Modelling activity-dependent changes of axonal spike conduction in primary afferent C-nociceptors

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Abbreviated title: activity-dependent conduction in C-nociceptors
Key words: activity-dependent slowing, recovery cycles, mechano-insensitive nociceptor, computer modelling
Abstract

Action potential initiation and conduction along peripheral axons is a dynamic process that displays pronounced activity dependence. In patients with neuropathic pain, differences in the modulation of axonal conduction velocity by activity suggest that this property may provide insight into some of the pathomechanisms. To date, direct recordings of axonal membrane potential have been hampered by the small diameter of the fibres. We have therefore adopted an alternative approach to examine the basis of activity-dependent changes in axonal conduction by constructing a comprehensive mathematical model of human cutaneous C-fibres. Our model reproduced axonal spike propagation at a velocity of 0.69 m/s commensurate with recordings from human C-nociceptors. Activity-dependent slowing (ADS) of axonal propagation velocity was adequately simulated by the model. Interestingly, the property most readily associated with ADS was an increase in the concentration of intraaxonal sodium. This affected the driving potential of sodium currents, thereby producing latency changes comparable to those observed for experimental ADS. The model also adequately reproduced post action potential excitability changes (i.e., recovery cycles) observed in-vivo. We performed a series of control experiments replicating blockade of particular ion channels as well as changing temperature and extracellular ion concentrations. In the absence of direct experimental approaches, the model allows specific hypotheses to be formulated regarding the mechanisms underlying activity-dependent changes in C-fibre conduction. As ADS might functionally act as a negative feedback to limit trains of nociceptor activity, we envisage that identifying its mechanisms may also direct efforts aimed at alleviating neuronal hyperexcitability in pain patients.
Introduction

Activity-dependent changes in neuronal excitability are of key interest for a variety of
diseases such as epilepsy or pain. While the plastic nature of signal processing at central
synapses has been studied extensively, excitability changes in peripheral axons have received
less attention. Pronounced activity dependent slowing of conduction velocity (ADS) has been
found to characterize axons of primary afferent C-nociceptors as compared to non-nociceptors
(Thalhammer et al. 1994) and it has been hypothesized that axonal characteristics would
reflect the functional requirements of the particular primary sensory afferent (Thalhammer et
al. 1994). Recently a direct correlation between activity dependent slowing of conduction
velocity and neuronal excitability has also been shown (De Col et al., 2012). In pain research,
there is a current controversy whether spontaneous pain (Bennett, 2012; Mogil, 2012) and
supra-threshold encoding behaviour of nociceptors would have higher predictive value over
studies of evoked pain read-outs which mainly would detect changed activation thresholds
from resting state. In this respect, activity-dependent modulation of conduction in C-fibres is
of major importance. Most interestingly, reduced ADS in peripheral C-nociceptors, leading to
reduced conduction failures of the axons at higher discharge frequencies has been described in
painful diabetic neuropathy in the rat (Sun et al. 2012). In addition, activation thresholds of
the DRGs were reduced and expression of NaV1.7 and NaV1.8 were found increased in the
neuropathic animals (Sun et al. 2012). A similar reduction of ADS in nociceptors accompanied
by reduced conduction failure could also be induced experimentally by intradermal injections
of nerve growth factor (NGF) in the pig (Obreja et al. 2011b). In humans, NGF injections led
to increased pain ratings upon electrical skin stimulation (Rukwied et al., 2010; Obreja et al.
2011a) and reduced activity-dependent slowing of mechano-insensitive nociceptors (Namer,
Obreja, unpublished data). Finally, in patients with neuropathic pain, reduced activity
dependent slowing of conduction has been assessed using microneurography (Serra et al. 2011; Schmidt et al. 2012) and recently a correlation between reduced activity dependent slowing and spontaneous activity of nociceptors was described in pain patients (Kleggetveit et al. 2012). Therefore understanding the physiological process of axonal action potential propagation is of considerable importance, particularly in relation to clinical pain states (Ørstavik et al. 2003; Serra et al. 2011) but also for neurodegenerative disorders (Kanai et al. 2006). Some of these changes can be determined experimentally in humans, offering a possibility to investigate diseases at the level of single human axons.

Unfortunately, intracellular recordings to determine the axonal membrane potential are difficult since unmyelinated fibres are thin (<0.5 µm) and hard to locate in the tissue they innervate. Therefore, we set out to identify the process(es) underlying activity-dependent changes in excitability in single unmyelinated nerve fibres using computational modelling. The aim was to determine the likely contribution of each of an array of candidate molecular targets (ion channel subtypes, ion pumps) to changes in axonal membrane dynamics. Several non-spatial (single compartment) C-fibre mathematical models representing DRG somata have been used to study excitability in somatosensory neurones (Scriven 1981; Herzog et al. 2001; Sheets et al. 2007; Maingret et al. 2008; Kouranova et al. 2008; Choi and Waxman 2011). The current model presents a more realistic framework representing a segment (12.5 cm long) of a C-fibre including the peripheral terminal branches. The model is thereby to our knowledge the first to address axonal propagation of action potentials in cutaneous C-fibres. Several features of activity-dependent changes in the conduction velocity of unmyelinated axons are used in the classification of C-fibre types (Weidner et al. 2000; Campero et al. 2004). In this work, we have focused on activity-dependent slowing (ADS) of axonal conduction velocity (Obreja et al. 2010) and recovery cycles (RC) (Weidner et al. 2000, 2002;
Bostock et al. 2003). For both ADS and RC the underlying mechanisms have not been fully resolved (Weidner et al. 2000, 2002; Bostock et al. 2003; George et al. 2007; Blair and Bean 2003; De Col et al. 2008; Serra et al. 2011, 2012). However, the findings presented here point to the importance of the intraaxonal sodium concentration in modulating axonal excitability and conduction velocity during activity.
Materials and methods

To study activity-dependent changes of excitability in peripheral C-fibres, a biophysical model was constructed based on our own experimental data as well as previously published data.

By virtue of their function, it is likely that the terminal region of C-fibres differs in some respects from the parent axon which connects to the spinal cord. The main goal of the present study was to replicate and understand results acquired by microneurographic recordings of electrically evoked action potentials. Since electrical stimulation directly generates an action potential we have excluded the process of sensory transduction within sensory terminals from this study. The model therefore consists of a terminal branch axon and a parent axon (Figure 1). The branch axon is rather thin (diameter 0.25 μm) and superficial and therefore cooler (i.e., 32 degrees), while the parent axon is thicker (diameter 1μm) and deeper (body temperature, 37 degrees). The branch and parent axons are connected by a linearly tapered (i.e. cone shaped) transition in diameter and temperature. Without a cone, there might be propagation failures due to a step change in area. To reduce model complexity and simulation time, we have modelled an unbranched axon and to avoid unwanted boundary contributions to the solutions we use a passive-end strategy, specifically by adding low-conductance segments to both ends of the fibre (not shown). The model is a detailed multicompartmental model implemented using the NEURON simulation software (Hines and Carnevale 1997). The number of compartments was 2430 and equations were solved using the variable time step method.
Passive properties

The peripheral portion of a C-fibre was represented by a cylindrical membrane with length, diameter and temperature as indicated in Figure 1. Passive parameters of the membrane and the initial ion concentrations are listed in Table 1. We implemented dynamics for changes in sodium and potassium concentrations according to Scriven (1981), as illustrated in Figure 1B (see Scriven (1981) for equations). To reduce periaxonal potassium accumulation we doubled the periaxonal space to theta=29 nm. Changes of ion concentration following an action potential can be seen in Figure 2.

Ion channel and pump models

Models for Hodgkin-Huxley-type ion channel currents were taken from the literature and are detailed below. Channel densities are presented in Table 2. Equations for ion channels and the pump are found in Supplementary information. For the rate constants, Q10 was set at 2.5 for the voltage-dependent sodium channels (Jonas, 1989; Lux et al. 1990; Russ et al. 1996), 3.3 for the potassium channels (Russ and Siemen 1996) and 3 for the HCN channels (Pena et al. 2006).

Nav1.7: Values taken from Sheets et al. (2007).

Nav1.8: Values taken from Sheets et al. (2007) for activation (m³) and fast inactivation (h), while for slow (s) and ultra-slow (u) inactivation we used the equations and values from Maingret et al. (2008), according to g = gbar·m³hsu. For the simulations presented in Figure 5F, an additional transition, from the open state of the fast activation gate to an inactivated state, was added. Rate constants for this transition were a=0.0043, b=0.00024.

Nav1.9: Values taken from Herzog et al (2001), which include three gates (activation, fast
inactivation, slow inactivation).

**K_{dr}:** Values taken from Sheets et al. (2007), with steady-state voltage dependence shifted by -10 mV in order to better fit the experimental data.

**K_{M}:** The voltage dependence of steady-state activation is from Maingret et al. (2008). The K\_M channel activation has a fast and slow a time constant described by Passmore et al. (2003). To account for the two time constants we implemented one fast (nf) and one slow (ns) gate, combined as follows: \( g = g_{\text{bar}} \cdot (n_s \cdot 1/4 + n_f \cdot 3/4) \). Time constants were as follows:

\[
\text{if (V_m<-60) } \{ \tau_{ns}=219 \}, \text{ else } \{ \tau_{ns}= 13 \cdot (V_m)+1000 \}
\]

\( \tau_{nf}=1/(a+b), \) with \( a=0.00395 \cdot \exp((V_m+30)/40) \) and \( b=0.00395 \cdot \exp(-(V_m+30)/20) \)

**K_{A}:** Values taken from Sheets et al. (2007), with steady-state voltage dependence shifted by -15 mV.

\**H:** Values taken from Kouranova et al. (2008), which includes one fast (m_{fast}) and one slow (m_{slow}) activation gate, added as follows: \( i_h = g_{\text{bar}} \cdot (0.5 \cdot m_{\text{fast}} + 0.5 \cdot m_{\text{slow}}) \).

**K_{Na}:** We modelled the K\_Na current according to data from Bischoff et al. (1998), assuming an instantaneous effect of Na\_in on gate opening: \( g_{KNa} = g_{\text{bar}}/\left(1+(38.7/Na_{\text{in}})^{3.5}\right) \).

**Leak currents:** Sodium and potassium leak (balancing) currents are included in the model to assure that the sum of sodium and potassium currents are each zero at the resting membrane potential.

\( i_{\text{leak}} = g_{\text{Kleak}} \cdot (V_m - E_K) + g_{\text{Naleak}} \cdot (V_m - E_{Na}) \)

\( g_{\text{Naleak}} = - \left( ina_{Nav1.7} + ina_{Nav1.9} + ina_{Nav1.8} + ina_h + ina_{NaKpump} \right) / (V_{rest} - E_{Na}) \)

\( g_{\text{Kleak}} = - \left( ik_{KM} + ik_{KA} + ik_h + ik_{Kdr} + ik_{NaKpump} + ik_{KNa} \right) / (V_{rest} - E_K) \)

**Na-K-ATPase:** Values taken from Scriven (1981) with a modified sodium dependence to
account for recent C-fibre data (Hamada et al. 2003): $I_{\text{K,pump}} = \frac{g\text{bar}}{(1+1/K_{sp})^2} \cdot (1.62/(1+(6.7/(N_{\text{Na}}+8))^3) + 1.0/(1+(67.6/(N_{\text{Na}}+8))^3))$

$INa_{\text{pump}} = -\frac{3}{2} \cdot I_{\text{K,pump}}$

$I_{\text{pump}} = I_{\text{K,pump}} + I_{\text{Na,pump}}$

Stimulation protocols

To study activity-dependent changes of action potential (AP) conduction an action potential was initiated in the distal-most compartment of the branch axon using current injection (5 ms, 0.1 nA). As in Weidner et al. (1999), Campero et al. (2004) and Obreja et al. (2010) we used two different protocols to assess the amount of activity-dependent slowing: a high frequency protocol (360 pulses at 2 Hz followed by 60 pulses at 0.25 Hz) and a low frequency protocol (20 pulses at 0.125 Hz, 20 pulses at 0.25 Hz, 30 pulses at 0.5 Hz, 20 pulses at 0.25 Hz). Using these paradigms, we measured activity-dependent changes of the propagation latency (Figure 3). To examine recovery cycles, axons were stimulated at constant frequency until the conduction latency had stabilised, at which point additional electrical stimuli were interposed at varying inter-stimulus intervals (ISI), similar to Weidner et al. (2000) and Bostock et al. (2003).

Extracellular single-fibre recordings in anesthetized pigs

Extracellular recordings from the saphenous nerve were performed according to the experimental protocol described previously (Obreja et al. 2010). Experimental procedures were approved by the Ministry Dept. Baden-Württemberg in Karlsruhe, Aktenzeichen 35-
9185.81/G-145/08 and by the central animal research unit at the University of Heidelberg.

Action potentials were evoked by constant current electrical stimulation (20 mA; 0.5 ms; DS7A, Digitimer Ltd., Hertfordshire, UK) applied at 0.25 Hz via intradermal non-insulated microneurography electrodes to the skin (FHC Inc., Bowdoin, ME, USA). The distance between stimulation needles and the recording electrode was used to determine conduction velocity (CV). When determined immediately after a 2-min pause all fibres in this study had CV values ≤ 2 m/s. Extracellular signals were amplified (Low-Noise Voltage Preamplifier Model 5113, Ametek Inc., TN, USA), filtered (bandwidth 100 – 3000 Hz; Model 3364, Krohn-Hite Corp., Brockton, MA, USA), audio monitored and digitized at a sampling rate of 32 kHz using DAPSYS 7.0, a joint hardware and software system designed for real-time acquisition, window discrimination and latency measurements of action potentials (Obreja et al., 2010).

During ongoing intradermal electrical stimulation (0.25 Hz), mechanical stimuli (150 mN; Semmes-Weinstein calibrated monofilaments) were applied to the skin to localize the peripheral receptive field of the unit under study. At mechano-sensitive spots, action potentials induced by mechanical stimulation led to “marking” of the electrically-evoked action potential, i.e. an increase in latency of the electrically-evoked action potential (Torebjörk and Hallin 1974; Schmelz et al. 1995). Fibres displaying marking upon stimulation with 150 mN but not activated by stimulation with a paintbrush were classified as mechano-sensitive nociceptors (CM; Obreja et al., 2010). Receptive fields of mechano-insensitive nociceptors (CMi) were determined using collision techniques (Meyer and Campbell, 1988).

Estimates of action potential width

Extracellularly recorded action potentials (AP) in slowly conducting nerve fibres typically
have a triphasic shape (2 positive peaks and 1 large negative peak). Time-locked latencies and spike shape were used to discriminate action potentials. To determine AP width, at least 20 time-locked, overlapping traces were averaged. Action potential width was calculated as the time between the 1st and 3rd (i.e., positive) peak.

Criteria used to set model parameters

Activity-dependent changes of conduction velocity have been measured in human control subjects (Weidner et al. 2000; Namer et al. 2009) as well as in patients with neuropathic pain (Schmidt et al. 2012; Kleggetveit et al. 2012; Ørstavik et al. 2003; Kanai et al. 2006; Serra et al. 2011) with similar results in pig (Obreja et al. 2010). Using the available experimental data, the model was adjusted to meet the following four criteria: (1) the model axon should show a similar magnitude of conduction velocity slowing as human C-fibres, both for the low-frequency protocol and high-frequency protocol, see below; (2) the model axon should hyperpolarize with activity in the high-frequency protocol (Moalem-Taylor et al. 2007); (3) the unstimulated (control) conduction velocity should lie between 0.6 and 1.3 m/s (Obreja et al. 2010); (4) the supernormal phase (SNP) magnitude should increase with increasing ADS (Weidner et al. 2002; Bostock et al. 2003); (5) the fibre should show a supernormal phase when slowing has been induced (Weidner et al. 2002) and (6) pre-conditioning pulses should not further increase SNP (Bostock et al. 2003).

Several parameters used in the model, notably ion channel densities, have not currently been determined. For other parameters (e.g. ion channels $V_{1/2}$), values observed experimentally need to be adjusted to account for differences in ion concentration, temperature etc. In the
process of constructing a model that fulfils the functional criteria, replicating e.g. experimental characteristics of ADS and recovery cycles, a large number of simulations were performed. After each simulation, changes were made to parameter values based on several aspects of model output (output on which performance according to functional criteria can be assessed), or by the use of an optimizer algorithm (Tigerholm and Fransén 2011). In the development of the model, we initially set the ion channel conductances according to experimental data from DRG somata (Gold et al. 1996; Winkelman et al. 2005; Blair and Bean 2002, 2003; Passmore et al. 2003; Hamada et al. 2003; Kouranova et al. 2008). These and a set of other ion channel parameters were subsequently changed in order for the model to adequately replicate ADS and recovery cycles. We constructed the model in the following steps: basic propagation of action potentials, basic ADS pattern, basic RC pattern, improved action potential propagation and wave form, improved ADS pattern, improved RC pattern, improved action potential propagation etc. Criteria regarding basic electrophysiological properties are described below.

**Basic electrophysiological properties**

**Resting membrane potential**

The membrane potential (Vm) of human C-fibres in situ is not known, and might vary among various fibres. We used Vm=-55 mV in this study which is consistent with experimental data from rat DRG somata (-63 to -41 mV; Fang et al. 2005) and has been recently confirmed by recordings from isolated neurites from rat DRG neurons suggesting a membrane potential of -60.2mV (Vasylyev and Waxman, 2012)
Passive membrane properties

The combination of passive membrane properties, ion channels and pumps described above result in a branch membrane time constant of around 1.3 ms and length constant of around 3 mm at the resting membrane potential.

Action potential waveform

Details regarding the shape of the axonal AP and the relative influence of the underlying currents in C-fibre axons are not known. However, the major sodium current during an AP in DRG somata is TTXr (Blair and Bean 2002), most probably driven by the activation of Nav1.8 channels (Akopian et al., 1996; Renganathan et al., 2001). This observation was used as a criterion when tuning the model. Note that the TTX-sensitive Nav1.7 current also contribute to AP generation and conduction velocity. A further criterion on the short time scale was that the AP width should be less than 5 ms.

Results from our experimental investigations from pig nociceptive C-fibres showed that the extracellularly recorded AP in C-type nociceptors was around 3 ms wide: C mechano-insensitive (CMi): 3.0 +/- 0.22; N= 26; C mechano-sensitive (CM): 2.94 +/- 0.2; N= 17). The intracellular AP width in the model was ~3.5 ms measured at -40 mV, see also Figures 2 and 4.

Refractory period

Preliminary experimental data from n= 4 pig polymodal nociceptors show that for 3 out of 4
nociceptors, twin pulses applied with suprathreshold current pulses can generate two
propagating action potentials at an ISI of 5 ms (at ~2 times current threshold), respectively 4
ms (at 15 times threshold). In the model, twin action potentials could be generated with ISI = 5
ms for suprathreshold (>1.4 times threshold) pulses, and with ISI = 4 ms for very
suprathreshold (>14 times threshold) pulses, which further supports the validity of our model.

Results

In this work, we have generated a mathematical model of a peripheral C-fibre axon (Figure 1)
in order to investigate the mechanisms involved in activity-dependent changes of action
potential propagation. The rationale for a computational approach was difficulties associated
with experimental intra-axonal recordings that have thus far precluded direct determination of
the intracellular membrane potential. The model replicates basic properties of the action
potential (Figure 2; see also Basic electrophysiological properties in the Methods section as
well as supplementary information (Figure 1; top, middle) for expanded plots of the action
potential). In particular, Nav1.8 and K_{dr} are the dominating currents of the action potential
(Figure 2A). These currents differentially affect intracellular sodium and periaxonal
potassium concentrations during the action potential in the branch vs. the parent axon (Figure
2B).

Activity-dependent slowing

Simulations of latency changes during action potential propagation along the simulated C-
fibre are shown in Figure 3. The intracellular action potential amplitude decreases 10-15%,
see Figure 3A, it becomes 10-15% wider and develops a 2-5 mV late depolarizing after-
potential during its propagation along the axon. For additional information about the action
potential shape and its after potential, see also Figure 2A, 6A, and Supplementary information
During repetitive stimulation the modelled axon conducts subsequent action potentials progressively slower with the magnitude of the slowing of conduction latency being more pronounced at higher rates of stimulation (Figure 3B-D). During repetitive stimulation at 2Hz for 180s, the propagation latency increased from 212 ms (corresponding to a CV in the parent axon of 0.69 m/s) to 289 ms corresponding to a 36% increase in propagation latency (see Figure 3C, D, upper panels). This is largely commensurate with experimentally obtained values for ADS in human and porcine C fibres (i.e., 36.7+-2.2%, respectively 30.1+-1.6%; Obreja et al. 2010). We also conducted a simulation with the low frequency protocol (Figure 3 C, D; lower panels). The resulting ADS magnitude after a stimulation at 0.125 Hz (20 pulses), followed by 0.25 Hz (20 pulses) and 0.5 Hz (30 pulses) was ~8%, which also corresponds well to the experimental results (Obreja et al. 2010). The slowing of axonal conduction speed is also associated with changes in action potential shape with the development of a prominent depolarizing afterpotential at the end of a 180s period of 2Hz stimulation (Figure 3B and Figure 4 and Supplementary information Figure 1 top, middle). The after potential is produced as an interplay between currents with slower kinetics (e.g. K\textsubscript{M} and h), currents that change when the Na-concentration changes (Na-K-ATPase, K\textsubscript{Na}) and currents that change over successive pulses due to accumulated inactivation or concentration change, see Figure 3 (top) in Supplementary information for further discussion.

In Figure 4, changes in ionic currents and membrane potential during ADS are depicted. For information about the net membrane current during an action potential, see Figure 2 in Supplementary information. Compared to the first pulse (4A) the membrane potential of the
360th pulse (4B) is hyperpolarised and the overshoot of the action potential decreases. The ionic basis for these changes is shown in Figure 4C, D with peak Nav1.8 current being much reduced. The magnitude of the Nav1.7 current also decreases, albeit to a lesser extent than the Nav1.8 current. Changes in currents before initiation of the AP are listed in Table 2. As shown in the table, the main difference is the increase in KNa current as a major factor contributing to membrane hyperpolarization. See also Figure 1 (top, middle) in Supplementary information for additional information about the hyperpolarization.

**Mechanism of ADS - accumulation of intracellular sodium**

The simulations indicate that the concentration of intracellular sodium may play a central role in ADS and thereby that changes in sodium reversal potential may be involved in the observed slowing. As can be seen in Figure 5A, clamping the reversal potential for sodium to its initial value abrogates ADS and the response latency stays constant during 2Hz stimulation. Note that in the model we are able to dissociate the reversal potential, which here was held fixed, from ion flux, which was unaltered and could affect other concentration dependent processes such as KNa and the pump. Demonstrable changes in the concentration of intra-axonal Na were observed during the ADS protocol (Figure 5B, green line) presumably because the diameter and thus volume of the axon is small and this results in a substantial reduction in the reversal potential for Na (E_{Na}, Figure 5C). In contrast periaxonal K remained relatively unaffected (Figure 5B, black line). To examine the contribution of individual Na conductances to the increase in intra-axonal Na, the Na reversal potential was clamped separately for the individual Na subtypes 1.7 and 1.8. The results are illustrated in Figure 5D and suggest that the increase in intra-axonal Na is largely attributable to the Nav1.8 current. With the reversal potential for Nav 1.8 held constant the amount of slowing is
reduced by approximately 80%, while clamping $E_{Na}$ for Nav1.7 results in an approximate 40% reduction in ADS.

Previous work has suggested that the entry of Nav channels into slow inactivated states can account for many of the features of ADS (Blair and Bean, 2003; De Col et al. 2008; Snape et al., 2010). We examined this by adding a slow inactivation state to the Nav1.8 channel while clamping Na and K concentrations (Figure 5E, red and green traces). Importantly, activity slows axonal conduction under these conditions (Figure 5E) however slow inactivation alone can only generate about 25% of the slowing attributable to intra-axonal Na accumulation (compare to blue traces in Fig. 5E). Note that the ADS amplitude attributable to Nav entry into slow inactivated states was more prominent at the cooler temperatures used experimentally in vitro. The rapid rise and early plateau of the slowing suggests that entry into slow inactivated states occurs rapidly in this model (Figure 5E), in particular at body temperatures.

Direct intracellular recordings from DRG somata have shown that the minimum current amplitude needed to trigger an action potential, increases during repetitive activation at 2Hz (Snape et al, 2010). In the simulated C-fibre axon presented here, the minimum current required to trigger an action potential was found to increase linearly with the number of preceding action potentials, Figure 5F (bottom). Moreover, we studied the relationship between slowing and the action potential threshold (Figure 5F, top). The findings suggest that ADS is indicative of changed axonal excitability with a reduction in axonal conduction velocity paralleling an increase in activation threshold (fig 5F, top).
**Recovery cycles**

The recovery cycle refers to changes in either latency or threshold seen in the period up to ca. 300 ms following a single action potential in a peripheral axon. Simulations were performed using stimulus pulses at variable inter-stimulus intervals (ISI) and at different base frequencies. Changes in membrane potential at the stimulation site (top) and at the end of the parent axon (bottom) are shown in Figure 6A.

In Figure 6B, characteristic changes in AP conduction latency are shown for the model axon over the 250 ms period immediately following the passage of the first AP. The basic form of the recovery cycle for the model axon is similar to that observed in recordings from peripheral unmyelinated axons in humans (Weidner et al. 2002; Bostock et al. 2003). An initial phase of reduced conduction velocity (i.e., subnormality) is followed by a period of supernormality (at ISI between 30 and 100ms, consistent with the experimental range of 20-250ms) and this subsequently reverses to a second late period of subnormality (Figure 6B). The timing of these three phases varies according to axonal subtype and for unmyelinated axons the extent and even the incidence of the supernormal phase is dependent upon the base rate of stimulation (Bostock et al, 2003). For human C-fibre axons in situ, the magnitude of supernormality typically increases in proportion to the magnitude of ADS (Bostock et al., 2003, their Figure 6). Similarly, for the simulated axon an increase in the magnitude of the supernormal phase was observed with increasing repetition frequency (fig. 6B).

**Control experiments**

To validate the model, the behaviour of the model was examined during a range of manipulations designed to replicate in vitro C-fibre experiments using pharmacological
agents, changes in ion concentrations and temperature. The results are shown in Figure 7. We also performed a parameter sensitivity analysis, found in Supplementary information (Figure 3). Since most phases of the spike and post spike periods show contributions from several currents, the model does not show indications of sensitivity. One exception is during the phase of the action potential where Nav1.8 and Kdr together amount to almost all variation, but this is really what is to be expected.

**h-channel block**

Since repetitive spiking-activity hyperpolarized the membrane potential, we assumed that hyperpolarization-activated channels (Ih) known to modulate the membrane potential, might change the amount or shape of ADS. We find that h-channel block, results in an increased ADS (Figure 7A), consistent with experimental data (Takigawa et al. 1998). In addition, full block of h-current decreased the initial latency, and resulted in a steep rise in slowing for the first ~50 pulses, followed by a much slower, essentially linear increase in latency (see Figure 7A, left top panel, magenta curve).

**Nav1.7 and Nav1.8 block**

Figure 7A also depicts ADS changes following blockade of the TTX-sensitive Nav1.7 and the TTX-resistant Nav1.8 channel. Blockade of Nav1.7 channels only slightly increased absolute latency and ADS, which is consistent with the results of De Col et al. (2008). Nav1.8 block, for which there is currently no experimental data for comparison, also increased latency, but decreased ADS.
Changing extracellular ion concentrations

Reduction of the periaxonal (i.e. extracellular) sodium concentration (Figure 7B) increased absolute latencies and increased ADS consistent with De Col et al. (2008).

Changing temperature

A temperature reduction of 10°C, from 37 to 27°C, increased both the absolute conduction latency and the magnitude of ADS (Figure 7C), in good agreement with De Col et al. (2008).

Discussion

The model presented here offers a framework to examine activity-dependent changes in axonal excitability and conduction velocity in C-fibres. The model allows various contributing influences, like membrane potential and ion concentrations, to be specifically and selectively dissociated from one another, a feature that is experimentally not possible. Moreover, the model provides a means of performing in-silico pharmacology which further allows for approaches that cannot be performed in real experiments such as changing reversal potentials only for a subset of ion channels. Arguments for the validity of the model include the replication of a variety of independent experimental findings from single fibre recordings in human, rat and pig. The key result is the identification of a specific contribution of intra-axonal Na accumulation to activity-dependent changes in the conduction velocity of C-fibres. In addition, the model provides insight into the relationship between axonal excitability and ADS with the degree of slowing being indicative of magnitude of stimulation current needed...
Accumulation of intracellular sodium can contribute to ADS

There have been several hypotheses regarding the mechanistic basis of ADS. For some time, axonal hyperpolarization driven by increased Na-K-ATPase activity accompanying action potential activity in unmyelinated axons (Rang and Ritchie 1960) was thought to result in the slowing of conduction speed. While changes in membrane potential most certainly contribute to activity-dependent changes in axonal conduction velocity, pharmacological blockade of the Na-K-ATPase increased the magnitude of ADS (De Col et al. 2008) casting doubt on a causal link between pump activity and ADS. A contribution of sodium channel entry into slow inactivated states was proposed to contribute to ADS (Blair and Bean, 2003; De Col et al. 2008) and was confirmed using current threshold measurements to examine excitability in DRG somata (Snape et al. 2010). Our simulations support the contribution of Nav channel inactivation to ADS (Figure 5E) but indicate that additional mechanisms are needed to fully explain the quantitative nature of ADS.

The results of the simulated C-fibre axon here contend that an accumulation of intracellular sodium, which would reduce the current passing through sodium channels, prominently affects axonal conduction velocity. Recordings from DRG somata are less affected by AP mediated changes in intracellular ion concentrations and experimental approaches to examine Na concentration in axons have not yet been realised but have been suggested by Endres et al. (1986). Decreased extracellular sodium reduces the sodium driving force and results in increased latency (Donnelly et al, 1998). Experimentally, indirect evidence for accumulation...
of intracellular sodium as a mechanism for increased activity dependent slowing is provided by increased slowing following block of the Na-K-ATPase activity by low extracellular potassium or by replacing extracellular sodium with lithium, that is not extruded by the Na-K-ATPase (De Col et al., 2008). Indeed, reducing extracellular sodium resulted in an increase in ADS in single dural afferents axons in the rat (De Col et al. 2008) consistent with the observed increase in ADS accompanying lowered extracellular Na in our model.

The physiological role of ADS remains speculative. Raymond (1979) showed that the electrical activation threshold of myelinated frog axons increases in proportion to ADS, results recently confirmed in porcine cutaneous C-fibres, where a strong correlation between activity-induced increased electrical thresholds, propagation failure and amount of ADS was documented (Obreja et al. 2011b). This has recently been corroborated using dynamic mechanical stimuli in single afferent axons in the rat cranial dura, which showed that mechanical activation threshold increased monotonically with axonal conduction latency (De Col et al. 2012). An increase in intracellular sodium concentration leading to a measurable slowing of axonal conduction might be regarded as form of accommodation, i.e. a negative feedback mechanism that prevents excessive discharge, in particular for small C-fibre neurones and their terminals exposed to restricted metabolic conditions.

Implications of conduction velocity, recovery cycles and ADS for pain

Standard experimental assessment of neuronal hyperexcitability mainly relies on excitation thresholds rather than on suprathreshold discharge. However, under clinical conditions spontaneous activity and discharge frequencies upon suprathreshold activation are crucial. In
this respect, reduced ADS in nociceptors in human pain patients (Orstavik 2003, 2006, Serra et al., 2012) and in particular the correlation between reduced ADS and spontaneous activity (Kleggetveit et al. 2012) suggests that reduced ADS can contribute to chronic pain states. As mechanistic studies in humans are limited, important contributions were provided by animal models of neuropathic pain. Reduced ADS and lower conduction failure in the nociceptive primary afferents nociceptors, in combination with increased expression of Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8 in the corresponding dorsal root ganglia were found in a rodent model of diabetic neuropathy (Sun et al., 2012). Our model would suggest that ADS is mainly determined by sodium influx via Na\textsubscript{v}1.8. The model also shows that Na\textsubscript{v} 1.7 in fact opposes ADS, and thus that decreased ADS would be associated with relatively larger increases in Na\textsubscript{v}1.7 as compared to Na\textsubscript{v}1.8. This is consistent with the increased protein levels found in the dorsal root ganglia (Sun et al., 2012). Furthermore, the model would also predict that additional changes are required to explain the decreased ADS in the hyperalgesic animals such as increased sodium potassium pump activity or depolarization of the peripheral axons. Thus, specific contributions of channel subtypes in combination with membrane potential and pump activity to neuronal hyperexcitability can be identified. This is of particular value to predict the effects of specific blockers and thus increase the probability for advances in drug development from a better understanding of mechanism of action. Future studies will link the model more closely to experimental data from pathophysiologic states such as nerve growth factor-induced reduction of ADS (Obreja et al, 2011b). Ultimately, spontaneous activity (Kleggetveit 2012) and increased discharge frequencies of nociceptors (Serra 2012; Schmidt 2012) as the key source of clinical pain in the peripheral nervous system have to be modelled.
Acknowledgements

This work was supported by Swedish VR 621-2007-4223, German Research Council (LA 2740/2-1), State Baden-Württemberg (Kompetenzzentrum Schmerz).

Disclosures

The authors acknowledge financial support from AstraZeneca R&D, Södertälje, Sweden.

Author contributions

Jenny Tigerholm: contributed in conceiving the project, model development, model simulations, analysis of results, and to manuscript writing

Marcus Petersson: contributed in conceiving the project, model development, model simulations, analysis of results and to manuscript writing

Otilia Obreja: performed pig experiments, contributed in conceiving the project, analysis of experimental data for parameter estimation and to manuscript writing

Angelika Lampert: contributed in conceiving the project, analysis of experimental data for parameter estimation and to manuscript writing

Richard Carr: contributed in conceiving the project and to manuscript writing

Martin Schmelz: contributed in conceiving the project and to manuscript writing

Erik Fransén: contributed in conceiving the project and to manuscript writing
References


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Figure legends

FIGURE 1 Model overview, geometry and temperature
A) Model consisting of a terminal branch axon and a parent axon, connected via a cone. The branch and parent axons differ in length, diameter and temperature.
B) Axon cross-section. The model consists of an intra-axonal space, a periaxonal space and extracellular fluid (where concentrations are assumed to be unaffected by activity), modelled according to Scriven (1981).

FIGURE 2 Currents and concentrations during an action potential.
Following a single current injection at the beginning of the branch, an action potential was generated and propagated along the axon.
(A) Membrane potential (Vm) and ionic currents recorded at centre of the branch (left) and centre of the parent (right). Time frame shown is expanded from simulation shown in B. Lower lane shows currents at a larger time scale.
(B) Intra-axonal (K_{in}, Na_{in}) and peri-axonal (K_{sp}, Na_{sp}) concentrations as well as resulting reversal potentials (K-rev, Na-rev).

FIGURE 3 Activity–dependent slowing of conduction velocity.
(A) Stimulus evoked action potentials at the start and (B) at the end of the repetitive stimulation (2Hz; 3 min). Time frame shown is expanded from simulations shown in C and D around the time of the first and last AP respectively. (C) Latency during repetitive stimulation
(upper panel: 360 pulses at 2 Hz, 60 pulses at 0.25 Hz; lower panel: 20 pulses at 0.125 Hz, 20 pulses at 0.25 Hz, 30 pulses at 0.5 Hz, 20 pulses at 0.25 Hz). (D) The latency during repetitive stimulation normalized to the initial latency. Relative latency changes for both the high-frequency (upper panel) and low-frequency (lower panel) protocols.

**FIGURE 4 Activity-dependent changes of the membrane potential and ionic currents.**

First (left) and 360th (right) action potential (AP) during the high-frequency ADS protocol. Membrane potential Vm (A-B) and ionic currents (C-D) are measured at the centre of the parent axon. Note the reduction in Vm and current peaks during the 360th AP compared to the 1st pulse. Also note that the 360th AP starts from a more hyperpolarized level (due to activity-dependent hyperpolarization).

**FIGURE 5 Activity-dependent slowing is induced by accumulation of intracellular sodium.**

(A) The relative latency during the high-frequency protocol (2 Hz), control (blue) and with clamped reversal potentials (black). (B) Intracellular concentration of sodium (green) and periaxonal potassium concentration (black). (C) Reversal potential of sodium. (D) The latency relative to the initial latency when the reversal potential was held constant, separately for Nav1.7 (green) and Nav1.8 (red), versus control (blue). (E) ADS resulting from Nav1.8 slow inactivation. Control condition (when the ADS developed from changes in Na concentration, blue) versus ADS when Na and K concentrations were held fixed and a slow inactivation transition was added to Nav1.8 (20 °C, green), (37 °C, red). (F) Relation between minimum current injection and degree of latency change (top) and pulse number (bottom). The
minimum current injection needed to trigger an action potential was measured over repetitive stimulations. Increased minimum current indicates decreased fiber excitability. Current injection was positioned on the midpoint of the branch segment.

FIGURE 6 Modeling recovery cycle velocity changes

The figure shows the slowing/speeding during the recovery cycle protocol. The frequency is 2 Hz and the inter-stimulus interval (ISI) vary between 10-250 ms (note that only 3 specimen are shown). (A) The membrane potential for different ISIs. The upper graphs represent the membrane potential at the beginning of the branch axon and the lower graphs show the membrane potential at the end of parent axon. (B) The slowing/speeding for different ISIs.

FIGURE 7 Model testing by changes of the temperature and ion concentration and by block of ion channels and the ion pump. We plot both absolute (top) and relative (bottom) latencies in simulations using the ADS protocol. L = absolute latency.

A. Block of ion channels: h channel (top, left), Nav1.8 (top, right), Nav1.7 (bottom, left). Control condition (black). Block (colours), steps of 10% (except for h-channel block where magenta represents full block).

B. Changes of extracellular ion concentrations. Decreasing concentrations of the periaxonal Na (Na\textsubscript{sp}, 10% blue, 20% green, 30% red) versus control concentration (black).
C. Changing temperature. Lower temperature (27°C, blue) compared to control temperature (37°C, black).
Appendix A

A1. Equations for ionic currents

Nav1.7:

\[ I_{\text{Nav1.7}} = g_{\text{Nav1.7}} m^3 h s (V_n - E_{\text{Na}}) \]

\[ m = m + (1 - \exp(-dt/\tau_m)) \cdot (m_{\text{inf}} - m) \]

\[ \alpha_m = 15.5 / \{1 + \exp [(V_n - 5)/(-12.08)]\} \]

\[ \beta_m = 35.2 / \{1 + \exp [(V_n + 72.7)/16.7]\} \]

\[ \tau_m = 1 / (\alpha_m + \beta_m) \]

\[ m_{\text{inf}} = \alpha_m / (\alpha_m + \beta_m) \]

\[ h = h + (1 - \exp(-dt/\tau_h)) \cdot (h_{\text{inf}} - h) \]

\[ \alpha_h = 0.38685 / \{1 + \exp [(V_n + 122.35)/15.29]\} \]

\[ \beta_h = -0.00283 + 2.00283 / \{1 + \exp [(V_n - 5.5266)/(-12.70195)]\} \]

\[ \tau_h = 1 / (\alpha_h + \beta_h) \]

\[ h_{\text{inf}} = \alpha_h / (\alpha_h + \beta_h) \]

\[ s = s + (1 - \exp(-dt/\tau_s)) \cdot (s_{\text{inf}} - s) \]

\[ \alpha_s = 0.0003 + 0.0092 / \{1 + \exp [(V_n + 93.9)/16.6]\} \]

\[ \beta_s = 132.05 - 132.05 / \{1 + \exp [(V_n + 384.9)/28.5]\} \]

\[ \tau_s = 1 / (\alpha_s + \beta_s) \]

\[ s_{\text{inf}} = \alpha_s / (\alpha_s + \beta_s) \]

Nav1.8:

\[ I_{\text{Nav1.8}} = g_{\text{Nav1.8}} m^3 h s u (V_n - E_{\text{Na}}) \]

\[ m = m + (1 - \exp(-dt/\tau_m)) \cdot (m_{\text{inf}} - m) \]

\[ \alpha_m = 2.85 - 2.839 / \{1 + \exp [(V_n - 1.159)/13.95]\} \]

\[ \beta_m = 7.6205 / \{1 + \exp [(V_n + 46.463)/8.8289]\} \]

\[ \tau_m = 1 / (\alpha_m + \beta_m) \]

\[ m_{\text{inf}} = \alpha_m / (\alpha_m + \beta_m) \]

\[ h = h + (1 - \exp(-dt/\tau_h)) \cdot (h_{\text{inf}} - h) \]

\[ \tau_h = 1.218 + 42.043 \cdot \exp \left\{ - \left[ (V_n + 38.1)^2 / (2 \cdot 15.19^2) \right] \right\} \]

\[ h_{\text{inf}} = 1 / \{1 + \exp [(V_n + 32.2)/4]\} \]

\[ s = s + (1 - \exp(-dt/\tau_s)) \cdot (s_{\text{inf}} - s) \]

\[ \tau_s = 1 / (\alpha_s + \beta_s) \]

\[ s_{\text{inf}} = \alpha_s / (\alpha_s + \beta_s) \]

\[ u = u + (1 - \exp(-dt/\tau_u)) \cdot (u_{\text{inf}} - u) \]

\[ \tau_u = 1 / (\alpha_u + \beta_u) \]

\[ u_{\text{inf}} = 1 / \{1 + \exp [(V_n + 45)/8]\} \]
\[
\beta_u = 0.0002 \cdot 1.9952 \div \{1 + \exp[-(V_m + 30.963) / 14.792]\}
\]

Nav1.9:

\[
I_{Nav1.9} = g_{Nav1.9} \cdot m_h s \cdot (V_m - E_{Na})
\]

\[
\alpha_m = 1.032 \div \{1 - \exp[(V_m + 6.99) / 14.8715]\},
\]

\[
\beta_m = 5.79 \div \{1 + \exp[(V_m + 130.4) / 22.9]\}
\]

\[
\alpha_h = 0.06435 \div \{1 + \exp[(V_m + 6.99)/14.8715]\},
\]

\[
\beta_h = 0.13496 \div \{1 + \exp[(V_m + 130.4) / 22.9]\}
\]

\[
\alpha_s = 0.00000016 \cdot \exp(-V_m / 12)
\]

\[
\beta_s = 0.0005 \div \{1 + \exp[-(V_m + 32) / 23]\}
\]

\*Q10_{Na} ?

Kdr:

\[
I_{Kdr} = g_{Kdr} \cdot n^4 \cdot (V_m - E_K)
\]

\[
dn/dt = (n_{inf} - n) / \tau_n
\]

\[
n_{inf} = 1 / \{1 + \exp[-(V_m + 45) / 15.4]\}
\]

\[
\tau_n = 1000 \cdot \{0.000688 + 1 / [\exp((V_m + 75.2) / 6.5) + \exp((V_m - 131.5) / -34.8)] \} \* Q10_K, \text{ if } V_m < -31 \text{ mV}
\]

\[
\tau_n = 0.16 + 0.8 \cdot \exp(-0.0267 \cdot (V_m + 11)) \* Q10_K, \text{ if } V_m > -31 \text{ mV}
\]

KM:

\[
I_{KM} = g_{KM} \cdot (n_s / 4 + 3 n_f / 4) \cdot (V_m - E_K)
\]

\[
dn_s / dt = (n_{inf} - n) / \tau_{ns}
\]

\[
dn_f / dt = (n_{inf} - n) / \tau_{nf}
\]

\[
n_{inf} = 1 / \{1 + \exp[-(V_m + 30) / 6]\}
\]

\[
if \ (V_m < -60) \{ \tau_{ns} = 219 \* Q10_K \}, \text{ else } \{ \tau_{ns} = 13 \cdot V_m + 1000 \* Q10_K \}
\]

\[
\tau_{nf} = 1 / (\alpha + \beta), \text{ with } \alpha = 0.00395 \cdot \exp[(V_m + 30) / 40] \text{ and } \beta = 0.00395 \cdot \exp[-(V_m + 30) / 20] \* Q10_K
\]

KA:

\[
I_{KA} = g_{KA} \cdot n_h \cdot (V_m - E_K)
\]

\[
dh / dt = (h_{inf} - h) / \tau_h
\]

\[
n_{inf} = \{1 / \{1 + \exp[-(V_m + 5.4 + 15) / 16.4]\}\}^4
\]

\[
\tau_n = 0.25 + 10.04 \cdot \exp(-[(V_m + 24.67) / 20] / 34.8) \* Q10_K
\]

\[
h_{inf} = 1 / \{1 + \exp[(V_m + 49.9 + 15) / 4.6]\}
\]

\[
\tau_h = 20 + 50 \cdot \exp([-[(V_m + 40)^2] / (2 \cdot 40^2)] \* Q10_K
\]

if \ \tau_h < 5 \text{ then } \tau_h = 5
\[ h: \]

\[ I_{h,Na} = 0.5 \cdot g_{h} \cdot (0.5n_{s} + 0.5n_{f}) \cdot (V_m + E_{Na}) \]

\[ I_{h,K} = 0.5 \cdot g_{h} \cdot (0.5n_{s} + 0.5n_{f}) \cdot (V_m + E_{K}) \]

\[ n_{s} = \frac{1}{1 + \exp((V_m + 87.2) / 9.7)} \]

\[ n_{f} = \frac{1}{1 + \exp((V_m + 87.2) / 9.7)} \]

\[ \frac{dn_{s}}{dt} = (n_{inf,s} - n_{s}) / \tau_{n,s} \]

\[ \frac{dn_{f}}{dt} = (n_{inf,f} - n_{f}) / \tau_{n,f} \]

\[ \tau_{n,s} = 300 + 542 \cdot \exp((V_m + 25) / -20) \cdot Q_{10H} \text{ if } V_m > -70 \]

\[ \tau_{n,s} = 2500 + 100 \cdot \exp((V_m + 240) / 50) \cdot Q_{10H} \text{ if } V_m < -70 \]

\[ \tau_{n,f} = 140 + 50 \cdot \exp((V_m + 25) / -20) \cdot Q_{10H} \text{ if } V_m > -70 \]

\[ \tau_{n,f} = 250 + 12 \cdot \exp((V_m + 240) / 50) \cdot Q_{10H} \text{ if } V_m < -70 \]

\[ K_{Na}: \]

\[ I_{KNa} = g_{KNa} \cdot w \cdot (V_m - E_{K}) \]

\[ w = \frac{1}{1 + (38.7 / N_{ain})^{3.5}} \]

\[ \text{Leak currents:} \]

\[ I_{leak} = g_{Kleak} \cdot (V_m - E_{K}) + g_{Naleak} \cdot (V_m - E_{Na}) \]

\[ g_{Naleak} = -(in_{Nav1.7} + in_{Nav1.9} + in_{Nav1.8} + in_{h} + in_{NaKpump}) / (V_{rest} - E_{Na}) \]

\[ g_{Kleak} = -(ik_{Na} + ik_{K} + ik_{h} + ik_{Kdr} + ik_{NaKpump} + ik_{KNa}) / (V_{rest} - E_{K}) \]

\[ \text{Na-K-ATPase:} \]

\[ I_{K,pump} = g_{pump} / \{(1 + 1 / K_{sp})^2 \} \cdot \{1.62 / [1 + (6.7 / (Na_{in} + 8))^3]\} + \]

\[ 1.0 / [1 + (67.6 / (Na_{in} + 8))^3]\} \]

\[ I_{Na,pump} = -3 / 2 \cdot I_{K,pump} \]

\[ I_{pump} = I_{K,pump} + I_{Na,pump} \]

\[ \text{A2. Sensitivity analysis} \]

Figure A1. Action potential for 1st and 360th pulse. The figure below shows action potential shapes for the 1st (left) and 360th pulse (right) in the parent axon. Intracellular membrane potential is plotted on a long (top: 400 ms), medium (centre: 10 ms) and short (bottom: 0.6 ms) time scale.

Figure A2. Net membrane current (sum of all transmembrane currents) for 1st and 360th pulse. The figure below shows net action potential current for the 1st (left) and 360th pulse (right) in the parent axon. Transmembrane current is plotted on a long (top: 400 ms) and medium (bottom: 10 ms) time scale.
Figure A3. First order sensitivity analysis. In the figure below, we show the relative influence of a range of currents during the AP phases shown above. Influences are shown as first order sensitivity indexes (Sobol 1990, Homma and Saltelli 1996), the contribution to the variation from a current. To compute the first order sensitivity index, all ionic conductances were varied by +/-20%. See Petersson (2012) for an in-depth discussion. Color legend: Nav1.8 (dark blue), Nav1.7 (medium blue), h (light blue), pump (turquoise), leak (green), KM (yellow), KA (orange), Kdr (red), KNa (brown). As can be seen, different currents contribute differently to the action potential (short time scale), after potential (intermediate time scale), and ADS (long time scale) phenomena. Since most phases show contributions from several currents, the model does not show indications of sensitivity. One exception is during a phase of the action potential where Nav1.8 and Kdr together amount to almost all variation, but this is really what is to be expected.

A3 References


Petersson M, Dendritic and axonal ion channels supporting neuronal integration: From pyramidal neurons to peripheral nociceptors, 2012, PhD thesis, KTH Royal Institute of Technology. (http://urn.kb.se/resolve?urn=urn:nbn:se:kth:diva-102362)

A

Branch axon
superficial: 32°

"Cone" 34.5°

Parent axon
deep: 37

B

cell membrane

intra-axonal

periaxonal space

extracellular fluid

Na_{in}, K_{in}

Na_{sp}, K_{sp}

D

Θ
Table 1. Values of basic parameters

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<tr>
<th>Parameter</th>
<th>Value</th>
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Table 2 Conductance and resting current densities (parent axon)

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