NMDA Channels Together With L-Type Calcium Currents and Calcium-Activated Nonspecific Cationic Currents Are Sufficient to Generate Windup in WDR Neurons

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Aguiar P, Sousa M, Lima D. NMDA channels together with L-type calcium currents and calcium activated nonspecific cationic currents are sufficient to generate windup in WDR neurons. J Neurophysiol 104: 1155–1166, 2010. First published June 16, 2010; doi:10.1152/jn.00834.2009. Windup is characterized as a frequency-dependent increase in the number of evoked action potentials in dorsal horn neurons in response to electrical stimulation of afferent C-fibers. This phenomenon was first described in the mid-60s, but the core mechanisms behind it still remain elusive. Several factors affecting its dynamics have been identified, but the distinction between modulating mechanisms from generating mechanisms is not always clear. Several mechanisms contribute to the excitation of dorsal horn neurons exhibiting windup, and one of our main aims was to help making this distinction. The approach presented here relies on mathematical and computational analysis to study the mechanism(s) underlying windup. From experimentally obtained windup profiles, we extract the time scale of the facilitation mechanisms that may support the characteristics of windup. Guided by these values and using simulations of a biologically realistic compartmental model of a wide dynamic range (WDR) neuron, we are able to assess the contribution of each mechanism for the generation of action potentials windup. We show that the key mechanisms giving rise to windup is the temporal summation of N-methyl-D-aspartate (NMDA) long-lasting postsynaptic responses taking place on top of a membrane potential cumulative depolarization. Calcium-activated nonspecific cationic currents driven by calcium influx from L-type calcium channels and synaptic currents support this cumulative depolarization and plateau formation in WDR neuron membrane potential. The effects of different nonhomogeneous stimulation protocols are explored, and their important role in clarifying many aspects of the windup generation is shown. The models are used to produce several predictions that can be tested experimentally.

INTRODUCTION

One of the most important features of the nociceptive system is the ability to adapt and change its functional responses to the same form of stimuli as a result of relevant sensorial experiences. This plastic capability is a key component in the system’s dynamics and plays a central role in nociceptive information processing. Comprehending the nociceptive system workings implies therefore a deep understanding of the numerous plasticity mechanisms involved in nociception. Here we present our work regarding one important plasticity mechanism present at the spinal cord: action potentials windup of deep dorsal horn neurons. Windup is defined as a frequency-depen-

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this work it is necessary to provide a precise definition: throughout the paper, we use the definition from (Mendell and Wall 1965), phrased above, where the responses are measured as the number of elicited action potentials.

This paper is organized as follows. We start by using typical windup profiles, and its stimuli frequency dependence, to analyze what should be the temporal features (time scales) of the mechanism that could give rise to the experimentally observed profiles. This is made without making any a priori assumption regarding the particular generating mechanism. These results are used to identify a reduced set of synaptic and membrane mechanisms that, isolated or combined, satisfy the time profile constrains. These mechanisms are, in turn, used to build a biophysically detailed compartmental model of a WDR neuron using the NEURON simulation environment (Hines and Carnevale 1997). Computer simulations of the WDR neuron model, under synaptic stimulation and intracellular current injection conditions, are used to identify the core mechanisms underlying C-fibers mediated windup.

**Methods**

The experiments described in this work refer to mathematical and/or computational analysis using detailed quantitative descriptions of the key biophysical elements involved in WDR neuron dynamics and action potentials windup. Two sets of models were used: one set, composed of a conceptual model for windup responses, was used to assess time domain properties, whereas a second set, consisting of biophysical models describing a WDR neuron, was used to assess the mechanisms underlying windup under detailed neurobiological constrains.

**Dissecting windup time profiles**

Typical windup profiles, measured as the monotonically increasing number of action potentials evoked by C-fiber stimulation, can be quantitatively well approximated by a single exponential process following Eq. 1. In this equation, \( R(n) \) represents the response to stimuli \( n \), where \( n \) is an integer and \( n \geq 1 \), \( T \) is the stimulation period, \( \tau_W \) is the windup time constant, \( R_1 \) is the first (bias) response, and \( R_w \) is the asymptotic response to a stimulation with constant frequency \( 1/T \). The profile of this curve is shown in Fig. 1A (black line) but notice that the observable values are discrete because \( n \) can only take integer values

\[
R(n) = (R_w - R_1) \left(1 - e^{-\frac{(n-1)T}{\tau_W}}\right) + R_1
\]

Although an infinite number of different facilitation mechanisms could give rise to the typical windup profile envelope, we take a simple yet reasonable hypothesis and assume that the underlying facilitation process can be described by a first-order equation with a single time constant (Eq. 2). This approximation is appropriate even if multiple time constants would be involved, as long one of them dominates by being much slower than all the others. The first-order equation involves a quantity \( X \) (dimensionless) that is incremented when stimulation \( n \) is induced at time \( t_0 \) and constantly decays with a time constant \( \tau_F \). The increment is state-independent and defined by \( X_1 \). Function \( S \), defined in terms of Dirac’s \( \delta \) function, takes nonzero values only at stimulation times \( t_0 \)

\[
\frac{dX}{dt} = -X + X_1 \cdot S(t)
\]

\[
S(t) = \sum n \delta(t - t_n)
\]

**Biophysical model for WDR neurons**

As previously mentioned, one of our main aims was to identify, from a list of candidates, what are the key mechanisms behind the generation of action potentials windup. To assess and quantitatively analyze the contributions of each component, we created a detailed biophysical compartmental model of a deep dorsal horn wide-dynamic range neuron. This model contains mathematical descriptions for the dynamics of synaptic mechanisms and membrane conductances that may, directly or indirectly, contribute to the establishment of C-fiber–mediated action potentials windup. Both construction and simulations of the WDR neuron model was performed using the NEURON simulation environment (Hines and Carnevale 1997).

**Morphology and connectivity**

The WDR neuron model consists of connected compartments representing dendrite, soma, axon initial segment, and axon. A single
cylindrical compartment was used for each structure. The dendrite was 500 μm in length and 4 μm in diameter; the soma was 20 μm in diameter and 20 μm in length; the axon hillock decreased in diameter from 2 to 1 μm along a length of 3 μm; and the axon was 1 μm in diameter and 1,000 μm in length. Experimental evidence points to the relevance of temporal, not spatial, features behind the generation of windup (for review, see Herrero et al. 2000) that motivates the use of a simple topology for the WDR neuron model.

Inputs from Aδ-fibers impinged directly on the WDR neuron, whereas the connection from C-fibers was mediated by a relay interneuron with the same morphological features as the WDR neuron (Fig. 2A). The activity in both types of fibers was modeled using a common artificial event generator that produced action potentials (spikes) at specified times, according to the particular experimental protocol. The Aδ-fiber and the relay interneuron established each 20 synapses with the WDR neuron. The interneuron received a single connection from the C-fiber. The delays on the arrival of Aδ-fiber signals at the WDR neuron follow a normal distribution with parameters 30 ± 5 (SD) ms. The total delays on the arrival of C-fiber polysynaptic signals also follows a normal distribution but with parameters 200 ± 20 ms. The higher variability in the C-fiber signals accounts for the higher dispersion produced by the polysynaptic connections (Sivilotti et al. 1993). All delays are predefined and used throughout all stimulations. For the purpose of computational simplification, the C-fiber signal total delays were placed after the relay neuron; i.e., the delay between C-fiber activation and signal arrival at the interneuron was set to 0 ms.

The stimulation protocol in the WDR neuron model consists in setting a periodic list of stimulus times. At each stimulus time, an event is generated on both fibers; after the delay associated with each fiber, the appropriate synapse(s) are activated. This is equivalent to produce a single action potential in the initial portion of each fiber, for each stimulus time, which takes a certain amount of time to propagate and activate the appropriate synapse(s). Notice that the typical experimental protocol involving a 500 μs current shock in the fibers, with an amplitude shortly above C-fiber threshold, aims for the same effect. The core difference is that in the model we do not actively simulate the action potentials propagation in the fibers. To further clarify the stimulation protocol and the inputs to the model, consider the example of a standard 1.0 Hz stimulation protocol: 1) the artificial event generator produces a single event (stimulus) on both fibers; 2) the C-fiber synapse to the interneuron is instantaneously activated after each stimulus, whereas the Aδ-fiber synapses are activated only after the associated predefined set of delays, specified for each individual synapse; 3) each spike produced by the interneuron is delivered at the interneuron to WDR neuron synapses after the other set of predefined delays, also specified for each individual synapse; and 4) the artificial event generator continues to produce a single event (stimulus) on both fibers with a period of 1 s.

Synaptic mechanisms

The following synaptic receptors were included in the model: NMDA, AMPA, NK1, and GABA<sub>A</sub>. All synapses were modeled as conductance changes following dual exponential profiles defined by rise time constants <em>τ<sub>r</sub></em> and decay time constants <em>τ<sub>d</sub></em>. The maximum conductance for each synapse was defined as γ. NMDA and NK1 receptors were subject to additional mechanisms to account for some receptor type specificities.

The NMDA conductance was subject to a dimensionless magnesium block multiplicative factor <em>f<sub>Mg</sub></em> following the detailed physiological model by Jahr and Stevens (1990). An extracellular magnesium concentration of 1 mM was used in all simulations. In addition, from the total nonspecific cationic current passing through the NMDA channels, a fraction of 10% was set as a calcium current (Burnashev et al. 1995). This inward calcium current played an important role increasing the intracellular calcium concentration, which in turn affected calcium-dependent currents.

The effects of the activation of NK1 receptors are not totally clear but they seem to include both a membrane conductance change and an increase in intracellular calcium concentration, which probably results from the release of calcium from intracellular buffers (Ito et al. 2002). Our NK1 model included these two major mechanisms, the slow conductance change generating a slow depolarizing nonspecific cat-

![WDR neuron model](image-url)
ionic current and the increase in calcium intracellular concentration that was generated using a fake (silent) calcium current proportional to the total nonspecific cationic current. As with the NMDA, the proportionality constant was set to 10%.

In the simulations, synaptic noise was used to introduce variability in the WDR neuron membrane potential. These synapses were driven by independent stochastic sources following a Poisson process and produced very small postsynaptic responses. Their parameters were set so that their cumulative net effect would be very close to zero. They did not affect the WDR neuron windup profiles and basically served solely the purpose of approximating the simulated membrane potential traces to the experimental characteristics. The reversal potential for AMPA and NMDA synapses was set to 0 mV, and the reversal potential for GABA_A synapses was set to −80 mV.

In one series of simulation experiments, synapses from C-fibers were also considered to be subject to short-term plasticity. This form of plasticity in the C-fibers is strongly supported by experimental evidence: C-fiber field potentials located in the superficial dorsal horn show response increases with similar frequency requirements as dorsal horn neurons windup (Schouenborg 1984); glutamate and substance P (SP) release is subject to facilitation mechanisms, leading to higher release efficacies at higher activation frequencies (Duggan et al. 1995; Mantyh 2002); it has also been identified the presence of presynaptic NMDA receptors that regulate, in a feedback manner, neurotransmitter release (Ahmadi et al. 2003; Liu et al. 1997; Martínez et al. 1997). The model used for the short-term dynamics of C-fibers synapses was adapted from the probabilistic model by Fuhrmann et al. (2002) where \( \tau_{\text{fac}} \) and \( \tau_{\text{rec}} \) are, respectively, the facilitation and the recovery time constants, \( U_{\text{SE}} \) is the amount of resources used in a release at time \( t \), \( U_1 \) is a constant determining the step increase in \( U_{\text{SE}} \), \( P \) is the probability of a vesicle being available for release at time \( t \). Function \( S \), as before, is built from Dirac’s delta functions and takes nonzero only at the activation times \( t_n \) (times associated with spike arrivals)

\[
\tau_{\text{fac}} \frac{dU_{\text{SE}}}{dt} = -U_{\text{SE}} + U_1 \cdot (1 - U_{\text{SE}}) \cdot S(t)
\]

\[
\tau_{\text{rec}} \frac{dP}{dt} = (1 - P) - U_{\text{SE}} \cdot P \cdot S(t)
\]

The product of \( U_{\text{SE}} \) and \( P \) define the probability of release \( P_r \) that is used as a multiplicative factor in the synaptic conductance profile.

### Table 1. Default synaptic parameters

<table>
<thead>
<tr>
<th>Source</th>
<th>Target</th>
<th>Receptor</th>
<th>( \tau, \text{ms} )</th>
<th>Parameters</th>
<th>( \tau, \text{ms} )</th>
<th>( g, \text{nS} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aδ-fiber</td>
<td>WDR</td>
<td>AMPA</td>
<td>0.1</td>
<td>5.0</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NMDA</td>
<td>2.0</td>
<td>100.0</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>C-fiber</td>
<td>Interneuron</td>
<td>AMPA</td>
<td>0.1</td>
<td>5.0</td>
<td>0.2</td>
<td></td>
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<td></td>
<td></td>
<td>NMDA</td>
<td>2.0</td>
<td>100.4</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nk1</td>
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<td>3,000.0</td>
<td>0.020</td>
<td></td>
</tr>
<tr>
<td>WDR*</td>
<td></td>
<td>Nk1</td>
<td>200.0</td>
<td>3,000.0</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>Interneuron</td>
<td>WDR</td>
<td>AMPA</td>
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<td>5.0</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NMDA</td>
<td>2.0</td>
<td>100.0</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GapA</td>
<td>0.1</td>
<td>10.0</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Noise</td>
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<td>AMPA</td>
<td>0.1</td>
<td>5.0</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GapA</td>
<td>0.1</td>
<td>5.0</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

Although there is no direct synaptic contact in the model between the C-fiber and the WDR neuron, the Nk1 receptor in the WDR neuron membrane* is triggered by C-fiber activity. The smaller peak conductance and the slower rise time constant of the WDR neuron Nk1 receptor, compared with the interneuron Nk1 receptor, intends to reflect substance P diffusion to deeper layers and the reduced expression of Nk1 receptor in WDR neurons (Marvizzón et al. 1997). WDR, wide dynamic range; NMDA, N-methyl-D-aspartate.

The parameters used for each type of synapse, in each type of connection, are summarized in Table 1. Unless stated, all simulations use synapses with fixed peak conductances (nonplastic). In the few simulations where C-fiber synapses were subject to short-term plasticity, equal parameters were used for glutamate and SP release: in synaptic facilitation, \( U_1 = 0.5, \tau_{\text{rec}} = 3,000.0 \text{ms} \) and \( \tau_{\text{rec}} = 0.1 \text{ms} \); in synaptic depression, \( U_1 = 0.07, \tau_{\text{rec}} = 0.1 \text{ms} \) and \( \tau_{\text{rec}} = 4,000.0 \text{ms} \). Inhibitory synapses were also added to the WDR neuron to analyze the implications in the windup profile of blocking such synapses. However, instead of modeling a specific inhibitory interneuron between C-fiber inputs and the WDR neuron, the relay interneuron activity was used also to trigger GABA_A responses in the WDR neuron. A delay of 1.0 ms was imposed in the GABA_A postsynaptic response.

**Membrane mechanisms**

The following membrane conductances, and respective model source, were used in our simulations: Hodgkin-Huxley currents consisting of a transient sodium current \( i_{\text{Na}} \) and a delayed rectifier potassium current \( i_{\text{K}} \) (Miles and Traub 1991), long-lasting high-threshold calcium current \( i_{\text{CaL}} \) (McCormick and Huguenard 1992), calcium-activated nonspecific cationic current \( i_{\text{CAN}} \) (Partridge and Swandulla 1988), calcium-dependent potassium current \( i_{\text{KCa}} \) (Dexteux et al. 1994), and persistent sodium current \( i_{\text{NaP}} \) (Prescott and Komnik 2005). All parameters in these imported models have been left untouched with the following three exceptions. In the \( i_{\text{CAN}} \) model, a time factor was introduced to slow down the activation time constant to 2 s. This change allowed the slowly decaying cumulative depolarization seen in the WDR neuron experimental traces when stimulation is interrupted. Without this change, the cumulative depolarization would decay in tenths of milliseconds. In addition, the concentration of intracellular calcium at which the \( i_{\text{Ca}} \) current is maximal has been halved from 1.0 to 0.5 \( \mu \text{M} \), allowing higher sensitivity to intracellular calcium concentration increases. The third change was in the \( i_{\text{KCa}} \) current, in the concentration of intracellular calcium at which the \( i_{\text{KCa}} \) current is maximal. This value has also been reduced from 3.0 to 1.0 \( \mu \text{M} \). The volume of the WDR neuron is very large, and this parameter reduction allows an increase in sensitivity of the intracellular calcium-dependent potassium current.

The conductances parameters resulted from a fine-tuning process to achieve membrane voltage traces in close agreement with published experimental data. Important experimental properties/results used to constrain the model were 1) low-frequency stimulation is capable of producing a large cumulative depolarization, which, in the absence of stimulation, decays back to baseline with a time constant in the order of seconds (Baranauskas and Nistri 1998; Sivilotti et al. 1993); 2) deep dorsal horn neurons expressing windup typically exhibit plateau potentials that are dependent on calcium currents (Morisset and Nagy 2000); 3) the sequence of elicited WDR neuron responses follows a monotonically increasing profile with a saturation ceiling that is frequency-dependent (Fossier et al. 2007); and 4) in a sequence of stimuli, the WDR neuron scatter plots show a progressive increase in number of spikes, as well as a dispersion in the spike times, in the C-fiber band; the spike times in the Aδ-fiber band remain relatively unchanged (Herrero et al. 2000). The distributions and parameters for all the membrane conductances in soma and dendrites were as follows (units are \( [g] = \text{ms} \cdot \text{cm}^{-2} \) and \( [p] = \text{cm} \cdot \text{s}^{-1} \) for WDR soma, \( g_{\text{Na}} = 80, g_{\text{K}} = 20, g_{\text{CaL}} = 10^{-4}, g_{\text{KCa}} = 0.1, \) and \( g_{\text{NaP}} = 0.1 \); for WDR dendrite, \( g_{\text{CaL}} = 3 \times 10^{-5}, g_{\text{CaN}} = 0.07, g_{\text{KCa}} = 1 \); for interneuron soma, \( g_{\text{Na}} = 80, g_{\text{K}} = 20, \) and \( g_{\text{KCa}} = 2 \); and for interneuron dendrite, \( g_{\text{KCa}} = 2 \). Axons (initial segment and whole fiber), in both WDR and relay interneuron, contained Hodgkin-Huxley conductances with the following parameters (in mS/cm²): \( g_{\text{Na}} = 100 \) and \( g_{\text{K}} = 40 \). The reversal potentials and the threshold adjustment parameter were, respectively (in mV), \( E_{\text{Na}} = 50, E_{\text{K}} = -70, \) and \( V_{\text{Na}} = -55 \).
All compartments contained a leakage (passive) conductance with a reversal potential of $-65 \text{ mV}$ and a maximum value of 0.042 mS/cm$^2$. The cytoplasmatic resistivity used in all compartments was 150 $\Omega$ cm. Finally, all compartments affected by inward calcium currents (somas and dendrites) included dynamics for the intracellular calcium concentration. The model used (Destexhe et al. 1993) contained a simple version of a calcium pump and a calcium concentration decay, which can be viewed as a simplified buffering mechanism. The equilibrium intracellular calcium concentration was set to 50 nM, and the decay time constants were set to 1 ms in the somas and 2 ms in the dendrites.

A note on the possible locations for windup

A diagram of possible sites where windup-generating mechanisms can take place is shown in Fig. 2A. Hypothesized locations include 1) C-fiber synaptic facilitation, 2) A$\delta$-fiber synaptic facilitation, 3) interneuron synaptic facilitation, and 4) WDR membrane conductance mechanisms leading to increased excitability. One of the most important experimentally established properties of windup is that it requires the activation of unmyelinated afferent C-fibers (Mendell and Wall 1965; Price et al. 1971; Schouenborg and Sjöland 1983). Although the number of A$\delta$-fibers evoked spikes can be subject to some increase, it is in the C-fibers delay region (100–500 ms) where most facilitation takes place. This makes A$\delta$-fiber synaptic facilitation as an improbable cause for windup. Naturally, we cannot exclude the possibility of complex interactions between A$\delta$-fiber synapses and C-fiber activity contributing for windup, but the lack of evidence and data hold back the computational analysis of hypothesis 2. A similar problem arises with hypothesis 3. Interneuron facilitation, coming from an increase in synaptic efficacy, could naturally lead to a progressive increase in the excitation provided to the WDR neuron. However, this hypothesis is also not supported by sufficient experimental data, which impairs the construction of a solid model.

The following sections concentrate therefore in candidate windup generating mechanisms which take place either at the C-fibers synapses or at the WDR neuron membrane, locations that have been the target of an extensive number of studies in the windup literature.

RESULTS

Dissecting windup time profiles

An assessment of the time scales associated with the windup mechanism was achieved by fitting Eq. 1 to a series of windup profiles acquired experimentally. The data, kindly supplied by P. Fossat and F. Nagy, consists in measurements of action potentials windup in the nociceptive flexion reflex in the rat. Each windup profile is the sequence of responses elicited by a single shock (500 $\mu$s) stimulus delivered at a constant frequency (Fossat et al. 2007). The tested frequencies were 0.1, 0.3, 0.5, 0.7, and 1.0 Hz. The response values were normalized to the first response in the series.

The nonlinear regression analysis was performed on the experimental sequences, allowing only two free parameters, $\tau_w$ and $R_w$; the parameter $R_1$ was fixed to 1 (the quality of the fits was scarcely improved by allowing $R_1$ to adjust). The normalized experimental value for $R_1$ for all first response measurements was 0.995 $\pm$ 0.026 (SD), showing that the first response can indeed be considered constant. The parameters $\tau_w$ assessing the time scale of the facilitation process underlying the windup mechanism, were calculated using the regression analysis results. A summary of the regression analysis results and calculated $\tau_w$ are shown in Table 2, and a graphical representation of the experimental windup profile curves with the fitting curves from Eq. 1 are shown in Fig. 1B. The agreement between observed and modeled values, shown through the high coefficients of determination in the regression analysis, shows that the windup time profile can be well approximated by a single exponential process. All regression results were taken into account in the following analysis with exception of the 0.1 Hz data: the extremely high time constant and the low percent increase in the responses, close to the measurements fluctuations, are indicators of a roughly constant response and therefore not subject to a facilitation process. The variability on the reminder values may reflect some interference with other mechanisms of plasticity while the stimulation frequency approaches 1.0 Hz (Sandkühler 2000). The results show some dependence with the stimulation frequency, but all values are in the same order of magnitude, and we conclude that the mechanism(s) that brings forth the windup profile works in a time scale of 2–11 s. The higher responses to increasing frequencies seem to result from a more efficient temporal summation. The average of these values, 5 s, was used as an influential estimator for the time scale of the mechanism(s) underlying windup generation. Mechanisms working on time scales much shorter than 5 s are unable to support temporal summation at stimulation frequencies as low as 0.3 Hz, whereas mechanisms working on time scales much longer than 5 s would lead to response increases beyond the experimentally observed. Notice that a time scale in the order of 5 s rules out many proposed mechanisms for the generation of windup, namely simple time summation of NMDA postsynaptic responses: NMDA conductance kinetics have an associated relaxation time constant in the order of a few hundred milliseconds; it is simply not possible to rely solely in this mechanism to generate the facilitation levels typically present in windup. In the presence of appropriate membrane conductances, however, these postsynaptic responses can be extended to the experimentally observed values of a few seconds (Sivilotti et al. 1993). This does not mean that NMDA receptors are not required for windup; it states, however, that NMDA receptor dynamics by themselves are not sufficient to support, or explain, the mechanism giving rise to the typical windup time profiles. Therefore they alone should not be seen as a windup generating mechanism.

Biophysical model for WDR neurons

The results from the windup profile model provided time scales and hence served as helpful guide lines in setting a short list of strong candidates for the mechanism(s) underlying the
generation of windup. These were short-term synaptic plasticity, time summation of long-lasting postsynaptic responses, plateau potentials, and cumulative depolarization generated by L-type calcium channels and calcium-dependent nonspecific cationic currents. All these mechanisms work in the time scales ranging from 2 to 11 s. The contribution of all these components was assessed by analyzing simulation results from different variants of the WDR biophysical model. Each model variant is achieved by changing a small number of parameters from the values defined in METHODS.

Figure 2C shows a membrane potential trace of the WDR neuron model for an eight-stimuli, 1 Hz periodic stimulation. Each stimulation gives rise to two sequences, or waves, of spikes in the WDR neuron model: the first one corresponding to the earlier Aδ-fiber input, and the second one elicited by the delayed C-fiber/interneuron input. The Aδ-fiber wave shows no increase in the number of elicited responses, whereas the C-fiber wave region shows a marked increase in the number of spikes. The cumulative depolarization decays slowly after stopping the stimulation. The raster plot for the same stimulation protocol, but without interruption after the eight stimuli, emphasizes the spike time dispersion in the C-fiber wave region. The response profiles for the WDR neuron model, as a function of the stimulation frequency, are shown in Fig. 2B. These profiles are in very close agreement with the profiles obtained for the experimental data, shown in Fig. 1B. The asymptotic maximal responses and the range of windup profile time constants are also in tight agreement with the results from Fig. 1B. As a note, a stimulation frequency of 0.6 Hz is presented instead of a 0.7 Hz (which would match the value in the regression analysis to the experimental data; see Table 2) because the response profile for an 0.7 Hz stimulation was already very similar to the 1.0 Hz stimulation.

Role of C-fibers short-term plasticity

It has been proposed that windup is caused by an increase in C-fibers synaptic efficacy (Schouenborg 1984). This idea is further supported by results showing that the release of substance P is subject to facilitation, reaching maximum release values at low frequencies (Duggan et al. 1995). Importantly, the observed facilitation lasts >1.5 s but <6 s (Duggan et al. 1995), which is in accordance with the time scales we calculated from the windup profiles. Although less is known regarding the release of glutamate, in principle, it may be also subject to short-term plasticity (Herrero et al. 2000). Although windup can be achieved without C-fiber synaptic facilitation, as shown in the control experiment in Fig. 2C, it is important to address the consequences of this mechanism. To assess the possible role of C-fibers synaptic facilitation in windup, we analyzed the WDR neuron model using the short-term plasticity dynamics in Eq. 3 to describe the C-fiber synaptic release. Both glutamate (acting on NMDA and AMPA receptors) and substance P (acting on NK1 receptors) releases were subject to short-term dynamics with the parameters described in METHODS.

As expected, facilitation of C-fiber synapses can enhance the level of windup but this is achieved through a progressive increase in the interneuron responses (Fig. 3). It is worth noticing that this progressive increase in the interneuron responses has the same time scale and profile as the responses windup in a WDR neuron. Recall that, in our model, the interneuron does not contain $i_{CaL}$ or $i_{CAN}$ conductances. The small cumulative depolarization seen in the interneuron membrane potential trace is driven by the long-lasting NK1 receptor response. After the stimulation sequence stops, the membrane potential relaxes to resting potential with the NK1 receptor decay time constant, which, in this model, has been set to 3 s. An increase to the NK1 receptor peak conductance, relatively to the other synaptic conductances, enhances the cumulative depolarization (data not shown). Notice that, in the control
situation, without facilitation, the interneuron does not show this progressive increase in its responses. This shows that C-fiber short-term facilitation alone could account for some of the distinctive properties of action potentials windup in the dorsal horn. However, and very importantly, in experimental studies, windup is mostly observed in the deep dorsal horn WDR neurons (Schouenborg and Sjölund 1983). To our knowledge, windup in interneurons from lamina II (which receive direct inputs from C-fibers) has not been reported thus far, despite measurements in this lamina. For this reason, we discard the possibility of C-fiber presynaptic facilitation being a key mechanism in the generation of windup. It should be noted nevertheless that there is the problem of sampling in electrophysiology experiments: because interneurons are smaller than projection neurons, they are harder to select; this fact may underestimate the existence of windup in interneurons at the more superficial layers of the dorsal horn (presynaptic to the WDR neurons).

Depression of C-fiber synapses, however, seems to be allied with a reduction in the C-volley responses for stimulation frequencies above 1–2 Hz (Fig. 3). Experimentally, this reduction seems to come not only from a effective decrease in the number of elicited responses but also from the increase in the dispersion of C-volley spikes: the progressive increase in the latencies push C-fiber evoked responses to later times, beyond the time of arrival of the next stimulus; for a 2.0 Hz stimulation, this corresponds to a border line at 500 ms. These phantom responses are not accounted for in the dot-raster plots. Notice that C-fiber short-term plasticity was used only in this section. All results outside this section use nonplastic synapses.

**Long-lasting synaptic responses**

Intracellular recordings show that the duration of WDR postsynaptic potentials (measured from resting potential) evoked by C-fibers is in the order of 6 s. The initial component (~2 s) is sensitive to antagonists of excitatory amino acid receptors, be they NMDA or non-NMDA, whereas the second component seems to result from metabotropic and NK1 receptors activation (Sivilotti et al. 1993).

The temporal evolution of all relevant synaptic currents in the WDR neuron model under a standard 1.0 Hz stimulation (control stimulation; Fig. 2C) is shown in Fig. 4. The changes between the response to the first stimulus and to the last stimulus in the sequence are measured as the changes in the areas under each curve (using a 1 s window). The total AMPA synaptic currents provided by the Aδ-fiber and by the interneuron for each stimulation is very similar in magnitude and do not vary much throughout the stimulus sequence: there is a 9 and 6% reduction of the total charge provided, respectively, by the Aδ-fiber and by the interneuron AMPA synapses. This reduction is a consequence of the decrease in the effective potential driving the synaptic currents, which results from the membrane cumulative depolarization (AMPA synapses reversal potential was set to 0 mV). Although also resulting from the membrane cumulative depolarization, the decrease in the magnesium block leads to an increase of the total charge provided by the NMDA synapses: in the sequence of stimulations, the Aδ-fiber NMDA synapses suffer an increase of 19%, whereas the interneuron NMDA synapses suffer an increase of 11%. Notice that the interneuron NMDA component is much larger than the Aδ-fiber NMDA component. This choice of parameters, namely the relative peak conductances, is important: if the Aδ-fiber NMDA component would be as large as the interneuron (C-fiber mediated) component, we would also see a strong increase in the number of elicited responses in the Aδ-fiber region in the WDR neuron traces. Although with a much smaller amplitude than NMDA and AMPA components, the long-lasting NK1 current has a noticeable effect on the WDR neuron dynamics; it is subject to an increase of almost 400%, measured in the 1 s interval after the stimulus. Recall that the NK1 current has a decay time constant of a few seconds. Also shown in Fig. 4 are the intracellular calcium concentration and the two plateau generating currents \(i_{CaL}^{\text{CAN}}\) and \(i_{CaL}^{\text{ICAN}}\). The changes in intracellular calcium concentration increase 22% throughout the sequence of stimulations as a result of more calcium available from NK1 dynamics and from NMDA- and \(i_{CaL}^{\text{Aδ}}\).
driven currents. The progressive increase in the number of WDR neuron spikes leads to an increase of 200% in the total calcium charge provided by $i_{\text{Ca}}$ currents. It is nevertheless the $i_{\text{Ca}}$ current that suffers the largest increase, 1,300%, and is a key element responsible for supporting the observed plateau potentials and cumulative depolarization. The role of $i_{\text{K,Ca}}$ and $i_{\text{Ca}}$ currents is assessed in more detail in the following section.

The NMDA current contribution to windup is analyzed in more detail in Fig. 5, where different NMDA components are selectively blocked. Although the $\Delta$-fiber NMDA synapses slightly affect the WDR neuron response profile for the control 1.0 Hz stimulation, blocking the interneuron NMDA synapses completely impairs action potential windup. Notice that plateau potentials and cumulative depolarization are also strongly reduced by blocking interneuron NMDA synapses: the calcium influx is impaired at the blocked NMDA channels and is reduced at the less active $i_{\text{Ca}}$ channels.

As mentioned above, NK1 receptors also contribute to windup, but instead of being responsible for its generation, they seem to have a role in amplifying it. The results of blocking NK1 receptors at both interneuron and WDR neurons, which is equivalent to blocking substance P release from the C-fiber, are shown in Fig. 6. Although there is a noticeable reduction in the WDR neuron model response profiles as in Budai and Larson (1996), the NK1 block did not impair windup. Also, plateau potentials and cumulative depolarization suffer only a small reduction. The effect of blocking NK1 receptors is slightly higher at lower stimulation rates, because at higher rates, the faster components of the postsynaptic potentials are sufficient to elicit temporal summation efficiently.

**Role of plateau potential generating currents**

The role of the calcium-dependent plateau potentials generated by the L-type calcium current and the calcium-activated nonspecific cationic current was assessed using independent and graded blocks of both these currents. The results are shown in Fig. 7. The WDR neuron membrane potential traces for different blocks are shown in Fig. 7A, together with the associated dot raster plots. The control trace (no block, $i_{\text{Ca}}$ at 100% and $i_{\text{Ca}}$ at 100%), corresponds to the trace in Fig. 2C. There is a direct relation between the level of reduction in the $i_{\text{Ca}}$ current and the level of windup produced. The sequence of membrane potentials immediately before the onset of the $\Delta$-fiber activity because the impaired $i_{\text{K,Ca}}$ current is one of the main drives to the activity control $i_{\text{K,Ca}}$ current. As seen in Fig. 7, $A$ and $C$, a reduction to 90% impairs windup generation. However, a further decrease in the $i_{\text{K,Ca}}$ current has the effect of leading to a higher WDR neuron activity because the impaired $i_{\text{K,Ca}}$ is unable to balance the level of excitation. By definition, windup is still reduced, because the ratio between each response and the first response is lower than in the control configuration, but the overall activity increases after $i_{\text{Ca}}$ block. This increase is not in accordance with some published results (Morisset and Nagy 2000). It may be the case that, although our WDR neuron model relies on $i_{\text{K,Ca}}$ currents for activity control, deep dorsal horn neurons may have other, more robust, mechanisms to control activity that do not rely strongly on $i_{\text{Ca}}$ and $i_{\text{K,Ca}}$ current interaction. In such a scenario, with $i_{\text{Ca}}$...
focused on driving $i_{CaL}$ currents, it is possible to restore the reduction in windup without an increase in activity after blocking the $i_{CaL}$ current.

**Different stimulation protocols**

A usual critique made to the windup literature targets the canonical periodic stimulation protocols that may be quite different from the real natural stimulation patterns. In this section, some results are presented regarding the WDR neuron model responses to nonstandard stimulation protocols.

Although experimentally it is difficult to activate C-fibers without activating the Aβ-fibers, in the WDR neuron model, it is possible to independently activate each fiber type. The traces shown in Fig. 8 take advantage of this possibility to emphasize windup C-fiber specificity. By selectively activating the C-fiber
alone, the WDR neuron model responds with spikes in the C-fiber delay region, which almost match the spikes generated by stimulating both fibers. The number of responses is nevertheless reduced, and the asymptotic maximal response in the response profile is 27% less than the control stimulation (from 2.75 to 2.0, in normalized responses). A stimulation protocol where the C-fiber wave is substituted by a 2nd Aδ-fiber stimulation with the appropriate delay. In this situation, there is neither a progressive increase in the number of elicited responses nor the formation of a cumulative depolarization. The stimulation with a single Aδ-fiber wave in each period also fails to produce either windup or cumulative depolarization (data not shown).

We also tested the behavior of the WDR neuron model under stimulation protocols consisting of stimulus grouped in doublets or triplets and under stochastic stimulation times. These novel stimulation protocols were compared with the 1.0 Hz control stimulation protocol. The doublets and triplets stimulation consists of a sequence of 2 or 3 fiber activations separated by 300 ms, followed by another sequence of activations after 2,000 or 3,000 ms, so that the average stimulus rate remains at 1.0 Hz. In the stochastic stimulation, the fibers activation times are drawn from a homogeneous Poisson process with a rate of 1.0 Hz. Notice that each drawn time is used in both fibers but with the different predefined fiber delays applied. Interestingly, all these alternative protocols produce more responses than the standard periodic protocol in the WDR neuron model. Cumulative counts of all responses produced (both Aδ-fiber and C-fiber mediated) in the WDR neuron model, under these nonstandard stimulation protocols, are shown in Fig. 9. For the homogeneous Poisson stimulation protocol, three possible cumulative counts pathways are shown. For a 1.0 Hz stimulation, the standard protocol leads to a WDR neuron average firing frequency of 14 Hz; the doublets produce a 20 Hz average firing rate, whereas the triplets produce a 24 Hz average firing rate. The homogeneous Poisson stimulation produces an average firing rate of 31 ± 9 Hz, which is double the average firing rate produced by the standard stimulation protocol.

Some remarks regarding the robustness of the WDR neuron model

The WDR neuron model is composed of a large number of parameters. The question of how robust the presented results are given the choice of parameters therefore arises naturally. Many of the parameters are defined within imported, published model dynamics that rely on detailed biophysical properties measured experimentally. The core free parameters that significantly affect the WDR neuron dynamics are therefore the synaptic and membrane conductances. With the exception of the relative sensitivity in the $i_{CaL}$ conductance parameter (addressed in Fig. 7), all other parameters support changes in the order of 10–20% without affecting the main properties of the WDR neuron responses. Most parameters are presented with a
single significant digit to emphasize this point. Moreover, many strong manipulations in the parameters produce response behaviors that are also observed experimentally. For example, if the dendritic $i_{\text{Ca}^+}$ conductance is doubled (from 0.07 to 0.14 mS/cm$^2$), the WDR neuron starts to exhibit long and strong afterdischarges after six to seven stimulations at 1.0 Hz. This type of membrane potential trace is observed experimentally (Herrero et al. 2000).

**DISCUSSION**

Action potential windup in dorsal horn neurons is a complex form of signal amplification involving many factors. Most of them, however, are solely modulation factors. Membrane excitation result from the contribution of many components, and firing activity is achieved in the regions where all these contributions combined lead the membrane to threshold. All these excitatory components give rise to an increase in the firing probability, but only some of them exhibit the dynamics and time scales consistent with the windup time profiles. This is the distinction between modulating and generating mechanisms emphasized in this study. From our descriptive model for the windup profile envelope, it was possible to infer the time scale of the mechanisms underlying windup. Facilitation time constants in the order of 2 to 11 s are the most compatible with the windup profiles obtained experimentally. However, many mechanisms operate in this time scale, but according to our WDR neuron model, the key mechanisms giving rise to windup is the temporal summation of NMDA long-lasting postsynaptic responses taking place on top of a cumulative depolarization. This cumulative depolarization is largely supported by calcium activated nonspecific cationic currents, which are, in turn, driven by calcium influx from L-type calcium channels and synaptic currents. Substance P and NK1 receptors affect the WDR neuron responses in the model and seem to play a role in amplifying windup instead of being responsible for generating it.

Despite identifying key mechanisms underlying the generation of windup, our model also showed some flexibility in achieving windup. This emphasizes the point that windup is achieved by the collective action of more than one mechanism. Shifting the contribution for membrane excitation from one mechanism to another does not impair the generation of windup. It is, however, important to stress the time constraints imposed in the facilitation mechanisms: only mechanisms with dynamics driven by time scales in the order of 2–11 s can be associated with windup-generating mechanisms. Slower mechanisms would lead to slower and larger windup time profiles, whereas faster mechanisms would simply fail to produce temporal summation of postsynaptic responses.

Our model also predicts the behavior of WDR neuron responses under different nonstandard stimulation protocols. In fact, we believe nonhomogenous stimulation to be crucial to clarify important details of the windup process, not just in the sense of approximating the experimental stimulation protocols to natural stimulation, but most importantly to explore conditions that may disambiguate the role of each mechanism. Finally, we would like to mention that the WDR neuron biophysical model presented here should be seen as an important tool for further quantitative analysis of the contributions of different mechanisms in shaping windup and for motivating future experiments.

The NEURON code for the WDR neuron model is available for download from http://www.fc.up.pt/pessoas/pauloaguiar/WDRmodel.zip.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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