes phased-locked to the DCC frequency. Indeed the voltage transients will trigger a spike more reliably than the slow decay of the after-hyperpolarization that follows the preceding spike. The interspike intervals thereby can only take values that are multiples of the DCC period, leading to the characteristic step-like pattern observed in the F-I curves.

Interestingly, motoneurons have a natural regime of firing where a similar step-like pattern can be observed in response to very slow current ramps. We have shown previously that, for a narrow range of current motoneurons exhibit subthreshold oscillations which alternate with spikes, producing a very irregular firing. This regime, called mixed-mode oscillations (MMOs) is responsible for the sub-primary firing range. These oscillations naturally emerge from a sodium to potassium ratio too weak to generate full blown spikes with high reliability; but when a spike is finally generated, it is locked to one of the oscillations (Iglesias et al., 2011). However, since the frequency of the MMOs is much lower (100–125 Hz, Manuel et al., 2009; Iglesias et al., 2011), and disappear when the firing reaches past the transition frequency between the sub-primary and the primary range (Iglesias et al., 2011), the resulting plateaus in the F-I curve are only apparent over the sub-primary range.

As shown above, the distortions caused by the DCC rate are primarily dependent on the time constant of the membrane. In spinal motoneurons, there is a strong relationship between membrane time constant and cell size, such that small, Slow-type (S) motoneurons have a longer membrane time constant than the larger, fast fatigable (FF) motoneurons (Gustafsson and Pinter, 1984). Consequently, FF motoneurons require an even higher DCC frequency than S motoneurons to obtain accurate measurements of their excitability. We have previously shown that mouse motoneurons have shorter time constants than cats (Manuel et al., 2009). Mouse

FF motoneurons have an average time constant of 2.1±0.2 ms, FR motoneurons 2.9±0.9 ms, while S motoneurons have a time constant of 4.0±0.7 ms (unpublished data from Martínez-Silva et al., 2018). Based on our present results, which show that a DCC frequency corresponding to at least 15 cycles per time constant is required to measure the excitability of the cell, FF motoneurons should be recorded with a DCC at at least 7 kHz, while S motoneurons can accommodate DCC frequencies as low as 3.75 kHz. Because of their size, FF motoneurons are also the cells that require the most current to fire. The impedance of the electrode is often highly non-linear, and both the resistance and the time constant of the electrode have a tendency to increase with the amount of injected current. Consequently, It is often difficult to record the firing of these cells at high DCC rates. Instead, it would be tempting, particularly in these cells, to lower the DCC rate to obtain proper settling of the electrode's IR drop, but, as we demonstrate here, doing so would lead to an overestimation of the cell's firing and excitability parameters. Moreover, in a mouse model of Amyotrophic Lateral Sclerosis, we have shown that the largest motoneurons become incapable of firing repetitively in response to a slow ramp of current (Martínez-Silva et al., 2018). Given the membrane time constants of these cells, it was essential to perform these recordings at high DCC rates (all of our recordings were performed in DCC at 7–9 kHz), since lower DCC rates have the potential to distort the firing of these cells, and even mistakenly transform a non-repetitively-firing motoneuron into a repetitively-firing motoneuron (Jensen et al., 2020).

# 4 Conclusions

In conclusion, the effects of inappropriate DCC switching rates on the apparent conductance of the cells is well known. However, the effect on the firing characteristics of the neurons are not often discussed. We show here that choosing a sub-optimal DCC rate may dramatically distort parameters that are classically used to define the

"excitability" of neurons: lower current onset, lower current offset, higher firing frequencies, higher F-I gains, and even the appearance of an artifactual "secondary range" of firing. Low DCC rates can therefore lead to a misrepresentation of neuronal excitability.

# 5 Methods

## 5.1 Animals

All procedures were approved by the Paris Descartes University ethics committee (CEEA34; authorization number 2018052100307589) and followed the European Directives (86/609/CEE and 2010-63-UE) and the French legislation on the protection of animals used for scientific purposes. C57BL/6 male mice (weight 29.4±1.3 g; N=5) were used in this study.

## 5.2 Experimental procedure

The surgical procedures have been described previously (Manuel et al., 2009; Manuel and Heckman, 2012). Briefly, atropine (0.20 mg/kg; Aguettant) and methylprednisolone (0.05 mg; Solu-Medrol; Pfizer) were given subcutaneously at the onset of experiment, to prevent salivation and oedema, respectively. Fifteen minutes later, anaesthesia was induced with an intraperitoneal injection of sodium pentobarbitone (70 mg/kg; Pentobarbital; Sanofi-Aventis). A tracheotomy was performed, and the mouse was artificially ventilated with pure oxygen (SAR-830/AP ventilator; CWE). The end tidal CO2 level was maintained around 4% (MicroCapstar; CWE). The heart rate was monitored (CT-1000; CWE), and the central temperature was kept at 37°C using an infrared heating lamp and an electric blanket. A catheter was introduced in the external jugular vein, allowing us to supplement the anaesthesia whenever necessary (usually every 20–30 min) by intravenous injections (sodium pentobarbitone, 6 mg/kg). The adequacy of anaesthesia was assessed on lack of noxious reflexes and

on the stability of the heart rate (usually 400–500 bpm) and end-tidal PCO2. A slow intravenous infusion (50 μL/h) of a 4% glucose solution containing NaHCO3 (1%) and gelatine (14%; Plasmagel; Roger Bellon) helped maintain the physiological parameters. The animal was paralyzed after the surgery with atracurium besylate (Kalceks; initial bolus was 0.1 mg, followed by a continuous infusion 0.01 mg/h). Additional doses of anaesthetics were then provided at the same frequency as before the paralysis, and adequacy of anaesthesia was assessed on the stability of the heart rate and of PCO2. The vertebral column was immobilized with two pairs of horizontal bars (Cunningham Spinal Adaptor; Stoelting) applied on the Th12 and L2 vertebral bodies, and the L3–L4 spinal segments were exposed by a laminectomy at the Th13–L1 level. The Triceps Surae nerve (containing the branches innervating the Medial Gastrocnemius, the Lateral Gastrocnemius and the Soleus) was dissected and placed on a bipolar electrode for stimulation. All other branches of the sciatic nerve were cut. The tissues in the hindlimb and the spinal cord were covered with pools of mineral oil. At the end of the experiments, animals were killed with a lethal intravenous injection of pentobarbitone (200 mg/kg).

## 5.3 Electrophysiological recordings

The motoneurons were impaled with micro-pipettes (tip diameter, 1.0–1.5 μm) filled with 3 M KCl (resistance 16.9±3.4 MΩ [13.0–26.0 MΩ], N=10). Recordings were performed using an Axoclamp 2B amplifier (Molecular Devices) connected to a Power1401 interface and using the Spike2 software (CED). The current ($I_m$) and voltage output ($10V_m$) of the amplifier were low-pass filtered at 10 kHz and sampled at 20 kHz. When recorded, the continuous output $I_1$ and $V_1$, and the DCC monitor output were sampled at 100 kHz. After impalement, identification of motoneurons rested on the observation of antidromic action potentials in response to the electrical stimulation of their axon in the triceps nerve. All care was taken to compensate for the microelectrode resistance and capacitance. No bias current was used to maintain

the resting membrane potential. All cells kept for anaysis had a resting membrane potential more hyperpolarized than −50 mV and an overshooting antidromic spike. As fully described previously (Manuel et al., 2009), the input resistance was measured using the peak response of a series of small-amplitude square current pulses (−3 to +3 nA, 500 ms) recorded in DCC mode (8 kHz). The membrane time constant was measured on the relaxation of the membrane potential after injection of small hyperpolarizing current pulses (−5 nA, 1 ms), recorded in Bridge mode. Slow triangular ramps of current were injected in DCC mode (switching rates as described in the text). A recovery period of at least 30s was left in between each repetition. The onset current was defined as the value of the injected current at which the first action potential was generated one the ascending phase of the ramp. The offset current was the current intensity corresponding to the last action potential on the descending phase of the ramp. The F-I gain was measured as the slope of the F-I relationship in the most linear part of the ascending phase of the ramp ("primary range"). The voltage threshold was measured at the point when the slope of the membrane voltage crosses 10 mV/s (Sekerli et al., 2004) just prior to the first spike of the ascending phase of the ramp.

## 5.4 Numerical simulations

Numerical simulations were conducted in Python v.3.7 using the SciPy ecosystem (v.1.5.0; Virtanen et al., 2020). The equation $C \cdot \frac{dV}{dt} = G \cdot (V_0 - V) + I_{inj}$ was integrated using a standard 4th order Runge-Kutta algorithm. For the cell, $G_{in}$ was set to 0.2 μS and $C$ to 0.001 nF to obtain a time constant $\tau_m$=5 ms. To model the IR drop through the electrode, parameters were chosen so that the electrode was 200× faster than the membrane time constant ($\tau_e$=$\tau_m$/200=25 μs). The equation above was solved with $G_e$=1 μS. Although not quite realistic, this value was chosen so that the response of the electrode would not completely dominate the graphs in Figure 2. Note however that the value of the resistance of the electrode is only relevant at high

DCC rates, when the IR drop through the electrode does not have time to vanish by sampling time (Figure 2). At lower switching rates, the resistance of the electrode is irrelevant since its contribution has fully dropped to zero at the end of the DCC period. All figures were drawn using matplotlib v.3.2.2 (Hunter, 2007).

## Competing interests

The author declares no conflict of interests.


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